



DETECTION OF AQUAREOVIRUS IN FARMED TASMANIAN ATLANTIC SALMON (*Salmo salar*)

by

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Statement of originality

The work presented in this thesis is, to the best of my knowledge and belief, original and my own work, except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other university.

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ABSTRACT

This thesis focused on the detection and identification of Tasmanian Atlantic salmon aquareovirus (TSRV), which is one of the few viral agents of Atlantic salmon endemic in Tasmania. Due to the low pathogenicity and ubiquitous nature of TSRV, there has been little interest in the significance of this virus. However, more recently, TSRV infections appeared to be associated with diseased fish and concerns about the negative impact of infection with this virus on aquaculture productivity have increased. Industry's concerns regarding the significance of TSRV have resurfaced and recent research has indicated that under certain conditions TSRV could cause disease. Thus better management and control is being considered by industry and regulators. Validation of diagnostic methods has been a major focus in this thesis. Intra-laboratory and inter-laboratory comparison of PCR and virus isolation on piscine cell lines were carried out to determine the most sensitive diagnostic method, using tissues of farmed Atlantic salmon from various aquaculture sites around Tasmania. A total of 144 fish from 9 sites (12-33 fish per site) were sampled from two regions (Tamar River & South-east Tasmania) during late spring to early summer of 2009 and the data were analysed using different statistical approaches. This study demonstrated the qPCR assay to be highly sensitive (95.2%) and highly specific (95.2%) for the detection of TSRV. The prevalence of TSRV ranged from 6-22% in both regions. Following this, the use of swabs in preference to organs as the sample collection method was evaluated for individual and pooled samples. The use of swabs was shown to be best for field surveillance and screening purposes when the only concern with the presence and absence of virus in the population.

The incidence of TSRV infections was investigated by undertaking a field investigation at farm sites located in South-east Tasmania. Throughout this field investigation, the incidence of TSRV infections was low (6.15%). The findings do not exclude the role of TSRV in influencing the host's susceptibility to other infections. Non-specific gross pathology and histopathological changes were observed in TSRV positive salmon and similar observations were present in TSRV negative salmon. On the basis of archival cases liver pathology has been identified as the predominant pathology caused by TSRV. As a basis for a preliminary characterisation study, fourteen isolates of TSRV originating from various locations in Tasmania, covering a 20-year period obtained from various host species, host tissues and isolated on different cell lines, were selected in an attempt to increase the probability of detecting virus variants. Typical and atypical variants of TSRV were identified based on genotypic and phenotypic characterisation of the different isolates. Electron microscopic examination demonstrated the existence of at least three variants based on viral particle size. This study revealed preliminary evidence of vertical transmission of TSRV from brood-stocks to eggs and horizontal transmission from farmed salmon to wild fish. Finally, this characterisation study demonstrated the existence of at least one variant TSRV isolate other than the more commonly isolated, typical TSRV in farmed Tasmanian Atlantic salmon. The use of different detection/diagnostic methods in this thesis, has improved the scope of the detection of TSRV.

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VIRUS ABBREVIATIONS

Virus Name	Abrev.
American grass carp reovirus	AGCRV
American oyster reovirus	13p2
Atlantic salmon reovirus	TSRV
Atlantic salmon reovirus	ASRV
Carp gill necrosis virus	CGNV
Channel catfish reovirus	CCRV
Channel catfish virus disease	CCVD
Chum salmon reovirus	CSRV
Cyprinid herpesvirus 2	CyHV-2
Epizootic epitheliotropic disease virus	EEDV
Epizootic Haematopoietic Necrosis	EHN
Gill associated virus	GAV
Golden shiner reovirus	GSRV
Grass carp hemorrhagic reovirus	GCHV
Grass carp reovirus	GCRV
Guppy aquareovirus	GPRV
Human papilloma virus	HPV
Ictalurid herpesvirus 2	IcHV-2
Infectious haemopoietic necrosis virus	IHNV
Infectious myonecrosis virus	IMNV
Infectious pancreatic necrosis virus	IPNV
Infectious salmon anaemia virus	ISAV
Koi herpesvirus	KHV
Largemouth bass virus	LMBV
Marine birnavirus	MBV
Nervous necrosis virus	NNV
<i>Oncorhynchus masou</i> virus	OMV
Pilchard herpesvirus	PHV
Piscine reovirus	PRV
Red sea bream iridovirus	RSIV
Salmon pancreas disease virus	SPDV
Sleeping disease virus	SDV
Spring viraemia of carp virus	SVCV
Striped bass reovirus	SBRV
Tasmanian aquabirnavirus	TAbV
Threadfin reovirus	TFRV
Turbot aquareovirus	TRV
Viral haemorrhagic septicaemia virus	VHSV
Virus nervous necrosis	VNN
White spot syndrome virus	WSSV

GENERAL ABBREVIATIONS

E	amplification efficiency
cDNA	complementary DNA
NTC	no-template-control
TCID ₅₀	tissue culture infective dose
ΔRn	reported dye fluorescence
μM	micromolar
AAHL	Australian Animal Health Laboratory
AFDL	AAHL Fish Diseases Laboratory
AGD	Amoebic gill disease
AHL	Animal Health Laboratory
AQRV	aquareovirus
AUC	area under curve
BF-2	bluegill fry
bp	base pair
BTV	blue tongue virus
CHSE-214	Chinook salmon embryo
CI	confidence interval
CPE	cytopathic effect
C _T	threshold cycle
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpi	days post inoculation
DPiPWE	Department of Primary Industries, Park, Water and Environment
ds	double-stranded
EMEM	Eagle's Minimum Essential Medium
EPC	<i>Epithelioma Papillosum Cyprini</i>
EtOH	ethanol
F	forward
FAM	6-carboxy-fluorescein
FBS	foetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
ICTV	International Committee on the Taxonomy of Viruses
IP	intraperitoneal
kDa	kilodalton

L-15	Leibovitz' cell culture medium
LB	Luria broth
LDS	lithium dodecyl sulphate
M	molar
mM	millimolar
MW	molecular weight
ND	non detected
NS	non-structural
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
R	reverse
RNA	ribonucleic acid
ROC	receiver operating characteristics
RT	reverse transcription
S10	segment 10
S2	segment 2
SAN	submission accession number
SDS	sodium dodecyl sulphate
TAGS	Test evaluation in the absence of gold standard
TAMRA	6-carboxy-tetramethyl-rhodamine
TRLO	Tasmanian rickettsia-like organism
TSHSP	Tasmanian Salmonid Health Surveillance Program
UNG	uracil-DNA glycosylase
VI	virus isolation
VIC	4,7,2'-trichloro-7'-phenyl-6- carboxyfluorescein

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CHAPTER 1

Introduction

1.1 Tasmanian Atlantic salmon industry

1.1.1 Brief History

Salmonid fish, particularly salmon and trout of the genera, *Salmo* and *Oncorhynchus* are valued very highly as table fish and while the origins of salmonid aquaculture can be traced back to the eighteenth century Europe, it was not until the mid-1900s that salmonid aquaculture of any significance had developed. The modern technique of farming salmon in floating sea-cages that is still used in all salmon-farming countries of the world was developed first in Norway in the 1960s. Salmonid aquaculture on a commercial level commenced in the 1970s and today is arguably the most successful aquaculture industry globally, valued in excess of US\$10 billion (FAO Fisheries Technical Paper 2007).

The Tasmanian Atlantic salmon industry was founded in the early 1980s using progeny derived from Atlantic salmon farmed at Gaden, New South Wales, Australia. The Gaden stock was originally imported from Phillip River, Nova Scotia, Canada in the 1960s. The majority of Tasmania's Atlantic salmon farms are located in the south east of the state in the Huon River, Port Esperance and D'Entrecasteaux Channel, and Tasman Peninsula areas. Other farms are located in Macquarie Harbour on the west coast, and there is one farm in the Tamar estuary on the north coast (Figure 1.1). The volume of Tasmanian farmed salmonids harvested in 2008-09 was 32,200 tonnes with a value of AU\$345 million, accounting for 30% of the state's gross value of primary production (Source: Food and Beverage Industry Score Card 2007-08, DPIPW, July 2010). In the 2009/10 period, the value of farmed salmonids

rose to AU\$410 million. In the early years of the Tasmanian salmon industry, the majority of product was sold to Japan for the sushimi market (Source: Tasmanian Salmonid Growers Association, 2007).

Nowadays, with the changing global markets, while Tasmanian products still exported to Japan, USA, Hong Kong, Singapore, Thailand, Indonesia, China, Taiwan, Vietnam, Guam, Malaysia, the Philippines and India, the majority of the salmon is sold domestically (Source: Tasmanian Salmonid Growers Association, 2007). However, the success achieved in any intensive farming industry often brings new and diverse disease problems and the Tasmanian Atlantic salmon industry is no exception. Disease management and assessment of farmed fish as well as wild stock have been a major concern to commercial aquaculturists and fisheries conservationists.

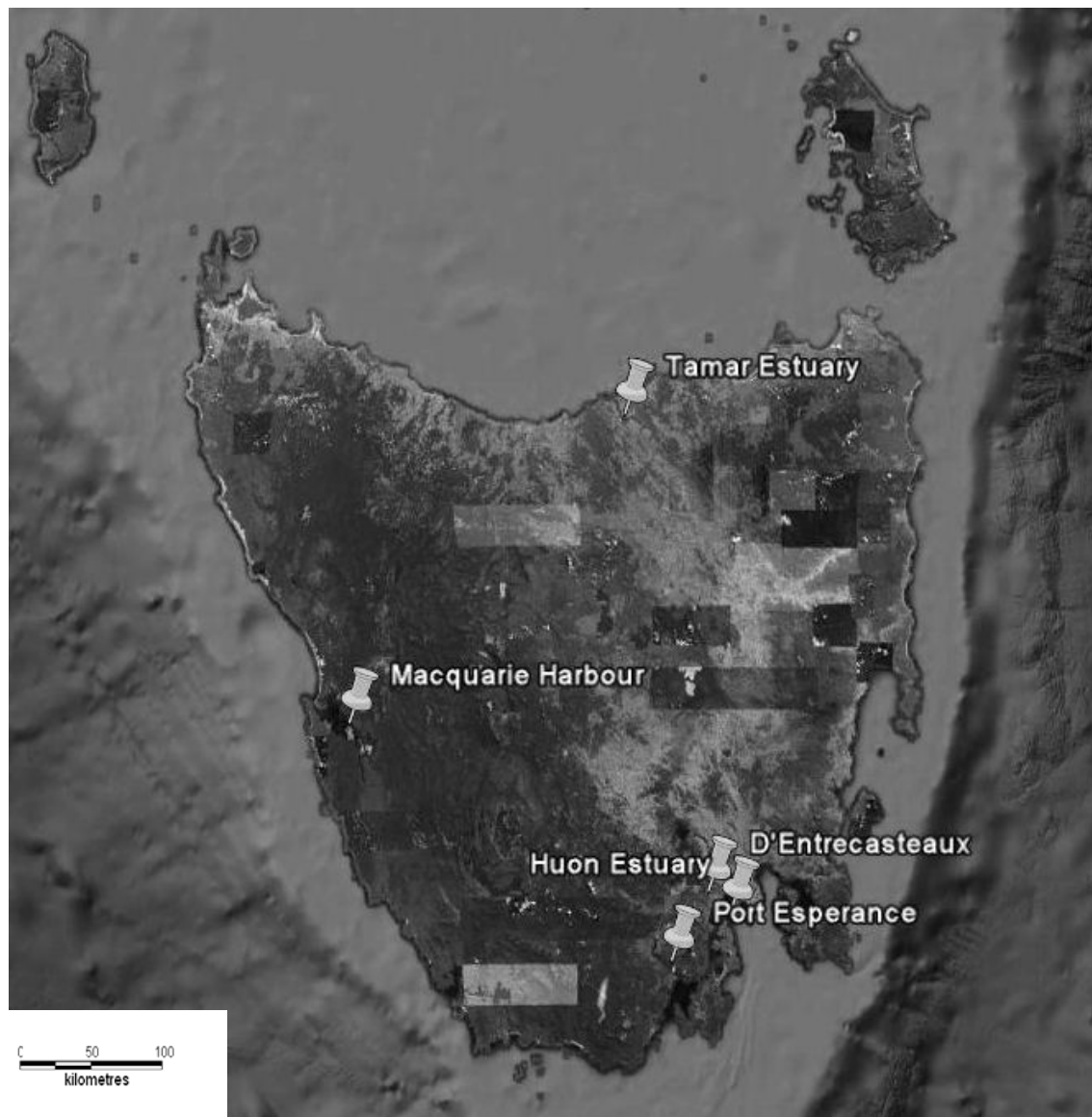


Figure 1.1 Commercial salmon production areas in Tasmania: Macquarie Harbour, Tamar Estuary and South-east region. The majority of production is conducted in the South-east of Tasmania.

The ability to demonstrate the presence or absence of specific pathogens in fish has vital economic benefits as it can help to establish good management practices, avoid significant economic losses (Barlic-Maganja *et al.*, 2002) and maintain market access. An additional benefit would be if the level of the infectious organism could be determined in the fish or the environment so that changes in pathogen abundance could be monitored (Overturf *et al.*, 2001). Most of the aquatic animal diseases listed by the World Organisation for Animal Health (OIE; Office International des Epizooties), including those of salmonids, are caused by viruses.

The OIE is an intergovernmental organization responsible for improving animal health worldwide and promoting trade in animal products. The missions of OIE include ensuring transparency in the global animal health situation, collecting, analyzing and disseminating veterinary scientific information, encouraging international solidarity in the control of animal diseases and safeguarding world trade by publishing health standards for international trade in animals and animal products. The health standards developed by OIE for protection from introduction of aquatic animal disease and pathogens are the *Aquatic Animal Health Code and Manual of Diagnostic Tests for Aquatic Animals*.

The OIE *Aquatic Code* is a reference document for use by veterinary authorities, import/export services, epidemiologists and all those involved in international trade of aquatic animals and their products. Part 1 of the OIE *Aquatic Code* contains provisions for aquatic animal disease diagnosis, surveillance and notification, on risk analysis and on the quality of competent authorities; it provides general recommendations on disease prevention and control; it sets out trade measures, import/export procedures and model health certificates and provides guidelines on the welfare of farmed fish during transport. Whereas, the

recommendations in the disease chapters of the *Aquatic Code* are intended to prevent the pathogen in question being introduced into the importing country, taking into account the nature of the traded commodity and the aquatic animal health status of the exporting country. Correctly applied, the recommendations provide for trade with an optimal level of animal health security, incorporating the latest scientific findings and available techniques.

Epizootic Haematopoietic Necrosis (EHN) is the only salmonid viral disease currently listed in the OIE *Aquatic Animal Health Code* that is present in some parts of Australia and is exotic to Tasmania. In fact, since its establishment in the mid-1980s, the salmonid industry in Tasmania has not experienced any significant outbreak of viral disease (Crane *et al.*, 2000). However, two potentially significant viruses, Tasmanian Atlantic salmon reovirus and Tasmanian aquabirnavirus, were isolated for the first time in the 1990s. Both these viruses have relatively low pathogenicity (Crane and Williams, 2007). The Tasmanian aquabirnavirus (TAbV) continues to be isolated, during the on-going Tasmanian Salmonid Health Surveillance program, on a regular but infrequent basis. The Tasmanian Atlantic salmon reovirus (TSRV) has also been isolated on a regular basis during the Tasmanian Salmonid Health Surveillance Program (Kevin Ellard personal communication) and, in recent years, isolations of TSRV appear to be occurring on a more frequent basis and possibly associated with co-infections with other pathogens.

1.1.2 Disease history of salmonid culture in Tasmania

Active surveillance of all farmed salmonid populations has been undertaken as part of the Tasmanian Salmonid Health Surveillance Program which has been in place since 1990. The program involves regular farm inspections and collection of samples for histopathology, bacteriology and virology. As a part of the surveillance program, several significant diseases of Tasmanian salmon have been described such as Amoebic gill disease (AGD), Tasmanian salmonid rickettsiosis (RLO), Yersiniosis, Vibriosis, Tasmanian aquabirnavirus infection and *Aeromonas septicaemia*.

Yersiniosis is a contagious bacterial disease of salmonids, eels, goldfish, sole, sturgeon and turbot caused by *Yersinia ruckeri*, a member of the family *Enterobacteriaceae*. *Yersinia ruckeri* is enzootic to Australia. In Australia, two biotypes of *Y. ruckeri* are known to occur: serotype O1b, biotype 1 and serotype O1, non-O1b, biotype 2. The virulent Hagerman strain, the cause of enteric red mouth in rainbow trout is exotic to Australia. *Y. ruckeri* is found in fish populations throughout Europe, North and South America, Australia and New Zealand. Infection with *Y. ruckeri* results in a bacterial septicaemia without specific clinical signs but is most commonly detected due to exophthalmos and blood spots in the eye. The severity of the disease is dependent upon the biotype of the bacterium involved. Acute infections in trout with the 'Hagerman' strain are usually florid but the milder form of the disease occurring in Atlantic salmon is termed Yersiniosis.

In Tasmania, infection with *Y. ruckeri* (Australian isolate serovar O1b) occurs predominantly in Atlantic salmon with very rare isolations from rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) (Carson and Wilson, 2009). In 2005, the occurrence of

Yersiniosis resulted in clinical expression of the disease post transfer to marine sites. The level of subclinical carriage in Atlantic salmon stock within hatcheries affected the level of disease expression post transfer to marine sites and occasionally was treated with chlortetracycline or trimethoprim (Jeremy Carson personal communication). While most Tasmanian Atlantic salmon have been vaccinated against Yersiniosis since 2006 (using Yersinivac-B, developed by DPIPWE, Launceston, Tasmania and manufactured by Intervet), significant outbreaks still occur. Yersiniosis killed half a million fish in just one hatchery during a 6-month period in 2007, resulting in a substantial shortfall for the entire Tasmanian salmon industry. A poor response to vaccination in juveniles, 1–5 g in weight, has led to the investigation of the suitability of the current formalin-killed, whole-cell vaccine Yersinivac-B and current research has highlighted the efficacy of double-dip vaccination for juveniles (Bridle and Nowak, 2010, unpublished data) and the potential use of trypsin to increase the efficacy of Yersinivac-B (Costa *et al.*, 2011).

Vibriosis due to *Vibrio anguillarum* (*Vibrionaceae*) infection is a major cause of mortality in farmed marine fish worldwide (Munday *et al.*, 1992). Diseases involving *Vibrionaceae* are usually caused by opportunistic infections. The pathogen may have an intimate association with the host as part of the normal flora but may exhibit pathogenicity if host defences are breached. This may occur either from stress events that lead to immune suppression, physical damage to the integument, or the emergence of aggressive biovars within a population of aquatic animals. Severity of disease may range from fulminating septicaemias, typical of disease outbreaks, through to chronic infections that affect just a few individuals (Carson and Wilson, 2009).

The Australian situation in relation to vibriosis vaccination of salmonids is probably unique as the genetic variation in these populations is restricted due to limited introductions, and, to date, only one serotype of *Vibrio anguillarum* (serovar O1) has been isolated from outbreaks of clinical vibriosis (Carson, 1990). The first Tasmanian isolation of *Vibrio anguillarum*, in 1985, involved disease outbreaks throughout salmonid marine farms which resulted in 30% mortality (Carson, 1990). Due to the severity of the outbreaks, a vaccine (Anguillvac-C) was developed against vibriosis by DPIWE (Launceston, Tasmania) which is now manufactured by Intervet. Vibriosis-affected fish have been traditionally treated with oxytetracycline prior to the development of vaccines for bacterial infections. In the following years, DPIWE has been working with salmon producers to assess modifications to the vaccination strategy (Munday *et al.*, 1992; Whittington *et al.*, 1994; Morrison *et al.*, 2000) in an attempt to address the occasional disease outbreaks despite vaccination. The impact of vibriosis on salmonid farms has been significantly reduced with the adoption of a modified vaccination strategy (Jeremy Carson pers communication).

Aeromonas salmonicida is a significant pathogen of many species of freshwater and marine fish and has been reported to occur in most countries worldwide. *Aeromonas salmonicida salmonicida* is the causative agent of the disease furunculosis in salmonid fish, a septicaemic condition that varies in severity. In Australia, an atypical form of *Aeromonas salmonicida* is the cause of goldfish ulcer disease (GUD), an ulcerative dermatitis prevalent in fish in the aquarium trade (Humphrey and Ashburner, 1993). Isolates of atypical *Aeromonas salmonicida* from GUD lesions were shown to be highly pathogenic for Tasmanian Atlantic salmon in an experimental infectivity trial (Carson and Handler, 1988).

During 1993, a n a typical *Aeromonas salmonicida* was r ecovered from j uvenile, hatchery-reared greenback flounder, *Rhombosolea tapirina*, and w ild c aught f ish he ld i n shore-based t anks i n T asmania (Carson, unpubl ished da ta). In a ddition, a f urther a typical *Aeromonas salmonicida* (biovar Acheron) was isolated in 2000 from an Atlantic salmon farm at M acquarie H arbour, Tasmania (Jeremy C arson p ers co mmunication). S ince 2000, t he incidence of clinical disease outbreaks caused by this pathogen and the negative impact it has on salmon production has increased. Oxytetracycline was used to treat the disease while the work on vaccine development was carried out by DPIPWE (Launceston, Tasmania). In 2006, vaccination of stock was introduced to marine sites with the development of AnguiMonas, a bivalent v accine d eveloped ag ainst *Aeromonas salmonicida* and *Vibrio anguillarum*. T he incidence of *Aeromonas salmonocida* was reduced significantly following vaccination and it has not b e en reported i n T asmanian s almon i ndustry s ince 2008 (Jeremy C arson p e rs communication).

Several o ther p athogens h ave b een i solated an d ch aracterized i n t he course o f t he Tasmanian surveillance program such as *Enterococcus*-like bacterium, causing 60% mortality in rainbow trout *Oncorhynchus mykiss* (Carson *et al.*, 1993), *Vagococcus salmoninarum* from salmonids (Schmidtke a nd C arson, 1994), *Flavobacterium* sp.(*Flexibacter psychrophilus*) from A tlantic s almon w hich c aused 0.01 % mortality/week a nd 80% m orbidity (Schmidtke and C arson, 1995) and *Lactococcus garvieae* from r ainbow t rout (Schmidtke a nd C arson, 2003).

In 2001, rickettsia-like organism (RLO) associated with Tasmanian salmonid rickettsiosis was identified in Atlantic salmon farmed in Tasmania (Corbeil *et al.*, 2003). Tasmanian RLO (TRLO) differs at the genetic and antigenic level from exotic *Piscirickettsia salmonis* isolates (Corbeil *et al.*, 2005). The disease has occurred periodically since 2001 and occurs in close association with TSRV infections in Atlantic salmon (Morrison and Carson, 2011). In 2006, significant and widespread outbreaks occurred during summer (in part due to elevated water temperature) in south-eastern Tasmania which resulted in mortalities (26.8%). Since the first outbreak of TRLO, this disease has been treated with the antibiotic, oxytetracycline (OTC). DPIPW (Launceston) has developed a vaccine, Corrovac for TRLO which is currently in the field trial stage (Jeremy Carson pers communication).

Amoebic gill disease (AGD) is probably the most economically important disease affecting cultured Atlantic salmon in the south east of Tasmania (Munday *et al.*, 1990; Nowak, 2001; Parsons *et al.*, 2001). Mortality rates of up to 50% in Atlantic salmon can occur if the disease is left untreated (Munday *et al.*, 1990). The predominant aetiological agent has been identified as *Neoparamoeba perurans* (see Young *et al.*, 2007) which can be isolated from the gills of infected Atlantic salmon. The prevalence of AGD is influenced by environmental conditions, with salinity considered an important factor in the occurrence of the disease (Nowak, 2001). During salmon grow-out season in Tasmania, AGD was first seen during the first summer period after transfer of smolt from freshwater to seawater sites (Langdon, 1990; Adams and Nowak, 2003).

Since then, research has been conducted to understand the pathogenesis of this disease, and the influence of fish husbandry practices in precipitating outbreaks, and to assist in control through early recognition and farm management (Munday *et al.*, 1990; Clark and Nowak, 1999; Parsons *et al.*, 2001; Powell *et al.*, 2000; Zilberg and Munday, 2000; Douglas-Helders *et al.*, 2001; Nowak, 2001; Zilberg 2001; Nowak *et al.*, 2002; Powell *et al.*, 2002; Clark *et al.*, 2003; Adams *et al.*, 2004; Morrison *et al.*, 2004; Douglas-Helders *et al.*, 2005a; Douglas-Helders *et al.*, 2005b; Gross *et al.*, 2005; Leef *et al.*, 2005a; Leef *et al.*, 2005b; Bridle *et al.*, 2006; Young *et al.*, 2007; Young *et al.*, 2008a; Young *et al.*, 2008b).

Currently, A GD is treated by bathing Atlantic salmon in freshwater, a labour-intensive, and expensive, treatment which often needs repeating several times per grow-out. Bathing significantly reduces the number of amoebae on the gills (Parsons *et al.*, 2001; Clark *et al.*, 2003). However, the inability to remove all amoebae during treatment and their presence in the environment, as well as on Atlantic salmon, eventually results in proliferation of the remaining amoebae on the gills leading to a requirement for further treatment. Freshwater bathing is not an ideal treatment. Apart from being costly, freshwater is in low supply and bathing is not without its risks, causing stress to the fish. For these reasons, vaccine development is a major research activity.

Tasmanian aquabirnavirus (TABV) was isolated in 1998 from an 18-month old 'pinhead' Atlantic salmon from a sea-cage in Macquarie Harbour, Tasmania (Crane *et al.*, 2000). It has never been associated with mortalities in freshwater hatcheries. Since then, there have been several further isolations from fish in Tasmania, but always restricted to Macquarie Harbour, and never from freshwater hatcheries (Crane *et al.*, unpublished data).

It has been isolated from healthy Atlantic salmon *Salmo salar*, rainbow trout *Oncorhynchus mykiss*, greenback flounder *Rhombosolea tapirina* and red cod (*Pseudophycis* sp) (Munday and Owens, 1998). *Aquabirnavirus* is one of the genera within the family Birnaviridae which includes icosahedral, double-stranded (ds), bisegmented RNA viruses with a non-enveloped capsid. The genus includes both virulent and avirulent viruses with the term ‘infectious pancreatic necrosis’ (IPN) virus being reserved for those isolates that are pathogenic for species within the Family Salmonidae.

No abnormal clinical signs have been reported in fish infected with TABV. Pancreatic lesions, consistent with IPN virus infection, have been noted in some fish naturally infected with TABV. However, virus was also isolated from some fish that had no histological lesions (McColl *et al.*, 2009). In recent years, it has been shown through laboratory investigation of submitted tissue samples that low mortalities have been associated with TABV infections in Atlantic salmon (Macquarie Harbour). Research on vaccine development has been initiated at DPIPWE (Launceston) but is still at its early stages (Jeremy Carson pers communication).

Thus for the significant bacterial diseases afflicting the Tasmanian salmon industry, stress factors precipitating the diseases have been identified, and until recently, treatment has involved the use of antibiotics. Currently, management practices and control strategies include use of vaccines, development of improved vaccines, better management of stress, and improved farm husbandry practices. It is well established that environmental factors and farm management practices can influence the severity of infections. TSHSP has been involved in early disease investigation and agent identification and these efforts have led to development of effective control measures with a positive impact of industry productivity and profitability.

To date, the emerging viruses (Tasmanian Aquabirnavirus and Tasmanian Aquareovirus) are thought to be of low pathogenicity and have not received much attention, similar to the situation with AGD when, in the early days, freshwater bathing appeared to be the solution and research on this pathogen was considered a low priority. However currently, AGD is the most significant health issue for the industry with a major impact on industry profitability. Thus, research on infectious agents should not wait for significant losses to occur but rather characterisation of newly emerging agents should be considered a priority particularly at the time of local climate changes. It is well established that adverse environmental conditions, which may be precipitated by climate change, can modulate pathogen populations and lead to the emergence of new viral variants (especially for RNA viruses such as Tasmanian Aquareovirus) which could be more problematical.

1.2 Tasmanian Atlantic Salmon Reovirus (TSRV)

1.2.1 The Reoviridae

The family Reoviridae includes 12 genera of RNA viruses (*Aquareovirus*, *Coltivirus*, *Cypovirus*, *Idnoreovirus*, *Fijivirus*, *Mycoreovirus*, *Orbivirus*, *Orthoreovirus*, *Oryzavirus*, *Phytoreovirus*, *Rotavirus*, *Seadornavirus*). These reoviruses (respiratory enteric orphan virus) are also known as orphan viruses – viruses that are not normally known to be associated with disease in any host organism. Although, initially, reoviruses commonly may have been associated with subclinical infections, it is now known that many are associated with disease (Crane and Carlile, 2008). Reoviruses have been isolated from a wide range of hosts including plants, vertebrates, insects, and other invertebrates (Urbano and Urbano, 1994).

According to Stone *et al* (1997), reoviruses of varying pathogenicity for salmonids exist within localised geographic ranges. A number of members of this family which are highly pathogenic include, Rotavirus A (Genus *Rotavirus*) (Dennehy, 2000), Mammalian orthoreovirus (Genus *Orthoreovirus*) (Lui *et al.*, 2000), Bluetongue virus (Genus *Orbivirus*) (Roy, 1996), Grass carp reovirus (Genus *Aquareovirus*) (Chen and Jiang, 1984) and Piscine Reovirus (Palacios *et al.*, 2010). Viruses belonging to the Reoviridae family are non-enveloped with a multi-segmented, double-stranded RNA genome, demonstrate an icosahedral symmetry and have a double capsid layer with 20 peripheral capsomeres (Mertens *et al.*, 2005).

The capsid layer is composed of an outer and inner shell termed the core (Francki *et al*, 1991). The genome of a typical Reovirus is made up of 10-12 segments of double-stranded RNA which are grouped into three classes (Nibert, 1998), based on segment size obtained by polyacrylamide gel electrophoresis and which are: large (segments 1-3), medium (segments 4-6), and small (segments 7-12) (Nibert and Schiff, 2001). Each segment encodes 1-3 structural or non-structural proteins. Virions are 60-80 nm in diameter and spherical in appearance (Francki *et al*, 1991). The genera of Reoviridae are divided into two subsets based on the presence or absence of a turret protein on the inner capsid; TSRV belongs to the turreted genus *Aquareovirus* (Figure 1.2).

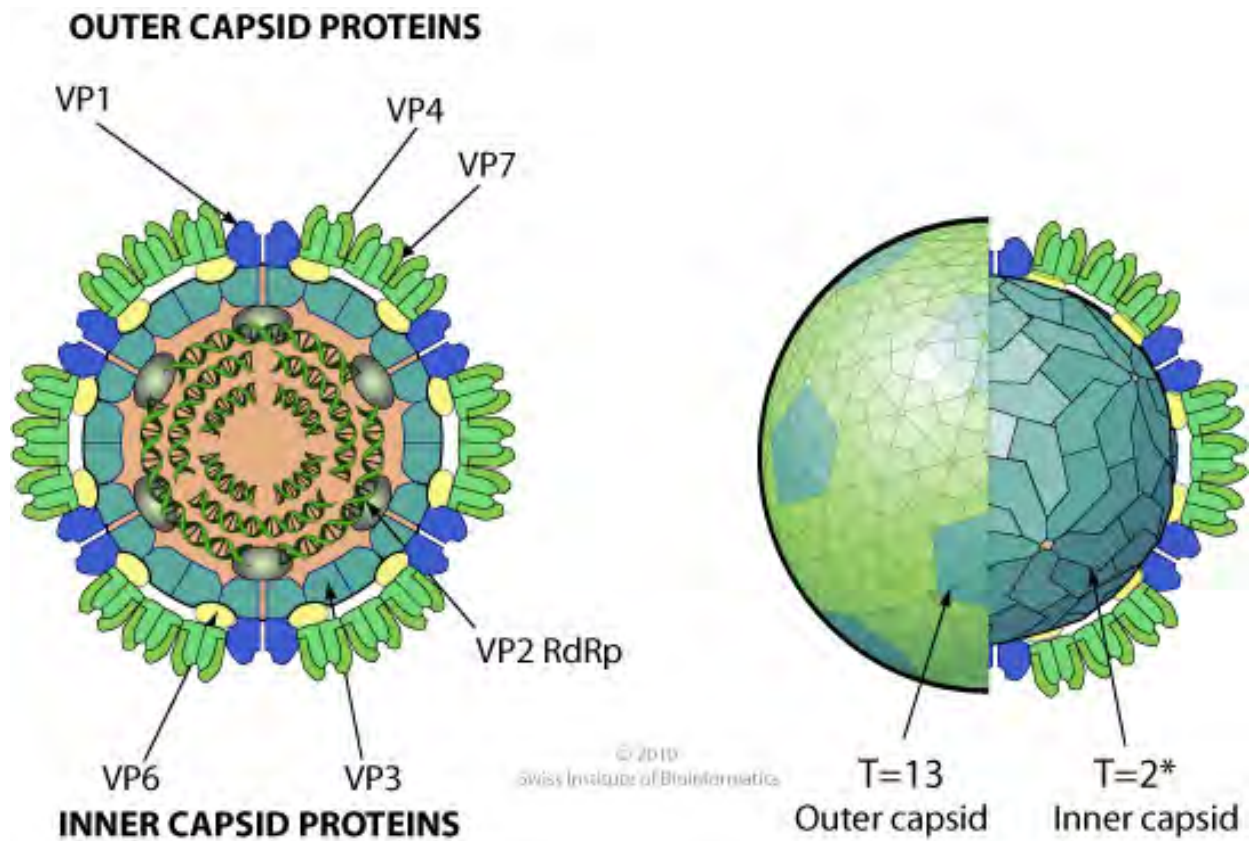


Figure 1.2 Non-enveloped, icosahedral Aquareovirus virion with a double capsid structure, outer capsid and inner capsid (Viral Zone Databases, Swiss Institute of Bioinformatics, 2010).

1.2.2 *Aquareovirus*

Aquareoviruses were first isolated in the 1970s from North American cyprinids (Jaafar *et al.*, 2008; Plumb *et al.*, 1979; Winton *et al.*, 1987). Since then, aquareoviruses have been isolated from a range of aquatic animals including finfish, crustaceans and molluscs from both freshwater and marine environments. The structural and morphological characteristics of the aquareovirus virion resemble those of other members of the Reoviridae family: the virions have a spherical, icosahedral symmetry and a double capsid layer, 80 nm in diameter. The genome of this genus consists of 11 double-stranded RNA segments that encode seven structural proteins and five non-structural proteins. There are six genogroups (A-F) within the *Aquareovirus* genus, based on analyses of RNA-RNA blot hybridization, RNA electrophoresis, antigenic properties, and nucleic acid sequence (Mertens *et al.*, 2005; Mohd Jaafar *et al.*, 2008; Lupiani *et al.*, 1995), and according to Rangel (1999), Genogroup A is the most heterogeneous group.

Aquareoviruses replicate in cell lines of piscine and mammalian origin (Samal *et al.*, 1998) and produce cytopathic effects that are characterised by the formation of large syncytia (Samal *et al.*, 1990; Winton *et al.*, 1989). Variable cytopathic effects can be observed based on the aquareovirus isolate and the cell line used for isolation (Crane and Carlile, 2008) (Figure 1.3 and Figure 1.4). Most aquareoviruses replicate at temperatures between 15-20°C; others that originate from, warm-water hosts have optimal replication at 25-30°C (Lupiani *et al.*, 1995).

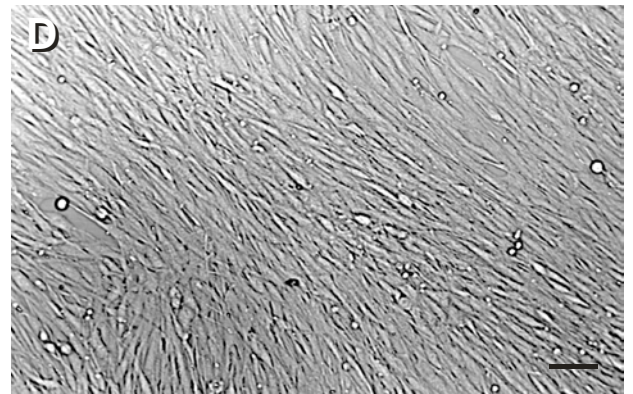
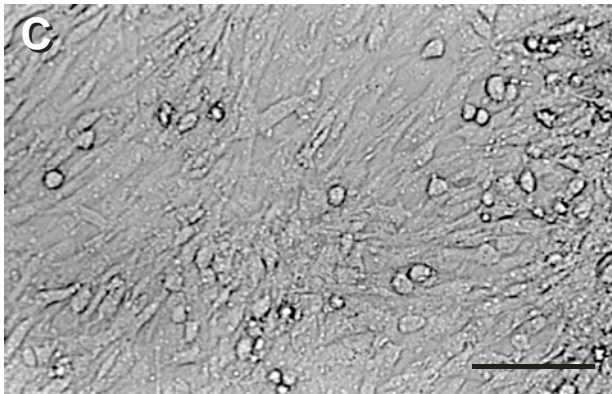
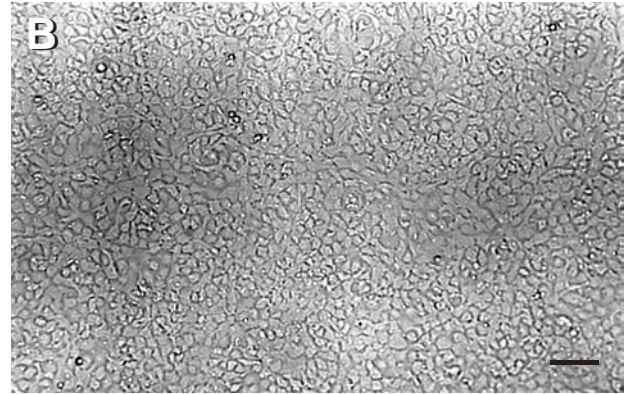
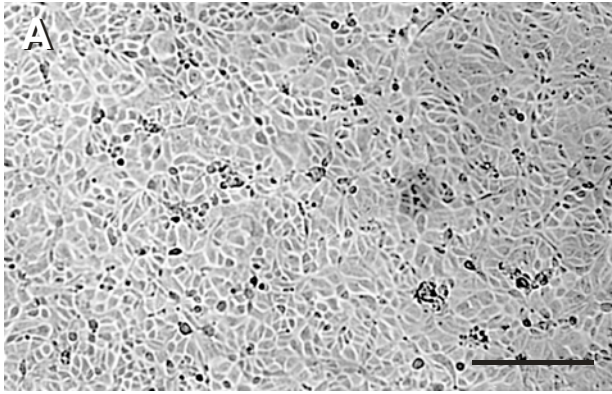


Figure 1.3 Normal uninfected CHSE-214 (A), EPC (B), BF-2 (C), and RTG-2 (D) cell cultures after 3-7 days incubation at 15°C. (Scale bars = 100µm). Courtesy of Nette Williams, Australian Animal Health, CSIRO Livestock Industries, Geelong, VIC, Australia.

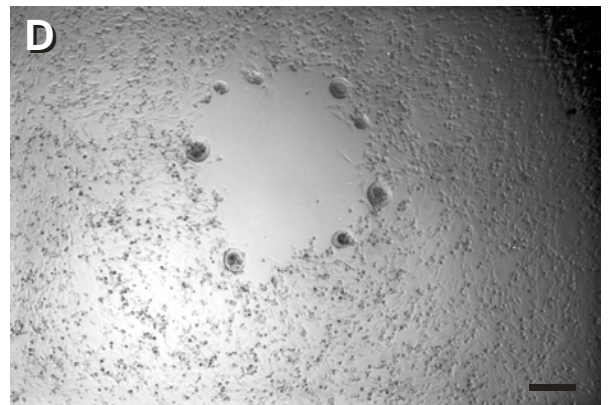
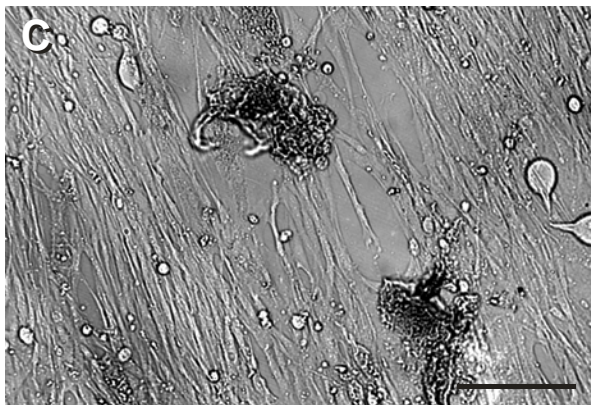
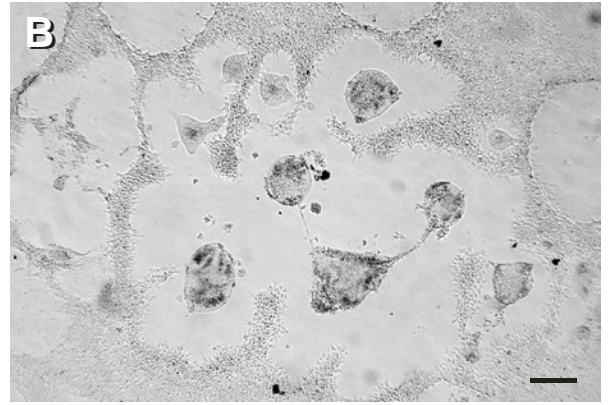
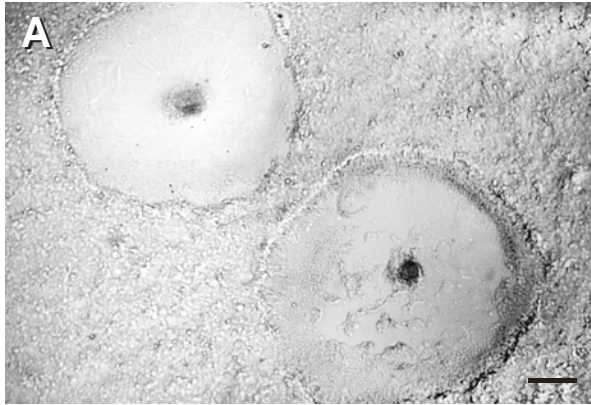


Figure 1.4 Aquareovirus-infected cell cultures and incubated at 15°C: (A) Atlantic salmon reovirus-infected CHSE-214 cell cultures at 4 dpi, (B) Atlantic salmon reovirus-infected EPC cultures at 5 dpi, (C) Atlantic salmon reovirus-infected RTG-2 cell cultures at 6 dpi and (D) Learmonth reovirus-infected BF-2 cell cultures at 10 dpi. (Scale bars = 100µm). Courtesy of Nette Williams, Australian Animal Health, CSIRO Livestock Industries, Geelong, VIC, Australia.

Aquareoviruses have been isolated from both healthy and diseased cold-water and warm-water fish species (Rangel *et al.*, 1999). However, several salmonid aquareoviruses, including the Tasmanian Atlantic Salmon Reovirus (TSRV), have been isolated from apparently healthy fish (Essbauer and Ahne, 2001). Although, initially, these viruses were thought to be associated with subclinical infections only, it is now known that some can cause significant clinical signs and even severe disease (Fang *et al.*, 1989). The presence of aquareoviruses is likely to be under-reported due to the fact that most isolations of aquareoviruses occurred incidentally during surveillance activities for other, more significant pathogens. It is during such surveillance activities that numerous aquareoviruses have been isolated from apparently healthy aquatic animals (Crane and Carlile, 2008).

The pathological characteristics shown by fish infected with aquareoviruses are non-specific and include: haemorrhages of internal organs, and pale liver and kidney (Plumb *et al.*, 1979; Meyers, 1980; Subramanian *et al.*, 1997; Cusack *et al.*, 2001; Attoui *et al.*, 2002; Seng *et al.*, 2002). Likewise, external signs exhibited by fish in disease outbreaks are also non-specific and include abdominal distension, lethargy, skin discolouration, haemorrhages of the skin, loss of equilibrium, inappetence, anorexia and abnormal swimming behaviour (Seng *et al.*, 2002; Isshiki *et al.*, 2003). Wolf (1988) categorized aquareovirus infections into varying degrees of pathogenicity which are dependent on not only the virulence of the specific reovirus but also on various environmental and host factors. Under non-stressful conditions, the fish's immune response is able to control infection with an aquareovirus such that disease is averted.

However, increase in stress due to, for example, poor water quality, high stocking density and/or increased ambient temperature, which can severely compromise the fish's ability to mount an effective immune response, can precipitate disease in reovirus-infected fish (Plumb *et al.*, 1979; Rivas *et al.*, 1996; McEntire *et al.*, 2003; Kongtrop *et al.*, 2004), as in fish infected with other viruses (Amend, 1970; Hetrick *et al.*, 1979; Arkoosh *et al.*, 1998; Inendino *et al.*, 2005; Arkush *et al.*, 2006). Viral infections in ectothermic vertebrates can be greatly influenced by temperature (Ahne *et al.*, 2002). Water temperature is known to influence the onset and severity of fish virus infections directly by altering virus replication and indirectly by augmenting the efficacy of the host immune response (Alcorn *et al.*, 2002; Bly and Clem, 1992). The influence of water temperature on outbreaks caused by fish viruses in other species is well established and include IHNV in rainbow trout *Oncorhynchus mykiss* (Hetrick *et al.*, 1979), RSIVD in rock bream *Oplegnathus fasciatus* (Jun *et al.*, 2009), SVC in common carp *Cyprinus carpio* and VHSV in Pacific sardine *Sardinops sagax* (Arkush *et al.*, 2006). Furthermore, poor water quality and over-crowding were demonstrated to influence the survival and condition of juvenile largemouth bass *Micropterus salmoides* infected with largemouth bass virus (LMBV) (Inendino *et al.*, 2005).

Other known aquareoviruses have been found to be highly pathogenic, including those isolated from grass carp (Jiang and Ahne, 1989), turbot (Lupiani *et al.*, 1989), threadfin (Seng *et al.*, 2002), golden shiner (Plumb *et al.*, 1979), chum salmon (Winton *et al.*, 1981), channel catfish (Amend *et al.*, 1984) and bluegills (Meyers, 1980). Chum salmon Reovirus (CSRV) was isolated from farmed salmonids (in Japan) involved in a disease outbreak associated with low mortality rates (Winton *et al.*, 1981). Similarly, chronic low mortality rates (5%) of farmed channel catfish in California were found to be due to reovirus (CCRV) infection (Amend *et al.*, 1984). Bluegill hepatic necrosis virus (13p₂ reovirus) induced 44% mortality in

experimentally infected bluegill, *Lepomis macrochirus* (Meyers, 1980). Similarly, Meyers (1983) reported subclinical disease in adults (56%) and juveniles (65%) of rainbow trout experimentally infected with 13p₂V reovirus (American oyster reovirus).

Turbot aquareovirus (TRV) was isolated in northwest Spain from turbot *Scophthalmus maximus* suffering a mixed bacterial and viral infection (Lupiani *et al.*, 1989), causing low but economically serious mortalities in a few turbot farms. Since then, the virus has been repeatedly isolated from the same and other fish farms. These later isolations were consistently made in fish after stress and/or concomitant with bacterial infections (Rivas *et al.*, 1996). Golden Shiner Reovirus (GSRV) was isolated from moribund bait fish during cool seasons, associated with losses of bait fish in USA (Plumb *et al.*, 1979). Mortality rates associated with GSRV infections are normally around 5% but, acute epizootics with mortality rates of 50-75% have been reported for outbreaks involving over-crowded conditions and elevated ambient temperatures (Plumb *et al.*, 1979).

GSRV is nearly identical to a Chinese isolate of GCRV (96-99% amino acid identity) (Attoui *et al.*, 2002a); a significant pathogen of farmed grass carp, *Ctenopharyngodon idellus* and fathead minnows, *Pimephales promelas* and has also been isolated from wild 'creek chub', *Semotilus atromaculatus* in the USA (Goodwin *et al.*, 2006). Reovirus had caused haemorrhagic disease in grass carp, *Ctenopharyngodon idellus* which resulted in 80% of farmed mortality in fingerlings and yearlings during periods of high water temperature (Jiang and Ahne, 1989). Grass carp reovirus (GCRV) produced mortality in the black carp *Mylopharyngodon piceus*, chub *Pseudorasbora parva* (Li *et al.*, 1997), and the rare minnow *Gobiocypris rarus* (Wang *et al.*, 1994). It can replicate subclinically in the silver carp *Hypophthalmichthys molitrix* and the Chinese minnow *Hemiculter bleekeri* in China (Ding

et al., 1991; June *et al.*, 1997). In North America, GCRV has been isolated from both moribund and healthy grass carp and golden shiner *Notemigonus crysoleucas* (Hedrick *et al.*, 1989; McEntire *et al.*, 2003).

The outbreaks of disease in both species cultured in the United States have been associated with the same virus (GCRV), and GSRV is considered a variant of GCRV having 96-100% similarity of genome sequences and 90-93% of amino acid identity (McEntire *et al.*, 2003). In addition, it has been concluded from these studies that GSRV and GCRV are more likely to produce disease in additional North American hosts when environmental conditions are poor (Goodwin *et al.*, 2006). Threadfin reovirus (TFRV) caused a mass mortality (100%) of cultured threadfin fingerlings in an aquaculture farm in Singapore and 62.5% of mortality in experimental infections (Seng *et al.*, 2002). Experimental infection of sea bass (*Lateolabrax niloticus*) with TFRV resulted in severe mortality (~ 65%). Guppy reovirus (GPV) was isolated from a moribund guppy in the year 2001 from a fish farm in Singapore. It caused severe mortalities in infected guppies and is a major threat to the local ornamental fish farming industry (Choo *et al.*, 2001). Vaccine development is being undertaken due to the devastating losses caused by TFRV and GPV in the fish farming industry of Singapore (Sim *et al.*, 2004; Seng *et al.*, 2005a; 2005b).

Piscine Reovirus (PRV) has been associated with heart and skeletal muscle inflammation (HSMI), a frequently fatal disease of farmed Atlantic salmon which was first recognized in one farm in Norway (Kongtropol *et al.*, 2004). Disease outbreaks were most common during spring and early summer (related to high water temperature). In Norway, this disease resulted in high morbidity and varied mortalities up to 20% (Kongtropol *et al.*, 2004). Husbandry-related stress increased the mortality rate and prolonged the healing process

during outbreaks (Kongtrop *et al.*, 2004). It has now been reported to cause 5 % of farm mortalities and outbreaks in 417 farms in Norway and United Kingdom (Gustavo *et al.*, 2010). In general, losses attributed to aquareoviruses are commonly associated with sub-optimal environmental conditions that suppress the host's immune response, or due to co-infections with other pathogens. This scenario has been likened to the viral equivalent of a “ticking time bomb” (Seng *et al.*, 2004).

1.2.3 TSRV

Tasmanian Atlantic salmon reovirus (TSRV) was the first reported identification of an aquareovirus in Australia and the original detection was based on unexplained cytopathic effect (CPE) in CHSE-214 cells. The demonstration of 11 segments of dsRNA by gel electrophoresis and ultrastructural characteristics by electron microscopic examination suggested that the CPE was caused by an aquareovirus (Gemma Carlile pers communication). Tasmanian Atlantic salmon reovirus (TSRV) is endemic within Tasmania and has been isolated from Atlantic salmon (*Salmo salar*) on a regular basis since 1989/90 when it was first detected (Crane *et al.*, 2000). TSRV belongs to the species group AQRV-A as it shares the closest homology to Chum salmon Reovirus (CSRV) (Gemma Carlile pers communication).

TSRV has been recorded in three locations in Tasmania: Tamar River, Macquarie Harbour and South-east Tasmania (Gemma Carlile pers communication). Based on a retrospective epidemiological investigation of TSRV emergence using data collected from the Tasmanian Salmonid Health Surveillance Program (TSHSP) between 1990 and 2005, highest prevalence of TSRV was observed in farms from South-east Tasmania (Gemma Carlile pers communication). TSRV is considered to be of low pathogenicity since natural infections do not seem to be associated with clinical disease or mortality.

Following its first isolation in the early 1990s, a preliminary infectivity trial indicated that TSRV was non-pathogenic to a approximately 1-year-old Atlantic salmon under experimental conditions (Humphrey *et al.*, unpublished). This first experimental study showed viral replication and persistence of TSRV in Atlantic salmon in the absence of clinical disease or severe pathological changes (Humphrey *et al.*, unpublished). Interestingly, a subsequent

experimental infection of TSRV in Atlantic salmon fry (less than 6 months old) resulted in relatively high mortality rates (Crane *et al.*, unpublished), thus, demonstrating that TSRV could be highly pathogenic under certain conditions.

Gross and behavioural signs such as distended abdomen, lethargy, loss of equilibrium and, at times, inappetence were observed during the trial (Gemma Carlile pers communication). Nevertheless, characteristic histopathology of TSRV infection was mild-to-severe, multifocal, acute hepatic necrosis, mild-to-severe, multifocal necrosis of the renal haematopoietic tissue, and moderate-to-severe, multifocal acute pancreatic necrosis with foci of liquefactive fat necrosis also observed in the most severe examples (Gemma Carlile pers communication). Factors that may influence the incidence of TSRV infections include fish species, fish age, salinity, geographical location and temperature (Gemma Carlile pers communication). Based on a retrospective epidemiological study using data collected between 1980 and 2005, the prevalence of TSRV in Tasmanian population was shown to be influenced by seasonal, spatial and temporal factors (Gemma Carlile pers communication). The highest prevalence of TSRV was found in Atlantic salmon from South-east Tasmania during summer, in two-year-old fish, and an increase in prevalence as sampling location moved from freshwater locations downstream towards the sea. Experimental infections of Atlantic salmon with TSRV have suggested that pathogenicity is influenced by water temperature, the higher the water temperature, the higher the mortality rate (Gemma Carlile pers communication).

Reports of dual infections involving Reovirus in disease outbreaks with mixed aetiology have been reported for striped bass (*Morone saxatilis*) (Baya *et al.*, 1989), turbot (*Scophthalmus maximus*) (Lupiani *et al.*, 1989), striped snakehead (*Channa striata*) (John *et al.*, 2001), halibut (*Hippoglossus hippoglossus*) (Cusack *et al.*, 2001) and cyprinid (Hoole *et*

al., 2001). Interestingly, *Neoparamoeba perurans*, aetiological agent of Amoebic Gill Disease (AGD) (Munday *et al.*, 1990; Nowak, 2001) and Tasmanian Rickettsia-like organisms (TRLO) which causes Tasmanian salmonid rickettsiosis (Corbeil *et al.*, 2003) have been frequently isolated from cultured Atlantic salmon in the same geographical range, South-east of Tasmania (Gemma Carlile pers communication).

Due to the fact that AGD and TRLO infections cause significant losses to the Tasmanian Atlantic salmon industry; the Tasmanian Salmonid Growers Association (TSGA) has invested a significant level of funding into vaccine development for both diseases. The occurrence of TSRV was found to be more prevalent in the same geographical regions as *N. perurans* and TRLO (Gemma Carlile pers communication). TSRV has been associated with TRLO infections in Tasmanian farmed Atlantic salmon. Atlantic salmon samples which were investigated for infection with Rickettsia-like organism were found to contain TSRV and it was suggested that TSRV infection may predispose salmon to infection with TRLO (Ellard pers. communication). Dual infection studies between AGD and TSRV failed to demonstrate that exposure to TSRV was a predisposing factor for AGD (Gemma Carlile pers communication). Tasmanian aquareovirus appears to have a significant effect on the health of Atlantic salmon, and the possibility of clinical disease being induced in juvenile fish, in fish subjected to stress factors such as high stocking density or elevated water temperatures, or in association with bacterial infection should be considered (Humphrey *et al.*, unpublished).

1.2.4 Detection method for TSRV

The isolation of TSRV in the late 1980s /early 1990s was one of the several considerations that prompted the Tasmanian salmon industry into cooperation with State

authorities to establish a formal and systematic Tasmanian salmonid health surveillance program which has been in place for the best part of 20 years. Fish (mostly farmed Atlantic salmon and rainbow trout) have been tested at the Australian Animal Health Laboratory (AAHL), Geelong (by virus isolation in cultures of fish cell lines as part of a salmonid health surveillance program coordinated by DPIPWE, Tasmania) for the presence of pathogenic viruses. Over this time period, TSRV has been detected in approximately 20% of the Atlantic salmon submissions and in < 1% of the rainbow trout submissions (Gemma Carlile pers communication).

Currently, virus isolation in cultures of piscine cell lines is considered the 'gold standard' for detection of TSRV infections (Gemma Carlile pers communication), similar to methods for other finfish viruses (OIE, 2011). Generally, the diagnosis of TSRV infections relies on virus isolation in cell culture followed by identification of the virus using either immunocytochemistry or PCR (Gemma Carlile pers communication). Immunocytochemistry serves as a confirmatory test for the presence of TSRV in cell cultures exhibiting typical cytopathic effect (CPE) (Figure 1.2). CHSE-214 (chinook salmon, *Oncorhynchus tshawytscha* embryo, ATCC catalogue No. CRL 1681) and EPC cell lines (*epithelioma papulosum cyprinid*, derived from fathead minnow *Pimephales promelas*) are the fish cell lines used in virus isolation of TSRV and are known to support its replication (Mark Crane and John Young, pers communication).

CHSE-214 is the most sensitive cell line for the isolation of IPN-like virus (Crane *et al.*, 2000) and it has been proven to be very effective in the detection and propagation of a range of salmonid fish viruses and is widely used in research of viral diseases of fish (Lannan *et al.*, 1984). Likewise, the temperature growth range, high replication rate and high virus

susceptibility make EPC a highly suitable cell line for propagation of some pathogenic viruses of Salmonidae (Fijan *et al.*, 1983). Used together, these cell lines have a wide range of fish virus susceptibility which includes T SRV, IHNV, IPNV, VHSV, ISAV, OMV and C SRV (Lupiani *et al.*, 1995; Crane and Williams, 2007).

Currently, virus isolation on fish cell lines is internationally recognised as the most appropriate technique for the detection of important viruses of salmonid species, and its use is of fundamental importance to the control of disease spread, health surveillance programs and in disease diagnosis (OIE, 2011). It is also recognised that virus isolation on cell lines requires the presence of replicating viruses, and involves costly cell culture and maintenance facilities, incubation time lag to initial observation of CPE (up to 21 days), requires skilled personnel (OIE, 2011) for the interpretation of cytopathic effects and additional tests for confirmation of CPE using either immunocytochemistry or PCR (Merrill, 2002). However, in recent years, PCR (polymerase chain reaction) techniques such as conventional RT-PCR (reverse-transcriptase polymerase chain reaction) and qPCR (real-time quantitative polymerase chain reaction) are becoming the standard diagnostic technique for detection and identification of those viruses that are not culturable, for example, WSSV and YHV of prawns (OIE Manual 2011). In addition, these molecular tests, due to their improving sensitivity, specificity and relative low-cost, are becoming popular even for the detection and identification of viruses that can be cultured.

Since the 1990s, PCR has been used as a detection method for viral diseases of fish and has become relatively inexpensive, safe and user-friendly (Table 1.1). Following the introduction of molecular tests, comparison of diagnostic methods particularly of classical

methods and molecular methods, has been carried out by various investigations to determine the most appropriate diagnostic method for viral diseases in salmonids (Table 1.2). PCR-based assays offer several advantages over cell culture assays in detecting viral pathogens, as a well-designed assay can be rapid, highly sensitive, and specific (Hafliger *et al.*, 1997), and less laborious and time-consuming (Arakawa *et al.*, 1990). Thus, in an attempt to overcome the limitations posed by cell culture, conventional hemi-nested RT-PCR and qPCR diagnostic tests were developed for identification of TSRV (Gemma Carlile pers communication). The conventional generic RT-PCR detects squareoviruses in general including CSRV, GSRV, Green River reovirus, Australian redfin Reovirus and TSRV. The conventional hemi-nested RT-PCR and qRT-PCR primers detect specific sequences of Atlantic salmon Reovirus (TSRV) only. The development of TSRV-specific PCRs represented a major improvement for the detection and identification of TSRV in infected Atlantic salmon and infected cell cultures (Gemma Carlile pers communication).

Table 1.1 Detection methods of viruses in farmed aquatic animals in different geographic regions, showing the trend of classical methods being replaced by PCR. Diagnostic (Y): methods classified as a diagnostic method for viruses, Detection (N): methods that are not classified as diagnostic method but been used for the detection of viruses.

VIRUS	FISH SPECIES	METHOD OF DETECTION (Diagnostic : Y / Detection : N)	LOCATION	REFERENCE
IHNV	Salmon & trout	Immunological methods (Y)	USA	Hsu and Leong 1985
IHNV, IPNV, VHSV	<i>Oncorhynchus tshawytscha</i>	Immunoblot Assay & ELISA (Y)	USA	McAllister <i>et al.</i> , 1986
IHNV	<i>Oncorhynchus mykiss</i>	RT-PCR & nucleic acid probe (Y)	USA	Arakawa <i>et al.</i> , 1990
	<i>Oncorhynchus nerka</i>			
	<i>Oncorhynchus tshawytscha</i>			
IHNV, VHSV	<i>Oncorhynchus mykiss</i>	RT-PCR & Semi nested PCR (Y)	Germany	Miller <i>et al.</i> , 1998
IHNV, IPNV	<i>Oncorhynchus mykiss</i>	RT-PCR (Y)	Spain	Alonso <i>et al.</i> , 1999
IHNV	<i>Oncorhynchus mykiss</i>	Real Time-PCR & fluorescent tagging (N)	USA	Overturf <i>et al.</i> , 2001
IHNV, IPNV	<i>Oncorhynchus mykiss</i>	RT-PCR & PCR-ELISA (Y)	Slovenia	Barlic-Maganja <i>et al.</i> , 2002
IHNV	<i>Oncorhynchus mykiss</i>	Real Time-PCR (N)	USA	Dhar <i>et al.</i> , 2008
IPNV	<i>Oncorhynchus kisutch</i>	RT-PCR (N)	Chile	Lopez-Lastra <i>et al.</i> , 1994
	<i>Oncorhynchus mykiss</i>			
IPNV	Salmonids	Dot Blot Hybridization (Y)	USA	Dopazo <i>et al.</i> , 1994
IPNV	Salmonids	ELISA & virus isolation (CHSE-214) (N)	Scotland	Davis <i>et al.</i> , 1994
IPNV	<i>Salmo salar</i>	RT-PCR, virus isolation (BF-2 and CHSE-214 cells) (Y)	Norway	Taksdal <i>et al.</i> , 2001
IPNV	<i>Salmo salar</i>	DNA-RNA-Hybridization (Y)	Norway	Rimstad <i>et al.</i> , 2002
IPNV	Rainbow Trout	Real time RT-PCR (N)	USA	Dhar <i>et al.</i> , 2007
	Wild & cultured salmonids			
IPNV	<i>Salmo salar</i>	Histology, immunocytochemistry & Real-time PCR (N)	UK	Ellis <i>et al.</i> , 2010
ISAV	<i>Salmo trutta</i>	RT-PCR (N)	Norway	Devold <i>et al.</i> , 2000
ISAV	<i>Salmo salar</i>	RT-PCR (N)	Norway	Mikalsen <i>et al.</i> , 2001
ISAV	<i>Salmo salar</i>	Nested RT-PCR (N)	Norway	Lovdal and Enger 2002
ISAV	<i>Salmo salar</i>	RT-PCR, IFAT, Histopathology & virus isolation (Y)	USA	Merrill, 2002

VIRUS	FISH SPECIES	METHOD OF DETECTION (Diagnostic : Y / Detection : N)	LOCATION	REFERENCE
ISAV	<i>Salmo salar</i>	RT-PCR, IFAT, virus isolation (SHK-1 cells) & light microscopy (Y)	UK	Snow <i>et al.</i> , 2003
ISAV	<i>Salmo salar</i>	Real-time PCR (N)	UK	Snow <i>et al.</i> , 2009
ISAV	<i>Salmo salar</i>	Real-time PCR (Y)	UK	Starkey <i>et al.</i> , 2006
ISAV	<i>Salmo salar</i>	Real-time PCR(Y)	Chile	Godoy <i>et al.</i> , 2010
SPDV	<i>Salmo salar</i>	CHSE-214 (Y)	Norway	Christie <i>et al.</i> , 1998
SPDV	<i>Salmo salar</i>	CHSE-214 & Histopathology (Y)	Scotland	Rowley <i>et al.</i> , 1998
CCVD	<i>Ictalurus punctatus</i>	ELISA(enzyme-linked immunosorbent assay) (N)	USA	Crawford <i>et al.</i> , 1999
CCVD	<i>Ictalurus punctatus</i>	RT-PCR (N)	USA	Gray <i>et al.</i> , 1999
	<i>Oncorhynchus mykiss</i>	RT-PCR (N)	France	Villoing <i>et al.</i> , 2000
Birnavirus	<i>Salvelinus fontinalis</i>	RT-PCR (N)	USA	Blake <i>et al.</i> , 1995
Birnavirus	<i>Salmo salar</i>	CHSE-214,EPC,RTG-2 & PCR (N)	Australia	Crane <i>et al.</i> , 2000
Rhabdovirus	Marine fishes	ELISA(enzyme-linked immunosorbent assay) (Y)	Great Britain	Dixon <i>et al.</i> , 1984
Novirhabdovirus	<i>Oncorhynchus mykiss</i>	Bioluminescent image technique (N)	France	Harmache <i>et al.</i> , 2006
Iridovirus (RSIV)	<i>Pagrus major</i>	RT-PCR(Y)	Japan	Oshima <i>et al.</i> , 1998
Iridovirus	<i>Pseudosciaena crocea</i>	Real Time PCR with molecular beacon (N)	China	Wang <i>et al.</i> , 2006
VHSV	Marine fishes	RT-PCR (Y)	Germany	Bruchhof <i>et al.</i> , 1995
VHSV	Marine fishes	RT-PCR, virus isolation (EPC, BF-2, CHSE-214, FHM cells) (N)	UK	Dixon <i>et al.</i> , 2003
VHSV	<i>Oncorhynchus mykiss</i>	Nested RT-PCR (Y)	Spain	L'opez-V'azquez <i>et al.</i> , 2006
VHSV & IHNV	<i>Salmo trutta</i>	RT-PCR (Y)	Germany	Knusel <i>et al.</i> , 2007
	<i>Oncorhynchus mykiss</i>			
VHSV	<i>Oncorhynchus mykiss</i>	RT-PCR & real-time RT-PCR (Y)	UK	Matejusova <i>et al.</i> , 2008
VHSV	<i>Clupea harengus</i>	Real-time PCR (N)	UK	Matejusova <i>et al.</i> , 2010

VIRUS	FISH SPECIES	METHOD OF DETECTION (Diagnostic : Y / Detection : N)	LOCATION	REFERENCE
IHHNV, WSV, SPDV	<i>Penaeus sp</i>	Real-time PCR (Y)	California	Dhar <i>et al.</i> , 2001
WSSV	<i>Penaeus monodon</i>	Nested RT-PCR (N)	India	Thakur <i>et al.</i> , 2002
GAV	<i>Penaeus monodon</i>	Real-time PCR (Y)	Australia	De la Vega <i>et al.</i> , 2004
IMNV	<i>Litopenaeus vannamei</i>	Real-time PCR (N)	Brazil	Andrade <i>et al.</i> , 2007
MBV	Penaeid shrimp	Real-time PCR (Y)	China	Yan <i>et al.</i> , 2009
MBV	<i>Seriola dumerili</i>	RT-PCR & Nested PCR (Y)	Japan	Suzuki <i>et al.</i> , 1997
	<i>Seriola quinqueradiata</i>			
	<i>Pagrus major</i>			
	<i>Lateolabrax japonicus</i>			
GCHV	<i>Ctenopharyngodon idellus</i>	RT-PCR (N)	China	Li <i>et al.</i> , 1997
NNV	<i>Dicentrarchus labrax</i>	RT-PCR (Y)	Italy	Valle <i>et al.</i> , 2000
SDV	<i>Salmo salar</i>	CHSE-214 cells (N)	UK	Lopez-Doriga <i>et al.</i> , 2001
TFRV	<i>Eleutheronema tetradactylus</i>	BF-2 cells (Y)	Singapore	Seng <i>et al.</i> , 2002
KHV	<i>Cyprinus carpio</i>	Real-time PCR (N)	USA	Gilad <i>et al.</i> , 2004
CGNV	<i>Cyprinus carpio</i>	Semi-quantitative PCR & Immunohistochemistry (N)	Israel	Eli <i>et al.</i> , 2004
VNN	Marine cultured fish	RT-PCR & Nested PCR (Y)	Japan	Gomez <i>et al.</i> , 2004
CyHV-2	<i>Carassius auratus</i>	Real Time-PCR (Y)	USA	Goodwin <i>et al.</i> , 2006
SVCV	<i>Cyprinus carpio</i>	Virus isolation (EPC cells) & RT-PCR (N)	Canada	Garver <i>et al.</i> , 2007
LMBV	<i>Micropterus salmoides</i>	Real-time PCR (N)	USA	Getchell <i>et al.</i> , 2007
PHV	<i>Sardinops sagax neopilchardus</i>	ISH, RT-PCR, Real-time PCR (N)	Australia	Crockford <i>et al.</i> , 2008
EEDV	<i>Salvelinus namaycush</i>	RT-PCR (Y)	USA	Kurobe <i>et al.</i> , 2009
IcHV-2	<i>Ameiurus melas</i>	Real-time PCR (Y)	USA	Goodwin <i>et al.</i> , 2010

Table 1.2 Comparison of diagnostic methods (classical versus molecular methods) for the detection of viruses in salmonids. Bolded methods are classical methods for each disease outbreak.

Virus	Fish	Method	Location	Author
IHN	Salmon & trout	Immunological methods	USA	Hsu and Leong, 1985
IHN, IPNV	<i>Salmo salar</i>	Virus isolation (EPC and BF-2 cell lines), IFAT, RT-PCR and ELISA	Slovenia	Barlic-Maganja <i>et al.</i> , 2002
IPNV	<i>Salmo salar</i>	Histology , immunocytochemistry and Real-time PCR	UK	Ellis <i>et al.</i> , 2010
Birnavirus	<i>Salvelinus fontinalis</i>	RT-PCR and virus isolation (CHSE-214)	USA	Blake <i>et al.</i> , 1995
ISAV	<i>Salmo salar</i>	clinical signs of disease, gross pathological and histological observations, IFAT, virus isolation (SHK-1 and CHSE-214 cell lines) and RT-PCR	USA	Opitz <i>et al.</i> , 2000
ISAV	<i>Salmo trutta</i>	RT-PCR, virus isolation (ASK cell lines) and IFAT	Norway	Devold <i>et al.</i> , 2000
ISAV	<i>Salmo salar</i>	RT-PCR, IFAT, Histopathology and virus isolation (SHK-1 and CHSE-214 cell lines)	USA	Merrill, 2002
ISAV	<i>Salmo salar</i>	RT-PCR, IFAT, virus isolation (SHK-1 cells) and light microscopy	UK	Snow <i>et al.</i> , 2003
ISAV	<i>Salmo salar</i>	Real-time PCR, virus isolation and IFAT	Canada	McClure <i>et al.</i> , 2005
ISAV	<i>Oncorhynchus mykiss</i>	RT-PCR, virus isolation (SHK-1 cell lines) and IFAT	Canada	Nerette <i>et al.</i> , 2005
ISAV	<i>Salmo salar</i>	Histopathology, Real-time PCR, and virus isolation (ASK cell lines)	Norway	Abayneh <i>et al.</i> , 2010
ISAV	<i>Salmo salar</i>	Real-time PCR, Histopathology and IHC	Chile	Godoy <i>et al.</i> , 2010
SAV	<i>Salmo salar</i>	Real-time PCR, Histopathology and serology	Norway	Jansen <i>et al.</i> , 2010

1.3 Thesis Aims

The Tasmanian Atlantic salmon industry has raised questions about the effect of TSRV on aquaculture production since its first detection in the late 1980s, and TSRV's significance remains unclear to the present day. Until recently, partly due to the perception that TSRV is non-pathogenic, TSRV has attracted little attention and the level of research activity on the characterisation of this virus has been low. Industry's concerns regarding the significance of TSRV have resurfaced mainly due to its association with low-level mortalities, and recent research by Carlile (2011) has indicated that under certain conditions TSRV could cause disease. Despite the fact that development of molecular methods for the detection and identification of TSRV was considered a step forward (Gemma Carlile pers communication) validation of such methods has not been carried out yet.

Validation is important for diagnostic tests because it determines the fitness of the assays and includes estimates of the analytical and diagnostic performance characteristics of the tests. Assay performance is affected by many factors that span from the earliest stages of assay development through the final stage of performance assessments that involve the application of tests to targeted population of animals (OIE, 2011). In addition, an assay cannot be considered validated unless the specific set of essential validation criteria/factors by OIE have been fulfilled, either quantitatively or qualitatively. The first four of these factors that affect assay performance have been addressed during the development of the diagnostic methods by Carlile (2011) which include the intended purpose of the assay, optimisation, standardization and robustness. The remaining eight factors which include reproducibility, repeatability, analytical sensitivity and specificity, threshold, diagnostic sensitivity and diagnostic specificity were evaluated and determined in this thesis.

Thus, validation of diagnostic methods has been a major focus in this thesis prior to any subsequent studies to assess specific aspects of TSRV such as epidemiology and pathogenesis. In order to progress further research on the characterisation of TSRV in an attempt to determine its significance for the Tasmanian salmon aquaculture, the principal aims of this thesis are:

1. To evaluate current diagnostic methods to determine the most sensitive and specific method for the detection and identification of TSRV infections in farmed Atlantic salmon
2. To evaluate sample collection methods (organs versus swabs) for TSRV detection in farmed Atlantic salmon
3. To undertake a field investigation to determine the incidence of TSRV infections with other pathogens
4. To undertake preliminary characterisation of a limited range of TSRV isolates.

CHAPTER 2

Comparison of two diagnostic methods, PCR and virus isolation on piscine cell lines for TSRV detection

2.1 Introduction

Atlantic salmon reovirus, formally designated as Tasmanian salmon reovirus - TSRV (Samal *et al.*, 2005), was first isolated in Australia in 1990. Following the initial isolation, TSRV has been isolated regularly during Tasmanian salmonid health surveillance (TSHSP) activities coordinated by the Tasmanian Department of Primary Industry, Parks, Water and Environment (DPIPWE). As with the diagnostic methods for other finfish viruses, virus isolation in cultures of piscine cell lines is considered the 'gold standard' for detection of TSRV infections (Gemma Carlile pers. communication) and has been used for all viral surveillance activities for the TSHSP to date.

The major drawbacks to the cell culture assay is that it is laborious, relatively expensive and time-consuming; normally, incubation (in low temperature incubators) of inoculated cell cultures for up to 3 weeks, together with at least one passage onto fresh cell cultures is required to confirm both positive and negative results. In addition, some fish samples may be cytotoxic resulting in an appearance of the cell cultures that can be confused with viral cytopathic effect (CPE) (Freshney, 2000). In contrast, viral detection using PCR has been demonstrated to be a rapid, sensitive and specific method for detection and identification of viruses in fish tissues (Devold *et al.*, 2000). Furthermore, real-time PCR (qPCR) assays have added advantages over conventional PCR: improved sensitivity and specificity (Overturf *et al.*, 2001), high sample throughput (Bustin *et al.*, 2005), simultaneous analysis of several targets facilitated by multi-channel processing and prevention of cross-

contamination by post-amplification elimination (Bustin, 2000; 2002 and Bustin *et al.*, 2005). While the advantages for using PCR can be clearly demonstrated, it should be noted that PCR detects the presence of nucleic acid only; it does not provide information on whether or not infectious virus is present in the test sample which can only be determined by either virus isolation in cell culture (if available) or bioassay in live fish.

Carlile (2011) developed conventional RT-PCR tests for the pan-specific detection of Aquareovirus and for Atlantic salmon reovirus (TSRV) specifically. The generic primers were able to amplify a 314 bp product using Chum salmon reovirus (CSRV), golden shiner reovirus (GSRV), Green River reovirus, Australian redfin reovirus and TSRV as target (Gemma Carlile pers communication). The specific primers were able to amplify a primary PCR product of 280bp and hemi-nested primers, a product of 141 bp in length. In addition to the conventional hemi-nested RT-PCR, a real-time RT-PCR (qPCR) was also developed to detect TSRV and for use as a diagnostic tool. The development of both conventional and real-time TSRV-specific PCRs represents a major improvement for the detection and identification of TSRV in infected Atlantic salmon as well as infected cell cultures (Gemma Carlile pers communication). Although, improved diagnostic techniques for TSRV detection have been developed, the analytical and diagnostic sensitivity and specificity of each test have not been assessed. Furthermore, the most effective diagnostic method for TSRV detection has not been determined.

The performance of a new diagnostic test is often evaluated by comparison with a perfect test (gold standard). According to Greiner and Gardner (2000), the gold standard refers to the methods or a combination of methods by which one can unequivocally classify animals as positive/infected or negative/uninfected for validation of a diagnostic test. The

ideal gold standard would be a perfect test that produces no misclassification (i.e. no false positive and no false negative), thus reflecting the true status of disease and provide a reference test of known and high accuracy.

Sensitivity and specificity of a test can be estimated directly with the availability of a gold standard (OIE, 2011). The determination of sensitivity and specificity of new diagnostic tests in comparison to a gold standard is known as the classical approach. However, in the absence of an appropriate gold standard, a variety of approaches (non-gold standard) can be used to estimate the sensitivity and specificity of diagnostic methods. These approaches involve the use of statistical analyses such as the latent-class models based on maximum likelihood estimation procedures (TAGS, Pouillot *et al.*, 2002) and Bayesian inference analyses (Frossling *et al.*, 2003). As stated above, virus isolation in cultures of piscine cell lines is considered the 'gold standard' for detection of TSRV infections.

This study encompasses the detection of TSRV in populations of Atlantic salmon in Tasmania. For a number of reasons (e.g. establishment costs) fish virology capability is not available at DPIWE, Tasmania and for the past three decades an agreement has been in place for all virology to be undertaken at AAHL Fish Diseases Laboratory, CSIRO, Geelong, Victoria. Thus, all surveillance specimens for virology are submitted to AAHL Fish Diseases Laboratory (AFDL) on a routine basis for virus isolation in cultures of piscine cell lines. The length of time required to obtain a result using classical virology (virus isolation in cell lines), the relative high cost of this testing, the availability of potential alternative tests (PCR) at DPIWE prompted this study to use two different methods, gold standard (classical approach) and non-gold standard (TAGS and Bayesian analyses), to assess the accuracy of the available diagnostic tests.

This chapter describes the comparison of available diagnostic techniques for TSRV detection in farmed populations of Tasmanian Atlantic salmon: virus isolation, conventional hemi-nested RT-PCR and qPCR. The main aim of this chapter is to determine which technique would be the most accurate with respect to specificity and sensitivity for the detection of TSRV infections as part of the TSHSP. To increase the level of confidence in the results, and therefore the conclusions, both intra-laboratory and inter-laboratory comparisons of PCR (conventional hemi-nested RT-PCR & qPCR) and virus isolation in cell culture using two finfish cell lines (CHSE-214 & EPC) were carried out for the detection of Tasmanian Atlantic Salmon Reovirus (TSRV) in tissues of farmed Atlantic salmon from various sites around Tasmania. These studies included collaboration between the National Centre for Marine Conservation and Resource Sustainability, University of Tasmania, Animal Health Laboratory (AHL), Mount Pleasant Laboratories (DPIPWE), Launceston, Tasmania and the AAHL Fish Diseases Laboratory (AFDL), located at CSIRO Australian Animal Health Laboratory (AAHL), Geelong, Victoria.

2.2 Materials and Methods

2.2.1 Sampling regime

Sampling was carried out from 29th September to 21st December 2009. Atlantic salmon were sampled from farm sites located in two regions, the Tamar River and South-east Tasmania. Table 2.1 shows the sampling sites (coded), numbers of fish sampled, fish size (for all the sampled fish, the mean weight was 1.2 kg and the standard deviation was 0.7 kg), and water temperature (taken at 5 metre depth) at the time of sample collection.

Table 2.1 Total number of fish sampled from different sampling sites in Tasmania, fish sizes and water temperature during the study. Site A : Tamar River and Sites B -I: South-east Tasmania.

Location	Fish number	Fish size (kg)	Water temperature (°C)
Site A	33	2.3	12
Site B	12	1.5	12
Site C	12	0.5	12
Site D	12	0.75	13
Site E	15	2	13.5
Site F	15	1.5	15
Site G	15	0.2	15
Site H	15	1.3	14
Site I	15	0.75	15
Total	144		

The South-east Tasmanian samples were collected as part of the Tasmanian Salmonid Health Surveillance Program (TSHSP) and the Tamar River samples were collected independently of TSHSP. All samples were sent to the Animal Health Laboratory, Mount Pleasant Laboratories (AHL) for further processing. The samples consisted of liver, spleen and kidney from moribund or freshly dead (77.1%) and healthy fish (22.9%). Gross pathological signs

observed in sampled fish included distended abdomen, enlarged/swollen organs (kidney, liver and spleen), pale liver and dark hindgut.

Samples were processed (see section 2.2.3) at AHL, prior to submission of duplicate samples to AFDL for inter-laboratory comparison of diagnostic methods of TSRV detection. For the intra-laboratory comparison, samples were examined by virus isolation as per standard operating procedures of AFDL (NATA accredited), conventional hemi-nested RT-PCR and qPCR (both methods developed by Gemma Carlile, pers communication) were done at AHL. Tests evaluated by AFDL were virus isolation and qPCR. The comparison between conventional hemi-nested RT-PCR and qPCR was undertaken for all the samples in AHL. In contrast, AFDL conducted conventional hemi-nested RT-PCR on several samples which were found to be negative by its qPCR but positive by qPCR done at AHL.

2.2.2 Cell culture (virus isolation)

CHSE-214 (chinook salmon, *Oncorhynchus tshawytscha* embryo, ATCC catalogue No. CRL 1681) and EPC (*epithelioma papulosum cyprinid*, derived from fathead minnow *Pimephales promelas* and provided to AFDL by CEFAS Weymouth Laboratory UK) (Fijan *et al.*, 1983; Winton *et al.*, 2010) cell lines were provided by AFDL and used as host cells for virus isolation at both AHL and AFDL. Stock cultures of CHSE-214 cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10 mM HEPES buffer, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU penicillin / mL and 100 µg streptomycin / mL (Invitrogen, Australia), and incubated at 22°C in an atmosphere of 5% CO₂/95% air.

Stock cultures of EPC cells were maintained in L-15 Leibovitz medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 100 IU penicillin / mL and 100 µg streptomycin / mL (Invitrogen, Australia), and incubated at 22°C in normal atmosphere. Both cell lines were maintained in 75 cm² plastic cell culture flasks. After they reached greater than 90% of confluency, stock cell cultures were sub-cultured as follows. The old culture medium from the cell cultures was decanted and the cell monolayer was rinsed with 3 mL PBSA (sterile phosphate buffered saline, pH 7.4 without Ca²⁺/Mg²⁺, Invitrogen, Australia).

PBSA was decanted into a discard vessel and 3 mL trypsin-versene [0.05%, 1X with EDTA 4 Na (Ethylenediaminetetraacetic acid disodium salt dihydrate), Invitrogen, Australia] was added. The cells were incubated at room temperature until the monolayer detached which normally took around 5-10 minutes. The detached cells were resuspended in 7 mL growth medium (as above, MEM for CHSE-214 and L-15 for EPC) and the cell suspension was transferred to a 10 mL sterile centrifuge tube and centrifuged at 5°C at 100 x g for 5 min. After centrifugation, the supernatant was decanted carefully into a discard vessel leaving the cell pellet in the tube.

The pellet was resuspended in 9 mL growth medium and the cell density (number of cells per mL) was estimated using a haemocytometer. The cells were seeded into fresh culture flasks at 4.5 million cells in 20 mL growth medium and incubated at 22 °C (atmosphere of 5% CO₂/95% air for CHSE-214 and normal atmosphere for EPC). For virus isolation, standard procedures (OIE 2010) were used; 24-well cluster plate monolayer cultures of CHSE-214 and EPC were prepared as follows. Stock cultures of the cell lines

were treated with trypsin-versene as above and the required volume of cells was determined based on the estimated cell count.

A total volume of 40 mL cell suspension (cells suspended in growth medium) was required to seed one 24-well plate and the plates were seeded at a cell density of 6 million CHSE-214 cells/plate (250,000 cells/1.5 mL/well) or 9 million EPC cells/plate (375,000 cells/1.5 mL/well). The cell suspension was mixed gently; 1.5 mL cell suspension was dispensed into each well using a sterile pipette and the culture plates were incubated at 22°C. On the following day and prior to sample inoculation, the cultures were checked by light microscopy to ensure that the cultures demonstrated a minimum of 90% confluency with mitotic figures (dividing cells) present, there was no microbial contamination and that the cell density and cellular morphology was consistent across all culture wells in each plate.

2.2.3 Sample processing

All tissue samples were processed for virus isolation within 24 hours of collection. The fish samples (pooled organ samples approximately 2.5 g from liver, kidney and spleen) were homogenised and resuspended in the transport medium (Hank's balanced salt solution supplemented with 10% FBS, Invitrogen, Australia) using individual frozen, sterile mortar and pestle. The mortars and pestles were autoclaved at 121°C and then stored frozen at -20°C, 24 hours prior to tissue processing. Additional transport medium was added, if necessary, to make the ratio of tissue weight (0.5 g) to supplemented HBBS volume (5 mL) 1:10. The homogenised tissues were then centrifuged at 2000-4000 x g for 15 min at 5°C to clarify. Duplicate aliquots (1.5 mL) of the supernatants were placed into 2 mL cryo-tubes (for

submission to AFDL) for inter-laboratory comparison study), for PCR and for virus isolation using cell lines.

For virus isolation at both AHL and AFDL, the homogenized tissue supernatants were diluted 1:10 and 1:100 with maintenance medium (growth medium with 2% instead of 10% (v/v) FBS, see section 2.3.2) and 150 μ L of the diluted preparations were inoculated onto duplicate cultures (i.e. 2 wells) of 24-well plate cultures of each cell line (see Figure 2.1 for the standard 24-well plate set-up). The inoculated cultures were incubated for 1 hour at 15°C to allow adsorption of virus particles onto the cell monolayer. After adsorption, 1.5 mL of EMEM for CHSE-214 and Leibovitz's L-15 for EPC cells (both enriched with 2% FBS, 2 mM L-glutamine, 100 IU penicillin / mL and 100 μ g streptomycin / mL), were added per well to yield final sample dilutions of 1:100 and 1:1000. Cultures were incubated at 15°C in either normal atmosphere (EPC cultures in L-15) or an atmosphere of 5% CO₂/95% air (CHSE-214 cultures in EMEM). On the following day, the cultures were examined by inverted light microscopy for any microbial contamination and/or tissue sample cytotoxicity. Subsequently, the cultures were examined at 3, 7 and 10 days post inoculation for the presence of viral CPE.

Figure 2.1 Standard 24-well plate set-up

Sample 1 1/10	Sample 2 1/10	Sample 3 1/10	Sample 4 1/10	Sample 5 1/10	Negative Control
Sample 1 1/10	Sample 2 1/10	Sample 3 1/10	Sample 4 1/10	Sample 5 1/10	Negative Control
Sample 1 1/100	Sample 2 1/100	Sample 3 1/100	Sample 4 1/100	Sample 5 1/100	Negative Control
Sample 1 1/100	Sample 2 1/100	Sample 3 1/100	Sample 4 1/100	Sample 5 1/100	Negative Control

At 7 days post-inoculation, for cultures displaying cytopathic effect (CPE), the tissue culture supernatants were pooled from the respective 4 inoculated wells for each sample producing CPE, filtered through 0.45 μ m membrane filters (Sartorius) and 150 μ L aliquots added onto each of 4 wells of 24 well plate cultures of fresh CHSE-214 and EPC cell lines prepared on the previous day as described above. The cultures were observed for a total of 21 days for completion of the assay. The results for the virus isolation were reported independently for inter-laboratory comparison of both cell lines. The sample was considered positive if CPE was observed in any of the duplicate cultures of either one of the cell lines (CHSE-214 or EPC) for each laboratory. The virus isolation results were pooled for the inter-laboratory and intra-laboratory comparison of diagnostic methods. For both inter-laboratory and intra-laboratory comparisons, the sample was considered to be negative if none of the cultures of the two cell lines showed any cytopathic effect.

2.2.4 Isolation of RNA from tissue samples

RNA was extracted from the homogenized tissue supernatants (samples which were processed and placed into 1.5 mL of cryo-tube, see section 2.3.3) using the High Pure Viral Kit (Roche, Germany). A total 400 μ L binding buffer supplemented with Poly (A) was added to 200 μ L sample (homogenized tissue supernatants) and transferred into the High Pure Filter assembly and centrifuged at 8,000 \times g for 15s. The flowthrough liquid and collection tube were removed and a new tube was used in each of the following steps. Under the buffer conditions used in the procedure, the viral RNA binds to the glass fleece in the High Pure tube, while contaminating substances (salts, proteins and other cellular contaminants) do not.

Inhibitor Removal Buffer (500 μ L) was added to the upper reservoir and the tube was centrifuged at 8,000 $\times g$ for one minute. After the removal of any inhibitors, the sample was washed (twice) by adding 450 μ L of Wash buffer and centrifuged at 8,000 $\times g$ for one minute. The addition of the Inhibitor Removal Buffer and brief wash-and-spin steps removed those contaminants. The Filter Tube-Collection tube assembly was left in the centrifuge and was spun at maximum speed (13 000 $\times g$) for 10s to remove any residual Wash Buffer. The remaining, purified RNA was eluted into a final volume of 50 μ L with Elution Buffer. The purified RNA was used as RNA templates for conventional hemi-nested RT-PCR (see section 2.2.5.1) and TaqMan real-time RT-PCR assay (qPCR) (see section 2.2.6.1).

2.2.5 Conventional hemi-nested RT-PCR

2.2.5.1 cDNA synthesis

cDNA synthesis was carried out using TaqMan Reverse Transcription Reagents with MultiscribeTM reverse transcriptase (Applied Biosystems, USA) in the following reaction mixture with a final volume of 10 μ L: 2 μ L RNA, 1 μ L reverse transcriptase buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.2 μ L magnesium chloride ($MgCl_2$) (25 mM), 2 μ L 0.5 mM each deoxynucleoside triphosphate (dNTP), 0.2 μ L (0.4 U / μ L) RNase inhibitor, 0.5 μ L random hexamer (primer) mix, 0.25 μ L MultiscribeTM reverse transcriptase and 1.85 μ L RNase free water. The total RNA was heat-denatured at 95°C for 40 seconds before addition of RT reagents and reverse transcription. The reaction was incubated for 10 minutes at 25°C followed by 30 minutes at 48°C and a final incubation at 95°C for 5 minutes to deactivate the reverse transcriptase.

2.2.5.2 Conventional hemi-nested RT-PCR Assay

The conventional PCR was carried out using primers that were designed by Carlile (2011) and based on the sequence alignment of the S10 gene of TSRV (GenBank accession No. S10E_F434979): Forward primer SpT10Fb (position 306-323: 5' - TTCCCTCTCTAAGACCC-3') and reverse primer SpT10Ra (position 567-585: 5'-GCCACCGGTAATAGTACG-3') for the primary reaction followed by forward primer SpT10FN (position 445-463: 5'-AATTGTGATCGCGCTCTC-3') and reverse primer SpT10Ra (position 567-585: 5'-GCCACCGGTAATAGTACG-3') in the hemi-nested reaction. A total of 23 μ L PCR mixture containing: 9.5 μ L 2X HotStar Taq Plus Master Mix (QIAGEN), 12.9 μ L RNase-free water, 0.3 μ L SpT10Fb and 0.3 μ L SpT10Ra were added to 2 μ L prepared cDNA.

For the primary reaction, the amplification was programmed as followed: 15 minutes at 95°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 40 seconds. A final extension of 72°C for 5 minutes terminated the thermal cycling reaction. The hemi-nested reaction was carried out with a total of 24 μ L PCR mixture containing: 9.5 μ L 2X HotStar Taq Plus Master Mix (QIAGEN), 13.9 μ L RNase-free water, 0.3 μ L SpT10FN and 0.3 μ L SpT10Ra were added to 1 μ L PCR product. The amplification was conducted with the following programme: 15 minutes at 95°C, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 40 seconds and a final extension of 72°C for 5 minutes. Both reactions were carried out using a thermal cycler MyCycler Thermal Cycler (Bio-Rad Laboratories, Australia). The amplified PCR products from both reactions were analysed by electrophoresis (30 minutes at 90 V) on 2% (w/v) agarose gel in TAE buffer and stained with

gel red (Biotium Inc, USA). A fluorescent nucleic acid gel stain, gel red is being used as substitute for the highly toxic ethidium bromide.

2.2.5.3 Development of plasmid internal control

A plasmid with the sequence of the PCR amplification product inserted was developed as an internal control for the conventional hemi-nested RT-PCR. For RNA extraction, 200 μ L TSRV at a titre of $10^{4.7}$ TCID₅₀/mL were used with the High Pure Viral Kit (Roche, Germany) and cDNA was synthesized according to section 2.2.5.1. PCR amplification was carried out as described in the section 2.2.5.2 and the amplified PCR product was analysed by gel electrophoresis. The TSRV PCR product was ligated using the pGEM[®]-T Easy Vector Systems (Promega, USA). The pGEM[®]-T Easy Vector is linearized with a single 3'-terminal thymidine at both ends. The ligation reactions were set-up as follows: 2.5 μ L 2X Rapid Ligation Buffer, 0.5 μ L pGEM[®]-T Easy Vector, 1.5 μ L TSRV PCR product and 0.5 μ L T4 DNA ligase.

The reaction mixture using the 2 X Rapid Ligation Buffer was incubated overnight at 4°C for maximum number of transformants (product containing both of vector and TSRV) as longer incubation times will increase the number of colonies after transformation. JM109 high-efficiency competent cells ($\geq 1 \times 10^8$ cfu/ μ g DNA) were used for transformation. The ligation of fragments with a single-base overhang can be inefficient, so it was essential to use cells with a transformation efficiency of at least 1×10^8 cfu/ μ g DNA to obtain a reasonable number of colonies. JM109 are *E. coli* competent cells, an ideal host for many molecular biology applications and were prepared according to a modified procedure of Hanahan (1985) by Promega (USA).

The frozen competent cells were thawed on ice for 10 minutes and the ligation mixture was centrifuged at 100 x g for one minute. A volume of 5 µL ligation reaction was added to 50 µL defrosted competent cells and incubated on ice for 20 minutes. Cells were then heat-shocked for one minute at 42°C and placed on ice for another 2 minutes. Then, 950 µL SOC medium (super optimal broth with catabolite repression (SOC) is a nutrient-rich bacterial growth medium used for the growth of *E. coli*) was added to the ligation reaction transformants and incubated for 1 hour at 37°C (with shaking at 225 rpm). Subsequently, 50 µL and 100 µL transformant culture were plated out on duplicate LB agar plates (with ampicillin) and incubated overnight at 37°C. Luria Bertani (LB) agar supplemented with ampicillin was used for the growth of *E. coli* which allows the growth of transformed cells with the plasmid insert.

Colonies from the LB agar plates were screened for the plasmid insert by PCR using the M13F/R primers (kindly supplied by Dr. Richard Morrison, AHL, DPIPWE, Tasmania). The pGEM®-T Easy vectors are high-copy-number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region (MCS). The M13 forward and reverse primers anneal to plasmid DNA that flanks the multiple cloning site. Thus, running a PCR using M13F/R primers on plasmid (hosted within the *E. coli*) would produce an amplicon size with a total size of 3095 bp (includes the insert ligated into plasmid and the DNA between the MCS and M13 annealing sites). Five colonies were selected from 8 plates and conventional PCR was run using this reaction mixture: 1 µL 10x buffer, 0.3 µL magnesium chloride (MgCl₂), 0.1 µL dNTP (100 mM), 0.8 µL M13 forward primer (100 mM), 0.8 µL M13 reverse primer (100 mM), 0.08 µL Bio Taq and 6.92 µL RNase free water.

The amplification was conducted with the following programme: 2 minutes at 94°C, followed by 25 cycles of denaturation at 94 °C for 10 seconds, annealing at 50 °C for 10 seconds and extension at 72 °C for 1 minute 40 seconds. The amplified PCR products were analysed by gel electrophoresis (30 minutes at 90 V) on 2% (w/v) agarose gel in TAE buffer and stained with gel red. Colonies which produced an amplicon with a band size of 3095 bp were inoculated into 4 mL LB broth (contained 50 µL/mL final concentration of Ampicillin) and incubated overnight at 37°C with shaking. The plasmid DNA insert was purified by using the QIAprep Spin Miniprep Kit (QIAGEN). Conventional hemi-nested RT-PCR was conducted on the purified plasmid inserts according to section 2.2.5.2 to ensure that the plasmid DNA contained the target sequence gene for TSRV. A plasmid DNA control template of 3095 bp was developed as an internal control for conventional hemi-nested RT-PCR assay to detect TSRV.

2.2.6 TaqMan Real-time RT-PCR Assay (qPCR)

2.2.6.1 qPCR Assay

The primers and probe for the qPCR assay were designed to amplify and detect an 82 nucleotide sequence of cDNA corresponding to 705–787 bp of the viral genome within segment 10 of TSRV (GenBank accession No. S10EF434979) (Gemma Carlile pers communication). The sequences for the primers were: Forward primer, TSRV-10F (position 705-725, 5' -GATCGAACCCGTCGTGTCTAA-3'), reverse primer, TSRV-10R (position 769-787, 5' -CGGTGCTCAGCTTGTCACA-3'). The TSRV probe (position 731 -748, 5' -CCC GAG C CA T CT GGG C GC-3') contained a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), located at the 5' end and the quencher, 6-carboxy-tetramethyl-rhodamine (TAMRA), located at the 3' end.

The qPCR assay also included 18S ribosomal (rRNA) to check for sample-specific RT and PCR inhibitors as well as to correct for variation in RT efficiency and template quantity. The primers and probes for 18S were as follows: Forward primer, 18F (5'-CGGCTACCACATCCAAGGAA-3'), reverse primer, 18R (5'-GCTGGAATTACCGCGGCT-3') and probe, 18S P probe (5'-TGCTGGCACCAGACTTGCCCTC-3') which contained a proprietary fluorescent reporter dye, (VIC) located at the 5' end and the quencher TAMRA located at the 3' end. The amplification protocol consisted of addition of RNA to a master mix as per the TaqMan[®] one-step RT-PCR master mix reagents kit (Applied Biosystems, USA).

The reaction mixture consisted of: 5.75 μ L RNase free water, 12.5 μ L TaqMan[®] 2 \times Universal PCR master mix no AmpErase[®] UNG (containing AmpliTaq Gold[®] DNA polymerase), 0.625 μ L 40 \times Multiscribe[™] and RNase inhibitor mix, 1.25 μ L Primer TSRV-10F (18 μ M), 1.25 μ L Primer TSRV-10R (18 μ M), 1.25 μ L TSRV-10 probe (5.0 μ M), 0.125 μ L 18S rRNA Primer F (10 μ M), 0.125 μ L 18S rRNA Primer R (10 μ M), 0.125 μ L 18S rRNA VIC labelled Probe and 2 μ L of denatured of viral RNA.

The amplification was carried out in ABI PRISM[™] 7500 Fast Real-Time System (Perkin-Elmer, Applied Biosystems, USA) with a total of 25 μ L reaction mixture. The RNA was amplified with the following programme: An initial 30 minutes at 48°C, followed by 10 minutes at 95°C, then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fixed Δ Rn and upper cut-off C_T value for TSRV specific products were set at 0.1 and 36.0 respectively based on relative quantification with 18S rRNA-specific products from all TSRV qPCR reactions (Gemma Carlile pers communication). These values were established in

experimental infections only, thus the C_T cut-off value for differentiating negative and positive samples needed to be determined based on natural TSRV infections in farmed populations of Atlantic salmon. Therefore, the C_T cut-off value for qPCR was determined based on intra-laboratory comparison with virus isolation which included pooled results from both cell lines, CHSE-214 and EPC. Positive C_T values obtained for qPCR were compared to the results for virus isolation from the same samples. A C_T value was considered to be positive if virus was isolated from the equivalent sample on both cell lines. A C_T value > 36.0 was considered negative if virus was undetectable in either on one or both cell lines when the equivalent sample inoculated onto both cell lines (to avoid false positives).

2.2.6.2 Absolute quantification of qPCR with synthetic dsRNA

TSRV synthetic dsRNA of 430 bp amplicon size was generated with sequence covering the region of both conventional semi-nested RT-PCR and qPCR. The synthetic dsRNA was developed by Dr. Nick Moody from AAHL Fish Diseases Laboratory (AFDL). The sequences for the primers were: Forward primer, TSRV SpT10Fb (5'-TTC CCT CTC TAA GAC CC -3') and reverse primer, TSRV-10R (5'-CGG TGC TCA GCT TGT CAC A -3'). Absolute quantification of qPCR was carried out in AAHL using the synthetic dsRNA provided by AFDL. For RNA extraction, 140 μ L synthetic dsRNA with the initial viral copy number of standard RNA molecules of 6.2×10^{12} molecules/ μ L was used.

The extraction was conducted using the QIAmp[®] Viral RNA kit (QIAGEN, Germany) according to the manufacturer's instructions. A total of 560 μ L prepared Buffer AVL containing carrier RNA was added into 140 μ L synthetic dsRNA. The sample-buffer was mixed well with vortex mixer for 15s, to ensure efficient lysis and to yield a homogenous

solution. The sample-buffer solution was incubated at room temperature for 10 min and the tube was centrifuged at $8,000 \times g$ for one minute to remove drops from the inside of the lid. A total of 560 μL 100% ethanol were added to the sample, mixed with a vortex mixer for 15s and the tube was centrifuged at $8,000 \times g$ for one minute. The solution (630 μL) was transferred into QIAmp[®] Mini column (in a 2 mL collection tube) without wetting the rim.

The sample was centrifuged at $6000 \times g$ for a minute. The QIAmp[®] Mini column was placed into a clean 2 mL collection tube and the tube containing the filtrate was discarded. Buffer AW1 (500 μL) was added to the tube and centrifuged at $6000 \times g$ for one minute. After centrifugation, a new collection tube replaced the initial tube. A total of 500 μL Buffer AW2 was added and the tube was centrifuged at $13,000 \times g$ for 3 min. The QIAmp[®] Mini column assembly was placed into a new collection tube and was centrifuged at maximum speed ($16,837 \times g$) for one minute to remove any residual Wash Buffer. The QIAmp[®] Mini column was placed into a clean 1.5 mL microcentrifuge tube and 60 μL Buffer AVE was added. It was incubated at room temperature for a minute and centrifuged at $6000 \times g$ for one minute. The synthetic dsRNA was eluted into a final volume of 60 μL Buffer AVE.

A stock of TSRV was produced by infecting a CHSE-214 cell culture with TSRV and then incubating the infected cells at 15°C to allow viral replication. When 100% CPE had developed (at 9 days of post-inoculation), the cell culture supernatant was harvested, centrifuged at low speed ($100 \times g$) for 15 min at 4°C to sediment cell debris and aliquots (250 μL) of the resultant supernatant which contained the replicated virus were stored at -80°C to be used as positive control material for PCR assays. This TSRV stock which had a qPCR C_T value of 14.0 and a $\text{TCID}_{50}/\text{mL}$ of $10^{4.7 \text{ to } 5.0}$ was used to prepare ten-fold dilution series from

10^{-1} to 10^{-10} as follows: a) 10^{-1} (40 μ L undiluted TSRV stock was added to 360 μ L supernatant) b) 10^{-2} (40 μ L 10^{-1} diluted TSRV stock added to 360 μ L supernatant) c) 10^{-3} (40 μ L 10^{-2} diluted TSRV stock added to 360 μ L supernatant) and so on through to i) 10^{-10} (40 μ L 10^{-9} dilution of TSRV stock added to 360 μ L supernatant).

Each dilution was mixed well with a vortex mixer prior to preparing the next dilution. For RNA extraction, 200 μ L each of the dilution was used with the High Pure Viral Kit (Roche, Germany) and extraction was conducted according to section 2.2.4. A ten-fold dilution series of the eluted synthetic dsRNA was prepared in the same manner: a) 10^{-1} (2 μ L undiluted synthetic dsRNA was added to 18 μ L molecular grade water) b) 10^{-2} (2 μ L 10^{-1} diluted synthetic dsRNA added to 18 μ L molecular grade water) c) 10^{-3} (2 μ L 10^{-2} diluted synthetic dsRNA added to 18 μ L molecular grade water) and so on through to i) 10^{-10} (2 μ L diluted synthetic dsRNA added to 18 μ L molecular grade water).

The RNA concentrations for the ten-fold dilution series of synthetic dsRNA and TSRV positive control were determined spectrophotometrically at 260 nm, and the copy number of standard RNA molecules was calculated using the following formula: $(X \text{ g}/\mu\text{L RNA} / [\text{transcript length in nucleotides} \times 340]) \times 6.022 \times 10^{23} = Y \text{ molecules}/\mu\text{L}$. This formula gives the molecules per μ L (Y), if the concentration of the RNA (X) is known in relation to the transcript length in nucleotides multiplied by a factor derived from the molecular mass and the Avogadro constant. The qPCR assay was performed in duplicates for both the ten-fold dilution serial of synthetic dsRNA and the TSRV positive control according to section 2.2.6.1. The synthetic dsRNA standard curve was generated by plotting C_T value against the logarithm of the calculated initial copy numbers. The unknown initial TSRV

positive control copy numbers were then automatically calculated from their C_T values, from the synthetic dsRNA standard curve.

2.2.6.3 Limit of detection of the PCR tests

To estimate the analytical sensitivity of TSRV-specific conventional hemi-nested RT-PCR and qPCR, a limit of detection estimation was carried out before the intra-laboratory and inter-laboratory comparison study of diagnostic methods. Each PCR was run with: a) a ten-fold dilution of TSRV cell culture supernatant, b) a ten-fold dilution of negative tissues spiked with TSRV cell culture supernatant, c) negative samples as extracted negative controls, d) known positive samples from a natural infection as extracted positive control, e) PCR water as PCR negative control and f) plasmid as an internal control.

The stock TSRV (frozen stock used as positive control material for PCR assays with $TCID_{50}/mL$ of $10^{4.7 \text{ to } 5.0}$) cell-culture supernatant was grown on CHSE-214 until 100% CPE and viral titration was performed in accordance with the Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) to determine the 50% tissue culture infective dose, $TCID_{50}/mL$. $TCID_{50}/mL$ is the quantity of virus that will produce cytopathic effect (CPE) in 50% of the cultures inoculated. 96-well microplates (Nunc, Roskilde, Denmark) were seeded with CHSE-214 cells at a cell density of 250,000 cells/1.5 mL/well in appropriate growth medium. Cultures were incubated at 22°C with 5% CO_2 /95% air for 24 hours, after which they were checked by light microscopy before inoculation to ensure there was a minimum of 75% confluency with mitotic figures present, and also consistent cell density and morphology in all culture wells.

Aliquots (50 μ L) of appropriate maintenance medium were dispensed into each sample well using a multi-stepper pipette (plate columns 1 to 10) and 100 μ L dispensed into each control well (columns 11 and 12). A ten-fold serial dilution of virus was prepared in maintenance medium and then 50 μ L dispensed into each well of the appropriate plate columns. Plates were sealed and placed in plastic containers and cultures incubated at 15°C with 5% CO₂/95% air. Cultures were incubated for 10 days and examined using a compact inverted microscope (Olympus CKX31, United Kingdom) at 3, 7 and 10 days post-inoculation. Culture wells that demonstrated development of viral CPE was scored as positive (+), and the final titre expressed as tissue culture infective doses, TCID₅₀/mL. The TCID₅₀/mL was calculated according to the method of Reed and Muench (1938). The TSRV cell-culture supernatant of TCID₅₀/mL value of 10^{4.7} was used in both ten-fold dilutions.

A total of 200 μ L TSRV cell-culture supernatant was diluted in Elution Buffer to produce ten-fold dilutions. The diluted TSRV was used to spike negative samples (pooled kidney, liver and spleen) taken from healthy (uninfected) Atlantic salmon. The negative samples were homogenised in cell culture growth medium and centrifuged at 2000-4000 $\times g$ for 15 min at 5°C to clarify. Each 200 μ L aliquot of ten-fold dilutions of TSRV and spiked negative tissues were removed for viral RNA extraction using High Pure Viral Kit (Roche, Germany). cDNA was synthesised, and conventional semi-nested RT-PCR was performed according to section 2.2.5.1 and 2.2.5.2 of this chapter. qPCR was conducted as per section 2.3.6.1 in this chapter using the extracted viral RNA. Both PCRs were run separately for ten-fold dilution of TSRV and spiked negative tissues.

2.2.7 Statistical analysis

To compare the performance of each diagnostic test, statistical analyses were performed using a number of different approaches including gold-standard (Classical) and non gold-standard (Bayesian and Maximum Likelihood) analyses for tests undertaken at AHL. The classical approach involved the comparison of virus isolation (gold standard) with the molecular methods. The test evaluation against a gold standard was based on EpiTools - epidemiological calculators developed by AusVet Animal Health Services (<http://www.ausvet.com.au>). This analysis was used to estimate sensitivity, specificity and positive and negative likelihood ratios of tests by comparison with a known reference (gold standard) test. Results were presented as estimates of sensitivity and specificity with specified confidence limits and point estimates of positive and negative likelihood ratios.

The sensitivity and specificity of virus isolation was assumed to be 100%. ROC (receiver operator characteristic) analyses were performed using Microsoft Med-Calc Version 11.6.1.0 for the determination of cut-off C_T value of qPCR assay using virus isolation as a gold standard. The second statistical approach was carried out by utilizing a program freely accessible via web interface for the evaluation of test accuracy known as TAGS (Test evaluation in the Absence of Gold Standard) software (Pouillot *et al.*, 2002) ([http://www.epi.ucdavis.edu/diagnostic tests](http://www.epi.ucdavis.edu/diagnostic%20tests)). The algorithm in the TAGS software follows the frequentist paradigm and utilizes Newton-Raphson and expectation-maximization (EM) algorithms to generate maximum likelihood estimates of population prevalence, sensitivity and specificity of a diagnostic test, in the absence of a gold standard.

The TAGS software used maximum likelihood which included best assumption based on expert opinion of sensitivity for all the tests: conventional hemi-nested RT-PCR: 80%, virus isolation: 99% and qPCR: 99%. The third means of analysis was Bayesian analysis which used a Gibbs sampler, an iterative Markov-chain Monte Carlo (MCMC) technique which approximates the marginal posterior densities of sensitivity, specificity and prevalence in the absence of a gold standard (Frossling *et al.*, 2003). In the Gibbs sampling, prior probabilities (“priors”) of the parameters of interest are incorporated into the simulation. These probabilities are specified as beta distributions described by two variables (α and β) presented in the form Beta (α , β) (Gelman, 1995). Priors can be non-informative, a uniform distribution of between 0 and 1, or informative, a distribution achieved by using past data, or by using expert opinion, or a combination of both.

In this study, the Gibbs-sampler simulations were performed for two analyses, one using non-informative priors (uniform distributions set to Beta (1, 1) and the other using informative priors based on expert opinion. In using the informative analysis, sensitivity of all tests was assumed to be 95% and there was a 95% confidence that the sensitivity would be greater than 90%, and specificity was assumed to be 99% with 95% confidence that the specificity would be greater than 95%. Non-informative priors were used for the analysis of prevalence of T SRV in this study. Using the non-informative and informative priors, point estimates (medians of posterior distributions) and 95% Bayesian credible intervals (2.5% and 97.5% percentiles of posterior distributions) of sensitivity and specificity for virus isolation, conventional hemi-nested RT-PCR and qPCR were calculated.

Point estimates and 95% credible intervals of prevalence of infection for Tamar River and South-east Tasmania population were also calculated simultaneously. A burn-in phase of 5,000 iterations was used and inferences were made based on a subsequent 20,000 iterations. Burn-in refers to the standard practice to discard the initial iterations of iterative simulation as they are too strongly influenced by starting values and do not provide good information about the target distribution (Gelman and Shirley, 2011). Data management for Bayesian analysis was performed using Microsoft Excel 2003. Beta distributions were calculated using online software (<http://www.ausvet.com.au/epitools/content.php?=&BetaParams1>) provided by AusVet Animal Health Services (Sergeant 2004).

The Gibbs sampler was run using WinBUGS software (Imperial College and Medical Research Council) (Spiegelhalter *et al.*, 1996) version 2.1. There are three main assumptions that must not be violated when using Bayesian techniques. The first is that the target population should at least consist of two populations with different prevalence of diseases. To meet this requirement, samples were analysed based on the collections from two areas: Tamar River and South-east Tasmania. Based on previous surveys, there are differences in TSRV prevalence between the two areas (Gemma Carlile pers communication). The second assumes independence between the diagnostic tests and the third is that the sensitivity and specificity of diagnostic tests are assumed to be constant across populations (both characteristics of a test should be the same regardless of different prevalence of infection) (Enoe *et al.*, 2000).

The diagnostic tests were considered to be conditionally independent because different biological measures are evaluated in each test. The best assumption and prior probabilities (for both TAGS and Bayesian analyses) for PCRs were based on expert opinion from AFDL and AHL, DPIPWE (Mark Crane and Nick Moody, AFDL; Marianne Douglas

and Teresa Wilson, AHL, personal communication). Virus isolation detects replicating virus particles, conventional hemi-nested RT-PCR detects a nucleic acid sequence within segment 2 of the TSRV genomes while qPCR amplifies a sequence within segment 10 of the TSRV genome. Kappa and corresponding *p*-values were calculated to compare the performance of two tests evaluated using samples from the same population (Tasmanian population) for the inter-laboratory study. This analysis was based on EpiTools - epidemiological calculators developed by AusVet Animal Health Services (<http://www.ausvet.com.au>).

Kappa is a statistical measure that assesses the reliability of agreement beyond chance (Fleiss, 1981). Kappa value can range from -1 to +1. The Kappa statistic is normally interpreted as follows: a value of -1.0-0 represents no agreement, values of 0-0.20 indicate slight agreement, values of 0.21-0.40 show fair agreement, values of 0.41-0.60 indicate moderate agreement, values of 0.61-0.80 indicate substantial agreement, values of 0.81-1.00 indicate almost perfect agreement, and a value of +1 represents perfect agreement.

Predictive values quantify the probability that a test result for a particular animal correctly identifies the condition of interest. Thus, positive predictive value (PPV) stands for the proportion of test positive animals which really have the disease (true positives) and negative predictive value (NPV) stands for the proportion of test negative animals which are truly negative (true negatives). The positive predictive value and negative predictive value reflects the sensitivity and specificity of a test (Pfeiffer 2002). Thus, predictive values were obtained in relation to virus isolation (<http://statpages.org>).

2.3 Results

2.3.1 Characteristic viral cytopathic effect (CPE) caused by TSRV

Following inoculation of the CHSE-214 cell cultures, the cell monolayers showed regions of bullseye-like CPE, characterised by syncytia formation which subsequently lifted off the culture plate substrate and rolled into clumps that were observed floating in the supernatant at 10 days of post-infection (Figure 2.2) – typical of TSRV infection (Crane and Williams, 2008). Similarly, the EPC cell cultures showed formation of large plaque/syncytia as the infection progressed and syncytia lifted off the culture substrate at 11 days of post infection (Figure 2.3). The immunocytochemical test undertaken as a confirmatory test by AFDL confirmed that the cytopathic effect observed in both cell lines was due to the presence of TSRV.

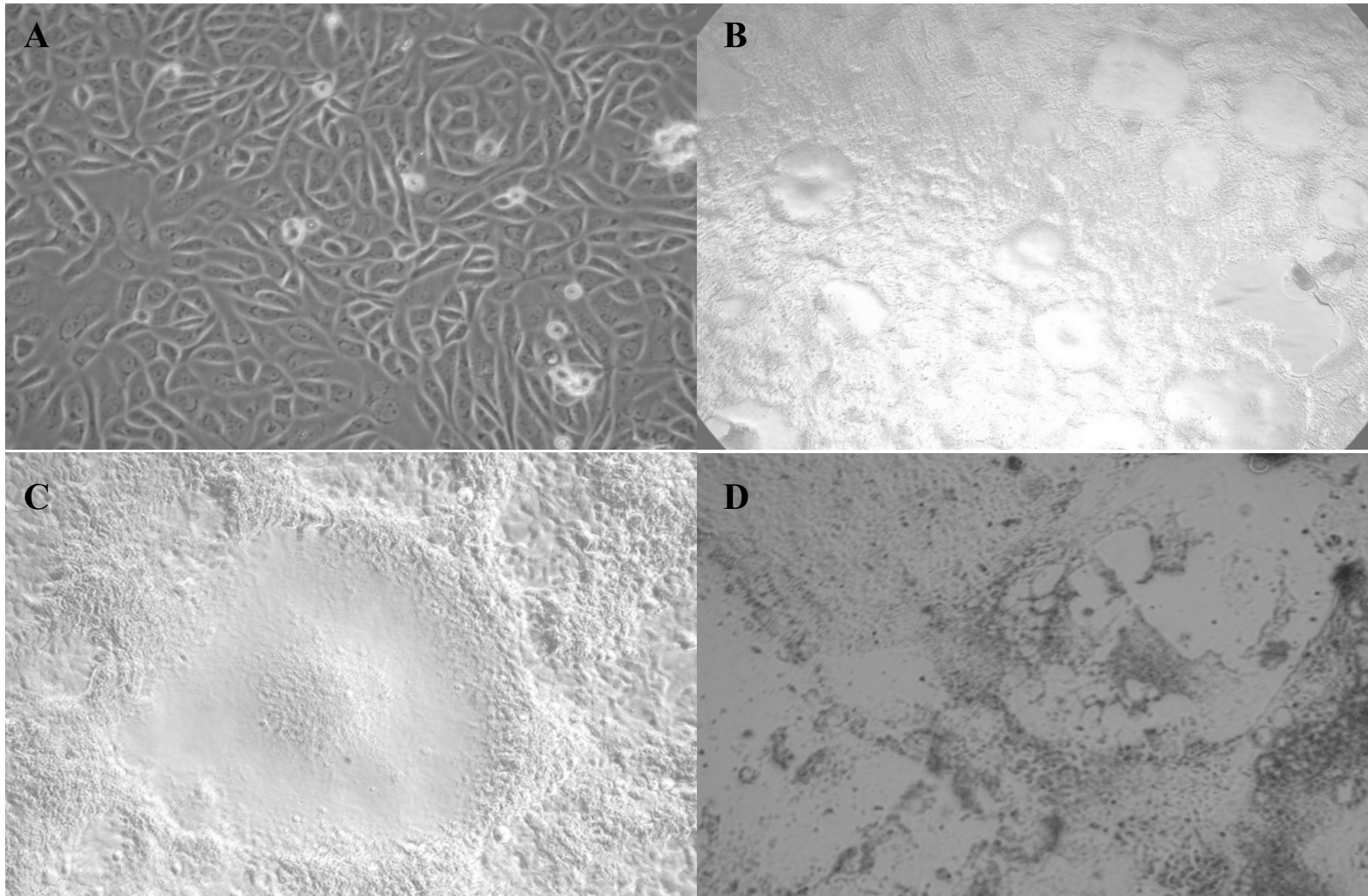


Figure 2.2 Photomicrographs of positive cytopathic effects (CPE) TSRV infection of CHSE-214 cell cultures: (A) Normal uninfected cells, (B) CPE at 9 days of post inoculation (dpi), (C) Large syncytial formation at 9 dpi at scale bars = 400 μm , (D) CPE at 10 dpi. (Scale bars = 100 μm).

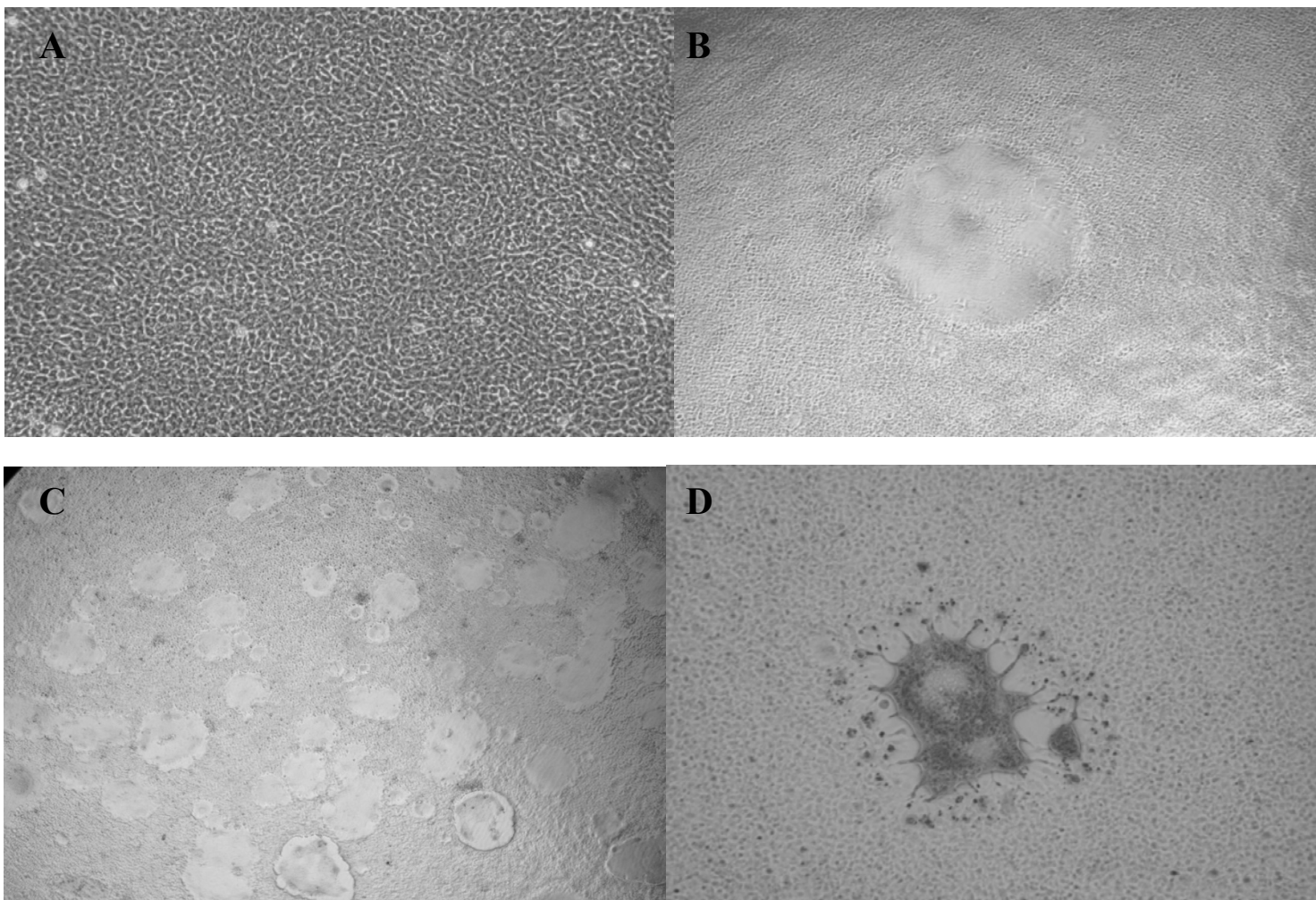


Figure 2.3 Photomicrographs of positive cytopathic effects (CPE) caused by TSRV infection of EPC cell cultures: (A) Normal uninfected cells, (B) Formation of plaque or syncytia at 9 days of post inoculation (dpi) (scale bars = 400 μm), (C) CPE at 10 dpi, (D) Syncytia lifting off the culture at 11 dpi. (Scale bars = 100 μm).

2.3.2 Inter-laboratory comparison of virus isolation on cell culture

The proportion of positive test results found using CHSE-214 cell cultures for both laboratories was higher than the proportion of positive test results found using EPC cell cultures (Table 2.2). Both cell lines showed significant Kappa agreement (Kappa agreement between CHSE-214 and EPC cell for each lab), AHL (0.698) and AFDL (0.678). The Kappa values for agreement (agreement between pooled results of cell lines) between AHL and AFDL was 0.9 indicating excellent inter-laboratory agreement.

Table 2.2 Inter-laboratory comparison of virus isolation, CHSE-214 cell and EPC cell between AHL (Mt.Pleasant, Tasmania) and AFDL (AAHL, Victoria), prevalence (Prev) estimation for each cell line and level of Kappa agreement. Kappa values represent the agreement between CHSE-214 cell and EPC cell for each laboratory.

		Positive	Negative	Prev (%)	Kappa
AHL	CHSE-214	22	122	15.2	0.698
	EPC	16	128	11.1	
AFDL	CHSE-214	20	124	13.8	0.6779
	EPC	11	133	7.6	
	ICC	23	121	15.9	

2.3.3 Inter-laboratory comparison of qPCR

qPCR demonstrated the highest prevalence of TSRV as shown by analyses undertaken in the two laboratories, AHL had higher prevalence (22.2%) than AFDL (12.5%) (Table 2.3). The Kappa value calculated for qPCR showed a substantial agreement between both laboratories.

Table 2.3 Inter-laboratory comparison of qPCR between AHL (Mt.Pleasant, Tasmania) and AFDL (AAHL, Victoria), prevalence (Prev) estimation for each PCR and level of Kappa agreement. Kappa value represents agreement of qPCR analyses between AHL and AFDL.

		Positive	Negative	Prev (%)	Kappa
AHL	qPCR	32	112	22.2	0.6667
AFDL	qPCR	18	126	12.5	

An excellent level of agreement of 0.8235 was demonstrated by the qPCR and virus isolation carried out in AHL compared to substantial Kappa agreement achieved by AFDL, 0.7686 (Table 2.4). These Kappa values represent the agreement between qPCR analysis and virus isolation for each laboratories.

Table 2.4 Level of agreement of qPCR with virus isolation (pooled results for both cell lines, CHSE-214 and EPC from each lab), AHL (Mt.Pleasant, Tasmania) and AFDL (AAHL, Victoria). The Kappa values represent the agreement between qPCR analysis and virus isolation for each laboratories.

		Positive	Negative	Prev (%)	Kappa
AHL	qPCR	32	112	22.2	0.8235
AFDL	qPCR	18	126	12.5	0.7686

2.3.4 Intra-laboratory comparison of diagnostic methods

A total of 24 positive results were obtained using virus isolation (on cell cultures of CHSE-214 and EPC cell lines) whereas conventional hemi-nested RT-PCR detected the lowest number of positive results, 14 (includes two false positive samples which were not positive by the other two diagnostic tests). qPCR demonstrated the highest proportion of positive results, 32 of the 144 Atlantic salmon tested. Nine samples were classified positive by all the diagnostic tests and qPCR yielded an additional five positives (Table 2.5).

Table 2.5 Intra-laboratory comparison of virus isolation (pooled results for both cell lines, CHSE-214 and EPC), conventional hemi-nested PCR (RT-PCR) and qPCR at Mount Pleasant Laboratories, Tasmania. + = positive, - = negative. Pop.1: Tamar River Pop.2: South-east Tasmania.

Virus isolation	RT-PCR	qPCR	Pop 1	Pop 2	Total
+	+	+	0	9	9
-	+	+	2	1	3
+	-	+	2	13	15
-	-	+	2	3	5
+	+	-	0	0	0
-	+	-	2	0	2
+	-	-	0	0	0
-	-	-	25	85	110
24*	14*	32*	33	111	144

* Total positive results for each diagnostic test

The results for conventional hemi-nested RT-PCR showed a lower prevalence of TSRV, 9.7% than the results of qPCR. Kappa value showed the level of agreement between PCR and virus isolation. Conventional hemi-nested RT-PCR had a moderate level of agreement of 0.4 with virus isolation compared to qPCR which had an excellent level of agreement (Table 2.6). Conventional hemi-nested RT-PCR by AFDL detected 9 out of 14 positive qPCR samples (Appendix I).

Table 2.6 Intra-laboratory comparison of conventional hemi-nested PCR (RT-PCR) and qPCR at Mt.Pleasant Laboratories, Tasmania, prevalence (Prev) estimation for each PCR and level of Kappa agreement with virus isolation. Kappa value showed the level of agreement between each PCR and virus isolation.

	Positive	Negative	Prev (%)	Kappa
RT-PCR	14	130	9.7	0.4
qPCR	32	112	22.2	0.8235

Predictive values for conventional hemi-nested RT-PCR and qPCR were obtained relative to virus isolation (VI) as VI is the current method of choice for detection of TSRV. The positive predictive value (PPV) for conventional hemi-nested RT-PCR was moderate: 0.643 (CI: 0.409-0.826) (Table 2.7). The positive predictive value (PPV) for qPCR was high: 0.750 (CI: 0.640-0.750) (Table 2.8). The negative predictive values (NPV) for both tests were high. The NPV for conventional hemi-nested RT-PCR was 0.885 (CI: 0.859-0.904) (Table 2.7) and for qPCR was 1.000 (CI: 0.976-1.000) (Table 2.8).

Table 2.7 Intra-laboratory comparison of conventional hemi-nested PCR (RT-PCR) to virus isolation with positive predictive value (PPV), negative predictive value (NPV), and 95 % confidence intervals (95% CI) of conventional hemi-nested PCR. + = positive, - = negative.

Virus isolation	RT-PCR	Total
+	+	9
+	-	15
-	+	5
-	-	115
PPV (95% CI)	0.643 (0.409-0.826)	
NPV (95% CI)	0.885 (0.859-0.904)	

Table 2.8 Intra-laboratory comparison of Real-time PCR (qPCR) to virus isolation with positive predictive value (PPV), negative predictive value (NPV), and 95% confidence intervals (95% CI) of conventional hemi-nested PCR. + = positive, - = negative.

Virus isolation	qPCR	Total
+	+	15
+	-	0
-	+	17
-	-	112
PPV (95% CI)	0.750 (0.640-0.750)	
NPV (95% CI)	1.000 (0.975-1.000)	

2.3.4.1 Limit of detection for the PCR tests

The analytical sensitivity of the TSRV - specific conventional hemi-nested RT-PCR and qPCR assays were evaluated by testing different types of template: TSRV cell culture supernatant, negative tissues spiked with TSRV cell culture supernatant, known positive tissue from a natural infection and plasmid positive control. The diagnostic methods were able to detect TSRV from a TCID₅₀/mL value of 10^{4.7} down to a dilution of: virus isolation (1:1000), conventional hemi-nested RT-PCR: TSRV cell culture supernatant (1:10 000) and spiked tissues (1:1000), qPCR: TSRV cell culture supernatant (1:100 000) and spiked tissues (1:10 000). The results were consistent with the limit of detection assay conducted by Carlile (2011) in which the diagnostic methods were able to detect TSRV from a original concentration of 6.32×10⁴ TCID₅₀/mL, to as low as 1.26×10² TCID₅₀/mL (1:100) for virus isolation, 1:10 000 for conventional hemi-nested RT-PCR and 1:100 000 000 for qPCR.

2.3.4.2 Determination of cut-off value for Real-time RT-PCR Assay

Samples with qPCR C_T values between 20 and 28 (high viral load) showed CPE in primary cultures in both cell lines between 4-8 dpi, whereas, samples with C_T values between 29 and 36 (low viral load) showed CPE in primary cultures in both cell lines between 11-21 dpi. Samples with C_T values of 36-45 did not produce CPE in either by one or both cell lines (Table 2.9).

Table 2.9 Determination of cut-off value for qPCR assay based on intra-laboratory comparison with virus isolation (v.i) (pooled results for both cell lines, CHSE-214 and EPC).
+ = positive v.i, positive qPCR, - = negative v.i, positive qPCR.

C_T value +	C_T value -		
Virus isolation +	Virus isolation -	Cut-off	
qPCR +	qPCR -		
20.06		C_T value < 35.8	Positive result
25.06		C_T value $35.8 < A < 36.13$	Ambiguous values
25.28	43.04	C_T value > 36.13	Negative result
26.56	41.8		
27.34	40.49		
28.81	40.18		
29.09	39.35		
29.11	38.65		
29.44	38.13		
29.59	37.96		
30.12	37.92		
32.02	37.39		
32.87	36.96		
33.47	36.76		
34.72	36.67		
35.33	36.25		
35.8	36.13		

ROC curve analysis was used to evaluate the discriminatory power of the qPCR assay (Figure 2.4). Of the 32 positive samples (based on qPCR result), qPCR assay classified 17 as positive relatively to virus isolation, that is, having a C_T value < 35.8 (Figure 2.3 A). If the 32 samples (based on qPCR results) were considered as positives, the cut off value C_T value would be at 43.04 (Figure 2.3 B). The qPCR assay obtained the highest combined diagnostic sensitivity, 100 (95% CI: 79.4 to 100.0) and specificity, 100 (95% CI: 97.1 to 100.0), at a cycle threshold value C_T of 35.8. In contrast, the qPCR assay obtained a lower specificity 94.92 (95% CI: 89.3 to 98.1) and similar sensitivity, 100 (95% CI: 86.3 to 100.0) at a cycle threshold value C_T of 43.04.

The area under the ROC curve is a global summary statistic of the diagnostic accuracy. Plots for diagnostic tests with perfect discrimination between negative and positive samples (i.e. no overlap of values of the 2 groups) pass through the coordinates 0 and 1, and represent 100% sensitivity and specificity. In accordance with an arbitrary guideline, one could distinguish between non-informative ($AUC = 0.5$), less accurate ($0.5 < AUC \leq 0.7$), moderately accurate ($0.7 < AUC \leq 0.9$), highly accurate ($0.9 < AUC < 1$) and perfect tests ($AUC = 1$) (Greiner *et al.*, 2000). The AUC of the qPCR assay was 0.988 at cut-off C_T value of 43.04 and 1.0 at cut-off C_T value of 35.8. Hence, this assay can be considered a highly accurate test.

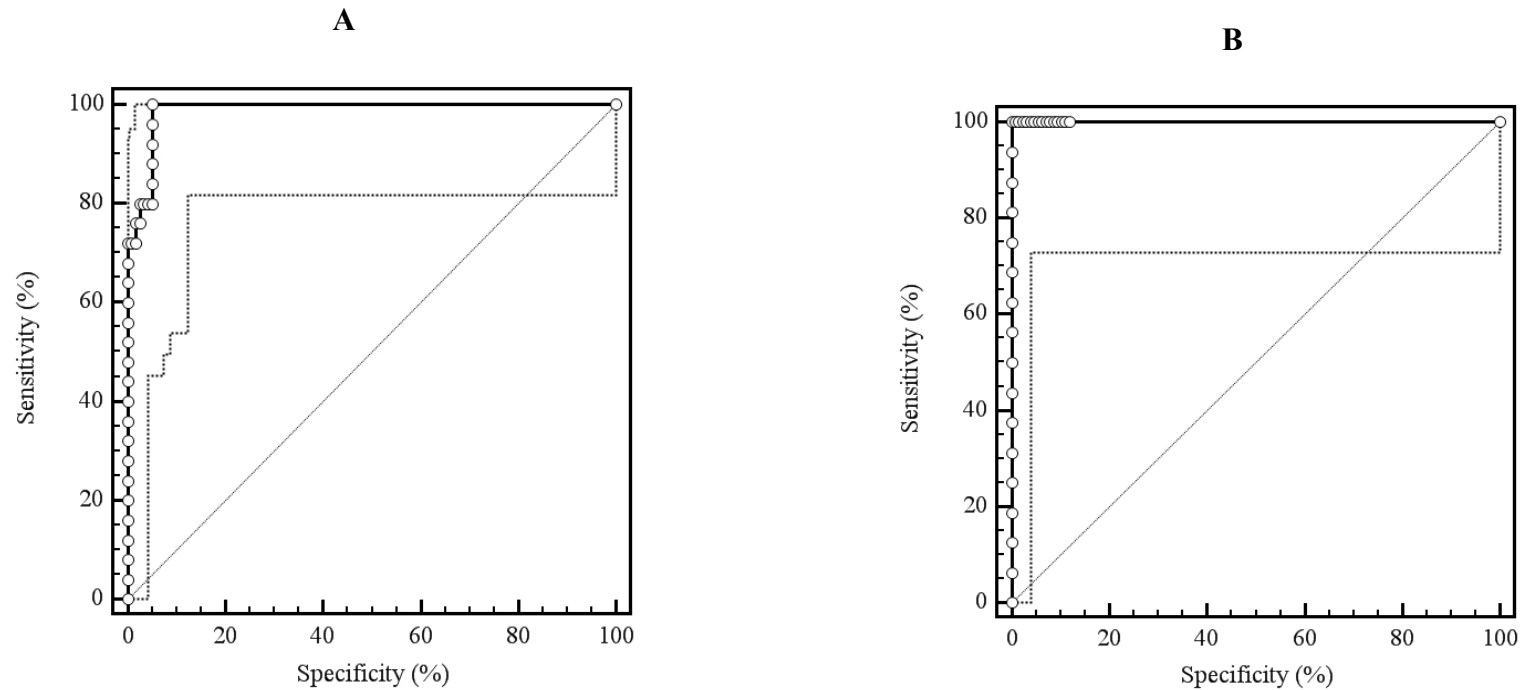


Figure 2.4 Receiver operator characteristic (ROC) curves (solid lines). The 95% confidence interval is indicated (fine dotted line). For reference, a ROC curve for a test that cannot discriminate between positive and negative samples is also shown (thick dashed line). **A:** Combined diagnostic sensitivity, 100 (95% CI: 86.3 to 100.0) and specificity, 94.92 (95% CI: 89.3 to 98.1), were obtained at a cycle threshold value C_T of 43.04 (see Fig. 2.6) **B:** The highest combined diagnostic sensitivity, 100 (95% CI: 79.4 to 100.0) and specificity, 100 (95% CI: 97.1 to 100.0), were obtained at a cycle threshold value C_T of 35.8 (see Fig. 2.6).

The interactive dot diagram (Figure 2.5) was used to illustrate the best separation (minimal false negative and false positive results) between the positive and negative samples as determined by virus isolation. At a 35.8 C_T cut-off value, shown as a horizontal line in Figure 2.5 B, the qPCR assay did not produce any false positive or false negative samples. However, the qPCR produced 6 ‘false positive’ in negative samples at a 43.04 C_T cut-off value (Figure 2.5 A).

A higher specificity is generally required for a confirmatory test. Figure 2.6 shows results for sensitivity and specificity for the qPCR assay at all possible cut-off values. Hence, the cut-off C_T value for qPCR assay was determined at 35.8 due to higher combined sensitivity and specificity of qPCR assay and better separation between positive and negative samples compared to cut-off C_T value of 43.04. C_T values lower than 35.8 were considered positive; C_T values greater than 36.13 were considered negative. C_T values in the range of 35.8-36.13 are classified as ‘ambiguous’ and the PCR assay for those samples were repeated to avoid false positives.

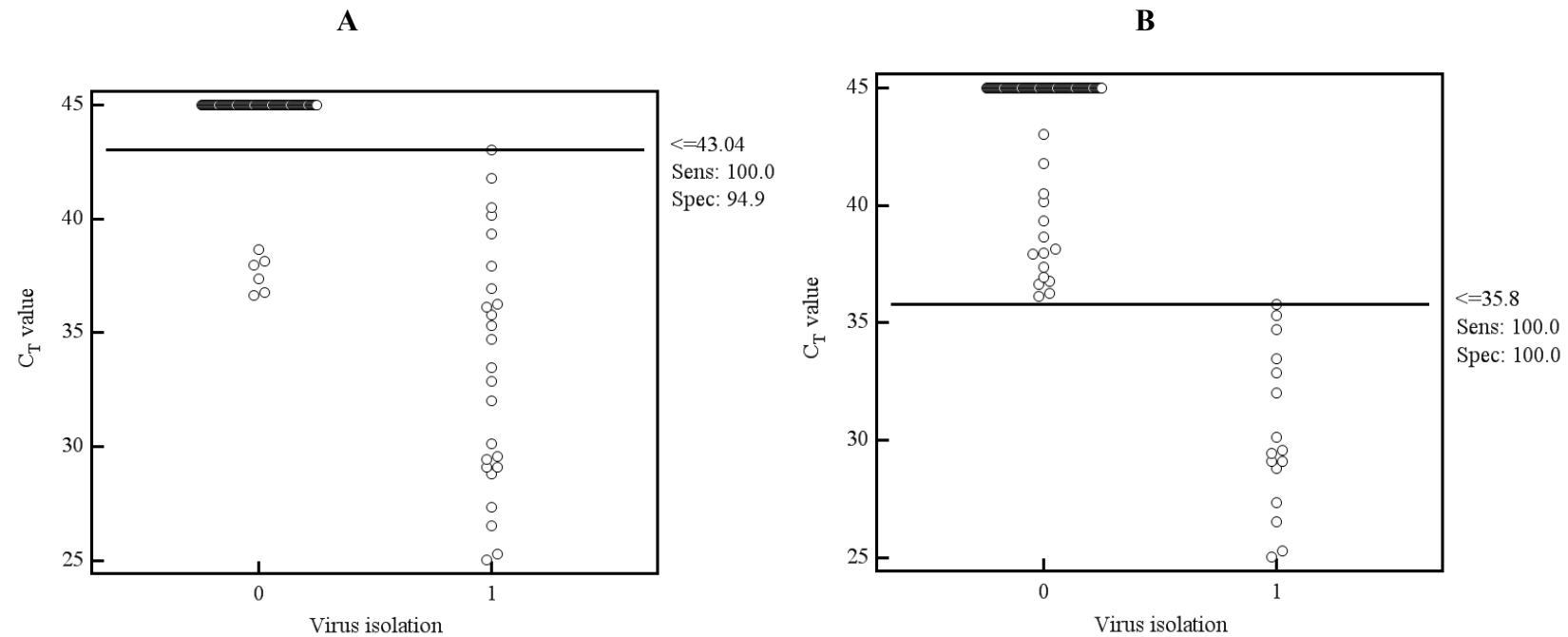


Figure 2.5 The relationship between virus isolation and qPCR assay results illustrated by interactive dot diagrams. **A:** This illustrates the separation (with false positives) between the positive and negative groups at a cut-off value of 43.04 the cycle threshold (C_T) (horizontal solid line) **B:** This illustrates the best separation (minimal false negative and false positive results) between the positive and negative groups at a higher cut-off value of 35.8 for the cycle threshold (C_T) (horizontal solid line). For virus isolation: 0 = negative, 1 = positive.

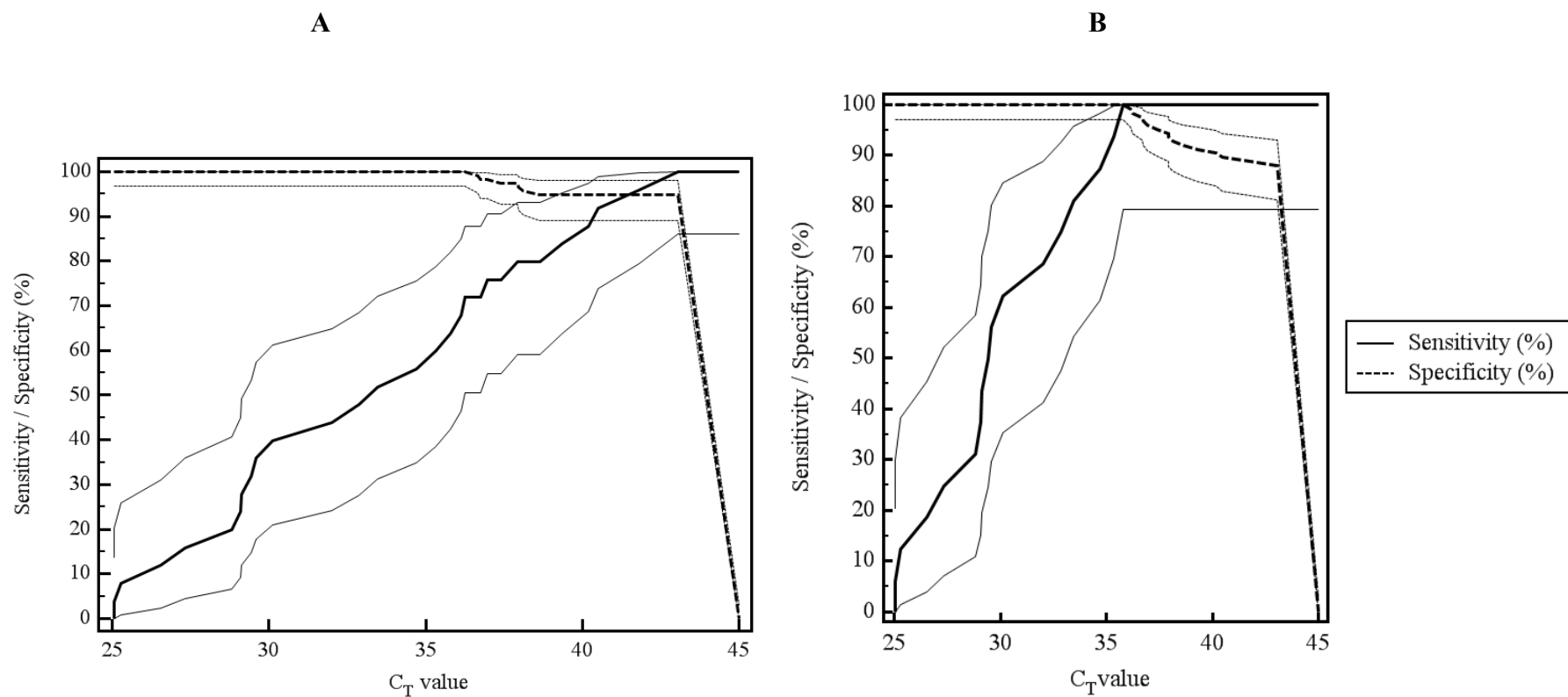


Figure 2.6 P lot versus c riterion graph illu strates th e c hanges in d iagnostic s ensitivity (thick s olid lin e) a nd s pecificity (thick d ashed l ine), including their 95% confidence intervals (sensitivity: solid fine lines; specificity: dashed fine lines), at different cut-off levels (**A**: 43.04 and **B**: 35.8) for the cycle threshold (C_T).

2.3.4.3 Absolute quantification of qPCR with synthetic dsRNA

Absolute quantification of qPCR was carried out using a synthetic dsRNA (RNA standard) as target to determine the viral copy numbers of TSRV in positive control material. The viral copy numbers (molecules/ μ L) was determined using a standard curve constructed from the serial dilution of TSRV positive control material and synthetic dsRNA. Table 2.10 shows the mean C_T value, standard deviation and RNA concentration (ng/ μ L) of the tenfold dilution series of TSRV positive control material and synthetic dsRNA. The dynamic range was wide (at least eight orders of magnitude) and both materials had a similar starting C_T values, 14.0. A strong linear relationship (slope = 3.3) with a correlation coefficient of $r^2 > 0.99$ was observed for both linear curves (Figure 2.7), generated when C_T value was plotted against the logarithm of the ten-fold dilutions.

The amplification efficiencies (E) of TSRV positive control and synthetic dsRNA were calculated according to the equation: $E = 10^{1/m} - 1$, where m is the slope of the linear curve and was found to be 98.0% and 98.3% respectively. The standard curve was generated for synthetic dsRNA and C_T values were plotted against calculated viral copy numbers (molecules/ μ L), with the initial viral copy numbers of 1.943×10^{10} molecules/ μ L (Figure 2.8). The detection limit of TSRV positive material (1.943×10^4 molecules/ μ L) was equivalent to synthetic dsRNA which was detected to 1.943×10^3 molecules/ μ L.

Table 2.10 Mean C_T values, standard deviation (S.D) and RNA concentration of ten-fold dilution of TSRV positive control synthetic dsRNA. TSRV 10e-1 till 10e-10: ten-fold dilution of TSRV positive control, synthetic dsRNA 10e-1 till 10e-10: ten-fold dilution of synthetic dsRNA, ND: Non-detectable.

Sample	Mean C_T values	S.D	RNA concentration (ng/ μ L)
TSRV 10e-1	14.35	0.18	302.56
TSRV 10e-2	17.9	0.06	24.69
TSRV 10e-3	21.6	0.22	2.6
TSRV 10e-4	25.02	0.12	ND
TSRV 10e-5	28.66	0.18	ND
TSRV 10e-6	31.83	0.25	ND
TSRV 10e-7	35.13	0.06	ND
TSRV 10e-8	37.63	ND	ND
TSRV 10e-9	ND	ND	ND
TSRV 10e-10	ND	ND	ND
Synthetic 10e-1	14.01	0.41	98.06
Synthetic 10e-2	17.68	0.33	8.02
Synthetic 10e-3	20.86	0.07	1.78
Synthetic 10e-4	24.27	0.40	ND
Synthetic 10e-5	28.02	0.46	ND
Synthetic 10e-6	31.16	0.08	ND
Synthetic 10e-7	34.01	0.19	ND
Synthetic 10e-8	35.8	ND	ND
Synthetic 10e-9	ND	ND	ND
Synthetic 10e-10	ND	ND	ND

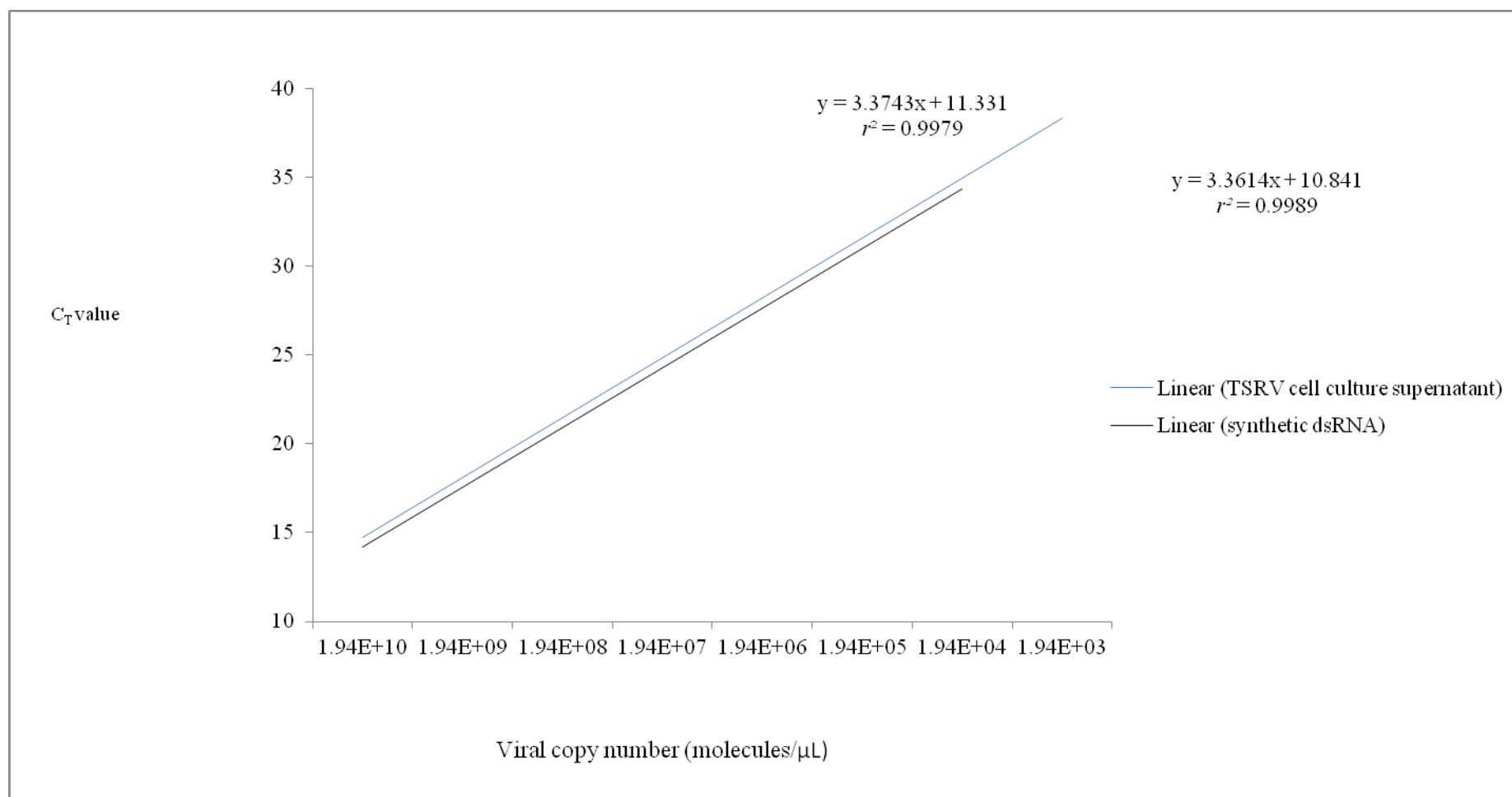


Figure 2.7 Relationship between C_T values (qPCR) and viral copy numbers (molecules/μL) obtained for ten-fold dilution of TSRV positive control (cell-culture supernatant) and synthetic dsRNA. Both linear curves showed good linear correlation (slope = 3.3) and high correlation coefficient ($r^2 > 0.99$).

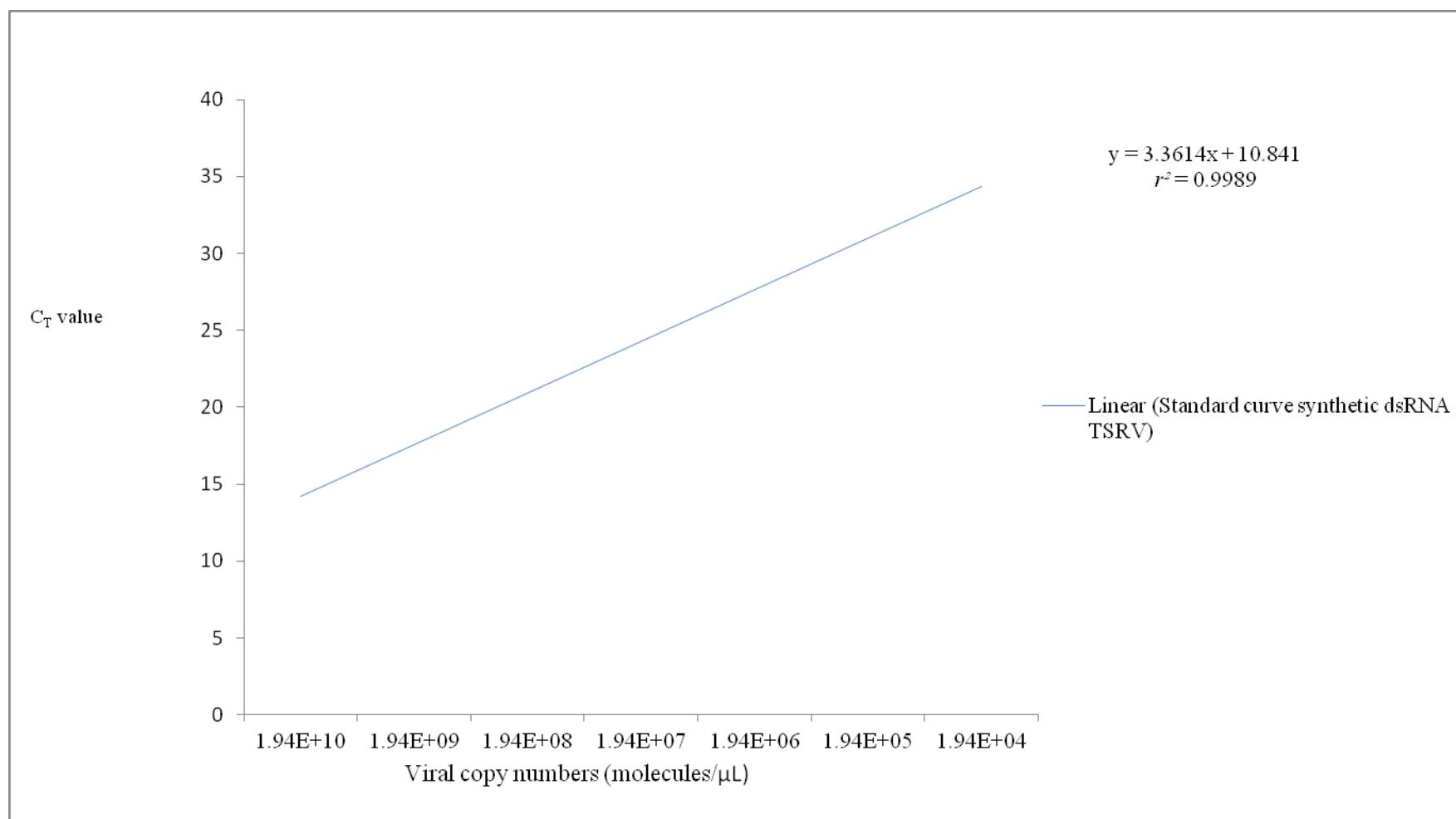


Figure 2.8 Standard curve generated for absolute quantification of T SRV positive control material (cell-culture supernatant) with synthetic dsRNA. C_T values plotted against calculated viral copy numbers (molecules/μL), with the initial copy numbers of 1.943×10^{10} molecules/μL.

2.3.5 Estimation of sensitivity and specificity of diagnostic methods

Three different approaches were used during the study to determine the prevalence of TSRV in each population and the sensitivity and specificity of each diagnostic method (Table 2.11). Analyses using TAGS software estimated a higher prevalence of infection than the Bayesian and Classical analyses in Population 1. The prevalence of TSRV in Population 2 was not significantly different between the analyses. The variation of prevalence between both populations could be due to the different numbers of samples collected from each region, Tamar River (33) and South-east Tasmania (111).

The TAGS analysis also estimated lower sensitivities of diagnostic tests than the Bayesian and Classical analyses except for qPCR which has a higher sensitivity of 99.0%. The classical and TAGS analyses estimated similar sensitivity for conventional hemi-nested RT-PCR. All the analyses showed high specificities for the diagnostic tests for TSRV detection in contrast to substantial differences of sensitivities. Bayesian analysis showed significant (statistically) differences of sensitivity for conventional hemi-nested RT-PCR between informative and non-informative priors distribution. The analyses estimated significantly higher sensitivity and specificity for qPCR in comparison to virus isolation and conventional hemi-nested RT-PCR.

Table 2.11 Comparison of estimation of sensitivity (Se) and specificity (Sp) of virus isolation, conventional hemi-nested PCR and qPCR by different statistical approaches and prevalence estimates (Prev) and 95% confidence intervals (95% CI) of two populations for each statistical approach at Mount Pleasant Laboratories, Tasmania. TAGS: Test evaluation in the Absence of Gold Standard (Pouillot *et al.*, 2002). Pop.1: Tamar River Pop.2: South-east Tasmania.

Analysis	Test	Se (%)	Sp(%)	Prev (%)	
		(95% CI)	(95% CI)	(95% CI)	
				Pop.1	Pop.2
Classical Approach	Virus isolation	100 (85.7-100)	100 (96.9-100)	6.1 (1.7-19.6)	19.8 (13.5-28.2)
	Hemi-nested PCR	37.5 (18.8-59.4)	95.8 (90.5-98.6)		
	qPCR	100 (85.7-100)	93.3 (87.3-97.1)		
TAGS	Virus isolation	78.4 (47.8-93.5)	99 (97.5-100)	16.1 (0.05-0.39)	22.8 (0.15-0.32)
	Hemi-nested PCR	39 (22.2-58.9)	98.2 (93.1-99.55)		
	qPCR	99 (97.5-100)	98.7 (27.4-99.0)		
Bayesian analysis (Informative)	Virus isolation	93.6 (88.6-97.33)	99 (97.0-99.8)	7.91 (0.01-0.19)	21 (0.13-0.29)
	Hemi-nested PCR	78.1 (70.6-84.8)	98.9 (96.9-99)		
	qPCR	95.2 (90.9-98.2)	95.2 (91.6-97.8)		
Bayesian analysis (Non-Informative)	Virus isolation	87.4 (62.0-99.6)	99.1 (96.0-99.0)	6.28 (0.001-0.23)	22.1 (0.14-0.30)
	Hemi-nested PCR	5.67 (0.16-18.6)	97.9 (94.0-99.0)		
	qPCR	95.9 (84.6-99.8)	92.8 (86.3-99.0)		

2.4 Discussion

While the use of molecular techniques is becoming more common and widespread (Overturf *et al.*, 2001; Mackay *et al.*, 2002; Niesters *et al.*, 2002; De la Vega *et al.*, 2004; Gilad *et al.*, 2004; Munir and Kibenge., 2004; Purcell *et al.*, 2006; OIE Manual, 2011), it is generally accepted that, for most viruses that are culturable, virus isolation is considered the preferred diagnostic method for detection of viral infections (OIE, 2011). It is for this reason that surveillance for TSRV infections in Atlantic salmon populations farmed in Tasmania is undertaken by virus isolation (VI) on fish cell lines (Kevin Ellard pers communication). Recently, however, some molecular (PCR) tests have been developed for the detection of TSRV (Gemma Carlile pers communication) which has raised the question whether these tests, which are faster, less expensive and possibly more accurate than VI, would be suitable for a surveillance program.

Thus the aim of this study was to determine which technique, classical virus isolation, or one of the more recently developed conventional hemi-nested RT-PCR and qPCR techniques would be the most sensitive and specific for diagnosis of TSRV infections in farmed populations of Atlantic salmon in Tasmania. To achieve this aim, two geographically separated populations of farmed Atlantic salmon were chosen as the source of samples used to evaluate each of the tests. Within these two regions, a total of 144 fish from 9 sites (12-33 fish per site) were sampled. While this number of samples is relatively low for test evaluation (OIE Manual 2011), the logistics of sample collection and processing for three different diagnostic tests were limiting and it was anticipated that this number would be sufficient for, at least, a preliminary analysis with statistical robustness.

In addition to comparing each of the three techniques within a single laboratory (intra-laboratory comparison), duplicate samples were submitted to a second laboratory for testing (inter-laboratory comparison) thereby providing an indication of reproducibility between laboratories. Newly developed molecular methods should be standardised and validated in comparison with the existing conventional methods (OIE, 2011). Validation is important for diagnostic tests because it determines the fitness of the assays and includes estimates of the analytical and diagnostic performance characteristics of the tests (OIE, 2011). Validation of a new test is a complex procedure which includes the development, evaluation and standardising of the test method; determining the repeatability; determining diagnostic and specificity and sensitivity; determining reproducibility; and statistical analysis of the results (OIE, 2011).

Diagnostic tests applied to individuals or populations aid in: documenting freedom from disease in a country or region, preventing spread of disease through trade, eradicating an infection from a region or country, confirming diagnosis of clinical cases, estimating infection prevalence to facilitate risk analysis, and classifying animals for herd health or immune status post-vaccination (OIE, 2011). The capacity of a positive or negative test result to predict accurately the infection and exposure status of the animal or population is the ultimate consideration of assay validation (OIE, 2011). Thus, diagnostic tests should be validated as the sensitivity and specificity estimates assist in determining the true status of disease and updating clinical inferences. Therefore, inter-laboratory comparison between Australian Animal Health Laboratory (AAHL) and Animal Health Laboratory, Mount Pleasant Laboratories (DPIPWE) emphasized the robustness (reproducibility) of newly developed conventional hemi-nested RT-PCR and qPCR for the detection of T SRV. A

molecular method that is robust and reliable for the detection of viral RNA in field samples is beneficial for both laboratories which regularly receive samples of this type.

The good inter-laboratory correlation for virus isolation and molecular methods for the detection of TSRV proof-read the results obtained for the intra-laboratory comparison of diagnostic methods. Based on the classical approach, the intra-laboratory comparison of diagnostic methods showed a similar trend to the inter-laboratory study in which qPCR was the most sensitive method and correlated well with virus isolation to the extent that all the positive samples by virus isolation were positive by qPCR. Abayneh *et al* (2010) concluded that qPCR was the most sensitive method for the detection of ISAV as it detected the most positives and correlated well with virus isolation. Virus isolation is less sensitive than qPCR and conventional hemi-nested PCR was the least sensitive method for detection of TSRV infections with only 14 positive samples.

Overall, qPCR assay had a high sensitivity and specificity in all the analyses, with a sensitivity of 95.2% and a specificity of 95.2%. All the diagnostic tests had high specificity for the detection of TSRV contrary to significant differences of estimated sensitivities between all of them and 95% CI of the sensitivities are much wider than those of the specificities. This could be due to two reasons, less room for variation is generated while the specificity of diagnostic methods is commonly close to 100% and estimated prevalence for the two sets of populations are small, thus sensitivity is estimated from a narrow range of the data (Abayneh *et al.*, 2010).

The high specificities of diagnostic tests are concurrently consistent with high negative predictive value obtained relative to virus isolation. Virus isolation is the most specific (99.0%) whereas qPCR is the most sensitive (95.2%) among the diagnostic tests. The estimated sensitivity of diagnostic methods varies substantially among the different statistical approaches except for qPCR assay. The classical approach over-estimated both test characteristics for virus isolation (gold standard) and sensitivity of qPCR compared to the other statistical analyses. In addition, this approach underestimated specificities of conventional hemi-nested PCR, qPCR and sensitivity of the former test. The prevalence estimation and 95% confidence interval by classical approach was consistent with the Bayesian analysis.

According to Basanez (2004), evaluation of diagnostic tests in the presence of a gold standard does underestimate prevalence, sensitivity and overestimates specificity. It has been reported that the assessment of diagnostic tests with a gold standard underestimated the prevalence of the disease, sensitivity and specificity (Alonzo and Pepe, 1999; Enoe *et al.*, 2000). The limitations portrayed by classical approach are that no gold standard is 100% accurate due to occurrence of measurement errors (Joseph *et al.*, 1995), a gold standard (in this study: cell culture) is nearly 100% specific but less than 100% sensitive (Alonzo and Pepe, 1999) and introduces biases in accuracy estimates of a new diagnostic test against the use of an imperfect gold standard (Alonzo and Pepe, 1999).

Bayesian analysis with prior information is more acceptable than the other statistical analyses for the validation of diagnostic tests. It has been demonstrated that a Bayesian analysis performs better than maximum likelihood-frequentist paradigm and classical approach in estimating prevalence of an infection, sensitivity and specificity of diagnostic

tests (Joseph *et al.*, 1995; Enoe *et al.*, 2000; Dunson, 2001; Basanez *et al.*, 2004; Geurden *et al.*, 2004). According to Dunson (2001), Bayesian models can easily accommodate unobserved variables such as an individual's true disease status in the presence of diagnostic errors. It has been established that this approach can produce improved inference with substantive prior information and accountable posterior probabilities of test characteristics (Joseph *et al.*, 1995 and Dunson, 2001), while maximum likelihood approach often gives crude estimates and standard errors (Basanez *et al.*, 2004). Prior probabilities of a test's sensitivity, specificity and prevalence estimate are chosen based on available evidence (expert's opinion & data from laboratory trials) to obtain subsequent probabilities, and this likelihood of the diagnostic test result (conditional on latent disease status) is incorporated into the analysis (Dunson, 2001).

The use of maximum likelihood-frequentist approach leads to results that depend on the truly unknown parameters (Se, Sp and Prev) which are chosen as known/best assumption and contains less degrees of freedom, while Bayesian approach takes into account all the unknown parameters and includes all degrees of freedom, thus minimising standard errors (Basanez *et al.*, 2004). Classical approach, TAGS and Non-informative Bayesian analyses showed large interval widths of 95% confidence limits for the estimation of all the diagnostic tests, resulting in less precise estimations of sensitivity, specificity and prevalence compared to the narrower 95% confidence limits obtained by Informative Bayesian analysis.

These results are consistent with Joseph *et al.* (1995) who demonstrated that non-informative analysis is undesirable as it results in wider interval estimation for all parameters and depending on whether the prevalence in a population is high/low; data will contain relatively less information on specificity and sensitivity. It has been reported that large

samples are required to achieve valid CIs based on maximum-likelihood approach contrary to Bayesian analysis (informative) which provides point estimates and intervals without the need of a large sample size (Enoe *et al.*, 2000 and Wallace *et al.*, 2008). If the sample size is large, Bayesian analysis using vague priors could yield results that are quite similar to maximum likelihood-based inferences (Dunson, 2001), and possibly larger numbers of samples are needed in order to obtain equivalent estimates of test characteristics between both analyses. If only either one of the statistical approaches (classical, TAGS or Non-informative Bayesian analyses) was performed, the estimation of the prevalence, sensitivity and specificity would be either underestimated or overestimated. Taking the advantages and disadvantages of the statistical approaches into consideration, both gold standard and non-gold standard approaches should be conducted to determine the best estimate of diagnostic tests characteristics.

Virus isolation is the most specific diagnostic method for the detection of TSRV. The intra-laboratory comparison result for virus isolation included pooled results using two cell lines, CHSE-214 and EPC. A sample was considered to be positive if virus was detected on either one or both cell lines; which increased the sensitivity of virus isolation. The lineage of the CHSE-214 cell line used in this study was found to be more sensitive in isolating TSRV from field samples in both laboratories than the lineage of the EPC cell line used. Following infection with TSRV, both cell lines showed formation of large syncytia/plaques which detached from the substratum as the infection progressed, a common cytopathic effect of aquareoviruses in epithelial and fibroblast-like cell lines (Winton *et al.*, 1987; Samal *et al.*, 1998; Cusack *et al.*, 2001). It is interesting to note that Cusack (2001) found that CPE was only produced in CHSE-214 cell cultures in comparison to EPC cell cultures in a study of aquareovirus in Atlantic halibut. Moreover, the lineage of CHSE-214 used in the present

study is the most sensitive cell line for the isolation of IPN-like virus (Crane *et al.*, 2000). In considering sensitivity to viral infection, it must be noted that sensitivities of cell lines to the various viral pathogens of finfish can vary between laboratories for any number of reasons. For example, during the maintenance of stock cell cultures by serial passage, cells are under constant selection pressure imposed by the culture conditions which are potentially highly variable (Nicholson, 1985; Freshney, 2000).

Important factors which can influence the survival of sub-populations of cells within a lineage are the type and batch of FBS used, the source of the cell culture medium used, temperature of incubation, the method of sub-culture, the time interval between sub-culture and so on (Nicholson, 1985; Freshney, 2000). An immunocytochemical test (using a polyclonal antiserum raised against T SRV) was conducted by AFDL as a confirmatory test for the presence of T SRV in association with the cytopathic effect exhibited by the cell cultures incubated with the test samples. A confirmatory test (such as an immunoassay or a nucleic acid-based test such as PCR) is required in most cases, since presence of CPE is not necessarily virus-specific. Some CPE can be induced by non-specific cytotoxicity of the test sample, rather than viral infection, but can be confused with viral infection (Nerette *et al.*, 2005). Cytotoxicity of samples can be influenced by the diet or environment (e.g. presence of toxic algae) of the fish from which the sample has been sourced. To an inexperienced operator microbial contamination can also be confused with viral CPE (Freshney, 2000).

Substantial agreement as shown by Kappa achieved between CHSE-214 and EPC cells shows that both cell lines are suitable for the isolation and replication of T SRV. An excellent inter-laboratory Kappa agreement of 0.9 confirmed the accuracy, precision and sensitivity of virus isolation results in this study. The lineages of the two cell lines originated

from the same stocks (the stocks were kindly supplied by AFDL and cultured by Sandra Zainathan at DPIPWE), which also contributed to the agreement between both laboratories. The difference in prevalence of positive samples in the population tested by the two laboratories could be caused by the heterogeneity of the virus distribution in tissue samples. The uneven distribution of the virus in the same sample played a minor role as both cell lines had excellent inter-laboratory agreement, similar observation been reported in the evaluation of diagnostic tests for ISAV in Atlantic salmon (Nerette *et al.*, 2005).

Thus, parallel use of both cell lines was more sensitive in detecting TSRV than the use of either CHSE-214 cells or EPC cells alone. This observation is consistent with Opitz (2000) who found that the parallel use of SHK-1 cells and CHSE-214 cells was more sensitive in detecting ISAV in Atlantic salmon than use of either one of the cell lines alone. The high specificity of virus isolation is due to the fact that it detects virus capable of replication, is in fact reliant on the presence of infectious virus, and that a positive result is generally considered to be the verification of the presence of infectious virus (Nerette *et al.*, 2005); it is highly unlikely that a false positive will occur, particularly when the virus induces a characteristic CPE, such as that induced by TSRV, that cannot be confused with some other infection. Virus isolation has been found to be as sensitive as RT-PCR for ISAV isolation (Blake *et al.*, 1995; Mikalsen *et al.*, 2001; Nerette *et al.*, 2005). Virus isolation on fish cell lines is the conventional technique for the detection of important pathogens of salmonids species and is considered the test of choice (the ‘gold standard’) by OIE. Its use is of fundamental importance to the control of disease spread, in health surveillance programs for virus exclusion, and in disease diagnosis is undeniable (OIE, 2011).

Conventional hemi-nested RT-PCR assay was more specific (98.9%) than qPCR but was the least sensitive diagnostic method with a sensitivity of 78.1%. The conventional hemi-nested RT-PCR assay was developed to be highly specific to detect TSRV gene fragments and this result is consistent with assay specificity conducted by Carlile (2011). TSRV-specific conventional hemi-nested RT-PCR was evaluated using CSRV, GSRV, Green River reovirus, Australian redfin reovirus, IPNV, ISAV, EHNV, IHNV, VHSV, TAB and RSIV as target and there was no non-specific amplification of nucleic acid from any other aquareoviruses or other aquatic viruses tested (Gemma Carlile pers communication). The evaluation of analytical sensitivity of the diagnostic tests through experimental infections of TSRV found that qPCR is the most sensitive assay followed by conventional hemi-nested RT-PCR and virus isolation was the least sensitive assay (Gemma Carlile pers communication).

The estimation of sensitivity of the tests in this study (intra-laboratory comparison of diagnostic tests) found that the conventional hemi-nested RT-PCR was less sensitive than virus isolation. Analytical sensitivity and analytical specificity are different from the diagnostic sensitivity and specificity (Saah and Hoover, 1997). Analytical sensitivity refers to measurement of the lowest level of pathogen that can be detected. Analytical specificity is the assay's ability to correctly identify a specific pathogen in order to minimize the tests cross-reactivity with other pathogens (Dohoo *et al.*, 2003). Therefore, the inconsistency between analytical estimation of sensitivity, specificity of conventional hemi-nested RT-PCR (Gemma Carlile pers communication) and estimation of both characteristics using field samples is relevant because it has been reported (Saah and Hoover, 1997) that high analytical sensitivity and specificity do not ensure acceptable diagnostic values.

Conventional hemi-nested RT-PCR was the least sensitive method for the detection of TSRV. This is in contrast to previous reports for other viruses showing that the PCR test was more sensitive than virus isolation (Blake *et al.*, 1995; Miller *et al.*, 1998; Devold *et al.*, 2000; Opitz *et al.*, 2000; Mikalsen *et al.*, 2001; Barlic-Maganja *et al.*, 2002; Snow *et al.*, 2003; McClure *et al.*, 2005; Nerette *et al.*, 2005; Lopez-vasquez *et al.*, 2006; Knusel *et al.*, 2007; McClure *et al.*, 2008). The performance of conventional hemi-nested RT-PCR under laboratory trials for TSRV has always been unreliable: TSRV positive controls/samples are only detected in the secondary reaction and weak bands are generally observed in secondary reaction. Primers are able to amplify PCR product of 280 bp and 140 bp for primary and secondary reactions, respectively.

The observations from laboratory trials are consistent with the intra-laboratory and inter-laboratory comparison study which involved field samples. The assay detected positive samples with high viral load only (equivalent to qPCR C_T values of 26.0-35.0) and PCR products from high dilution showed very faint bands in some cases. Interpretation of the results was confirmed with duplicate samples and repetition of the PCR assays. It had been suggested the usage of conventional RT-PCR should be used only for detection of samples with high viral loads such as in cases of overt disease (Rimstad *et al.*, 1999; Andrade *et al.*, 2007). The lower sensitivity of conventional hemi-nested RT-PCR could also be caused by low viral titre of TSRV in samples, it has been reported that if fish has a very low viral titre, virus would not be detectable with available tests, which results in a loss of sensitivity (McClure *et al.*, 2008).

Conventional hemi-nested RT-PCR had a moderate agreement of positive predictive value and a large confidence interval width. The wide interval of positive predictive value: 0.643 (0.409-0.826) implies poor precision of conventional hemi-nested RT-PCR. This is due to the observation that conventional hemi-nested RT-PCR detected the lowest proportion of positive samples of T SRV. The high negative predictive value obtained by conventional hemi-nested RT-PCR is consistent with high numbers of negative conventional hemi-nested RT-PCR/negative virus isolation (high specificity). Predictive values depend on sensitivity and specificity of a test (Thrusfield, 1995); therefore predictive value of conventional hemi-nested PCR is related to its sensitivity, specificity and prevalence of T SRV. As the positive predictive value decreases and negative predictive value increases, the prevalence of infection in a population decreases (Thrusfield, 2005). This PCR assay showed high negative predictive values which corresponded to the low prevalence of T SRV previously detected in farmed populations of Atlantic salmon, 10 -25% (based on retrospective epidemiological study undertaken by Carlile (2011) and 6-22% (this study).

The q PCR assay exhibited high sensitivity and specificity using the different statistical approaches. qPCR assay detected the highest proportion of positives in inter-laboratory and intra-laboratory comparison studies, produced high predictive values, and excellent Kappa agreement with virus isolation. Various authors have applied qPCR tests for the detection of fish pathogens due to its high sensitivity, specificity, rapidity, reproducibility and high throughput sample processing (Bustin, 2000; 2002; Mackay *et al.*, 2000; Dhar *et al.*, 2001; Overturf *et al.*, 2001; Gilad *et al.*, 2004 ; De la Vega *et al.*, 2004; Bustin *et al.*, 2005; Plarre *et al.*, 2005; Goodwin *et al.*, 2006; Wang *et al.*, 2006; Andrade *et al.*, 2007; Getchell *et al.*, 2007; Crockford *et al.*, 2008; Dhar *et al.*, 2008; Matejusova *et al.*, 2008; Snow *et al.*, 2009; OIE, 2011; Abayneh *et al.*, 2010, Ellis *et al.*, 2010, Goodwin *et al.*, 2010).

This PCR assay also demonstrated high positive and negative predictive values. These values were consistent with the narrow confidence interval and the high proportion of positive samples detected by qPCR. High positive predictive value of qPCR demonstrated the existence of good correlation with virus isolation and the number of positive samples by qPCR but negative by virus isolation does not indicate false positive reactions; rather the likelihood of a better sensitivity of qPCR (Douglas *et al.*, 2001). The high predictive values of qPCR confirmed that it is an appropriate diagnostic method for TSRV infections as a test with high sensitivity (positive predictive value) and specificity (negative predictive value) is useful whether the prevalence of infection is either high or low (Newbound *et al.*, 1995; Noga *et al.*, 1988).

The specificity of the qPCR assay for detection of TSRV was tested experimentally with other aquareoviruses including CSRV, GSRV, Green River reovirus, Australian redfin reovirus, and other significant viral pathogens of fish: IPNV, ISAV, EHNV, IHNV, VHSV, TAB and RSIV. Only CSRV and TSRV were amplified demonstrating the close relationship between these two viruses which share 91% nucleotide identity (Gemma Carlile pers communication). It should be noted that CSRV is absent in Atlantic salmon in Australia.

Excellent Kappa agreement for qPCR at both laboratories showed that it is as accurate as virus isolation in detecting TSRV in farmed populations. There was substantial inter-laboratory correlation (Kappa value) of qPCR between both laboratories which showed that the method is reproducible and consistent. The difference of prevalence estimate achieved by qPCR between the two laboratories could be caused by the heterogeneity of the virus distribution in tissue samples (Nerette *et al.*, 2005). The usage of deionised formamide with

extracted viral RNA prior to amplification by AFDL could account for the differences of prevalence detected by qPCR in both laboratories (Nick Moody pers communication).

qPCR assays have been developed and validated by many investigations to provide quantitative results, to distinguish between the high viral loads characteristic of an active infection (clinical) and low viral loads that may be related to latent (subclinical) infection (Dhar *et al.*, 2001; Overturf *et al.*, 2001; Gilad *et al.*, 2004; De la Vega *et al.*, 2004; Goodwin *et al.*, 2006; Andrade *et al.*, 2007). As the prevalence of T SRV is low (6-22%) in the population of farmed Atlantic salmon and as it varies accordingly to spatial, seasonal and temporal factors (Gemma Carlile pers communication), it is vital to consider the varying viral loads between samples. Variation of viral loads between samples would provide quantitative results regarding status of an infection (clinical/subclinical) and a acknowledgement of such status is vital as T SRV natural infections are not associated with clinical disease and mortality.

Sensitivity of diagnostic methods may vary according to clinical/subclinical status of the population being tested, however qPCR is more likely to detect pathogens that are undetectable by virus isolation particularly if a varying spectrum of infection levels exist (Branscum *et al.*, 2005). In order to relate the C_T cut-off values of qPCR assay to disease, the determination of C_T cut-off value was based on the comparison of virus isolation and qPCR. This comparison is reliable due to the fact that virus isolation detects infectious virus particles compared with qPCR which detects a specific nucleic acid sequences whether it is derived from either infectious virus or non-infectious material (Nerette *et al.*, 2005). Although, qPCR is known to be more sensitive than virus isolation in detecting carrier fish (Clementi *et al.*, 2000; Opitz *et al.*, 2000; Dhar *et al.*, 2001; Mackay *et al.*, 2002; Plarre *et al.*, 2005; Goodwin

et al., 2006), virus isolation is the only practical method available, to detect infectious virus particles and to exhibit levels associated with active infection.

The determination of the C_T cut-off value of 35.8 based on intra-laboratory comparison is consistent with previous work based on experimental infection (estimation of analytical sensitivity and specificity) of Atlantic salmon with T SRV (Gemma Carlile pers communication). Initially, the selection of a C_T cut-off value was based on analytical evidence which was primarily justified by setting a reliable limit of detection and selection of a threshold level directed by the software associated with the thermocycler (Gemma Carlile pers communication). These justifications were based on selection of cut-offs for qPCR assay at the bench level (analytical approaches), illustrated by Caraguel *et al* (2011). Following these criteria, Carlile (2011) found that the qPCR was able to detect T SRV from an original concentration of 6.32×10^4 TCID₅₀/mL which had an average of 1.94×10^{10} molecules/ μ L down to 1.94×10^3 molecules/ μ L.

Similarly, the estimation of limit of detection carried out prior to this study demonstrated that the qPCR were able to detect T SRV from an original concentration of TCID₅₀/mL value of $10^{4.7}$ (1.94×10^8 molecules/ μ L) to 1.94×10^2 molecules/ μ L. The qPCR assay in this study (intra-laboratory comparison) used a maximum of 45 cycles so that a low copy number of targets would be amplified and detected. The initial purpose of this test was to optimize the estimation of prevalence of T SRV infections in farmed Atlantic salmon population. Thus, high C_T values less than 36.0 were considered positive for inter-laboratory and intra-laboratory comparison study of diagnostic method and for the determination of C_T cut-off value for qPCR assay.

The remainder of positive samples with C_T values of 36-38 (low viral loads) which were detected only by qPCR, indicated that this assay is capable of detecting subclinical infections. The circumstances of qPCR assay detecting T SRV genomic DNA (36.0-43.04) may result in positive qPCR results with corresponding negative virus isolation. In the present study, such data were classified as false positives and resulted in an underestimation of diagnostic specificity. However, based on the ROC curve analysis, interactive dot diagram and plot versus criterion, the C_T cut-off value of 35.8, rather than 43.04, was established since at this C_T value the qPCR test showed a higher *combined* diagnostic sensitivity and specificity.

Phillips *et al* (2009) determined the median C_T value between negative ELISA (gold standard) with positive qPCR as a C_T cut-off value for diagnosis of group A Rotavirus. Phillips *et al* (2009) concluded that qPCR was able to detect subclinical infections and it was more effective to use a real-time platform with a viral load cut-off equivalent to traditional method. A C_T cut-off value equivalent to viral load was selected to improve the specificity of qPCR in diagnosing rotavirus A infections associated with clinical disease. Taking this into consideration, qPCR can quantify viral RNA load precisely even though it is not directly associated with the number of infectious virus particles (De la Vega *et al.*, 2004); by determining a C_T cut-off value according to the diagnostic purpose.

Setting a higher C_T cut-off value was reported to increase the specificity of diagnostic test for ISAV (Gustafon *et al.*, 2005). The increase in specificity will result in decrease of sensitivity of the test and the setting of the appropriate cut-off level also depends on the diagnostic strategy (Pfeiffer, 2002; Araguel *et al.*, 2011). According to OIE requirements, the fitness for a specific purpose of an assay must be demonstrated for

validation and a test should be validated according to how well it performs for a specific purpose (Jansen *et al.*, 2010; Caraguel *et al.*, 2011). For the purpose of diagnostic confirmation of suspect or clinical cases which includes the confirmation of positive screening test, the test validation should prioritize diagnostic specificity (PPV) over diagnostic sensitivity (Caraguel *et al.*, 2011).

The main purpose of diagnostic work for TSRV detection is to ensure that every test positive is truly infected, thus, a diagnostic test with high specificity and good sensitivity is acceptable. Determination of C_T cut-off value at 35.8 is more suitable for this purpose due to the above mentioned reasons. As qPCR for the detection of TSRV is highly specific and sensitive, the sensitivity of the test would not be compromised. Thus, establishment of the cut-off value for qPCR through a parallel comparison will improve the specificity (reduces false-positives), and reliability of the assay thus providing an accurate diagnosis. It is apparent that in farmed fish, dual analysis could detect emerging epidemics and allows earlier implementation of remediation measures (Nance *et al.*, 2010). In a naïve outbreak of disease/diagnostic work, where it is more important to detect every single positive sample (high PPV value), and the most sensitive diagnostic method would be appropriate: qPCR. Any positive outcomes from qPCR assay should be confirmed with virus isolation as it the most specific diagnostic method for detection of TSRV.

The viral copy number of the TSRV positive control material (cell-culture supernatant) was determined using a synthetic dsRNA preparation as a standard. In this absolute quantification, the quantity of the unknown TSRV positive control material was interpolated from a serial dilution of the synthetic dsRNA preparation which had the equivalent viral copy number of 6.2×10^{12} molecules/ μ L. Various authors have applied

absolute quantification of qPCR using RNA standards for RNA quantification and determination of viral loads (Souazé *et al.*, 1996; Freeman *et al.*, 1999; Martell *et al.*, 1999; Bustin *et al.*, 2000; Fronhoffs *et al.*, 2002; Liu and Saint, 2002; Pfaffl, 2004; Ozoemena *et al.*, 2004; Wong *et al.*, 2005). Synthetic dsRNA (RNA standard) can be defined as an in vitro-transcribed synthetic RNA that shares the same primer binding sites as the native RNA (Freeman *et al.*, 1999).

RNA standards are suitable for absolute quantification as a RNA standard can be used to demonstrate the variability during the RT step and is most likely to have the similar RT and PCR efficiencies (Freeman *et al.*, 1999). The accuracy of the quantification results in this study is supported by comparison with the preliminary absolute quantification conducted by AFDL (Nick Moody personal communication). The standard curve constructed with synthetic dsRNA can be used to determine the target (TSRV) quantity in an unknown sample (surveillance and diagnostic) in the future. The degree of sensitivity along with the wide dynamic range added confidence that the TSRV positive material can be used to determine the viral copy number (molecules/ μ L) based on the standard curve.

The prevalence estimation of Population 1 (Tamar River) was higher by TAGS software (maximum-likelihood) (16.1%) than Classical (6.1%) and Bayesian analysis (6.28% and 7.91%). Taking this into account, Bayesian analysis provides better estimates of prevalence than maximum likelihood and Classical analyses, as prevalence of TSRV infection in farmed population of Atlantic salmon in Tamar River is considered to be 6.28% - 7.91%. The prevalence of Population 2 (South-east Tasmania) was not significantly different by all the analyses but was higher (21% - 22.8%) than Population 1. The prevalence of TSRV in Tasmanian population has been reported to be influenced by seasonal, spatial and temporal

distribution based on a retrospective epidemiological study by Carlile (2011) undertaken from 1980 till 2005.

Based on that study, the prevalence of TSRV in the two different geographic locations, South-east Tasmania and Tamar River was 32.1% and less than 5% respectively. TSRV was reported only once in the Tamar River region (2003) within the study as opposed to 90 positives in South-east Tasmania. TSRV has been found to be ubiquitous in South-east Tasmania. As it is the most intensively farmed region, this is likely to contribute, in part, to the high prevalence of TSRV in this region. Further studies have been suggested by Carlile (2011) to be undertaken to demonstrate those parameters that influence the presence of TSRV in this region. As the sampling of the current study was conducted during late spring (September) to early summer (December), the prevalence of TSRV was expected to be 10-25% based on seasonal factors due to differences in water temperature.

Prevalence of TSRV was found to be higher during summer presumably due to the increased water temperature than winter (Gemma Carlile pers communication). In this study, the water temperature was 15°C in farms from South-east Tasmania, higher than the Tamar River region (12°C), which may have led to the higher prevalence of TSRV found in South-east Tasmania. These observations and estimated prevalence of TSRV in both regions are consistent with the retrospective epidemiological study by Carlile (2011). It has also been reported that elevated water temperature is very influential in determining the severity of viral disease in cold-water aquatic animals, with higher temperatures leading to increased mortality. Temperature has been documented as an important environmental variable affecting the pathogenicity of some fish viruses, including GSRV (Schwedler and Plumb, 1982), infectious hematopoietic necrosis virus (IHNV) (Amend, 1970; Hetrick *et al.*, 1979)

and viral hemorrhagic septicemia virus (VHSV) (Arkush *et al.*, 2006). Thus it can be argued that as climate change occurs, any increase in water temperature may be expected to exacerbate TSRV infections to levels that may precipitate disease.

In the present study, the true prevalence of TSRV in farmed populations of Atlantic salmon could be over-estimated due to insufficient sample numbers which were limited to allow the primary objective of this study which was to validate available diagnostic methods. True prevalence is the proportion of a population that is actually infected (Thrusfield, 1995). Hence, the overestimation of prevalence could be caused by the fact that the sampling was largely based on moribund and mort fish, and prevalence values can vary depending on the stage of infection. This study has shown that moribund fish were positive more often than samples from healthy fish. The virus concentration in moribund and freshly dead fish would be significantly higher than in healthy fish (Pfeiffer, 2002). Thus, a better estimation of true prevalence of TSRV needs to be undertaken utilising random sampling and a larger sample size as well as the most accurate diagnostic test. Locksley (2008) emphasized the importance of sample size in determining the precision of final estimates of prevalence and that the accuracy of case identification and precision estimates are also dependent on the diagnostic test characteristics (sensitivity and specificity).

This study has demonstrated that the most effective and accurate diagnostic method for the detection of TSRV in farmed population of Atlantic salmon is the qPCR assay. Both cell lines, CHSE-214 and EPC, were suitable for the isolation and replication of TSRV and parallel use of both cell lines was more sensitive than the use of either one only for virus isolation. The molecular methods developed for detection of TSRV which are conventional

hemi-nested and qPCR were validated through inter-laboratory and intra-laboratory comparison analyses. Conventional hemi-nested RT-PCR was not an effective diagnostic method relative to qPCR and the use of conventional hemi-nested RT-PCR for diagnostic and surveillance purposes should be limited for the detection of TSRV. In contrast, conventional hemi-nested RT-PCR can be used as a confirmatory test to differentiate between genotypes for sequencing purposes due to its high specificity. Real-time PCR (qPCR) is a consistent, robust, highly sensitive, and highly specific test, and was found to be as accurate as virus isolation in detecting TSRV. This assay has been adopted as a diagnostic method at Animal Health Laboratory, Mount Pleasant Laboratories (DPIPWE), Tasmania. Future research on TSRV should involve: development of a case definition of TSRV and an epidemiological investigation using qRT-PCR assay to obtain the true prevalence of TSRV.

CHAPTER 3

Comparative study of sample collection (organs vs swabs) for TSRV detection in farmed Atlantic salmon

3.1 Introduction

Sample collection is a critical factor in making an accurate diagnosis (Swenson *et al.*, 2001). If the sample is not collected correctly or if it is handled improperly after collection or not dispatched as soon as possible, test results may not accurately reflect the disease status of the animal sampled. According to the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2011), samples of 5 mm³ of liver, kidney and spleen are collected for diagnostic virology and are considered sufficient to detect most viruses in diseased fish. Generally, these organs are sampled for fish that exceed 6 cm in length (OIE, 2011). The choice of sample collection method for diagnosis or surveillance depends on the status of an infection and diagnostic methods appropriate for detection of the virus. In the case of clinical infection, organs to be sampled are anterior kidney, spleen, heart and brain. Samples can be combined in pools of no more than ten fish per pool for the detection of subclinical carriers of a virus (OIE, 2011). In Australia, fish virus isolation (in cell culture) is available at the Fish Diseases Laboratory at the Australian Animal Health Laboratory (AAHL), the national reference laboratory for animal diseases, and some state laboratories, which have cell lines appropriate for endemic viruses.

The diagnostic method of choice for the detection of Atlantic salmon reovirus (TSRV) is virus isolation using two finfish cell lines (CHSE-214 & EPC). The surveillance activities for the detection of TSRV are coordinated by the Animal Health Laboratory (AHL), Department of Primary Industries, Park, Water & Environment (DPIPWE) at Launceston. As a part of the Tasmanian Salmonid Health Surveillance Programme (TSHSP), DPIPWE staffs collect samples aseptically from fish at various farm sites across the state, and send them to AAHL Fish Diseases Laboratory (AFDL), located at CSIRO Australian Animal Health Laboratory (AAHL), Geelong, Victoria for virus isolation. In 2009, qPCR (quantitative one-step reverse transcriptase polymerase chain reaction) for TSRV, based on the method of Carlile (2011), was implemented at the Animal Health Laboratory, DPIPWE, Launceston. The test was introduced for the purpose of disease investigation and diagnosis to enhance service capability in Tasmania in support of the TSHSP. Routine samples for the detection of TSRV using qPCR included pooled liver, kidney and spleen. The choice of kidney, liver and spleen for detection of fish viruses is due to the fact that for systemic infections virus is likely either to be accumulated in or infect these organs (Handler, 2008). Selection of these organs for the detection of TSRV is supported by experimental work which showed that these organs were similarly affected in fish following cohabitation with fish infected with TSRV (Gemma Carlile pers communication). The infected fish did not show any clinical signs other than distended abdomen but demonstrated mild to severe histological changes in kidney, liver and spleen (Gemma Carlile pers communication).

Samples of organs less than 0.5 cm in any dimension are required to be placed in 5 mL RNA *later*TM at the time of collection to stabilise the sample RNA for testing by qPCR for TSRV. Preservation of samples for detection of TSRV in RNA *later*TM is required prior to despatch of samples from farm sites because TSRV is a dsRNA virus and therefore is more labile than DNA viruses (Lupiani *et al.*, 1995; Nason *et al.*, 2000). The samples are removed from RNA *later*TM, cut and homogenized in Extraction Buffer (tissue homogenizer) prior to viral RNA extraction and PCR amplification.

Sample collection on-farm and subsequent processing in the laboratory is laborious and time consuming particularly when large numbers of samples are processed for surveillance or disease investigation purposes. There are now known commercial sources of swabs which are commonly used for sample collection from a variety of organs, and for the subsequent isolation of infectious agents (bacteria, fungi, or viruses). The use of swabs as an effective and practicable sample collection method has been reported for veterinary diagnosis (Austin and Hayment *et al.*, 1985; Weiblen *et al.*, 1992; McCaw *et al.*, 1995; Kamp *et al.*, 1996; Hoie *et al.*, 1997; Calsamiglia *et al.*, 1999; Kim *et al.*, 2000; Swenson *et al.*, 2001; Choi *et al.*, 2002; Komar *et al.*, 2002; Rice *et al.*, 2003; Pearce and Bolton, 2005; Young *et al.*, 2008b; Goyal *et al.*, 2010). Modern molecular biology methods, such as the detection of viruses by PCR, requires specific approaches in the downstream handling of a sample collected by swab. The goal is to recover as much of the material adhering to the swab as possible without further diluting it and without introducing potentially inhibitory substances (Biochemica, Roche Applied Science, 2002).

The use of swabs compared to organ samples as sample collection method for qPCR to detect TSRV could make the process less laborious, easier to perform, less time-consuming and overall more practical. Thus, this chapter describes the comparison of sample collection methods using either organ samples or swabs for the detection of TSRV in farmed Atlantic salmon. Evaluation of these two approaches was carried out to determine the sensitivity of the collection methods. The effect of pooling samples, collected as swabs or organ samples, on the detection of TSRV by qPCR was investigated. While the use of swabs has been described for collecting samples to detect viruses by PCR for humans and terrestrial animals (Mahony and Chernesky, 1985; Ahluwalia *et al.*, 1987; Roelofsen *et al.*, 1999; Boivin *et al.*, 2000; Cloud *et al.*, 2002; Jordens *et al.*, 2002; Farhat *et al.*, 2001; Echevarria *et al.*, 2003; Uhl *et al.*, 2003; Warren *et al.*, 2004; Issa *et al.*, 2005), this approach has not been evaluated for the detection of fish viruses by PCR.

3.2 Materials & Methods

3.2.1 Experiment 1 – comparative study of individual sampling collection (organs vs swabs)

3.2.1.1 Atlantic salmon

Stock Atlantic salmon parr maintained at the Animal Health Laboratory, Mount Pleasant Laboratories (DPIPWE) were used as a source of uninfected samples (previously been used as a source for extracted negative controls for PCR). The fish were euthanased by placing them in a solution of AQUIS (0.5 mL/20 L water) (AQUIS[®], New Zealand). The entire kidney, liver and spleen were removed from each fish and placed in a 5 mL tube for each fish (TechnoPlas, Australia). The pooled organs from ten fish were kept at -80°C until required.

3.2.1.2 Preparation of ten-fold dilutions of tissues spiked with TSRV

The pooled organs (from ten fish) were homogenised using a frozen, sterile mortar and pestle and viral transport medium (Hank's balanced salt solution supplemented with 10% FBS, Invitrogen, Australia). The mortar and pestle were autoclaved at 121°C for 15 minutes and frozen at -20°C, 24 hours prior to sample processing. The homogenate was resuspended in supplemented HBBS and the ratio of sample weight (2.5 g) to supplemented HBBS volume (25 mL) was 1:10. The homogenised samples were then centrifuged at 5°C at 3000 x g for 15 min. The supernatant was harvested and aliquots (360 µL) were placed in 1.5 mL Eppendorf tubes. These were spiked with cell culture supernatant harvested from TSRV-infected CHSE-214 cell cultures demonstrating 100% CPE.

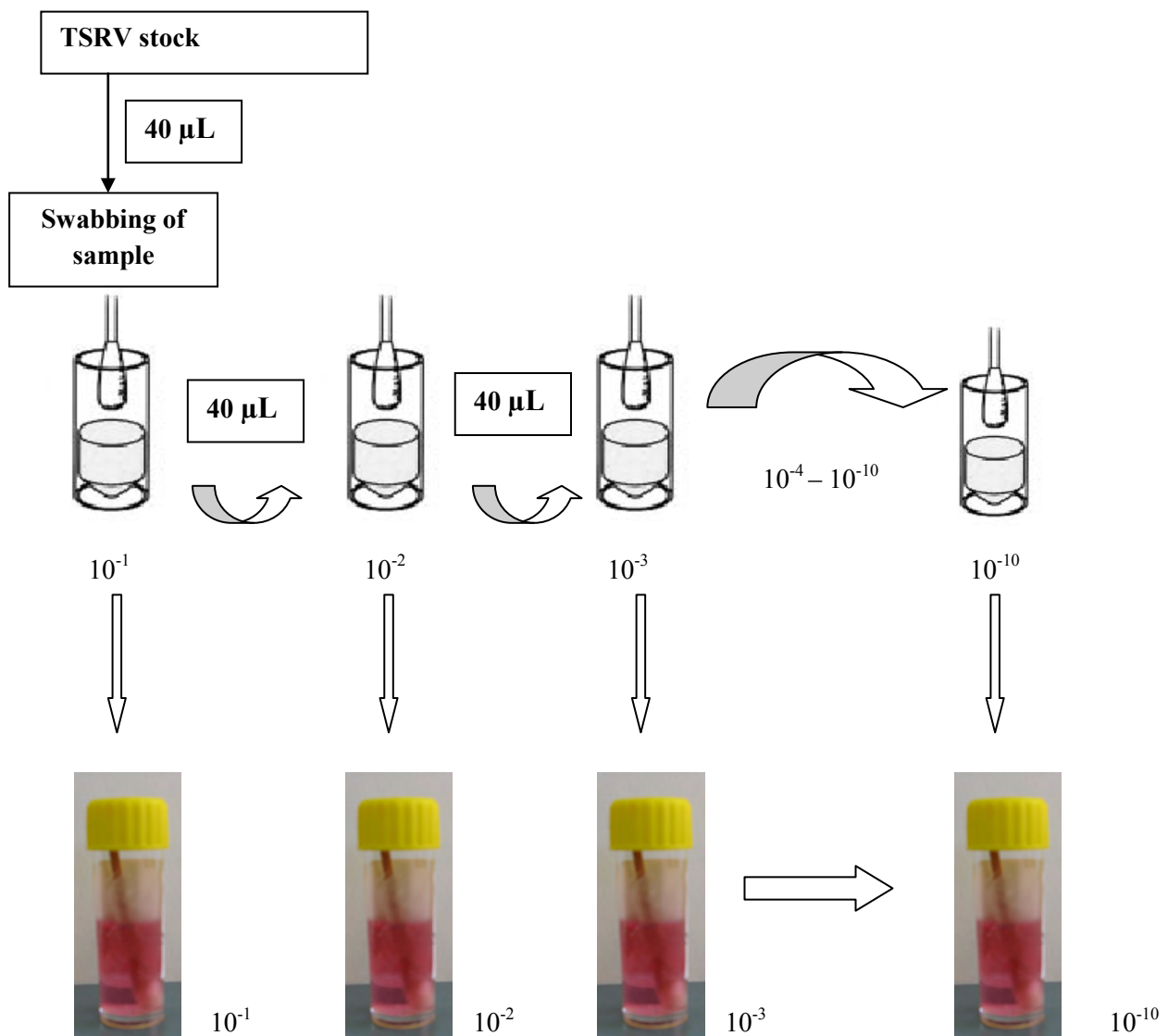
A stock of TSRV was provided by AAHL Fish Diseases Laboratory (AFDL), initially isolated from clinically healthy Atlantic salmon during routine health surveillance in Australia. The stock of TSRV (AFDL SAN NO: 0802-17-0321 initially expanded on CHSE-214 and harvested after the first passage) was expanded by infecting CHSE-214 cell cultures which were incubated at 15°C until 100% CPE developed. The cell culture supernatant was harvested, centrifuged at low speed (100 x g) to sediment cell debris and aliquots (250 µL) of the resultant supernatant were stored at -80°C to be used as positive control material for qPCR assays. This TSRV stock which had a TCID₅₀/mL of 10^{4.7 to 5.0} was used to prepare a ten-fold dilution series from 10⁻¹ to 10⁻¹⁰ as follows: a) 10⁻¹ (40 µL undiluted TSRV stock was added to 360 µL supernatant) b) 10⁻² (40 µL 10⁻¹ diluted TSRV stock added to 360 µL supernatant) c) 10⁻³ (40 µL 10⁻² diluted TSRV stock added to 360 µL supernatant) and this procedure was continued through to i) 10⁻¹⁰ (40 µL 10⁻⁹ dilution of TSRV stock added to 360 µL supernatant).

Each dilution was mixed vigorously with a vortex mixer prior to preparing the next dilution. Each of the tubes making up the ten-fold dilution series of spiked tissue supernatants was “swabbed” using sterilized wooden cotton-tipped swabs (Livingstone International, Australia), as follows. For each dilution, a swab was immersed in spiked supernatant for a few seconds, removed and then shaken vigorously into a corresponding tube containing 3 mL of viral transport medium (VTM) as shown in Figure 3.1. The wooden handle of the swab was broken off at the height of the tube which was then capped ready for storage. Prior to storage, an aliquot (200 μ L) of each of the ten-fold dilutions of spiked organs and swabs (VTM medium) was taken for RNA extraction immediately ($t = 0$). The swabs in VTM were shaken lightly before removing 200 μ L from the bottom of the tube for RNA extraction. The swabs, still in VTM, were stored at -4°C and a further sample was taken for RNA extraction after 24 hours ($t = 24\text{h r}$). This experiment was repeated three times (using freshly made replicates of spiked supernatants) to ensure consistency and repeatability of the results.

3.2.1.3 Isolation of RNA from organ samples

RNA extraction was carried out using the High Pure Viral RNA Kit (Roche, Germany). Poly (A) carrier RNA (2 mg) provided in the buffer kit was dissolved in 0.4 mL elution buffer prior to the extraction. A total 396 μ L binding buffer supplemented with 4 μ L poly (A) was added to 200 μ L sample (homogenized sample supernatants) and transferred into the High Pure Filter assembly and centrifuged at $8,000 \times g$ for 15s, using a microcentrifuge.

The flowthrough liquid and collection tube were removed and a new tube was used in each of the following steps. Under the buffering conditions used in the procedure, the viral RNA binds to the glass fleece in the high pure tube, while contaminating substances (salts, proteins and other cellular contaminants) do not (Roche Applied Science, 2008). Inhibitor Removal Buffer (500 μ L) was added to the upper reservoir and the tube was centrifuged at 8,000 $\times g$ for one minute. After the removal of any inhibitors, the sample was washed twice by adding 450 μ L Wash Buffer and centrifuged at 8,000 $\times g$ for one minute. The addition of the Inhibitor Removal Buffer and brief wash-and-spin steps removed those contaminants. The filter tube-collection tube assembly was left in the centrifuge and was spun at maximum speed (13,000 $\times g$) for 10s to remove any residual wash buffer. The remaining, purified RNA was eluted into a final volume of 50 μ L with Elution Buffer.



Legend:



360 μL Supernatant (pooled organs) spiked with ten-fold dilution of TSRV



Storage of swabs in 3 mL VTM

Figure 3.1. Overview of experimental design of Experiment 1: comparative study of individual sample collection method (organs versus swabs).

3.2.1.4 TaqMan qPCR assay

cDNA was synthesised for qPCR using TaqMan[®] one-step RT-PCR master mix reagents kit (Applied Biosystems, USA). The primers and probe for the qPCR assay were designed to amplify and detect an 82 nucleotide sequence of cDNA corresponding to positions 705–787 of the viral genome within segment 10 of TSRV (Gemma Carlile pers communication). The sequences for the primers were: forward primer (TSRV-10F): 5'-GATCGAACCCGTCGTGTCTAA-3' Positions 705-725, reverse primer (TSRV-10R): 5'-CGGTGCTCAGCTTGTCACA-3' positions 769-787. The TSRV probe (positions 731-748): 5'-CCC GAG C CA T CT GGG C GC-3' contained a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), located at the 5' end and the quencher, 6-carboxy-tetramethyl-rhodamine (TAMRA), located at the 3' end. The primers and probe for 18S control were: forward primer (18F): 5'-CGGCTACCACATCCAAGGAA-3', reverse primer (18R): 5'-GCTGGAATTACCGCGGCT-3' and probe (18S Probe): 5'-TGCTGGCACCAGACTTGCCCTC-3' which contained a proprietary fluorescent reporter dye, (VIC) located at the 5' end and the quencher TAMRA located at the 3' end.

The reaction mixture (25 µL total volume) consisted of: 5.75 µL RNase free water, 12.5 µL TaqMan[®] 2 × Universal PCR master mix no AmpErase[®] UNG (containing AmpliTaq Gold[®] DNA polymerase), 0.625 µL 40 × Multiscribe[™] and RNase inhibitor mix, 1.25 µL Primer TSRV-10F (18 µM), 1.25 µL Primer TSRV-10R (18 µM), 1.25 µL TSRV-10 probe (5.0 µM), 0.125 µL 18S rRNA Primer F (10 µM), 0.125 µL 18S rRNA Primer R (10 µM), 0.125 µL 18S rRNA VIC labelled Probe and 2 µL denatured viral RNA. The amplification of samples in duplicates was carried out in an ABI PRISM[™] 7500 Fast Real-Time System (Perkin-Elmer, Applied Biosystems, USA).

The RNA was amplified with the following programme: an initial 30 minutes at 48°C, followed by 10 minutes at 95 °C, then 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The cut-off value for qPCR for the detection of TSRV was set at $C_T = 35.8$ (see section 2.3.4.2 in chapter 2). C_T values lower than 35.8 are considered positive and C_T values greater than 36.13 are considered negative. C_T values in the range of 35.8-36.13 are classified as 'ambiguous' and the PCR assay for those samples were repeated to avoid false positives. TSRV viral copy numbers and corresponding qPCR C_T values generated based on a standard curve for absolute quantification of qPCR with synthetic RNA (see section 2.3.4.3 in chapter 2). The standard curve was generated by plotting the C_T values against logarithm of the calculated initial copy numbers. The unknown initial sample copy numbers for each dilution (both collection methods) were then automatically calculated from their C_T values, as compared to the standard curve.

3.2.2 Experiment 2- Comparative study of pooled sample collection (organs vs swabs)

3.2.2.1 Atlantic salmon

Samples of Atlantic salmon parr organs were obtained and stored as described in 3.2.1.1.

3.2.2.2 Preparation of ten-fold dilution series of pooled tissues spiked with TSRV

The pooled organs were homogenized using viral transport medium (Hank's balanced salt solution supplemented with 10% FBS, Invitrogen, Australia) in frozen, sterile mortar and pestle. The homogenate was resuspended in supplemented HBBS and the ratio of sample weight (5.0 g) to supplemented HBBS volume (50 mL) was 1:10. The homogenised tissues were then centrifuged at $3000 \times g$ for 15 min at 5°C and the supernatants were harvested. Aliquots (3.6 mL) of the supernatants were placed in 15 mL sterile centrifuge tubes (Iwaki, Crown Scientific, Australia). TSRV cell-culture supernatant, which had a $\text{TCID}_{50}/\text{mL}$ of $10^{4.7}$ to $10^{5.0}$, was used to prepare ten-fold dilution series from 10^{-1} to 10^{-10} as follows: a) 10^{-1} (40 μL undiluted TSRV stock was added to 360 μL supernatant) b) 10^{-2} (40 μL 10^{-1} diluted TSRV stock added to 360 μL supernatant) c) 10^{-3} (40 μL 10^{-2} diluted TSRV stock added to 360 μL supernatant) and so on through to i) 10^{-10} (40 μL 10^{-9} dilution of TSRV stock added to 360 μL supernatant). Each dilution was mixed with a vortex mixer prior to preparing the next dilution. Replicate aliquots (200 μL) of spiked supernatants of each of the TSRV dilutions were placed in Eppendorf tubes (1.5 mL) and used as replicate positive controls. Similarly, replicate aliquots of non-spiked supernatants of tissue homogenates were used as negative controls.

3.2.2.3 Experimental design

Each experimental pool of each sample type (organs versus swabs) consisted of five organ samples comprising different combinations of spiked and un-spiked preparations. Six organ combinations were prepared: a) C1: all positives b) C2: 4 positives and 1 negative c) C3: 3 positives and 2 negatives d) C4: 2 positives and 3 negatives e) C5: 1 positive and 4 negatives f) C6: all negatives (Figure 3.2). Each set of organ combinations was spiked with one of the dilutions of TSRV as described.

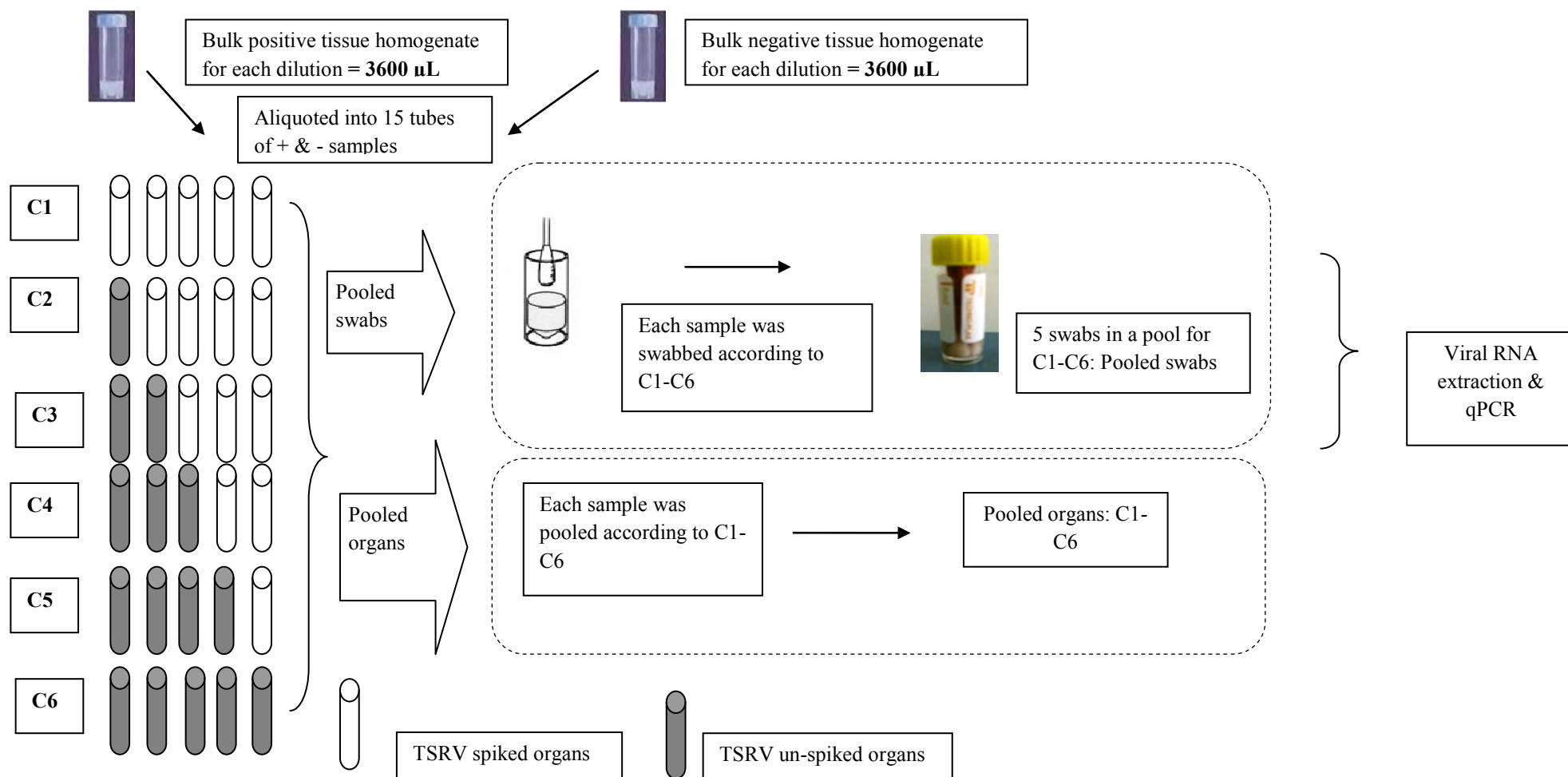


Figure 3.2 Experimental designs for Experiment 2: Six organ combinations of positive (spiked) and negative (un-spiked) of 5 samples in a pool for every dilution. In total, 6 pools of organ combination for every combination, dilution and sample collection method (organs versus swabs). C1: All positives; C2: 4 positives + 1 negative; C3: 3 positives + 2 negatives; C4: 2 positives + 3 negatives; C5: 1 positives + 4 negatives; C6: All negatives.

3.2.2.4 Sample processing

The sample processing for each dilution were processed individually, starting from the lowest dilution, 10^{-6} and C6. For every combination and dilution, samples were collected with a wooden, cotton-tipped swab (Livingstone International, Australia). A swab was immersed in a spiked tissue homogenate for a few seconds, removed, placed into a corresponding tube containing 3 mL viral transport medium (VTM) by breaking off the swab to the height of the tube. The tube was then shaken vigorously and the five swabs for a particular tissue combination were pooled into the same 3 mL tube of VTM.

After samples had been collected by swab, the organ homogenates were pooled to form pools for every dilution and tissue combination. A total of 200 μ L were taken from each of the pooled organs and swabs (every combination and dilution) for RNA extraction. Just prior to the extraction, the swabs in VTM were shaken lightly again before removing 200 μ L from the bottom of the tube for the extraction. The RNA extractions were conducted according to section 3.2.1.3. The qPCR assay was performed in duplicate samples according to section 3.2.1.4.

3.2.3 Statistical analysis

Kappa values were calculated to assess the agreement between the two methods of sample collection (organs and swabs). The results for both experiments were pooled for the assessment of Kappa values (Appendix II). This analysis was undertaken using EpiTools - epidemiological calculators developed by Aus-Vet Animal Health Services (<http://www.ausvet.com.au>). Effect of the trials and sample collection methods were determined for each viral copy numbers using two-way ANOVA analysis. Values were identified as significantly different if $p < 0.05$. The results of the three trials were pooled for each sample collection method if there was no significant effect between the trials.

Regression analysis was used to determine if there was a significant relationship between viral copy numbers and PCR results. Linearity of the data provides confidence that the dilutions have been prepared accurately and indicates that the PCR is working consistently with minimal variation between replicates. Linearity is accepted if r^2 reaches an acceptable p value, $p < 0.05$. One-way ANOVA was performed to analyse mean differences between the tissue combinations for both pooled organs and swabs. Values were identified as significantly different if $p < 0.05$. The Tukey HSD *post hoc* test was applied at a significance level of $\alpha \leq 0.05$, to determine differences between the explanatory variables. The regression analysis and one-way ANOVA were performed using SPSS® Statistics software, version 17.0.

3.3 Results

3.3.1 Comparative study of individual sample collection methods (organs versus swabs)

The sensitivity of both sample collection methods was evaluated based on positive samples obtained for the ten-fold dilution series and viral copy numbers. The C_T values shown in bold are positive, demonstrating that these samples contain quantifiable T SRV nucleic acid. Pooled results of the 3 trials as replicates for each viral copy numbers demonstrated that the use of swabs as sensitive as sampling organs, where the limit of detection was down to 1.94×10^4 molecules/ μ L with initial viral copy numbers of 1.94×10^7 molecules/ μ L (Table 3.1).

Table 3.1 Comparison of qPCR pooled results (3 trials) using organs and swabs spiked with a ten-fold dilution series of T SRV. Bolded mean C_T values (\pm standard error) are positive samples. t = 0: RNA extraction conducted directly after spiking, t = 24hr: RNA extraction conducted after 24 hours of spiking, ND: Non-detectable, SE: standard error. SE* values represent estimation from two trials only.

Trials	Dilutions	Viral copy numbers molecules/ μ L	Mean C_T values	Mean C_T values	Mean C_T values
			\pm SE	\pm SE	\pm SE
			Organs t = 0	Swabs t = 0	Swabs t = 24 hours
Pooled results (3 trials)	Initial value	1.94E+07	23.91 \pm 2.99	26.61 \pm 2.99	25.54 \pm 2.91
	10 ⁻¹	1.94E+06	27.21 \pm 2.80	28.42 \pm 3.36	28.46 \pm 3.66
	10 ⁻²	1.94E+05	30.96 \pm 3.10	34.7 \pm 3.19	33.32 \pm 3.59
	10 ⁻³	1.94E+04	34.05 \pm 3.20	35.99 \pm 3.39*	34.04 \pm 3.47
	10 ⁻⁴	1.94E+03	36.44 \pm 3.42	38.98	37.5 \pm 3.73*
	10 ⁻⁵	1.94E+02	ND	ND	ND

The C_T values obtained for viral copy numbers were consistent for the collection methods and the decrease in C_T value between each copy numbers was 3 to 4 C_T values (Figure 3.4), which is close to the theoretical value of 3.322 ($\log_2 10$). A good linear relationship and high correlation coefficient, $r^2 > 0.9$ (slope ≈ 3.3) between viral copy numbers and C_T value was shown, for all the collection methods. There was no significant interaction between the different trials and collection methods ($F = 0.130$, d.f. = 4, $p = 0.970$), which allowed the results of the three trials to be pooled for each collection method. Based on the C_T values obtained down to the limit of detection for the collection methods, there was no significant differences between the three trials for each sample collection methods, $p = 0.520$. Similarly, no significant mean differences were found between the sample collection methods, $p = 0.958$ (Figure 3.3).

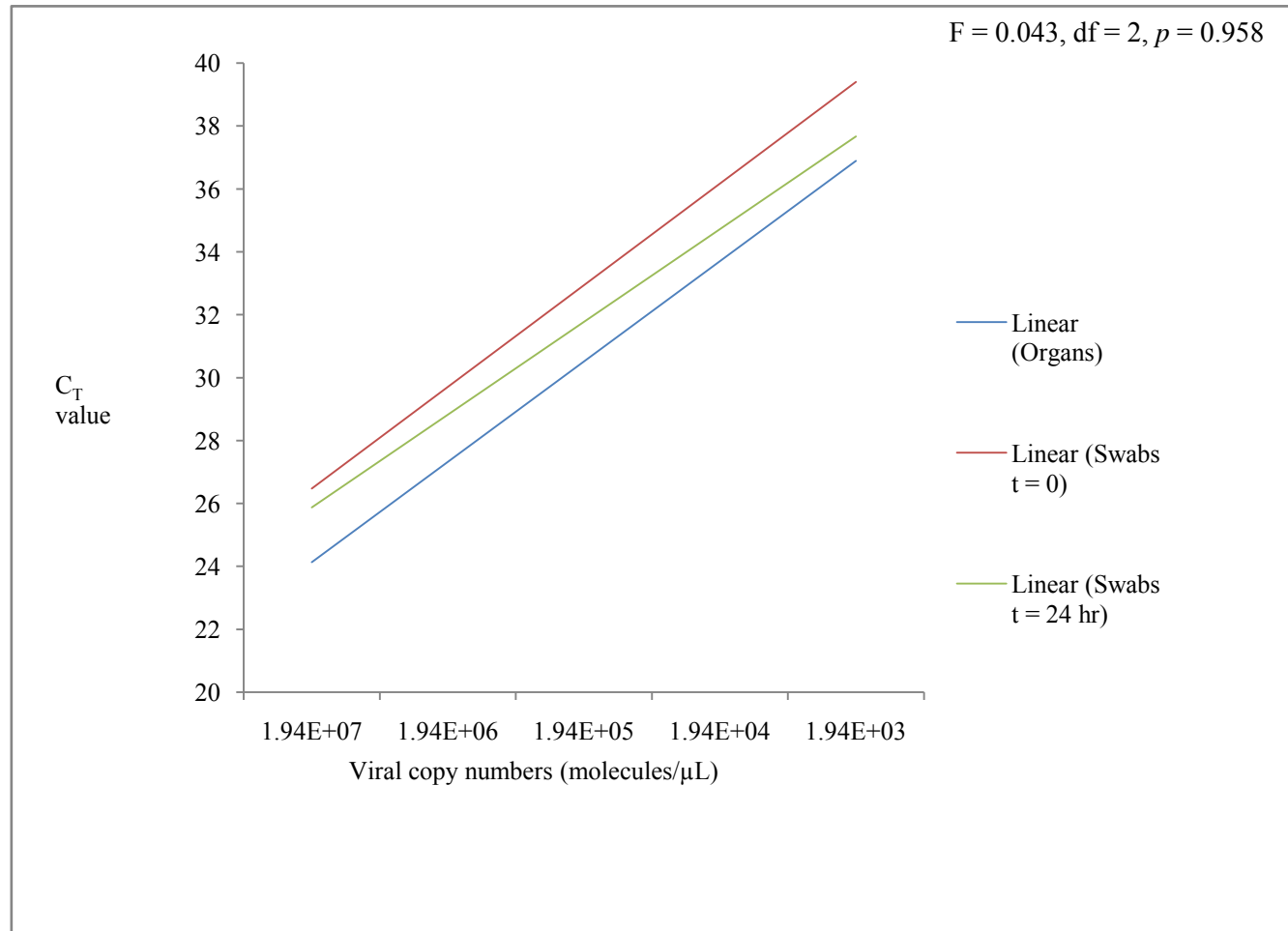


Figure 3.3 Relationship between mean C_T values (qPCR) and viral copy numbers obtained for the pooled results of the three trials conducted for the comparison of individual sample collection methods (organs versus swabs). Each trial was conducted on a different day using independently prepared serial dilutions. No significant differences were observed between the three trials for each sample collection methods, $p = 0.958$ by two-way ANOVA.

There was a good linear correlation and significant p values for sampling organs and swabs based on the pooled results of the three trials. This indicates minimum variation between the replicates in each trial (Figure 3.3 & Table 3.2).

Table 3.2 Linear equation, correlation coefficient and p values calculated for pooled results (3 trials) of comparative study of individual sample collection (organs versus swabs). p value for regression analysis and significant at the 0.05 level. Bolded values are significant.

Trials	Sample type	Equation of slope	r^2	p value
Pooled results (3 trials)	Organs	$y = 3.19x + 20.94$	0.99	< 0.001
	Swabs $t = 0$	$y = 3.23x + 23.247$	0.95	0.004
	Swabs $t = 24\text{hr}$	$y = 2.95x + 22.92$	0.97	0.003

The Kappa value (0.8571), the proportion of agreement, between organs and swabs showed a perfect agreement (Table 3.3). This indicates an excellent correlation between both collection methods for the detection of T SRV. The organs had a mean C_T of 30.0 cycles compared to a mean of C_T of 32.2 cycles by the swabs obtained at time, $t = 0$.

Table 3.3 Kappa value calculated between individual sample collection methods (organs versus swabs) based on pooled results of the three trials at time, $t = 0$.

		Organs		
		+	-	Total
Swabs	+	10	0	10
	-	2	18	20
Total		12	18	30
Kappa value				0.8571

The Kappa value (0.5928), for agreement of collection of individual swabs between $t = 0$ and $t = 24$ hours based on pooled results of three trials showed moderate agreement (Table 3.4). The swabs at different time points had similar mean C_T values of 32.2 cycles ($t = 0$) and 31.1 cycles ($t = 24$ hours).

Table 3.4 Kappa value calculated based on pooled results of the three trials between individual swabs collection at different times, $t = 0$ and $t = 24$ hours.

		Swabs $t = 0$		
		+	-	Total
Swabs $t = 24$hours	+	10	3	13
	-	3	14	17
Total		13	17	30
Kappa value				0.5928

3.3.2 Comparative study of pooled sample collection (organs versus swabs)

Sampling of pooled organs demonstrated a sensitivity of one ten-fold dilution higher than sampling of pooled swabs for the detection of TSRV, detecting down to the viral copy number of 1.94×10^4 molecules/ μL for organs compared to swabs which detected 1.94×10^5 molecules/ μL (Table 3.5). Both pooled sample collection methods were able to detect TSRV from C1 (all positives) to C5 (1 positive and 4 negatives), indicating that pooling four negative fish with one positive fish did not affect the sensitivity of the method. Pooled organs detected TSRV from C1 to C5 down to the viral copy numbers of 1.94×10^5 molecules/ μL whereas pooled swabs were able to detect TSRV down to 1.94×10^6 molecules/ μL .

Based on the C_T values obtained down to the limit of detection for both pooled organs and swabs, there was no significant differences between the different organ combinations (C1-C5), $p = 0.881$ for organs and $p = 0.946$ for swabs. Figure 3.4 and 3.5 illustrate the relationship between C_T value (qPCR) and viral copy numbers generated for each \log_{10} dilution of pooled organs and pooled swabs for TSRV, respectively, for every tissue combination. The C_T values for pooled organs dropped as soon as there were only one or two positive samples in a pool (C4-C5) for the greater copy numbers (1.94×10^7 to 1.94×10^5 molecules/ μL). For the more diluted samples (1.94×10^4), at least three positive samples needed to be in a pool (C3) to detect TSRV (Figure 3.4). The C_T values for pooled swabs increased as soon as there were only two positive samples in a pool (C4) for diluted samples (1.94×10^5 to 1.94×10^4) whereas there were no significant differences for the more potent dilutions (1.94×10^7 to 1.94×10^6 molecules/ μL , Figure 3.5).

Table 3.5 Comparison of qPCR results of pooled samples between organs and swabs spiked with ten-fold dilutions of TSRV at different tissue combinations. TSRV viral copy numbers generated based on absolute quantification of qPCR with synthetic RNA. Bolded mean C_T values are positive samples. Positive tissues were pooled tissues spiked with ten-fold dilutions of TSRV. Negative tissues were pooled tissues from naïve fish. C1: All positives; C2: 4 positives + 1 negative; C3: 3 positives + 2 negatives; C4: 2 positives + 3 negatives; C5: 1 positive + 4 negatives; C6: all negatives. ND: Non-detectable.

Ten-fold	Initial viral copy numbers	Tissue	C_T values	C_T values
Dilutions	(molecules/ μ L)	Combinations	Pooled organs	Pooled swabs
10^{-1}	1.94E+07	C1 (5+ 0-)	22.9	26.8
		C2 (4+ 1-)	24.17	27.45
		C3 (3+ 2-)	24.75	28.05
		C4 (2+ 3-)	24.14	29.04
		C5 (1+ 4-)	25.56	30.11
		C6 (0+ 5-)	ND	ND
10^{-2}	1.94E+06	C1 (5+ 0-)	27.49	30.97
		C2 (4+ 1-)	27.78	29.46
		C3 (3+ 2-)	27.78	30.84
		C4 (2+ 3-)	28.35	33.62
		C5 (1+ 4-)	29.55	32.44
		C6 (0+ 5-)	ND	ND
10^{-3}	1.94E+05	C1 (5+ 0-)	30.44	33.66
		C2 (4+ 1-)	30.27	34.11
		C3 (3+ 2-)	30.68	33.96
		C4 (2+ 3-)	32.13	35.03
		C5 (1+ 4-)	32.92	ND
		C6 (0+ 5-)	ND	ND

Ten-fold	Initial viral copy numbers	Tissue	C _T values	C _T values
Dilutions	(molecules/ μ L)	Combinations	Pooled organs	Pooled swabs
10^{-4}	1.94E+04	C1 (5+ 0-)	34.72	36.69
		C2 (4+ 1-)	33.97	36.26
		C3 (3+ 2-)	35.59	37.95
		C4 (2+ 3-)	37.72	37.13
		C5 (1+ 4-)	36.49	37.96
		C6 (0+ 5-)	ND	ND
10^{-5}	1.94E+03	C1 (5+ 0-)	37.36	ND
		C2 (4+ 1-)	ND	ND
		C3 (3+ 2-)	36.97	ND
		C4 (2+ 3-)	37.62	ND
		C5 (1+ 4-)	38.22	ND
		C6 (0+ 5-)	ND	ND
10^{-6}	1.94E+02	C1 (5+ 0-)	ND	ND
		C2 (4+ 1-)	ND	ND
		C3 (3+ 2-)	ND	ND
		C4 (2+ 3-)	ND	ND
		C5 (1+ 4-)	ND	ND
		C6 (0+ 5-)	ND	ND

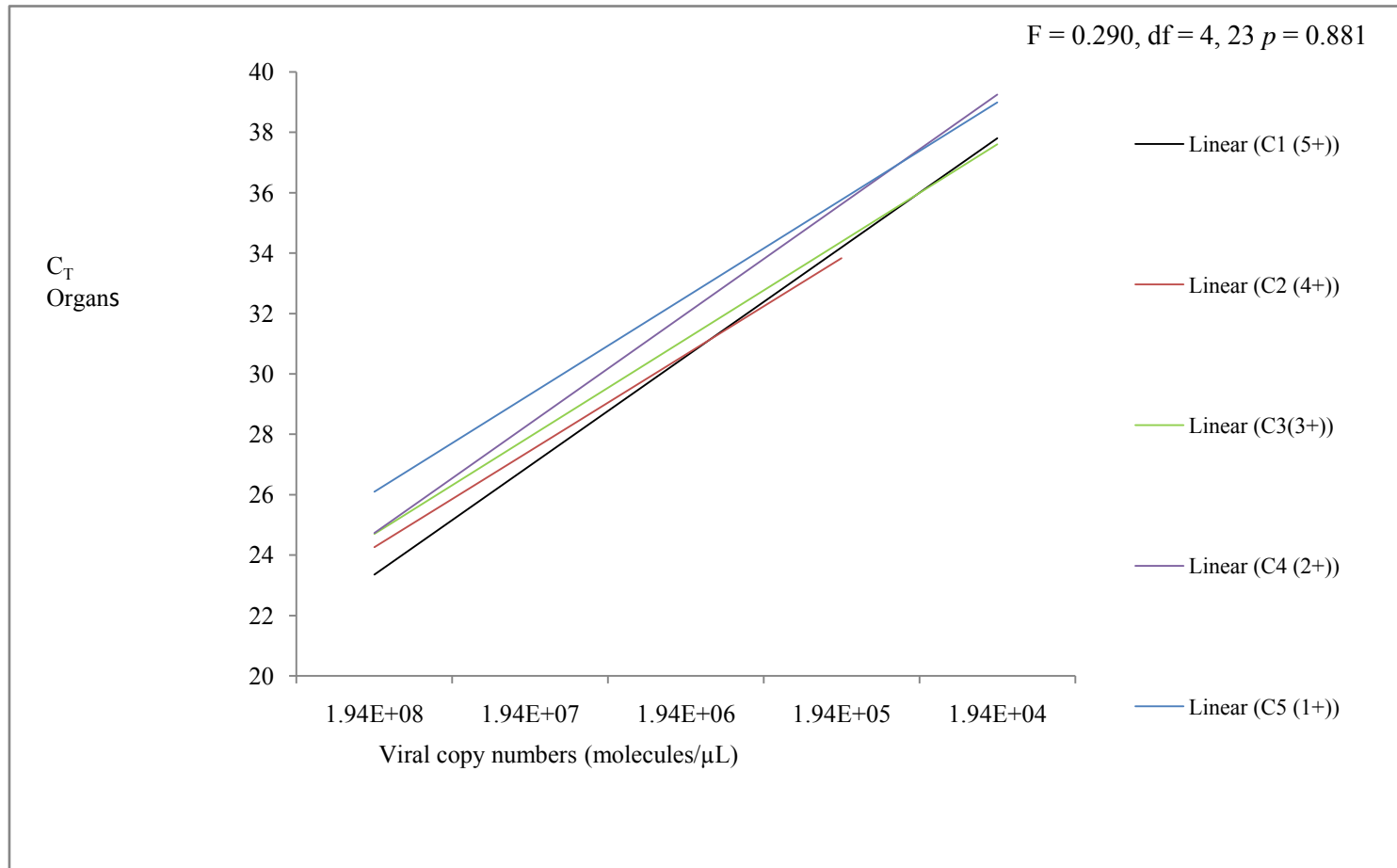


Figure 3.4 Relationship between C_T value (qPCR) and viral copy numbers obtained for each \log_{10} dilution of 10^{-1} to 10^{-5} TSRV of pooled organs for every tissue combinations. (C1/5+: All positives C2/4+: 4 positives + 1 negative C3/3+: 3 positives + 2 negatives C4/2+: 2 positives + 3 negatives C5/1+: 1 positives + 4 negatives). No significant differences were observed between the different tissue combinations (C1-C5) for pooled organs ($p = 0.881$) by one way ANOVA.

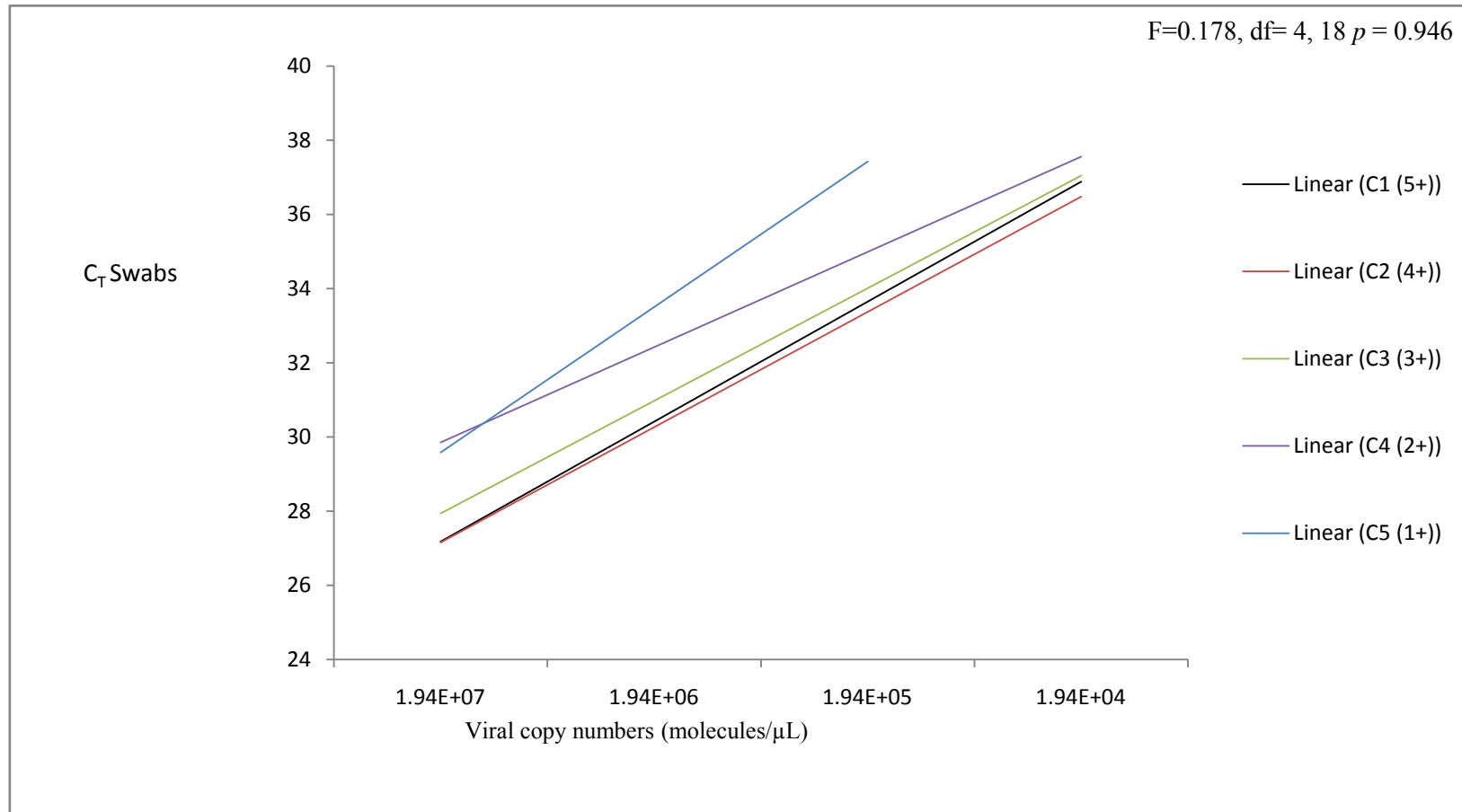


Figure 3.5 Relationship between C_T value (qPCR) and viral copy numbers for each \log_{10} dilution of 10^{-1} to 10^{-4} TSRV of pooled swabs for every tissue combinations. (C1/5+: All positives C2/4+: 4 positives + 1 negative C3/3+: 3 positives + 2 negatives C4/2+: 2 positives + 3 negatives C5/1+: 1 positives + 4 negatives). No significant differences were observed between the different tissue combinations (C1-C5) for pooled swabs ($p = 0.946$) by one way ANOVA.

A significant correlation between the 5 different tissue combinations and high correlation coefficient, $r^2 > 0.95$ was shown for both pooled sample collection methods (Table 3.6). These values indicated that the variation among the five-fold dilution was minimal for both methods and for all of the tissue combinations except C5 for pooled swabs. The insignificant p value obtained for C5 (1 positive and 4 negatives) in pooled swabs indicates that pooling four negative fish with one positive produces higher variation compared to C5 (lower dilution samples) in pooled organs.

Table 3.6 Linear equation, correlation efficient (r^2) and p values calculated for the comparative study of pooled sample collection methods (organs versus swabs) for each tissue combination (Figure 3.4 and 3.5). (C1: All positives C2: 4 positives + 1 negative C3: 3 positives + 2 negatives C4 : 2 positives + 3 negatives C5: 1 positives + 4 negatives). p value = p value for regression analysis and significant at the 0.05 level. Bolded values are significant.

Collection method	Tissue combination		Linear equation	r^2	p value
Pooled organs	C1	(5+ 0-)	$y = 3.61x + 19.74$	0.99	<0.001
	C2	(4+ 1-)	$y = 3.19x + 21.08$	0.99	0.003
	C3	(3+ 2-)	$y = 3.23x + 21.48$	0.99	0.001
	C4	(2+ 3-)	$y = 3.63x + 21.09$	0.97	0.005
	C5	(1+ 4-)	$y = 3.23x + 22.87$	0.99	0.001
Pooled swabs	C1	(5+ 0-)	$y = 3.24x + 23.94$	0.99	0.005
	C2	(4+ 1-)	$y = 3.11x + 24.05$	0.99	0.013
	C3	(3+ 2-)	$y = 3.04x + 24.09$	0.97	<0.001
	C4	(2+ 3-)	$y = 2.57x + 27.28$	0.97	0.033
	C5	(1+ 4-)	$y = 3.92x + 25.65$	0.98	0.147

The Kappa value (0.7778) for sampling collection methods between pooled organs and pooled swabs (based on pooled results – each dilution for each tissue combination was considered as a sample) showed a substantial agreement indicating a good correlation (Table 3.7).

Table 3.7 Kappa coefficient of agreement between pooled sample collection methods (organs versus swabs), based on pooled results – each dilution for each tissue combination was considered as a sample.

		Organs		
		+	-	Total
Swabs	+	14	0	14
	-	4	18	22
Total		18	18	36
		Kappa value		0.8571

3.4 Discussion

The detection of TSRV in individual samples was as sensitive when organs were sampled compared to swabs. The detection of TSRV in individual samples has a limit of detection of 1.94×10^4 molecules/ μ L (viral RNA copy number). The experiment for comparing swabs with organs was designed to replicate existing methods for sample collection of TSRV but required known concentration of the virus. After collecting a sample by swab, the swab was placed in 3 mL of VTM, diluting the sample 15-fold compared to the organ sample. However, the dilution effect from using a swab for sample collection did not affect the sensitivity of swabs and yielded similar results (1.94×10^5 molecules/ μ L) as when samples were collected directly from organs.

The evaluation of Kappa coefficient showed a perfect agreement between sampling organs and swabs indicating that the using swabs were as good as sampling organs in detecting TSRV positive fish. While the C_T was higher for swab samples, there was a good correlation with organ samples, to the extent that only two additional positives samples (which were at lower dilution) detected with organ samples, were not detectable by swabs. Based on the sensitivity estimates for RT-PCR, blood swabs were as sensitive as serum (88%) for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in swine (Rovira *et al.*, 2007). Although, blood swab samples contained smaller amount of PRRSV than serum at each collection time, blood swabs performed similarly to serum. In another study, nasal swabs showed good to excellent Kappa coefficient agreement compared to lung tissues for the detection of swine influenza virus (SIV) which causes porcine respiratory disease complex (PRDC) in swine (Swenson *et al.*, 2001).

Oral and cloacal swabs have been proven as effective sample collection methods compared to brain tissues for the detection of West Nile virus (WNV) in dead birds (Komar *et al.*, 2002). The concentrations of WNV infectious particles in *Corvus brachyrhynchos*, *Corvus ossifragus*, and *Cyanocitta cristata* were detected equivalently ($5.7 - 8.2 \times 10^5$ PFU) by the three different sample collection methods (Komar *et al.*, 2002). These findings are in agreement with the detection of TSRV by swabs in this study. It been reported that swabs performed as well as the gold standard method of sample collection in these studies; intestine (Fox *et al.*, 2000), tissue biopsy (Pelizzer *et al.*, 2001), nasopharyngeal aspirate (Heikkinen *et al.*, 2002) and Virocult[®] swab (Valette *et al.*, 2010).

A preliminary study was conducted by Douglas, Wilson & Zainathan (unpublished, see Appendix III) to evaluate the performance of swabs to collect field samples for TSRV. Parallel samples were collected using swabs and organ samples from farm sites in the south east of Tasmania. For the study, samples were collected from Atlantic salmon and a total of 73 and 154 samples were tested independently by qPCR and conventional hemi-nested RT-PCR, respectively. Swabs performed better than organ samples in these studies and both collection methods demonstrated a moderate kappa agreement (Douglas, Wilson & Zainathan, unpublished, Appendix III). Swabs detected additional 7 positive samples by qPCR and 27 positive samples by conventional hemi-nested RT-PCR. This preliminary field study was conducted based on a larger sample size (73 and 154 samples) compared to the experimental trials conducted in this chapter (30 for individual and 36 for pooled collection method). Small sample sizes have been reported to pose challenges to any statistical analyses and result in decreased predictive values compared to larger sample sizes (Enoe *et al.*, 2000 and Wallace *et al.*, 2008).

Among the advantages of using swabs for sample collection is the swiftness and ease of application which makes this method adaptable to field sampling, suitable for large sample sizes (Smalley and Campanella, 2006; Hyatt *et al.*, 2007; Le Vin *et al.*, 2011), low cost (Wuthiekanun *et al.*, 2001) and preservation of sample integrity (Hyatt *et al.*, 2007) . In addition, swabs have been described to provide the best DNA recovery while ensuring minimal risk of contamination and ease of use within the laboratory (Hyatt *et al.*, 2007). The storage of a swab in a tube provides a physically compact and enclosed device, which is resistant to breakage or damage, especially during transportation from farm sites (Johnson *et al.*, 1984).

There was no significant difference in the mean C_T values obtained at $t = 0$ and $t = 24$, when samples were held between 2-8°C. Samples for surveillance and diagnostic work are collected from farm sites located in the south-east, northern and western regions of Tasmania and sent to the Animal Health Laboratory of DPIWE laboratory for testing within 24 hours. Therefore, the time points at $t = 0$ and $t = 24$ hours were selected because the samples typically are received by the laboratory within 24 hours of collection. This was important to determine since TSRV, an *Aquareovirus*, is a double-stranded RNA virus and labile (Lupiani *et al.*, 1995; Nason *et al.*, 2000). The outer capsid layer of Aquareoviruses' virions has been reported to be labile and prone to degradation (Lupiani *et al.*, 1995; Nason *et al.*, 2000) . Samples collected in the field are kept cool by packing them in an insulated box with ice bricks before being despatched. The decay rate of viral infectivity, and hence integrity is influenced by temperature (Johnson, 1990); therefore keeping samples cool once collected is important in maintaining integrity of the virus, consistent with OIE (2011) recommendations. Experimentally, samples were held between 2-8°C and it is not known if field samples could be kept within this temperature range using an insulated container with ice bricks, particularly

in summer time. Moderate level of agreement was obtained between $t = 0$ and $t = 24$ suggesting that the swabs can be stored in VTM for 24 hours and still yield similar qPCR results.

The stability of TSRV for 24 hours is assumed to be at least partly due to the storage of swabs in VTM. VTM, based on Hank's balanced salt solution (HBBS) supplemented with 10% FBS, is the recommended medium for the preservation and transport of viral samples from the field to laboratory (Crane and Williams, 2008). The apparent stability of TSRV in VTM is likely to be due to the Hank's balanced salt solution because of its ability to stabilise osmotic pressures, important to maintaining virus integrity (Johnson, 1990). Protein, supplied by the FBS, is also important for virus stability (Johnson, 1990). In a study of transport medium to recover human respiratory viruses, it was found that 90% of viruses could be recovered where the transport medium was based on HBBS (Huntoon *et al.*, 1981).

Vigorous shaking in VTM appeared to be a reproducible process for releasing TSRV from swabs given consistent C_T values at both $t = 0$ and $t = 24$. In this study with TSRV, once the sample was collected by swab, it was shaken vigorously in VTM to increase the likelihood of virus release from the sample and reduce the effects of entrapment in the swab. Bacteria collected using swabs in transport medium have been shown to be viable for at least 48 hours from the time of sampling, confirming the adequacy of swabs as a n effective collection method (Mahony and Chernesky, 1985; Farhat *et al.*, 2001). Reduced recovery of samples from swabs in transport medium has been attributed to organism entrapment, particularly where the number of trapped organisms was too low to be adequately released once the swab was applied to the surface of the bacteriological culture medium (Collee *et al.*,

1974; Sng *et al.*, 1982). Based on research carried out to study the recovery of bacteria from swabs, a agitation of a loaded swab in sterile broth released larger numbers of organisms compared to a passive process where swabs were not agitated (Collee *et al.*, 1974). Overall, these findings provided confidence that VTM preserves the integrity of TSRV held at 4°C for 24 hours and that swabs can be used as a convenient sampling method.

Pooled sample collection (organs versus swabs)

This study indicated that pooling four negative samples with one positive sample did not affect the limit of detection of the collection methods for dilutions containing high concentrations of TSRV (C_T values < 32.0 containing 1.94×10^5 molecules/ μ l viral copy numbers). However, pools of five organ samples or five swabs at lower dilutions had higher C_T values (> 35.8) for the tissue combinations than higher dilutions. This is because pooling of individual fish reduces the likelihood of detecting virus at low prevalence/viral load (Cowling *et al.*, 1999; Wuthiekanun *et al.*, 2001; Grmek-Kosnik *et al.*, 2005; Gomes-Keller *et al.*, 2006). In a study of salmonid alphavirus (SAV), an ssRNA virus, samples which had an individual C_T value greater than 35 (low viral loads) had a viral load that failed to be detected by qPCR when analysed in pools for SAV (Jansen *et al.*, 2010). This shows that pooling reduced the likelihood of detection of SAV, which is in agreement with this study. Pooling of specimens (pooled swabs) decreased the sensitivity of MRSA (methicillin-resistant *Staphylococcus aureus*) detection compared with processing each swab separately, particularly in swabs with a low number of colony-forming units (Grmek-Kosnik *et al.*, 2005). In another study, the use of pooling of buccal swabs for the detection of FeLV (Feline leukaemia virus), an ssRNA virus, demonstrated a limitation of the method due to loss of sensitivity because of the dilution effect in samples (Gomes-Keller *et al.*, 2006).

According to the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2011), samples can be pooled for no more than 10 fish per pool for detecting subclinical carriers of virus. When disease is rare (prevalence < 10%), pooled testing offers a cost-effective alternative to testing samples from individual fish, if the goal of a study is to estimate incidental prevalence (Cowling *et al.*, 1999). The favourable method to estimate individual level of prevalence from pooled samples depends on many factors including the proportion of positive pools in the sample, pool size, number of pools tested, prevalence of the disease, clinical status of the disease, purpose of the study, sensitivity and specificity of diagnostic methods (Cowling *et al.*, 1999; Rovira *et al.*, 2007; OIE, 2011).

Pooling reduces material and labour costs during sample collection and processing (Thoesen, 1994; Kapala *et al.*, 2000; Morre *et al.*, 2001; Mumford *et al.*, 2005; Gomes-Keller *et al.*, 2006; Mertz *et al.*, 2007), resulting in an increase in effective sample size (Thoesen, 1994) and a reduction of workload by allowing the testing of a larger number of samples (Rovira *et al.*, 2007; Engelsma *et al.*, 2009). Pooling of samples can be successful if the diagnostic test has a high analytical sensitivity (Rovira *et al.*, 2007). Although, they were not compared directly, the individual and pooled collection methods demonstrated similar limits of detection, 1.94×10^4 molecules/ μL (viral RNA copy number) for organ samples and 1.94×10^5 molecules/ μL for swabs. A pooling strategy would be adequate for the collection of samples for TSRV for these reasons: the qPCR used for the detection of TSRV is highly sensitive and specific (see Chapter 2 of this thesis); the prevalence of TSRV in Tasmanian population of Atlantic salmon is less than 10% (Gemma Carlile pers communication; see Chapter 2 of this thesis); and the decrease in sensitivity can be compensated by an increased potential to use and accommodate a larger number of samples.

The advantages of using swabs as a preferred sample collection method for the detection of TSRV compared to organ samples are evident from these experimental trials. Swabs appear well suited as the sample collection method for surveillance and screening where the purpose of testing is to establish presence or absence of TSRV in a population. The use of organ samples as the sample collection method would be more suitable for the determination of freedom from disease, because it is critical to detect every single positive sample. Further studies should be done to validate the use of swabs in field trials using a larger number of samples and to evaluate different types of swab material that could be used for viral sample collection. The studies described in this chapter have led to the development of a rigorous and defined sample collection method for TSRV. Following these studies described swabs have been adopted as the diagnostic sample collection method for the detection of TSRV by the Fish Health Unit, Animal Health Laboratory, DPIWE, Launceston, Tasmania.

CHAPTER 4

Field investigation on incidence of TSRV infections with other pathogens

4.1 Introduction

Reovirus infections have been reported in disease outbreaks with mixed aetiology (Baya *et al.*, 1989; Lupiani *et al.*, 1989; John *et al.*, 2001; Cusack *et al.*, 2001; Hoole *et al.*, 2001). This does not only demonstrate the uncertainty about the virulence and pathogenesis of aquareoviruses in these co-infections, but also about the potential role of aquareovirus infections in other disease outbreaks. Isolation of aquareovirus often occurs during investigations that demonstrate conditions with a mixed or unknown aetiology, involving either other viruses or a bacterial infection. In mixed infections with bacteria, treatment of the bacterial infection with antibiotics does not always resolve the condition, suggesting that reovirus may play some role in the disease (Lupiani *et al.*, 1989; Crane and Carlile, 2008). An infection with a virus may adversely affect the resistance of a host to other infectious agents by influencing the host's immune response to other pathogens (Phuoc *et al.*, 2009).

The Tasmanian Atlantic salmon industry has for many years experienced several diseases associated with different pathogens such as Tasmanian Atlantic salmon Reovirus (TSRV), Tasmanian Aquabirnavirus (TAB), Tasmanian Rickettsia-like organism (TRLO), *Yersinia ruckeri*, *Vibrio* sp., *Aeromonas salmonicida* and *Neoparamoeba perurans*. The co-occurrence of diseases in the production of salmonids is likely to be a result of a combination of factors which could include environmental changes, weakening of the immune system of the host, thus, increasing the susceptibility of the host to co-infections with different pathogens in the environment (Nylund *et al.*, 2011). The occurrence of TSRV, predominantly in South-east Tasmania, has raised questions concerning the potential role of TSRV in the

outbreaks of two different commercially important diseases found only in South-east Tasmania, Amoebic gill disease (AGD) and Rickettsiosis.

AGD is an economically important disease (Munday *et al.*, 1990; Nowak, 2001; Parsons *et al.*, 2001) caused by *Neoparamoeba perurans* (see Young *et al.*, 2007) which can be isolated from the gills of infected Atlantic salmon. Mortality rates of up to 50% in Atlantic salmon can occur if the disease is left untreated (Munday *et al.*, 1990). The prevalence of *N. perurans* is influenced by environmental conditions such as seasonal water temperature and salinity; both considered to be important factors in the occurrence of the disease (Nowak, 2001). Rickettsiosis (caused by TRLO infections) has occurred periodically since 2001 and occurs in close association with T SRV infections in Atlantic salmon (Morrison, 2011). In 2006, significant and widespread disease occurred during summer in South-east Tasmania which resulted in significant mortalities (Morrison, 2011). Since the first outbreak of rickettsiosis, this disease has been treated with oxytetracycline (OTC). DPIPWE (Launceston) has developed an experimental vaccine, Corrovac for TRLO infections, which is currently undergoing field trials (Jeremy Carson, DPIPWE pers communication). It has been implied that Atlantic salmon are exposed simultaneously to T SRV, *N. perurans* and TRLO when introduced as smolts into the marine environment (Mark Crane pers communication). Co-infections with these pathogens either naturally or by experimentation has not been investigated systematically.

Experimental infections of Atlantic salmon with TSRV suggested that TSRV was of low pathogenicity (Humphrey *et al.*, 1993; Crane *et al.*, 1993), a finding confirmed by the frequent isolation of TSRV from farmed fish in the absence of clinical signs of disease. Moreover, experimental TSRV infections trials demonstrated inconsistent results. An early infection trial using 11-month-old Atlantic salmon, infected by intraperitoneal inoculation (IP), showed viral replication and persistence in Atlantic salmon in the absence of clinical disease or severe pathological changes (Humphrey *et al.*, 1993). Histological examination of tissues sampled from experimental fish showed moderate focal mononuclear cell infiltrations of the liver and vascular congestion in only a few of the infected fish and no histological changes were observed in the remaining infected salmon (Humphrey *et al.*, 1993). No mortalities were recorded among inoculated fish for the experimental duration of 29 days (Humphrey *et al.*, 1993). However, 5- and 8-month-old Atlantic salmon inoculated with a similar titre ($TCID_{50}/\mu L: 10^{3.3}$) of TSRV showed necrotic and degenerative lesions in the liver, pancreas, spleen, kidney and gastrointestinal tract at 9 days post-inoculation (Crane *et al.*, 1993). Atlantic salmon held at two temperatures, 8-10°C and 18-22°C, were infected via intraperitoneal (IP) inoculation. No mortalities were recorded for Atlantic salmon held at 8-10°C with very few recorded in the 18-22°C group, suggesting that Atlantic salmon at 5 months of age had a low susceptibility to TSRV (Crane *et al.*, 1993). Further TSRV infection trials showed that the histological changes included mild-to-severe, multifocal, acute hepatic necrosis, mild-to-severe, multifocal necrosis of the renal haematopoietic tissue and moderate-to-severe and multifocal acute pancreatic necrosis with foci of liquefactive fat necrosis in the most severe cases (Gemma Carlile pers communication). There is a need for further research on the gross pathology and histological changes associated with TSRV infections in farmed Atlantic salmon.

This chapter focused on co-infections of *N. perurans*, T RLO and T SRV and the hypothesis that a primary infection with T SRV would increase the susceptibility of infected fish to infections with other agents. This was investigated through a field study and analyses of archival data. Comparison between gross pathology and histological changes associated with infection of T SRV was described.

4.2 Materials & Methods

4.2.1 Study site and sampling

Atlantic salmon were sampled from farm sites located in South-east Tasmania between 19th January and 2nd March 2011. A total of 195 fish were collected from 3-5 cages from two or three sites during each sampling. Each time, at least 45 salmon were sampled; these included live, apparently healthy fish (used as controls), moribund fish and freshly dead fish. The largest proportion of freshly dead salmon were collected on 2nd March since moribund fish were unavailable, whereas the smallest proportion of dead fish was collected on 16th February. Some of the salmon (fish numbered from 151 to 189) on 2nd March 2011 by intra-peritoneal injection with the bivalent vaccine *Aeromonas* against *Vibriosis* and *Aeromonas septicaemia*, as these salmon were initially destined for Macquarie Harbour which, in contrast to South-east Tasmania, requires (by legislative regulations) the fish to receive this vaccine (Macquarie Harbour is known to be a risk factor for these infections). Table 4.1 shows the sampling dates, numbers of fish sampled according to the status of fish, fish size, number of cages per site, number of sites and Figure 4.1 shows the water temperature (taken at 5 metres depth) at the time of sample collection. For all the sampled fish, the mean weight was 1.59 kg (standard error 1.11 kg) and the mean length was 44.85 cm (standard error 8.17 cm).

Table 4.1 Total number of fish sampled according to fish status, number of sites, number of cages per sites on each sampling dates from farm sites in South-east Tasmania, fish sizes and water temperature during the study. NA: Not available.

Sampling dates (2011)	Number of sites	Number of cages	Fish mean weight (kg) & standard error	Fish mean length (cm) & standard error	Water temperature (°C)	Fish status	Fish number
19th January	3	3	2.10 (1.43)	41.9 (7.20)	15.5	Control	11
						Moribund	20
						Dead	19
1st February	2	4	1.62 (1.26)	47.6 (8.33)	15.6	Control	11
						Moribund	18
						Dead	21
16th February	2	5	1.65 (0.66)	48.3 (7.16)	16.5	Control	21
						Moribund	17
						Dead	7
2nd March	2	3	0.99 (1.10)	41.6 (10.0)	16.7	Control	15
						Moribund	NA
						Dead	35
						Total	195

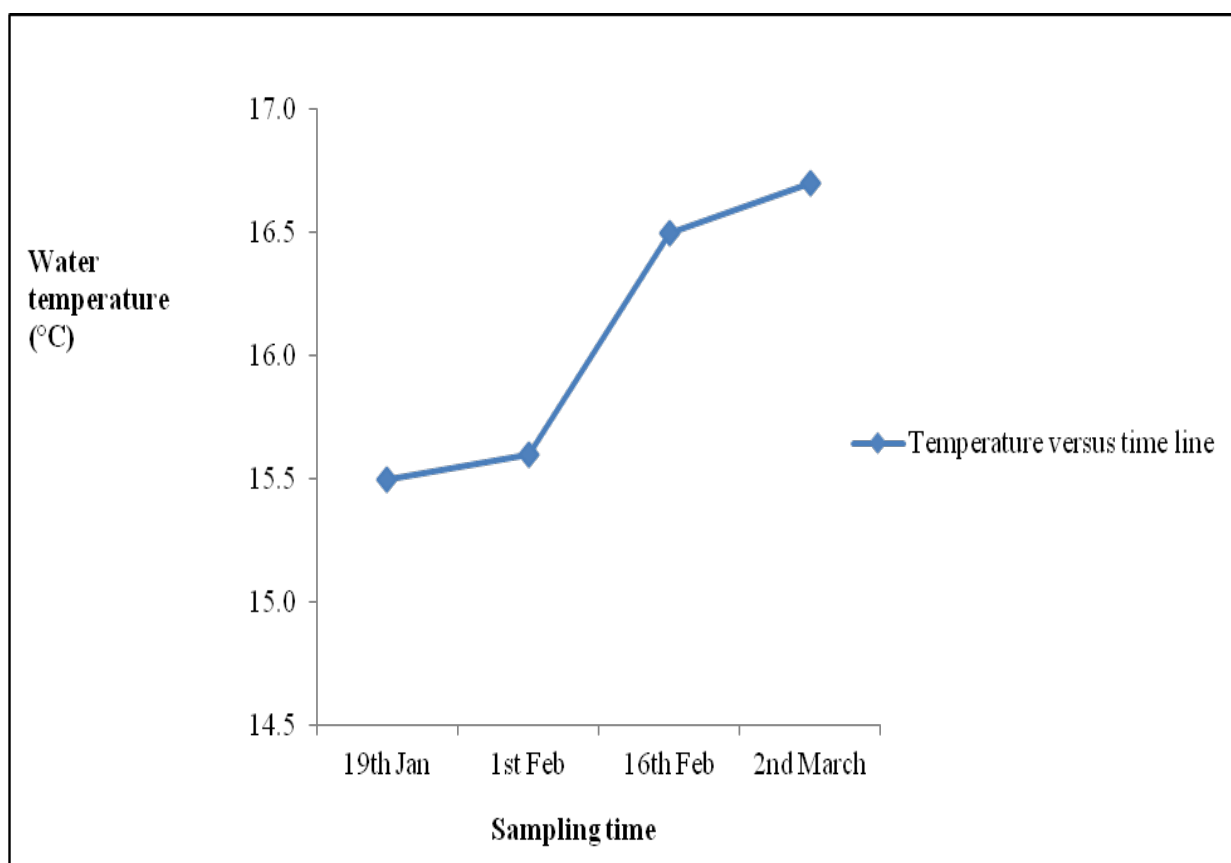


Figure 4.1 Water temperature taken at 5 m depth at the study area during sampling time between 19th January and 2nd March 2011.

To examine the nature of infection with TSRV in Atlantic salmon, gross signs were observed and samples for histology, microbiology and molecular diagnostics were collected. The fish were euthanized using a solution of AQUI-S (0.5 mL/20 L water) (AQUI-S[®], New Zealand). The weight and length were measured, and gross signs and clinical history were recorded. Presence of other pathogens was investigated. For each fish, the kidney sample was collected using a sterile loop, a thin smear was prepared on slides for all the samples and air-dried for Gram staining. Another kidney sample collected using a loop was streaked out on sheep's Blood Agar (blood agar base no. 2, Oxoid, Adelaide, enriched with 7% defibrinated sheep's blood) and TCBS Agar (thiosulphate citrate bile sucrose agar, Oxoid) and the agar plates were kept on ice. Thiosulphate-citrate-bile salts-sucrose agar (TCBS) is a type of selective agar medium that is used to isolate *Vibrio* spp. Blood Agar is a bacterial growth medium that can distinguish normal from pathogenic bacteria based on the effect of bacterial haemolytic exotoxins on red blood cells (Pfeffer and Oliver, 2003).

Samples for TSRV isolation were obtained from kidney, liver and spleen. The organs were swabbed with sterile wooden cotton-tipped swabs (Livingstone International, Australia) for a few seconds and then the swabs were shaken vigorously into a corresponding tube containing 3 mL viral transport medium (VTM). The wooden handle of the swab was broken off at the height of the tube which was then capped ready for transport. For the testing of Tasmanian *Rickettsia*-like organism (TRLO), pooled samples of kidney, liver and spleen were excised aseptically and placed in a sterile container. The samples for TRLO and TSRV were kept on ice and packed securely in an insulated box with ice bricks.

For the testing of *N. perurans*, samples were collected from Atlantic salmon gills using swabs and stored in RNA later™. Upon processing, the swabs were removed from RNA later™ and placed directly in Tissue and Cell Lysis Solution (Epicentre, Wisconsin, USA). The DNA extraction and qPCR assay were performed as reported by Bridle *et al.* (2010). For histological examination, samples of gills (arch 2 and 4), kidney, liver, spleen, skin and muscle through the lateral line, gut, pyloric caeca, heart and brain were fixed in 10% Neutral Buffered Formalin (NBF). Each time, 90% to 100% control fish and 30% to 94% moribund fish, out of the total proportion of fish collected in this investigation were subjected to histological examination. None of the dead fish were sampled for histology (due to tissue autolysis compromising tissue integrity). The samples for *N. perurans* (swabs) were sent to National Centre for Marine Conservation and Resource Sustainability, University of Tasmania for further processing. All the other samples were taken to the Animal Health Laboratory, Mount Pleasant Laboratories (AHL) for receipt in 24 hours and further processing.

4.2.2 Histology

The fixed samples were transferred to 70% ethanol (EtOH) and a mid-sagittal section of each sample was then cut, trimmed into histology cassettes and processed using a Leica tissue processor (Leica Microsystems, Australia). Once processed, the cassettes were transferred to the embedding console and the samples were embedded in paraffin wax (Leica Microsystems, Australia). Sections of 5 µm thickness were cut from each block using a microtome (Microm, Cambridge Scientific, USA) and the prepared slides were dried in the 60°C hot air oven for at least 15 minutes. Once dried, the sections were stained with haematoxylin and eosin and viewed using a Nikon Optiphot (Optotek, USA) microscope. The

sections were photographed with a Leica DC300f camera (Wetzlar, Germany) mounted on a light microscope (Olympus BH2, Hamburg, Germany) and images were modified using Image Manager Version 1.20 software (Leica IM50).

4.2.3 Microbiological examination

Smears were stained by modified Hucker's method (Hendrickson & Krenz, 1991) using dilute carbol fuchsin as the counterstain (Preston & Morrell, 1962). The tissue smears were heat-fixed by passing the smear face up for 2-3 minutes through a Bunsen flame and then cooled on the bench for 2 minutes prior to the staining procedure. The culture plates were incubated at 25°C for 48 hours. Growth on culture plates was assessed for purity and colonies were subcultured to appropriate media for subsequent identification. Cultures were assessed by Gram stain to determine cell morphology, cell arrangements and Gram reaction. For Gram negative rods, oxidase reaction was determined by Kovács' method (Cowan, 1974). Gram negative rods that were oxidase negative were presumptively allocated to the *Enterobacteriaceae* and identification undertaken using the MicroSys E24 phenotyping panel (DPIPWE, Launceston).

Phenotypic profiles were assessed by probabilistic identification software, PIBWin (Bryant, 2004) and data matrix described by Farmer (1995). Isolates identified as *Yersinia ruckeri* were assessed against the criteria described by Carson and Wilson (2009) to determine serotype and biotype. Gram negative rods that were oxidase positive were presumptively allocated to the *Vibrionaceae* and identified using the MicroSys V36 phenotyping panel (DPIPWE, Launceston). Phenotypic profiles were assessed using PIBWin

and the VibEx7 data matrix (Carson *et al* 2008). An identification was accepted when the Willcox probability value $P \geq 0.99$ and the modal likelihood score was ≥ 0.001 .

The isolates that were not represented in the data matrix were reported as a *Vibrio* species, not identified. They had characteristics conforming to those that define the Vibrionaceae family: Gram negative rods, oxidase positive, glucose fermentative and sensitive to the pteridine compound O/129 at 150 µg. Identification of isolates as *Nocardia* species was made on the basis of Gram reaction, cell morphology and arrangement, colony morphology and acid-fastness by the modified Ziehl-Neelsen stain method. Identification to species level for *Nocardia* sp. was not undertaken.

4.2.4 TaqMan qPCR assay for TSRV

4.2.4.1 RNA extraction

RNA extraction was carried out using the MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems, USA) and processed using the MagMAX™ Express 96 magnetic particle processor (Applied Biosystems, USA). The swabs in VTM were shaken lightly before removing 70 µL from the bottom of the tube for RNA extraction. The Lysis/Binding solution was prepared by adding 1.1 µL carrier RNA in 75 µL Lysis/Binding buffer; 75 µL 100% isopropanol was added to this mixture (carrier RNA and Lysis/Binding buffer) and mixed thoroughly. Each extraction reaction for a sample required 20 µL RNA Binding Bead Mix. RNA Binding Bead Mix consisted of RNA binding beads and Lysis/Binding Enhancer. The RNA binding beads were mixed well with a vortex mixer at a moderate speed to form a uniform suspension. A total of 10 µL RNA binding beads (10 mg/mL) were added to 10 µL Lysis/Binding Enhancer, mixed with a vortex mixer and kept on ice until needed.

The MagMAX[™] processing plates were prepared according to the manufacturer's instructions (Appendix IV). Deep-well plates were used for all the processing steps except elution (the last stage) when an elution plate with lid was used. The processing plates were loaded into appropriate loading stations in the magnetic particle processor (MagMAX[™] Express 96) and the extraction was carried for 30 minutes. The elution plate was sealed with plate-sealing tape and stored at -20°C till PCR amplification.

4.2.4.2 qPCR assay

The qRT-PCR detection for TSRV was conducted using the specific primer and probe set developed by Carlile (2011). The primers and probe for the qRT-PCR assay were designed to amplify and detect an 82 nucleotide sequence of cDNA corresponding to 705–787 bp of the viral genome within segment 10 of TSRV. The sequences for the primers were: Forward primer, TSRV-10F (position 705–725, 5'-GATCGAACCCGTCGTGTCTAA-3'), reverse primer, TSRV-10R (position 769–787, 5'-CGGTGCTCAGCTTGTCACA-3'). The TSRV probe (position 731–748, 5'-CCCGAGCCATCTGGCGC-3') contained a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), located at the 5' end and the quencher, 6-carboxy-tetramethyl-rhodamine (TAMRA), located at the 3' end.

The qRT-PCR assay also included 18S ribosomal (rRNA) primers and probe set to check for sample-specific RT and PCR inhibitors as well as to correct for variation in RT efficiency and template quantity. The primers and probe for 18S were as followed: Forward primer, 18F (5'-CGGCTACCACATCCAAGGAA-3'), reverse primer, 18R (5'-GCTGGAATTACCGCGGCT-3') and probe, 18S Probe (5'-TGCTGGCACCAGACTTGCCCTC-3') which contained a proprietary fluorescent reporter

dye, (VIC) located at the 5' end and the quencher TAMRA located at the 3' end. The amplification protocol consisted of addition of RNA to a master mix as per the TaqMan[®] one-step RT-PCR master mix reagents kit (Applied Biosystems, USA).

The reaction mixture consisted of: 5.75 µL RNase free water, 12.5 µL TaqMan[®] 2 × Universal PCR master mix no AmpErase[®] UNG (containing AmpliTaq Gold[®] DNA polymerase), 0.625 µL 40 × Multiscribe[™] and RNase inhibitor mix, 1.25 µL Primer TSRV-10F (18 µM), 1.25 µL Primer TSRV-10R (18 µM), 1.25 µL TSRV-10 probe (5.0 µM), 0.125 µL 18S rRNA Primer F (10 µM), 0.125 µL 18S rRNA Primer R (10 µM), 0.125 µL 18S rRNA VIC labelled Probe and 2 µL viral RNA. The amplification was carried out in ABI PRISM[™] 7500 Fast Real-Time System (Perkin-Elmer, Applied Biosystems, USA) with a total of 25 µL reaction mixture. The RNA was amplified with the following programme: an initial 30 minutes at 48°C, followed by 10 minutes at 95°C, then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The cut-off value for qRT-PCR for the detection of TSRV was set at $C_T = 35.8$ (see section 2.3.4.1 in chapter 2), hence, C_T values < 35.8 were considered positive, C_T values between 35.8-36.13 were ambiguous values and C_T values > 36.13 were considered negative.

4.2.5 TaqMan qPCR assay for RLO

4.2.5.1 DNA extraction

DNA was extracted from Atlantic salmon liver, kidney and spleen using the MagMax-96 DNA multi-sample kit (Applied Biosystems, USA) and processed using the MagMAX[™] Express 96 magnetic particle processor (Applied Biosystems, USA). A total of 92 μ L PK buffer and 8 μ L 100 mg/mL Proteinase K were added to a sterile 1.5 mL microcentrifuge tube. Each organ (approximately 20 mg) was excised using sterile scalpel and forceps and placed in the prepared sterile microcentrifuge tube. The samples were incubated at 55°C overnight for complete lysis.

Once the samples were lysed, DNA binding bead mix was prepared for each sample; 8 μ L DNA binding beads (10 mg/mL) were added to 12 μ L nuclease-free water. A total of 200 μ L Lysis Buffer and 100 μ L of the sample were added to each well of the sample plate; prior to adding 150 μ L 100% isopropanol. The plate was sealed and shaken for 3 minutes (600 rev/min) on a plate shaker. The MagMAX[™] processing plates were prepared according to the manufacturer's instructions (Appendix). Deep-well plates were used for all the processing steps except elution (the last stage) when elution plates with lids were used. The processing plates were loaded into appropriate loading stations in the magnetic particle processor (MagMAX[™] Express 96) and extraction was carried for 30 minutes. The elution plate was sealed with plate-sealing tape and stored at -4°C until PCR amplification.

4.2.5.2 qPCR assay

The TRLO primers and probe were based on the 23S rDNA gene, a relatively conserved genomic region of TRLO (Corbeil *et al.*, 2003). Primer and probe sequences were as followed: Forward primer, F-760 (5'-TCT GGG AAG TGT GGC GAT AGA-3'), reverse primer, R 836 (5'-TCC CGA CCT ACT CTT GTT TCA TC-3') and probe, PS23S (FAM-TGA TAG CCC CGT ACA CGA AAC GGC ATA-TAMRA). The primers and probe for 18S were as followed: Forward primer, 18SF (5'-CGG CTA CCA CAT CCA AGG AA-3'), reverse primer, 18SR (5'-GCT GGA ATT ACC GCG GCT-3') and probe, 18S Probe (VIC-5'-TGC TGG CAC CAG ACT TGC CCT C-3'-TAMRA).

The reaction mixture consisted of: 5.37 μ L RNase free water, 10.0 μ L TaqMan[®] 2 \times Universal Fast Master Mix, 0.75 μ L Primer F-760 (24 μ M), 0.75 μ L Primer R836 (24 μ M), 0.75 μ L probe (6.66 μ M), 0.1 μ L 18SF (12.5 μ M), 0.1 μ L 18SR (0.1 μ M), 0.18 μ L 18S Probe (3.5 μ M) and 2 μ L bacterial DNA. The amplification was carried out in ABI PRISM[™] 7500 Fast Real-Time System (Perkin-Elmer, Applied Biosystems, USA) with a total of 20 μ L reaction mixture. The DNA was amplified with the following programme: an initial 2 minutes at 50°C, followed by 10 minutes at 95°C, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The positive C_T cut-off value is < 35.8, C_T values between 35.8-37.0 are ambiguous and C_T values > 37.0 are considered negative. A positive test result indicates that DNA from *Piscirickettsia* sp. is present in the submitted samples. The PCR primers are directed at the 23S rDNA region and are specific for both *Piscirickettsia salmonis* and the Tasmanian Rickettsia-like organism. It should be noted however that *P. salmonis* is an exotic pathogen and has to-date not been detected in Australia. Tasmanian Rickettsia-like organism differs at the genetic and antigenic level from *P. salmonis* (Corbeil and Crane, 2009).

4.2.6 Statistical analysis for field sampling

The effects of time of sampling and fish status on the prevalence of TSRV and TRLO were determined using two-way ANOVA analyses. These results were presented as mean C_T value \pm standard error based on pooled data. Two way ANOVA analyses were conducted to test the effect of sampling time and fish status on the size, length and condition factor of the fish. The results were presented as mean weight/length/condition factor \pm standard error. ANOVA analyses were performed using SPSS[®] statistic software version 19.0. Values were identified as significantly different if $p < 0.05$. The Tukey HSD *post hoc* test was applied at a significance level of $\alpha \leq 0.05$, to determine differences between the explanatory variables. Statistical analyses were not performed for *Yersinia ruckeri* and *Nocardia* sp. due to their low prevalence. Goodness of fit Chi-square tests (χ^2) were conducted to determine difference in frequencies of presence/absence of *N. perurans* and *Vibrio* sp. according to sampling time and fish status. Chi-square Test of Independence was conducted to determine the hypothesis whether TSRV predisposes fish to other infections. Chi-square Test of Independence and Goodness of fit Chi-square tests (χ^2) were performed using SPSS[®] statistic software version 19.0 and values were identified as significantly different if $p < 0.05$.

4.2.7 Archival cases

Data from archival cases of diagnostic submissions were analysed to describe the co-occurrence of TSRV and TRLO based on results of available diagnostic methods (virus isolation, conventional hemi-nested RT-PCR, and qPCR) and histopathological changes coinciding with TSRV infections in farmed Tasmanian Atlantic salmon. These data were obtained from submissions of diagnostic cases from the Fish Health Unit, Animal Health Laboratory, DPIPWE, Launceston, Tasmania. Data collation was difficult as the data set was incomplete and it was important to identify all available data that could be analysed. The purpose of diagnostic submission for the detection of TSRV and TRLO was found variable throughout the years (Table 4.2). Most of the diagnostic samples were a part of the salmonid health surveillance program which was initiated by DPIPWE three decades ago.

Diagnostic submissions were also initiated by incidences of sudden death of fish or low mortalities associated with high water temperature, overcrowding, acclimatization after transfer from freshwater to seawater and post-transfer mortalities. These diagnostic samples were submitted to investigate whether presence/absence of pathogens (TRLO/TSRV) coincided with any stress factors. Data analysed to describe the co-occurrence of TSRV and TRLO were based on total cases of diagnostic submissions for pathogens, total cases of presence for both pathogens, total cases of presence for individual pathogen (TSRV or TRLO alone) and total cases of presence of TSRV in the absence of TRLO. The total cases of presence for both pathogens and presence of TSRV in the absence of TRLO were analysed from the same cases. The sample of origin for both pathogens were analysed further whether the occurrences of positives were either from the same sample or population using the following formula: $(\text{total cases from the same sample} / \text{total cases of presence for both})$

pathogens) x 100% and (total cases from the same population/total cases of presence for both pathogens) x 100%.

Table 4.2 The purpose of submission of diagnostic cases for the detection of TSRV and TRLO between 2001 and June 2011. None = no submission of diagnostic cases.

Year	Diagnostic submission (Total cases)		Purpose of diagnostic submission
	TSRV	TRLO	
2001	14	11	Salmonid surveillance (first outbreak of TRLO in 2001)
2002	6	8	Salmonid surveillance
2003	9	5	Salmonid surveillance and stress factor was high temperature (17°C)
2004	3	5	Salmonid surveillance
2005	None	None	None
2006	3	11	Salmonid surveillance (TRLO testing)
2007	10	10	Salmonid surveillance
2008	11	8	Salmonid surveillance
2009	23	12	Salmonid surveillance and stress factors were associated with high temperature, acclimization and transport
2010	22	20	Sudden death, low mortality due to stress factors: high temperature (16°C-21°C) and overcrowding
2011	7	6	Salmonid surveillance

4.3 Results

4.3.1 Effect of sampling time and fish status on prevalence of pathogens and size, length and condition factor of fish

A total of 189 (96.9%) out of the 195 fish sampled showed presence of pathogens including TSRV, *Yersinia ruckeri*, *Vibrio* sp., TRLO (Tasmanian Rickettsia-like organism), *Nocardia* sp. and *N. perurans* or a combination of these pathogens. Significant differences ($p < 0.001$) were observed in size of the sampled fish (both length and weight) between different sampling dates (Figure 4.2- 4.3). No significant differences were observed for the condition factor based on the sampling time and fish status (Figure 4.4). Table 4.3 illustrates the presence of different pathogens found in Atlantic salmon during this investigation. *N. perurans* (causative agent of AGD) was the most prevalent pathogen followed by TRLO, other bacterial infections and TSRV. Only one fish (number 174, dead) which was positive for TSRV had co-infection with TRLO and *N. perurans* on the last day of sampling, 2nd March. Thus, this co-infection was present in 0.5% of all sampled fish. Similarly, only one fish (number 34, dead) which was positive for TSRV had co-infection with *Yersinia ruckeri*. All the salmon which were positive for TSRV (6.15% of all sampled salmon) had co-infection with *N. perurans*. None of the sampled fish had co-infections with all the pathogens. Four (2.0%) of the positive samples for TSRV, including three freshly dead and a moribund were infected by *Vibrio* sp.

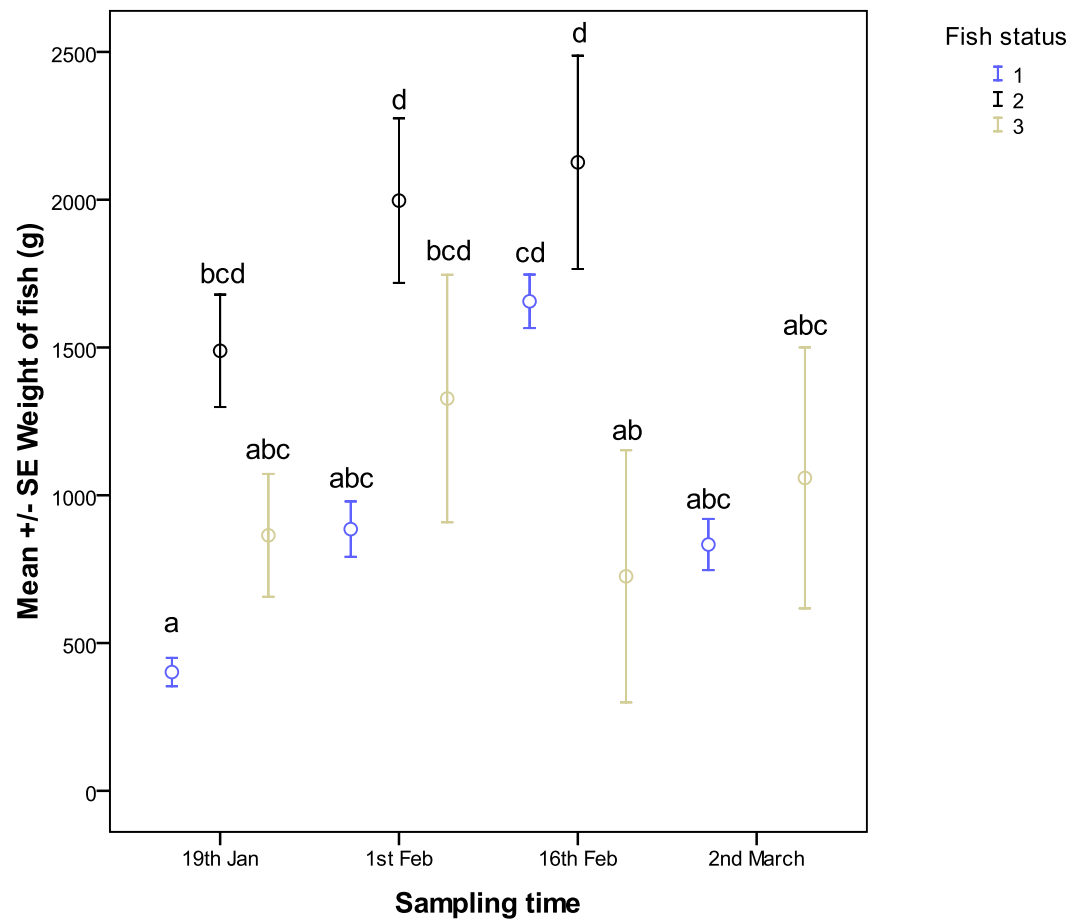


Figure 4.2 Mean (\pm S E) w eight of fish (g) a ccording t o s ampling t ime a nd f ish s tatus. Different lower case letters denote significant differences at $p < 0.05$. SE: standard error. Fish status: 1: Control, 2: Moribund and 3: Dead.

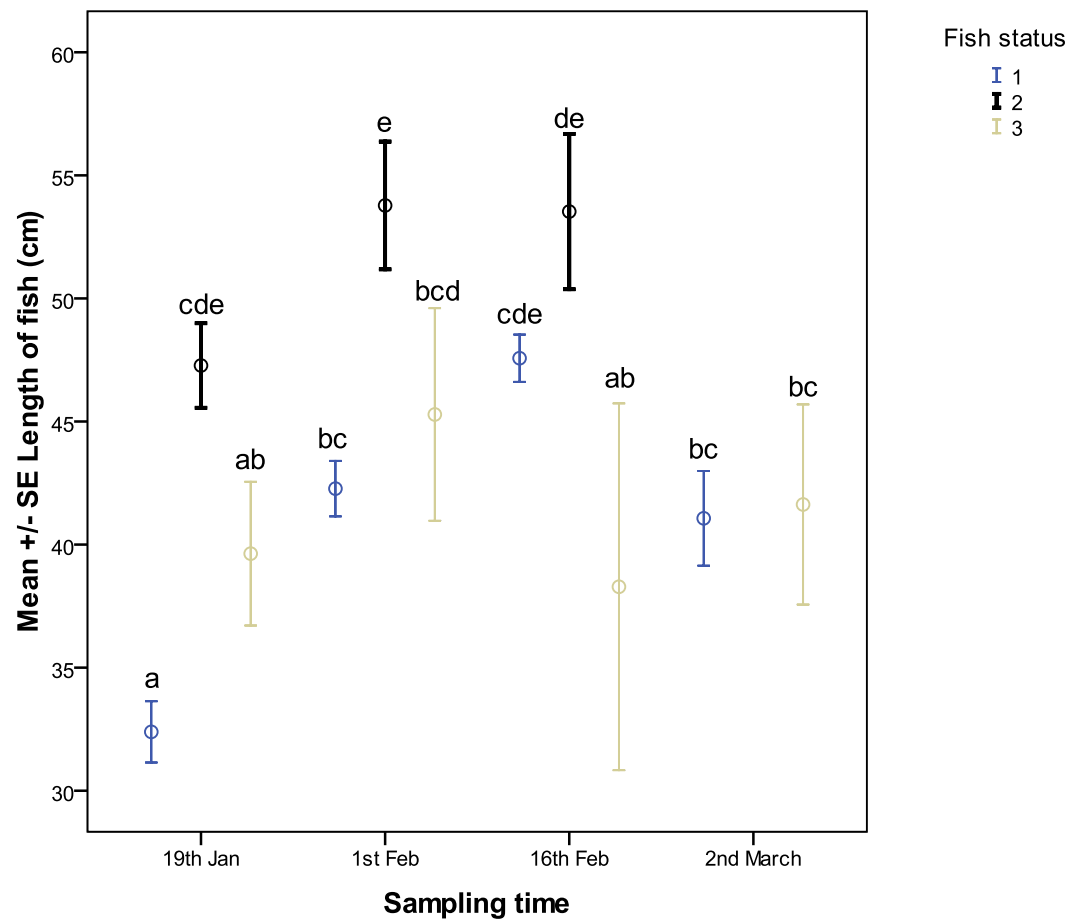


Figure 4.3 Mean (\pm SE) length of fish (cm) according to sampling time and fish status. Different lower case letters denote significant differences at $p < 0.05$. SE: standard error. Fish status: 1: Control, 2: Moribund and 3: Dead.

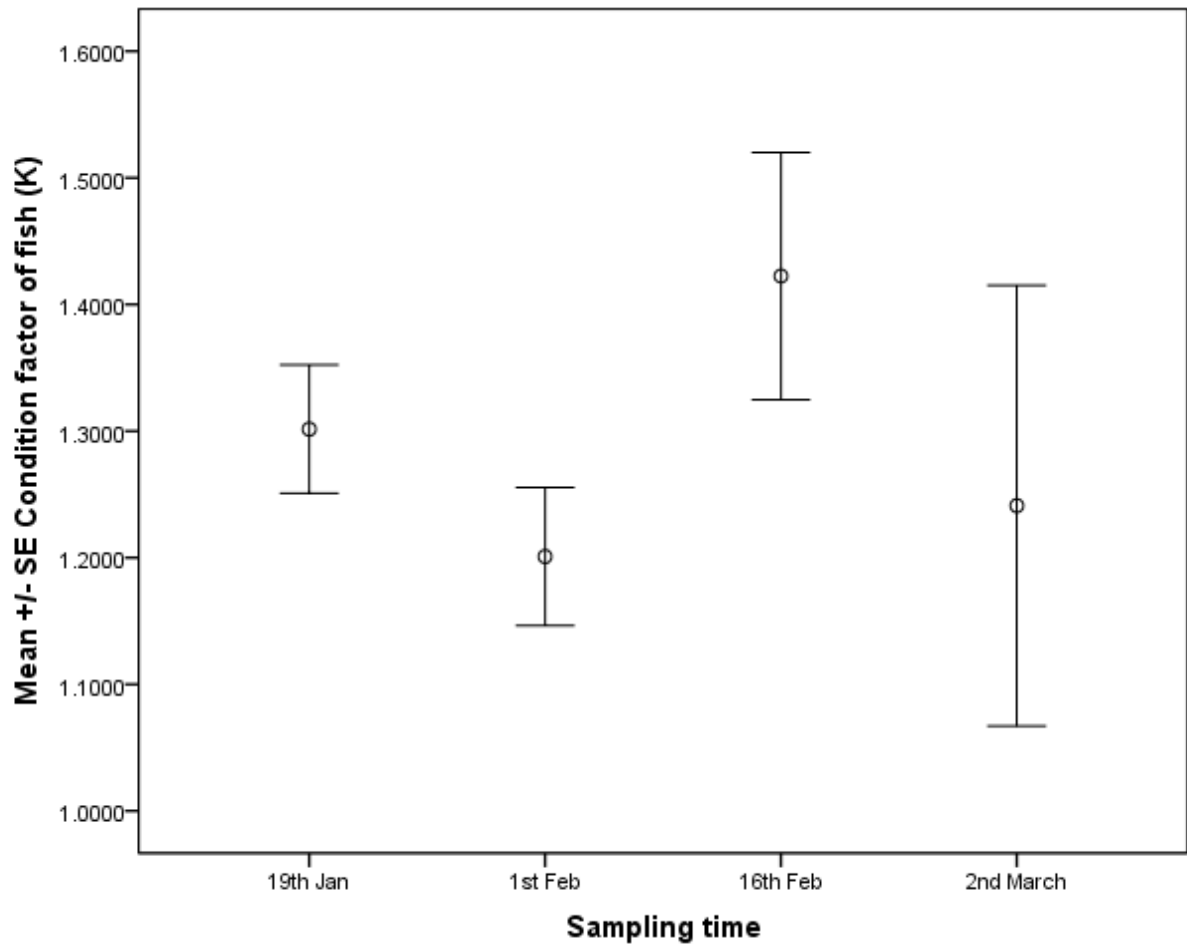


Figure 4.4 Mean (\pm SE) condition factor (K) according to sampling time and fish status. Different lower case letters denote significant differences at $p < 0.05$. SE: standard error. Fish status: 1: Control, 2: Moribund and 3: Dead.

Table 4.3 Co-infections with different pathogens found in Atlantic salmon. Positive C_T values obtained for the detection of TSRV and TRLO by qPCR, positive results for the detection of *N. perurans* by qPCR and bacterial infections by Gram staining and cultures. Bold letters show fish co-infected with TSRV and other pathogens.

Fish number	Fish status	TSRV (qPCR-C _T values)	TRLO (qPCR-C _T values)	<i>N. perurans</i> (qPCR)	Other pathogens
12	Dead	Negative	Negative	Positive	<i>Yersinia ruckeri</i>
13	Dead	Negative	Negative	Positive	<i>Yersinia ruckeri</i>
14	Dead	Negative	Negative	Positive	<i>Yersinia ruckeri</i>
15	Dead	Negative	Negative	Positive	<i>Yersinia ruckeri</i>
34	Moribund	31.931	Negative	Positive	Negative
38	Dead	34.14	Negative	Positive	Negative
44	Dead	29.83	Negative	Positive	<i>Yersinia ruckeri</i>
68	Moribund	35.807	Negative	Positive	Negative
83	Dead	33.227	Negative	Positive	Negative
85	Dead	Negative	19.72	Positive	Negative
86	Dead	Negative	31.00	Positive	Negative
87	Dead	35.08	Negative	Positive	<i>Vibrio tasmaniensis</i>
90	Dead	30.68	Negative	Positive	Negative
91	Dead	32.26	Negative	Positive	<i>Vibrio</i> sp
92	Dead	29.75	Negative	Positive	<i>Vibrio</i> sp
122	Control	35.04	Negative	Positive	<i>Vibrio splendidus</i>
123	Control	Negative	Negative	Positive	<i>Vibrio</i> sp
129	Dead	Negative	32.84	Positive	<i>mixed growth inc Vibrio</i> sp
130	Dead	Negative	Negative	Positive	<i>Vibrio splendidus, V. tasmaniensis, Vibrio</i> spp.
132	Dead	Negative	33.38	Positive	<i>Nocardia</i> sp.
133	Dead	Negative	22.34	Positive	Negative
135	Control	Negative	30.54	Positive	Negative
137	Control	Negative	34.31	Positive	Negative
138	Control	Negative	Negative	Positive	<i>Vibrio</i> sp.
141	Control	Negative	31.88	Positive	Negative

Fish number	Fish status	TSRV (qPCR-C _T values)	TRLO (qPCR-C _T values)	<i>N. perurans</i> (qPCR)	Other pathogens
142	Control	Negative	36.53	Positive	Negative
145	Control	Negative	35.00	Positive	Negative
155	Control	Negative	34.72	Positive	Negative
160	Control	Negative	32.62	Positive	Negative
164	Control	Negative	35.43	Positive	Negative
165	Control	Negative	28.56	Positive	Negative
166	Dead	Negative	26.41	Positive	Negative
167	Dead	Negative	26.21	Positive	<i>Vibrio</i> sp.
168	Dead	Negative	19.40	Positive	<i>Vibrio</i> sp.
169	Dead	Negative	31.55	Positive	Negative
171	Dead	Negative	28.16	Positive	Negative
172	Dead	Negative	22.16	Positive	Negative
173	Dead	Negative	34.66	Positive	Negative
174	Dead	35.12	22.74	Positive	Negative
175	Dead	Negative	27.80	Positive	Negative
176	Dead	Negative	14.61	Positive	Negative
177	Dead	Negative	25.51	Positive	<i>Vibrio</i> sp.
178	Dead	Negative	21.44	Positive	<i>Vibrio</i> sp.
180	Dead	Negative	18.84	Positive	Negative
181	Dead	Negative	17.60	Positive	Negative
182	Dead	Negative	21.59	Positive	Negative
183	Dead	Negative	22.04	Positive	Negative
184	Dead	34.27	Negative	Positive	Negative
185	Dead	Negative	30.33	Positive	<i>Vibrio</i> sp.

Fish number	Fish status	TSRV (qPCR- C _T values)	TRLO (qPCR-C _T values)	<i>N. perurans</i> (qPCR)	Other pathogens
186	Dead	Negative	18.27	Positive	Negative
187	Dead	Negative	18.65	Positive	Negative
188	Dead	Negative	21.76	Positive	Negative
189	Dead	Negative	33.87	Positive	Negative
190	Dead	Negative	28.18	Positive	Negative
191	Dead	Negative	32.41	Positive	Negative
192	Dead	Negative	Negative	Positive	<i>Vibrio</i> sp.
194	Dead	Negative	28.84	Positive	Negative
195	Dead	Negative	23.61	Positive	Negative
196	Dead	Negative	23.10	Positive	Negative
197	Dead	Negative	23.03	Positive	Negative
198	Dead	Negative	35.62	Positive	<i>Vibrio</i> sp. phenon 45
199	Dead	Negative	27.38	Positive	Negative
200	Dead	Negative	Negative	Positive	<i>Vibrio</i> sp.
Total positives		12	44	190	22
Total samples		195	195	194	195
Total prevalence (%)		6.15	22.00	97.9	11.28

Total prevalence of pathogens was influenced by the different proportion of fish of different status, with more dead salmon (n=82) sampled than controls or moribund fish. There were differences in the proportion of different status salmon at each sampling time, making comparisons between the sampling times difficult. Comparison of total positives for the different pathogens showed higher prevalence in freshly dead salmon than in live controls or moribund for each sampling time (Table 4.4). TRLO was found in live controls on 19th January only, whereas *Yersinia ruckeri* and *Nocardia* sp. were found only in freshly dead fish on 19th January and 16th February, respectively. Moribund salmon and freshly dead fish were positive for T SRV more often than the control fish. Comparison of total positives for pathogens based on the sampling date showed highest prevalence of pathogens at the last time of sampling, 2nd March 2011, most likely due to the highest proportion of dead salmon (n=35 out of 50) on that day (Table 4.4).

Table 4.4 Total positives for T SRV (Tasmanian Salmon R eovirus), T RLO (Tasmanian Rickettsia-like organism), *N. perurans*, *Vibrio* sp., *Yersinia ruckeri* and *Nocardia* sp. according to the sampling time and fish status. Results presented as prevalence. Prev: Prevalence.

Sampling	Fish	Prev (%)					
time	status	TSRV	TRLO	<i>N.perurans</i>	<i>Vibrio</i> sp.	<i>Y.ruckeri</i>	<i>Nocardia</i> sp.
19th Jan	Control (n=11)	0.0	9.1	90.9	0.0	0.0	0.0
	Moribund (n=20)	5.0	0.0	100.0	0.0	0.0	0.0
	Dead (n=19)	10.5	0.0	100.0	0.0	26.3	0.0
1st Feb	Control (n=11)	0.0	0.0	100.0	0.0	0.0	0.0
	Moribund (n=18)	5.6	0.0	94.4	0.0	0.0	0.0
	Dead (n=21)	23.8	9.5	100.0	14.2	0.0	0.0
16th Feb	Control (n=21)	4.8	23.8	90.5	3.0	0.0	0.0
	Moribund (n=17)	0.0	0.0	88.2	0.0	0.0	0.0
	Dead (n=7)	0.0	42.8	100.0	28.6	0.0	14.7
2nd Mar	Control (n=15)	0.0	26.6	100.0	0.0	0.0	0.0
	Moribund (n=0)	NA	NA	NA	NA	NA	NA
	Dead (n=35)	6.3	82.8	100.0	3.0	0.0	0.0
Total prevalence	Control (n=58)	1.72	15.52	96.4 (n=57)	5.17	0	0
	Moribund (n=55)	3.63	0	94.5	0	0	0
	Dead (n=82)	10.97	42.68	100.00	15.85	6.09	1.21

Fish status (live controls, moribund and freshly dead) had a significant effect on the prevalence of TRLO (ANOVA, $p < 0.001$). However, there was no significant effect for TSRV (Table 4.5 and Figure 4.5). The presence of *N. perurans* ($\chi^2 = 6.889$, df 2, $p = 0.032$) and *Vibrio* sp. ($\chi^2 = 6.250$, df 1, $p = 0.012$) depended on fish status, with more positives found in dead salmon than in live controls or moribund fish (Figure 4.6).

Table 4.5 qPCR results of TSRV (Tasmanian Salmon Reovirus) and TRLO (Tasmanian Rickettsia-like organism) in Atlantic salmon shown as mean C_T value \pm SE for each fish status (pooled data). p values were calculated using one way ANOVA to determine mean differences of the prevalence of the pathogens according to fish status. p value significant at the 0.05 level. SE: standard error.

Fish status	Mean C_T value \pm SE	
	TSRV	TRLO
Control	35.04 \pm 0.00	33.50 \pm 0.80
Moribund	33.85 \pm 1.95	ND
Dead	32.64 \pm 0.82	25.44 \pm 0.96
p value (ANOVA)	0.579	< 0.001

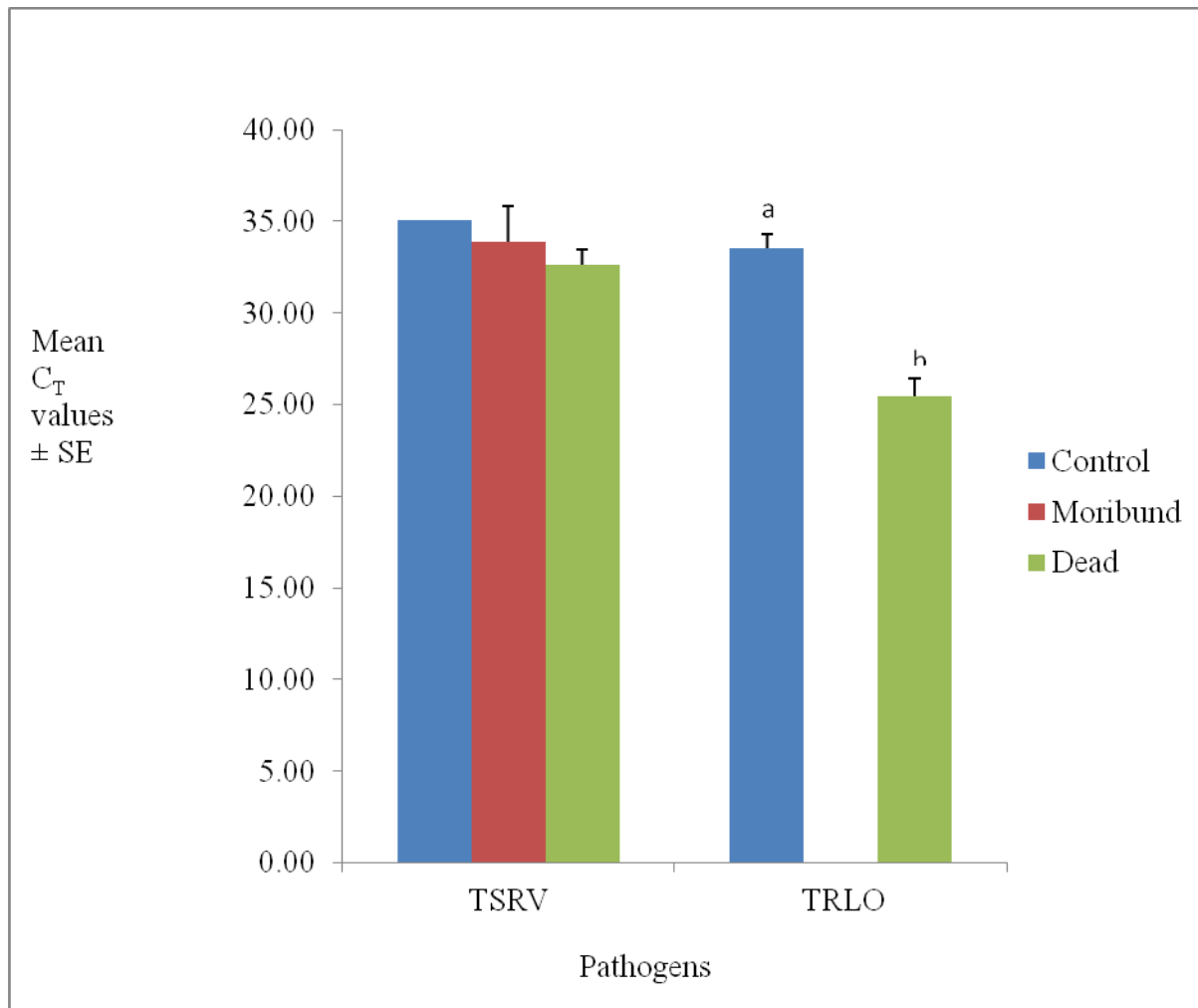


Figure 4.5 qPCR results of TSRV (Tasmanian Salmon Reovirus) and TRLO (Tasmanian Rickettsia-like organism) in Atlantic salmon shown as mean C_T value \pm SE for each fish status (pooled data). p values were calculated using one way ANOVA to determine differences in the prevalence of the pathogens according to fish status. Different lower case letters denote significant differences at $p < 0.05$. SE: standard error.

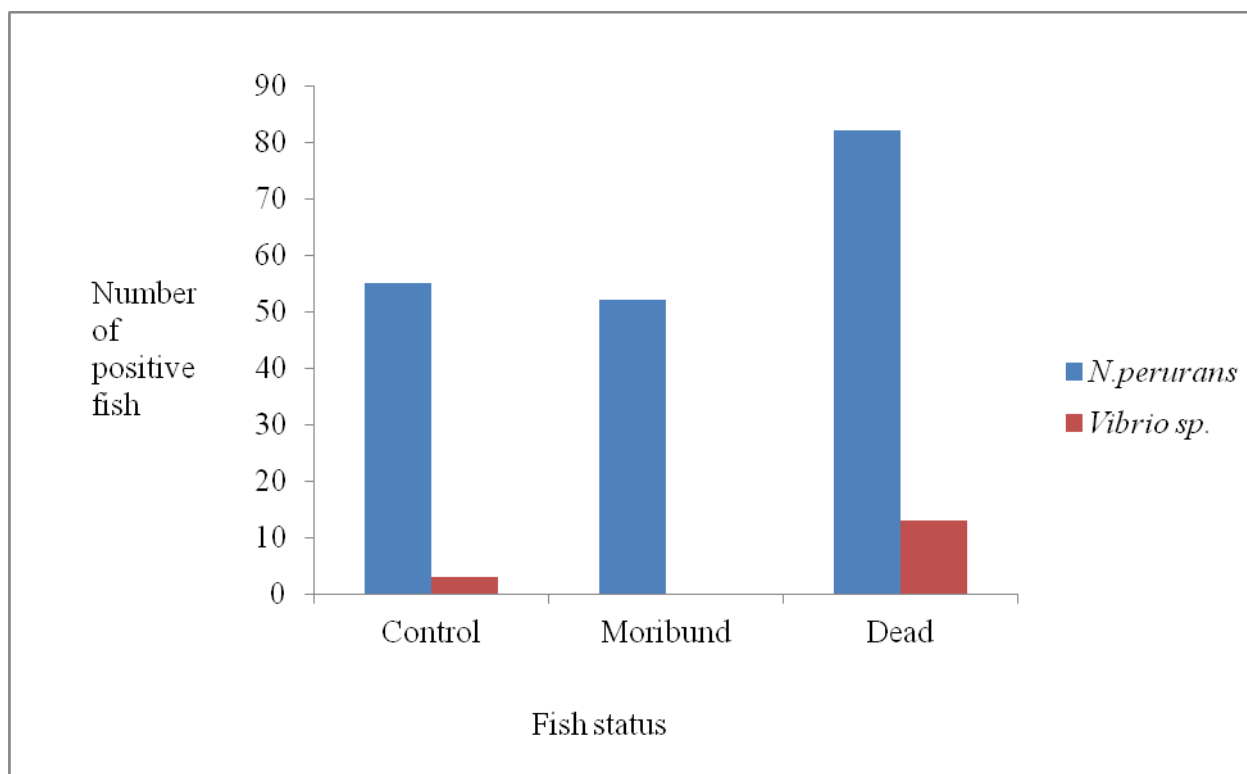


Figure 4.6 The number of positive fish (frequencies) for *N. perurans* and *Vibrio sp.* according to fish status (pooled data).

Table 4.6 illustrates the occurrence of the pathogens, starting with the most prevalent *N. perurans* (98.00%) and *Yersinia ruckeri* (10.00%) on 19th January, followed by TSRV (12.00%) and AGD (100.00%) on 1st February, single occurrence of *Nocardia sp.* on 16th February and occurrence of TRLO (66.00%) and *Vibrio sp.* (16.00%) on 2nd March. *N. perurans* demonstrated the highest prevalence throughout the sampling. The prevalence of TSRV and *N. perurans* was consistent with the proportion of dead salmon collected at each sampling time, where the prevalence of both pathogens increased on 1st February, dropped on the 16th February and peaked on the last day of sampling, 2nd March. The prevalence of TRLO and *Vibrio sp.* increased gradually and reached highest prevalence at the last sampling time.

Table 4.6 Comparison of total positives of TSRV (Tasmanian Salmon Reovirus), TRLO (Tasmanian Rickettsia-like organism), *N. perurans*, *Vibrio* sp., *Yersinia ruckeri* and *Nocardia* sp. according to the sampling time. Bolded prevalence indicates the highest prevalence for each pathogen according to time of sampling. Results were presented as prevalences.

Sampling time(2011)	Prevalence (%)					
	TSRV	TRLO	<i>N. perurans</i>	<i>Vibrio</i> sp.	<i>Yersinia ruckeri</i>	<i>Nocardia</i> sp.
19th January (n=50)	6.00	2.00	98.00	0.00	10.00	0.00
1st February (n=50)	12.00	4.00	100.00 (n=49)	6.00	0.00	0.00
16th February (n=45)	2.22	17.77	91.11	11.11	0.00	2.22
2nd March (n=50)	4.00	66.00	100.00	16.00	0.00	0.00

The sampling time did not have any significant effect on the prevalence of T SRV (ANOVA, $p = 0.486$) and T RLO (ANOVA, $p = 0.714$, Figure 4.7). The presence of *N.perurans* ($\chi^2 = 1.116$, df 3, $p = 0.773$) and *Vibrio* sp. ($\chi^2 = 2.375$, df 2, $p = 0.305$) was not dependent on the sampling time (Figure 4.6). The Chi-square test of independence showed that there was no significant difference between positive and negative fish infected by TSRV and total fish sampled in this field investigation ($\chi^2 = 0.380$, df 1, $p = 0.537$). This result indicated that TSRV does not predispose fish to other infections.

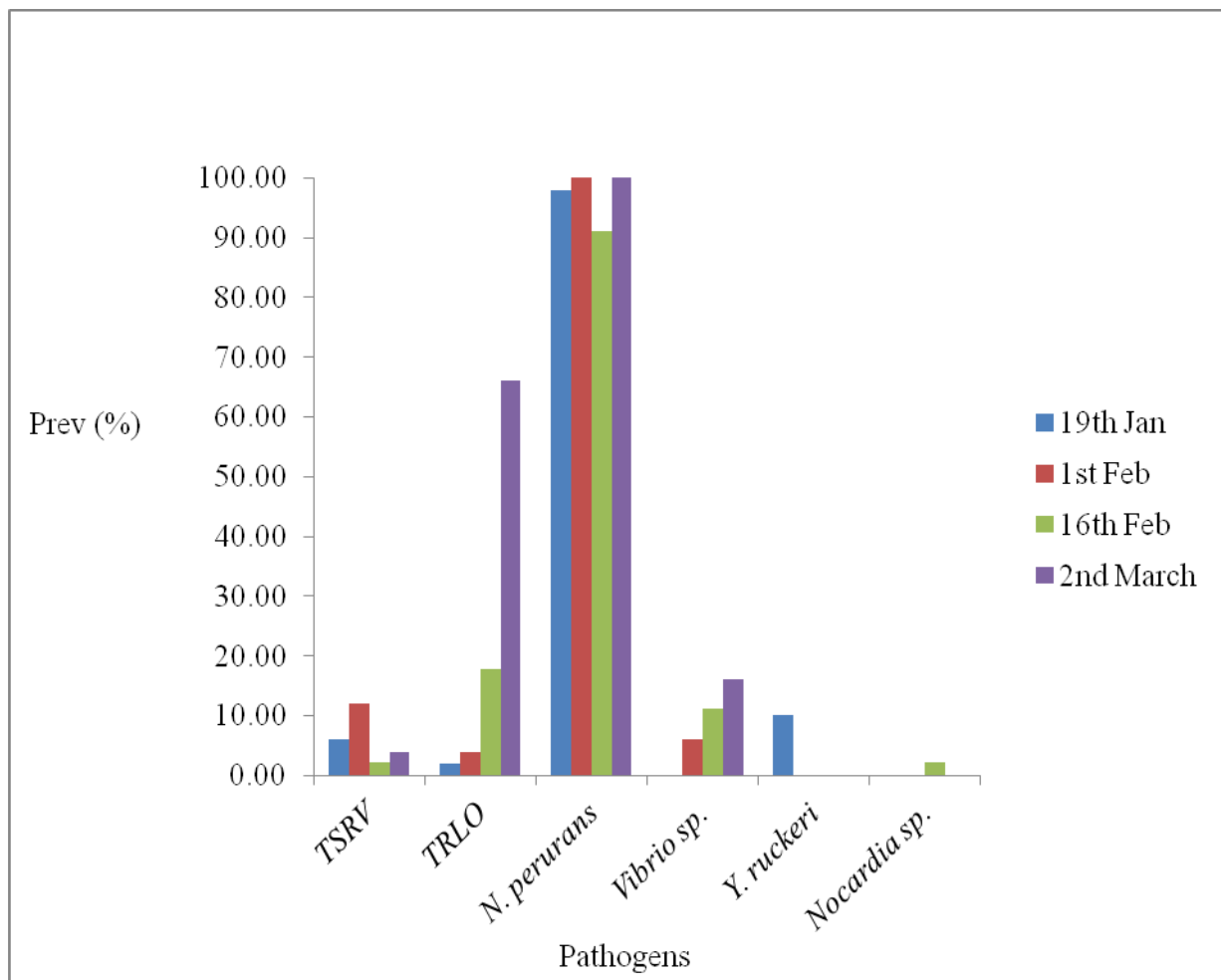


Figure 4.7 Comparison of total positives of T SRV (Tasmanian Salmon Reovirus), T RLO (Tasmanian Rickettsia-like organism), *N. perurans*, *Vibrio* sp., *Yersinia ruckeri* and *Nocardia* sp. according to sampling time. Prev: Prevalence, *Y. ruckeri*: *Yersinia ruckeri*.

4.3.2 Gross pathology

The following gross signs were observed in some of the sampled fish; enlarged spleen and liver, pale liver, liver abscess, granular spleen, dark hindgut, haemorrhages of internal organs, multifocal lesions in pyloric caecal, congestion and adhesions of internal organs and ascites (Table 4.7). A total of 101 salmon (51.7%) showed gross clinical signs during sampling and only 47.5 % were associated with the presence of pathogens. A total of 94 salmon (48.3%) did not show any gross clinical signs and 96.8% of those salmon were positive for at least one pathogen. Some of the salmon sampled from one cage on 19th January had gross AGD lesions and mucus on gills. A total of 25.0% TSRV positives showed gross signs such as pale liver and haemorrhaging of internal organs and muscle. A total of 75.0% TSRV positive salmon did not show any gross signs and only 14.3% TSRV negative salmon showed gross signs. Figure 4.8 shows some of the apparent gross signs observed in most of the sampled fish. Internal adhesions were observed in some of the salmon which were vaccinated by intra-peritoneal injection.

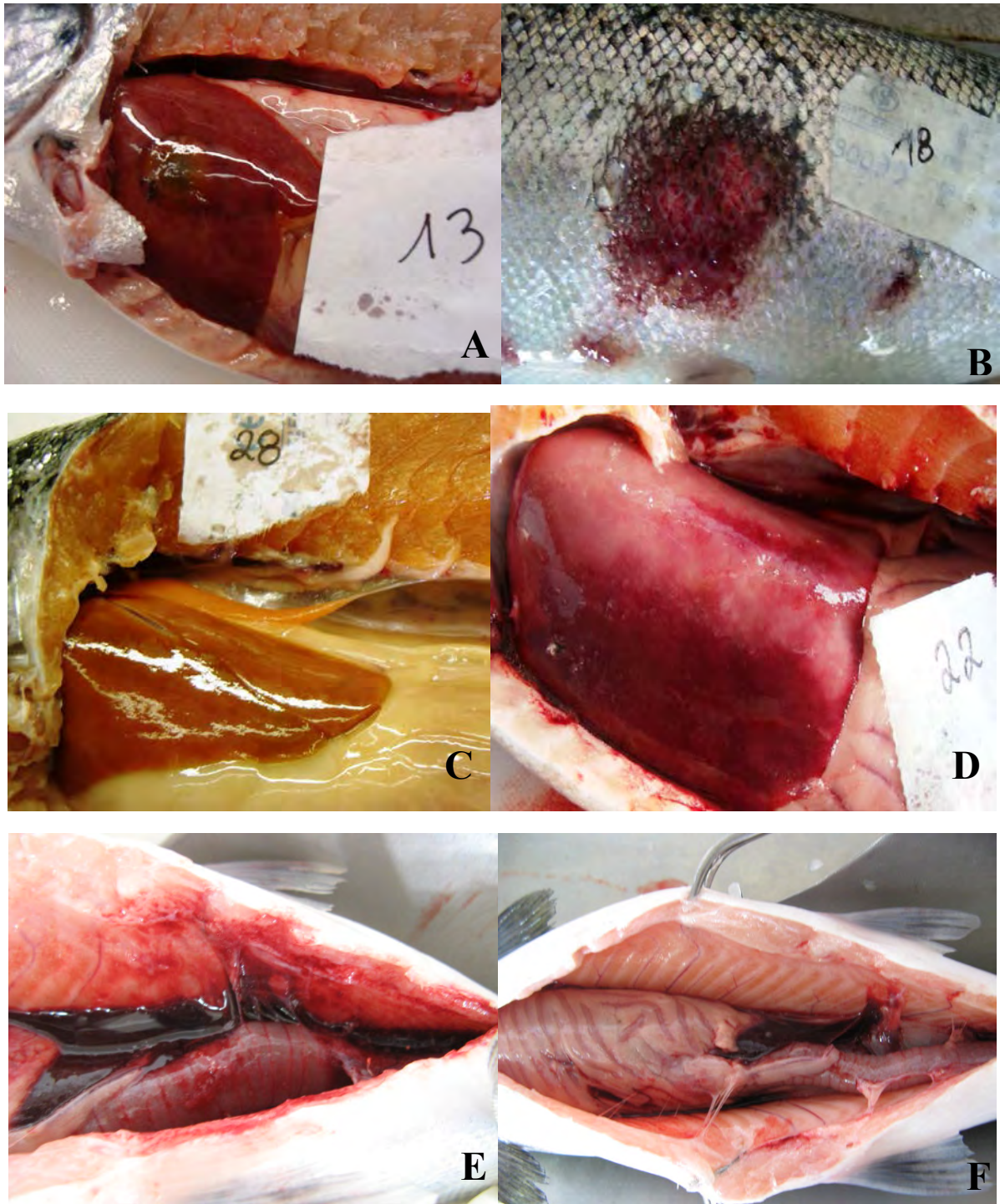


Figure 4.8 Apparent gross pathological signs present in some sampled fish: (A) liver abscess, (B) skin erosion, (C) pale liver, (D) enlarged liver (E) extensive haemorrhage and congestion of internal organs and (F) internal adhesions in intra-peritoneally vaccinated fish.

Table 4.7 Description of apparent gross pathological signs present in sampled fish based on date of sampling, fish status and association of gross pathological signs with the presence of pathogens.

Sampling time	Fish status	Gross pathology	Pathogens
19th Jan	Dead	Petechiae in the ventral side, pelvic, skin and eyes, enlarged liver and spleen	<i>Y. ruckeri</i>
	Dead	Haemorrhages in pelvic and pectoral fins and belly, liver abscess, opaque swim bladder and adherence to peritoneum	<i>Y. ruckeri</i>
	Dead	Haemorrhagic in eyes, pelvic fins and ventral side	<i>Y. ruckeri</i>
	Dead	Haemorrhagic on ventral side and caudal fin	<i>Y. ruckeri</i>
		Haemorrhagic ventral area, lateral/pectoral muscle petechiae	TSRV
1st Feb	Dead	Multifocal white round lesions in pyloric caecal	TSRV
	Moribund	Pale liver	TSRV
	Dead	AGD lesions	<i>N. perurans</i>
	Dead	Petechiae in internal organs	TRLO
	Dead	Damaged skin	<i>Vibrio</i> sp.
	Dead	Damaged skin, skin erosions	<i>Vibrio</i> sp.
16th Feb	Dead	Extensive skin erosion	TRLO, <i>Vibrio</i> sp.
	Dead	Skin erosion	<i>Vibrio</i> sp.
	Dead	Extensive haemorrhagic and congestion of internal organs	TRLO, <i>Nocardia</i> sp.
	Dead	Blood spots in eyes	TRLO

Sampling time	Fish status	Gross pathology	Pathogens
16th Feb	Control	Ascites	TRLO
	Control	Very large quantity of ascites and water belly	<i>Vibrio</i> sp.
	Control	Ascites	TRLO
2nd March	Control	Internal adhesions (due to intra-peritoneal vaccination)	TRLO
	Control	Internal adhesions (due to intra-peritoneal vaccination)	TRLO
	Dead	Internal adhesions (due to intra-peritoneal vaccination)	TRLO
	Dead	Internal haemorrhaging	TRLO, <i>Vibrio</i> sp.
	Dead	Internal haemorrhaging	TRLO, <i>Vibrio</i> sp.
	Dead	Red vent	TRLO
	Dead	Ascites	TRLO
	Dead	Red eyes	TSRV, TRLO
	Dead	Red eyes, ascites	TRLO
	Dead	Tail lesion	TRLO, <i>Vibrio</i> sp.
	Dead	Skin abrasion	TRLO, <i>Vibrio</i> sp.
	Dead	Red neck	TRLO
	Dead	Internal adhesions	TRLO
	Dead	Internal adhesions	TSRV
	Dead	Red popped eyes	TRLO, <i>Vibrio</i> sp.,
	Dead	Skin abrasion	TRLO
	Dead	Mouth erosion	TRLO
	Dead	Fin erosion	TRLO
	Dead	Internal adhesions (due to intra-peritoneal vaccination)	TRLO
	Dead	Internal adhesions (due to intra-peritoneal vaccination)	TRLO
	Dead	Skin erosion	<i>Vibrio</i> sp.

4.3.3 Microbiology

Microbiological screening for the presence of pathogenic bacteria in Atlantic salmon showed multiple infections of the affected fish (Table 4.8). A total of 22 salmon (11.3%) were infected with *Yersinia ruckeri* (2.5 %, fish number 12 to 15), *Nocardia* sp. (0.5%, fish number 132), *Vibrio* sp. (6.1%), *Vibrio tasmaniensis* (1.0%), *Vibrio splendidus* (1.0%) or *Vibrio* sp. phenon 45 (0.5%).

Table 4.8 Results of positive Gram-staining and quantity of bacterial colonies cultured on SBA (Sheep Blood Agar) and TCBS agar from sampled salmon. Quantity: \pm = occasional, + = small, ++ = moderate and +++ = large.

Fish number	Gram staining	Quantity	Cultures	Quantity
12	Gram negative rods	\pm	<i>Yersinia ruckeri</i>	++
13	Gram negative rods	\pm	<i>Yersinia ruckeri</i>	\pm
14	No bacteria detected	-	<i>Yersinia ruckeri</i>	++
15	Gram negative rods	++	<i>Yersinia ruckeri</i>	+++
44	Gram negative rods	+	<i>Yersinia ruckeri</i>	++
87	No bacteria detected	-	<i>Vibrio tasmaniensis</i>	\pm
91	No bacteria detected	-	<i>Vibrio</i> sp.	+
92	No bacteria detected	-	<i>Vibrio</i> sp.	+
122	No bacteria detected	-	<i>Vibrio splendidus</i> I	+
123	No bacteria detected	-	<i>Vibrio</i> sp.	\pm
129	Gram negative rods	\pm	Mixed growth inc <i>Vibrio</i> sp. Mixed growth inc <i>Vibrio</i> sp., <i>Vibrio splendidus</i> I, <i>Vibrio</i> <i>tasmaniensis</i>	++ +++
130	No bacteria detected	-	<i>Nocardia</i> sp.	++
132	No bacteria detected	-	<i>Vibrio</i> sp.	+
138	No bacteria detected	-	<i>Vibrio</i> sp.	+++
167	No bacteria detected	-	<i>Vibrio</i> sp.	\pm
168	No bacteria detected	-	<i>Vibrio</i> sp.	++
177	No bacteria detected	-	<i>Vibrio</i> sp.	++
178	No bacteria detected	-	<i>Vibrio</i> sp.	++
185	No bacteria detected	-	<i>Vibrio</i> sp.	++
192	No bacteria detected	-	<i>Vibrio</i> sp.	++
198	No bacteria detected	-	<i>Vibrio</i> sp. phenon 45	+++
200	No bacteria detected	-	<i>Vibrio</i> sp.	+++

4.3.4 Histopathology

Histological examination of the sampled fish revealed predominant pathology in gills, liver, kidney, spleen and heart. Table 4.9 summarises histopathological changes according to the date of sampling, fish status and association with the presence of pathogens. A total of 22.1% of the 81 salmon sampled for histology showed histopathological changes associated with the presence of pathogens. Out of the proportion of salmon which demonstrated histological changes associated with the presence of pathogens, 20.6% were sampled from control, 1.2% from dead and 9.0% from moribund. The moderate-to-severe granulomatous peritonitis present in some of the fish sampled on 2nd March was probably caused by post-vaccination responses. Random foci of oedematous separation of cardiomyofibres were observed predominantly in the outer layer of myocardium of the heart. There were moderate to high numbers of mixed lymphoid and white blood cell aggregates along the pericardium, inner layer and outer compact layer of myocardium and inflammation of pericardium suggestive of pericarditis.

Histopathological changes observed in TSRV positive salmon (detected as positive by qPCR) included mild inflammation in liver (8.3% out of TSRV positive salmon, $n = 1$) (Figure 4.9). However, similar observations were found in 2.2% TSRV-negative salmon (out of total negative salmon for TSRV detected by qPCR) which included histopathological changes such as congested kidney and spleen. Thus, these results indicated that changes found in TSRV infected salmon ($n =$ one salmon) were not associated with TSRV infection and the changes observed could be caused by any other diseases or factors. Histopathological changes in TRLO infected fish were found to be more severe and included; severe lesions in liver, kidney and spleen (Figure 4.10 -4.11), granulomatous peritonitis and congested internal

organs. Diffuse mild congestion and mild to moderate extensive fibrosis were observed in the spleen. There was moderate to severe loss of cells cuffing the ellipsoid and increased fibrous stroma proliferation with some granulomatous peritoneal tags. The most common pathology found in the kidney of positive TRLO fish included; severe congestion, occasional oedema, and moderate depletion of haematopoietic tissues with few foci of flattened tubular epithelium and dilated tubules.

Table 4.9 Description of histopathological changes found in sampled fish according to sampling time, fish status and presence of pathogens.

Sampling time	Fish status	Histopathology	Pathogens
19th Jan	Control	Congested lamellae, multifocal lamellar hyperplasia and clubbing of secondary lamellar tips and vascular damage of the gills (gill pathology)	<i>N. perurans</i>
	Control	Gill pathology, liver pathology, congested kidney and spleen	TRLO, <i>N. perurans</i>
	Control	Gill pathology	<i>N. perurans</i>
	Moribund	Gill pathology	<i>N. perurans</i>
	Moribund	Gill pathology	<i>N. perurans</i>
	Moribund	Gill pathology	<i>N. perurans</i>
	Moribund	Gill pathology	<i>N. perurans</i>
1st Feb	Moribund	Mild inflammation in liver, kidney and spleen, gill pathology	TSRV, <i>N. perurans</i>

Sampling time	Fish status	Histopathology	Pathogens
16th Feb	Control	Mild multifocal patch fusion of gills	<i>N. perurans</i>
	Control	Diffuse mild spleen congestion	TRLO
	Control	Diffuse mild spleen congestion and mild to moderate extensive fibrosis	<i>Vibrio</i> sp.
	Control	Diffuse mild spleen congestion and mild to moderate extensive fibrosis	TRLO
	Control	Diffuse mild spleen congestion and mild to moderate extensive fibrosis	TRLO
	Control	Clear and cloudy intracytoplasmic liver vacuoles	TRLO
2nd March	Control	Gill pathology	<i>N. perurans</i>
	Control	Gill pathology	<i>N. perurans</i>
	Control	Liver pathology, random foci of oedematous separation of cardiomyofibres (heart), congested kidney and spleen.	TRLO
	Dead	Liver pathology, random foci of oedematous separation of cardiomyofibres (heart), congested kidney, moderate depletion of haematopoietic tissues hepatitis, severe kidney and spleen lesion.	TRLO

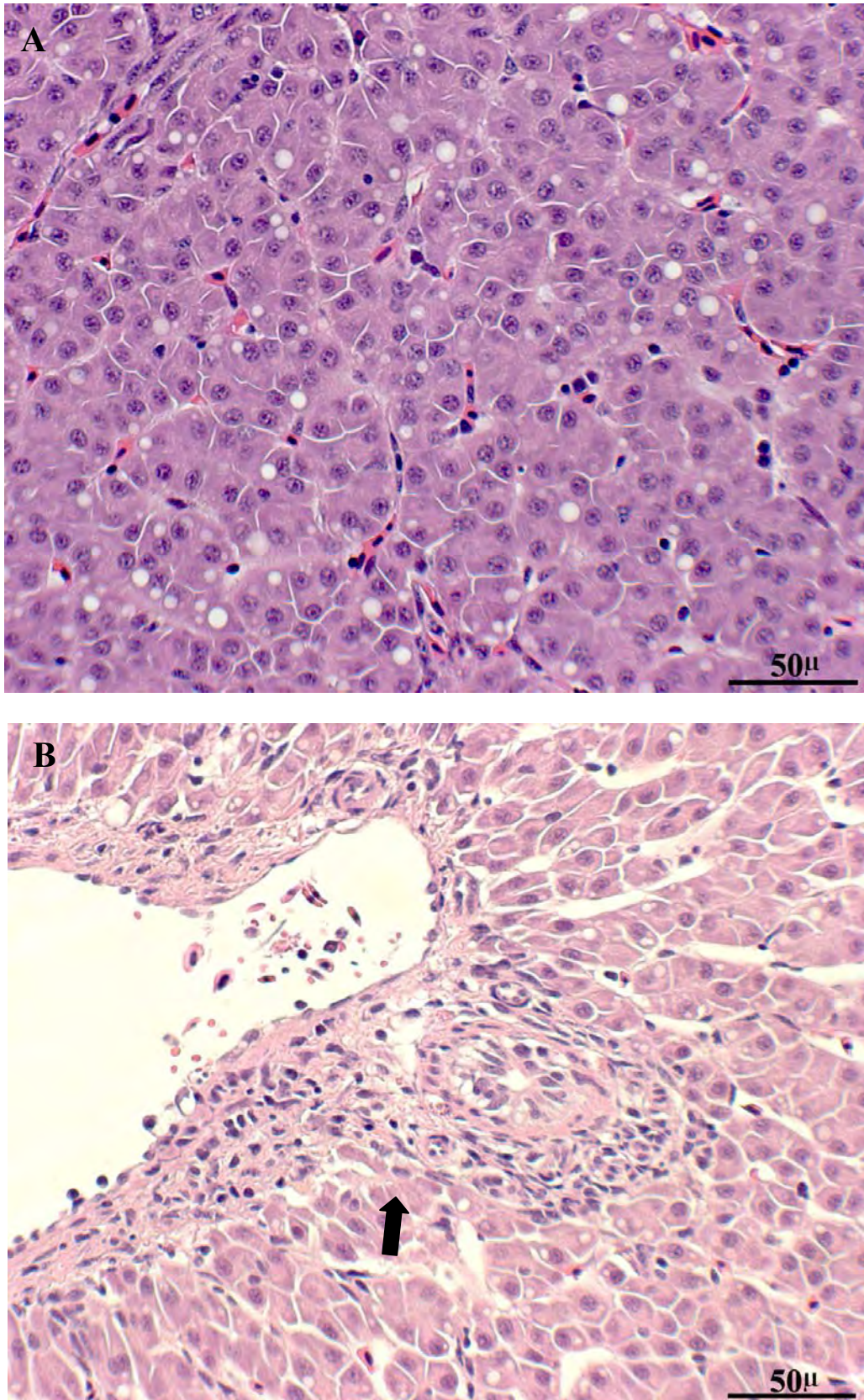


Figure 4.9 Liver of a TSRV negative and TSRV positive fish. (A) Normal uninfected liver from TSRV negative fish, (B) Mild inflammation around blood vessels observed in TSRV positive fish (arrow).

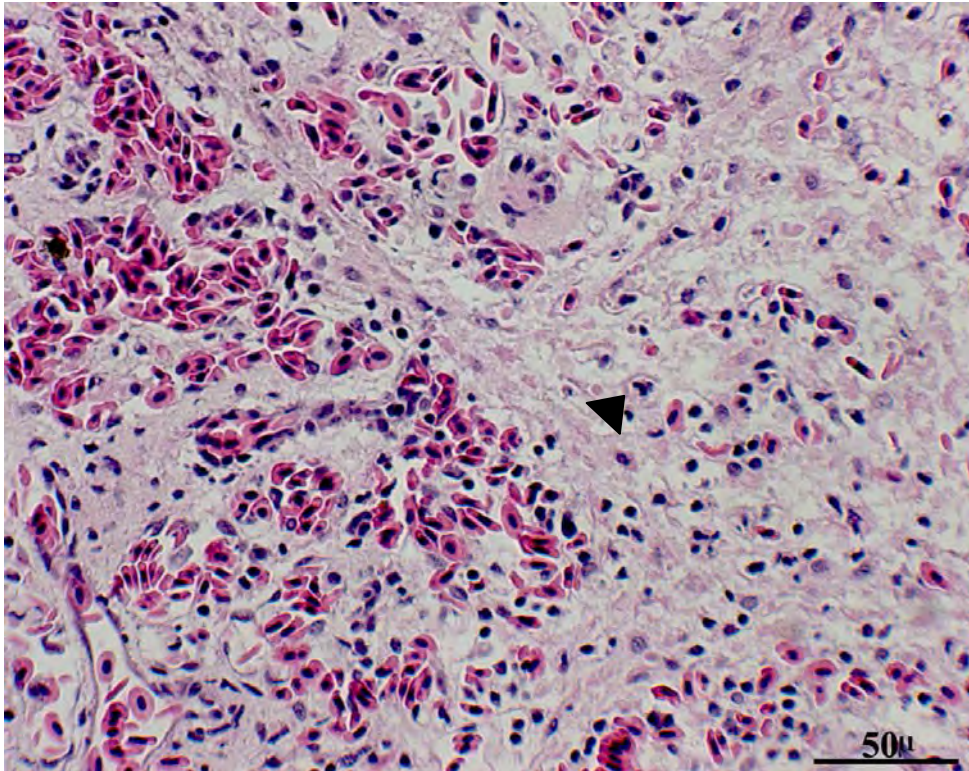


Figure 4.10 Marked depletion of ellipsoids and marked fibroplasias (arrow head) in TRLO positive spleen.

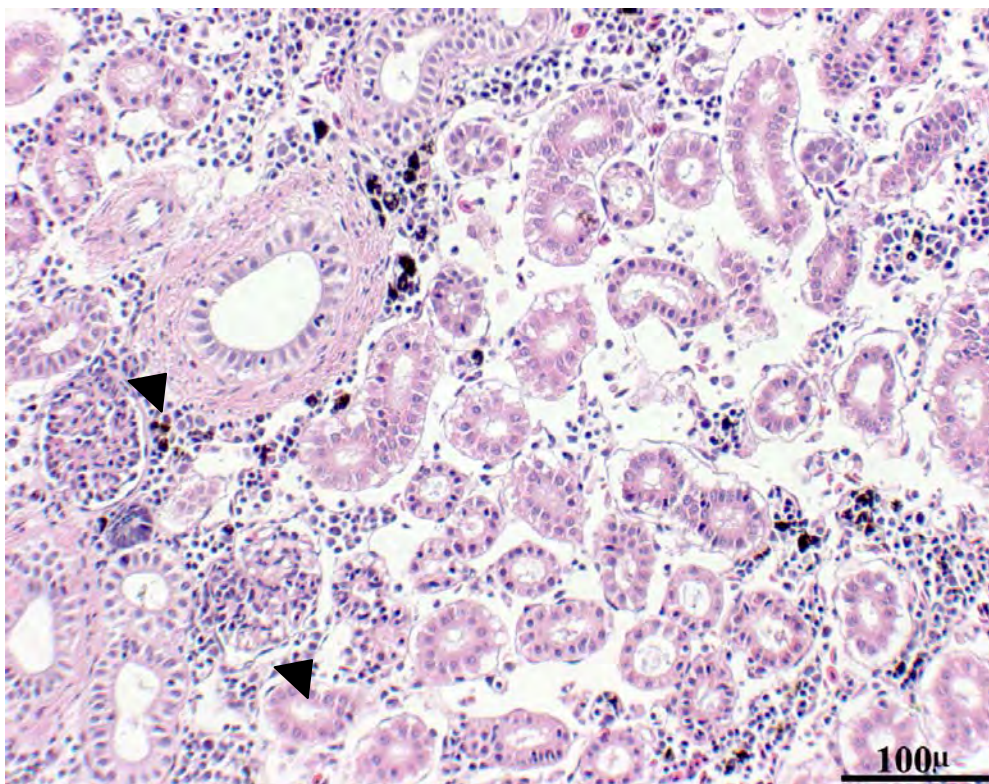


Figure 4.11 Severe depletion of haematopoietic tissues and dilated tubules in TRLO positive kidney (arrowheads).

4.3.5 Comparison of TSRV infection and pathology

Relationship between T SRV C_T values (qPCR) and pathology was investigated (Table 4.10). The C_T cut-off value established for the collection method of organs (see section 2.3.4.1 in chapter 2) was the same for the swabs. Thus, C_T values ≤ 35.8 were considered positive compared to C_T values greater than 36.13 which were considered negative. C_T values between 35.8 and 36.13 were classified as ambiguous and the PCR assay for those samples were repeated to avoid false positives. Hence, the C_T values obtained for qPCR were compared to the results for histopathology from the same samples. Histology samples were not collected for the 11 samples which were detected by qPCR as positives due to the lack of apparent gross and clinical signs during the sampling process, and histology samples were not collected from dead fish. The histopathological changes in these salmon could not be observed and they tested positive for TSRV by qPCR demonstrating the high sensitivity of qPCR as a detection method. A sample obtained from only one salmon was marginally positive for T SRV by qPCR and this fish was also sampled for histology examination. Due to these circumstances, positive qPCR results could not be compared directly with histological changes observed in T SRV positive salmon. There was no histopathology evident in the salmon that were negative by qPCR except sample 16. As similar gross pathology and histological changes were observed in a positive TSRV and other negative salmon, these results indicated that the changes were not associated with TSRV infection.

Table 4.10 Relationship between TSRV C_T values (qPCR) and pathology obtained from field samples using swabs as collection method. Bolded values: C_T values ≤ 35.8 are considered positive. NA: samples were unavailable for histology.

Fish number	Fish status	TSRV(C _T values)	Gross Pathology	Histopathology
92	Dead	29.75	Skin erosions	NA
44	Dead	29.83	No obvious gross signs	NA
90	Dead	30.68	Damaged skin	NA
34	Moribund	31.93	Haemorrhagic ventral area, petechiae lateral/pectoral muscle	NA
91	Dead	32.26	Damaged skin	NA
83	Dead	33.23	No obvious gross signs	NA
38	Dead	34.14	Multifocal white round lesions in pyloric caecae	NA
184	Dead	34.27	Adhesions of internal organs	NA
122	Control	35.04	No obvious gross signs	NA
87	Dead	35.08	Damaged skin	NA
174	Dead	35.12	Red eyes	NA
68	Moribund	35.8	Pale liver	Vacuolated liver and mild multifocal liver inflammation, normal kidney and spleen
73	Control	36.02	No obvious gross signs	Normal liver, kidney and spleen
8	Control	36.52	Enlarged spleen	Multifocal lymphoid infiltrates around blood vessels
154	Control	36.52	No obvious gross signs	Congested liver and spleen
55	Moribund	37.16	Pale liver	Normal liver, kidney and spleen
79	Control	37.31	No obvious gross signs	Normal liver, kidney and spleen
158	Control	37.97	No obvious gross signs	Congested liver, kidney and spleen
143	Control	38.33	No obvious gross signs	Mild congestion of spleen
105	Moribund	38.81	Skin lesion	Normal liver, kidney and spleen
152	Control	39.04	Pale liver	Congested liver and spleen
16	Moribund	39.10	Granular spleen	Congested kidney and spleen

4.3.6 Archival cases

4.3.6.1 Co-occurrence of TSRV and TRLO

Table 4.11 illustrates the co-occurrence of both pathogens based on archival cases from diagnostic submissions between 2000 and June 2011. The total number of cases of diagnostic submissions represents the total positive cases of TRLO and TSRV. The estimated overall apparent prevalence of TSRV and TRLO are considered to be 21%-22.8% (Carlile, 2011) and 20.3%-23.5% based on archival cases. The occurrence of TSRV infection correlated well with the occurrence of TRLO; both pathogens were found in at least one case per year except in 2005. The occurrence of a single case of either TSRV or TRLO is only 30% compared to total cases per year. In 2005, no samples were submitted for diagnosis of TSRV and TRLO. Most commonly TSRV and TRLO were detected from the same sample (50-100%) and in few cases, both pathogens were isolated from the same population of Atlantic salmon.

Table 4.11 Co-occurrences of TSRV and TRLO based on submission of diagnostic cases between 2000 and June 2011, total cases of positives for TSRV and TRLO based on results of available diagnostic methods (virus isolation, conventional hemi-nested RT-PCR and qPCR), total cases of presence for both pathogens (same cases), total cases of presence for individual pathogen (TSRV or TRLO alone), origin of presence (occurrence of positives either from the same sample or population) and total cases (same cases) of presence of TSRV in the absence of TRLO. None = no submission of diagnostic cases.

Year	Total cases TSRV	Total cases TSRV alone	Total cases TRLO	Total cases TRLO alone	Both present (positives)/ Total cases	Origin of presence Same sample	Origin of presence Same population	TSRV present TRLO absent/ Total cases
2000	6	6	0	0	None	0.00%	0.00%	None
2001	14	9	11	6	3/5	66.70%	33.30%	2/5
2002	6	1	8	3	1/5	100.00%	0.00%	4/5
2003	9	4	5	0	2/5	100.00%	0.00%	4/5
2004	3	1	5	3	1/2	100.00%	0.00%	1/2
2005	None	None	None	None	None	None	None	0
2006	3	0	11	8	3/3	66.70%	33.30%	0/3
2007	10	5	10	5	1/5	100.00%	0.00%	4/5
2008	11	6	8	3	2/5	50.00%	50.00%	3/5
2009	23	12	12	1	3/11	60.00%	40.00%	8/11
2010	22	2	20	0	17/20	70.60%	29.40%	3/20
2011	7	4	6	3	1/3	100.00%	0.00%	2/3

4.3.6.2 Archival results of histopathology of TSRV infections

The major changes in the archival cases of TSRV positive fish were congestion of internal organs, liver pathology, pericarditis and epicarditis (Table 4.12). The initial few years (1994-1997) of histological examination of samples did not indicate any significant pathology associated with TSRV infections. Among the earliest signs of pathology in 1998 included the vacuolation and congestion of liver; a review of histopathology against virology results confirmed an association of lymphoid infiltrates in liver with reovirus infections. This histology report in 1998 was based on histological samples submitted for TSRV infections in Atlantic salmon, prior to any occurrence of TRLO infections. The most severe histological changes were found in fish in the year 2001 (due to the outbreak of TRLO) and in 2010 (most likely due to relatively high water temperature). The highest numbers of diagnostic cases were investigated for the occurrence of TSRV in 2010 due to the high water temperature and TRLO outbreaks.

Table 4.12 Histological changes associated with TSRV infections in Atlantic salmon based on submission of diagnostic case between 1994 and June 2011. The total number of fish which showed the following histological changes in each submission are shown in brackets and the numbers vary according to the diagnostic methods used for TSRV detection. Each entry illustrates a single submission which demonstrated histological changes in TSRV infected samples in each year.

Year	Method	Histological changes associated with TSRV infections in Atlantic salmon
1994	Virus isolation	No significant pathology
1995	None	No submission of diagnostic cases for TSRV
1996	None	No submission of diagnostic cases for TSRV
1997	Virus isolation	No significant pathology
1998	Virus isolation	Focal congestion in liver, mild necrotic liver (2 pool of samples/6 fish)
1998	Virus isolation	Occasional mononuclear foci in liver/pancreas, vacuolation in liver, review of histopathology against virology results confirms an association of lymphoid infiltrates in liver, pancreas with reovirus infections (mild lesions) (1 pool of samples/8 fish)
1998	Virus isolation	Lymphoid foci in liver (1 pool of samples/8 fish)
1998	Virus isolation	Liver nodules, occasional mononuclear foci in liver (1 pool of samples/10 fish)
1998	Virus isolation	Vacuolated and congested liver (1 pool of samples/5 fish)
1998	Virus isolation	Liver congestion (1 pool of samples/5 fish)
1998	Virus isolation	Small mononuclear foci liver, vacuolated liver (1 pool of samples/5 fish)
1998	Virus isolation	Focal renal fibrosis, liver monofoci, small mononuclear foci liver (1 pool of samples/5 fish)
1998	Virus isolation	Liver congestion (1 pool of samples/5 fish)
1999	Virus isolation	Vacuolated liver, chronic inflammatory foci (1 pool of sample/10 fish)
1999	Virus isolation	Vacuolation in liver, large single vacuoles, multifocal necrosis (1 pool of sample/11 fish)
2000	Virus isolation	No significant pathology
2000	Virus isolation	Mild focal liver damage (1 pool of sample/10 fish)
2000	Virus isolation	Rare liver reactive nodule (1 pool of sample/6 fish)
2001	Virus isolation	Liver changes (2 pools of samples/5 fish)
2001	Virus isolation	Liver necrosis (1 pool of sample/11 fish)
2001	Virus isolation	No significant pathology

Year	Method	Histological changes associated with TSRV infections in Atlantic salmon
2001	Electron microscopy	No significant pathology
2001	Virus isolation	Liver lesions (concurrent with TRLO outbreak) (5 pools of samples/5 fish)
2002	Virus isolation	Liver congestion and focal liver necrosis (1 pool of samples/5 fish)
2002	Virus isolation	No significant pathology
2003	Virus isolation	Congestion of internal organs (1 pool of samples/5 fish)
2003	Virus isolation	No significant pathology
2003	Virus isolation	Liver lesions and focal necrosis, pericarditis (3 samples)
2003	Virus isolation	Liver periportal fibrosis (2 pools of samples/10 fish)
2004	Virus isolation	No significant pathology
2005	None	No submission of diagnostic cases for TSRV
2006	None	No submission of diagnostic cases for TSRV
2007	Virus isolation	No significant pathology
2008	Nested PCR	No significant pathology
2008	Nested PCR	Mild hepatocellular liver necrosis(2 samples)
2008	Virus isolation	Splenic and hepatic congestion and mild necrosis (2 pools of samples/10 fish)
2008	Virus isolation	Splenic congestion (2 pools of samples/10 fish)
2008	Virus isolation	Liver congestion (1 pool of samples/5 fish)
2009	Nested PCR	No significant pathology
2010	Nested PCR	Rare hepatocellular liver necrosis, pericarditis (3 samples)
2010	Nested PCR	No significant pathology
2010	Nested PCR	Mild hepatocellular necrosis, congestion of intestine, pericarditis (4 samples)
2010	Nested PCR	Moderate multifocal midzonal necrosis of hepatocytes (1 sample)
2010	qPCR	Congestion of spleen, epicarditis (6 samples)
2010	qPCR	Hepatocellular necrosis (2 samples)
2010	qPCR	Mild liver congestion & liver necrosis (2 samples)

4.4 Discussion

This study is the first attempt to gain a more complete picture of the pathogens that are involved in disease outbreaks during production of Atlantic salmon in Tasmania with a main focus on TSRV, TRLO and *N. perurans*. These pathogens were selected due to their co-occurrence in the same geographical location, South-east Tasmania. In this field investigation, the co-occurrence of pathogens was dependent on the different fish status where more positives were found in freshly dead salmon than in control or moribund fish. Neither obvious clinical signs nor histological changes were observed in most of the collected salmon. The co-occurrence of TSRV and TRLO in the same sample and population was defined based on this field investigation and archival data.

Gross signs and pathology typical of TSRV infections were non-specific and observed in both infected and uninfected salmon. The gross signs observed in TSRV infected salmon during this field investigation were; congestion and enlargement of internal organs, pale liver and petechial hemorrhages on the body surfaces which were consistent with changes reported from other species of fish from which aquareoviruses have been isolated. The only pathology coinciding with TSRV infection in Atlantic salmon in this field investigation was mild multifocal necrosis and inflammation of liver. The absence of pathology observed in kidney and spleen of the salmon sampled in this investigation contradicted the reported changes with other reovirus infections. Non-specific clinical signs including skin discoloration, abdominal distension, haemorrhages in the skin, liver, spleen, kidney, musculature and intestine, and pale liver or kidney were reported from aquareovirus infections, including both natural disease outbreaks and experimental infections (Plumb *et al.*, 1979; Meyers, 1980; Jiang and

Ahne, 1989; Lupiani *et al.*, 1989; Chew-Lim *et al.*, 1992; Subramanian *et al.*, 1997; Cusack *et al.*, 2001; Attoui *et al.*, 2002; Seng *et al.*, 2002).

Vascular congestion of the distal intestinal mucosa, splenic hypertrophy and congestion were observed in 25-33% of Atlantic salmon sampled between 5-16 days post inoculation in initial experimental infection trial with TSRV (Humphrey *et al.*, 1993). However, distended abdomen was the only gross sign present in subsequent experimental infections of Atlantic salmon with TSRV (Gemma Carlile pers communication). It is noteworthy that several other aquareoviruses isolated from apparently healthy finfish with no gross external signs have been shown to cause low-level pathology in experimental infections.

Based on archival cases, the most apparent histopathology coinciding with TSRV infections was congestion of internal organs, liver pathology, pericarditis and epicarditis, and these observations were consistent with the descriptions of pathological changes for other aquareoviruses. Some of the sampled fish which were positive for TSRV infection (detected by qPCR) did not demonstrate any histological changes. The discrepancies between pathology associated with TSRV infection and infection with other aquareoviruses could be due to the low number of TSRV positive salmon sampled for histology which could potentially introduce a bias in this study, and the low pathogenicity of TSRV; concurrent with low C_T values (29.75-35.80) detected in all the positives.

Unlike TSRV, other reported aquareoviruses were isolated from disease outbreaks and experimental infections that caused at least 40%-65% of mortality (Meyers, 1980; Meyers, 1983; Isshiki *et al.*, 2003). When aquareoviruses were isolated from disease

outbreaks pathological changes were associated with the liver, shown by the formation of hepatic lesions with varying degrees of severity and syncytial giant cell formations of hepatocytes. Diffuse multifocal hepatitis with large syncytial cells, coagulation of necrosis of hepatocytes, fibrous connective tissue, scattered lymphocytes and bile duct hyperplasia were observed in bluegill (*Lepomis macrochirus*) fingerlings and rainbow trout experimentally infected with 13p2 virus (American Oyster Reovirus) (Meyers, 1980; Meyers, 1983). Similarly, focal hepatic necrosis was also the main pathology described in experimental infection of chum salmon with CSRV (Winton *et al.*, 1989a). Histopathological lesions due to aquareovirus infections have been reported in kidney, intestine, eye and brain (Cusack *et al.*, 2001; Isshiki *et al.*, 2003). Severe multifocal hepatocellular necrosis and acute necrosis of proximal renal tubules were consistently found in Atlantic halibut (*Hippoglossus hippoglossus*) experimentally infected with halibut reovirus, isolated from a large-scale mortality event (Cusack *et al.*, 2001). Isshiki *et al.* (2003) also reported pathological changes in other organs including the intestine, eye and brain displayed by marbled sole (*Pleuronectes yokohamae*) with a reovirus infection.

Based on archival cases, severe pathological changes have been observed in fish with TSRV infection concurrent with TRLO infections or high water temperature (17-21°C). However, it is possible that these severe pathological changes in samples co-infected by TSRV and TRLO could be due to TRLO infection alone. This hypothesis could not be tested in this investigation because only one fish was positive for both TSRV and TRLO. This fish did not show any significant gross signs and was not sampled for histology. There is a need for further investigation to determine the specific histopathological changes caused by TSRV alone and TSRV-TRLO co-infection. Due to the low incidence of natural TSRV infections observed during the course of this study this aspect could not be investigated.

Based on this field investigation, TSRV does not predispose fish to other infections. Co-infection of TSRV with *N. perurans* was higher than with the other pathogens, consistent with high prevalence of *N. perurans* in this field investigation. *N. perurans* has been reported to be ubiquitous in the water column around Atlantic salmon cages in South-east Tasmania (Bridle *et al.*, 2010; Douglas-Helders *et al.*, 2003). The detection of *N. perurans* in this study was based on the presence/absence of *N. perurans* genomic DNA, detected by qPCR assay. The obtained prevalence was not based on development of AGD lesions, but on the presence of *N. perurans* as only a few of the samples were subjected to histopathological examination. The salmon which were negative for TSRV were positive for *N. perurans*. Thus, there is no evidence of direct interaction between both pathogens. A preliminary study of a controlled dual infection with TSRV and *N. perurans* in Atlantic salmon smolt was also unable to demonstrate a clear interaction between both pathogens (Gemma Carlile pers communication).

Moribund and freshly dead fish were positive for TSRV more often than healthy fish. As prevalence of a virus in a population varies according to the stage of infection, the virus concentration in moribund and freshly dead fish would be significantly higher than in the healthy fish (Pfeiffer, 2002). However, this does not seem to be case with the occurrence of other pathogens, positives for TRLO and other bacterial infections were found more often in live and dead salmon than in the moribund fish. This is in contrast to reports of experimental co-infections of marine birnavirus (MABV-F) and bacteria (*Vibrio harveyi* and *Edwardsiella tarda*) in olive flounder, *Paralichthys olivaceus* (Oh *et al.*, 2006). The virus and bacteria were re-isolated from all dead and moribund fish and not from live control fish. It has been shown that amoebae numbers increase on the gills of dead fish up to at least 30 h post-mortem,

consistent with the higher numbers of *N. perurans* found in dead samples in this study (Douglas-Helders *et al.*, 2000).

The predominant bacterial infections in farmed Atlantic salmon were caused by *Vibrio* sp. although a few of the salmon were positive for *Yersinia ruckeri*. The co-occurrence of TSRV and both of these pathogens was not significant due to the low number of co-infections observed in this study, and co-infections of this type have not been reported previously as a significant threat to salmonid production in Tasmania. *Yersinia ruckeri* is enzootic to Australia and Yersiniosis in fish is a significant bacterial septicemia in freshwater (Carson and Wilson, 2009). This organism appears to have a wide geographical distribution and it is found in many countries that farm salmonids under intensive conditions. Fish most at risk are those subject to stress caused by poor management or environmental changes such as elevated water temperature and poor water quality (Carson and Wilson, 2009). The co-infection of TSRV and *Yersinia ruckeri* most likely represented the infection of a few individual fish. As *Yersinia ruckeri* is a freshwater species, the presence of this pathogen in salmon in marine environment is most likely due to survivors of *Yersinia ruckeri* infection from the hatchery. Asymptomatic carriage of *Yersinia ruckeri* is known to occur in Atlantic salmon and the isolation of *Yersinia ruckeri* in this study could have been from sub-clinical carriers (Carson and Wilson, 2009).

Vibrio spp. are ubiquitous in the environment and can be present with the host's normal flora. Vibriosis may occur when the host's immune system is compromised (Crane and Williams, 2009) from stress events, physical damage to the integument, or the emergence of aggressive biovars within a population of fish. In this investigation, *Vibrio* sp. was mostly found in freshly dead fish, concurrent with TRLO infection. The isolation of *Vibrio* sp. with

aquareovirus has been reported in a large-scale mortality of larval and juvenile halibut *Hippoglossus hippoglossus* which occurred at a semi-commercial halibut farm in Atlantic Canada (Cusack *et al.*, 2001). An increase in the mortality levels despite the use of an antibiotic to reduce the concurrent bacterial infections supported the hypothesis that the primary pathogen was of viral origin. The culture of *Vibrio* sp. was sensitive to the antibiotic and it seems that the antibiotic was effective in controlling *Vibrio* sp. infections (Cusack *et al.*, 2001). The co-occurrence of *Vibrio* sp. with viral infection in penaeid shrimp (*Penaeus monodon*) has been reported previously (Saulnier *et al.*, 2000). Most outbreaks of shrimp vibriosis occur in combination with physical stress or following primary infection with other pathogens (Sung *et al.*, 2001). Co-infections of viruses and *Vibrio* spp. seem to increase the mortality/infection level due to the opportunistic nature of *Vibrio* sp. and increased susceptibility of the host to bacterial infection (Johansen and Sommer, 2001; Phuoc *et al.*, 2009).

The co-occurrence of TSRV with TRLO was unexpectedly low (one sample) but both pathogens were isolated from the same site throughout the sampling. Archival data showed that high mortalities in Atlantic salmon were recorded due to either TRLO infection alone or TRLO-TSRV co-infection and that TSRV alone did not cause any mortality events. To investigate whether TSRV causes immuno-suppression leading to increased susceptibility to infections by TRLO, immune responses in Atlantic salmon should be measured in experimental infections. Co-occurrence of viral and bacterial pathogens in fish has been reported to increase mortality. In addition, immuno-suppression caused by a primary infection is hypothesized to be the cause of multiple bacterial infections (Baya *et al.*, 1990; Hament *et al.*, 1999; Cusack *et al.*, 2001; Johansen and Sommer, 2001; St-Hilaire *et al.*, 2001; Mohan *et al.*, 2002; Romero and Jimenez, 2002; Pakingking *et al.*, 2003; 2004; 2005;

Selvin and Lipton, 2003; Oh *et al.*, 2006; Overturf and LaPatra, 2006; Samuelson *et al.*, 2006; Rosado *et al.*, 2007; Phuoc *et al.*, 2009; Xu *et al.*, 2009; Lovoll *et al.*, 2010; Mitchell and Rodger, 2011 ; Nylund *et al.*, 2011). Experimental co-infections of Olive flounder, *Paralichthys olivaceus* with marine birnavirus (MABV-F) and bacteria (*Vibrio harveyi* and *Edwardsiella tarda*) indicated that the mass mortality was due to the bacterial infection (Pakingking *et al.*, 2003). Higher mortalities were hypothesized to occur due to co-infection of MABV-F and bacteria although MABV-F alone did not cause direct mortality (Pakingking *et al.*, 2003).

In this study, the mortality of Japanese flounder, *Paralichthys olivaceus*, increased to 84% and 76% by secondary infection with *Streptococcus iniae* or *Edwardsiella tarda* at 1-week post-MABV infection, while the mortality rates of the fish, which had bacterial infection alone, was 52% and 44%. The mechanisms behind the viral-bacterial interactions in this particular study were unknown. It was suggested that viral infections are accompanied by general immuno-suppression that may affect the phagocytic activity of fish leukocytes (Pakingking *et al.*, 2003). Active replication of the virus could have resulted in a transient immuno-suppression that reduced the bactericidal activity of flounder phagocytes that led to mortality by the subsequent bacterial infection (Pakingking *et al.*, 2003). An IPNV carrier condition with low virus titres in Atlantic salmon did not affect their general health or lower their susceptibility to diseases (Johansen and Sommer, 2001). However, an increased mortality was observed at higher IPNV titres inducing acute IPN which was accompanied by a severe secondary infection by *Vibrio salmonicida* (Johansen and Sommer, 2001).

The interactions between viral and bacterial infections in a host are complex and the defence mechanisms in a fish are different towards viral or bacterial infections (Johansen and

Sommer, 2001) . Bacterial invasion which elicits a respiratory burst response in fish phagocytes and the decreased oxygen-production in acute IPNV affected fish were taken into account for the increased mortality due to the co-infections. These findings are consistent with higher mortalities coinciding with co-infection of TSRV and TRLO based on archival data examined during the present study. However, further research needs to be undertaken to demonstrate whether T SRV infections in salmon cause immune-suppression and lead to secondary infection by TRLO or other bacteria. To date, natural infection with T SRV is characterised by low pathogenicity and subclinical carriage in Atlantic salmon. However, experimental infections have shown that T SRV could cause mortality under certain environmental conditions (Gemma Carlile pers communication). Thus, current findings have raised more questions on the effect of possible acute TSRV infection and in combination with other pathogens.

One significant observation is the co-occurrence of T SRV with other pathogens reported in this investigation and potential involvement of other factors. There is a definite paucity of knowledge on synergisms between these pathogens and the potential interactions between these agents and variations in environmental factors. This is particularly relevant with the increased water temperature associated with climate change. Based on the archival data, prevalence of T SRV was influenced by high water temperature during the summer of 2009-2010. In Tasmania, Atlantic salmon is normally farmed at high temperatures (summer water temperatures can exceed 19°C) relative to northern hemisphere production sites. As with other viral infections of temperate aquaculture fish species, temperature has been shown to have a substantial effect on the pathogenicity of T SRV, that is, the higher the water temperature, the higher the mortality (Gemma Carlile pers communication). Similarly, clinical disease and mortality due to AGD are most commonly reported as temperature rises

beyond 16°C, and temperature has been recognized as one of the major risk factors for AGD outbreaks (Munday *et al.*, 2001; Douglas *et al.*, 2001; Mitchell and Rodger, 2011). As alluded to, temperature has been documented as an important environmental variable affecting the pathogenicity of some fish and shellfish viruses, including golden shiner reovirus (GSRV) (Schwedler and Plumb, 1982), grass carp reovirus (GCRV) (Jiang and Ahne, 1989), infectious hematopoietic necrosis virus (IHNV) (Amend, 1970; Hetrick *et al.*, 1979), CV-TS-1, birnavirus (Chou *et al.*, 1994), golden ide reovirus (GIRV) (Neukirch, 2000), marine aquabirnavirus (MABV) (Pakingking *et al.*, 2003) and red sea bream iredrovirus (RSIV) (Jun *et al.*, 2009).

In conclusion, it is likely that the co-occurrence of infections with TSRV and other pathogens in Tasmanian farmed Atlantic salmon is influenced by elevated water temperature. The significance of these infections is likely to increase with prevalence of other pathogens, adverse changes to environmental factors and the virulence of TSRV. Non specific gross pathology and histopathology were observed in TSRV positive salmon, however, they were also present in some TSRV negative salmon. Pathology associated with TSRV infection is still inconclusive due to the low number of TSRV positive samples observed in this study in general, and low number of positives sampled for histology. On the basis of archival samples, liver lesions have been identified as the predominant pathology caused by TSRV in Tasmanian Atlantic salmon. It is also apparent that the presence of TRLO is concurrent with TSRV infections and associated with higher water temperatures during summer.

CHAPTER 5

Preliminary characterisation of Tasmanian Aquareovirus isolates

5.1 Introduction

Members of the genus *Aquareovirus* possess a non-segmented dsRNA genome, similar to that of the members of the genera *Mycoreovirus* and *Rotavirus*, within the family *Reoviridae*. The classification of the aquatic reoviruses into a separate genus has been justified based on criteria such as host range, RNA sequence and serological differences (Fauquet *et al.*, 2005). Currently, aquareovirus isolates are classified into six genogroups (A-F). This classification is based on reciprocal RNA-RNA blot hybridization, RNA electrophoresis, antigenic properties and nucleotide sequence analysis. The recent use of nucleotide sequence analysis has helped to refine classification of aquareoviruses, as inadequacies of traditional methods have hampered it. For example, based on RNA-RNA hybridization, it had been suggested that GCRV may represent a seventh species group (G) (Subramanian *et al.*, 1997; Rangel *et al.*, 1999) but subsequent nucleotide sequence analysis has indicated that it should be placed within genogroup C, as being synonymous with golden shiner reovirus (GSRV) (Attoui *et al.*, 2002; McEntire *et al.*, 2003). The sequence data for many of the aquareoviruses are still inadequate, thus, limiting the phylogenetic analysis for each species group. It had been suggested that further research is needed to clarify the phylogenetic relationship within the genus *Aquareovirus* and to assist in a better understanding of the role each segment plays in viral pathogenesis (Seng *et al.*, 2005; Crane and Carlile, 2008).

Segments 2 (S2) and 10 (S10) of TSRV genome were characterised previously and the gene sequences deposited in the GenBank [database under accession numbers EF434978 and EF434979, respectively (Gemma Carlile pers communication)]. Sequence and phylogenetic studies of two gene segments and their deduced proteins allowed definitive classification of the virus, some speculation on its origin, and the development of diagnostic tools. Sequence and phylogenetic analysis confirmed the assignment of TSRV to species group *AQORV-A* as described by Lupiani *et al* (1995) (Gemma Carlile pers communication). Phylogenetic analysis for S2 and S10 has shown high sequence identity between the two aquareoviruses, 95% and 94%, respectively, indicating that TSRV is closely related to CSRV. Initially, a lack of available sequence information and access to other aquareoviruses limited further comparison of TSRV with other related isolates.

For the diagnosis of TSRV infections, immunocytochemistry serves as a confirmatory test for the presence of TSRV in cell cultures exhibiting typical cytopathic effect (CPE). A positive reaction for the presence of TSRV by immunocytochemistry using rabbit anti-TSRV polyclonal antibody, is indicated by the presence of grainy, focal and brick-red staining in infected cells. Throughout the years, immunocytochemistry reactions for the detection of TSRV in infected cells demonstrated different staining intensity of plaques; with some demonstrating intense staining and others with plaques without staining (John Young pers communication). The phenotypic differences observed between TSRV samples by isolation on different cell lines and immunocytochemistry raised questions regarding the existence of variants among TSRV isolates from different geographical locations in Tasmania, host species, host tissues. Since its first isolation in 1990s, TSRV has been isolated from different regions of Tasmania, Tamar River estuary in the north, Huon River estuary in the south-east, and Macquarie Harbour.

Studies have shown sequence divergence among isolates of *Aquareovirus* from different geographical location (Goodwin *et al.*, 2006; Lupiani *et al.*, 1993; Samal *et al.*, 1991; R angel *et al.*, 1 999; M cEntire *et al.*, 2003; A ttoui *et al.*, 200 2). RNA v iruses (including aquareovirus) have been shown to exhibit relatively high mutation rates and RNA virus populations are extremely heterogenous which allow for great adaptability and rapid evolution of RNA genomes (Steinhauer and Holland, 1987). A number of medically important viruses including HIV, hepatitis C virus and influenza, have RNA genomes, replicate with extremely high mutation rates and exhibit significant genetic diversity (Lauring and Andino, 2010). The instability and high mutation rate of the RNA genome in reovirus were reported by Fields and Joklik (1969). RNA viruses demonstrate high mutation rates and self-copying error due to the lack of proofreading mechanisms during viral replication (Steinhauer and Holland, 1987; Sanjuan, 2008) . This genetic diversity allows a viral population to rapidly adapt to dynamic environments and evolve resistance to vaccines and antiviral drugs (Lauring and Andino, 2010). Quasispecies theory was initially formulated to understand and describe the evolutionary dynamics of RNA viruses (Eigen, 1971). According to Lauring and Andino (2010), a quasispecies is a cloud of diverse variants that are genetically linked through mutation, interact cooperatively on a functional level, and collectively contribute to the characteristics of the population. As TSRV is a dsRNA virus, there was a possibility that TSRV isolates could represent a quasispecies similar to any other RNA viruses; adapt to their environment, replicate with high mutation rate and exhibit genetic diversity. To further investigate these hypotheses, this chapter describes preliminary characterisation of specific viral isolates of TSRV that were selected based on genotypic and phenotypic characteristics.

5.2 Materials and Methods

5.2.1 Selection of isolates for characterization of TSRV

As a basis for this characterisation study, 14 isolates, originating from various locations in Tasmania, covering a 20-year period obtained from various host species and host tissues, and isolated on different cell lines, were selected in an attempt to increase the probability of detecting virus variants (Table 5.1). TSRV isolates were obtained from an archive collection, maintained at AAHL, of laboratory submissions from the Tasmanian Salmonid Health Surveillance Program (TSHSP) carried out between 1990 and 2010. They were generally taken from freshly dead or moribund fish. With the exception of isolate 10 and isolate 11 (which was derived from Atlantic salmon brood-stock), all isolates were obtained from juvenile finfish. Isolate 10 was obtained from jack mackerel (*Trachurus declivis*) sampled at the time of a wild fish mortality (of unknown cause) found in salmon cages. As part of the TSHSP, all samples were processed for virus isolation on fish cell lines. Cultures that developed viral-like cytopathic effect (CPE) were further processed for confirmation of viral identification. In the early days of the TSHSP, confirmation was by electron microscopy and later by immunocytochemistry using virus-specific antibodies. More recently, and currently, confirmation of presence of virus is undertaken using PCR (Mark Crane pers communication). Thus, for the early isolates, confirmation of TSRV infection of cell cultures that demonstrated aquareovirus-like CPE was determined by immunocytochemistry using an aquareovirus-specific polyclonal rabbit antiserum developed at AAHL. From 2008, the PCR tests, developed by Carlile (2011), have been used at AAHL for confirmation of TSRV identification of viruses isolated in cell culture.

Table 5.1 Fourteen viral isolates isolated for the detection of TSRV in Tasmania during a 20-year period between 1990 and 2010. The isolates originating from various locations obtained from various host species, tissues, year classes and isolated on different cell lines. SE Tas: South-east Tasmania, RLO: Rickettsia-like organism.

Viral isolate	Sample No	Host Species	Year Class	Year Isolated	Cell line	Tissue	Location
90-0388	1	Atlantic salmon	1989	1990	CHSE-214	Kidney, liver & spleen	SE Tas
91-0130	2	Atlantic salmon	1988	1991	BF-2	Kidney, liver & spleen	SE Tas
91-0280	3	Atlantic salmon	1986	1991	BF-2	Ovarian fluid	SE Tas
97-0786	4	Atlantic salmon	1995	1997	CHSE-214	Kidney, liver & spleen	SE Tas
98-0885	5	Atlantic salmon	1996	1998	EPC	Kidney, liver & spleen	SE Tas
99-1980	6	Atlantic salmon	1997	1999	EPC	Kidney, liver & spleen	SE Tas
00-0357	7	Atlantic salmon	1997	2000	CHSE-214	Kidney, liver & spleen	SE Tas
01-1575	8	Atlantic salmon	1999	2001	EPC	Kidney, spleen & brain	SE Tas
03-1746	9	Atlantic salmon	2001	2003	CHSE-214	submitted for RLO testing	SE Tas
06-0412	10	Jack Mackerel	Wild fish	2006	EPC	Liver , pyloric caeca & heart	SE Tas
07-1692	11	Atlantic salmon	Broodstock	2007	EPC	Ovarian fluid	North Tas
08-0321	12	Atlantic salmon	2007	2008	CHSE-214	Kidney, liver, spleen & heart	SE Tas
08-0823	13	Atlantic salmon	2006	2008	EPC	Kidney, liver & spleen	North Tas
10-0356	14	Atlantic salmon	2009	2010	CHSE-214	Kidney, liver, spleen, brain and heart	SE Tas

5.2.1.1 Sample preparation

Each of the selected isolates of aquareovirus (see Table 5.1) were recovered from frozen storage and expanded in cultures of the CHSE-214 (chinook salmon, *Oncorhynchus tshawytscha* embryo, ATCC catalogue No. CRL 1681) cell line. The cell cultures were established in 25 cm² tissue culture flasks (Nunc, Roskilde, Denmark) using Eagle's minimal essential medium (EMEM) supplemented with 10 mM HEPES buffer, 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU penicillin / mL and 100 µg streptomycin / mL (Invitrogen, Australia) as the growth medium and the cultures were incubated at 22°C in an atmosphere of 5% CO₂/95% air until 90% confluency was attained (usually within 2 days of seeding the cultures). Immediately prior to virus infection the medium was changed to "maintenance medium", which had the same ingredients as growth medium except that it contained only 2 % FBS (use of reduced serum concentration assists in reducing the rate of cellular replication, allowing sufficient time for viral replication prior to any culture over-growth).

The cell cultures (one for each viral isolate) were inoculated with 200 µL of the thawed viral stock and then incubated at 15°C (this lower temperature is permissive for viral replication and also reduces the rate of cellular replication) in an atmosphere of 5% CO₂/95% air until 100% CPE was observed. The supernatants were decanted into 10 mL centrifuge tubes and clarified by centrifugation at 100 x g at 4°C for 20 minutes. For each isolate, an aliquot (140 µL) of the supernatant was taken for nucleic acid extraction (see 5.2.1.4) and the remainder of each supernatant was loaded into Beckman ultra-clear

SW41 centrifuge tubes and ultracentrifuged using a SW41 rotor at 100,000 x g at 5°C for 1.5 hours. After ultracentrifugation, the supernatants were decanted and as much fluid as possible was removed by inverting the tube on tissue paper. The pellets containing replicated virus were resuspended in the remaining supernatant (approximately 30 µL). For each isolate, the 30 µL of resuspended viral pellet was mixed well with NuPAGE® LDS sample buffer (Invitrogen, Australia), boiled for 5 minutes and stored at -20°C until further use. NuPAGE® LDS Sample Buffer contains lithium dodecyl sulphate at a pH of 8.4, which allows for maximal activity of the reducing agent.

5.2.1.2 Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% (w/v) acrylamide gels was carried out according to the method described by Laemmli (1970). Following electrophoresis, gels were stained using 1% (w/v) Coomassie brilliant blue R-250 and destained using 40% methanol with 7% acetic acid solution to visualize the polypeptide bands.

5.2.1.3 Western blotting

Western blotting was performed as described by Towbin et al. (1979). The isolates (resuspended viral pellet with LDS sample buffer) were thawed, heated at 99°C and micro-centrifuged. The isolates were resolved on gradient NuPAGE® Novex® 4-12% Bis-Tris Gels (1.0-mm thick, 12-well with NuPAGE® MOPS SDS Running Buffer) at 200V for 52 minutes. NuPAGE® MOPS (3-(N-morpholino) propanesulfonic acid) SDS

(sodium dodecyl sulphate) Running Buffer is used for separating medium to large sized proteins.

Following electrophoresis, the viral proteins were transferred to 0.45 μ m nitrocellulose membrane (Hybond C) at 100 V for 1 hour using the Mini Trans-Blot cell (BioRad) with Towbin transfer buffer. For immune-detection, the membrane was blocked in 5% (v/v) skim milk (SM) in 0.01 M Tris-HCl pH 7.4, 0.15 M NaCl for 30 minutes at room temperature. The membrane was then washed once with Tris-saline (pH 7.4), before incubation with rabbit anti-TSRV (diluted 1:200) serum in SM solution at room temperature on a slow-speed, flat-bed orbital shaker for 1 hour. The membranes were washed twice with 0.01 M Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% (v/v) Tween 20 and once with 0.01 M Tris-HCl pH 7.4, 0.15 M NaCl for 5 mins. The membranes were incubated at room temperature with freshly prepared goat-anti-rabbit IgG (whole molecule) conjugated with horseradish peroxidase (HRPO) (Sigma) (diluted 1:1000 with SM solution) using the same incubation conditions used for the primary antibody step.

The membranes were washed again as described previously and colour was developed using 4-chloro-1-naphthol as substrate with incubation at room temperature for 15 to 30 min to obtain optimal staining with a minimum of background. The substrate was prepared by dissolving 60 mg 4-chloro-1-naphthol in 20 mL methanol on ice; just before use this was mixed with 100 mL in 0.01 M Tris-HCl pH 7.4, 0.15 M NaCl to which 60 μ L of 30% (v/v) H_2O_2 had been previously added. Images of Western blots were digitised with a Nikon Cool Pix P2. Approximate molecular weights of viral

proteins were determined by comparison with molecular weight standards (See Blue[®] Plus2 Pre-Stained Standard, Invitrogen, Australia) using LaserSoft[®] SilverFast[®] SE software included in the Epson Perfection 2450 Photo scanner.

5.2.1.4 qPCR

RNA extraction was carried out on the 140 μ L aliquots obtained previously (See 5.2.1.1) using the QIAmp[®] Viral RNA kit (QIAGEN, Germany) according to the manufacturer's instructions. A total 560 μ L of prepared Buffer AVL containing carrier RNA was added to 140 μ L of each sample. The sample-buffer was mixed well using a vortex mixer for 15s, to ensure efficient lysis and to yield a homogenous solution. The sample-buffer solution was incubated at room temperature for 10 min and the tube was centrifuged at 6000 x g for one minute to precipitate any fluid from the inside of the lid. A total 560 μ L 100% ethanol were added to the sample, mixed with a vortex mixer for 15s and the tube was centrifuged at 6000 x g for one minute. The solution (630 μ L) was transferred to a QIAmp[®] Mini column (in a 2 mL collection tube) without wetting the rim. It was centrifuged at 6000 x g for one minute. The QIAmp[®] Mini column was placed into a clean 2 mL collection tube and the tube containing the filtrate was discarded. Buffer AW1 (500 μ L) was added to the tube and centrifuged at 6000 x g for one minute. After centrifugation, a new collection tube was used for a second run. A total of 500 μ L Buffer AW2 was added and the tube was centrifuged at 13000 x g for 3 min. Then the QIAmp[®] Mini column assembly was placed into a new collection tube and was centrifuged at maximum speed for a minute to remove any residual Wash Buffer.

The QIAmp[®] Mini column was placed into a clean 1.5 mL microcentrifuge tube and 60 µL Buffer AVE was added. It was incubated at room temperature for one minute and centrifuged at 6000 x g for one minute. The sample was eluted into a final volume of 60 µL AVE buffer. The qPCR detection for T SRV was conducted using the specific primers and probe developed by Carlile (2011). The primers and probe for the qPCR assay were designed to amplify and detect an 82 nucleotide sequence of cDNA corresponding to 705–787 bp of the viral genome within segment 10 of T SRV. The sequences for the primers and probe were as follows: Forward primer (T SRV-10F; position 705–725): 5'-GATCGAACCCGTCGTGTCTAA-3', Reverse primer (T SRV-10R; position 769–787): 5'-CGGTGCTCAGCTTGTCACA-3', Probe (position 731–748): 5'-CCC GAG CCA TCT GGG CGC-3'. The probe contained a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), located at the 5' end and the quencher and 6-carboxy-tetramethyl-rhodamine (TAMRA) located at the 3' end.

The amplification protocol consisted of addition of RNA to a master mix as per the TaqMan[®] one-step RT-PCR master mix reagents kit (Applied Biosystems, USA). The reaction mixture (25 µL) consisted of: 6.125 µL RNase free water, 12.5 µL TaqMan[®] 2 × Universal PCR master mix no AmpErase[®] UNG (containing AmpliTaq Gold[®] DNA polymerase), 0.625 µL 40 × Multiscribe[™] and RNase inhibitor mix, 1.25 µL Primer TSRV-10F (18 µM), 1.25 µL Primer TSRV-10R (18 µM), 1.25 µL TSRV-10 probe (5.0 µM) and 2 µL viral RNA. The amplification was carried out in an ABI PRISM[™] 7500 Fast Real-Time System (Perkin-Elmer, Applied Biosystems, USA). The RNA was amplified using the following programme: an initial 30 minutes at 48°C, followed by 10

minutes at 95°C, then 45 cycles of 95°C for 15 seconds and 60°C for one minute. The cut-off C_T value for detection of TSRV was set at 35.8 (see Chapter 2.3.4.1), hence, C_T values < 35.8 are considered positive, C_T values between 35.8 and 36.13 are ambiguous values (i.e. too close to call either positive or negative) and C_T values > 36.13 are considered negative.

5.2.1.5 Conventional hemi-nested RT-PCR

RNA extraction was carried out according to the manual instructions for the QIAamp[®] Viral RNA Mini Kit (Qiagen, Germany). The conventional hemi-nested RT-PCR was carried out using primers the design of which was based on the sequence alignment of the S10 gene of TSRV and developed by Carlile (2011): Forward primer SpT10Fb (position 306 -323: 5'-TTCCCTCTCTAAGACCC-3') and reverse primer SpT10Ra (position 567 -585: 5'-GCCACCGGTAATAGTACG-3') for primary reaction followed by forward primer SpT10FN (position 445 -463: 5'-AATTGTGATCGCGCTCTC-3') and reverse primer SpT10Ra (position 567 -585: 5'-GCCACCGGTAATAGTACG-3') in the hemi-nested reaction. The amplification was carried out using the SuperScript[®] III One-Step RT-PCR System with Platinum[®] Taq (Invitrogen, Australia). A total of 23 µL PCR mixture containing: 12.5 µL 2X Reaction Mix (QIAGEN), 9.0 µL RNase-free water, 1 µL SuperScript III/ Platinum Taq Mix, 0.25 µL SpT10Fb and 0.25 µL SpT10Ra was added to 2 µL prepared RNA.

For the primary reaction, the amplification was programmed as followed: 15 minutes at 95°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 40 seconds. A final extension of 72°C for 5 minutes terminated the thermal cycling reaction. The hemi-nested reaction was carried out with a total of 23 µL PCR mixture containing: 12.5 µL 2X HotStar Taq Plus Master Mix (QIAGEN), 9.5 µL Rnase-free water, 0.5 µL SpT10FN and 0.5 µL SpT10Ra were added to 2 µL PCR product. The amplification was conducted using the following programme: 15 minutes at 95°C, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 40 seconds and a final extension of 72°C for 5 minutes. Both reactions were carried out using a MasterCycler (Eppendorf, Australia) thermal cycler. The amplified PCR products from both reactions were analysed by electrophoresis (50 minutes at 90 V) on 1.5% (w/v) agarose gel in TAE buffer and stained with SYBR Safe DNA Gel Stain (Invitrogen, Australia).

5.2.1.6 DNA sequencing

To produce templates for DNA sequencing, amplicons were excised from the agarose gels and purified using a QIAquick® Gel Extraction kit (QIAGEN) according to the manufacturer's protocols. Purified PCR products were then sequenced using Big Dye® Terminator Sequencing kits (Applied Biosystems, USA) according to the manufacturer's instructions. An ABI PRISM® 377 DNA Sequencer (Applied Biosystems, USA) was used to obtain sequence. Chromatograms were analysed using Chromas

Version 2.01 (TechneLysium) and aligned using ClustalW (Larkin *et al.*, 2007) with Geneious ProTM software.

5.2.2 Characterisation of representative isolates of TSRV

Details on the isolate and origin (sources of viral isolates)

Based on the preliminary study (section 5.2.1) conducted to select the isolates for the characterisation of TSRV, initial results using Western blot and PCR analyses indicated that there was variation between TSRV isolates. Therefore a sub-set of isolates of particular interest (Isolate 6: 99-1980; 7: 00-0357; 10: 06-0412; 11: 07-1692; 13: 08-0823) were regrown in CHSE-214 cell cultures according to section 5.2.1.1. Isolates 6, 7 and 10 appeared to be representative of “typical” TSRV whereas isolates 11 and 13 demonstrated “atypical” characteristics based on the preliminary results. Thus, Western blot, PCR and sequencing analyses were conducted on these isolates of interest to confirm previous results.

5.2.2.1 Western blot

The Western blot analysis was conducted according to section 5.2.1.3.

5.2.2.2 qPCR

The RNA extractions and qPCR assay (duplicate samples) were conducted according to section 5.2.1.4.

5.2.2.3 Conventional generic RT-PCR

RNA extraction was carried out according to the instruction manual for QIAamp[®] Viral RNA Mini Kit (Qiagen, Germany). The generic aquareovirus PCR was carried out using degenerate primers (GARV-F and GARV-R) the design of which was based on the sequence alignment of the S2 gene of GCHV (AF284502), GCRV (AF260512), CSRV (AF418295), and GSRV (AF403399) as developed by Carlile (2011). The primers design was based on the conserved regions located within the polymerase gene, amplifying a PCR product of 314bp. The sequences for the primers were: Forward primer, GARV-F (position 1788-1812: 5'-TAAAGCTTGCGACGCCTCCATCAC-3') and reverse primer GARV-R (position 2082-2102: 5'-TGCTCGGTGGAGGTGACAGT-3'). The amplification was carried out using SuperScript[®] III One-Step RT-PCR System with Platinum[®] Taq (Invitrogen, Australia). A total of 23 µL PCR mixture containing: 12.5 µL 2X Reaction Mix (QIAGEN), 9.0 µL RNase-free water, 1 µL SuperScript III/ Platinum Taq Mix, 0.25 µL GARV-F and 0.25 µL GARV-R was added to 2 µL prepared RNA.

The amplification was programmed as followed: 30 minutes at 55°C, 2 minutes at 94°C, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C

for 30 seconds and extension at 68 °C for 45 seconds. A final extension of 68 °C for 7 minutes terminated the thermal cycling reaction. The amplified PCR products from the reaction were analysed by electrophoresis (50 minutes at 90 V) on 1.5% (w/v) agarose gel in TAE buffer and stained with SYBR Safe DNA Gel Stain (Invitrogen, Australia).

5.2.2.4 Segment 10 TSRV T10 conventional RT-PCR

RNA extraction was carried out according to the instruction manual for QIAamp[®] Viral RNA Mini Kit (Qiagen, Germany). This conventional RT-PCR was carried out using primers the design of which was based on segment 10 of TSRV, T10Fc and T10Rc - amplifying a PCR product of 263bp. The sequences for the primers were: Forward primer, T10Fc (position 669-690: 5'-TAAAGCTTGCGACGCCTCCATCAC-3') and reverse primer T10Rc (position 912-931: 5'-TGCTCGGTGGAGGTGACAGT-3'). The amplification was carried out using SuperScript[®] III One-Step RT-PCR System with Platinum[®] Taq (Invitrogen, Australia). A total of 23 µL PCR mixture containing: 12.5 µL 2X Reaction Mix (QIAGEN), 9.0 µL RNase-free water, 1 µL SuperScript III/ Platinum Taq Mix, 0.25 µL T10Fc and 0.25 µL T10Rc was added to 2 µL prepared RNA. The amplification was programmed as followed: 30 minutes at 55 °C, 2 minutes at 94 °C, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 68 °C for 45 seconds. A final extension of 68 °C for 7 minutes terminated the thermal cycling reaction. The amplified PCR products from the reaction were analysed by electrophoresis (50 minutes at 90 V) on 1.5% (w/v) agarose gel in TAE buffer and stained with SYBR Safe DNA Gel Stain (Invitrogen, Australia).

5.2.2.4.1 DNA Sequencing

The DNA sequencing was conducted according to section 5.2.1.6.

5.2.2.5 Electron microscopy

Duplicate cultures of CHSE-214 cells, grown in 25cm² cell culture flasks, were infected with typical isolate 10 and atypical isolates (isolate 11 and 13) of TSRV. After development of CPE affecting 50 - 100% of the cell monolayer, the tissue culture supernatant (TCSN) and cells were harvested. The cells were pelleted by centrifugation for 5 min at 840 x g. The supernatant was retained for negative contrast electron microscopy (NCEM) and the cells were used for ultrathin section preparation. Preparation of the specimens for electron microscopic examination was undertaken by staff of the Australian Microscopy & Microanalysis Research Facility, AAHL. The cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer for 40 min at room temperature, washed in PBS (3 x 20 min), post-fixed in 1% (w/v) osmium tetroxides for 1 hour and washed in reverse osmosis (RO) water (4 x 5 min).

The specimens were dehydrated in a graded ethanol series and infiltrated with 50% Spurr's resin in 100% ethanol for 30 min followed by 100% Spurr's resin for 1 hour. The sample was placed in a resin capsule (Better Equipment for Electron Microscopy, Inc.) and embedded with resin at 65°C overnight. The resin block was cut into ultra-thin sections using a Leica/Reichert Ultracut E microtome (Leica), transferred to plastic-

filmed grids and stained with 0.5% uranyl acetate and 0.25% lead citrate for 7 and 3 min, respectively. For NCEM, the TCSN was transferred to sterile tubes and centrifuged at 53 000 x g for 30 min. The supernatant was harvested and centrifuged at 230 000 x g for 1 hour. The virus pellet was resuspended in PBSA and adsorbed to a grid for 5 min followed by staining with 2% 12-tungstophosphoric acid (PTA) in RO water for 3 min. Stained grids from both NCEM and ultra-thin sections were examined by transmission electron microscopy (TEM) using a Philips electron microscope model CM120 at 75 kV.

5.2.2.6 Immunocytochemistry (ICC)

Immunocytochemical testing for confirmation of the presence of TSRV was undertaken using a standard test developed at AFDL. Cultures of CHSE-214 cell line were established in 24-well culture plates and infected with either a typical isolate (isolate 10) or an atypical isolate (isolate 11) of TSRV. Once TSRV-like CPE was observed the cultures were fixed in preparation for immunocytochemistry (ICC). Cell cultures were fixed by adding 20% formalin in PBS so that the final formalin concentration when added to the cultures was 4%. Thus for 24-well plate cultures, 500 µL of 20% formalin in PBS was added to each well. The plate cultures were incubated at room temperature for 1 hour and then fixative was removed and the fixed cultures rinsed 3 times with PBSA and stored (with PBSA covering the fixed monolayers) at 4 °C until used.

For ICC staining, the PBSA was removed from the stored fixed cell cultures which were then rinsed (2X) with 0.05% (v/v/ PBSA) Tween solution (PBST). The

primary positive (rabbit anti-TSRV antiserum) and negative (normal rabbit antiserum) antibodies were diluted to 1/5000 in 1% (w/v PBSA) skimmed milk solution (PBSA-SM) and, for each diluted antibody, were added to a set of duplicate wells of the fixed TSRV-infected and non-infected (control) cell cultures.

These were incubated at 37°C for 1 hour on a plate shaker at 100-200 rpm. Following incubation, the primary antibody solutions were removed and the cultures rinsed (3X) with PBST. The secondary antibody (biotinylated donkey anti-rabbit IgG antiserum) was diluted (1/500) in PBSA-SM (0.1%) solution and 200 µL were added to all wells. These were incubated at 37°C for 1 hour on a plate shaker at 100-200 rpm. Following incubation, the secondary antibody was removed from the wells which were rinsed (3X) with PBST. Streptavidin-peroxidase conjugate was diluted (1/500) in 0.1% SM solution and 200 µL added to each well. These were incubated at 37°C for 1 hour (in a humidified chamber) on a plate shaker at 100-200 rpm. Following incubation, the conjugate was removed from the wells which were rinsed (3X) with PBST. One tablet of 3-amino-9-ethyl-carbazole (AEC) substrate was dissolved in 2.5 mL of dimethyl formamide to make the stock solution which was then diluted in 47.5 mL acetate buffer (pH 5.0). Just before use, 25 µL 30% hydrogen peroxide was added to the AEC solution which was then added to each well. These were incubated at room temperature for 20 minutes. Following incubation, the substrate solution was removed and the wells rinsed (2X) with deionised water to stop the reaction. The monolayers were counterstained with Mayer's haematoxylin for one minute and rinsed with deionised water. Scott's tap water (50 µL) was added to each of the wells which were then rinsed with deionised water and

allowed to air dry. The interpretation of the results were based on these criteria: a) positive reaction: granular-like, focal, brick-red staining of cells indicates presence of virus and b) negative reaction: no red staining apparent - all cells should be stained pale blue due to counter staining.

5.3 Results

5.3.1 Selection of isolates for characterisation of TSRV: Preliminary results

5.3.1.1 SDS Polyacrylamide gel electrophoresis of viral polypeptides

Analysis of the protein profiles showed consistent bands for most of the samples. Figure 5.1 (a and b) demonstrates electrophoretic profiles of polypeptides derived from cell cultures infected with TSRV samples (sample 1-14). It was possible to identify some of the viral proteins in TSRV isolates compared to the three negative controls (uninfected CHSE-214, E PC & B F-2 cell cultures). SDS-PAGE of the virion proteins revealed putative viral polypeptides at approximately 130, 127, 116.3, 29, 14, 13.9 and 6.5 kDa in size; observed in TSRV samples only.

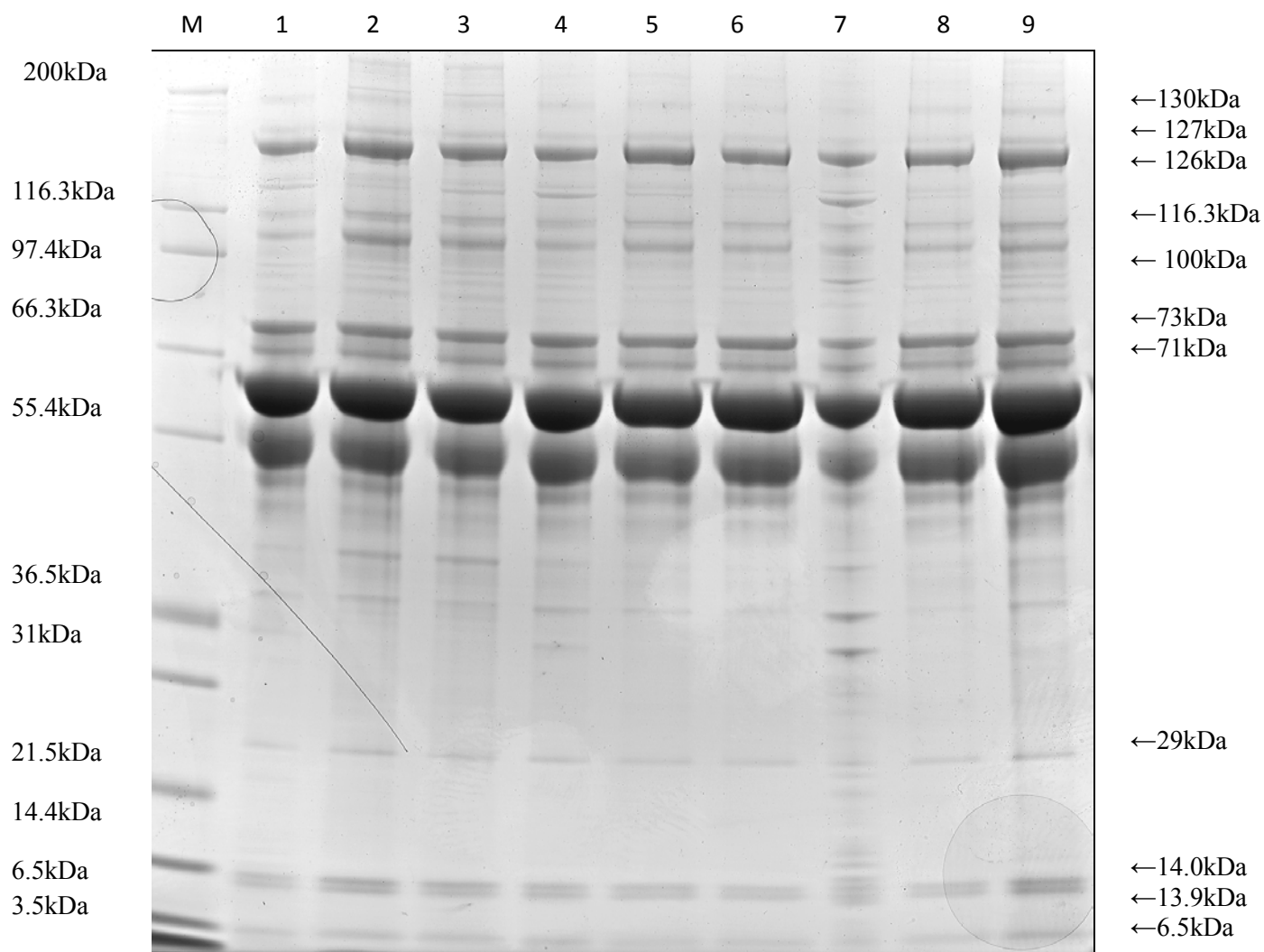


Figure 5.1a Electrophoretic profile of polypeptides derived from cell cultures infected with TSRV samples. Analysis of putative viral polypeptides on a 4-12% gradient NuPAGE[®] Bis-TRIS gel. The gel was stained with 1% Coomassie Brilliant Blue. Lane M: Mark 12[™] unstained standard (Invitrogen) as molecular marker, Lane 1-9: samples 1-9 (see table 5.1).

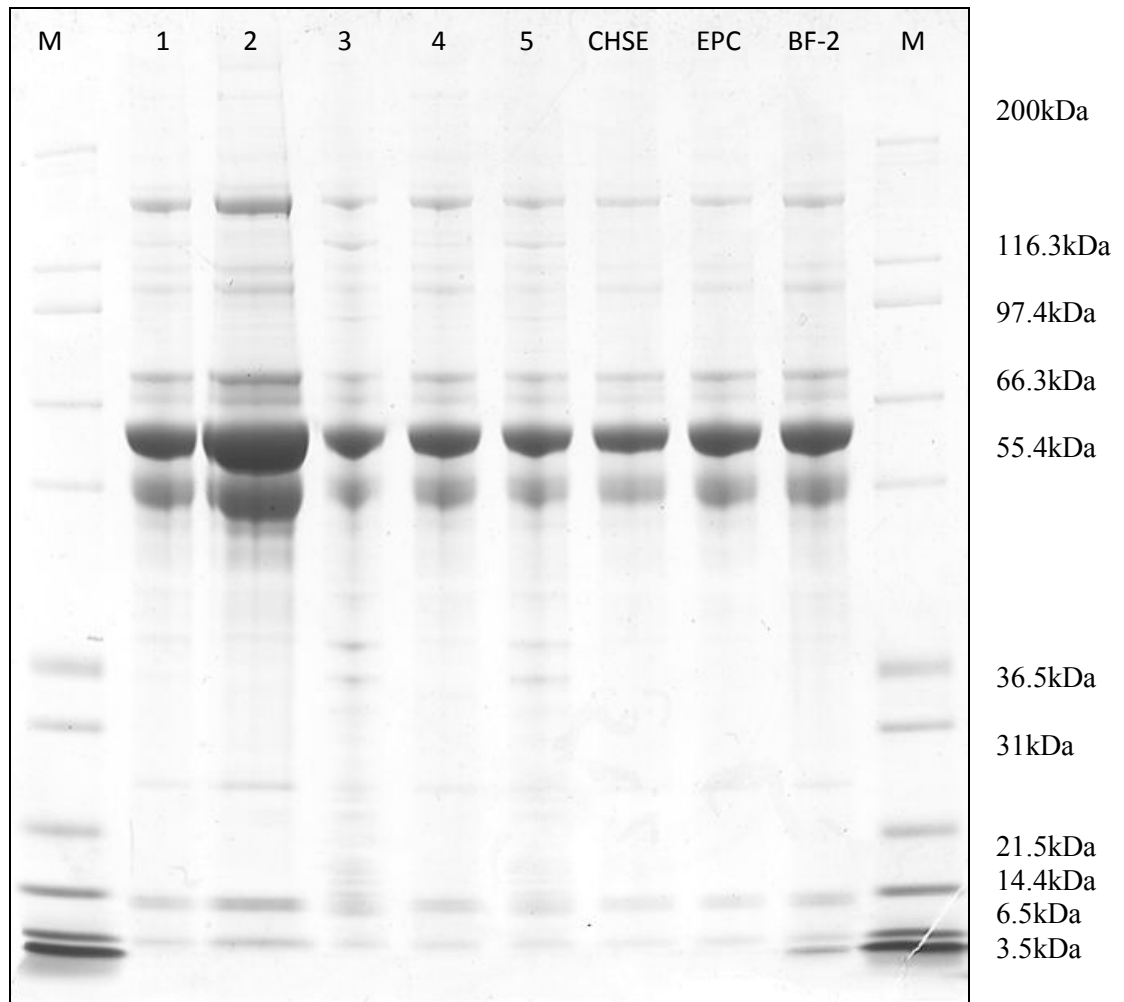


Figure 5.1b Electrophoretic profile of polypeptides derived from cell cultures infected with T SRV samples. Analysis of putative virion polypeptides on a 4-12% gradient NuPAGE® Bis-TRIS gel. The gel was stained with 1% Coomassie Brilliant Blue. Lane M: Mark 12™ unstained standard (Invitrogen) as molecular marker, Lane 1-5: Samples 10-14 and cell lines (CHSE-214, EPC & BF-2) as negative controls.

5.3.1.2 Western blotting

Western blot analysis showed the presence of three major protein bands in TSRV, with apparent molecular weights of approximately 130, 57 and 35 kDa that were identified by the rabbit anti-TSRV polyclonal antibody (Figure 5.2 -5.4). The three major groups of aquareoviral proteins can be classified as large, medium and small polypeptides. The presence of these bands were visualised in all of the isolates except isolate 6. Isolates 11 and 13 showed a typical protein bands compared with the other isolates, where both of these isolates showed the presence of bands at the following estimated molecular weights, 55 kDa and 32 kDa i.e. lower molecular weights than for the typical aquareovirus isolates. Of these bands, the 57 kDa band was present at highest intensity. Isolate 6 did not react with the rabbit anti-TSRV polyclonal antibody in all of the analyses. In addition some vague bands were visualised for isolate 1 and 4 with an estimated molecular weight of 60 kDa (Figure 5.4). The cell lines (uninfected CHSE-214, EPC & BF-2 cell cultures) which were included as negative controls did not demonstrate any detectable protein bands.

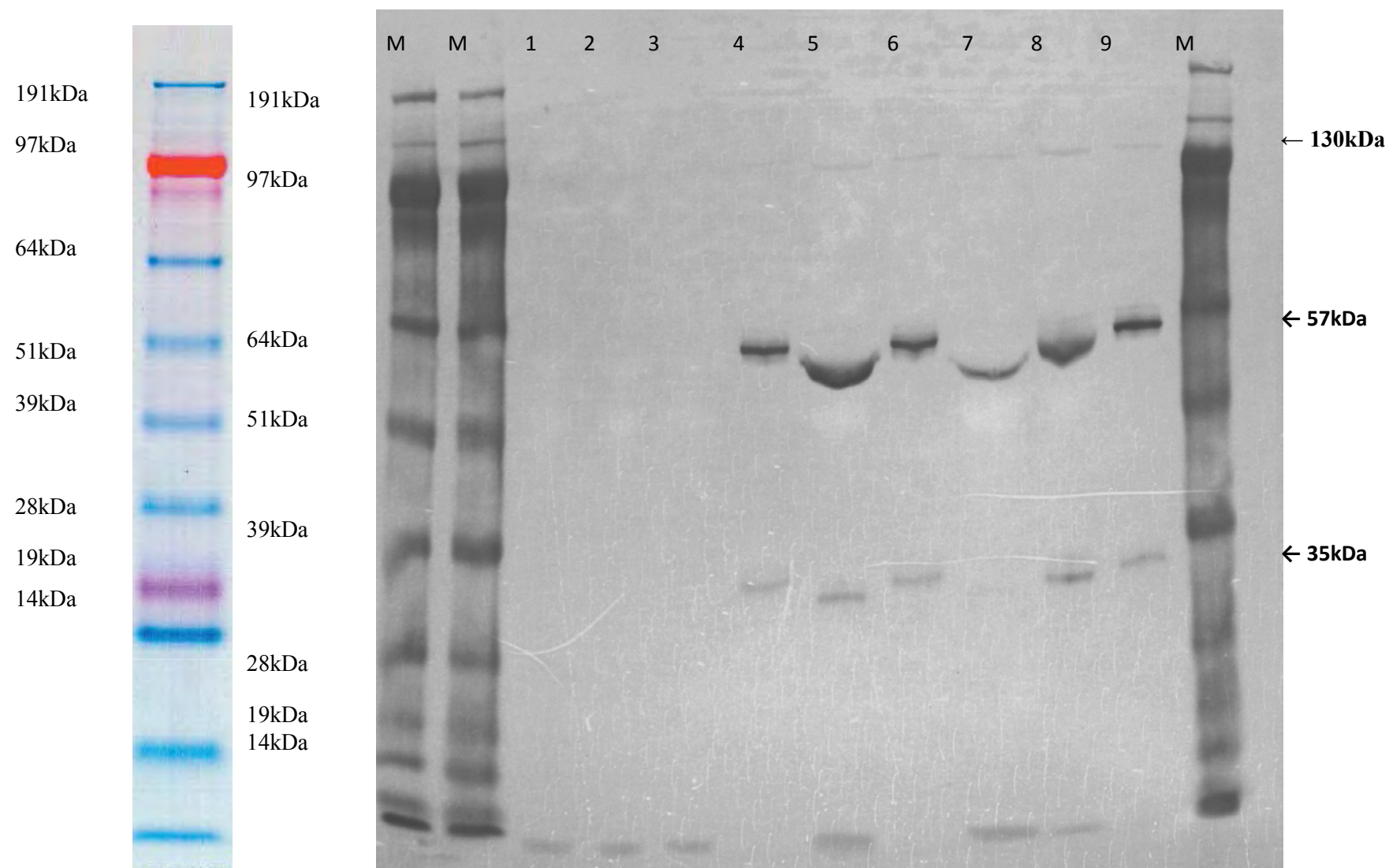


Figure 5.2 Western blot analysis of TSRV using rabbit antiserum against TSRV. Lane M: See Blue[®] Prestained Protein standard marker (Invitrogen), Lane 4-9: samples 14, 13, 12, 11, 10, 7 and Lane 1-3: cell lines as negative controls: BF-2, EPC and CHSE-214.

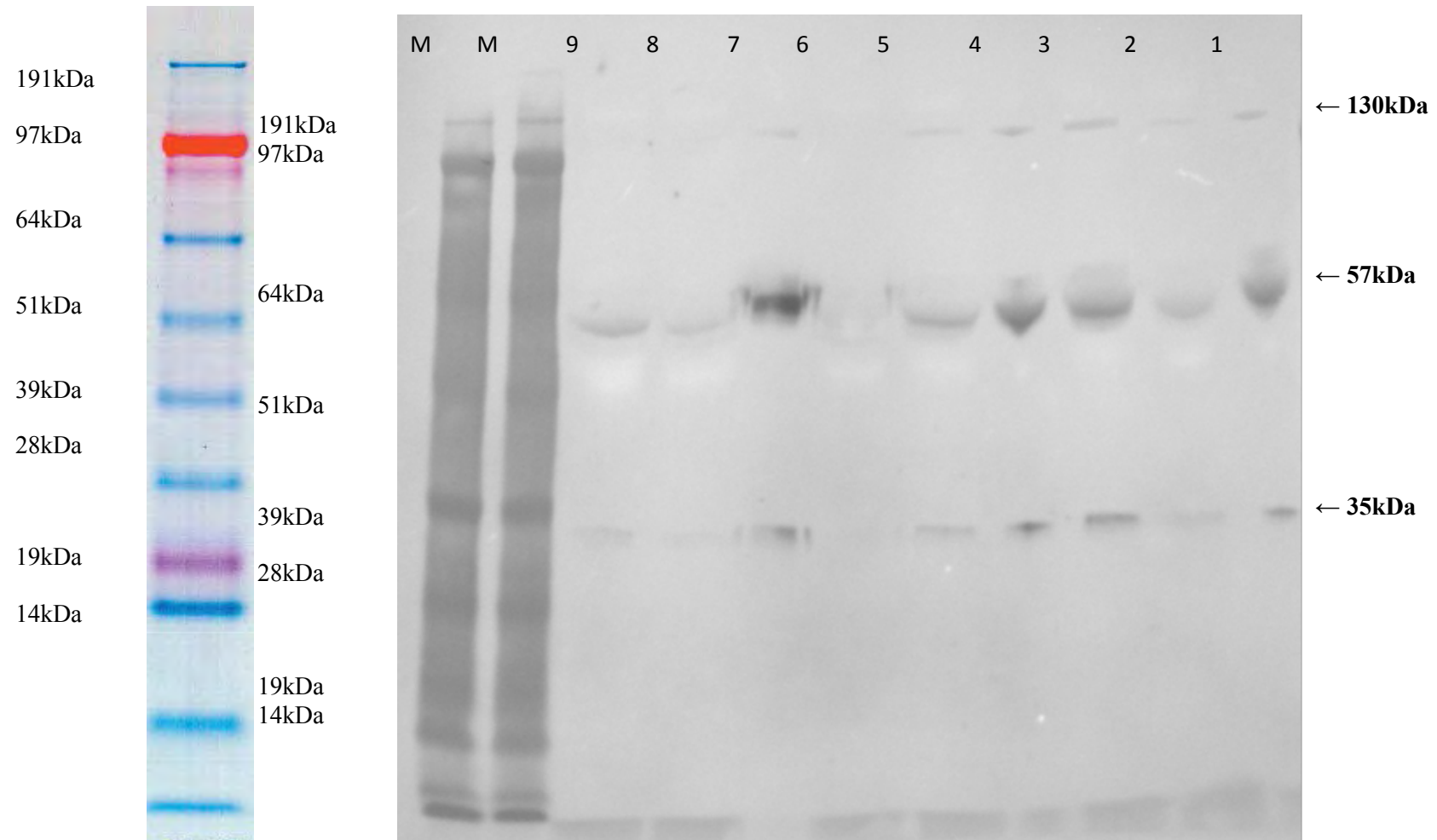


Figure 5.3 Western blot analysis of TSRV using rabbit antiserum against TSRV. Lane M: See Blue[®] Prestained Protein standard marker (Invitrogen) and isolates 1-9.

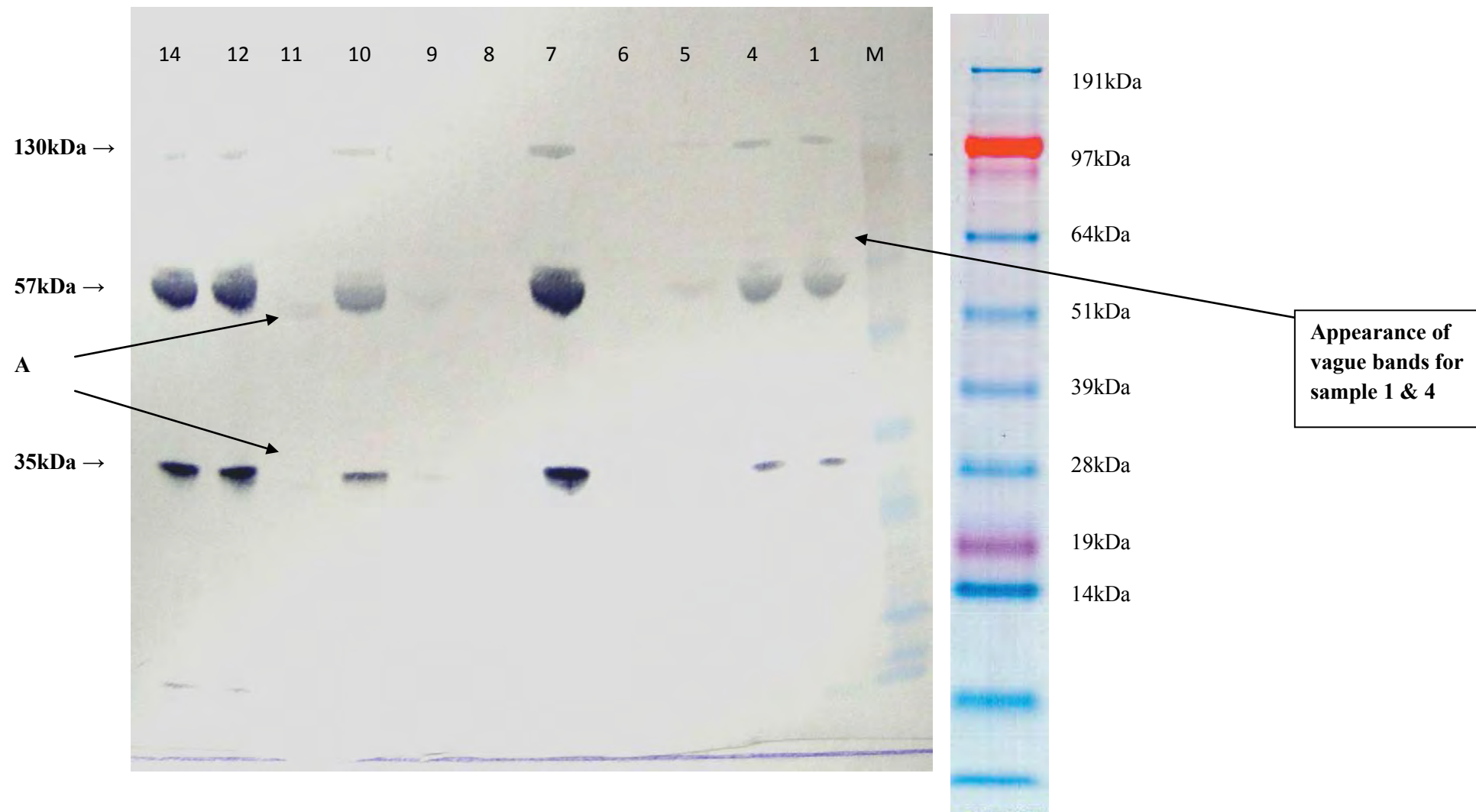


Figure 5.4: Western blot analysis of TSRV using rabbit antiserum against TSRV. M: See Blue[®] Prestained Protein standard marker (Invitrogen), Isolates 1-12 and 14. All the isolates had similar polypeptide profiles except a) isolate 6 which was negative (no staining) and b) **A**: isolate 11 which demonstrated a different polypeptide profile.

5.3.1.3 qPCR

The qPCR analyses demonstrated that all but two (11 and 13) of the isolates were positive for TSRV (Table 5.2). qPCR was conducted on all the isolates in duplicate and was repeated twice to confirm the negative results for isolates 11 and 13. Isolate 6 had a relatively high C_T value (approximately 26.34) which correlated with the negative reaction on Western blotting. The viral copy number for each isolate preparation (Table 5.2) was estimated using a standard curve generated by performing qPCR with a ten-fold dilution series of TSRV synthetic dsRNA as target (Table 5.3; Figure 5.5). C_T values were plotted against the calculated viral copy numbers with the initial copy number of 6.02×10^{10} molecules/ μ l. For each of the isolates, the relative viral concentration derived from the calculated copy number (molecules/ μ l) was consistent with the intensity of the viral protein bands on Western blotting.

Table 5.2 Mean C_T values, standard deviation (S.D) and viral copy numbers (molecules/ μ l) of TSRV isolates generated based on qPCR results which was conducted (twice) on all the isolates in duplicates. The viral copy numbers (molecules/ μ l) were derived from the inclusion of synthetic dsRNA as a positive control. ND: Non-detectable.

Isolates	Mean C_T values	S.D	Mean C_T values	S.D	Viral copy numbers (molecules/ μ l)
	First run		Second run		
1	17.17	0.06	17.77	0.01	6.02E+08
2	22.63	0.09	23.34	0.10	6.02E+06
3	21.72	0.18	21.72	0.18	6.02E+07
4	19.63	0.01	20.53	0.03	6.02E+07
5	18.95	0.05	19.72	0.01	6.02E+07
6	25.32	0.29	26.36	0.02	6.02E+05
7	15.45	0.02	16.35	0.08	6.02E+08
8	21.56	0.01	22.23	0.06	6.02E+07
9	20.37	0.65	21.44	0.01	6.02E+07
10	18.64	0.05	19.26	0.01	6.02E+07
11	ND	ND	ND	ND	ND
12	15.42	0.08	16.24	0.02	6.02E+08
13	ND	ND	ND	ND	ND
14	15.96	0.01	16.65	0.06	6.02E+08

Table 5.3 Mean C_T values, standard deviation (S.D) and viral copy numbers (molecules/ μ l) of ten-fold dilution of synthetic dsRNA. Synthetic dsRNA 10e-1 till 10e-10: ten-fold dilution of synthetic dsRNA, ND: Non-detectable.

Synthetic dsRNA	Mean C_T values	Viral copy numbers (molecules/ μ l)
Synthetic 10e-1	7.58	6.02E+10
Synthetic 10e-2	11.36	6.02E+09
Synthetic 10e-3	14.81	6.02E+08
Synthetic 10e-4	18.53	6.02E+07
Synthetic 10e-5	22.59	6.02E+06
Synthetic 10e-6	25.97	6.02E+05
Synthetic 10e-7	29.68	6.02E+04
Synthetic 10e-8	32.96	6.02E+03
Synthetic 10e-9	35.57	6.02E+02
Synthetic 10e-10	ND	6.00E+01

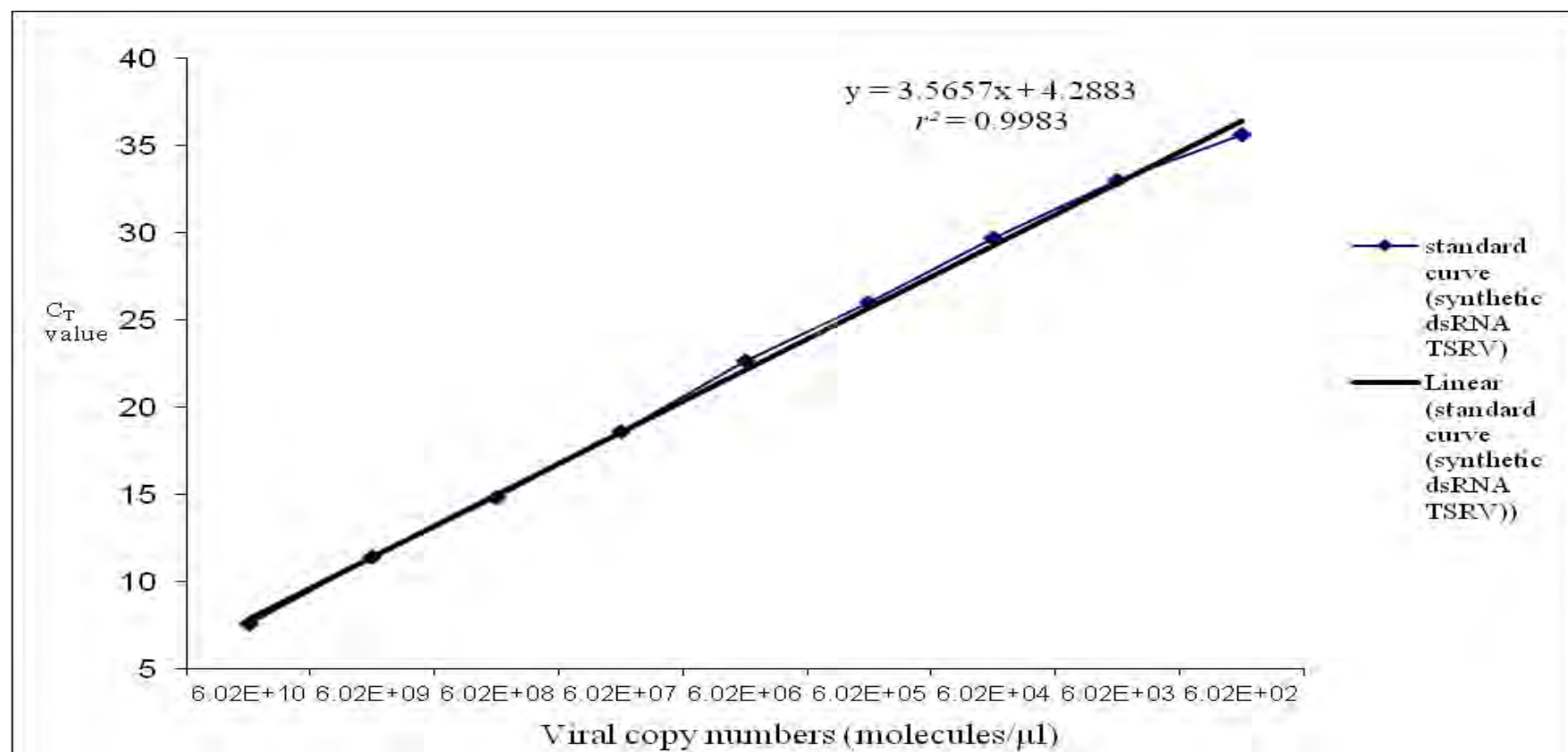


Figure 5.5 Standard curve generated for synthetic dsRNA TSRV to determine the TCID₅₀ (viral copy numbers) of isolates. C_T values plotted against calculated viral copy numbers (molecules/μl), with the initial copy numbers of 6.02×10^{10} molecules/μl.

5.3.1.4 Conventional hemi-nested RT-PCR

The conventional hemi-nested RT-PCR analysis produced amplicons of the expected size for both primary (280 bp) and secondary reactions (140 bp) (Figure 5.6). Bands were visualised for all but two (11 and 13) of the isolates. Isolate 6 showed visible bands in the secondary reaction only, consistent with the relatively high C_T value obtained for this isolate by qPCR (Table 5.2).

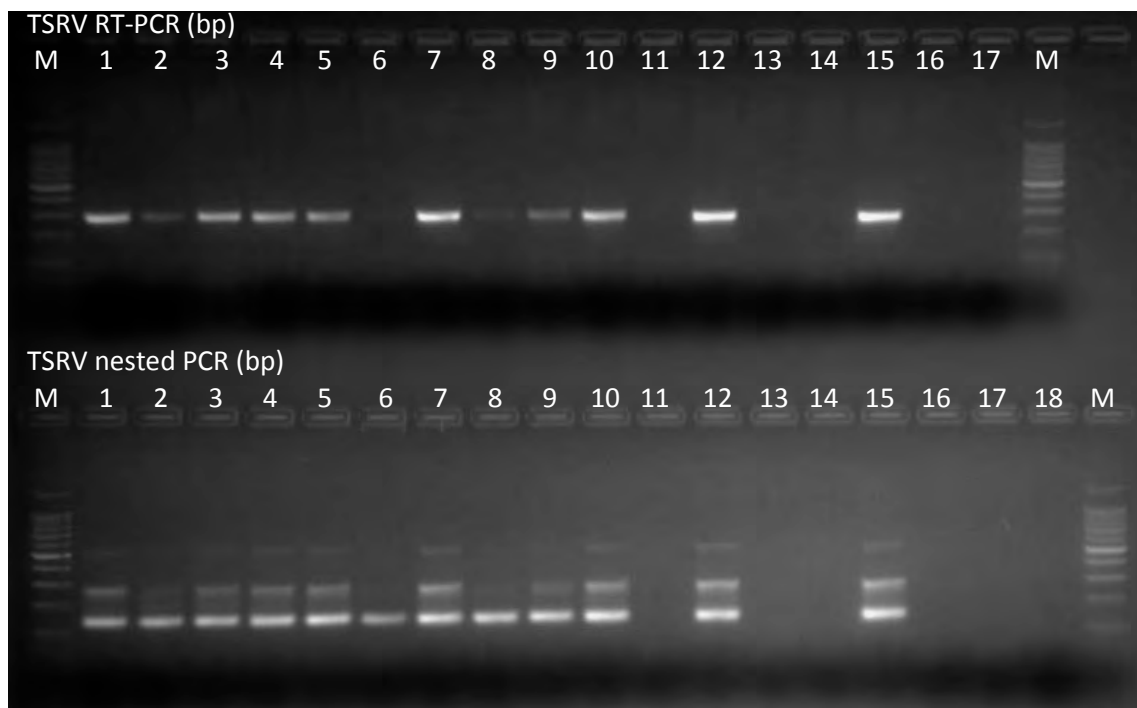


Figure 5.6 Conventional hemi-nested RT-PCR results for TSRV isolates. M: 100bp DNA ladder as a molecular weight marker, Lane 1-12: Isolate 1-12, Lane 13 & 14: Isolate 13, Lane 15: Isolate 14, Lane 16: CHSE-214 cells as negative control, Lane 17: Water as a negative control for primary reaction and Lane 18: Water as a negative control for secondary reaction.

Table 5.4 summarises the preliminary results for the characterisation of TSRV isolates based on the comparison of virus isolation, qPCR, western blot and conventional hemi-nested RT-PCR analyses. The development of cytopathic effect in cell cultures for each isolate correlated well with C_T values obtained by qPCR analysis and results by conventional hemi-nested RT-PCR. Cytopathic effects of more than 80% (at the time of harvest) resulted in low C_T values (= high viral copy number) and positive results in both reactions by conventional hemi-nested RT-PCR. Isolate 6 produced a negative reaction on western blotting and positive bands only in the secondary reaction on conventional hemi-nested RT-PCR which correlates with the low viral copy number (C_T value of 26.36). Despite the fact that isolate 11 and 13 produced 60% and 80% viral cytopathic effect on cell cultures, respectively, these isolates showed an atypical reaction on Western blotting and were non-detectable by qPCR and conventional hemi-nested RT-PCR.

Table 5.4 Summary of preliminary results for the characterisation of T SRV isolates and the description of virus isolation, qPCR, western blot and conventional hemi-nested RT-PCR analyses on all the isolates. Bolded isolates represent the atypical isolates in western blot and qPCR analysis. CPE: cytopathic effect on cell lines, dpi: days of post inoculation, RT-PCR: conventional hemi-nested RT-PCR.

Cell lines harvested				qPCR C _T values	Western blot	RT-PCR
Isolate #	Cell line	(Days of post-inoculation)	CPE			
1	CHSE-214	7dpi	80%	17.77	Positive	Positive (both reactions)
2	BF-2	12dpi	marginal	23.34	Positive	Positive (both reactions)
3	BF-2	12dpi	60%	21.72	Positive	Positive (both reactions)
4	CHSE-214	7dpi	100%	20.53	Positive	Positive (both reactions)
5	EPC	11dpi	100%	19.72	Positive	Positive (both reactions) Positive (2nd reaction only)
6	EPC	11dpi	60%	26.36	Negative	only)
7	CHSE-214	13dpi	100%	16.35	Positive	Positive (both reactions)
8	EPC	11dpi	80%	22.23	Positive	Positive (both reactions)
9	CHSE-214	16dpi	100%	21.44	Positive	Positive (both reactions)
10	EPC	11dpi	80%	19.26	Positive	Positive (both reactions)
11	EPC	11dpi	60%	ND	Atypical	Negative
12	CHSE-214	13dpi	100%	16.24	Positive	Positive (both reactions)
13	EPC	12dpi	80%	ND	Atypical	Negative
14	CHSE-214	13dpi	100%	16.65	Positive	Positive (both reactions)

5.3.1.5 Sequencing analysis

Sequences covering the 245 bp region of segment 10 of TSRV for the primary reaction were determined for all but two (11 and 13) of the isolates (Figure 5.7). Thus, only amplicons from isolate 1 to 10, 12 and 14 were excised for sequencing and phylogenetic analysis to verify any variation between the isolates. No variation was observed between the nucleotide sequences of the isolates. For the hemi-nested reaction, sequences covering 104 bp region of segment 10 of TSRV were determined for isolate 2, 6, 8 and 9. The alignment of these nucleotide sequences of the amplified PCR products confirmed that isolates were from the same lineage without any variations.

Consensus

1. TSRV-01_Consensus
2. TSRV-02_Consensus
3. TSRV-03_consensus
4. TSRV-04_consensus
5. TSRV-05_consensus
6. TSRV-06_consensus
7. TSRV-07_consensus
8. TSRV-08_consensus
9. TSRV-09_consensus
10. TSRV-10_consensus
11. TSRV-11_consensus
12. TSRV-12_consensus
13. TSRV-13_consensus
14. TSRV-14_consensus
15. TSRV-15_consensus

1 10 20 30 40 50 60
TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA
 TGTCAGGCGCCGGAAATCAGATGACTGAGGAAGCATTGTGCTGACATCAAGGCGACTATCAAGAA

Consensus

1. TSRV-01_Consensus
2. TSRV-02_Consensus
3. TSRV-03_consensus
4. TSRV-04_consensus
5. TSRV-05_consensus
6. TSRV-06_consensus
7. TSRV-07_consensus
8. TSRV-08_consensus
9. TSRV-09_consensus
10. TSRV-10_consensus
11. TSRV-11_consensus
12. TSRV-12_consensus
13. TSRV-13_consensus
14. TSRV-14_consensus
15. TSRV-15_consensus

70 80 90 100 110 120
GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT
 GGCCAGGGCCGGTAAGGTCAACAAGGTTATGAGTCTTGACCGCATTGTGCGGTGCCGTCAATTGTT

	130	140	150	160	170	180	190
Consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
1. TSRV-01_Consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
2. TSRV-02_Consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
3. TSRV-03_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
4. TSRV-04_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
5. TSRV-05_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
6. TSRV-06_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
7. TSRV-07_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
8. TSRV-08_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
9. TSRV-09_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
10. TSRV-10_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
11. TSRV-11_consensus	GATCGCGCTCTCACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
12. TSRV-12_consensus	-----ACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
13. TSRV-13_consensus	-----ACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
14. TSRV-14_consensus	-----ACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						
15. TSRV-15_consensus	-----ACTTTTCTATGGCCGGGACCTCGCCAGTCATCCATTGGTAACCGGGGCGACTT						

	200	210	220	230	240	245
Consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
1. TSRV-01_Consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
2. TSRV-02_Consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
3. TSRV-03_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
4. TSRV-04_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
5. TSRV-05_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
6. TSRV-06_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
7. TSRV-07_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
8. TSRV-08_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
9. TSRV-09_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
10. TSRV-10_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
11. TSRV-11_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
12. TSRV-12_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
13. TSRV-13_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
14. TSRV-14_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					
15. TSRV-15_consensus	CCTTCCGTACGGAGATTGAGGAGATCACCGGTGAGAAGTTGGCACGTGTCCAG					

Figure 5.7 Comparison of nucleotide sequence based on the alignment of Segment 10 of TSRV, SpT10Fb and SpT10Ra (primary reaction) for all the isolates (TSRV-01 to TSRV-11) except isolate 11 and 13. TSRV-12 to TSRV-15 represents the comparison of nucleotide sequence based on the alignment of Segment 10 of TSRV for the hemi-nested reaction, SpT10FN and SpT10Ra. No variation was observed between the nucleotide sequences of the isolates. TSRV-01_consensus to TSRV-05_consensus: Isolate 1 to 5, TSRV-06_consensus to TSRV-09_consensus: Isolate 7 to 10, TSRV-10_consensus: Isolate 12, TSRV-11_consensus: Isolate 14 and TSRV-12_consensus to TSRV-15_consensus: Isolate 2, 6, 8, & 9 (hemi-nested reaction).

5.3.2 Representative isolates of typical and atypical TSRV

Based on the preliminary analysis, representative isolates of “typical” (isolates 6, 7 and 10) and “atypical” (isolates 11 and 13) TSRV were selected for further analysis. These were regrown in CHSE-214 cell cultures, harvested and processed for Western blotting and PCR as described in methods section. Western blotting and PCR was repeated using the original preparations as well as the newly processed preparations to confirm preliminary results.

5.3.2.1 Western blotting

Although there was some variation between the isolates, Western blot analysis showed the presence of similar major protein bands in TSRV, approximately 130, 57 and 35 kDa, identified by the rabbit anti-TSRV polyclonal antibody (Figure 5.8). The three major groups of viral protein can be classified as large, medium and small. The presence of these bands were visualised in all of the isolates except the original preparations of isolates 6 and 11. Isolates 11 and 13 showed typical protein profile similar to the other isolates. Of these bands, the 57 kDa band was present at highest intensity. An additional band was present for the newly prepared isolates (7, 10, 11 and 13) which had an estimated molecular weight of 60 kDa.

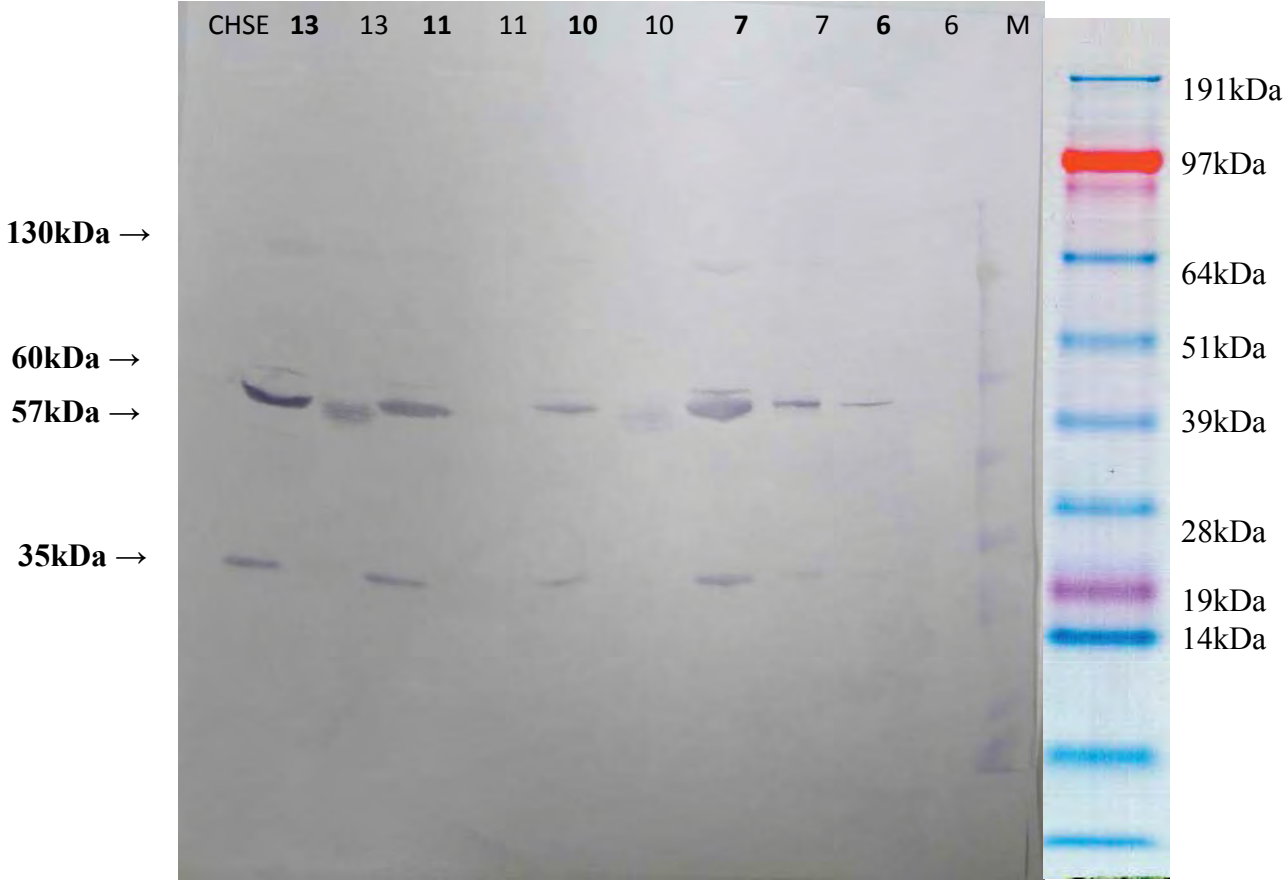


Figure 5.8 Western blot analysis of T SRV using rabbit antiserum against T SRV. M : See Blue® Prestained Protein standard marker (Invitrogen); Isolates 6, 7, 10, 11 and 13. Bolded isolates are newly regrown isolates derived from initial isolates used for preliminary study (see section 5.3.1). The rest of the isolates (not bolded) were the initial isolates used for preliminary study.

5.3.2.2 qPCR

The qPCR analyses demonstrated that all but two (11 and 13) of the isolates were positive for TSRV (Table 5.5), confirming the previous (preliminary) results. The original preparation of isolate 6 showed a high C_T value relative to the newly grown isolate 6.

Table 5.5 Mean C_T value and standard deviation (S.D) generated based on qPCR results; which was conducted (twice) on all the TSRV isolates in duplicates. Bolded isolates are regrown isolates derived from initial isolates used for preliminary study (see section 5.3.1). The remaining isolates (not bolded) were the initial isolates used for preliminary study. ND: Non-detectable.

Isolates	Mean C_T values	S.D
6	27.14	0.07
6	21.39	0.04
7	16.97	0.02
7	17.34	0.10
10	20.25	0.00
10	18.47	0.02
11	ND	ND
11	ND	ND
13	ND	ND
13	ND	ND

5.3.2.3 GARV Generic RT-PCR (Aquareovirus)

The conventional generic RT-PCR analysis produced amplicons of the expected size (340bp) and bands were visualised for all but two (11 and 13) of the isolates (Figure 5.9). In addition, it is noteworthy that the newly grown isolate 6 showed visible bands in contrast to the original preparation of isolate 6 used in the preliminary study. The new isolates were re-analysed using different volumes of template (2µL, 4 µL and 8 µL) for reconfirmation of the results (Figure 5.10). Bands were visible for isolate 6, 7 and 10 at template volumes of 4 and 8 µL. However, isolate 11 and 13 did not produce any amplicons despite increasing the volume of template for PCR.

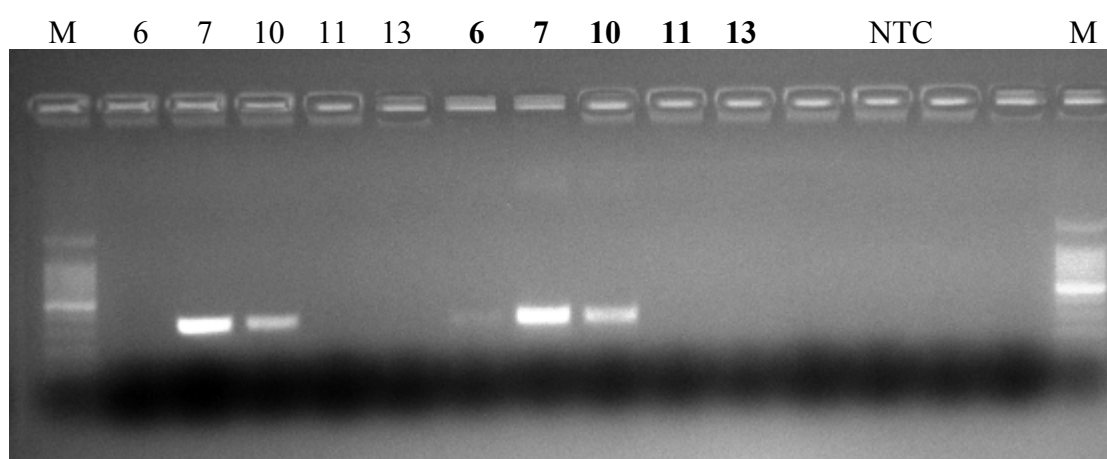


Figure 5.9 Conventional GARV generic RT-PCR results for TSRV isolates. M: 100bp DNA ladder as a molecular weight marker, 6, 7, 10, 11 & 13: TSRV isolates and NTC: No template control. Bolded isolates are regrown isolates derived from initial isolates (not bolded) used for preliminary study (see section 5.3.1).

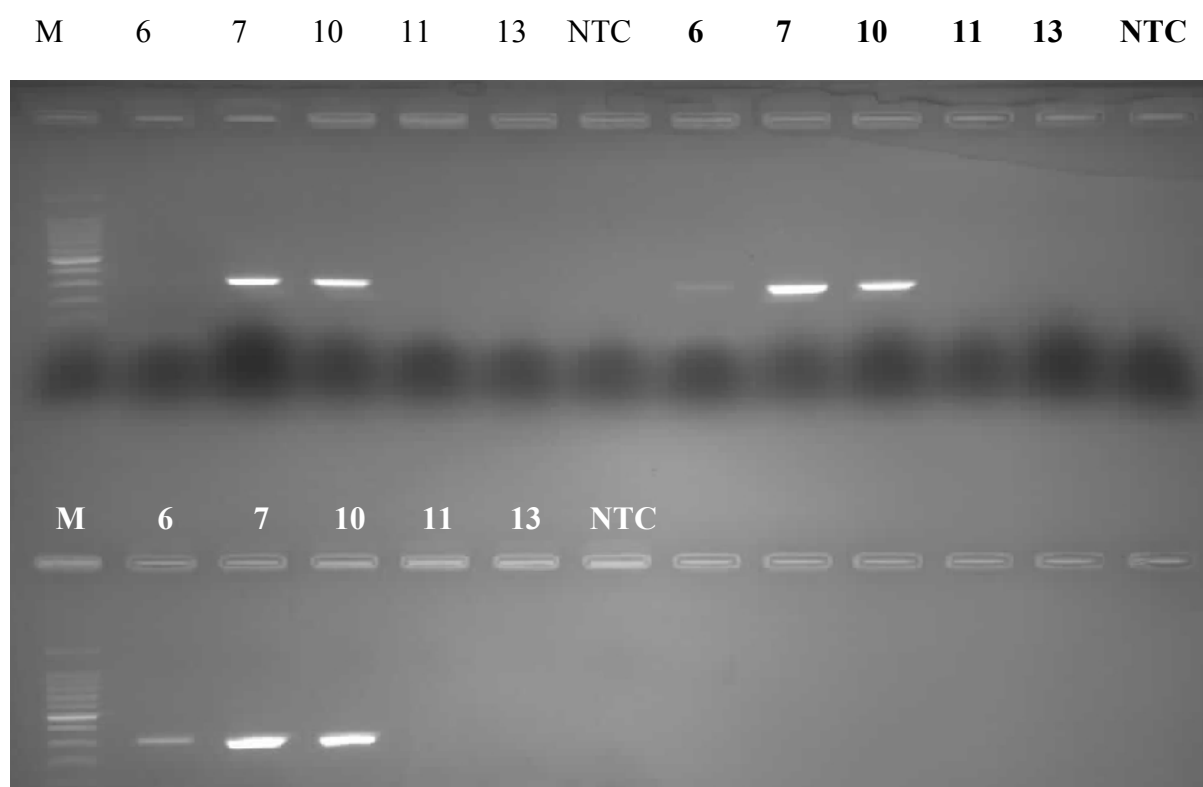


Figure 5.10 Conventional GARV generic RT-PCR results for T SRV re grown isolates. M: 100bp DNA ladder as a molecular weight marker, Row 1: Isolate 6, 7, 10, 11 & 13 (2 μ L of template), Row 1 (bolded isolates): 4 μ L of template, Row 2: 8 μ L of template and NTC: No template control.

5.3.2.4 Segment 10 TSRV T10 conventional RT-PCR

The T10 RT-PCR produced amplicons of the expected size of 263 bp for isolate 6, 7 and 10 in duplicate (Figure 5.11). Isolate 11 produced an amplicon of approximately 125 bp in size. Isolate 13 showed two faint bands with estimated sizes of approximately 263 bp and 125 bp.

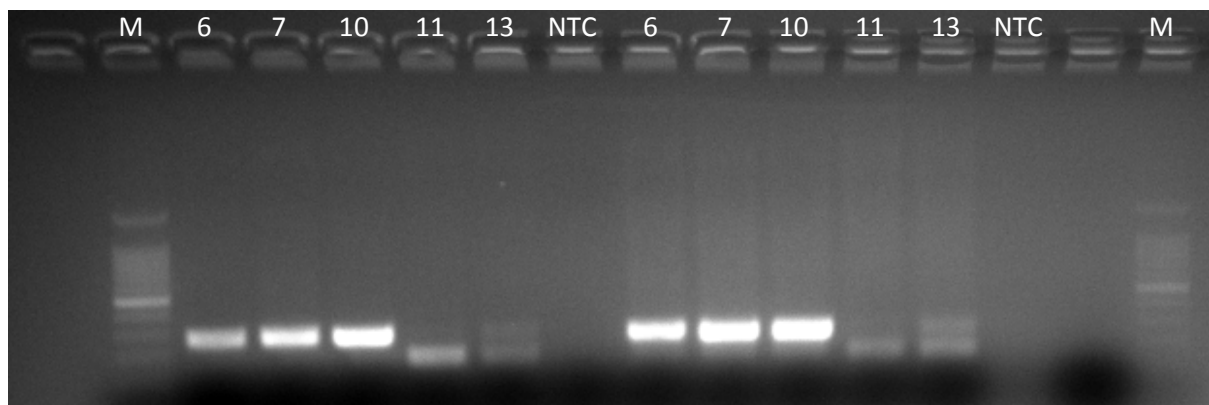


Figure 5.11 Conventional RT-PCR results for segment 10 of TSRV regrown isolates M: 100bp DNA ladder as a molecular weight marker, 6, 7, 10, 11 & 13: TSRV isolates in duplicates and NTC: No template control.

5.3.2.5 Sequence and phylogenetic analysis

Sequences covering the 263 bp region of segment 10 of TSRV were determined for all the isolates. However, isolate 11 and 13 produced bands of different molecular weights compared to isolate 6, 7 and 10. Thus, only amplicons from isolate 10 (as an example of typical TSRV isolate) and isolate 11 (as an example of atypical TSRV isolate) were excised for sequencing and phylogenetic analysis to verify any variation between the two isolates. Alignment of nucleotide sequences of the amplified PCR products confirmed that both isolates were from the same lineage with minor variations among the typical and atypical isolates. As shown in Figure 5.12, a typical isolate 11 showed variation in sequences of at least 30 nucleotides compared to the typical isolate 10.

BLASTN search (Altschul *et al.*, 1997) of 125 bp sequence of typical and atypical TSRV were compared with complete sequence of segment 10 (TSRV), EF434979, and other representative isolates from GenBank accession (Table 5.6). EF434979 is a direct deposit of Segment 10 of TSRV by Carlile (2011). Comparison of TSRV typical isolate revealed 100% nucleotide sequence identity to EF434979 and shared 85-98% to the other representative isolates. BLASTN search of the TSRV typical isolate also showed high sequence identity with the major outer capsid protein (VP7) of ASV (Canadian isolate, 2009) and SBRV.

In contrast, the atypical TSRV isolate only shared 96% nucleotide sequence identity to complete sequence of segment 10 of TSRV. Atypical TSRV isolate shared 96% nucleotide sequence identity to CSRV and 87% nucleotide sequence identity to the other representative isolates. This analysis showed that typical isolate 10 was more closely related to EF434979

and the other representative isolates than a typical isolate 11 with the exception of turbot reovirus. Both analyses demonstrated that isolate 11 is a variant TSRV.

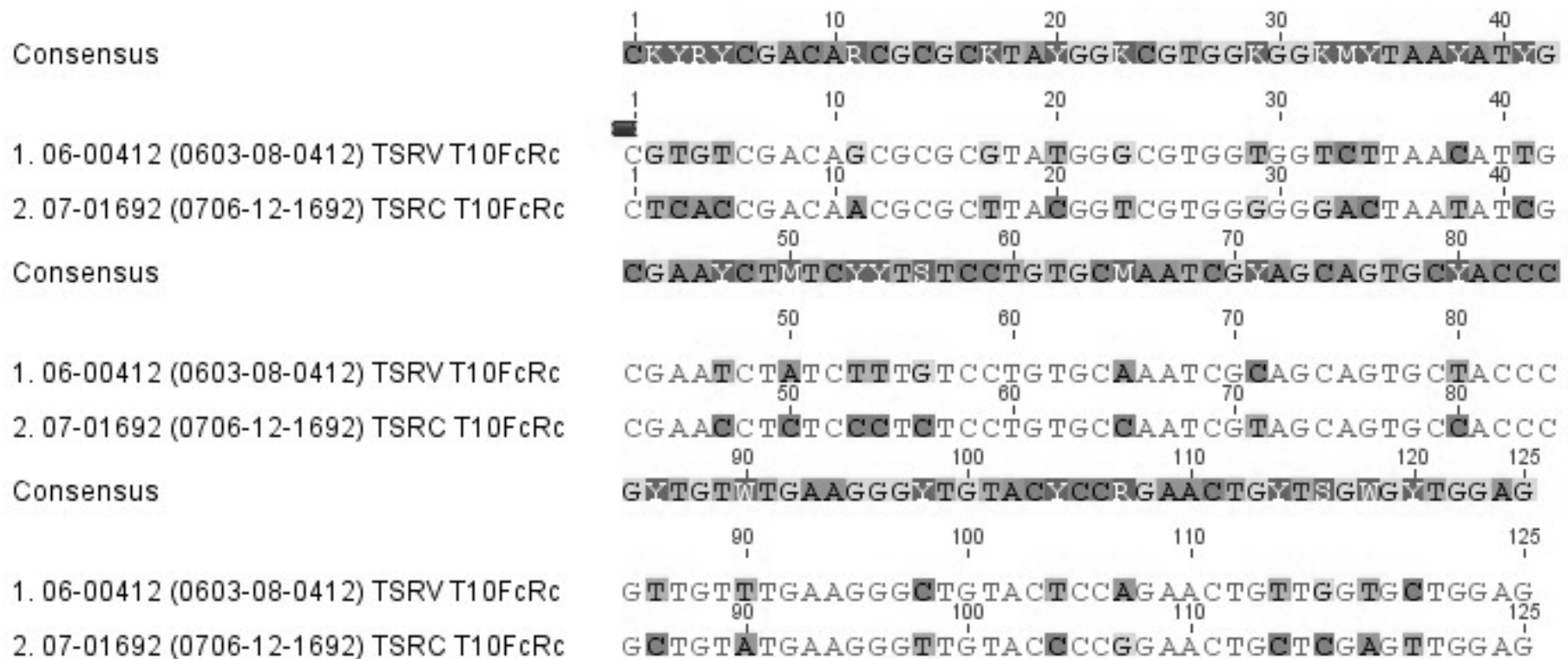


Figure 5.12 Comparison of nucleotide sequence based on the alignment of Segment 10 of TSRV, T10Fc and T10Rc for isolate 10 (06-00412) and isolate 11 (07-01692). Variation between the nucleotide sequences of the isolates are indicated with shading.

Table 5.6 Percent nucleotide sequence identities of the “typical” TSRV isolate 10, “atypical” TSRV isolate 11 and representative isolates from GenBank accession. Comparison of TSRV isolates were based on the 125 bp sequence using the T10Fc/T10Rc primer set. EF434979 is a direct deposit of Segment 10 of TSRV by Carlile (2011).

GenBank accession	Description of sequences producing significant alignments:	Isolate 10 Typical TSRV	Isolate 11 Atypical TSRV
EF434979.1	Tasmanian Atlantic salmon reovirus TSRV segment 10, complete sequence	100%	96%
AF418303.1	Chum salmon reovirus CSV segment 10, complete sequence	98%	95%
AY236219.2	Threadfin reovirus TFRV segment 10, complete sequence	88%	87%
FJ652576.1	Atlantic salmon reovirus ASV Canada - 2009, outer capsid protein (VP7) gene, complete sequence	92%	87%
AF450322.1	Striped bass reovirus SBV segment 10, outer capsid protein (VP7) gene, complete sequence	92%	87%
U83396.1	Striped bass reovirus SBV major outer capsid glycoprotein (VP7) mRNA, complete sequence	92%	87%
HM989939.1	<i>Scophthalmus maximus</i> , turbot reovirus TRV segment S10, complete sequence	85%	87%

5.3.2.6 Immunocytochemistry

Both typical and atypical isolates demonstrated positive reaction by immunocytochemistry using rabbit anti-TSRV polyclonal antibody, displaying grainy, focal and brick-red staining in infected cells indicating the presence of TSRV. Immunocytochemical staining of typical isolate 10 demonstrated intense staining of plaques (Figure 5.13) with most, if not all, plaques exhibiting a strong reaction. In contrast, cultures infected with atypical isolate 11 demonstrated less intense staining as well as plaques without staining (Figure 5.14 A and B), which correlates with the Western blot results.

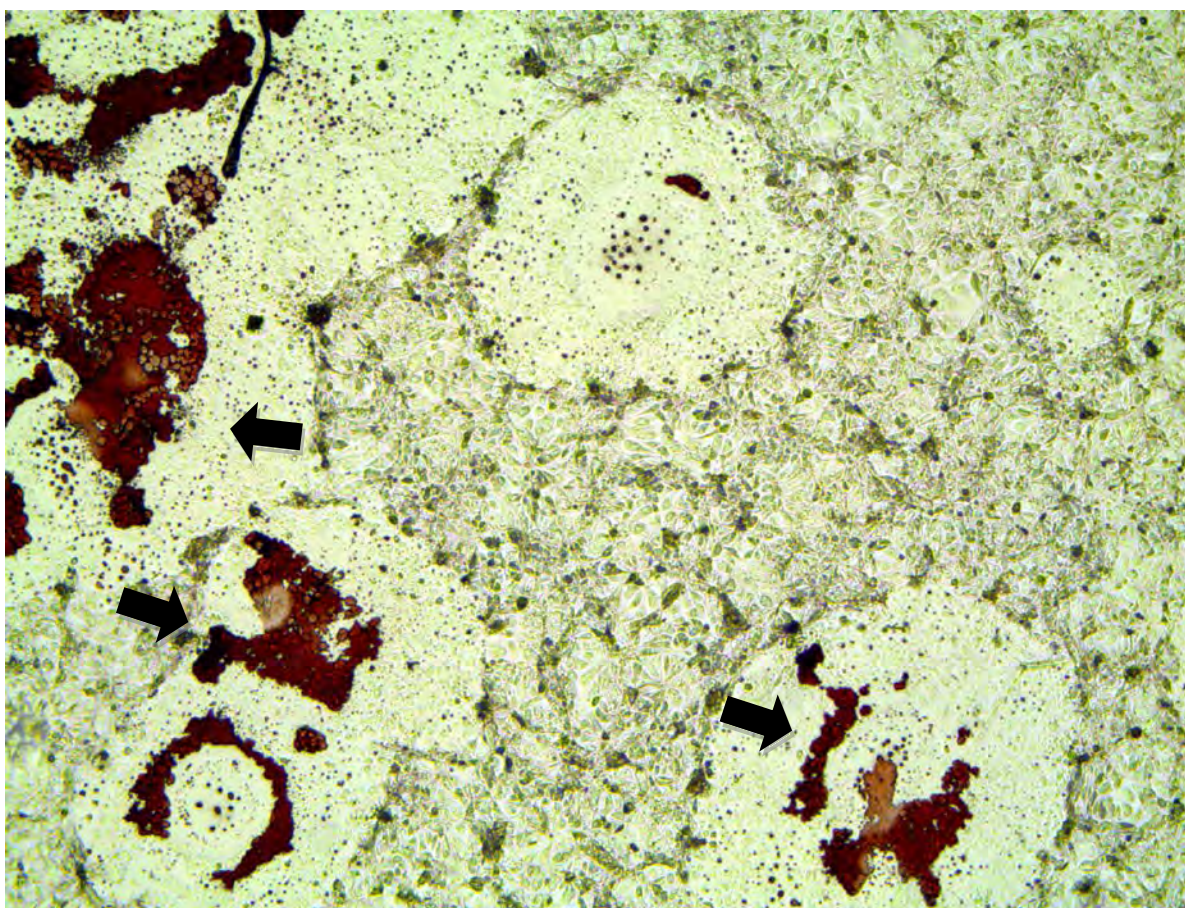


Figure 5.13 Immunocytochemical staining of “typical” isolate 10 of TSRV from Atlantic salmon. A positive reaction was observed as grainy, focal, brick-red staining in cytopathic effects produced in cell cultures (arrow).

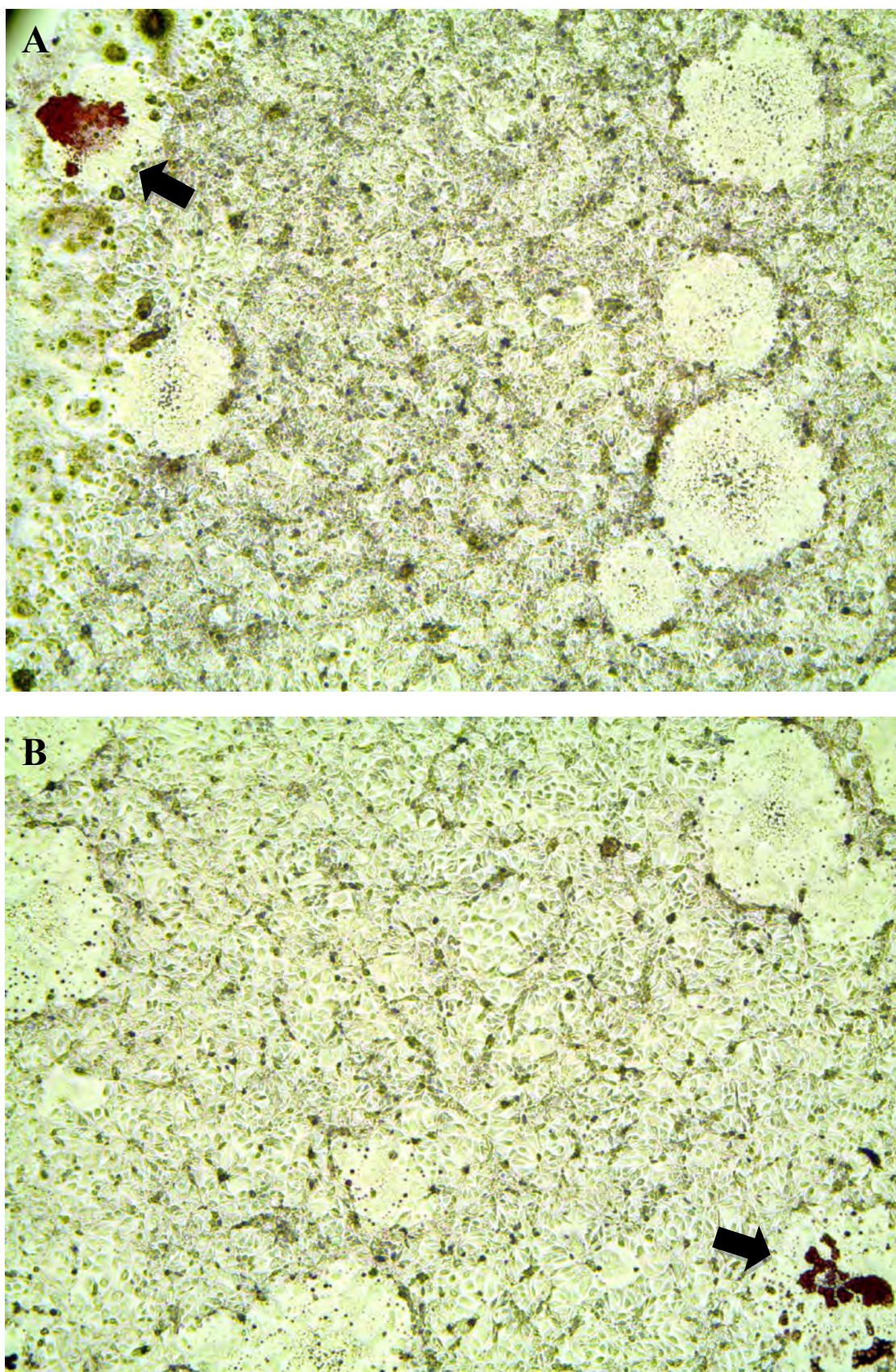


Figure 5.14 A & B Immunocytochemical staining of “atypical” isolate 11 of TSRV from Atlantic salmon. A positive reaction was observed as grainy, focal, brick-red staining in cytopathic effects produced in cell cultures (arrow).

5.3.2.7 Electron microscopy

The negatively stained virions and ultrathin sections of typical isolate 10 and atypical isolate 11 demonstrated that they belonged to the family Reoviridae (Table 5.7 and 5.8). In negatively stained preparations, spherical particles, approximately 50 - 70 nm in diameter, with a double capsid shell were observed (Figure 5.15-5.16). A prominent white ring between the outer capsid and inner core was evident in negatively stained electron micrographs of the isolates. Two populations of TSRV were observed in tissue culture supernatant of ‘atypical’ isolate 11 which differed in size (Figure 5.16). Whereas, no virus was observed in tissue culture supernatant of ‘atypical’ isolate 13 submitted for negative contrast electron microscopic examination (NCEM) (Table 5.7). The ultrathin sections of ‘atypical’ isolate 11 demonstrated a single array of virus particle, putative of family Reoviridae (Table 5.8 and Figure 5.17). Ultrathin sections of ‘atypical’ isolate 13 showed some ultra-structural changes, which indicated early stages of viral morphogenesis, but the identification of the isolate is still indeterminate (Table 5.8).

Table 5.7 Test result of electron microscopy for negatively-stained (NCEM) ‘typical’ TSRV isolate 10, ‘atypical’ TSRV isolate 11 and ‘atypical’ TSRV isolate 13. Virus pellet of ‘atypical’ isolate 11 was resuspended in PBSA and had been frozen before EM examination. Tissue culture supernatants were derived from CHSE-214 cells inoculated with isolate 10 and 13.

Isolates	Test result
Virus pellet 'atypical' TSRV (Isolate 11)	Virus belonging to the family Reoviridae observed. Two populations of particles observed. (Figure 5.16) Population one is larger in size than population two.
Tissue culture supernatant of 'typical' TSRV (Isolate 10)	Virus belonging to the family Reoviridae observed. (Figure 5.15)
Tissue culture supernatant of 'typical' TSRV (Isolate 13)	No virus observed.

Table 5.8 Test result of examination of ultrathin sections originating from infected CHSE-214 cells inoculated with ‘typical’ TSRV isolate 10, ‘atypical’ TSRV isolate 11 and ‘atypical’ TSRV isolate 13.

Isolates (cell pellet)	Test result (Thin sections - Spurr's)
‘Typical’ TSRV (Isolate 10)	Virus belonging to the family Reoviridae observed.
‘Atypical’ TSRV (Isolate 11)	A single array of virus particles observed, putative family Reoviridae. (Figure 5.17)
‘Atypical’ TSRV (Isolate 13)	Indeterminate. Some slight ultra structural changes were observed, which may be the early stages of viral morphogenesis.

Population 1 of ‘atypical’ isolate 11 is larger in size at approximately 74 nm in diameter, compared to population 2 (53 nm) (Table 5.9 and Figure 5.16). Typical isolate 10 was smaller in size than population 1 (atypical isolate 11), but larger in size than population 2, at approximately, 62 nm (Table 5.9 and Figure 5.15). The core inner diameter for the isolates varied from 39 to 49 nm.

Table 5.9 Whole outer diameter and core inner diameter for ‘typical’ TSRV isolate 10, ‘atypical’ TSRV isolate 11 and ‘atypical’ TSRV isolate 13 based on examination of ultrathin sections originating from infected CHSE-214 cells. A typical isolate 11 contained two populations of TSRV based on differences in size.

Isolates	Whole outer diameter ± standard deviation (nm)	Core inner diameter ± standard deviation (nm)
Virus pellet 'atypical' TSRV (Isolate 11, Population 1)	74 ± 3.5 (n=24)	49 ± 2.8 (n=24)
Virus pellet 'atypical' TSRV (Isolate 11, Population 2)	53 ± 2.8 (n=6)	39 ± 0.9 (n=3)
CHSE cells inoculated with 'typical' TSRV (Isolate 10)	62 ± 2.7 (n=12)	44 ± 2.3 (n=12)

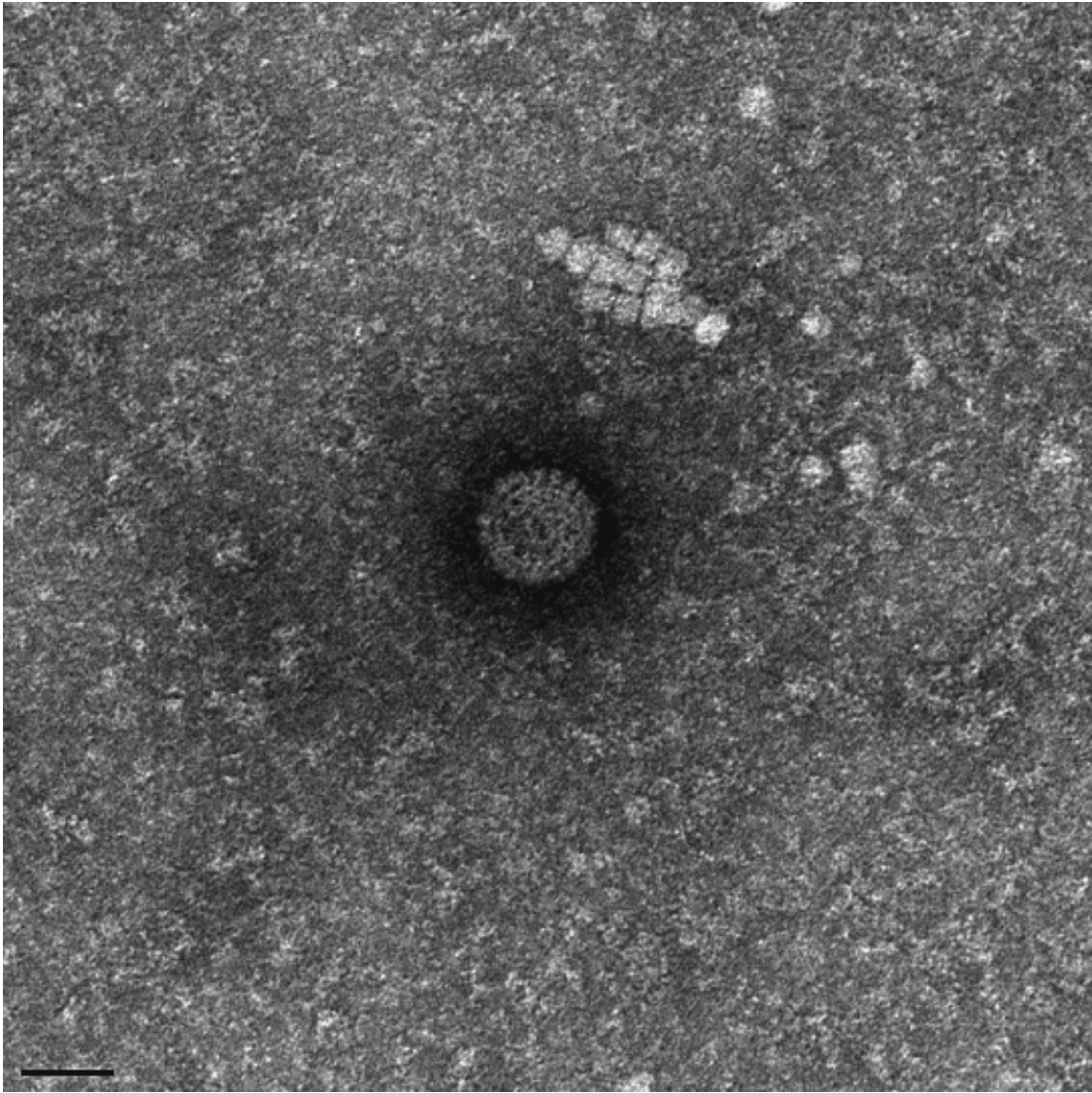


Figure 5.15 Negative contrast EM. Electron micrograph of negatively-stained TSRV ‘typical’ virions (Isolate 10), inoculated in CHSE-214 cells. The virion is approximately 62 nm in diameter. Bar marker represents 50 nm. Courtesy of Australian Microscopy & Microanalysis Research Facility, Australian Animal Health, CSIRO Livestock Industries, Geelong, VIC, Australia.

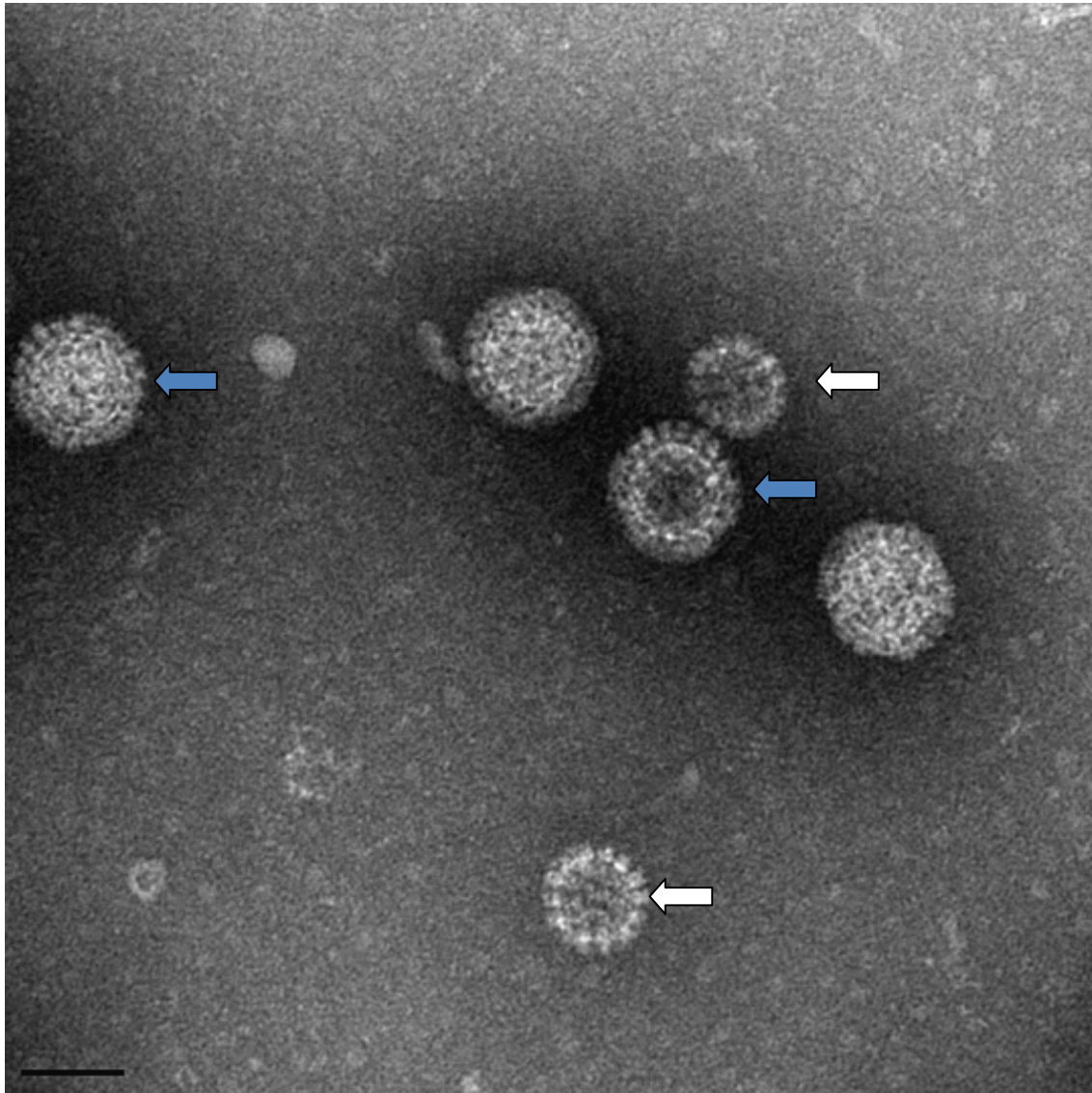


Figure 5.16 Negative contrast EM. Electron micrograph of negatively-stained of the TSRV 'atypical' virions (Isolate 11), inoculated in CHSE-214 cells. The presence of a double capsid protein shell can be seen from the image. Isolate 11 demonstrated two populations of TSRV based on size differences, population 1 (blue arrows) and population 2 (white arrows). The virions are approximately 74 nm and 53 nm in diameter, respectively. Bar marker represents 50 nm. Courtesy of Australian Microscopy & Microanalysis Research Facility, Australian Animal Health, CSIRO Livestock Industries, Geelong, VIC, Australia.

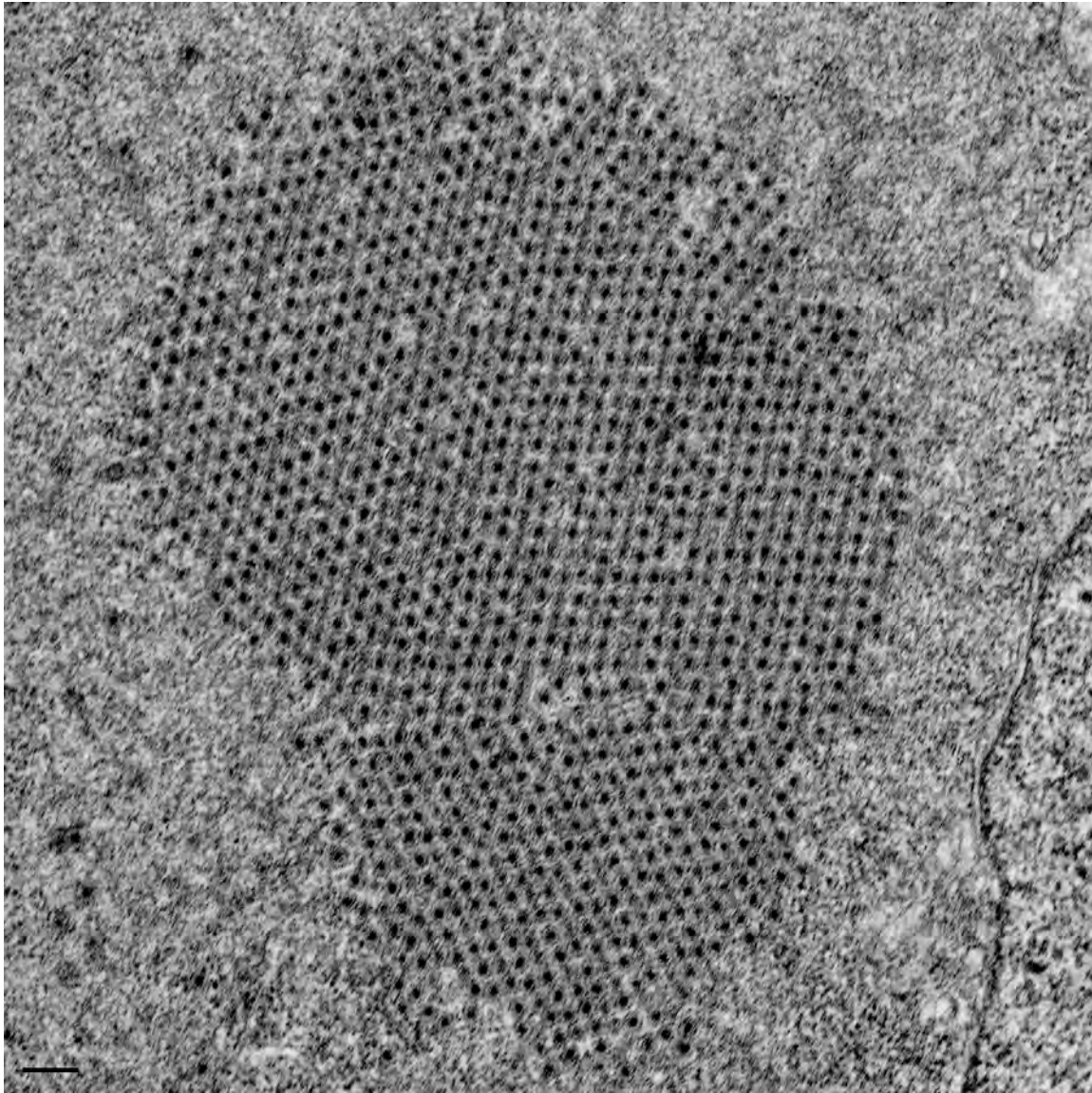


Figure 5.17 Electron micrograph of a single array of 'atypical' TSRV particles inoculated in CHSE-214 cells. Bar marker represents 200 nm. Courtesy of Australian Microscopy & Microanalysis Research Facility, Australian Animal Health, CSIRO Livestock Industries, Geelong, VIC, Australia.

5.4 Discussion

In this chapter, typical and atypical variants of TSRV were identified based on genotypic and phenotypic characterisation of the different isolates. Electron microscopy examination demonstrated the existence of at least three variants based on size of the viral particles. Alignment of nucleotide sequences of the amplified PCR products confirmed that typical and atypical isolates were from the same lineage with minor variations. A typical TSRV isolate shared 96% nucleotide sequence identity to complete sequence of segment 10 of TSRV, 95% to CSRV and 87% nucleotide sequence identity to the other representative isolates. Typical TSRV isolate (100% sequence homology to complete sequence of segment 10 of TSRV) originated from wild fish, Jack mackerel. Interestingly, one of the atypical isolates was found to be originated from brood-stock and freshwater.

The differences between typical and atypical TSRV were observed in all of the analyses (Table 5.10), genotypically; GARV Generic RT-PCR, conventional hemi-nested RT-PCR, qPCR, Segment 10 TSRV T10 conventional RT-PCR, sequencing analysis, and phenotypically; immunocytochemistry, EM analysis, SDS-PAGE and Western blot analysis. Atypical isolates were undetectable by GARV Generic RT-PCR, the primers for which were designed based on a conserved region of S2 located within the polymerase gene, which were very specific to a small region of genome in Aquareovirus. The S2 primers were unable to detect atypical TSRV due to variation in nucleotide sequences. In this study, PCR tests were used to obtain amplicons for sequence analysis so that the typical and atypical could be compared. The atypical isolates were undetectable using the conventional hemi-nested RT-PCR and qPCR due to the variation in nucleotide sequences. Atypical isolates were detectable by TSRV Segment 10 primers FcRcc (263bp) because these primers include the

binding sites for both qPCR primers and conventional hemi-nested RT-PCR. In contrast to molecular methods, atypical isolates were detected by both Western blot analysis and immunocytochemistry. Similarly, isolates of VNN in sea bass, *Dicentrarchus labrax*, were undetectable by PCR-based diagnostic method but demonstrated a positive reaction using rabbit polyclonal antibody, raised against purified virus, by immunohistochemistry (Thiery *et al.*, 1999). Several epitopes of the nodavirus capsid protein were recognized by the polyclonal antibody, whereas primers used for PCR are highly specific for a small portion of genome. Likewise, similar hypotheses could explain the differences between the PCR results and Western blot analysis obtained for atypical isolates of TSRV in this study.

Phenotypic differences were observed between typical and atypical isolates by EM analysis, Western blot analysis and immunocytochemistry. EM analysis revealed that typical and atypical isolates contained icosahedral, non-enveloped 50-70 nm diameter virus particles. The presence of a double capsid layer and a prominent white ring between the outer capsid and inner core suggest that the atypical isolate belongs to the family Reoviridae (Francki *et al.*, 1991), similar to typical isolate. These structural observations are consistent with published molecular characterization of genus *Aquareovirus* (Lupiani *et al.*, 1995; Shaw *et al.*, 1996). The aquareovirus virions are spherical in appearance, approximately 60-80 nm in diameter, with two icosahedrally distinct shells enclosing an ordered RNA layer (Lupiani *et al.*, 1995; Shaw *et al.*, 1996). EM analysis demonstrated that the atypical isolate 11 contained two different TSRV populations based on size. Further characterization of the two variants needs to be resolved by plaque-purification, thus, the nucleotide sequence of each sub-population, origin and pathogenicity are still unknown.

Table 5.10 Summary of genotypic and phenotypic differences between typical and atypical TSRV isolates based on the different analyses conducted in this study. EF434979 is a direct deposit of Segment 10 of TSRV by Carlile (2011). NA: Not available.

Analyses		Typical TSRV Isolate 10	Atypical TSRV Isolate 11	Isolate 13
Genotypic difference	Conventional hemi-nested RT-PCR	Positive: 140 bp	Negative	Negative
	GARV Generic RT-PCR	Positive: 340 bp	Negative	Negative
	TSRV T10 conventional RT-PCR	Positive: 263 bp	Positive: 125 bp	Positive: 263 bp & 125 bp
	qPCR Sequencing	Positive 100% sequence match to EF434979	Negative 96% sequence match to EF434979	Negative NA
Phenotypic difference	Virus isolation	80 - 100% CPE	60% CPE	80% CPE
	Western blot	Typical viral proteins bands	Atypical proteins bands	Atypical proteins bands
	Immunocytochemistry	Intense staining	Moderate staining	NA
	Electron microscopy (Whole outer diameter)	62 nm	Population 1 : 74 nm Population 2 : 53 nm	NA

SDS-PAGE of the virion proteins revealed putative viral polypeptides at approximately 130, 127, 116.3, 29, 14, 13.9 and 6.5 kDa in size; observed in TSRV samples only. As purification of the virus isolates was not carried out, the polyacrylamide gel electrophoresis showed more than 11 viral polypeptides, the others being derived from cellular and culture medium (serum) components. As the focus of this study was to increase the probability of identifying variant isolates, further work on characterisation of viral proteins was not pursued since this would require plaque-purified virus being obtained which was beyond the scope of this thesis. However, it was possible to identify TSRV viral proteins based on published literature for aquareoviruses (Lupiani *et al.*, 1993; Winton *et al.*, 1987; Subramanian *et al.*, 1994; Fauquet *et al.*, 2005; Chen *et al.*, 2011). The electropherotypes of 19 different aquareoviruses, isolated from all over the world were compared by SDS-PAGE (Lupiani *et al.*, 1993). This study revealed distinct variations of electrophoretic profiles of the isolates including ASV (Atlantic salmon reovirus) and CSV (Chum salmon reovirus). The electrophoretic profiles of TSRV were consistent with the published profiles of ASV and CSV. Twelve proteins, with molecular weight of 130, 127, 126, 97, 73, 71, 46, 39, 35, 29, 28 and 15 kDa were present in infected cells of SBRV (Subramanian *et al.*, 1994). Electrophoresis of purified TFRV revealed 11 segments of dsRNA and 5 major structural polypeptides of approximately 136, 132, 71, 41, and 33 kDa (Seng *et al.*, 2002). SDS-PAGE analysis of GCRV virion proteins identified seven structural protein components with molecular weight at about 138, 137, 136, 79, 67, 43 and 34 kDa, respectively.

Isolate 11 and 13 showed atypical protein bands in Western blot analysis compared to typical isolates which is in agreement with reported literature. While SDS-PAGE of TSRV virion proteins is of limited value because of the unavailability of purified virus isolates, Western blot analysis provided the ability to localize virus-specific proteins. Western blot analysis of TSRV isolates demonstrated three major protein bands in comparison to five major protein bands identified in other aquareoviruses, demonstrating the limited specificity of the antiserum used and maybe due to the conformational nature of epitopes in these proteins (Lupiani *et al.*, 1997; Kibenge *et al.*, 2000; Seng *et al.*, 2002). The bands in the Western blot of around 130, 57 and 35 kDa are consistent with the size of viral proteins previously reported in TFRV: 132-136, 41 and 33 kDa (Seng *et al.*, 2002); GSRV: 130 and 35 kDa (Fauquet *et al.*, 2005); SBRV: 130 and 34 kDa (Subramanian *et al.*, 1994; Fauquet *et al.*, 2005).

Immunocytochemistry test showed intense staining and production of more numerous plaques for typical isolates than atypical isolate. Unlike the typical isolate, the atypical isolate did not produce 100% CPE in cell culture. Phenotypic differences have been found among ISAV strains, a ssRNA virus pathogen in Atlantic salmon (Kibenge *et al.*, 2000) and IPNV strains (dsRNA virus) (McDonald and Gower, 1981; John and Richards, 1999). Thirteen isolates of ISAV (11 from Canada, one from Norway and one from Scotland) were studied for their replication in the CHSE-214 cell line compared with that in the SHK-1 cell line (Kibenge *et al.*, 2000). This study indicated that there were differences between ISAV isolates (genotypically different) with respect to their ability to replicate in CHSE-214 cells, similar to CPE differences observed between TSRV typical and atypical isolates.

Phylogenetic analysis showed that the typical and atypical TSRV isolate were from the same lineage with minor variations. However, as the sequence identities of the two different populations in a typical isolates are still unknown, it is difficult to conclude the origin of the isolates. The presence of three populations indicates that both typical and atypical isolates may have evolved from a common ancestor a long time ago rather than one evolving from the other. The isolation of VHSV, a RNA virus in *Oncorhynchus mykiss*, at new geographical locations and in new hosts have been reported to reflect either the spread of the virus to new ecological niches or the existence of viral populations that have not previously been discovered (Einer-Jensen *et al.*, 2005). Likewise, the discovery of at least three populations of TSRV could reflect the existence of viral populations that have not previously been discovered due to the sensitivity and specificity of the current detection method. Various authors have reported existence of sequence differences between Aquareovirus isolates (Dopazo *et al.*, 1992; Lupiani *et al.*, 1993; McEntire *et al.*, 2003). Similarly, sequence divergence have been demonstrated for other RNA viruses in fish; ISAV in *Salmo salar* (Blake *et al.*, 1999; Cunningham and Snow, 2000), IHNV in salmonids (Garver *et al.*, 2003), Aquabirnavirus in wild fish (Romero-Brey *et al.*, 2009), Betanodavirus in Australian finfish (Moody *et al.*, 2009) and Aquabirnavirus in *Salmo salar* (Davies *et al.*, 2010).

TSRV typical isolate also showed high sequence identity with the major outer capsid protein (VP7) of ASV (Canadian isolate, 2009) and SBRV. High homology between these isolates most likely reflects the homology present among viruses of the same species group classification, with TSRV, ASV and SBRV, all belonging to AQRV-A (Attoui *et al.*, 2002; Fauquet *et al.*, 2005).

Isolate 11 (atypical) was detected from ovarian fluids of brood-stock population from a freshwater hatchery in Northern Tasmania. Detection of TSRV in Atlantic salmon in the hatchery could be considered evidence of vertical transmission. In the past, anecdotal reports from Tasmanian Salmonid Health Surveillance Programme (TSHSP) suggested that TSRV infection does not occur in freshwater fish. However, a retrospective epidemiological study by Carlile (2011) revealed that TSRV submitted samples from inland hatchery sites tested positive for TSRV. It was reported that most of the samples were isolated from mature fish of brood stock age that had been translocated from the marine environment to the freshwater hatcheries. The prevalence of TSRV was reported to be 4.6% between 1990 and 2005, where isolate 11 was obtained, from the similar freshwater hatchery in Northern Tasmania (Gemma Carlile pers communication). Occurrence of TSRV in this hatchery is a separate occurrence than the prevalence published in the retrospective epidemiological study, as isolate 11 was detected in 2007.

To date, vertical transmission of TSRV has not been demonstrated. Detection of AGCRV in golden shiners present in hatchery was concluded as evidence of vertical transmission (Goodwin *et al.*, 2010). The author questioned the viability of AGCRV detected in the eggs because PCR assays could not distinguish between live and dead virus. However this is not the case with isolate 11, because it was positive for TSRV by virus isolation on cell lines and immunocytochemistry. Thus, these findings ruled out the possibility of detection of dead virus in the submitted TSRV samples. Vertical transmission has been demonstrated for the transmission of IPNV (Roberts and Pearson, 2005) and ISAV in Chile (Vike *et al.*, 2009) and Norway (Nylund *et al.*, 2007).

Isolation of isolate 11 raises questions regarding the presence of a freshwater TSRV variant which has been proven to be atypical in this study. The origin of the brood-stocks in the hatchery is still unknown. It is speculated that the brood-stocks could be transferred from the marine cages from South-east Tasmania; where the prevalence of TSRV is higher than Northern Tasmania. Nonetheless, the existence of a freshwater TSRV variant is indicated, similar to the detection of other Aquareoviruses from other freshwater fish species; AGCRV in *Ctenopharyngodon idella* (Goodwin *et al.*, 2010), GSV in *Notemigonus crysoleucas* (Plumb *et al.*, 1979), CCRV in *Ictalurus punctatus* (Amend *et al.*, 1984), GC RV in *Ctenopharyngodon idella* (Jiang and Ahne, 1989), TNRV in *Tinca tinca* (Ahne and Kolbl, 1984) and CHRV in *Leuciscus cephalus* (Ahne and Kolbl, 1984).

It has been further shown that Isolate 13 could be another variant of TSRV with mixed infections of typical and atypical isolates. This isolate was found in samples from Northern Tasmania, similarly to isolate 11. The multiple faint bands obtained for TSRV Segment 10 primers FcRcc with estimated sizes of approximately 263 bp and 125 bp, were similar to bands obtained for both typical (Isolate 10) and atypical (Isolate 11). However, further sequencing analysis could not be conducted because the amplicons obtained for isolate 13 were too weak to purify for further sequence analysis. Further investigation is required in the future to confirm the phylogeny of this isolate.

Typical TSRV isolate (100% sequence homology to complete sequence of segment 10 of TSRV) originated from wild fish, jack mackerel (*Trachurus declivis*). This isolate was sampled at the time of wild fish mortality (of unknown cause) found in salmon cages. Further investigation on the samples showed cytopathic effect consistent with infection by TSRV in EPC cultures and the presence of TSRV was confirmed by immunocytochemistry. The

samples demonstrated reddening of the pyloric fat and skin erosion, similar to pathological signs observed in TSRV infected samples. These findings are existing evidence of potential horizontal transmission of TSRV from farmed salmon to wild fish and/or vice-versa, wild to farmed salmon. Based on the retrospective epidemiological investigation using TSHSP data collected between 1990 and 2005, TSRV never been detected in any of the 33 samples (17 fish species) from wild-caught fish (Gemma Carlile pers communication), however, jack mackerel has not been sampled for the detection of TSRV till 2006.

Knowledge of the disease prevalence in wild populations is important when considering the role wild populations may play as reservoirs of disease agents. Evidence exists for the potential transmission of disease from wild to farmed animals in the cases of ISAV and VHS, and farmed to wild fish in the case of IPNV (Raynard *et al.*, 2007). The source of transmission of TSRV from farmed salmon to jack mackerel is still unknown; it could be transferred through contact with discharges of TSRV from dead or moribund fish into waters around the farm. There is increasing evidence that IPNV is transferred from farmed to wild fish through contact with discharges and products from IPNV-contaminated farms (Sonstegard *et al.*, 1972; Munro *et al.*, 1976; Bucke *et al.*, 1979; Hastein and Lindstad 1991; McVicar *et al.*, 1993; Mortensen *et al.*, 1993; McAllister and Bebak 1997; Wallace *et al.*, 2005b). Moreover, GSRV, a nquareovirus, could be recovered from water within 48 hours from infected golden shiners being exposed to crowding stress, which suggested viral shedding by the fish (Schwedler and Plumb 1982). Analysis of the water for the presence of TSRV was not conducted in the previous studies (Gemma Carlile pers communication) and in this study, but it has been demonstrated that horizontal transmission of TSRV can occur experimentally (Gemma Carlile pers communication).

Several aquareoviruses have been shown to infect a number of wild fish, raising questions about the primary host and wild reservoir of the virus. GCRV has been isolated from apparently healthy wild creek chub (*Semotilus atromaculatus*) (Goodwin *et al.*, 2006). Another example of this is virus 13p2, which was originally isolated from healthy American oysters (*Crassostrea virginica*) but later shown experimentally to infect and cause mortality in bluegill fry (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*) (Meyers, 1979; 1980; 1983). Though these diseases are highly virulent and pathogenic compared to TSRV infection, understanding the prevalence of virus/disease in wild populations is important for disease management. Future testing of the wild populations of fish for TSRV may be important to better understand their role in the distribution of TSRV.

Finally, this characterisation study demonstrated the existence of at least one variant TSRV isolate other than the more commonly isolated typical TSRV in farmed Tasmanian Atlantic salmon. This study revealed preliminary evidence of vertical transmission of TSRV from brood-stocks to eggs and horizontal transmission from farmed salmon to wild fish. Further research should include: biological characterisation of isolate 13, molecular characterisation of two populations in isolate 11, experimental infections of TSRV in wild fish and improvement of PCR methods to detect both typical and atypical TSRV. Further characterisation studies would add important data about the characteristics of the variants. Knowledge of this information will enable optimisation of diagnostic methods and improvement of management procedures and farm husbandry practices to reduce the impact of TSRV infections on salmon farms and the environment.

CHAPTER 6

General Discussion

This thesis focused on the detection and identification of Tasmanian Atlantic salmon aquareovirus (TSRV), which is one of the few viral agents of Atlantic salmon endemic to Tasmania. The results demonstrated genotypic differences between TSRV and other strains within the *Aquareovirus* A species of the genus *Aquareovirus*. For aquareoviruses, the primary species demarcation criteria are RNA cross-hybridization, antibody-based cross-neutralization, RNA sequence analysis and electropherotypes analysis (Fauquet *et al.*, 2005; Attoui *et al.*, 2011). Based on these criteria, there are six distinct species (*Aquareovirus* A to *Aquareovirus* F) originally identified by reciprocal RNA–RNA hybridization studies, but which now can also be distinguished by nucleotide sequence analyses (Attoui *et al.*, 2011). According to this species demarcation criterion in a genus, viruses within different species should have low levels of sequence homology among the cognate genome segments based on RNA sequence analysis (Fauquet *et al.*, 2005; Attoui *et al.*, 2011). For example, genome segment 10 that encodes the major outer capsid, VP7, will show >45% sequence variation between viruses from two different species. These nucleotide sequence differences should be reflected in the amino acid sequence variation (>64%) of the VP7 proteins (Fauquet *et al.*, 2005; Attoui *et al.*, 2011). A BLAST search of the TSRV S10 segment showed sequence identity with the major outer capsid (VP7) with CSRV (94%), SBRV (82%) and 20-37% with CRV, GSRV and GCRV (Carlile, 2011). TSRV VP7 protein showed high amino acid sequence identity to CSRV (94%), SBRV (82%) and moderate identity to CSV (37%), and relatively low identity to VP7 of GSRV (20%) and GCRV (20%) (Carlile, 2011). TSRV shares 92% nucleotide sequence identity with outer capsid protein (VP7) of ASV (Canadian, Genbank accession no: FJ652576.1), and both aquareoviruses infect the same species of

host, *Salmo salar* (Chapter 5). TSRV shares close nucleic acid and amino acid sequence identities with CSRV, ASV and SBRV as these viruses represent different strains, belonging to the same species from the same genogroup aquareovirus, AQRV-A (Gemma Carlile pers communication), whereas, GCRV and GCRV belong to different species groups, genogroup AQRV-C, and CRV belongs to AQRV-B (Fauquet *et al.*, 2005; Attoui *et al.*, 2011).

The preliminary characterisation study undertaken in Chapter 5 revealed the presence of at least three viral variants based on the size of the viral particles. There is a possibility of existence of another variant of TSRV (Isolate 13) with mixed infections of typical and atypical isolates. Questions still remain regarding the origin and pathogenicity of the TSRV atypical isolates. Molecular characterization of aquareoviruses has been focused mainly on highly virulent isolates such as GCRV (McEntire *et al.*, 2003; Goodwin *et al.*, 2006), GSRV (McEntire *et al.*, 2003), AGCRV (Jaafar *et al.*, 2008; Goodwin *et al.*, 2010), TFRV (Seng *et al.*, 2005), SBRV and GIRV (Attoui *et al.*, 2002). It is well-established that viral nucleic acid sequence has an important role in the determination of the taxonomy of viruses, their relationships and gene functions (Seng *et al.*, 2005). Due to the highly virulent nature of these viruses, a good deal of work has been carried out on pathogenesis (Arun *et al.*, 2009; 2010), production of vaccine (Sim *et al.*, 2004; Seng *et al.*, 2005; He *et al.*, 2011a), immunogenicity and function of virion proteins for such purposes (He *et al.*, 2011b; Shao *et al.*, 2011). Sequence information from geographically and chronologically diverse isolates may lead to further insights into origin of TSRV and the route of spread of the pathogen at regional level. Further characterisation of the three variants would assist in understanding the pathogenicity of TSRV and may lead to the design and development of vaccines (Sim *et al.*, 2004; Seng *et al.*, 2005; He *et al.*, 2011a) should this be a priority for the salmonid industry in Tasmania.

The pathogenicity of the typical isolates of TSRV is known to be low under the current conditions and farming practices, but the pathogenicity of the atypical isolates, identified here, remains unknown. The importance of genetically diverse infections with fish viruses in influencing viral evolution and virulence has raised attention; different variants have been shown to exhibit different levels of virulence and pathogenicity in their hosts (Purcell *et al.*, 2009; Shinmoto *et al.*, 2009; Snow *et al.*, 2010; Brudeseth *et al.*, 2008). Characterization of IHNV field isolates from North America has established three main genogroups (U, M and L) that differ in host-specific virulence (Purcell *et al.*, 2009). The study indicated that the low virulent strain (of the M genogroup) entered the host, replicated and spread in the host tissues, to a lesser extent than the high virulent strain (U genogroup). Although, both strains induced a host response, the high virulent strain continued to persist despite this immune response, whereas the genogroup M strain was cleared below detectable levels (Purcell *et al.*, 2009). *In vivo* studies of RSIV (Red sea bream iridovirus) in *Pagrus major* demonstrated that the isolates exhibited different virulence characteristics (Shinmoto *et al.*, 2009). These results revealed that variant characteristics exist in the same phylogenetic location in emergent iridoviruses. Strain variation would expand the host range and different levels of protection occurred in the immunization and challenge experiments (Shinmoto *et al.*, 2009). Similarly, atypical TSRV isolates could be either less or more pathogenic than the typical isolate. Thus, pathogenesis of both isolates should be investigated to increase the understanding of the biology of TSRV.

The possible existence of a freshwater variant of TSRV has raised questions regarding the pathogenicity of this isolate in the freshwater hatchery situation. VHSV strains have been isolated from several marine species and it is a concern that marine strains of VHSV could be a potential source of infection for farmed *Oncorhynchus mykiss*. Two strains of VHSV in

Oncorhynchus mykiss including a marine isolate and a freshwater isolate demonstrated different virulence characteristics (Brudeseth *et al.*, 2008). As the low virulent marine isolate was genetically closely related to the highly virulent freshwater isolate, the possibility of a disease outbreak due to the marine isolate was not ruled out (Brudeseth *et al.*, 2008). A recent outbreak on a marine farm indicated possible adaptation of the marine isolate of VHSV to a new host, *Oncorhynchus mykiss* (Lyngstad *et al.*, 2008). Immersion challenge trials of this isolate resulted in high mortality rates in the host species and provided the first report of a virulent marine isolate (Lyngstad *et al.*, 2008). Due to these reasons, future studies including investigations of the infectivity and virulence of the different isolates of TSRV in marine and freshwater environments should be pursued.

The initial aims of the field investigation in Chapter 4 were to develop a case definition for infection by TSRV in farmed Atlantic salmon and to determine the incidence of TSRV infections with other pathogens. According to OIE (2011), case definition is a set of criteria used to distinguish case and non-case animals. A case refers to an individual aquatic animal infected by a pathogen (positive for the pathogen), with or without clinical signs (OIE, 2011). Development of a case definition for TSRV infections was not undertaken due to the low prevalence of TSRV (6.15%) observed during this study, most likely caused by the relatively low water temperature (even in summer) during this sampling. Due to limited resources, collection of samples for histology focused on the fish that showed gross signs, in order to increase the probability of detecting histological changes caused by TSRV. Apparent gross signs and pathology caused by TSRV infection were non-specific and could not be fully investigated due to the low number of TSRV confirmed samples and the low number of TSRV positives sampled for histology (Chapter 4). Neither obvious clinical signs nor histological changes were observed in most of the collected field samples, which confounded

the effort towards developing a case definition. Therefore, the field investigation aimed to determine the incidence of TSRV infections with other pathogens and focused on the hypothesis that a primary infection with TSRV would increase the susceptibility of virus-infected fish to other infections.

The gross signs observed in this field investigation in salmon infected with TSRV were congestion and enlargement of internal organs, pale liver and petechial hemorrhages on the body surfaces. The only pathology found in TSRV infected Atlantic salmon were mild multifocal necrosis and inflammation of liver. Similar gross signs and pathology were observed in TSRV negative salmon, demonstrating the non-specific nature of the pathology caused by TSRV infections. Non-specific clinical signs demonstrated by fish infected with aquareoviruses have been reported by various authors (Plumb *et al.*, 1979; Meyers, 1980; Subramanian *et al.*, 1997; Cusack *et al.*, 2001; Attoui *et al.*, 2002; Seng *et al.*, 2002). TSRV infections are known to be of low pathogenicity with the absence of clinical signs of disease (Humphrey *et al.*, 1993; Crane *et al.*, 1993; Gemma Carlile pers communication). Experimental trials have indicated that TSRV infections can cause mortality and clinical signs of disease under conditions of relatively high temperature (18-22°C) (Crane *et al.*, 1993; Gemma Carlile pers communication). Based on the archival cases in Chapter 4, TSRV infections did not show any apparent or consistent pathology although, the salmon were positive for the presence of TSRV by qPCR and virus isolation. Aquareoviruses have been reported to be either non-pathogenic, low in pathogenicity or to cause low-level mortalities, sub-clinical disease, or initiate a carrier state, in both natural and experimental infections (Winton *et al.*, 1981; Amend *et al.*, 1984; Hsu *et al.*, 1989; Lupiani *et al.*, 1989; Yoshimizu, 1988; Baya *et al.*, 1990; Varner and Lewis, 1991; Lupiani *et al.*, 1995; Mohd Jaafar *et al.*, 2008; Palacios *et al.*, 2010).

CSRV (chum salmon reovirus), an aquareovirus closely related to TSRV, based on phylogenetic analysis, show low pathogenicity to chum salmon (Winton *et al.*, 1981). Experimental infections of rainbow trout and chum, kokanee and chinook salmon with CSRV showed focal necrotizing hepatitis in chum and chinook salmon (Winton *et al.*, 1989). No histological lesions were detected in kokanee salmon and rainbow trout. However, small necrotic foci in liver of chum salmon were evident from 8 dpi to 21dpi. By day 21 dpi, the liver began to recover and less severe lesions were observed in the liver (Winton *et al.*, 1989). The results of this study indicate that pathogenicity varies among strains of aquareoviruses (Winton *et al.*, 1989). In another study, an IHNV isolate from a North American non-pathogenic genogroup did not cause mortality in experimentally-challenged groups (Purcell *et al.*, 2009). The virus was unable to persist *in vivo* and there was no necrosis detected in internal organs of the challenged fish. Based on this study, the lack of mortality and lack of detectable viral particles, suggested that the fish cleared the virus and recovered from any pathology early in the infection (Purcell *et al.*, 2009). Similar to these observations, good immune response in the fish could be responsible for the lack of pathology observed in TSRV infected salmon. However, the pathogenicity of TSRV infection and clearing of the virus in immunocompetent fish still need to be investigated.

TSRV exists as a sub-clinical infection under the current environmental conditions. However, the presence of TSRV infections in Atlantic salmon without apparent pathology should not be taken lightly, because like any other aquareoviruses, TSRV has been shown to cause clinical disease due to changes in temperature (Crane *et al.*, 1993; Crane and Carlile, 2008). The isolation of virus from a apparently healthy fish, without any obvious signs of infection should be considered as an indicator of virus presence (Stone *et al.*, 1997). At the same time, it must be emphasized that the presence or detection of any infectious agent does

not imply the presence of disease (LaPatra *et al.*, 1998). Although, the virulence of a pathogen (viral factor/s) is an important component in the manifestation of disease, other variables, environmental factors such as water quality, temperature, and host factors such as immune-competence, are important in influencing pathogenicity (Snieszko, 1974).

Methods for the detection and identification of TSRV, specifically molecular tests based on PCR were investigated in this thesis. However, the question still remains whether qPCR assay can justifiably replace virus isolation, the “gold standard” for the detection of fish viruses. While virus isolation is considered the “gold standard” for the detection of many viral agents, the resources required for the establishment and maintenance of an aquatic animal virology laboratory are considerable and virus isolation can be labour-intensive, time-consuming and expensive (Merrill, 2002). With the recent advances in molecular technology, alternative approaches to the detection and identification of viral agents are now available (OIE, 2011). Moreover, while PCR is considered a highly sensitive technology it is also highly specific (Hafliger *et al.*, 1997); PCR technology is based on recognition of specific nucleotide sequences and therefore mutation(s) in the target recognition sites of the PCR primers could lead to a test failure (false negative) with significant consequences. The use of virus isolation in this study as a confirmatory test for qPCR results provided confidence with respect to the presence and absence of TSRV as detected by qPCR. The isolation of TSRV on piscine cell lines demonstrates the presence of replicating virus rather than merely viral nucleic acid. While qPCR has been recommended for screening and monitoring of TSRV in known positive populations, virus isolation is still the recommended detection method in health surveillance programs for virus exclusion, control of disease spread and disease diagnosis (OIE, 2011).

The comparison of available diagnostic methods for the detection of TSRV showed that qPCR assay is the most effective and accurate test due to its high sensitivity and specificity. The type of detection method used in specific identification of a disease/pathogen depends on the clinical status of the disease (clinical versus subclinical infection) (OIE, 2011). As qPCR relies on the detection of specific genomic sequences of the viral nucleic acid segments and is capable of detecting subclinical TSRV infections, it is currently the most suitable assay (Dhar *et al.*, 2001; Overturf *et al.*, 2001; Gilad *et al.*, 2004; De la Vega *et al.*, 2004; Goodwin *et al.*, 2006; Andrade *et al.*, 2007). A higher C_T cut-off value was chosen because TSRV is endemic in Tasmania and priority was given to choosing a cut-off value that detects every single positive and to minimize false negatives. Thus for diagnostic confirmation of suspect or clinical cases of TSRV infection, the test evaluation prioritized diagnostic specificity over diagnostic sensitivity (Caraguel *et al.*, 2011). The use of a quantifiable synthetic dsRNA standard for qPCR assay also enabled subsequent studies (chapter 3 and 5) in this thesis to determine the target (TSRV) quantity in an unknown sample (surveillance and diagnostic).

The use of different diagnostic methods targeting different components of the pathogen (e.g. nucleic acid versus protein) provides confirmatory data and a high level of confidence in the results. The identification of atypical variants of TSRV based on genotypic and phenotypic characterisation of the different isolates studied in Chapter 5 revealed the importance of availability of different diagnostic methods. The study demonstrated that the atypical TSRV isolate was detected by virus isolation and Western blot analyses only; qPCR assay was unable to detect the presence of different sequences (due to mutations). The presence of cytopathic effects on piscine cell lines, typical of TSRV infections confirmed that the atypical isolate was in fact an aquareovirus (confirmed by electron microscopy).

Subsequently, conventional PCR and sequencing analyses identified the origin of atypical isolate to be a variant of TSRV. The use of PCR assays alone could not have determined the identification of variants, as mutations at the primer recognition sites resulted in a negative result. This is of particular importance for detection of RNA viruses since mutations appear to be common (Fields and Joklik, 1969; Steinhauer and Holland, 1987). Thus the different diagnostic tests used in this study such as virus isolation, PCR, Western blotting, electron microscopy, complemented each other and provided a clearer identification of the viruses involved and demonstrates the importance of using different tests in the diagnostic context.

Clearly, subclinical infections, such as most cases of TSRV infection, are more difficult to detect and may require the use of a variety of diagnostic tests, as discussed previously. Presumptive diagnosis of a disease indicated by positive PCR test results must be confirmed by other indications such as clinical signs or other laboratory tests such as virus isolation on cell cultures (OIE, 2011). As mentioned earlier, TSRV infections do not demonstrate consistent clinical signs and no mortalities have been reported due to natural infections. Due to these reasons, a case definition for TSRV infections could not be developed based on pathology or clinical signs. However, a case definition for infection with TSRV could still be determined by qPCR assay followed by confirmation by virus isolation. For these reasons, virus isolation should not be excluded as the gold standard for the detection of infectious TSRV. For laboratories that rely on PCR for front-line diagnosis (surveillance and agent identification), it is important to have more than one PCR test that target different regions of the genome to reduce the incidence of false negative results. The evaluation of qPCR in this thesis led to the routine use of qPCR as the diagnostic method (instead of virus isolation) for the detection of TSRV in Tasmania. Evaluation of diagnostic methods utilising two separate laboratories [Australian Animal Health Laboratory (AAHL)

and Animal Health Laboratory, Mount Pleasant Laboratories (DPIPWE)] provided increased confidence during the conduct of this research.

Future studies should assess other aspects of TSRV such as molecular characterisation and improvement of molecular diagnostic methods. Firstly, further characterization of the two atypical variants needs to be undertaken. However, purified populations of each of the variants, probably by plaque-purification, are required as the first step. Then full genome sequencing would allow comparison at the nucleic acid level and determination of the relationships between the various variants circulating in farmed salmonids in Tasmania. Currently, it is not clear whether the variants are closely related to each other (i.e. members of a quasispecies) or whether they represent distantly related aquareoviruses that, for example, were introduced into the environment on separate occasions or via different host species. Nevertheless, following this approach, specific PCR tests for each variant should be developed with the potential for multiplexing. Characterisation of the plaque-purified viruses would provide important information on the significance of these variants to the salmonid industry in Tasmania.

The effect of TSRV infections on the immune response of Atlantic salmon has not been studied yet. Thus, experimental infections to determine the pathogenesis of TSRV variants and the effect on immune response of Atlantic salmon can be carried out according to Purcell *et al* (2009). This study involved the characterization of virus-host dynamics that contribute to genogroup specific virulence. Gene expression analysis by qPCR was used to concurrently assess viral load and host interferon (IFN) in affected fish (Purcell *et al.*, 2009). Experimental infections should investigate the co-occurrence of TSRV with other pathogens, particularly TSRV-TRLO. Following this study, immune response of Atlantic salmon

experiencing co-infections of TSRV with other pathogens could be determined once the effect of TSRV infections on immune response of Atlantic salmon is known.

In this thesis, detection methods for TSRV infection have been evaluated and the qPCR assay has been proven to be effective, reproducible and rapid for the detection of TSRV. The development of new sample collection method improves the detection of TSRV by qPCR assay. Currently, TSRV infections are still of low-pathogenicity, demonstrate non-specific pathology and can occur in co-infections with other pathogens. The existence of TSRV variants demonstrated that TSRV is likely to occur as a quasispecies similar to any other RNA viruses. Presence of genetic variants also confounds diagnosis and demonstrates the need for research in this area. The use of different detection/diagnostic methods in this thesis, has improved the scope of the detection of TSRV.

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APPENDICES

Appendix I

qPCR & Conventional hemi-nested RT-PCR results – Chapter 2

AHL	AFDL	
qPCR	qPCR	RT-PCR
C _T value	C _T value	
38.13	Negative	Positive
38.65	Negative	Positive
Negative	Negative	Positive
35.8	Negative	Positive
37.92	Negative	Positive
36.67	Negative	Positive
36.96	Negative	Positive
40.49	Negative	Positive
43.04	Negative	Positive

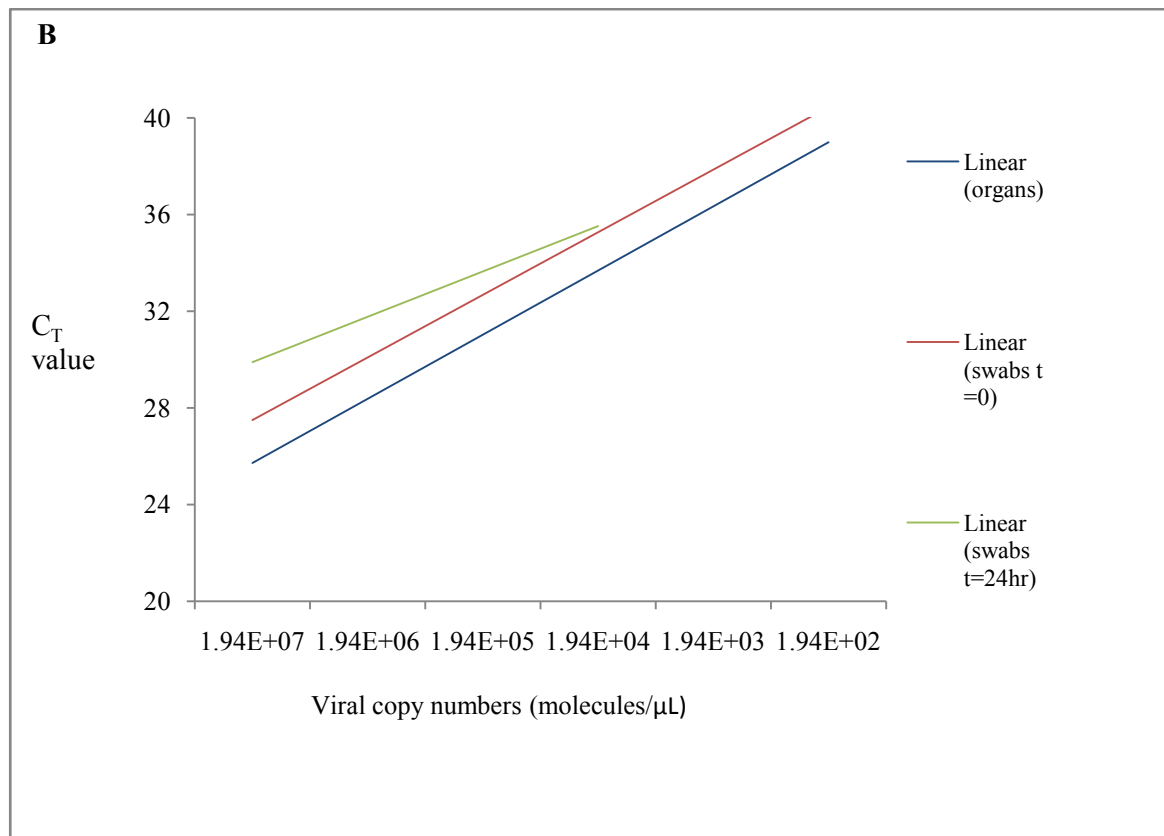
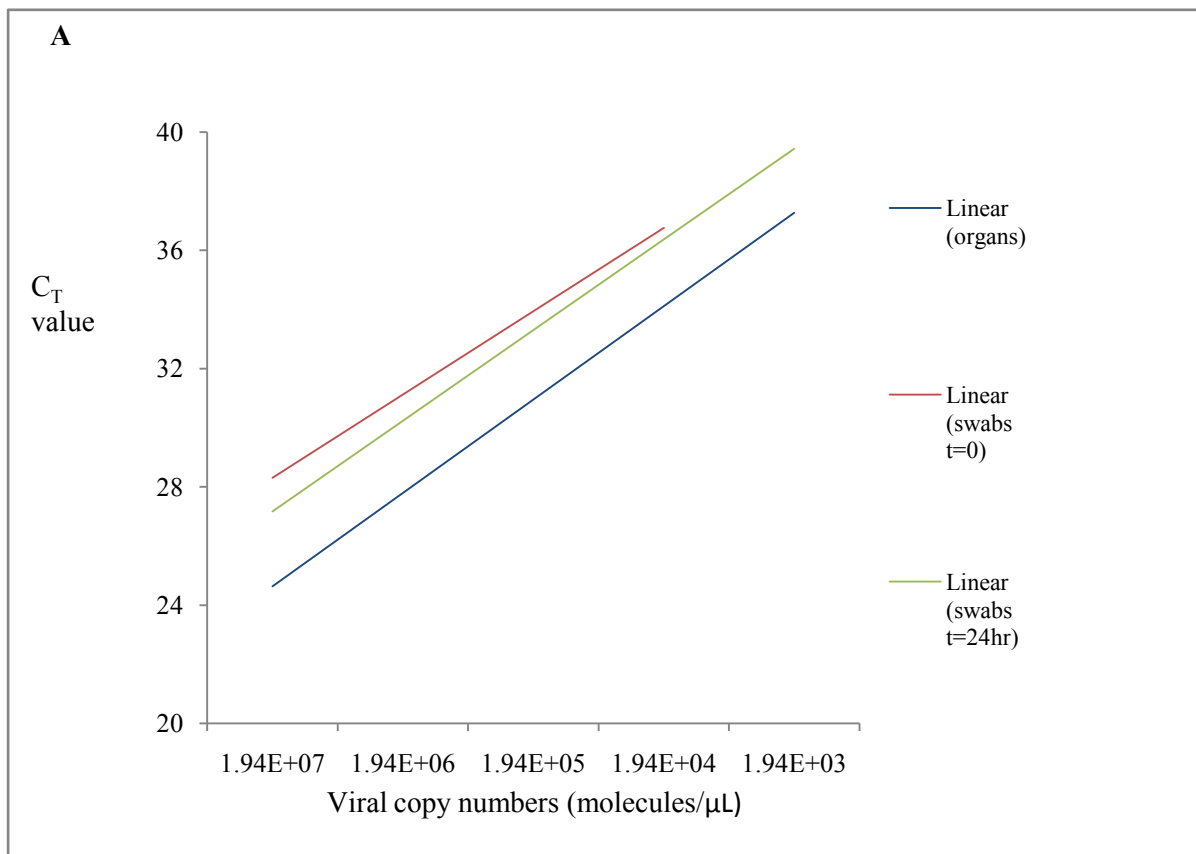
Appendix II

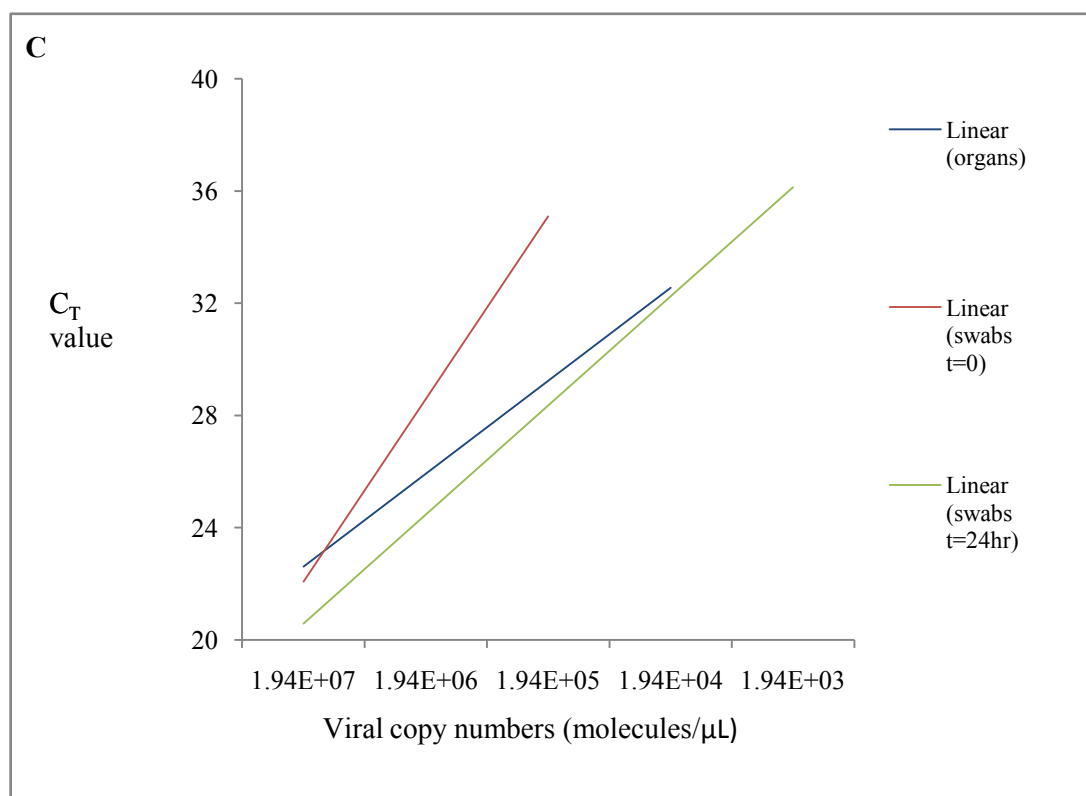
qPCR results of 3 trials – Chapter 3

Comparison of qPCR results of 3 trials using organs and swabs spiked with a ten-fold dilution series of T SRV. Bolded C_T values are positive samples. $t = 0$: RNA extraction conducted directly after spiking, $t = 24\text{hr}$: RNA extraction conducted after 24 hours of spiking, ND: Non-detectable.

Trials	Dilutions	Viral copy numbers molecules/ μ L	C_T values Organs t = 0	C_T values Swabs t = 0	C_T values Swabs t = 24 hours
Trial A	Initial value	1.94E+10	12.81	12.81	12.75
	10^{-1}	1.94E+07	24.99	28.1	29.1
	10^{-2}	1.94E+06	28.12	27.85	32.33
	10^{-3}	1.94E+05	31.42	34.65	34.94
	10^{-4}	1.94E+04	35.47	34.76	34.48
	10^{-5}	1.94E+03	36.26	38.98	ND
	10^{-6}	1.94E+02	37.88	39.523	ND
	10^{-7}	ND	ND	ND	ND
	10^{-8}	ND	ND	ND	ND
	10^{-9}	ND	ND	ND	ND
	10^{-10}	ND	ND	ND	ND
Trial B	Initial value	1.94E+09	18.69	18.69	17.99
	10^{-1}	1.94E+07	23.94	28.86	27.45
	10^{-2}	1.94E+06	28.32	30.47	28.82
	10^{-3}	1.94E+05	31.34	33.56	34.23
	10^{-4}	1.94E+04	34.52	37.23	37.62
	10^{-5}	1.94E+03	36.63	ND	38.39
	10^{-6}	1.94E+02	ND	ND	ND
	10^{-7}	ND	ND	ND	ND
	10^{-8}	ND	ND	ND	ND
	10^{-9}	ND	ND	ND	ND
	10^{-10}	ND	ND	ND	ND

Trials	Dilutions	Viral	C _T values	C _T values	C _T values
		copy numbers	Organs	Swabs	Swabs
		molecules/ μ L	t = 0	t = 0	t = 24 hours
Trial C	Initial value	1.94E+09	16.8	16.8	15.75
	10 ⁻¹	1.94E+07	22.8	22.89	20.08
	10 ⁻²	1.94E+06	25.2	26.96	24.25
	10 ⁻³	1.94E+05	30.12	35.9	30.79
	10 ⁻⁴	1.94E+04	32.2	ND	30.04
	10 ⁻⁵	1.94E+03	ND	ND	36.62
	10 ⁻⁶	1.94E+02	ND	ND	ND
	10 ⁻⁷	ND	ND	ND	ND
	10 ⁻⁸	ND	ND	ND	ND
	10 ⁻⁹	ND	ND	ND	ND
	10 ⁻¹⁰	ND	ND	ND	ND





Relationship between C_T values (qPCR) and viral copy numbers obtained for the three replicate trials (A-C) conducted for the comparison of individual sample collection methods (organs versus swabs). Each trial was conducted on a different day using independently prepared serial dilutions.

Linear equation, correlation coefficient and p values calculated for the three trials (A-C) of comparative study of individual sample collection (organs versus swabs). p value for regression analysis and significant at the 0.05 level. Bolded values are significant.

Trial	Sample type	Equation of slope	r^2	p value
Trial A	Organs	$y = 2.65x + 23.06$	0.97	0.001
	Swabs $t = 0$	$y = 2.58x + 24.91$	0.95	0.003
	Swabs $t = 24\text{hr}$	$y = 1.88x + 28.03$	0.91	0.091
Trial B	Organs	$y = 3.16x + 21.48$	0.99	<0.001
	Swabs $t = 0$	$y = 2.82x + 25.48$	0.99	0.014
	Swabs $t = 24\text{hr}$	$y = 3.07x + 24.09$	0.97	0.021
Trial C	Organs	$y = 3.31x + 19.30$	0.98	0.013
	Swabs $t = 0$	$y = 6.50x + 15.57$	0.98	0.136
	Swabs $t = 24\text{hr}$	$y = 3.88x + 16.69$	0.96	0.008

Appendix III

Preliminary study of evaluation of the performance of swabs as sample collection method for detection of TSRV in field samples (Douglas, Wilson & Zainathan, 2011) – Chapter 3

		Organs		
		+	-	Total
Swabs	+	13	16	29
	-	5	120	125
Total		18	136	154
Kappa value qPCR				0.4779

		Organs		
		+	-	Total
Swabs	+	32	27	59
	-	3	11	14
Total		35	28	73
Kappa value				0.2349
Conventional hemi-nested RT-PCR				

Swabs performed better than organ samples in these studies and both collection methods demonstrated a moderate Kappa agreement. Swabs detected additional 16 positives samples by qPCR and 27 positive samples by conventional hemi-nested RT-PCR.

Appendix IV

MagMAX™ processing plates procedures (MagMAX™ Express 96) – Chapter 4

Processing plates procedure for RNA extraction (TSRV)

Plate Position	Reagent		Volume	Plate to Use
1	Sample Plate			Deep Well Plate
2	Wash Buffer 1 – First Wash		160 ul	Deep Well Plate
	Wash Buffer 1 – Second Wash		160 ul	Deep Well Plate
3	Wash Buffer 2 – First Wash		150 ul	Deep Well Plate
4	Wash Buffer 2 – Second Wash		240 ul	Deep Well Plate
5	Elution	Elution Buffer 1	50 ul	Elution plate with lid
6	Tip Comb Plate	Deep Well Tip Comb in Plate		Deep Well Plate

Processing plates procedure for DNA extraction (TRLO)

Plate Position	Reagent		Volume	Plate to Use
1	Sample Plate			Deep Well Plate
2	Wash Buffer 1		150 ul	Deep Well Plate
3	Wash Buffer 2		150 ul	Standard or DW Plate
4	Wash Buffer 2		250 ul	Deep Well Plate
5	Elution	Elution Buffer 1	50 ul	Elution plate with lid
		<i>Elution Buffer 2</i> <i>Add EB2 when prompted by instrument</i>	<i>50 ul</i>	
6	Tip Comb Plate	Deep Well Tip Comb in Plate		Deep Well Plate