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Identification Of A Second Putative Receptor For Infectious Pancreatic Necrosis Virus

Khrystal C. McGrant
North Carolina Agricultural and Technical State University

IDENTIFICATION OF A SECOND PUTATIVE RECEPTOR FOR INFECTIOUS PANCREATIC NECROSIS VIRUS

by

Khrystal C. McGrant

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School of Graduate Studies
North Carolina Agricultural and Technical State University

This is to certify that the Master's Thesis of

Khrystal C. McGrant

has met the thesis requirements of
North Carolina Agricultural and Technical State University

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Dr. Doretha B. Foushee Major Professor	Dr. Andrew Goliszek Committee Member
Dr. Tracy Hanner Committee Member	Dr. Joseph Whittaker Committee Member
Dr. Mary A. Smith Department Chairperson	Dr. Sanjiv Sarin Interim Dean of Graduate Studies

BIOLOGICAL SKETCH

Khrystal C. McGrant, the daughter of Janet McGrant and Johnny Gregory, was born on October 16, 1985 in Charlotte, NC. She earned her high school diploma from Garinger High School in Charlotte, NC and was a participant in the medical magnet. Khrystal continued her education at Winston-Salem State University where she received her Bachelor's degree and began on the road to research. While attending Winston-Salem State University, she became a member of the Health Career Opportunity Program (HCOP) and shadowed Dr. Jeffery Overhault in "Understanding the Intracellular Activity of a Cell, in Response to O_2 and CO_2 Using Calcium Ion Channels". Khrystal, later joined Dr. Johanna Porter-Kelley's research laboratory as a participant of the Summer Undergraduate Research Experience (SURE) program and studied "The Role of the Adaptor Protein Complex 3 and the Morphological Transformation of *Leishmania* Major". After graduating from Winston-Salem State University, Khrystal was accepted into the Master of Science in Biology program at North Carolina Agricultural and Technical State University (NCA&TSU). Khrystal excelled tremendously academically and preformed research on "Identification of A Second Putative Receptor for Infectious Pancreatic Necrosis Virus" in the laboratory of Dr. Doretha B. Foushee. Khrystal has attended and presented her research findings at several conferences and is currently working on the completion of her first publication. Khrystal has decided to further her education by earning a Doctor of Philosophy degree (Ph.D.) in Virology.

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LIST OF ABBREVIATIONS

µl	microliter
Ala	Alanine
ASK	Atlantic Salmon Kidney
ATCC	American Type Culture Collection
BF-2	Blue Gill Fry Cells
CCA	Cell Culture Adapted
CHSE-214	Chinook Salmon Embryonic Cells
CPE	Cytopathic Effect
DIH_20	Deionized Water
DNA	Deoxyribonucleic Acid
dsRNA	double stranded RNA
EMEM	Eagle's Minimal Essential Medium
FBS	Fetal Bovine Serum
FHM	Fathead Mannose Cells
$HepG_2$	Human Hepatocellular Liver Carcinoma Cells
His	Histidine
IBVD	Infectious Bursal Disease Virus
IEF	Isoelectric Focusing
IHNV	Infectious Hematopoietic Necrosis Virus
IPNV	Infectious Pancreatic Necrosis Virus

kDA	Kilodalton
mAb	Monoclonal Antibody
Mins	Minutes
ml	Millimeter
O.I.E.	Office of International Education
ºC	Degrees Celsius
ORF	Open Reading Frame
pVP	Proviral Protein
RNA	Ribonucleic Acid
RTG-2	Rainbow Trout Gonad
RTS	Rainbow Trout Serum
SDS-PAGE	Sodium Dodecyl Sulfate –Polyacrylamide Gel Electrophoresis
SHK	Salmon Head Kidney
T	Threonine
TBS	Tris-Buffer Saline
TBS-T	Tris-Buffer Saline with Tween
Thr	Threonine
Tyr	Tyrosine
VHSV	Viral Hemorrhagic Septicemia Virus
VOPBA	Virus Overlay Protein Binding Assay
VP	Viral Protein
VPg	Viral Protein Genome Linked

HRP	Horseradish Peroxidase
nm	Nanometer
AChR	Alpha Subunit of The Acetylcholine Receptor
ASMx1 Protein	Atlantic Salmon Mx1 Protein
Mx1	Protein-Myxovirus = influenza resistance

ABSTRACT

McGrant, Khrystal C. IDENTIFICATION OF A SECOND PUTATVE RECEPTOR FOR INFECTIOUS PANCREATIC NECROSIS VIRUS.
(Major Advisor: Foushee, Doretha B.), North Carolina Agricultural and Technical State University.

Infectious pancreatic necrosis virus (IPNV) is a bi-segmented, double stranded RNA virus that causes high mortality in young salmonid fish worldwide. As an obligate intracellular parasite, the first stage of the IPNV infectious cycle is the binding of the virus to a receptor in the host cell membrane. Recently, research has focused on identifying the IPNV receptor, and several scientists have reported binding of IPNV to a protein of approximately 220-250 kDa. The focus of this research was to investigate and reaffirm the identity of the IPNV receptor(s) for IPNV serotypes VR-299 and Sp in Chinook Salmon Embryo (CHSE-214) cells and Rainbow Trout Gonad (RTG-2) cells. We hypothesized that both serotypes VR-299 and Sp utilize the same receptors. Membrane proteins from CHSE-214 and RTG-2 cells were extracted using the Calbiochem Proteo Extract Native Membrane Protein Extraction Kit. The Virus Overlay Protein Binding Assay (VOPBA) was used to identify IPNV-protein interactions. Our results confirmed the binding of IPNV serotypes Sp and VR-299 to a protein approximately 250kDa in the CHSE-214 and RTG-2 cells. However, our results also revealed binding of both IPNV serotypes Sp and VR-299 to two additional proteins of approximately 60kDa and 65kDa in the CHSE-214 and RTG-2 cells and a third protein at 140kDa in CHSE-214 cells.

CHAPTER 1

INTRODUCTION

Over the last two decades, infectious pancreatic necrosis (IPN), which is caused by infectious pancreatic necrosis virus (IPNV), has been recognized as a major disease in salmonid fry and post-smolt (Rønneseth, Wergeland, Devik, Evensen, & Pettersen, 2007). Infectious pancreatic necrosis is a highly contagious viral disease that has a wide geographical distribution including North and South America, Europe, Asia, and South Africa. The disease is known to affect various species of fish and cause an economic burden to those working in the fish farming industry. It has been reported that IPN has caused large economical losses worldwide. Christie (1997) estimated an economic loss of 60 million United States dollars yearly on fish farms in Norway. According to an overview of aquaculture in the United States (U.S) published in October of 1995 by the Center of Epidemiology and Animal Health, the U.S. is the largest exporter of fish products in the world. In 1993, approximately 10 billion dollars of fisheries products were imported into the U.S., while approximately 7 billion dollars of fisheries products were exported out the U.S. In 1993, recorded imports and exports ranged from approximately 5 to 6 billion dollars. It was found that farmed-raised Atlantic, Chinook, and Pacific Salmon made up 78% of the salmon imports in the U.S. These imports were transported from Canada or Chile.

Infectious pancreatic necrosis is caused by infectious pancreatic necrosis virus (IPNV). Infectious pancreatic necrosis virus is a bisegmented double stranded RNA

virus with a non-enveloped icosahedral shape and a diameter of 60-70nm. This virus is a member of the *Birnaviridae* family, which consists of three genera: *Aquabirnavirus, Avirbirnavirus,* and *Entomobirnavirus.* Infectious pancreatic necrosis virus has been identified as affecting *salmonid* species, such as salmon and trout, less than 6 months of age. Although IPNV can infect older fish, it does not cause disease in older fish. Mortalities range from less than 10% to more than 90% in infected fish that are less than 6 months of age; however, older fish may show no clinical signs (Graham). The following fish have been found to be infected by IPNV: Rainbow Trout (*Oncorhynchus mykiss*), Brook Trout (*Salvelinus fontinalis),* Brown Trout (*Salmo trutta),* Atlantic Salmon (*Salmo salar*), and several Pacific Salmon species (*Oncorhynchus spp*) (Roberts & Pearson, 2005). Moreover, the virus has also been shown to infect animal species as different as invertebrates such as mollusks, insects, and rotifers (Coulibaly et al., 2005).

As intracellular parasites, viruses must have a way of gaining entry into host cells. This access is provided by a receptor found on the plasma membrane that is specifically recognized by a ligand, in this case proteins on the capsid of a virus. The presence or absence of a viral receptor will determine whether the cell can be infected by the virus. However, it is also possible that other structures located on the cell's surface to which the virus binds serve as a means for facilitating viral entry. Those structures may assist in efficient entry of the virus by affecting cellular metabolism, or inhibiting the activation of an immune response. Viruses contain repeating subunits, and many copies of the viral attachment proteins are present on the virion surface, allowing for a single virion to interact with several receptor sites on the cell surface (Tardieu, Epstein, & Weiner, 1982).

Viral receptors that have been identified include: the receptor for the rabies virus, nicotinic acetylcholine receptor, and the putative co-receptors for Dengue 4 Virus. Receptors have also been identified for Influenza H1N1, Human Immunodeficiency Virus (HIV), Epstein Bar Virus, Herpes Simplex Virus 1 and 2, and poliovirus (Schneider-Schaulies, 2000).

The purpose of this research was to confirm the identity of the putative receptor for IPNV in Chinook Salmon Embryo cells (CHSE-214), which was reported by Dobos (1995), and to identify and characterize the receptor(s) for IPNV VR299 and Sp serotypes in Rainbow Trout Gonad (RTG-2) cells. We sought to identify and characterize the putative receptors(s) in CHSE-214 and RTG-2 cells by performing several proteomic techniques that include the Virus Overlay Protein Binding Assay and protein sequence analysis.

We hypothesized that IPNV binds to the 220-250kDa protein in CHSE-214 cells as reported by Dobos (1995), and that IPNV serotypes VR-299 and Sp bind to the same 220-250kDa protein in the CHSE-214 and RTG-2 cells.

CHAPTER 2

LITERATURE REVIEW

Infectious Pancreatic Necrosis was first described by M'Gonigle in 1941 as acute catarrhal enteritis. The viral etiology of IPN was established as infectious pancreatic necrosis virus (IPNV) by Wolf and his co-workers in 1960. In 1981, Dorson described infectious pancreatic necrosis virus as a nonenveloped virus with an icosahedral capsid and a diameter of 60 to 70nm. More recently, Dobos (1995) identified the capsid as consisting of 180 structural subunits that make up 92 capsomers on the surface of the virion. Comprehensive reviews of IPNV have been published by Wolf (1972, 1976), Roberts (1978), McAllister (1979), Munro and Duncan (1977), Pilcher and Fryer (1980), Dorson (1982), and McCallister (1983).

Infectious pancreatic necrosis virus is a member of the *Birnaviridae* family which contains three genera: (i) Aquabirnavirus, the genus to which infectious pancreatic necrosis virus (IPNV) belongs, which infects salmonid fish; (ii) Avibirnavirus, the genus to which infectious bursal disease virus (IBVD) belongs, which infects young chickens; and (iii) Entomobiranavirus, the genus to which the Drosophila X virus belongs, which infects Drosphila, commonly called the fruit fly (Villanueva, Galaz, Valdes, Jashes, & Sandino, 2004). Infectious pancreatic necrosis virus is known to replicate in a variety of continuous cell lines from teleost fish at temperatures between 14-24ºC; however, the virus replicates best at 20-24ºC (Dobos & Rowe, 1977).

Infection is initiated by the binding of virus particles to one or more receptors on the host cell surface followed by replication in the cytoplasm. A single cycle of replication occurs between 16-20 hours at 22ºC, resulting in a cytopathic effect (CPE) in which apoptosis precedes the pathological changes of necrosis (Dobos, 1995). The virus is sensitive to high temperatures and low pH, which causes it to become inactive (Graham). Signs and symptoms of infection in fish include: pale gills, distended abdomen, darkening pigmentation, whirling swimming motion, presence of clear or milky mucus in the stomach, and /or long thin white fecal matter. Organs other than the pancreas that are infected in fish include the spleen, kidney, liver, and heart (O.I.E., September 2000).

The first case of IPN occurred in 1941, affecting brook trout fingerlings in Canada. The symptoms that characterized infectious pancreatic necrosis in Canada were catarrhal enteritis, causing inflammation along the intestinal tract, vascular congestion, and increased mucus in the intestinal lumen. The first isolation of the virus was in the United States from Brook Trout (*Salvelinus fontinalis*) in 1957. This prototype isolate was deposited with the American Type Culture Collection (ATCC) in 1963 and given the reference number VR-299 (Hill & Way, 1995). The first noted outbreak of the virus in Europe occurred in rainbow trout (Oncorhynchus mykiss) in 1964 in France (Hill & Way, 1995). In 1968, IPNV was detected in Denmark in rainbow trout by Jorgensen and Bregnballe who reported the isolate now referred to as the Sp strain. In 1970, another isolate of IPNV was identified in Denmark and referred to as the Ab strain. It was thought that two serotypes, Sp and Ab, existed in Danish trout farms (Hill & Way, 1995).

The next documented outbreak of IPNV occurred in Norway in 1975 in the trout species, and later in 1985 a second outbreak occurred in the salmon species. That same year Chile also described its first outbreak (Schering Plough Animal Health, 2010). Earlier studies of IPNV placed all isolates in three serogroups: Sp, Ab, and VR-299. All of the European isolates of IPNV were members of the Sp and Ab serogroups. The other serogroup, VR-299, consisted of all of the North American isolates (Cutrin, Olveira, Barja, & Dopazo, 2000). Only the Sp serogroup is reported to cause disease in seawater-adapted salmonid fish (Santi, Song, Vakharia, & Evensen, 2005). Using monoclonal antibodies, IPNV strains were placed into eleven serogroups: VR-299, Sp, Ab, West Buxton (WB), Hecht (He). Tellina (Te), Canada 1 (C1), Canada 2 (C2), Canada 3 (C3), Jasper (Ja), and N1 (Christie, Ness, and Djupbik., 1990). Christie, Ness, and Djupbik (1990) reported that all the IPNV serotypes showed some degree of cross reaction in reciprocal neutralization test with rabbit antiserum.

Most Norwegian isolates are of Sp serotype and also cause mortality in Atlantic Salmon, *Salmosalar* L., post-smolts. Although mortalities have been seen during outbreaks, the mortalities vary considerably and have been ascribed to differences in genetic susceptibility of the host and differing levels of environmental stress (Santi, Vakharia, & Evensen, 2004).

2.1 Viral Transmission

Infectious pancreatic necrosis virus can be transmitted horizontally and vertically. Horizontal transmission of IPNV can be defined as the spread of the virus to other

freshwater or marine environments by a variety of reservoirs and vectors. A vector can be defined as an intermediate agent that transmits a pathogen to a susceptible host. Reservoirs are carriers for a pathogen which can remain unharmed while acting as a potential source of infection. Horizontal infection can be due to infected water, contaminated nets, equipment, feces, urine, secretions from infected fish, and by infected embryonated ova, which can introduce the virus to healthy ova. Vertical transmission is the transmission of the virus from mother to offspring.

2.2 Viral Prevention

Once IPNV is introduced into a fish hatchery or fish farm, there is no method of eliminating the virus other than to kill all of the stock and decontaminate the hatchery. After this procedure, it is necessary to restock the hatchery. This is a very costly method; therefore, most fish farmers practice preventive methods. Several countries carry out regular health checks on fish farms that include routine inspection of propagated fish and diagnostic examinations which, ultimately, may help prevent the spread of infection (Milne, Gallacher, Cash, & Porter, 2006). Preventive measures to protect against vertical transmission have been implemented such as recombinant vaccines against IPNV *in-vivo*; however, production cost hinders further vaccine development. Lastly, others have tried breeding fish that have been identified as genetically resistant to IPNV. These fish have all been rainbow trout (Guy et al., 2006). Health inspection of fish farms and screening of brood stock brought into fish farms from outside sources are the most widely used prevention strategies, because they are less expensive to implement.

2.3 Viral Genome

Infectious pancreatic necrosis virus contains a bi-segmented, double stranded RNA (dsRNA) genome with two genomic segments, segment A and segment B (Espen, Rimstad, et al., 2003). The genes on these segments encode five proteins designated as viral protein 1 (VP1), viral protein 2 (VP2), viral protein 3 (VP3), viral protein 4 (VP4), and viral protein 5 (VP5) (Espen, Rimstad, et al., 2003). Segment A consists of 3,100 base pairs and contains two partially opened reading frames. The short frame of segment A encodes a 17kDa arginine rich nonstructural protein, VP5. The long frame of segment A encodes a precursor 106kDa polyprotein. The viral protease, VP4, self cleaves from this 106kDa polypeptide to form a 29kDa VP4. Viral Protein 4 protease then generates a precursor of the major capsid protein, pVP2 (62kDa) and Vp3 (31kDa). The pVP2 (62kDa) is further cleaved to VP2 (54kDa) during virus maturation. Viral Protein 2 has been found to be the major outer capsid protein, and specific type neutralizing antibodies have been produced against this protein (Duncan et al., 1987; Manning and Leong, 1990; Mayar and Dobos, 1994; Saint Jean et al, 2003). The VP3 is an internal capsid protein that binds to virus RNA, forming the ribonucleoprotein's core structure. The VP3 also forms trimeric subunits that wrap the inner face of the capsid (Villanueva, et al., 2004). Segment A has also been found to encode an arginine-rich minor 17kDa nonstructural protein called VP5 from a small ORF. Viral Protein 5 has been detected in infected cells; however, it is not essential for virus replication in vitro (Song, Santi, Evensen, & Vakharia, 2005). The order of the virus proteins in the IPNV precursor polyprotein is NH2-pVP2-NS protease/VP4-VP3-COOH (Espen Rimstad, 2003). Segment B has been

found to consist of 2,900 base pairs that encode a minor internal polypeptide VP1 (94Kda), which has been said to be the putative viral RNA-dependent RNA polymerase. The VP1 has been identified within the virion in two forms: as a polypeptide acting as a RNA with RNA dependent polymerase associated activity and as a genome linked protein, VPg (Villanueva, et al., 2004).

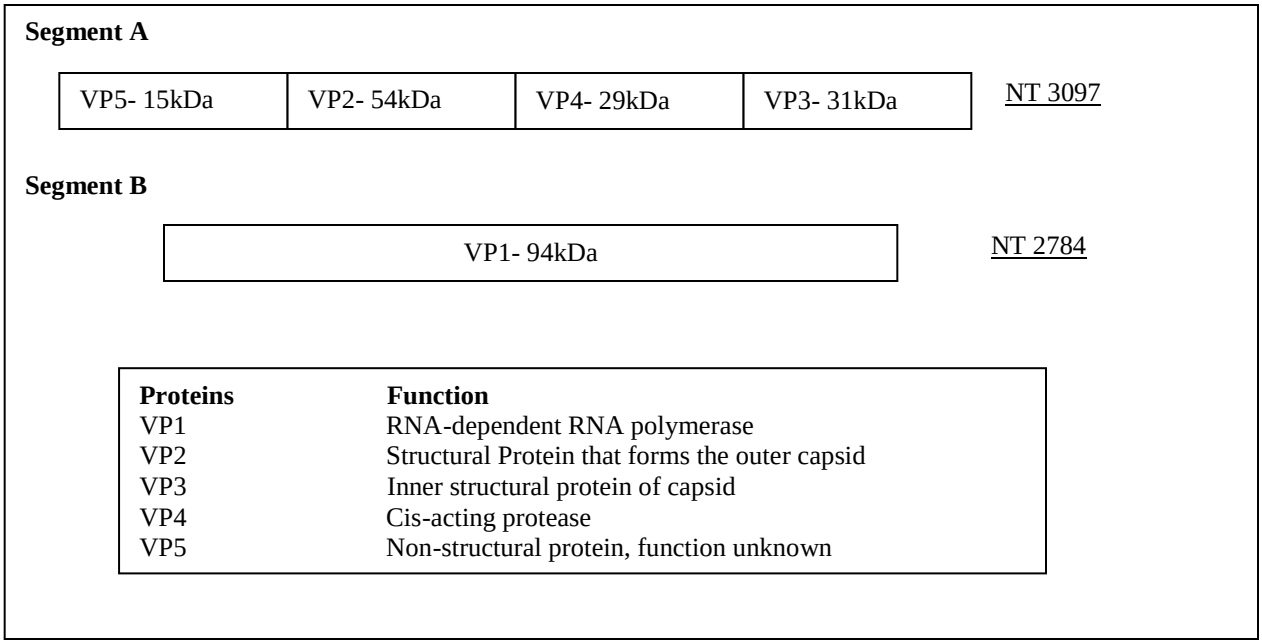

Figure 2.1 Description of proteins encoded by segment A and segment B of IPNV. The two genomic segments of IPNV, all 5 viral proteins, their molecular weights, and function, are shown.

Virulence of IPNV has been shown to be associated with segment A and not with segment B, which encodes VP1 (Song, et al., 2005). Scientific studies revealed that certain amino acids within the VP2 sequence are important factors in determining the virulence of IPNV strains. These studies indicated that Threonine (T) and Alanine (A) in amino acid positions 217, 221, and 247 lead to high virulent strains of the virus, while

proline in these positions lead to low virulent strains (Skjesol, 2009; Song et al 2005). Song et al. (2005) stated that virulent strains typically encode a 12 kDa VP5 and could also contain residues Tyrosine (Tyr) and Histidine (His) at the 217, 221, 247, and 500 amino acid positions in the VP2 gene leading to highly virulent strain.

2.4 IPNV Replication

Infectious pancreatic necrosis virus is similar to other non-enveloped animal viruses, meaning that it is able to enter the host cells by using receptor mediated endocytosis after specific attachment to subceptible cells. Dobos & Marshall (1995) conducted binding experiments using CHSE-214 cells and labeled IPNV, which revealed that binding of the virus took place in 2-3 hr at 4ºC. The binding studies were completed using soluble radio labeled virus specific polypeptides and CHSE-214 cells at 4ºC and indicated that the VP2 is the cell attachment protein of the virion. Dobos (1995) then proposed that VP2 is responsible for attachment of the virus to the host cell. Once the virus enters the host cell, IPNV proceeds directly with transcription, allowing replication to take place in the cytoplasm. Transcription is primed by VP1 binding to RNA followed by strand-displacement on the positive RNA-strand. Skjesol (2009) stated that recent studies have shown that the negative RNA strand is used as a template for genomic replication. However, during a single replication cycle, two different kinds of particles are formed: a larger one that contains uninfectious particles termed a provirion and a smaller one known as the mature or infectious virion. The capsid, however, is composed of both mature and immature viral polypeptides.

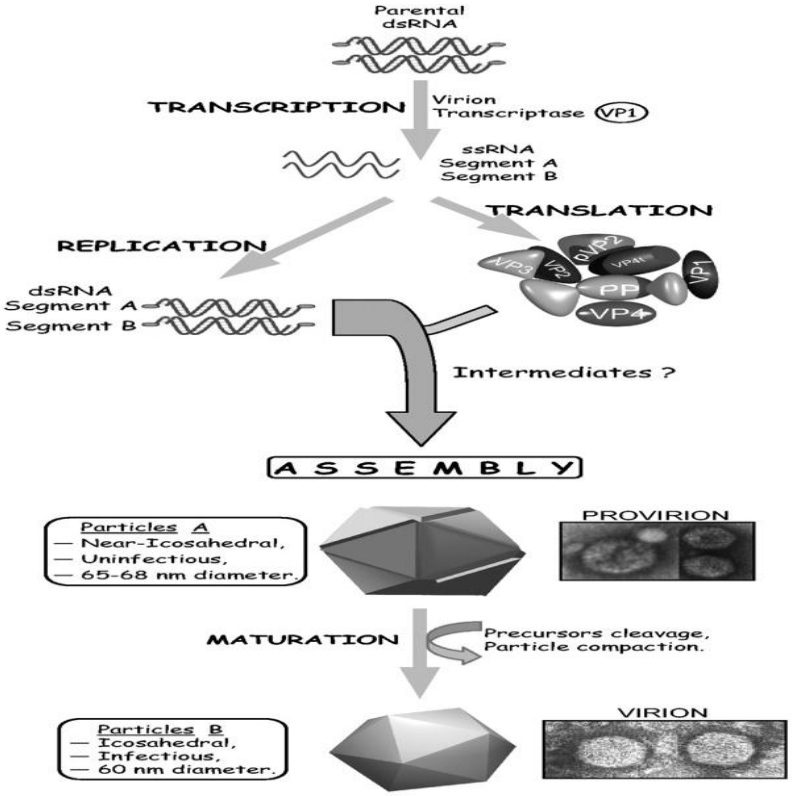

Figure 2.2 Schematic of the viral replication cycle of infectious pancreatic necrosis. Segment A and segment B as well as viral proteins aiding in viral replication are illustrated (Villanueva, et al., 2004).

2.5 Prior Research Discussing Glycosylation

There is much debate about whether glycosylation of IPNV is a general trait or simply a consequence of cultivation in special cell lines. Segment A sequence data by Jasper indicated four potential N-glycosylation sites in the pVP2 gene. By labeling IPNV with 3H-mannose, it was found that VP2 contains carbohydrate residues, and that it is a glycosylated protein (Estay et al 1990; Perez, Chiou, & Leong 1996). The general rule is that only viral proteins embedded in the envelopes are glycosylated. Since IPNV is a non-enveloped virus, these findings have been greatly disputed. Dobos (1995) reported

the results of a study in which radioactivity was counted in gel-slices and stated that the "single-slice peak" representing labeled VP2 was not very convincing. The glycosylation pathway for a protein in a cell involves translocation to the endoplasmic reticulum and the Golgi apparatus with addition of carbohydrates. However in a study by Perez, Chiou, & Leong (1996), no single peptide sequence in the VP2 protein was found to be glycosylated, nor was there evidence of the VP2 being inserted into the cytoplasmic membranes. In a different study using influenza hemagglutinin signal sequence to pVP2, proteins were shown to be synthesized in the rough endoplasmic reticulum and led to glycosylation of pVP2, which shifted the molecular weight from 41kDa to 60kDa. Native pVP2, on the other hand, was cytoplasmic and remained unglycosylated. There are two potential glycosylation sites near the amino termini of VP3 of IPNV.

Studies have shown that there are two known ways to add carbohydrates to virus proteins, either by N-linking or by O-linking. Hjalmarsson, Carlemalm & Everitt (1999) propagted IPNV in RTG-2 cells and found evidence of the O-glycosylation binding lectin recognizing sugar moieties of N- acetylgalactosamine, which consist of mannose and fructose, on the major structural protein VP2. However, Hjalmarsson et al. (1999) stated that Perez et al. (1996) tried to complete a glycosylation study using the same cell line and no glycosylated proteins were found. The following lectins and carbohydrate specifities listed were used in the study: *Bandeiraea simplicifolia* lectin II (BSL) α- or β-linked GlcNAc, Concanavalin A (ConA) α-linked mannose, *Dolichos biflorus* agglutinin (DBA) α-linked mannose, *Datura stramonium* lectin (DSL) (β-1,4)-linked GlcNAc, *Erythrina cristagalli* lectin (ECL) Galactosyl (β-1,4)-linked GlcNAc, Jacalin (Jac) O-

linked galactosyl (β-1,3)-linked GalNAc, *Lycopersicon esculentum* lectin (LEL) GLcNAc, Peanut Agglutinin (PNA), Galactosyl (β-1,3)-linked GalNAc, *Ricinus communis* agglutinin (RCA) Galactose, *Solanum tuberosum* lectin (SBA), GalNAc, STL GlcNAc, *Ulex europaeus* agglutinin (UEA) α-linked fucose, *Vicia villosa* agglutinin (VVA) α- or β-linked GalNAc, and WGA GlcNAc. Out of all 14 lectins, ECL was found to have a very strong signal at the VP2 position. Additional sugars present on VP2 were fructose and mannose binding to UEA. Earlier studies have shown that mannose is present in IPNV replicated in CHSE-214 cells by reactions with the lectin ConA (Hjalmarsson et al.,1999).

2.6 Viral Inhibition

A vital area of IPNV research focuses on viral inhibition. Two publications were identified that focused on viral inhibition, OGUT (2004) and Larsen, Rokenes, & Robertsen, (2004). The culturing of IPNV susceptible cells in medium containing serum taken from rainbow trout that had not been exposed to IPNV for approximately eleven passes were shown to result in these cells becoming resistant to IPNV. This viral inhibition is thought to be due to an antibody like non-virus induced protein which has a sedimentation rate of 6S. OGUT (2004), investigated the ability of rainbow trout serum (RTS) to inhibit the replication of IPNV isolates belonging to various serogroups in cell lines in which IPNV is normally able to replicate. Four virulent isolates were studied *in vitro* (in brook trout) in the presence and absence of RTS to determine whether passing changed the level of virulence. Passage eleven times in the brook trout resulted in loss of

virulence both in the presence and absences of RTS. Fathead Minnow (FHM) cells were infected with IPNV Sp. OGUT (2004) found that only 1-2% of the cells could be prevented from viral replication, allowing RTS to infect 98% of the cells. However, when looking at incubation of FHM cells with the virus in the presence of 1.3% RTS placed on the cells for 2 hours, 97% of cells remained free from viral replication, causing a 3% viral replication. In the absence of RTS, 45% of the FHM cells were infected by the virus, leaving 55% of the cells free from viral replication. OGUT. (2004) noticed that when using the virulent strain of IPNV Sp, and Rainbow Trout Serum, the Sp serotype became avirulent, which indicated that the Sp strain became inactivated by RTS. His results indicated that the isolates that contained more than 50% virulence were found resistant to RTS, and the isolates that had low virulence or no virulence were inhibited effectively by 1% RTS. He also stated that when the RTS is high, the virulence of virus tended to be low and that supporting evidence by Jorgensen (1972) and Dorson et al. (1975) suggest that avirulent isolates of IPNV were found to be inhibited from viral replication with usage of RTG-2 cells after one passage *in vitro* in the presence of RTS. In conclusion, OGUT stated that their findings supported the conclusion of Kelly and Nielsen (1985), which reported that serum inhibition is not dependent on the virus serotype.

The Mx protein is synthesized by a gene that encodes interferon-inducible proteins that lead to non-specific protection against viral infection in mammals (Heppell & Davis 2000) and belongs to a superfamily of large GTPases. Several mammals, including mice, rats, and humans, as well as birds and teleost fish, possess a subtype of

the Mx protein. Infectious Pancreatic Necrosis Virus inhibition was correlated with levels of Mx protein expression in interferon (IFN)-stimulated salmon cells (Larsen, Rokenes, & Robertsen, 2004). Antiviral activity from IFN has been demonstrated amongst a number of fish species in-vitro and in-vivo. Interferon has been shown to play a key role in inducing antiviral effects against the infectious pancreatic necrosis virus in salmon cells; however, understanding why and how is not well understood. When looking more specifically at the rainbow trout fish Mx proteins, and it's inhibitory effects on the replication of infectious hematopoietic necrosis virus (IHNV), a virus in the same family as IPNV, it was found that these proteins are expressed through transient transfection and have no inhibitory effect against replication of the virus. Infectious pancreatic necrosis virus has been found to be inhibited in salmon cells expressing high levels of Mx proteins after treatment with IFN or poly (I-C), a synthetic polymer which is not made to resemble the RNA of the infectious virus and which is used to stimulate the production of interferon. Larsen et al. (2004), expressed Atlantic salmon Mx1 (ASMx1) in CHSE-214 cells. Experiments using these cells revealed antiviral activity against IPNV. This antiviral activity was contributed to the ASMx1 protein. It was found that ASMx1 caused the cells to reduce transcription of viral RNA and protein synthesis and inhibited CPE.

2.7 Vaccine Development

Vaccination is an effective strategy used worldwide for controlling the spread of infectious diseases among many species. Heppell and Davis (2000) stated that chemicals

and antibiotics have been and can be used to control bacterial and parasitic diseases in fish, but may lead to harsh side effects in animals and contaminate the aquatic environment. There are three major ways to vaccinate fish: injection, immersion, and oral delivery. The injection method is usually the most immunologically effective; however, it is very labor intensive.

The first commercially licensed vaccine for fish was a killed vaccine against *Yersiniaruckeri*, which was delivered by immersion. *Yersiniaruckeri*, the causative agent of Enteric Red Mouth Disease, is a bacterial infection that occurs in fish such as rainbow trout and is characterized by subcutaneous hemorrhaging of the mouth, fins, and eyes. Later, scientists saw the success of the vaccine for enteric red mouth disease and formulated a formalin killed immersion vaccine for vibriosis, a gram negative bacterium found in trout and salmon (Shoemaker, Klesius, Evans, & Arias, 2009).

Most of the presently used vaccines in the Atlantic salmon industry are produced against multiple antigens in oil-adjuvants and delivered to the fish in one injection. These vaccines have been successful and have reduced the use of environmentally unfriendly chemicals. The United States currently has three modified licensed live vaccines which are used against bacterial kidney disease, enteric septicemia of catfish disease, and columnaris disease (Shoemaker, et al., 2009).

The cost of vaccine production is a major concern for manufactures and for aquatic farmers; therefore, different methods have been used for vaccine development. One of the methods used is the production of modified live bacterial vaccines. This method is low to moderate in cost; however, the vaccine must be injected into each and

every individual fish that is in the Atlantic Salmon industry triggering a very high labor cost. Other options include DNA vaccines. Heppell & Davis (2000) stated that "in theory, any gene coding for a protein of the pathogen that induces a protective immune response in a host can be used in DNA vaccines as long as there is expression within the fish." The production of DNA vaccines is a more expensive technique, but it has been successfully used in the treatment of two viruses in different species of fish.

Heppell & Davis (2000) researched three major ways to immunize fish: immersion, oral delivery, and injection. Injection would be the best way to inhibit viral replication. It was suggested that immersion and the oral delivery method were ideal, because they were inexpensive and did not cause stress to the animals. They found that the administration of oral and immersion vaccination in fish had some drawbacks, because it was impossible to determine the amount of vaccine ingested by the fish, and that oral injection or immersion appeared to be less effective than injection in vaccinating fish. However, they found that immersion had a greater effect over the oral method due to better absorption of the antigen through the skin and /or gills compared to the gut were the vaccine could undergo degradation in the digestive tract. Heppell & Davis also preformed research with a specific type of fish, rainbow trout, and made antibodies to the G protein of viral haemorrphagic septicemia virus (VHSV). The antibody to the virus was detected 23 days after injection with a plasmid that encoded the G gene. Heppell & Davis also found that the concentration of the serum antibody peaked at 3-8 weeks and remained constant for several weeks.

After evaluating the safety to the consumer, Heppell & Davis found the only potential risk was associated with ingestion of plasmid DNA remaining in the injected fish. When observing the risks of DNA vaccines absorbed into the environment of other fish, they concluded that most of the injected plasmid failed to enter the nucleus and cytoplasm.

DNA vaccination has been used in Atlantic salmon fish to protect against IHNV, as well as to protect rainbow trout against Viral Hemorrhagic Septicemia Virus (VHSV) (Shoemaker, et al., 2009). There are currently limited treatments available for aquatic viral infections. Unfortunately, most research being conducted on viral inhibition in fish concentrate their efforts more on prevention rather than treatment. When reviewing available vaccines for the aquatic industry, most commercially available vaccines are those that protect fish against bacterial diseases. These vaccines are made from inactivated bacteria and applied by either immersion or injection with an oil adjuvant.

2.8 The Virus Overlay Protein Binding Assay

Many scientists perform a procedure called the virus overlay protein binding assay (VOPBA) to test or study virus-receptor interaction. This assay uses protein extracts from cells that are separated by gel electrophoresis, transferred to nitrocellulose or some other membrane support, and probed with a labeled virus preparation or unlabeled virus followed by reaction with a labeled antibody against the virus (Gastka, Horvath, & Lentz, 1996). This procedure allows for the identification of an unknown virus receptor.

Gastka et al. (1996) used VOPBA to investigate the rabies virus and its binding pattern and reported that the virus binds to proteins of 40kDa and 51kDa in the alpha subunit of the acetylcholine receptor (AChR) in the Torpedo californica electric organ membranes. Binding of the rabies virus to the 40kDa protein was said to be inhibited by unlabeled α –bungarotin, which irreversibly and competitively binds to the acetylcholine receptor and is a neurotoxin found in the elapid snake.

The VOPBA was also used by Jindadamrongwech & Smith (2004) to identify the presence of dengue virus binding protein expressed on the surface of HepG2 human liver cells using three different strains: DEN-2 (16681), DEN-3 (16562), and DEN-4 (1036). This method was also used to identify any serotype specificity differences in the binding heterogeneity of a dengue virus protein on HepG2 human liver cells using serotypes 2, 3, and 4. The results of this study include the following: dengue virus strain DEN-3 bound to proteins of approximately 78-80kDa, 90kDa, 98kDa, and 102kDa. Strain 4, DEN-4, bound to proteins of approximately 90kDa and 130kDa. The protein to which strain 2, DEN-2, binds was not identified.

Puig, Araujo, Jofre, & Frias-Lopez (2001) studied *Bacteroides fragilis*, a gram-negative obligate anaerobe. The lytic bacteriophage B40-8 was used as a model to examine the interactions of phages with *B. fragilis*. The purpose of this study was to identify the receptors on the B. *fragilis* HSP-4 to which the phage B40-8 binds using the VOPBA. The results indicated that at least two membrane proteins, BactA, and BactB, were involved in the adsorption of bacteriophage B40-8 to the surface of B. f*ragilis*.

Orpetveit, Gjoen, Sindre & Dannevig (2008) viewed interactions between IPNV, Sp serotype isolated from Atlantic salmon and various cell lines, including the Blue Gill Fry (BF-2), Chinook salmon embryo (CHSE-214), Salmon head kidney (SHK-1) and Atlantic salmon kidney (ASK), using the VOPBA. Orpetveit et al. (2008) reported their results as follows: IPNV was found to bind to a membrane protein of approximately 220kDa in CHSE-214, SHK-1, and ASK cell lines, whereas in the BF-2, which is a non-salmonid cell line, binding occurred to a membrane protein of approximately 190kDa. These results were obtained by the use of two different IPNV antibodies: a monoclonal antibody, anti VP3 (IPN-VP3-C12), and a polyclonal antibody anti-IPNV. However, they did indicate that other weak bands were shown at approximately 55kDa within the RTG-2 and CHSE-214 cell lines, as well as faint bands at approximately 100kDa in the SHK-1 and ASK cell lines. Recombinant strains (rnV115, rNVI15VP2, and rNVI15C) were all said to bind in all cell lines at the 220kDa and 190kDa. However, when reviewing this paper we identified several gaps that were not well addressed in the paper. The blots displayed in the paper reveal several binding sites with the usage of the monoclonal antibody against the virus, which were shown in several of the cell lines. Based on the information given, it is not clear how many binding sites exist for the virus in each cell line based on the data generated using their field isolates and recombinant isolates.

While investigating the virus receptor, Dobos & Marshall (1995) used a purified IPNV to show that IPNV binds to a high molecular weight polypeptide of (100-200 kDa) in western blots in CHSE-214, CEP, RTG-2 and FHM cells. It was also found that

monoclonal antibodies (mAb) to CHSE-214 cells protected these cells from IPNV infection by binding to the same polypeptide, but binding competition between the labeled virus and the mAb could not be demonstrated, which was said to be due to the mAb binding to a different region of the putative receptor than the virion (Dobos, 1995).

This study further investigated the receptor for IPNV-VR-299 and IPNV-Sp in CHSE-214 and RTG-2 cells. The VOPBA was used in this study because it is the most efficient, economical, sensitive, and easily preformed assay utilized to date to study virus receptors. The Putative receptor(s) will also be analyzed mass spectrometry.

CHAPTER 3

MATERIALS AND METHODS

3.1 Specific Aim 1: Identification of the Protein to which IPNV Binds

3.1.1 Cells and Viruses

Chinook salmon embryo (CHSE-214) and Rainbow Trout Gonad (RTG-2) cells were purchased from the American Type Culture Collection (ATCC) (CRL-1681 and CCL-55 respectively). All cells were maintained in Eagle's Minimal Essential Medium (EMEM) containing Hanks salts and supplemented with 10% fetal bovine serum (FBS) and 5% gentamicin at 20ºC. The VR-299 and Sp strains of IPNV were also purchased from ATCC (VR-299 and VR-1318 respectively).

3.1.2 Antibodies

A primary polyclonal antibody (Rabbit Anti-IPNV), made against the VP-2 of the virus, was a kind gift from Dr. Vikhram Vakharia and was used at a 1:400 dilution with a 1:15,000 dilution of Fetal Bovine Serum (FBS). Both dilutions were made in Tris Buffer Saline with 0.05% Tween-20 (TBS-T) and incubated for 1hr on the membrane. A second primary antibody was also used to perform the VOPBA. The second primary antibody was a monoclonal antibody (Mouse Anti-IPNV) made against the VP-2 of the virus and was a kind gift from Tim Hewison (Microtek). The Monoclonal antibody was diluted 1:500 in TBS-T and was incubated for 1hr on the membrane.

3.1.3 Mass Production of IPNV

Cells were grown to 90% confluency and were washed in serum free media and incubated with 1ml of a 1:400 dilution of the IPNV Sp or VR-299 for 20 minutes at room temperature. Fiftteen milliliters of media without serum was added to each 75cm^2 flask and the flasks were incubated for 4 or 5 days at 15ºC. Once 90% CPE was achieved, the flasks were placed in the -80º freezer. After three freeze thaw cycles, the clarified virus was obtained by combining the medium from the flasks in 50ml centrifuge tubes and centrifuging at 10,000 rpm for 15 minutes. Supernants were removed, aliquoted into 1ml aliquots, and stored at -80ºC.

3.1.4 Protein Extract

The CHSE-214 and RTG-2 cell membrane proteins were extracted using the Proteo Extract Native Membrane Protein Extraction Kit (Calbiochem catalogue #444810) following the company's directions. At 80% confluency, the cells were washed with 5ml of ice cold wash buffer. Thirty microliters of protease cocktail inhibitor was added to the flasks along with 6ml of ice cold extraction buffer-1. The flasks were incubated for 10 minutes at 4ºC with gentle agitation. The supernatant was poured into a 15ml conical tube and placed on ice. Three milliliters of ice cold extraction buffer-2 was added to the flasks, which were then incubated on ice for 30 minutes at 4ºC with gentle agitation. The extraction buffer-2 solution from all the flasks, which now contained the integral cell membrane proteins, was transferred into a new 15ml conical tube. The membrane proteins were then placed into a 50ml centrifugal filter tube (Millipore catalogue #UFC905024) and centrifuged at 4ºC for 15 minutes at 5,000rpm. The filtrate was

discarded, and the retentate was poured into a clean 15ml conical tube, labeled, and stored at -80ºC for future use.

3.1.5 Protein Quantification

The proteins were quantified using the Quick Start Bradford Protein Assay (Bio-Rad catalogue #500-0207, 500-0205), following the company's instructions. Briefly, one milliliter of the Bradford dye reagent and 20mls of protein sample were mixed in a cuvette. The optical density (O.D) was measured in a UV spectrometer (Beckman) at a wavelength of 280nm after a five minute incubation.

3.1.6 Virus Overlay Protein Binding Assay

Cell membrane proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using 7.5% polyacrylamide precast ready gels (Bio-Rad catalog #456-1023). Membrane proteins were mixed in a 1:1 dilution with the Laemmli Sample Buffer (Bio-Rad catalog #161-0737). The mixture was heated for 3mins at 100ºC. Casting tray and wells were filled with Tris-Glycine-SDS buffer at a pH of 8.3. Five microliters of Precision Plus Protein WesternC Marker (Bio-Rad catalog# 161-0376) was loaded in lane 1 while 27µl of the 1:1 mixture of membrane proteins and loading dye was loaded into the remaining wells. Gels were run at 150vlts for 60mins. Once complete, the gel, filter paper, and nitrocellulose membrane were soaked in Tris-Glycine buffer with a pH of 8.3 for 40mins and placed in the cassette, which was placed into the tank blotting apparatus (Bio-Rad) containing Tris-Glycine buffer at a pH of 8.3. The proteins were transferred from the gels to nitrocellulose membranes at 100vlts for 75mins. The membranes were then incubated for 45mins at

room temperature (RT) in 1x TBS-T containin 5% nonfat dry milk. The blocked membranes were placed in a solution containing either Sp or VR-299 diluted 1:400 in TBS-T. The membranes were incubated with the virus for 3hrs at RT. All unbound virus was removed by washing the membranes 4 times for 5mins in 1x TBS–T at RT. The membranes were reacted with either polyclonal primary antibody (Rabbit Anti-IPNV), or monoclonal primary antibody (Mouse Anti-IPNV). The rabbit primary antibody was diluted to 1:500 in TBS-T containing 0.66µl fetal bovine serum (FBS). The mouse primary antibody was diluted 1:500 in TBS-T. The membranes were incubated in either of these primary antibody solutions for 1hr at RT and washed 4 times for 5mins using TBS-T. The secondary antibodies used were Goat Anti Rabbit or Goat Anti-Mouse with Streptactin (Bio-Rad) used at a 1:15,000 dilution. The membranes were incubated in secondary antibodies at RT for 1hr, washed 4 times for 5mins in TBS-T, and a final time with TBS before reacting with Immun-Star HRP Chemiluminscent solutions (Bio-Rad) diluted 1:1 for 5 minutes in the dark. The membranes were exposed on Flurochem HD2 (Alpha Innotech) for 5mins or to film for 1min.

3.2 Specific Aim 2: Charachterization of the Putative Receptor for IPNV.

3.2.1 Sequences Analysis of Putative IPNV receptors

Chinook salmon cells and rainbow trout gonad total membrane proteins were run on a 7.5% precast polyacrylmide gel for 1hr at 150 vlts. The precast gel was then placed in DIH_20 and washed four times for 5mins. The gel was stained with Simply Blue (invitrogen catalogue #LC6060) for 1hr. After staining, the gel was destained by washing four times for 15mins in DIH_20. The gel was sent to the Michael Hooker

Proteomic Center at the University of North Carolina at Chapel Hill's where the 250kDa, 65kDa, 60kDa proteins were extracted and sequence analysis and homology were carried out by Mass Spectrometry.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction of Membrane Proteins

The Chinook salmon (Oncorhynchus tshawytscha) is the largest species in the salmon family and is also known as the king of salmon**.** The Chinook salmon embryo cells were purchased from American Type Culture Collection (ATCC) and are adherent cells that were grown in T-75cm^2 flasks and subcultured once a week. Extraction of total membrane proteins from CHSE-214 cells using the Calbiochem Proteo Extract Native Membrane Protein Extraction Kit yielded a protein concentration of approximately 1.25µg/µl per two 75cm^2 flasks. Proteins were concentrated to 2.0-4.0µg/µl (Figure 4.1).

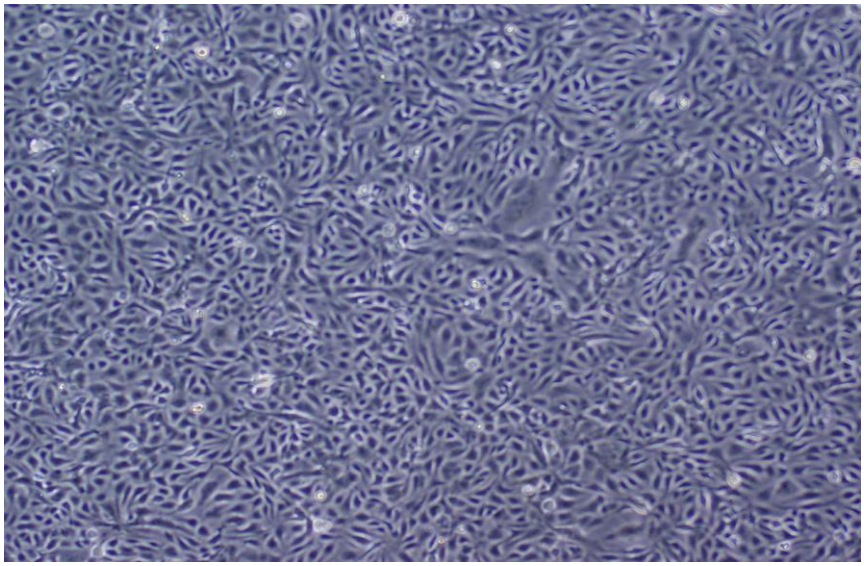

Figure 4.1 CHSE-214 cells in culture. CHSE cells in culture yielded a protein concentration of approximately 1.25µg/µl per extraction of every two 75cm flasks. Proteins were concentrated to 2.0-4.0 µg/µl.

Rainbow Trout (*Oncorhynchus mykiss*) are coldwater fish that live in healthy mountain streams and lakes in North America, Asia, Europe, and Africa. Rainbow trout have been found to live well and thrive in fish hatcheries, which have led to the introduction of rainbow trout into many hatcheries in the United States. The Rainbow trout gonad cells were purchased from ATCC and are adherent fibroblasts cells. Membrane proteins were extracted from the RTG-2 cells using the Calbiochem Proteo Extract Native Membrane Protein Extraction Kit and yielded a protein concentration of approximately .90µg/µl per two 75cm^2 flasks (Figure 4.2). These results indicate that the RTG-2 cells yield a lower concentration of extracted protein in comparison to the CHSE-214 cells. However, all protein extracted were concentrated to 2.0-4.0µg/µl for use in further studies.

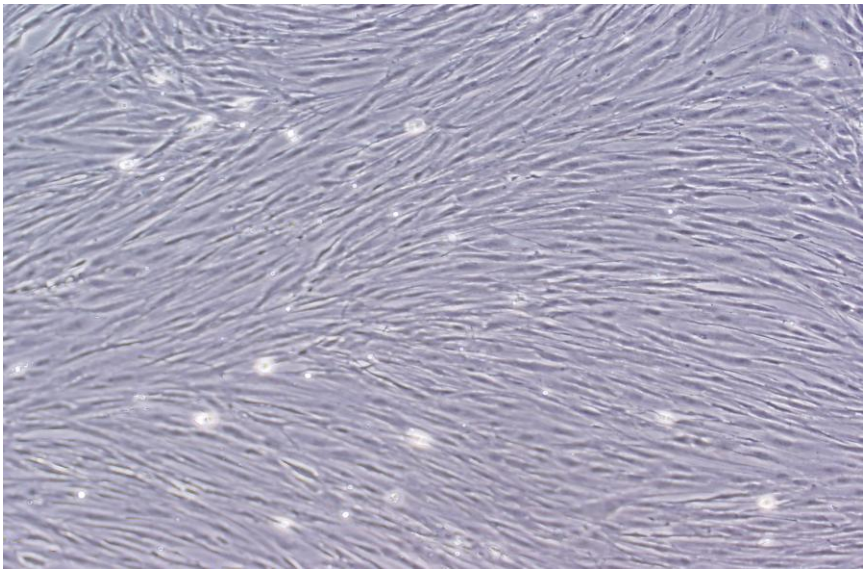

Figure 4.2 RTG-2 cells in culture. RTG cells yielded a protein concentration of approximately 0.90µg/µl per two 75cm^2 flasks. Proteins were concentrated to 2.0-4.0µg/µl

4.2 Mass Production of IPNV

The CHSE-214 cells were incubated for 4 or 5 days at 20ºC after infection with VR-299 serotype of IPNV until cells revealed 90% CPE. Figure 3.3 reveals CHSE-214 cells undergoing CPE. The morphology of the cells changed, resulting in disorientation, shrinkage, and death of the CHSE-214 cells as a result of replication of IPNV VR-299. Mass production of the virus resulted in 30ml of clarified virus.

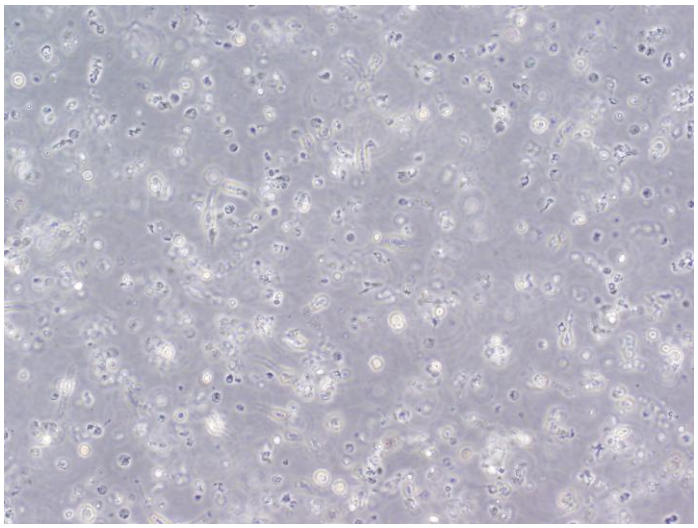

Figure 4.3 CHSE-214 cells infected with IPNV. CHSE-214 cells infected with IPNV VR-299 resulting in cytopathetic effect.

4.3 CHSE-214 and RTG-2 Total Protein

Total Membrane Protein Extracts from CHSE-214 and RTG-2 cells were separated by SDS-PAGE on 7.5% polycramide gels. The CHSE-214 total membrane protein extracts yielded cleaner and clearer bands in comparison to the RTG-2 total protein extracts (Figure 4.4) and (Figure 4.5). When comparing the CHSE-214 and RTG-

2 total membrane proteins separated on polyacrylamide gels, it was clearly visible that the CHSE-214 total membrane protein extracts contained a greater number of proteins than did the RTG-2 total membrane protein extracts. The greater number of proteins in CHSE-214 may make virus binding to the receptor and entry into the cell more efficient.

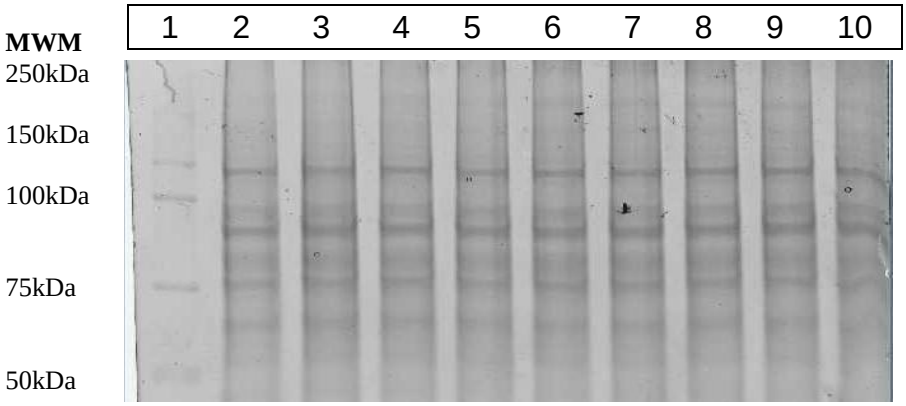

Figure 4.4 CHSE-214 total protein expression. Lane 1 contains the molecular weight markers and lanes 2-9 contain total protein extract of CHSE-214 cells.

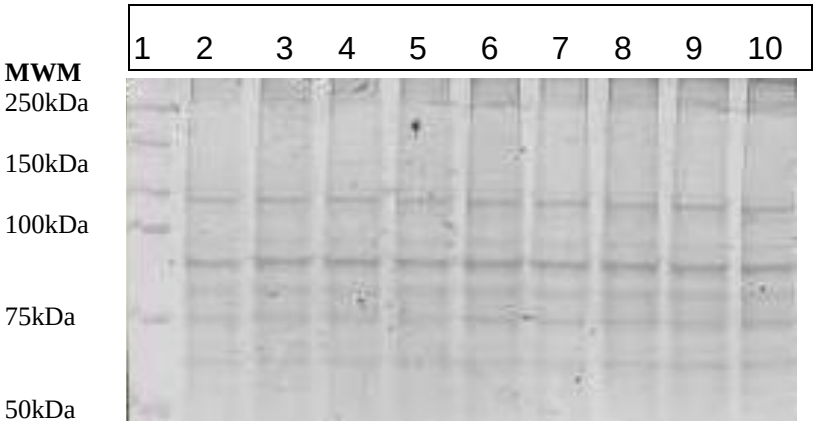

Figure 4.5 RTG-2 total protein expression. Lane 1 contains the molecular weight markers and lanes 2-9 contain total protein extract of RTG-2 cells

4.4 The Virus Overlay Protein Binding Assay

The VOPBA was completed to identify the protein(s) in CHSE-214 and RTG-2 to which IPNV VR-299 and Sp bind. Several studies, including Orpetveit et al. (2008) and Dobos (1995), have used the VOPBA to identify the receptor(s) to which IPNV binds. Orpetveit et al. (2008) used the VOPBA to identify the receptor to which IPNV Sp binds in several cell lines, including CHSE-214, BF-2, SHK-1, and ASK. Orpetveit et al. (2008) identified IPNV binding to the membrane proteins at approximately 220kDa in CHSE-214, SHK-1, and ASK cell lines whereas in the BF-2, which is a non-salmonid cell line, the binding was to a membrane protein approximately 190kDa. These results were obtained by using two different IPNV antibodies, a monoclonal antibody, anti VP3 (IPN-VP3-C12), and a polyclonal antibody anti-IPNV using the Sp serotype of the virus. However, they did indicate that other weak bands were shown approximately at 55kDa within the RTG-2 and CHSE-214 cell lines as well as other faint bands of 100kDa in the SHK-1 and ASK.

Our results reveal different viral-protein interactions than that of Orpetveit et al. (2008). Based on our experiments, there were viral-protein interactions at approximately 250kDa and 65kDa using CHSE-214 and RTG-2 total membrane protein extracts with the use of a polyclonal antibody against the VP2 protein of the virus in both the VR-299 and Sp serotypes of IPNV (Figures 4.6-4.8). These differences could be due to the fact that Orpetveit et al. (2008) used a monoclonal antibody to the VP3 that binds to the inner structural protein of the capsid, while both antibodies used in this study were to VP-2, the outer structural proteins on the outer layer of the capsid. It could be that the virus uses

certain proteins for attachment of the virus at VP2 that are different from the proteins found in the inner structure of the capsid in the VP3. The VP2 protein is expected to be used for initial viral attachment while the VP3 protein is used as a co-receptor or for initiation of the virus just before replication.

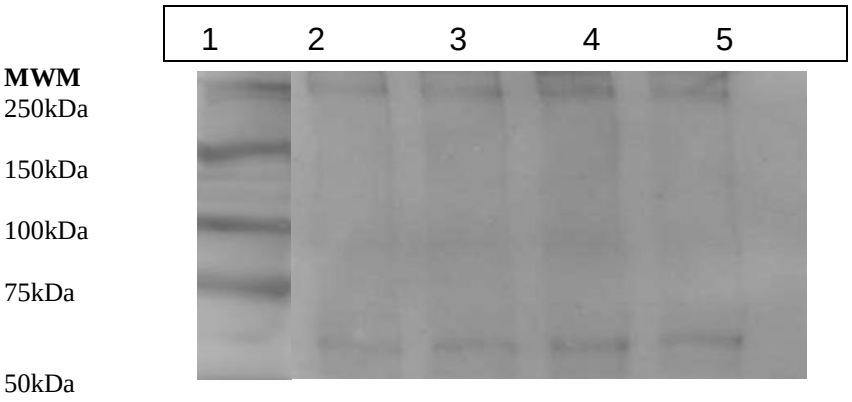

Figure 4.6 VOPBA using IPNV VR-299. Lane 1 consists of molecular weight marker and lanes 2-5 contain CHSE-214 protein. Serotype VR-299 binds to proteins at 250kDa and approximately the 65kDa.

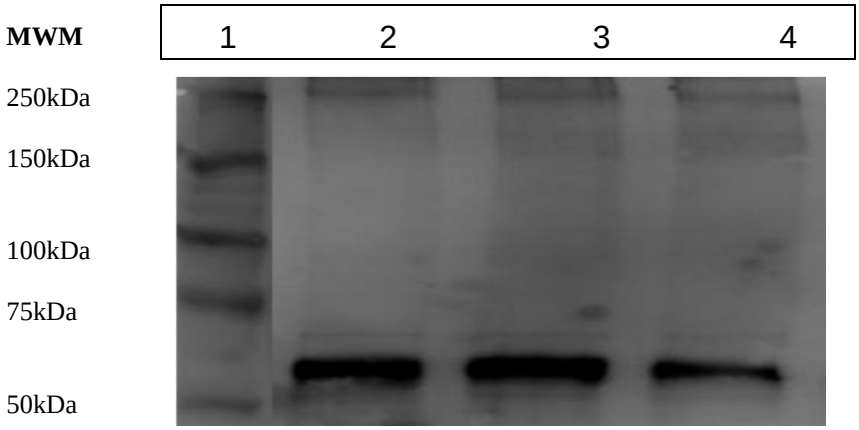

Figure 4.7 VOPBA using IPNV Sp. Lane 1 consists of molecular weight markers and lanes 2-4 contain CHSE proteins. Serotype Sp binds to proteins at 250kDa and proteins at approximately the 65kDa using anti-IPNV polyclonal antibody.

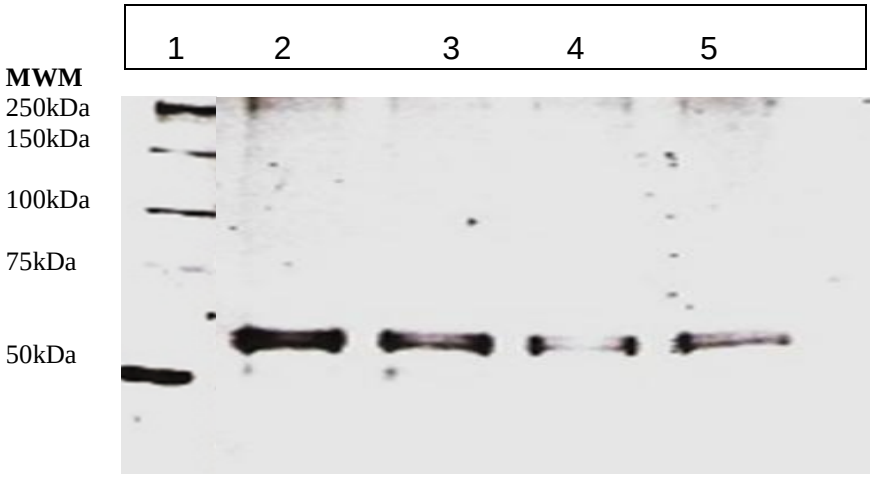

Figure 4.8 VOPBA using IPNV Sp. Lane 1 consists of molecular weight markers and lanes 2-5 contain RTG-2 proteins. Serotype Sp binds to proteins at 250kDa and approximately 65kDa using anti-IPNV polyclonal antibody.

Dobos & Marshall (1995) found that IPNV binds to a high molecular weight polypeptide of 100-200kDa in western blots in CHSE-214, CEP, RTG-2 and FHM cells. When comparing the findings of Dobos & Marshall (1995) to our findings, there are some differences. Dobos & Marshall (1995) stated that IPNV binds to a high protein of 100-200kDa using western blot analysis, leaving the study with a broad range of proteins to which the virus may bind as a putative receptor; however, we were able to look more closely and identify specific bands.

We used the VOPBA to investigate viral-protein interactions between IPNV VR-299 and IPNV Sp. CHSE-214 and RTG-2 total membrane protein extracts were used for the VOPBA. A monoclonal antibody to the VP2 of the virus was used. The VR-299 and Sp serotypes reacted with three proteins at approximately 140kDa, 65kDa, and 60kDa in CHSE-214 membrane extracts (Figure 4.9). The RTG-2 membrane proteins did not show

binding at the 140kDa as did the CHSE-214 with the use of the monoclonal. Furthermore, when using the RTG-2 total membrane proteins, IPNV VR-299 and IPNV Sp bound to the 65kDa and 60kDa proteins as identified using a monoclonal antibody generated against the VP2 of the virus.

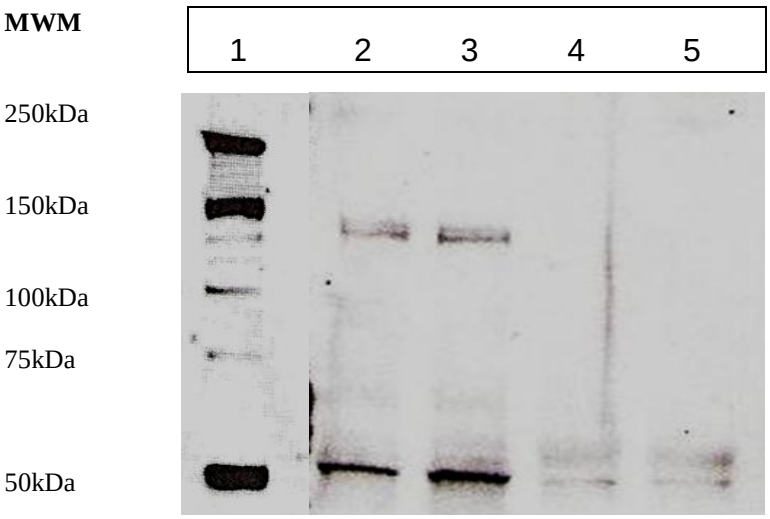

Figure 4.9 VOPBA on nitrocellulose incubated with IPNV. Lane 1 consists of the molecular weight markers, lanes 2-3 contain CHSE-214 proteins and lanes 3-4 contain RTG-2 proteins. Lanes 2 and 3 were reacted with serotype VR-299 and lanes 3 and 4 were reacted with SP. Viral proteins bound at approximately 140kDa, 65kDa, and 60kDa using an anti-IPNV monoclonal antibody to VP2.

4.5 Proteins with Homology with the Putative Receptors

The CHSE-214 and RTG-2 total membrane proteins were separated by SDS-PAGE on a 7.5% precast polyacrylamide gel. The gel was stained with simply blue destained, and sent to the Michael Hooker Proteomic Center at the University of North Carolina at Chapel Hill's for protein analysis. The 250kda, 65kDa, and 60kDa proteins were extracted and analyzed for homology with known proteins in DNA data bases by

mass spectrometry. Protein functions were identified using two search engines, protein prospector and Mascot. The CHSE-214 and RTG-2 250kDa, 65kDa, 60kDa bands were labeled A-1 toA6 (Figure 4.12). The 250kDa protein in CHSE-214 contains a protein homology with myosin heavy chain 9 non-muscle in Danio Rerio (Zebra fish) (Table 4.1). Myosin is a superfamily of proteins that are classified into two categories, myosin I and myosin II. Myosin II, has been found to be an actin-binding protein composed of myosin heavy chains, regulatory light chains and essential light chains. When Myosin II is expressed in non-muscle tissues, it is associated with cellular movement, muscle contractions, and cell division (Park et al., 2011; Takubo et al., 2003).

The 65kDa protein in CHSE-214 ,membrane extracts was found to have sequence homology with disulfide isomerase, disulfide-isomerase associated 3 (A3) precursor, and 60kDa heat shock protein/mitochondrial precursor (Table 4.1). Disulfide isomerase is an enzyme that has diversified metabolic functions. The main function of disulfide isomerase is to allow for proper protein folding through the breakage of disulfide bonds within the peripheral proteins attached to the cell membrane; however, disulfide isomerase is mostly associated with the endoplasmic reticulum. It is because disulfide isomerase has the ability to fold and unfold proteins that many conclude that the protein acts as a chaperone protein, which is involved in checking the correct folding of the proteins being synthesized in the endoplasmic reticulum (D'Aloisio et al., 2010). Disulfide isomerase A3 precursor is the inactive form of disulfide isomerase, which becomes active after post-translational modification. The heat shock protein 60kDa is a derivative of heat shock proteins. Heat shock proteins are named according to their

molecular weight. Overall, heat shock proteins are found in eukaryotes and bacteria cells. In eukaryotes, heat shock proteins are proteins that are trigged and become active in stress conditions such as inflammation and infection. Heat shock proteins are able to act as chaperones and aids in transferring proteins across membranes within the cell into the mitochondrion (Borges, 2005).

The 60kDa protein in CHSE-214 membrane extract is similar to the alpha 2-HS glycoprotein precursor of Bos Taurus (cattle) (Table 4.1). Alpha 2-HS glycoprotein precursor is a glycoprotein that is present in serum and is synthesized in the liver. Alpha 2-HS glycoprotein precursor is the inactive form of the alpha 2-HS glycoprotein, that has been associated with physical development of tissue and the mineral development in bone.

The 250kDa protein in RTG-2 membrane extract contains a protein homology with myosin heavy chain 9 non- muscle of Danio Rerio (Zebra fish). The function of the homologous protein was previously described in discussion of the 250kDa protein, in the CHSE-214 membrane extract.

The 65kDa protein in the RTG-2 membrane extract has homology with the alpha -1 antiproteinase precursor, alpha 2 HS glycoprotein precursor, and serum albumin precursor of Bos Taurus (cattle) (Table 4.1). The alpha-1 antiproteinase precursor is the inactive form of the alpha-1 antiproteinase. The alpha-1 antiptoreinase's function is to act as an inhibitor of serine proteases, trypsin, and chymotrypsin, and becomes an activator for plasminogen. The serum albumin precursor is one of the major proteins

found in plasma and is the inactive form of serum albumin. Once serum albumin becomes active, it functions in regulating osmotic pressure of the blood.

The 60kDa protein in the RTG-2 membrane extract was shown to have protein homology with the alpha-1 antiproteinase precursor and serum albumin precursor of Bos Taurus (cattle) (Table 4.1).

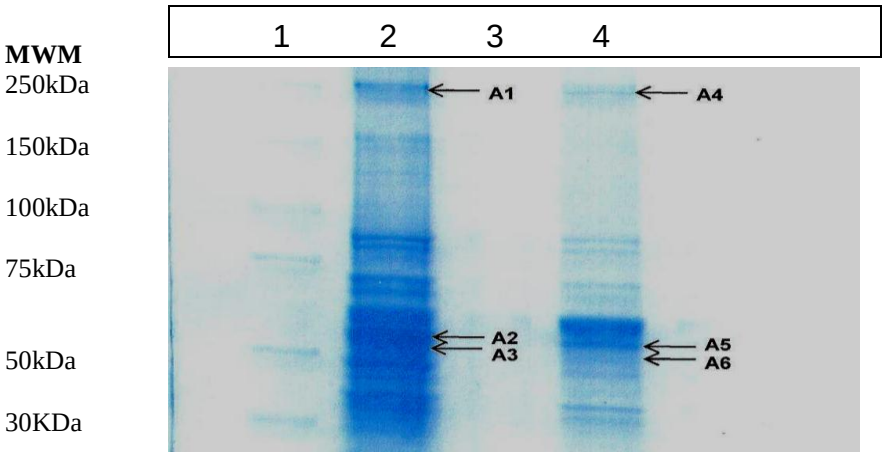

Figure 4.10. Polyacrylamide Gel of CHSE-214 and RTG-2 total membrane proteins labeled for protein analysis. Lane 1 consists of molecular weight markers. Lane 2 consists of CHSE-214 total membrane proteins. Lane 3 is empty and Lane 4 consists of RTG-2 total membrane proteins.

Table 4.1 Protein Homology and Species Information

Sample Band Spot	Well	Protein Name	Species	Database Accession ID	MW(kDa)	Peptide Count[2]
1	**A1**	Predicted: Myosin, heavy chain 9, non muscle	Danio Rerio	gil 189516653	204.11kDa	29

Table 4.1 (Cont.)

2	**A2**	Disulfide-isomerase A3 precursor (Salmo Salar)	Salmo Salar	gil 209156144	54.712kDa	14
2	**A2**	Disulfide-isomerase A3 precursor (Salmo Salar)	(Salmo Salar)	gil 209153384	54.97kDa	11
	A2	Disulfide-isomerase precursor (Salmo Salar)	(Salmo Salar)	gil 224613274	47.29kDa	6
	A2	60kDa heat shock protein, mitochondrial precursor	(Salmo Salar)	gil 223649224	61.04kDa	9
3	**A3**	Alpha 2-HS glycoprotein precursor (Bos Taurus)	(Bos Taurus)	gil 27806751	38.39kDa	5
4	**A4**	Predicted: Myosin, heavy chain 9, non muscle	(Danio Rerio)	Gil 189516653	204.11kDa	29
5	**A5**	Alpha -1 antiproteinase precursor (Bos Taurus)	(Bos Taurus)	gil 27806941	46.07kDa	12
	A5	Alpha 2-HS glycoprotein precursor (Bos Taurus)	(Bos Taurus)	Gil 27806751	38.39kDa	8
	A5	Serum Albumin (Bos Taurus)	(Bos Taurus)	gil 30794280	69.27kDa	13

Table 4.1 (Cont.)

6	**A6**	Alpha 2-HS glycoprotein precursor (Bos Taurus)	(Bos Taurus)	gil 27806751	38.39kDa	
	A6	Serum Albumin (Bos Taurus)	(Bos Taurus)	gil 30794280	69.27kDa	
	A6	Alpha -1 antiproteinase precursor	(Bos Taurus)	gil 27806941	46.07kDa	8

Table 4.1. Protein Homology and Species Information. The bands that were labeled on the polyacrylamide gel correspond with the letter and number labeled in the chart. The unknown proteins were labeled A1-A6. The unknown proteins sequences were matched with known protein sequences to identify the function. Corresponding Database ID numbers are listed as well as the molecular weights for the known proteins.

4.6 Protein Sequence Comparison

CHSE-214 and RTG-2 putative receptors were labeled A1-A6. The unknown proteins were sequenced and the sequences were searched through two databases for protein homology matched with known protein sequences to identify the function. Table 4.2 reveals the labeled sample bands, the function of the known protein, the C.I% that reveals the percentage of homology, the species in which the known protein was identified, and lastly the amino acids sequence in the order in which the homology occurs in the known and unknown protein. Some sample bands may have more than one possible function and some of the identified functions in the known protein may have more than one homologous sequence. Further studies will need to be conducted to confirm the function of the putative receptors.

Table 4.2 Sequence Homology of the Putative Receptors

Sample Band	Function of known Protein	C.I.%	Species of known Protein	Homology Sequences between unknown protein and known proteins
A1	Predicted: Myosin, heavy chain 9, non muscle	------	(Danio Rerio)	VQAQMK
A2	Disulfide-isomerase A3 precursor (Salmo Salar)	99.9%	(Salmo Salar)	MDATANDVPSQYEVR
A2	Disulfide-isomerase precursor (Salmo Salar)	50%	(Salmo Salar)	FFPAGDEHK
A2	60kDa heat shock protein, mitochondrial precursor	--------	(Salmo Salar)	APGFGDNR
A2	60kDa heat shock protein, mitochondrial precursor	----------	(Salmo Salar)	LVQDVANNTNEEAGDGT
A2	60kDa heat shock protein, mitochondrial precursor	----------	(Salmo Salar)	GANPVEIR
A3	Alpha 2-HS glycoprotein precursor	100%	(Bos Taurus)	TPIVGQPSIPGGPVR

Table 4.2 (Cont.)

A4	Predicted: Myosin, heavy chain 9, non muscle	----------	(Danio Rerio)	QGFPNR
A5	Alpha -1 antiproteinase precursor	100%	(Bos Taurus)	NLYHSEAFSINFR
A5	Alpha -1 antiproteinase precursor	96%	(Bos Taurus)	YASSANLHLPK
A5	Alpha -1 antiproteinase precursor	87.86%	(Bos Taurus)	LVDTFLEDVK
A5	Alpha -1 antiproteinase precursor	50%	(Bos Taurus)	DFHVDEQTTVK
A5	Alpha 2-HS glycoprotein precursor	99.88%	(Bos Taurus)	QDGQFSVLFTK
A5	Alpha 2-HS glycoprotein precursor	98.96%	(Bos Taurus)	HTLNQIDSVK
A5	Alpha 2-HS glycoprotein precursor	98.96%	(Bos Taurus)	HTLNQIDSVK
A5	Alpha 2-HS glycoprotein precursor	98.00%	(Bos Taurus)	HTFSGVASVESSSGEAF
A5	Serum Albumin (Bos Taurus)	97.45%	(Bos Taurus)	VPQVSTPTLVEVSR

Table 4.2 (Cont.)

A6	alpha-2-HS-glycoprotein precursor	100%	(Bos Taurus)	TPIVGQPSIPGGPVR
A6	alpha-2-HS-glycoprotein precursor	99.91%	(Bos Taurus)	QDGQFSVLFTK
A6	alpha-2-HS-glycoprotein precursor	99.17%	(Bos Taurus)	HTLNQIDSVK
A6	serum albumin precursor	0%	(Bos Taurus)	HLVDEPQNLIK
A6	serum albumin precursor	0%	(Bos Taurus)	YLYEIAR
A6	alpha-1-antiproteinase precursor	95%	(Bos Taurus)	DFHVDEQTTVK
A6	alpha-1-antiproteinase precursor	93%	(Bos Taurus)	NLYHSEAFSINFR

Table 4.2. Sequence Homology of the Putative Receptors. The unknown proteins were labeled A1-A6. The unknown proteins sequences were matched with known protein sequences to identify the function.

CONCLUSION

The purpose of this research was to confirm the identity of the putative receptor for IPNV in Chinook Salmon Embryo cells (CHSE-214), that was reported by Dobos (1995), and to identify and characterize the receptor(s) for IPNV VR299 and Sp serotypes in Rainbow Trout Gonad (RTG-2) cells. We sought to identify and characterize the putative receptors(s) in CHSE-214 and RTG-2 cells by performing several proteomic techniques that include the Virus Overlay Protein Binding Assay and protein sequence analysis.

Our results, which were obtained using the virus overlay protein binding assay, confirmed the results of Dobos (1995) which stated that IPNV serotypes VR-299 interacts with a protein of approximately 220-250kDa in the cell membrane of CHSE-214 cells. Our results also revealed IPNV-VR-299 and IPNV-Sp binding at the 65kDa in CHSE-214 membrane proteins extracts as well.

When the VOPBA was performed using CHSE-214 and RTG-2 cell membrane proteins and a monoclonal antibody generated against VP2 of IPNV, VR-299 and Sp were shown to interact with proteins of approximately 140kDa, 65kDa and 60kDa in CHSE-214 cells. Virus protein interactions were only revealed at proteins of approximately 65kDa and 60kDa for IPNV-VR-299 and Sp in RTG-2 cell membrane proteins. Both VR-299 and Sp serotypes did not show evidence of virus cell membrane protein interaction with the 140kDa protein in RTG-2 cell membrane proteins. Neither VR-299 nor SP showed evidence of binding to the 220-250kDa protein in CHSE-214 and

RTG-2 cells when the monoclonal antibody against the VP2 of IPNV was used as the primary antibody for the VOPBA.

Variations of viral protein interactions revealed by polyclonal and monoclonal antibodies could be due to the virus having more than one epitope that interacts with proteins in the membrane of CHSE-214 and RTG-2 cells. Binding to the 220-250kDa receptor apparently leads to a conformational change in the virus that causes the epitope against which the monoclonal antibody is generated to be unavailable for binding, whereas, binding to the 140kDa, 60kDa, and 65kDa proteins leaves the epitope against which the monoclonal antibody is generated exposed and available for binding. The polyclonal antibody, which contains antibody generated against all of the epitopes of the virus, can bind to viral epitopes that remain exposed after the virus binds to the 220-250kDa protein

We completed our study by sequencing the putative viral receptors using Mass Spectrometry. We were able to identify protein sequence homology between the putative receptors and known proteins by searching several databases. We concluded that the functions of our putative receptors may be the same or similar to the homologous proteins. However, we do not have enough data to definitively support this conclusion. Furthermore, by identifying the function of similar proteins, several high throughput techniques can be performed to make further analysis of the putative receptors such as: small interfering RNA (siRNA), which can be used to identify gene function; microRNA (miRNA), which can be used to silence genes and further understand their function; and cloning of the gene for the putative receptors for further studies of the protein structure

and identification of binding sites in the protein structure. This knowledge would increase our ability to design vaccines and to develop other methods of inhibiting both horizontal and vertical transmission of the virus.

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