ABSTRACT

MILLER JR, OTIS. Molecular Epidemiology of Outbreaks of Spring Viremia of Carp Virus in North America, Europe and Asia. (Under the direction of F. J. Fuller.)

Spring Viremia of Carp Virus (SVCV) or *Rhabdovirus carpio* is the causative agent of the fish disease, Spring Viremia of Carp (SVC). Genetic relationships between 35 spring viremia of carp virus (SVCV) genogroup Ia isolates were determined based on the nucleotide sequence of the phosphoprotein (P) gene and glycoprotein (G) genes. Phylogenetic analysis based on P-gene sequences revealed two distinct subgroups within the SVCV genogroup Ia, designated SVCV Iai and Iaii, and suggests at least two independent introductions of the virus in the USA in 2002. Combined P - and G - sequence data support the emergence of SVCV in Illinois, USA and Lake Ontario, Canada, from the initial outbreak in Wisconsin, USA, and demonstrate a close genetic link to viruses isolated during routine import checks on fish brought into the UK from Asia. The data also showed a genetic link between SVCV isolations made in Missouri and Washington, USA, in 2004 and the earlier isolation made in North Carolina, USA, in 2002. However, based on the close relationship to a 2004 UK isolate, the data suggests that the Washington isolate represents a third introduction into the US from a common source, rather than a re-emergence from the 2002 isolate. There was strong phylogenetic support for an Asian origin for 9 of 16 UK viruses isolated either from imported fish, or shown to have been in direct contact with fish imported from Asia. In one case, there was 100% nucleotide identity in the G-gene with a virus isolated in the Peoples Republic of China.

The remaining portion of this dissertation deals with biosecurity on ornamental fish farms.

A good biosecurity program results in control of disease transmission by eliminating pathogen introduction onto farm(s) and dissemination between farms or between ponds on any farm. Each method of possible pathogen movement to new susceptible animals is addressed by a biosecurity plan and prevention is implemented by education and appropriate actions of employees. A biosecurity plan seeks to control: 1. people carrying pathogens on person including visitors and employees, 2. inspection and quarantine of incoming fish, 3. contaminated inanimate objects like vehicles, nets, and other equipment, 4. wild animals that may act as vectors for the pathogen, and 5. water source. Daily operations of a farm must focus on these controls to prevent disease incidence, or if disease has already occurred, its spread. This paper will introduce, through phylogenetic analyses, countries of interest for disease risk to SVCV and introduce a risk assessment tool that can identify biosecurity measures for establishing a biosecurity plan suitable for the control and prevention of one of the industries' disruptive and economical devastating infectious disease, spring viremia of carp.

MOLECULAR EPIDEMIOLOGY OF OUTBREAKS OF SPRING VIREMIA OF CARP VIRUS IN NORTH AMERICA, EUROPE AND ASIA

by

OTIS MILLER, JR.

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

COMPARATIVE BIOMEDICAL SCIENCES

Raleigh, North Carolina

2007

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DEDICATION

And in the end, it's not the years in your life that counts.
It's the life in your years.
-Abraham Lincoln

It is my honor to dedicate this dissertation to my family.

The numerous occurrences that the obtaining of this degree has directly impacted their lives is worthy of recognition. To my wife and sons, first cousin, who is more like a sister, my wife's mother, my parents, although they did not see me earn this degree on this side, these are the ones responsible for instilling in me the desire for education.

Ultimately, I thank my LORD and SAVIOR JESUS CHRIST. I know, more than anyone else, without HIS divine intervention, favor, and supernatural ability I would not have been able to pass these tough courses, find a research proposal with funding and end up with such a fine graduate committee and a great advisor. It is not easy earning a PhD and when you consider that I was 50 years old when I entered into this program and now I am 53, this is amazing indeed. This is HIS amazing grace upon my life. And I am most grateful for HIS kindness shown to me and my family.

In Christ Jesus, the most precious name.

BIOGRAPHY

Otis Miller Jr., is a Tuskegee University Veterinary Medical Doctor graduate who has served in small animal medicine and various USDA agencies for over 25 years. He will earn a Ph.D in aquatic epidemiology on August 8, 2007. He has earned a Masters of Science in avian medicine and is a Senior Executive Service candidate currently serving as a Regional Aquatic Animal Epidemiologist in the Animal and Plant Health Inspection Service, Veterinary Services Eastern Regional Office in Raleigh, NC.

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Miller and Cipriano 2003. International response to infectious salmon anemia: prevention, control, eradication: proceedings of the symposium; 3-4 September; New Orleans, LA. Tech Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service. 194 p.

Miller O, Fuller FJ, Gebreyes WA, Lewbart GA,& eight others 2007. Phylogenetic analysis of spring viremia of carp virus reveals distinct subgroups with common origins in North America and the United Kingdom. Diseases of Aquatic Organisms. 76:193-204

Miller 2007. A biosecurity risk assessment tool for ornamental fish farms. Global Aquaculture Alliance Magazine. Submitted for publication.

ACKNOWLEDGMENTS

I sincerely thank USDA for Grant no. 52533 that funded this research and to F.J Fuller, W.A. Gebreyes, G.A. Lewbart, J.F. Levine, P. Dixon, D.M. Stone, J. Thorne, R. Anholt, B. Wiegmann, C. and V. Soden, and D. Franco.

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1. INTRODUCTION

The need to prevent the introduction of transboundary aquatic animal pathogens will escalate with increased globalization of trade of live aquatic animals and their products (FAO 2006).The European Union is the largest importer, followed by the United States, developing countries, and Japan in the trade of fish and their products. However, developing countries alone export almost half of the fish trade volume (Figure 1).

Each year more than 1 billion ornamental fish, consisting of over 4000 freshwater and 1400 marine species are traded internationally (Whittington and Chong 2007). The US imports $44 million of ornamental fish annually (FAO 2006). In 2004, the production value of world aquaculture was $70.3 billion and of that amount $16.3 billion were from the sale of cyprinids, the largest freshwater fish species group (FAO 2006) (Figure 2). This cyprinid group is also the most susceptible to Spring Viremia of Carp (SVC). The purpose of the literature review in chapter one is to provide the most current knowledge of SVC with emphasis

on molecular epidemiology . The dissertation's focus, contained in chapter 2, pertains to the use of molecular epidemiology, DNA sequencing and phylogenetic analysis to obtain characterizations that identify specific genotypic strains as likely trace sources of SVC outbreaks. Finally, a component of epidemiology is included that deals with intervention strategies and mitigation techniques. Recommendations to prevent introductions and control the spread of SVCV from international and domestic shipments of SVC[1] susceptible fish are outlined in chapter 3.

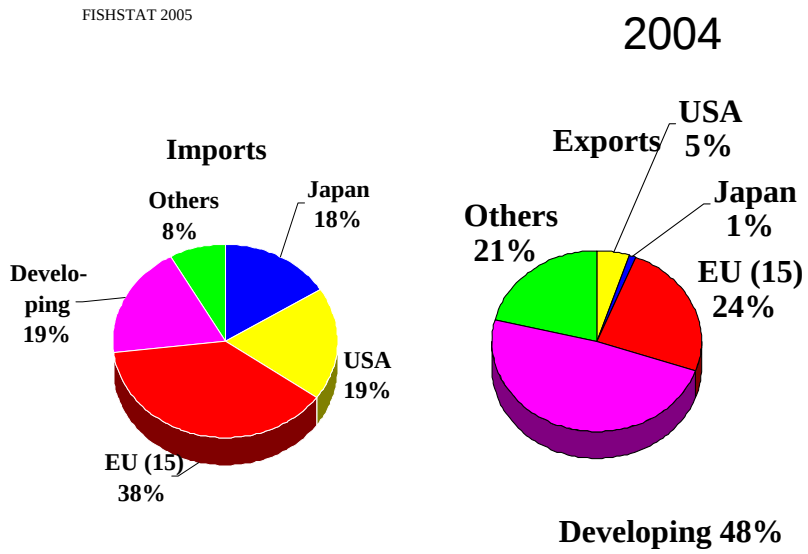

Figure 1.International fish trade and the impact of export from developing countries (FAO 2005).

1 In this dissertation SVC refers to the disease and SVCV refers to the virus.

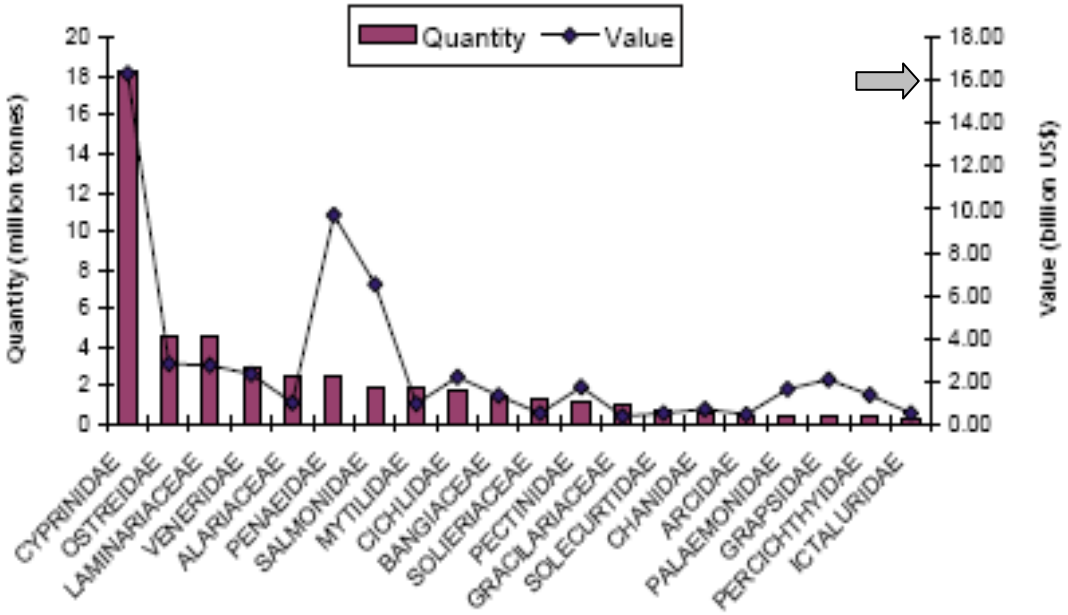

Figure 2. Top taxonomic families used in global aquaculture (production of more than 250 000 tonnes) with corresponding values in 2004.
Source: FAO 2006 State of World Aquaculture

LITERATURE REVIEW

"The use of sound epidemiological principles and logical and science-based approach to identify and manage risks comprise two of the most important components of an effective biosecurity program." Rohana Subasinghe (Subasinghe 2005)

GEOGRAPHICAL DISTRIBUTION OF SPRING VIREMIA OF CARP

Spring viremia of carp virus (SVCV) or *Rhabdovirus carpio* has been isolated during outbreaks of disease in more than 30 countries including the US (Fijan et al. 1971, World Organization for Animal Health [OIE] 2006a). The disease is endemic in parts of continental Europe, Russia, and states of the former USSR (Figures 3, 4). Estimates in Europe alone suggest annual losses attributable to SVC of around 4000 metric tons (Fijan 1999; Zhang 2002). Mortality in young carp can be as high as 70 percent and the severity of the disease is reflected in its inclusion on the OIE list of notifiable viral diseases of finfish.

The Office International des Epizooties (OIE) now recognized as the World Organization for Animal Health is intergovernmental, created in 1924, and currently includes 167 member countries. It has the mandated responsibility under the World Trade Organization, and the Application of Sanitary and Phytosanitary Measures to safeguard world trade by publishing health standards for international trade in animals and their products. The OIE ensures transparency in the reporting of global animal disease situation through the reporting of animal diseases detected in member countries (http://www.oie.int/eng/OIE/en_about.htm?e1d1).

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"First report" introductions of SVC to the OIE occurred, in Spain 1991, in Switzerland 2001, in the US 2002, in Denmark 2002, in Moscow Province 2003, in China 2004, and in Canada 2006 (Marcotegui et al. 1992; Bernet 2002; Goodwin 2002; Dikkeboom 2004; Liu et al. 2004; Shchelkunov et al. 2005a;

http://www.oie.int/wahid-

prod/public.php?page=disease_immediate_summary&selected_year=2006).

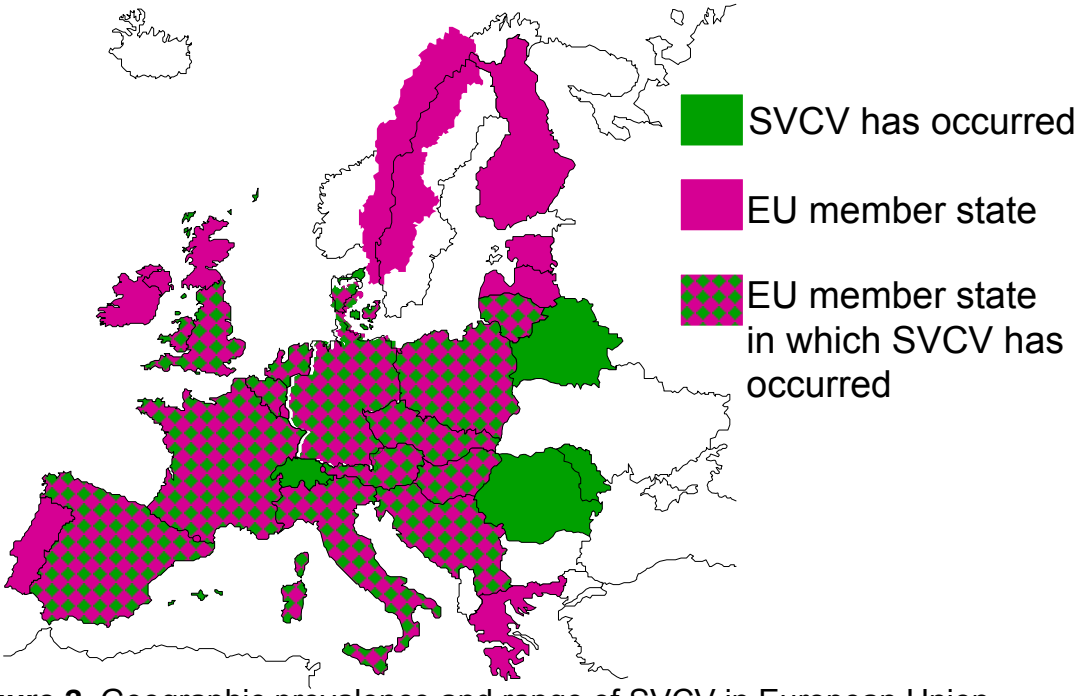


Figure 3. Geographic prevalence and range of SVCV in European Union. Courtesy of P. Dixon.

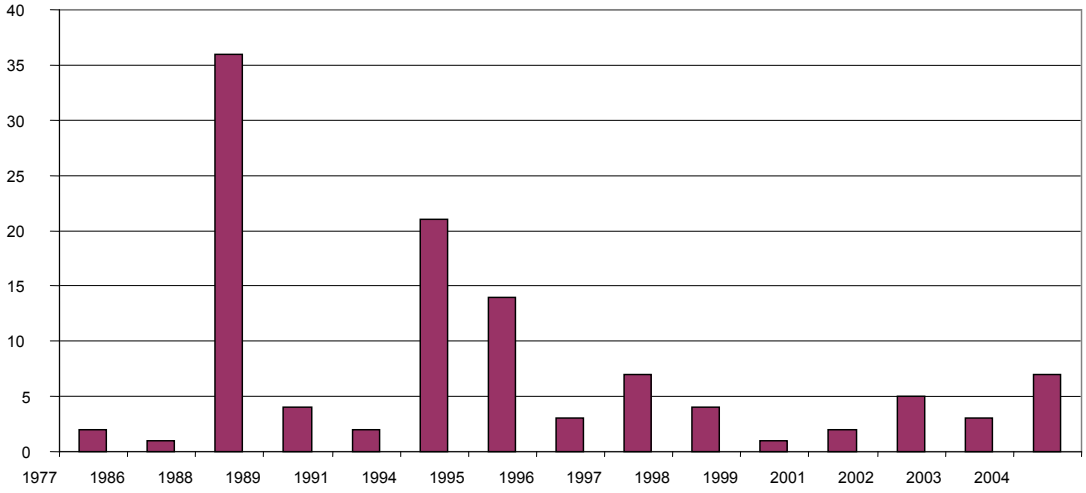


Figure 4. Number of new cases of SVCV in United Kingdom 1977-2004

According to OIE (2006a) from 2002 to 2006 there have been five US cases of SVCV reported in the US. Between 2002 and 2004, four outbreaks of SVC in koi and goldfish were reported in the US in breeder farms in North Carolina and Virginia, (July 2002), in a public lake (Cedar Lake experienced a 10 ton die-off of common carp) in Wisconsin (August 2002), in a backyard pond in Washington (June 2004), and in koi breeder farms in Missouri (July 2004). The fifth case of SVCV occurred from routine surveillance in a non-clinical case of common carp found in the Cal Slag Illinois River (Summer 2003). Subsequently, on June 28, 2007, APHIS confirmed SVCV in the upper Mississippi river between Onalaska Wisconsin and Dresbach, Minnesota from feral common carp samples submitted by the US Fish and Wildlife Service who were investigating a carp kill that occurred on May 7, 2007 (USFWS 2007-www.fws.gov/midwest/news/release07-68.html, Startribune 2007-www.startribune.com/531/v-print/story/1277118.html).

In the US, the 2002 SVC outbreak costs for eradication, depopulation, cleaning and disinfection, surveillance and indemnity reached approximately $11 million (Bondad-Reantaso et al. 2005). Affected and unaffected countries must be keenly aware of the exportation and importation risks (circulating strains of SVCV) and take steps to proactively minimize the spread of the disease through international movements of SVCV susceptible fish.

PATHOGENICITY OF SVCV

Spring Viremia of Carp Virus (SVCV) or *Rhabdovirus carpio* is the causative agent of the fish disease, Spring Viremia of Carp (SVC) (Fijan et al. 1971). *Rhabdovirus carpio* belongs to the genus *Vesiculovirus*, from the family *Rhabdoviridae*. The family *Rhabdoviridae* contains the genera *Vesiculovirus, Lyssavirus, Ephemerovirus, Cytorhabdovirus, Nucleorhabdovirus* and *Novirhabdovirus* (Tordo et al. 2005).

Although the disease was first described in Yugoslavia in 1971 by Fijan, there is evidence that the disease, which affects mainly common carp, and ('nishikigoi') koi carp (*Cyprinus carpio),* has been present in Europe for at least 50 years. Before SVC was recognized as a disease and diagnosed primarily in cyprinid fish capable of causing high morbidity and mortality in other susceptible fish, it was variously called, infectious dropsy, infectious ascites, hemorrhagic septicemia, or rubella (Ahne and Wolf 1977; Wolf 1988; Ahne et al. 2002). The virus is readily shed in feces, urine, and gill mucus of infected fish and by carriers. It is environmentally stable, and possibly transferred between surface waters and aquaculture farms by waterfowl, fish parasites, and fomites (Fijan et al. 1971; Fijan 1988; Fijan 1999; Ahne et al. 2002). The clinical signs of SVC are not pathognomonic and could be observed in any 'sick' fish. However, more commonly seen are, changes in behavior patterns, darkening of skin, swollen abdomen (from ascites), exophthalmia, hemorrhages in skin, gills and anterior eye chamber, anemia and pale gills, and a protruding vent with fecal casts (Fijan 1999) (Figures 5,6).

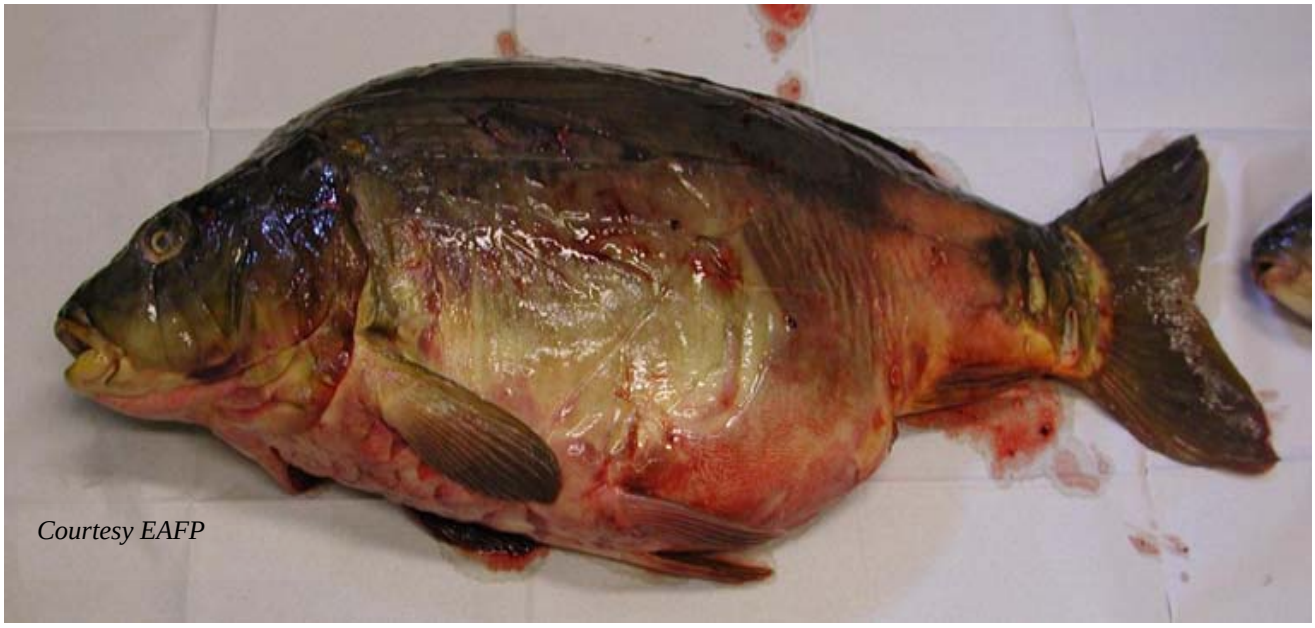


Figure 5. External clinical signs of SVC show extensive hemorrhage.

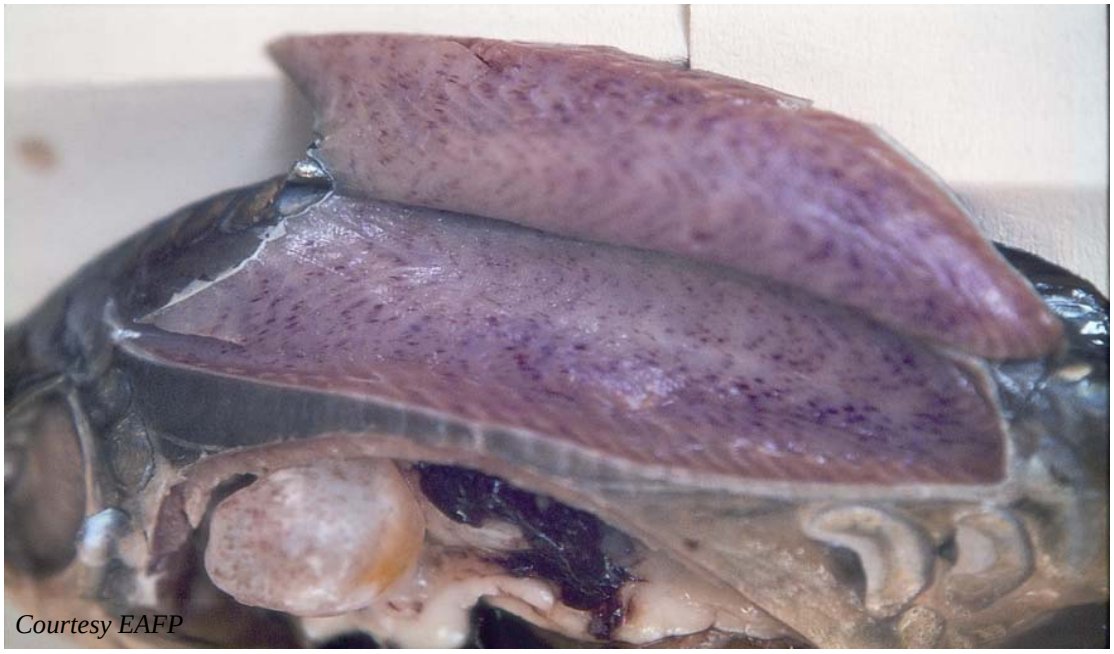


Figure 6a. Internal clinical signs of SVC showing edema, and hemorrhages of organs.

Figure 6b. Affected swim bladder showing mucosal hemorrhages.

Temperature and seasonality. Research has shown that optimal temperatures for virus replication in experimentally infected carp are between 16 and 17° C and in natural infections between 11-15° C (Fijan et al. 1971; Fijan 1988; Fijan 1999; Sanders et al. 2003). At these experimental temperatures (16 and 17° C), 90% of fish die within 5 to 17 days after being infected. At 11-15° C, the percent of fish mortality was similar but the mortality was delayed (2-3 weeks). Mortality was reduced at temperatures between 17 and 26° C. The optimum temperature *in vitro* virus replication is 20-22° C (Ahne et al. 2002). Other experiments have investigated the influence of increasing and decreasing temperatures on the rate of disease in carp (Fijan 1999) and zebra fish as a model for SVC (Sanders et al. 2003). Baudouy et al. (1980) demonstrated that a gradual decrease of temperature (11° down to 5° C) caused low mortality while increasing temperature back to 20° C caused very high mortality.These laboratory results correspond with the field observations that most SVCV outbreaks occur in the spring with warming temperatures.

When water temperatures rise above 15-18° C, carp produce interferon and neutralizing antibodies that suppress viral replication (Kennedy-Stoskopof 1993). Thus in the countries where SVCV has been reported, there are only sporadic reports in June and July (Fijan 1999). The temperature constraints make tropical and subtropical climates unfavorable for SVC outbreaks. The replication of virus as temperatures rise also has implications for detecting virus in fish populations. Beskesi and Csontos (1985) noted that all of their viral isolation were from

samples taken in May when the water temperature was between 10 to 19° C. Although new SVCV infections typically occur in the fall and winter during decreases in both water temperatures and immune system activity, virus isolation likely would be more difficult during the summer (due to active immune systems that reduce virus) and winter seasons (due to low levels of virus replication) of the year (Ahne and Wolf 1977; Ahne 1978; Ahne 1986; Ahne et al. 2002).

PROPERTIES OF SVCV

SVC virus particle structure. Spring viremia of carp virus has the typical rhabdovirus bullet-shaped morphology with an inner nucleocapsid measuring about 50 nm in diameter. The virion particle measures about 80-180nm in length and 60-90 nm in diameter (Figure 7). *Rhabdovirus carpio* can remain viable for longer than 4 weeks in 10° C water and more than 6 weeks in pond mud at 4°C. However, inactivation of the virus can occur in 30 minutes at 60° C, in 10 minutes by pH 12, in 3hrs by pH 3, in 10 minutes by formalin (3%), chlorine (500 ppm), iodine (0.01%), NaOH (2%), UV (254nm) and gamma irradiation (103 krads) (Ahne 1976,1982, Ahne et al. 2002).

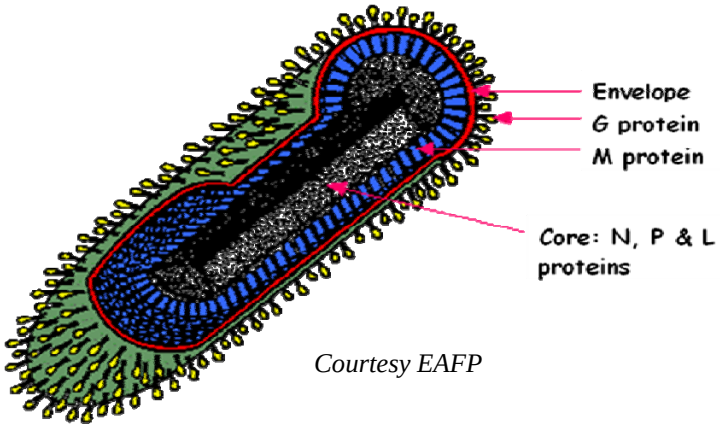


Figure 7. SVC virion showing location of proteins.

SVCV GENOME

Molecular analysis of the genome of spring viremia of carp virus (SVCV) identified the virus as a single molecule of linear, negative sense (*negative:* implies that the RNA needs to first undergo transcription and then translation before being used to form structural proteins and enzymes), single stranded RNA containing five major open reading frames (ORFs) encoding predicted proteins to five major genes in the order 3'N (nucleocapsid)-P (phosphoprotein)-M (matrix)-G (glycoprotein)-L (polymerase) 5' (Ahne et al. 2002) (Figure 8).

Further work along these lines resulted in the sequencing of the entire SVCV genome. It is 11,019 nucleotides (nt) in length (Hoffmann et al. 2002). This allowed SVCV to be distinguished from other fish rhabdoviruses in different genera and to demonstrate similarities of the virus to the prototype rhabdovirus, vesicular stomatitis virus. Phylogenic analyses using the full amino acid sequences of the five proteins demonstrated the close relationship to the

Vesiculovirus genus (Bjorklund et al. 1996; Johansson et al. 2001; Hoffmann et al. 2002, Teng t al. 2007).

Figure 8. SVCV RNA genomic organization (11019 nt).

Glycoprotein gene. The glycoprotein (G) gene of SVCV is 1588 nt long, encodes 509 amino acids (aa), forms trimeric peplomers (spikes) on the virus surface that bind to cellular receptors and induce uptake of virus particles by endocytosis (Coll 1995; Oreshkova et al. 1995; Bjorklund et al. 1996; Ahne et al. 2002). Hill et al. (1975) demonstrated that the glycoprotein surface protein determines the serologic properties of rhabdoviruses. Research results using the G gene include development of new molecular diagnostic tools, taxonomic groupings, molecular characterizations and phylogenic analysis of rhabdoviruses.

Phosphoprotein gene. The P gene is 930 nt long, extends from positions 1407 to 2336 with respect to the SVC genome and encodes a protein of 309 amino acids (aa). The phosphoprotein P (formerly NS) is a component of the rhabdovirus nucleocapsid that, in association with L and N proteins, is required for transcription and replication, whereas the G and M proteins are not essential for these functions (Emerson and Yu 1975; Roy 1981; Banerjee 1987; Oreshkova et al. 1999; Ahne et al. 2002; Shchelkunov et al. 2005b). While the N gene is highly conserved, a higher degree of genetic variation is found in the P

gene. In comparison, G and P gene nucleotide differences between base pairs of European and Asian sequences of SVCV (Table 1) were 7.52 and 10.54 % while their amino acid differences were 4.52 and 9.70%, respectively (D. Stone unpubl. data). The P gene is also the least conserved among *Lyssavirus* genes, and the genes of VHSV strains. Therefore, analysis of the P gene could provide a higher degree of virus strain differentiation for epidemiological study than either the G or N genes of SVCV.

Table 1. Percent nucleotide and amino acid differences between the European and Asian SVCV gene sequences**.**

Gene	base pairs	No of differences bp	% Difference
N	1257	68	5.44
P	930	98	10.54
M	672	44	6.60
G	1530	115	7.52

Gene	Amino acids	No of differences AA	% Difference
N	418	9	2.20
P	309	30	9.70
M	223	5	2.24
G	509	23	4.52

Courtesy of D. Stone.

GENOTYPIC STRAIN TYPING

Compared to phenotypic strain typing, genotypic strain typing offers the advantages of typeability, reproducibility, discriminatory power, and high throughput (Riley 2004). Genotypic strain typing is also the least affected by growth conditions and laboratory manipulations, while including the use of basic established analytic procedures such as gel electrophoresis, hybridization and nucleic acid sequencing. Nuclei acid content and gene sequence polymorphisms are the basis used in analysis of nucleic acid sequence differences between strains (Riley 2004). However, in order to use nucleic acid sequencing several major steps must precede them. They are (i) propagation of virus,(ii) extraction of viral RNA, (iii) reverse transcription, (iv) polymerase chain reaction (PCR), (v) TA cloning and (vi) and plasmid purification. After these steps and procedures are completed, the final step, phylogenetic analysis can be performed using the obtained DNA sequences.

MOLECULAR EPIDEMIOLOGY

Molecular epidemiology is the study of the distributions and characterization of determinates of disease that utilizes molecular biology methods. The focus is not merely taxonomy nor phylogeny by itself but the organism itself and its interactions with the host and the environment (Riley 2004). Molecular epidemiology has become an essential tool in disease surveillance and investigations of outbreaks, due to its ability to trace and identify possible infection sources (Ostroff 1999). More molecular biology techniques are being

used to reduced detection time, account for limited resources and to analyze data at levels not previously available.

Molecular epidemiology has become well established and plays a role in most infectious disease outbreak investigations (Ostroff 1999). For example, Bastos et al. (2003) conducted molecular epidemiological studies using 42 South African Territories type 3 foot and mouth disease virus isolates representing seven eastern and southern African countries and traced the origin for outbreaks of FMD in cattle to certain African buffalo from specific geographical regions. In the U.S., Frankhauser et al. (1998) used molecular data to confirm and/or dispute classic epidemiologic investigations from 90 nonbacterial gasteroenteritis outbreaks analyzed to characterize Norwalk-like viruses and determine their strain distribution and biogeography. Future molecular characterizations of pathogens to determine specific genotypic strains as likely trace sources of outbreaks should prove extremely useful in aquatic epidemiological investigations.

Molecular techniques for SVCV. PCR assays, RT-PCR, nucleotide sequencing, ribonuclease protection assays, hybridization, and phylogenetic analysis are the more common methods of molecular techniques used to describe SVCV. Molecular epidemiology allows the use of genetic characterization of SVCV strains to make geographical assignments of subtype and calculation of the degree of relatedness between isolates. Few reports are published characterizing sequence diversity between strains; this is considered

an area of active investigation in several labs and presents opportunities for future epidemiological research (Ahne et al. 2002, Stone et al. 2003).

The majority of prior studies have concentrated on the glycoprotein G gene for diagnostic purposes, vaccine development, and taxonomy relationships, (Coll 1995; Bjorklund et al. 1996; Johnson et al. 1999; Oreshkova et al. 1999; Johansson et al. 2001; Ahne et al. 2002; Koutna et al. 2003; Stone et al. 2003; Liu et al. 2004). This is mainly because the G gene is responsible for reacting with neutralizing antibodies, and for virus attachment to cell receptors. However, characterizing the genetic diversity of the entire P gene using, virus purification, RNA extraction, RT-PCR methods, nucleotide sequencing and phylogenetic analysis to isolate and identify the P-gene of the various outbreaks of putative isolates of SVCV both domestic and internationally has not been done.

Phylogenetic analysis. Phylogenetic analysis is a powerful tool for analyzing genotypic data essential for molecular epidemiology (Hall and Barlow 2006). Phylogenetics is the study of evolutionary relatedness among various groups of organisms (e.g., species, populations). Phylogeny is the study of lines of descent or the origin and evolution of a set of organisms, usually a set of species. When genotyping data is combined with phylogenetic analysis using nucleotide sequence data, the order of descent of related strains can be determined (if the phylogenetic tree is rooted). This is useful for molecular

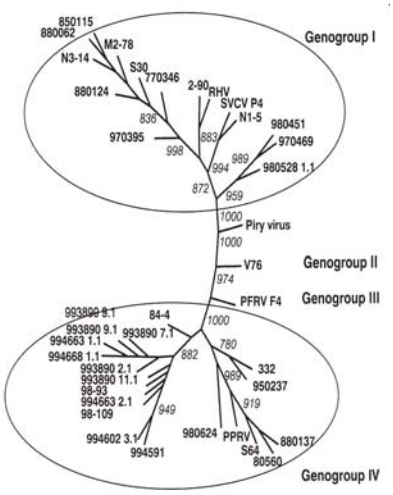
epidemiology, for often the strain or source of an outbreak of an infectious disease can be traced or identified (Hall and Barlow 2006).

Misinterpretation of phylogenetic trees can occur when recombination is present. True intermolecular recombination occurs frequently with certain RNA viruses but infrequently with others. This recombination could result in a tree displaying bifurcations rather than networks leading to a false conclusion about the organisms' relatedness. Based on the literature, intermolecular recombinations of negative strand RNA genomes (particularly rhabdoviruses) are rare (Nadine-Davis 2000; Kurath et al. 2003; Hall and Barlow 2006).

Molecular epidemiology of SVCV in Europe, and Asia. Studies conducted to date have largely focused on analysis of the glycoprotein (G) gene for characterization of SVCV (Johnson et al. 1999; Oreshkova et al. 1999; Ahne et al. 2002; Koutna et al. 2003; Stone et al. 2003; Liu et al. 2004). Johnson and co-workers (1999) demonstrated that rhabdovirus of penaid shrimp found in Hawaii was virtually identical to the European isolate of SVCV. Phylogenetic analysis of the G gene of SVCV by Liu et al. (2004) and Warg et al. (2007) confirmed the work of Stone et al 2003 as to designation of the Asian genogroup Ia for SVCV isolates from outbreaks in P.R. China and the US, respectively.

Glycoprotein gene. A phylogenetic analysis of 36 partial 550 bp G gene sequences of SVCV and pike fry rhabdovirus (PFRV) from a variety of fish species (Stone et al. 2003) identified four distinct genogroups (I to V) (Figure 9A). In Stone's study, all 15 SVCV isolates were designated as a group (I) that could be further sub-divided based on geographic origin of the isolates examined (Ia to d). Asian isolates were classified within group Ia. Moldovan, Ukrainian and Russian isolates were assigned to genogroups Ib and Ic, while isolates presumably from the UK and other European countries were classified in genogroup Id (Figure 9B) (Way et al. 2003; Hoffmann et al. 2005).

A



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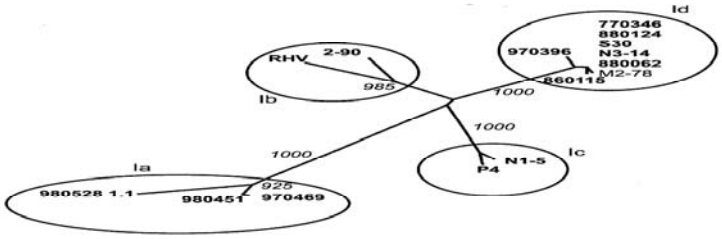

Figure 9. Phylogenetic trees (550bp G gene) generated by **(A)** maximum parsimony method showing 4 genogroups: I SVCV, II PFRV V76, III PFRV F4, IV PFRV and (**B**) Neighbor-Joining method showing 4 subgenogroups (1a to1d) of SVCV genogroup I. Used by permission of DOA (Stone et al. 2003).

Nucleoprotein gene. Other SVCV genes however, offer discriminatory potential and additional evidence for phylogenetic analysis. Indeed, using the SVCV N gene as a target, Shchelkunov et al. (2005b) developed a restriction fragment length polymorphism assay (RFLP analysis) that distinguished two major and several intermediate minor genogroups among more than 20 European SVCV isolates. A clear geographic correlation was found for some of the genogroups.

Phosphoprotein gene. Additional researchers have amplified the nucleoprotein N gene, and the polymerase L gene. The first characterization of SVCV RNA was performed with sequences of the matrix protein M gene in 1984 (Kiuchi and Roy 1984; Bjorklund et al. 1995; Oreshkova et al. 1999; Johansson et al. 2001; Bourhy et al. 2005; Shchelkunov et al. 2005b). At this writing, only one researcher (Hoffmann et al. 2005), was recorded in GenBank with publications using the complete SVCV phosphoprotein P gene.

Prior studies highlight the genetic diversity that exists among SVCV isolates and suggest that further molecular analyses of the SVCV P gene are needed to differentiate the strains that have been isolated and to determine the feasibility of using the P gene in comparison to the G gene for inferring phylogenetic analysis and epidemiologically useful information.

Molecular epidemiology of SVCV in US. Histological samples from a wild common carp kill in the Pentenwell Flowage Wisconsin River in 1989 produced RT-PCR products that indicated the presence of a SVCV virus of European

genotype (subgenogroup 1d) (Le Deuff, Marcquesnski and Dixon 2003, unpublished). Johnson and co-workers. (1999) also reported a European genotype SVCV from penaeid shrimp in Hawaii. This demonstrated that different strains (besides genogroup Ia) of SVCV exist in the US, suggesting further independent introductions of this virus.

In a recent publication, Warg et al. (2007) phylogenetic analysis of the G gene of five US isolates of SVCV, confirmed the work of prior genogroup designations (Stone et al. 2003). While partial P, M, N, and complete G genes were used in constructing the primers, only the 550 bp of G gene was used for inferring the phylogenetic tree. The US isolates were all from Asian genotype, (subgenogroup 1a). However, the bootstrap support values for this tree were less than 70%, not allowing identification of further subgroups in genogroup Ia.

PREVENTION AND CONTROL OF SVCV

Biosecurity and aquaculture. Biosecurity plays a very important role in prevention and spread of disease, knowledge of likely geographical disease-risk sources coupled with health certifications demonstrating freedom of disease from SVCV risk areas would be important in preventing new SVCV introductions.

The USDA APHIS was unable to recover SVCV from their volunteer SVCV susceptible broodstock surveillance survey covering over 30 States since its inception in 2002 (Miller unpubl. data). However, SVCV outbreaks have occurred

after 2002 in SVCV susceptible broodstock outside of the USDA surveillance survey.

The UK regulates the importation of fish, and the only hosts of SVCV that can be legally imported are ornamental fish such as goldfish, koi carp. There has been SVCV surveillance in the UK since the first major outbreak in 1988.

Currently, the majority of international trade of SVCV-susceptible fish consists of imports into the Europe Union and the US. Asia contributes over 90% of the world aquaculture production (Bondad-Reantaso et al. 2005). Identifying trace country of origin from point source outbreaks could avert further introductions and spread of SVCV. Thus, the recent implementation of the USDA APHIS interim rule (a rule made effective prior to public comment due to the urgency of the issue), in the autumn of 2006 establishing SVC import protocol, as other countries require, could reduce importation of SVCV infected fish into the US (*Federal Register* 2006). The US import protocol requires that "live fish, fertilized eggs or gametes of SVC-susceptible species must be accompanied by an import permit issued by APHIS and must be imported within 30 days of the proposed arrival date stated in the import permit."

Given the information above, along with the increased globalization of trade of live aquatic animals and their products, aquaculture has the capability to contribute to the transmission of pathogens or diseases that are highly

contagious with the potential for very rapid spread across national borders. Inadequate or poorly implemented biosecurity measures have led to significant losses due to aquatic animal diseases around the world. For example, Japan-Koi herpes virus (KHV), $16.4 Million; Ecuador-Whitespot disease (WSD), $280.5M; USA-SVC,$11.7M; and USA-Infectious salmon anemia (ISA), 8.3M (Bondad-Reantaso et al. 2005). An increased need for improved biosecurity will parallel the rising intensification and commercialization of aquaculture development. This is called transboundary aquatic animal disease/pathogens (TAAPs/TAADs) similar to transboundary animal disease in terrestrial livestock or (TADs) (Bondad-Reantaso et al. 2005). As a result, "APHIS concluded that the SVC outbreaks in US farmed fish were linked to the importation of SVC-infected fish (*Federal Register* 2006)."

The US with its mainly open aquatic import policies, huge seafood trade deficits and fish farmers who may not be convinced about either the cost benefit or the effectiveness of certain biosecurity measures (Delabbio et al. 2005), should increase international regulatory oversight. Import protocols, inspections and quarantines to minimize introductions of foreign aquatic pathogens should be implemented for aquatic species. Aquaculture in the US needs more infrastructure investment, more incentives for disease control programs, and adequate indemnity for required depopulations. Sound research that demonstrates the effectiveness' of biosecurity methods, and less domestic regulatory oversight e.g., streamlining the permitting process for a fish farmer to

operate a production facility), especially when that oversight is duplicative is also needed at the federal, State, and local levels.

SVC-Free aquaculture establishments. The OIE has specific criteria for declaring countries, zones and aquaculture establishments free of SVC. The International Aquatic Animal health Code and the Diagnostic Manual for Aquatic Animal Diseases have complete details on all of the requirements; some of the general criteria are presented here. A country declared free must meet these conditions: (1) no recorded outbreaks of SVC for at least 2 years; (2) no detection of virus in any of the susceptible fish species tested during an official surveillance scheme during the past 2 years; and (3) requirements met for importing live fish from other countries (OIE 2006b,OIE 2007).

For a zone to be declared free of SVC both aquaculture establishments and wild populations containing susceptible fish species must have been tested in an official surveillance scheme and SVC must not have been detected in the past 2 years.

The zone must also be one or more entire water catchment areas or be part of a catchment's areas where upstream migration of fish from downstream areas cannot occur.

For an aquaculture establishment to be declared free of SVC, it may be part of a free country or zone. An aquaculture establishment in an infected area can still be declared free if it (1) has been tested under an official health surveillance

scheme for at least 2 years without detection of SVCV; (2) is supplied by water from a spring, well or borehole only and is free from wild fish; and (3) is not connected to a watercourse or there is a natural barrier that prevents the migration upstream of fish from downstream stretches of the waterway.

The OIE Diagnostic Manual for Aquatic Animal Disease has specifications for surveillance programs to achieve and maintain SVC-free health status. Briefly, fish culture units on aquaculture establishments must be inspected twice annually for 2 years. Each inspection should be conducted in order to detect 2 percent prevalence with 95 percent confidence level. This represents collection of approximately 150 appropriate-age fish at times of the year clinical signs are most likely to be observed and isolating pathogens is the easiest. To maintain free status, twice-yearly inspections continue at a sample collection reduced to 30 fish. Wild fish populations require 150 specimens collected once a year for 2 years (OIE 2007). Additional recommendations for preventing and controlling SVC from becoming established on commercial farms are detailed in chapter 3 of this dissertation.

Vaccines for SVCV. Although, the future of SVCV DNA vaccines hold promise (Kim et al. 2000; Kanellos et al. 2006), currently, there is no commercially available vaccine to protect against SVC. In Canada, the first commercial DNA fish vaccine against infectious hematopoietic necrosis virus (IHNV) is now available (Adams and Thompson 2006). In the US, the Veterinary Services,

APHIS, USDA Center of Veterinary biologics (CVB) assures that “veterinary biologics for commercial use are produced at a USDA-approved establishment, and be demonstrated to be pure, safe, potent, and efficacious.” Currently, there is only one licensed biologic for fish, a killed viral vaccine against ISA, approved by CVB.

Ironically, the first viral fish vaccine, commercially produced by Bioveta, was against SVCV in 1982. However, this inactivated SVCV vaccine is no longer commercially available (Dixon 1997). Worldwide, there are 6 commercial vaccines that are currently available: infectious pancreatic necrosis virus, pancreas disease virus, ISA, grass carp hemorrhage disease virus, red sea bream iridoviral disease, and IHNV (Sommerset et al. 2005). Twenty-five years later, considering the substantial amount of research and the availability of an acceptable zebrafish model (Sanders et al. 2003), a commercial vaccine for this disease should emerge.

Since there is no treatment for this disease, a safe, potent, and efficacious SVC vaccine would greatly assist the establishment of an effective control program against SVCV. Vaccination programs for broodstock, fingerlings and incoming SVC susceptible fish could be developed that would ensure increased production and yield without risk from the introduction and spread of SVCV.

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2. PHYLOGENETIC ANALYSIS OF SPRING VIREMIA OF CARP VIRUS REVEALS DISTINCT SUBGROUPS WITH COMMON ORIGINS FOR RECENT ISOLATES IN NORTH AMERICA AND THE UNITED KINGDOM

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Published in international journal *Disease of Aquatic Organisms*

ABSTRACT

Genetic relationships among 35 spring viremia of carp virus (SVCV) genogroup Ia isolates were determined based on the nucleotide sequences of the phosphoprotein (P) gene and glycoprotein (G) genes. Phylogenetic analysis based on P gene sequences revealed two distinct subgroups within the SVCV genogroup Ia, designated SVCV Iai and Iaii, and suggests at least two independent introductions of the virus into the USA in 2002. Combined P and G sequence data supports the emergence of SVCV in Illinois, USA and in Lake Ontario, Canada, from the initial outbreak in Wisconsin, USA, and demonstrate a close genetic link to viruses isolated during routine import checks on fish brought into the UK from Asia. The data also showed a genetic link between SVCV isolations made in Missouri and Washington, USA, in 2004 and the earlier isolation made in North Carolina USA, in 2002. However, based on the close relationship to a 2004 UK isolate the data suggest that the Washington isolate represents a third introduction into the US from a common source, rather than a reemergence from the 2002 isolate. There was strong phylogenetic support for an Asian origin for 9 of 16 UK viruses isolated either from imported fish or shown to have been in direct contact with fish imported from Asia. In one case, there was 100% nucleotide identity in the G gene with a virus isolated in China.

KEY WORDS: Spring viremia of carp. SVCV. Phosphoprotein gene. Glycoprotein gene. Phylogenetic analysis. Molecular epidemiology

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INTRODUCTION

Spring viremia of carp virus (SVCV) or *Rhabdovirus carpio* has been isolated during outbreaks of disease in more than 30 countries including the USA (Fijan et al. 1971, World Organization for Animal Health [OIE] available at: www.oie.int/fdc/eng/en_fdc.htm). Spring viremia of carp disease (SVC) is endemic in parts of continental Europe, Russia, and states of the former USSR. Estimates in Europe alone suggest annual losses attributable to SVC of around 4000 metric tons (Fijan 1999, Zhang 2002). Mortality in young carp can be as high as 70 % and the severity of the disease is reflected in its inclusion on the OIE list of notifiable viral diseases of finfish (Wolf 1988, Ahne et al. 2002).

Phylogenetic analysis of 36 partial G gene sequences of SVCV and the related pike fry rhabdovirus (PFRV) identified four distinct genogroups (Stone et al. 2003). All of the SVCV isolates formed a genetic cluster and were assigned to a single genogroup (genogroup I) that could be further sub-divided based on both phylogenetic and geographic origin of the isolates examined (Ia to d). Asian isolates were classified within group Ia. Moldovan, Ukrainian and Russian isolates were assigned to genogroups Ib and Ic, while isolates presumably from the UK and other European countries were classified in genogroup Id. Other SVCV genes also offer discriminatory potential. Using the SVCV N gene as a target, Shchelkunov et al. (2005) developed a restriction fragment length polymorphism (RFLP) assay that distinguished two major and several intermediate minor genogroups among more than 20 European SVCV isolates and a clear geographic correlation was found for some of the genogroups.

The first isolation of a group Ia SVC virus was made in 1997 from a mirror carp (*Cyprinus carpio carpio*), followed in 1998 by isolations from asymptomatic koi carp (*Cyprinus carpio koi*) and goldfish (*Carassius auratus*) during routine import checks of ornamental fish imported into the UK from China (People's Republic of China). An SVC virus was isolated in the previous year from common carp held at the wholesaler's site, with fish originating from the same source. Based on partial glycoprotein gene sequence, this isolate was also assigned to Genogroup Ia (Stone et al. 2003).

A comprehensive surveillance program was undertaken by China for exit-entry and quarantine during 1998 to 2002, with no evidence of SVCV. However, following the association of Genogroup Ia viruses with the SVC outbreaks in North Carolina, USA (Goodwin 2002), and Wisconsin, USA (Dikkeboom et al. 2004), the authorities adopted a stricter SVCV surveillance program throughout China during 2003. During this more stringent testing, two strains of SVCV were isolated separately from koi carp (isolate 890) and common carp (isolate 992) in the Tianjin region of northern China in 2003 (Liu et al. 2004). Since these initial findings, a number of SVCV isolations have been made in both the USA and the UK, and for the latter there is supporting documentation in most cases to link the virus isolation with fish imported directly from China or to facilities holding fish recently imported from China.

In the USA, there have been six case reports of isolation of SVCV in cyprinids during the spring season, (OIE website available at: www.collabcen.net/toWeb/aq2.asp). Four of these were associated with disease

outbreaks occurring in koi and goldfish breeder farms in the states of North Carolina (NC) and Virginia (VA) (reported in July 2002), in feral carp in a public lake (Cedar Lake, Wisconsin [WI] August 2002), in a backyard pond of koi and goldfish (Washington [WA], June 2004), and in koi breeder farms (Missouri [MO], July 2004). The fifth isolate originated from a non-clinical case in feral carp sampled during a routine surveillance (Cal Slag Illinois Channel, Illinois [ILL], summer 2003) (available at: www.fws.gov/news/NewsReleases/showNews.cfm?newsId=E5DE11CB-EF51-49E8-A147A9955185C7C9). Subsequently, on June 28, 2007, SVCV was confirmed in the upper Mississippi river between Onalaska Wisconsin (WI) and Dresbach, Minnesota (MN) from feral common carp samples submitted by the US Fish and Wildlife Service who were investigating a carp kill on May 7, 2007 (USFWS 2007- available at: www.fws.gov/midwest/news/release07-68.html, Startribune 2007-www.startribune.com/531/v-print/story/1277118.html).

Tracebacks conducted by the Animal and Plant Health Inspection Service (APHIS) of the commercial breeder farms in NC and MO and the backyard pond owner, who bought fish from a pet store, indicated that each of these outbreaks followed the introductions of fish imported from an Asian production facility of unknown SVC-status or imported from Asian countries known to have SVC (*Federal Register* 2006). Additionally, SVCV was isolated from wild common carp in Hamilton Harbor on Lake Ontario in an open production system (September 2006) (OIE World Animal Health Information available at: www.oie.int/wahid-prod/public.php?page=disease_immediate_summary&selected_year=2006)

following a routine export screening for viral hemorrhagic septicemia virus (VHSV) prior to export to France (B. Souters pers. comm.). All seven of these case reports were determined to be in SVCV group Ia based on partial G gene sequences.

In the UK, a further isolation of SVCV was made in 1998 from tench *Tinca tinca* held at a site that had recently received fish from China. In 2001, SVCV was isolated from koi carp in a hobbyist's pond, and from koi carp taken from a mixed stock of fish originating from China and Japan and kept at the supplier's site. Similarly, in 2002, SVCV was isolated from koi carp and golden orf *Leuciscus idus* in a hobbyist's pond. Although in this case no SVCV positive fish were identified on the supplier's site, the supplier and a fish retail site shown to be positive in the same year had a common wholesaler who regularly received fish from China.

In 2004, SVC was isolated from consignments of goldfish, koi carp and common carp imported from Italy. The virus was isolated in the same year from goldfish in a mixed stock of fish including carp received from China.

In 2005, isolations were made from goldfish and common carp but in both cases there was no supporting documentation to suggest a possible source of the infection.

During the period 2002 to 2004 numerous SVCV sequences were submitted to Genbank/EMBL by Y. Jiang of the Chinese Exit-entry and Quarantine Bureau (Liu et al. 2005, Teng et al. 2007).

Molecular epidemiology has become an important tool in disease surveillance and investigations of outbreaks, due to its ability to trace and identify possible

infection sources (Ostroff 1999). The SVCV genome comprises 11019 nucleotides (nt) of negative sense single stranded RNA that encodes five major proteins in the order 3'N (nucleoprotein) -P (phosphoprotein) -M (matrix) -G (glycoprotein) -L (polymerase) 5' (Bjorklund et al. 1996, Hoffmann et al. 2002, Teng et al. 2007). Studies conducted to date have largely focused on analysis of the glycoprotein (G) gene for characterization of SVCV (Johnson et al. 1999, Oreshkova et al. 1999, Johansson et al. 2001, Ahne et al. 2002, Koutna et al. 2003, Stone et al. 2003, Liu et al. 2004, Dikkeboom et al. 2004).

The P gene of SVCV is 930 nt long, extends from positions 1407 to 2336 with respect to the genome, and encodes a protein of 309 amino acids (aa). The phosphoprotein P (formerly NS) is a component of the rhabdovirus nucleocapsid that, in association with L and N proteins, is required for transcription and replication, whereas the G and M proteins are not essential for these functions (Emerson & Yu 1975, Roy 1981, Banerjee 1987, Oreshkova et al. 1999, Ahne et al. 2002, Shchelkunov et al. 2005).

While the N gene is highly conserved, there is a higher degree of variation in the P gene. G and P gene nucleotide differences between base pairs of European and Asian sequences of SVCV were 7.52 and 10.54%, while their amino acid differences were 4.52 and 9.70%, respectively (D. Stone unpubl. data). The phosphoprotein is also the least conserved among *Lyssavirus* genes, and among the genes of VHSV strains (Johansson et al. 2002). Therefore, analysis of the P gene may be a more sensitive indicator of genetic diversity than either the G or N genes of SVCV, providing a more useful molecular

epidemiological tool. In this study, we explored for the first time the use of the nucleotide sequence information of the SVCV phosphoprotein P gene for phylogenetic and epidemiological analysis. We combined the analysis of partial G gene and P gene sequences to establish the genetic relationship among the entire available group Ia SVCV isolates from North America and UK and those viruses isolated in China.

MATERIALS AND METHODS

This publication is the combined work of two laboratories, each using their own 'in house' methods to generate sequence data. Where methods differ, details of both protocols have been provided.

Viruses and cell lines. The US (American) SVCV isolates were obtained from A.E. Goodwin, University of Arkansas at Pine Bluff; K. Snekvik and J. Thompson, Washington Animal Diagnostic Disease Laboratory, and J. Warg, USDA APHIS National Veterinary Services Laboratory, Ames Iowa. The Canadian SVCV isolate was obtained from B. Souter, Department of Fisheries and Oceans, Manitoba and the remaining viruses were obtained from the OIE SVC Reference Laboratory, The Centre for Environment, Fisheries, and Aquaculture Science (CEFAS), Weymouth UK (Table 2). Reference viruses used to determine specificity of primers were: S30 (Genogroup I), pike fry rhabdovirus (PFRV) F4 (Genogroup III), and tench rhabdovirus (TenRV) 9946631.1, 950237 and 84-4 (Genogroup IV).

Epithelioma papulosum cyprini (EPC) cells were used for propagation of the US SVC viruses, except for isolate PB02-131 for which bluegill *Lepomis macrochirus* fry cells (BF-2) were used. The cells were grown in monolayer cultures in L-15 Leibovitz RS medium with 5% fetal bovine serum, infected with American SVCV isolates at a multiplicity of infection of 0.1 and incubated at 20°C until 80% cytopathic effect was observed. Lyophilized international SVCV and other reference isolates were reconstituted in 0.5ml L-15 Leibovitz medium for direct viral RNA extraction and P gene amplification.

Table 2. *Rhabdovirus carpio.* Isolates of SVCV P gene sequence data analyzed. Publication details are given in parentheses; other sequence data are unpublished ND: no data.

Isolate	Date of Isolation	Country of Isolation	Host species	Genbank Accession no. P gene	Genbank Accession no. G gene	P-gene subgroup
970469	1997	UK*[1]	Common carp (*Cyprinus carpio carpio*)	DQ916049	AJ538067	Iai
980528	1998	UK[±]	Goldfish (*Carassius auratus*)	DQ916050	AJ538066	Iai
980451	1998	UK*[1]	*Koi carp* (*Cyprinus carpio koi*)	ND	AJ538065	Iai
980548	1998	UK[+]	Tench (*Tinca tinca*)	DQ916052	ND	Iai
980619	1998	UK[±]	Ghost carp (*Cyprinus carpio*)	DQ916051	AM501515	Iai
D-148	2001	UK*[2]	*Common carp*	DQ916055	AM501513	Iai
D-120	2001	UK*[2]	Koi carp	DQ916056	AM501514	Iai
PB02-46 (212364)	2002	NC, USA	Koi carp	DQ904366	DQ227501 (Warg *et al* 2007))	Iaii
PBO2-131 (207194)	2002	WI, USA	Common carp	DQ904368	DQ227500 (Warg *et al* 2007)	Iai
E208	2002	UK	Koi carp,Golden Orf (*Leuciscus idus*)	ND	AM501516	Iaii
E232	2002	UK	Koi carp	ND	AM501512	Iai
266921	2003	ILL, USA	Common carp	DQ904369	DQ227502 (Warg *et al* 2007))	Iai
PB04-1664 (322383)	2004	MO, USA	Koi carp	DQ904370	DQ227504 (Warg *et al* 2007)	Iaii
04-5061 (316715)	2004	WA, USA	Goldfish	DQ90436	DQ227503 (Warg *et al* 2007)	Iaii
GO67	2004	UK*[1]	Goldfish	DQ916053	AM501522	Iai
G083	2004	UK[‡]	Goldfish	DQ916048	AM501521	Iaii
G108	2004	UK[‡]	Goldfish	DQ916047	AM501520	Iaii
G144 1.2	2004	UK[‡]	Goldfish	ND	AM501527	Iaii
G144 2.8	2004	UK[‡]	Goldfish	ND	AM501523	Iaii
G144 3.1	2004	UK[‡]	Koi carp	ND	AM501519	Iai
G144 4.1	2004	UK[‡]	Koi carp	ND	AM501518	Iai
G144 5.2	2004	UK[‡]	Common carp	ND	AM501517	Iai
H243	2005	UK	Common carp	DQ916054	AM501511	Iai
H264	2005	UK	Goldfish	ND	AM501510	Iai
HHOCarp06	2006	Canada	Common carp	EF216718	EF194065	Iai
S30	1969	Yugoslavia	Common carp	DQ916041	AJ538061 (Stone et al. 2003)	
N3-14	1986	Ukraine	Grass carp (*Ctenopharyngodon idella*)	ND	AJ538062 (Stone et al. 2003)	
880163	1988	UK	Common carp	DQ916043	EU003618 (Stone et al. 2003)	
940626	1994	UK	Tench	DQ916044	EU003617 (Stone et al. 2003)	
P4	1983	Russia	Common carp	EF417826	AJ538074 (Stone et al. 2003)	
N1-5	1986	Ukraine	Bighead carp (*Aristichthys nobilis*)	AM501526	AJ538064 (Stone et al. 2003)	

Table 2 (continued**)**

Isolate	Date of Isolation	Country of Isolation	Host species	Genbank Accession no. P gene	Genbank Accession no. G gene	P gene subgroup
N3-14	1986	Ukraine	Grass carp	ND	AJ538062 (Stone et al.2003)	
RHV	1989	Ukraine	Rainbow trout	AM501525	AJ538074 (Stone et al. 2003)	
2/90	1990	Moldova	Common carp	AM501524	AJ538060 (Stone et al. 2003)	
01-01V1592	1999	Germany	Koi carp	AY424883 –[88] (Hoffman et al. 2005)	ND	
01-01v1621	2000	Germany	Koi carp	AY424884	ND	
19-0052/94	1994	Germany	Koi carp	AY424885	ND	
19-0059/95	1995	Germany	Koi carp	AY424886	ND	
19-0073/94	1994	Germany	Koi carp	AY424887	ND	
17/00-47/3	2000	Germany*[3]	Common carp	AY424888	ND	
F177	2003	UK	Crucian carp *(Carassius carassius)*	EF417828	ND	
F183	2003	UK	Koi carp	EF417829	ND	
F193	2003	UK	Crucian carp	EF417832	ND	
F223	2003	UK	Koi carp	EF417830	ND	
G151	2004	UK	Koi carp	EF417831	ND	
G221	2004	UK	Koi carp	EF417827	ND	
992	2003	Tianjin, China	Common carp	ND	AY842489 (Liu et al. 2005)	laii
890	2003	Tianjin, China	Koi carp	ND	EU049487	lai
AI	2006	China	Common carp	DQ097384	DQ09738 (Teng et al. 2007)	laii
A2	2006	China		DQ491000	DQ491000	laii
978	2004	China	Common carp	ND	AY842488 (Liu et al. 2005)	laii
926	2004	China	Common carp	ND	AY842487 (Liu et al. 2005)	lai
772	2004	China	goldfish	ND	AY842486 (Liu et al. 2005)	lai
461	2004	China	Common carp	ND	AY842484 (Liu et al. 2005)	lai
464	2004	China	Koi carp	ND	AY842485 (Liu et al. 2005)	lai

*[1] Isolated from a mixed stock of fish including carp recently received fish from the Peoples Republic of China
*[2] Isolated from carp in a mixed stock containing fish from the both Peoples Republic of China and Japan
*[3] Isolated from carp imported from Czech Republic
± Isolated during routine check of imports from the Peoples Republic of China
+ The tench were cohabited with koi carp from a number of countries including the Peoples Republic of China.
‡ Isolated from fish imported from Italy

Virus clarification and RNA extraction. Culture fluid from infected cell cultures was harvested and cell debris removed by centrifugation at 4000x g for 5 min at room temperature. Viral RNA was extracted from 140 µl of the supernatant using the QIAamp® Viral RNA Mini kit (Qiagen) according to manufacturer's protocol. Alternatively, RNA was extracted from 100µl of the supernatant using Trizol Reagent™ (Invitrogen) according to the method of Strommen & Stone (1997).

Primer design. Specific oligonucleotide primers were designed to amplify the entire coding region of the P gene from genomic viral RNA. Conserved primer annealing sites were identified by alignment of published nucleotide sequences of the phosphoproteins of SVCV (GenBank accession nos. AY424883-AY424888, Hoffman et al. 2005). Consensus primers were designed using Vector NTI Advance 10 DNA and protein analysis software (VectorNTI, Invitrogen) to amplify the P gene of the European strains. The DNA sequence of the P gene of the Asian strain 980528 was used to manually design primers to amplify the P gene from Asian strains. Specific oligonucelotide primer pairs designed to amplify P gene from Asian SVCV isolates were designated Asian primers and those that amplified the P gene of the European SVCV isolates were designated European primers (Table 3).

Reverse transcription and Polymerase Chain Reaction amplification. In most cases the complete P-gene sequence was amplified according to the protocol in the One Step RT-PCR Kit (Qiagen) using primer sets SVCVPAF/ SVCVPAR and SVCVPF/ SVCVPR (Table 2). This was repeated independently

at least twice for each virus isolate in order to obtain sequence information that was not subject to PCR bias. The reaction mix for RT-PCR consisted of 3 µl of viral RNA and 47 µl of the master mix in each PCR tube. The reactions were conducted in a GeneAmp PCR System 9600 thermocycler programmed to conduct a single cycle of reverse transcription at 50°C for 30 min, followed by 35 cycles of denaturing at 95°C for one min, annealing at 50°C for one min, and elongation at 72°C for one min, and a single final elongation at 72°C for 10 min. PCR products were either purified using the QIAquick Spin PCR Purification Kit according to protocol for direct nucleotide sequencing (Qiagen) or inserted into an appropriate cloning vector. PCR products of 930 bp were checked by 1% agarose gel electrophoresis with a 1kb DNA ladder (Promega) with positive and negative controls. Alternatively, the complete P gene and flanking sequences were amplified as three overlapping fragments using primer sets MD7/MD8, MD9/MD10 and MD11/MD12 (Table 3). Again, the amplifications were performed in duplicate to avoid errors introduced by the Taq polymerase, and the products were prepared for direct sequencing using the Freeze and Squeeze Kit (BioRad) following the manufacturer's instructions. The G gene sequences products were generated using Primers SVCV R2 and SVCV F1 using the method of Stone et al. (2003).

Table 3. Oligonucleotide primer sets used to amplify and sequence the SVCV P genes showing the relative position of the primers based on the published sequence for SVCV genome (Hoffman et al. 2002; AJ318079).

Oligonucleotide	Sequence	Location
SVCVPAF-5'	CTGATGTCTCTACATTCG	1407 – 1422
SVCVPAR	CTGCTACAACCTATATTTTTG	2336 – 2318
SVCVPF-5'	GTGATGTCTCTACACTC	1407 – 1421
SVCVPR	GCGCTATAACCTGTATTTTTG	2336 – 2318
MD7	ATATTTTCTTCATCATCAACTATC	1360 – 1383
MD8	CACCGAGGCCGTTATAGCG'	1818 – 1800
MD9	ACGGCAGACACAGTTTGGTAC	1762 – 1782
MD10	AATCTCGAATGGTCAGGCTTAG	2210 – 2190
MD11	AACTAGGGACCTTTCTTCTAC	2161 – 2181
MD12	AACTCCCTTGCACCTTGTTAG	2730 – 2710

TA cloning. The PCR products of the P gene from the 5 US isolates (PBO4-1664, PB02-131, PBO2-46, 266921, 04-5061) and 4 non-US isolates (G108,G067,G083,H243) mentioned previously were cloned into vector pCR2.1® using the TOPO TA Cloning kit according to manufacturer's protocol (Invitrogen). The US isolates and the most recent non-US isolates were cloned for further studies e.g. gene expression and vaccine development.

Positive recombinant plasmids were purified and isolated using the Promega Plus SV Mini-preps DNA Purification System according to protocol, except that 50µl of nuclease-free water was used for the final elution instead of the recommended 100µl. The presence of a 1 kb insert and 3.9 kb vector in purified plasmids was verified by restriction enzyme digestion using *Eco*RI.

Sequencing analysis. M13 primers (forward and reverse) were used for sequencing clones. At least two clones, one from each of the independent amplifications, were used to construct nucleotide consensus sequences. The primers used in the initial amplification were used for direct sequencing reactions of P and G gene products. Cycle sequencing was performed using the Applied Biosystems 3130/ 3131x/ Genetic Analyzers, Big Dye Terminator V1.1 Cycle Sequencing Kit. Editing and assembly of sequence trace (ABI) files and alignments of contigs were performed using either the VectorNTI software or Sequencher program from Gene Codes Corporation.

Phylogenetic analysis. Nucleotide sequence alignments were performed by AlignX contained within the VectorNTI software package or Clustal X program version1.83 (Thompson et al. 1997). Pairwise and multiple alignment gap opening and gap extension settings were 15.00 and 6.66, respectively. Since PCR primers used to amplify the complete P gene anneals with the first 15 (Asian), or 14 (European) nucleotides and the last 17 nucleotides (both Asian and European) it may not reflect virus-strain specific nucleotide variation in this region, we excluded these nucleotides from our phylogenetic analysis. Phylogenetic analyses were performed using Paup*4.0 (Swofford 2000) and MEGA version 3.1 (Kumar et al. 2004). Phylogenetic trees were generated by the Neighbor-Joining (NJ) method (Saitou & Nei 1987) on uncorrected p-distances or corrected under a general time reversible model of nucleotide substitution with site rate variation estimated using the discrete-gamma correction (0.4663) with four rate categories (GTR+G), empirical base frequencies, and without a term for proportion of invariable sites (Gu et al. 1995, Swofford et al. 1996). The GTR+G model was identified by likelihood ratio test and the Akaike Information Criterion as the best-fit to the data using MrModeltest 2.2 (Nylander 2004) in conjunction with PAUP*4.0. Three phylogenetic analyses were conducted to: (1) conduct an independent comparative analysis between 11 SVCV P genes (930 bp Asian and 930 bp European region) and 14 SVCV G genes (426 bp region) to confirm the P gene's ability to detect Genogroups Ia to Id, (2) assess the genetic diversity of the P gene to discriminate among 34 SVCV Genogroups Ia, Ic and Id sequences, and (3) determine the genetic relationship between 35 spring viremia of carp

virus (SVCV) Genogroup Ia isolates based on the nucleotide sequence of the P gene and G gene sequences.

Nonparametric bootstrapping was performed to assess node support with a minimum of 1000 and maximum of 2000 re-samplings from the original data set to confirm 95% reproducibility in the resulting trees (Hall & Barlow 2006). Tree diagrams were displayed using TreeView win32 software (Page 1996).

RESULTS

PCR primer pairs distinguish Asian, European and Russian SVCV strains

To analyze the genetic diversity among SVCV isolates, an initial total of 59 (54 SVCV; 1 Piry, 1 PFRV and 3 TenRV) rhabdoviruses from 12 countries during the period 1969 to 2006 were tested (Table 2). The oligonucelotide primer pairs designed in this study specifically amplified the entire P gene coding region (codon 1 ATG - codon 930 TAG) (data not shown). Detection of the amplified (930 bp) PCR products of the P gene of US isolates was visualized by agarose gel electrophoresis.

It was possible to amplify the P gene from all SVCV isolates using either the Asian or European primer pairs. The SVCV isolates in Genogroup Ia (18 out of 34) were amplified by the Asian primers (SVCPAF/SVCPAR), while 16 SVCV isolates (Genogroups Ic and Id) were amplified by the European primers (SVCPF/SVCPR), (data not shown). Neither Asian nor European primer pairs could amplify the P gene from PFRV isolate F4 (Genogroup III), or from TenRV isolates 9946631.1, 950237 and 84-4 (Genogroup IV). The Asian primer pair did

not amplify the European P genes nor did the European primer pair amplify the Asian P genes (data not shown).

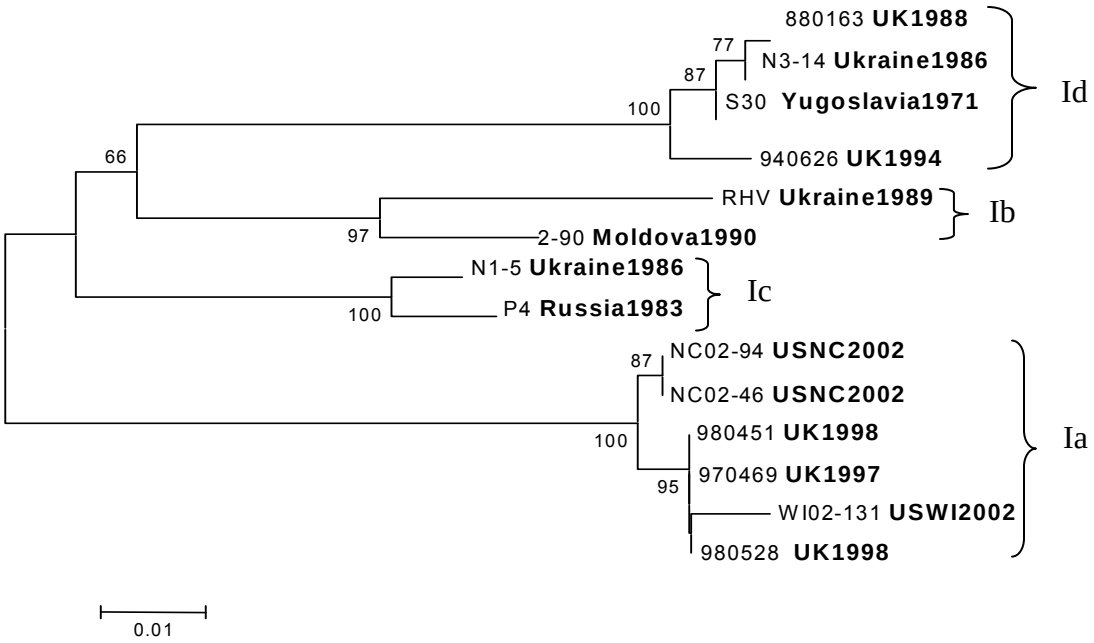
Comparison of P and G gene sequences reveals similar topologies among same virus strains

Twenty-nine complete SVCV P genes and 12 partial G genes were deposited into GenBank database under accession numbers DQ904366-DQ904370, DQ9106041, DQ916043-DQ916044, DQ916047-DQ916056, EF216718, EF417826-EF417832, AM501524-26, AM501510-23, and AM501527, respectively (Table 2).

We were interested in determining whether P gene sequences displayed relationships among SVCV strains similar to those that have been previously demonstrated by analyses of G gene sequences. Neighbor–joining analysis based on the P gene sequence produced a similar tree showing reciprocal monophyly for gene lineages and highly similar phylogenetic topologies to those described previously for the G gene (Stone et al. 2003, Dikkeboom et al. 2004, Warg et al. 2007). There were four distinct genogroups of SVCV (Ia-Id) with the US isolates clustering in the Ia group together with the viruses isolated in the UK from ornamental imports from China (Figure 10). The isolates RHV and 2-90 clustered together (genogroup Ib) as did N1-5 and P4 (genogroup Ic). The SVCV reference strain (S30) was assigned to genogroup Id. Separation into the four genogroups based on the G-gene sequences was supported by bootstrap values of ≥ 97%.

A G gene

n=14



B P gene

n=11

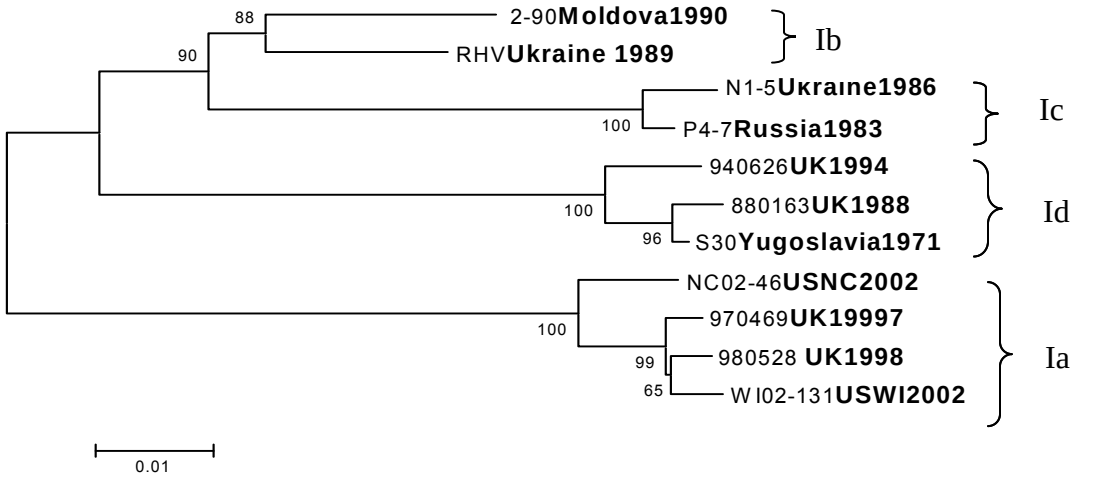


Figure 10. Phylogenetic trees generated by neighbor-joining analyses of (A) a 426 base pair partial glycoprotein gene sequence and (B) the 898 base pair phosphoprotein gene sequence of Genogroup Ia to Id SVC virus isolates. The P gene sequences were generated in this study. The G gene sequences for isolates 940626 and 88163 were generated in this study; all other G gene sequences were published previously by Stone et al. (2003). Analyses were done on 1000 bootstrapped data sets and values of >70 are shown on the trees. See Table 2 for details of isolates.

Phylogenetic analysis of SVCV strains reveals 2 clades, 3 genogroups and 2 subgroups

The NJ phylogram including P gene sequences of 29 international (non-US) and 5 US SVCV isolates identified 2 major clades, 3 genogroups and 2 subgroups (Figure 11). Bootstrap values of 100% indicate strong support for the distinct clades (A and B). Each clade corresponds to a specific geographical region in Asia (clade A) or Europe (clade B). In keeping with the genogroup identification of Stone et al. 2003, the clades clustered in their designation of Genogroup I, Subgenogroups Ia, Ic and Id for SVCV. The range of percent identity within Genogroups Ia, Ic and Id were 100 to 97.5%, 93.5 to 89.1%, and 100 to 88.1%, respectively.

Two subgroups (Iai and Iaii) with high support values (90% and 89%) of Asian and American SVCV isolates were contained in Genogroup Ia and there were two subgroups with high bootstrap values (100% to 83%) in Genogroup Id of SVCV nucleotide sequences from Germany, Czech Republic, UK, and Yugoslavia (Figure 11). However the subgroups in Id formed only one cluster with 76% bootstrap support using the GTR+G model (data not shown). Focusing within the Asian clade, Italy import (G083) shared 100% nucleotide identity with WA isolate (04-5061) and clustered with the China isolates (A1 DQ097384, 98.8%, A2 DQ491000, 99.6%), respectively. Italy import (G108) shared 99.5% nucleotide identity with NC (PB02-46). UK import (970469) clustered among ILL (266921), WI (PB02-131) and Canada (HHOCarp06) isolates sharing nucleotide identities of 99.2%, 99.3% and 99.2%, respectively. The Canada (HHOCarp06)

isolate shared nucleotide identities of 99.5% with WI (PB02-131) and 99.4% with ILL (266921).

Bootstrap values ≥ 89% further support isolates from China (A1 and A2), and Italy imports, (G083, G108) as likely trace sources of the WA and NC outbreak strains along with isolate 970469 as a likely trace source of the Canada, WI and ILL strains (Figure 11). Although isolate 980619 was an import from an unknown source, it clustered (sharing 100% nucleotide identities) with an import from China (980528) and 99.8% with UK (980548) that was thought to have originated in the UK. However, the genetic analysis and additional epidemiological information suggests that the above imports may have ultimately originated from the same region of China.

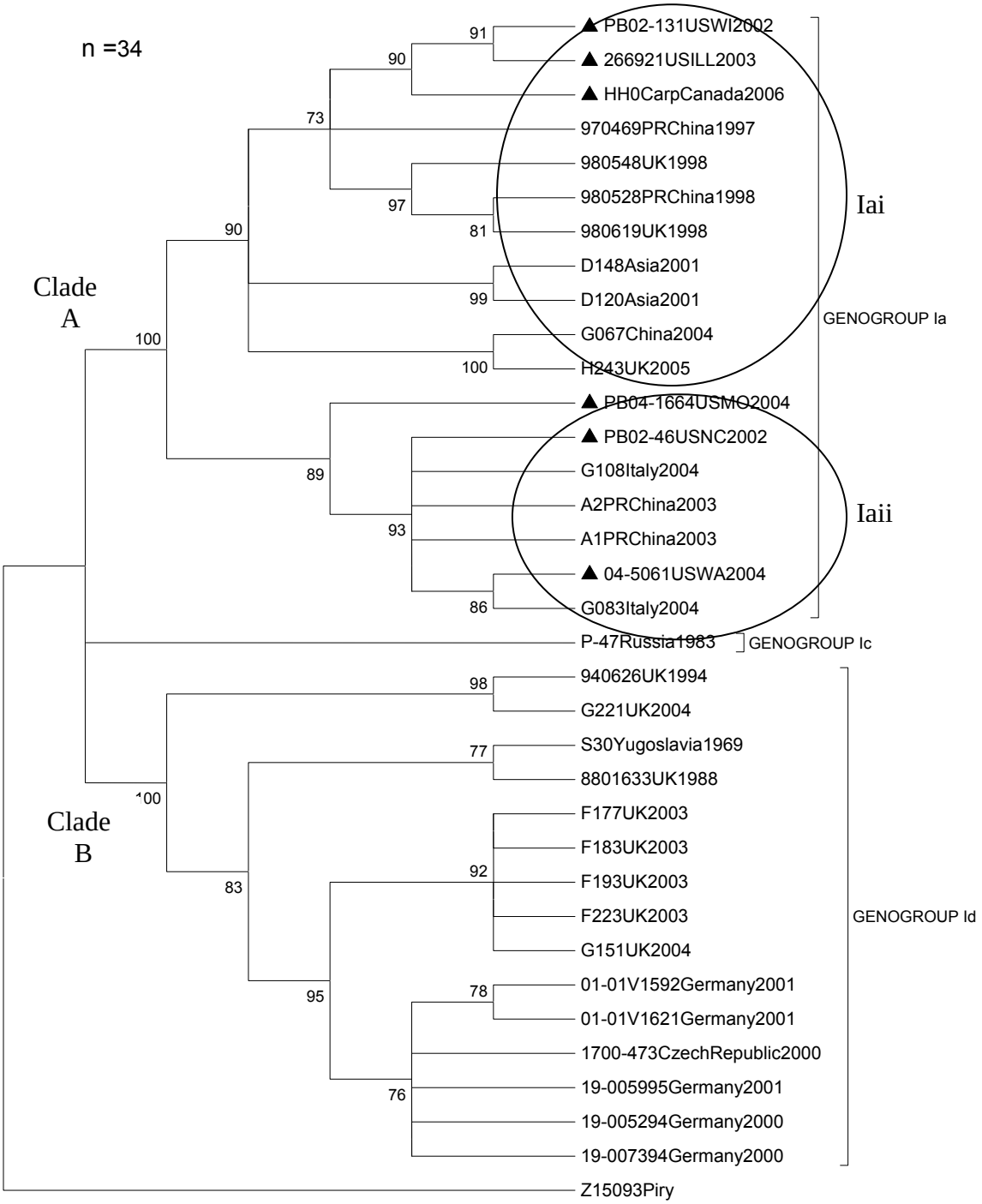


Figure 11. Condensed tree generated by neighbor-joining analyses of the 898 base pair phosphoprotein (P) gene sequence of Genogroup Ia, Ic and Id SVC virus isolates showing topology only; distinct subgroups Iai& Iaii are circled. Bootstrap percentages are displayed beside the corresponding nodes and are based upon 2000 resamplings with values of ≥70 shown on the tree. (Piry virus rooted as outgroup). All P genes were generated except for A1,A2,01-01v1592,01-01V1621,AY424888,19-0052995,19-005294,and 19-00739. ▲ indicates North America SVCV isolates.

Expanded analysis of the Ia genogroup based on P and G gene sequences

Based on our results (Figure 11), which indicated two distinct subgroups in the Ia genogroup, we decided to expand our P and G gene analysis with all available Ia genogroup SVCV isolates from North America and the United Kingdom. Multiple alignment of the 898bp P gene and 426bp G gene sequences of the SVCV Ia virus isolates revealed a high degree of sequence divergence, with between 0- and 21 nucleotide substitutions (97.75 to 100% nucleotide identity) and 0 to 11 nucleotide substitutions (95.5 to 100% nucleotide identity) in the P and G genes, respectively.

Phylogenetic analysis based on the P gene suggested a further division within the SVCV Ia clade (Iai and Iaii) supported by bootstrap values of >72% (Figure 12). Analysis based on a more comprehensive data set from the G gene revealed a number of discrete clusters of virus sequences, with clustering of isolates that was consistent with the P gene analysis; but in many cases the divisions within the SVCV Ia clade of the G gene were not supported by bootstrap analysis (Figure 13).

Based on the G gene sequence (Figure 13), strong support (81%) was provided by bootstrap analysis for the clustering of isolates D120 and D148 from 2001 with isolates from 2004 (G067 and G144 3.1), 2005 (H243 and H264) and sequence data for an isolate purported to have come from China (isolate 464 [AY842485]). There was also good support (79%) for a genetic link between E232, a UK isolate from 2002 and the sequence data for isolates 772 and 926 from China, and also for a link (79%) between two UK isolates (G144 1.2 and

2.8) from 2004 and the Chinese isolate 890. In the latter case, the three viruses shared identical nucleotide sequences.

The analysis of the P gene (Figure 12) provided good support (71%) for Subgroup Iaii cluster, (unlike the similarity observed cluster using the G gene sequence) placing the isolates from NC (NC02-46), MO (PB04-1664) and WA (04-5061) with the UK isolates from 2004 (G083 and G108) and further published and data for virus isolates from China (A1 DQ097384, A2 DQ491000), respectively. In addition, there was good support (77%) for (Subgroup Iai) clustering of the virus isolates from WI (WI02-131), ILL (266921) and the UK isolates from 1997 and 1998 (970469, 980528 1.1, 980548 and 980619) with strong support of 91% within Canada, WI and ILL. However, the G gene bootstrap values did not support the distinction of Subgroup Iai.

n=19

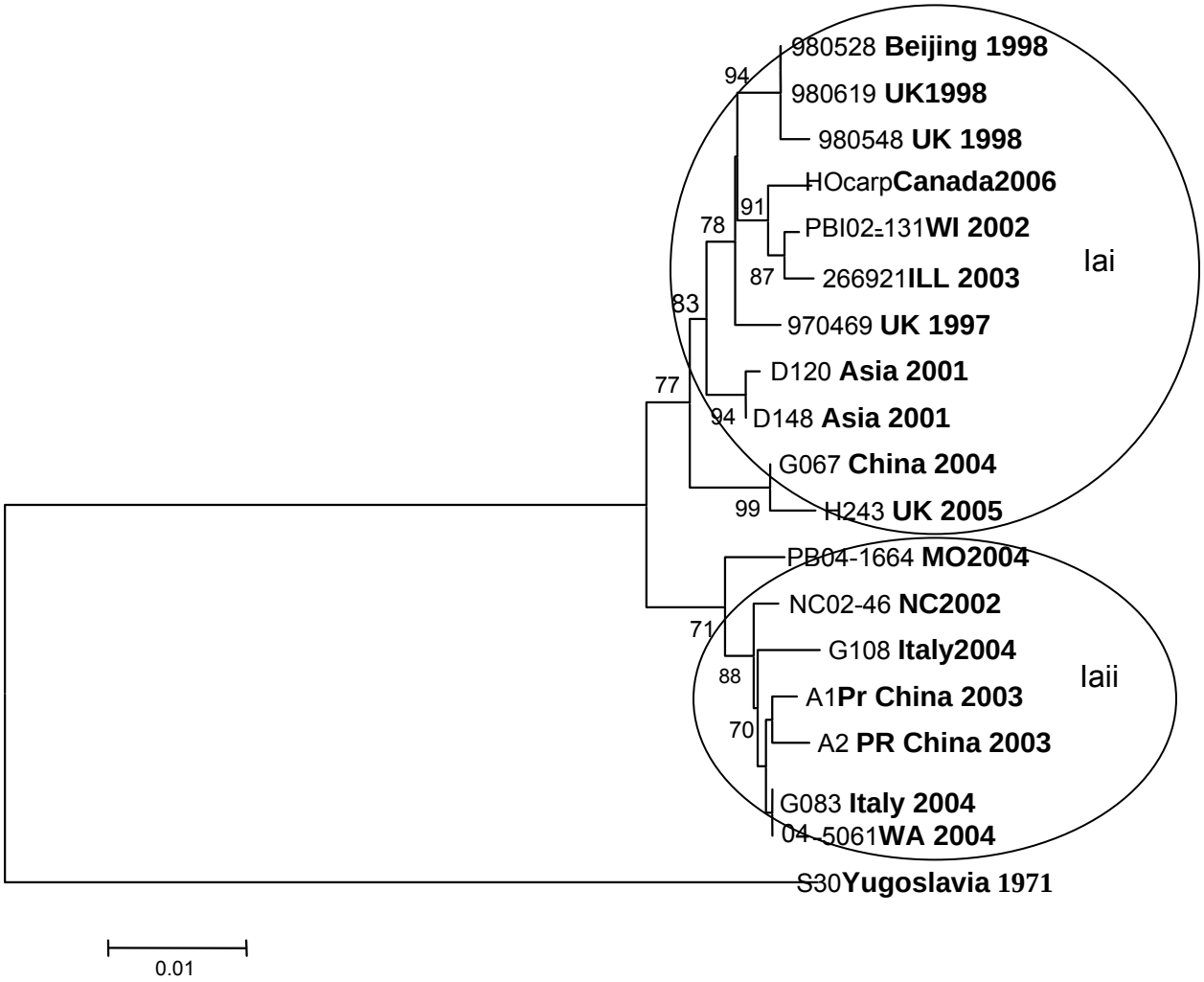


Figure 12. Phylogenetic trees generated by neighbor-joining analyses of the 898 base pair phosphoprotein (P) gene sequence of Genogroup Ia SVC virus isolates recovered in the US, UK and China. Details of the viruses used in the analysis are given in Table 2. Isolates A1 and A2 represent sequences submitted to Genbank from the People's Republic of China. Designated Subgroups (Iai and Iaii) are circled. Sequences labeled by isolate number followed by country of importation and year of isolation. Analyses were done on 1000 bootstrapped data sets and values of >70 are shown on the tree.

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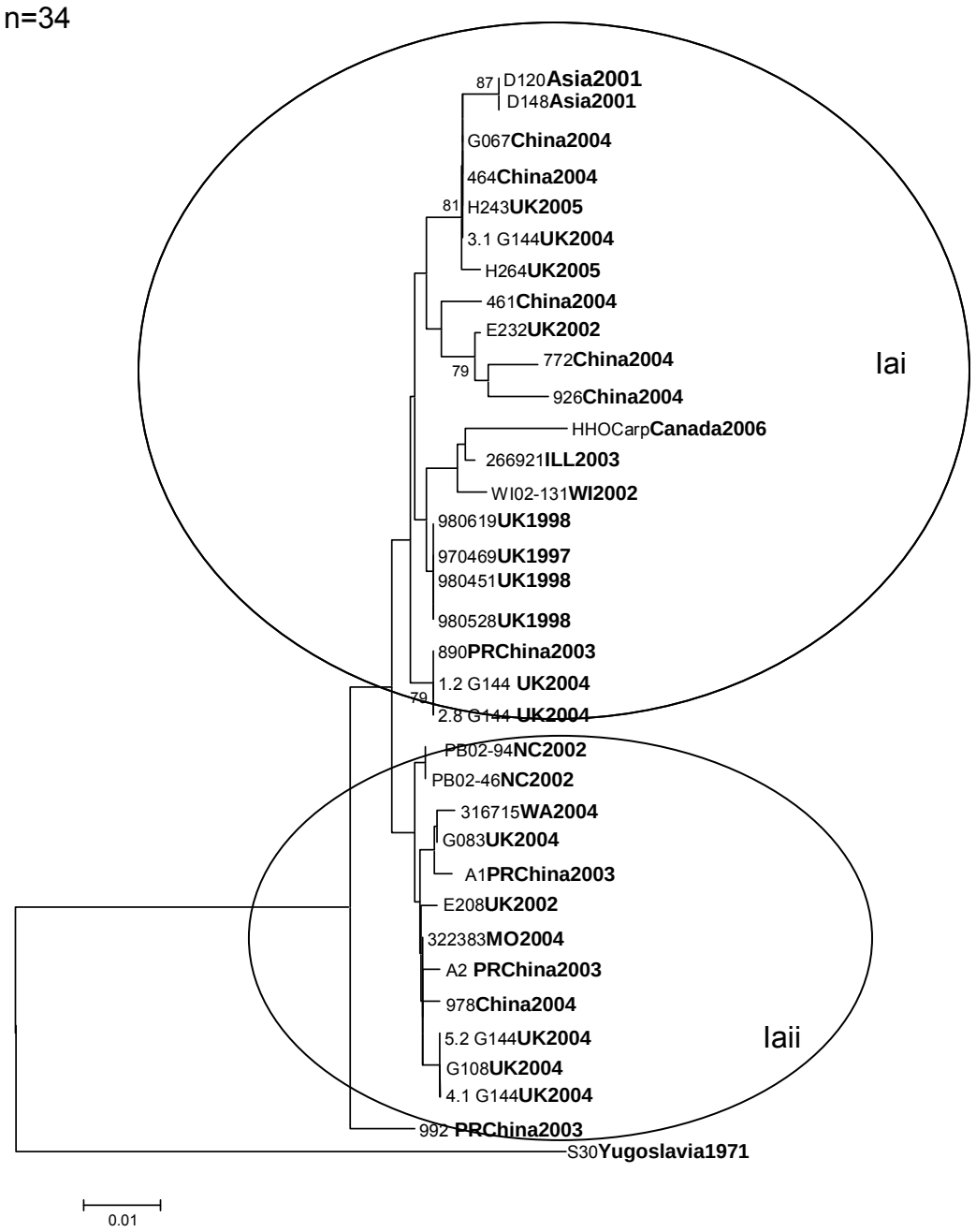

Figure 13. Phylogenetic trees generated by neighbor-joining analyses of the 426 base pair glycoprotein (G) gene sequence of Genogroup Ia SVC virus isolates recovered in the US, UK and the Peoples Republic of China. Details of the viruses used in the analysis are given in Table 2. Isolates 461, 464, 772, 926, 978, and A2 represent sequences submitted to GenBank (AY842484, AY842485, AY842486, AY842487, AY842488, DQ097384 and DQ491000) from the China. Designated Subgroups (Iai and Iaii) are circled. Sequences labeled by isolate number followed by country of importation and year of isolation. Analyses were done on 1000 bootstrapped data sets and values of >70 are shown on the tree.

It appears from epidemiological data that these sequences also clustered according to geographic location and fish species from which the virus was isolated. Isolates from WI, ILL and Canada were taken from feral common carp while isolates from MO, NC, and WA were from commercially farmed koi carp and goldfish. This species difference was not noted in Stone et al. 2003 and Hoffman et al. 2005. Imported breeder production stocks appear to have SVCV strains in Subgroup Iaii different from those in certain feral carp in Subgroup Iai in the USA.

DISCUSSION

This is the first epidemiological study to examine the diversity of SVCV nucleotide sequences of the phosphoprotein gene in comparison with the glycoprotein gene. Our studies examined 43 (35 Ia and 8 Ib to Id) isolates from different geographic regions worldwide. Our results indicate that previous SVCV taxonomic groupings (Genogroups Ia, Ib, Ic and Id) identified with G gene sequences are also identifiable using P gene sequences. Phylogenetic analysis of the P gene sequences revealed 2 distinct clades (A and B), 4 Genogroups (Ia, Ib, Ic and Id) and 2 subgroups (Iai and Iaii). Both P and G gene sequence analysis of genogroup Ia demonstrated distinct subgroups (Iai and Iaii) within Group Ia. However, the P gene had greater resolution and was supported by higher bootstrap support values. In the USA and Canada, all 6 homologous SVCV isolates belong to Genogroup Ia, confirming the G gene phylogenetic analysis of Warg et al. 2007. In addition, they are now designated into Subgroups

Iai and Iaii (Table 2). This offers the potential for novel and additional information in phylogenetic analyses of SVCV molecular epidemiology.

Over the last decade, the majority of reported isolations of SVCV have been of the Ia genotype. Although there is good documentation to support the origins of UK isolates from 1998, some of the remaining group Ia viruses were isolated from fish held on sites that import fish from a number of countries including China, and there are no data to support a direct link between the virus and the fish of Chinese origin. As part of an ongoing epidemiological investigation, our current study aimed to establish the genetic relationship between the group Ia SVCV isolates from North America and UK and those viruses isolated in China.

The phylogenetic data suggest that the occurrences of group Ia SVCV in the USA and the UK are likely to have arisen following multiple independent introductions of the virus. The data (1) provides evidence of a genetic link between the SVCV isolates from the UK and one of the two recognized isolates from China (Liu et al. 2004), (2) establishes genetic links between the 1998 isolates from the UK and isolations made in the USA and Canada, and (3) establish a link between USA and UK isolates and sequence data for isolates purported to have been recovered in China.

Phylogenetic analysis of US isolates based on the P gene identified two main subgroups (Iai and Iaii), which support at least two independent introductions of SVCV. The viruses from Wisconsin in 2002 (PB02-131), Illinois in 2003 (266921) and the more recent outbreak in Canada in 2006 (HHOcarp06) clustered with the UK isolates from 1998 in Subgroup Iai. From the data it can be inferred that all

three isolates in North America and the UK isolates share a common ancestor, and since the UK isolates were recovered from fish intercepted at the point of entry into the UK from Beijing, it is most likely that the common ancestor originated from a related source in China. It remains unclear, however, whether the viruses isolated in North America were introduced directly from China or entered the country via a third party.

The North Carolina (PB02-46), Missouri (PB02-1664) and Washington isolates (04-5061) were assigned to Subgroup Iaii together with UK isolates from 2004. Indeed, based on the 100% nucleotide identity to a 2004 UK import from Italy (isolate G083) the data suggest that the 2004 Washington isolate (04-5061) represents a third introduction into the USA from a common source rather than a reemergence from the 2002 NC isolate (PB02-46). More significantly, sequences for two Chinese viruses (A1 and A2; DQ097384 and DQ491000) isolated in 2006 cluster within this subgroup. Two UK isolates from goldfish imported into the UK via an Italian supplier in 2004 were also assigned to the same group. Similar relationships were obtained from analysis of the G gene sequence data, but in contrast to the P gene sequence data the clusters were not supported by the bootstrap analysis.

Based on the G-gene data, two of the UK isolates (G144 1.2 and 2.8) from goldfish from 2004 shared 100% nucleotide identity with 890, a virus isolated from koi carp in Tianjin, China in 2003 (Liu et al. 2004). This finding is surprising, given that the infected farm site in China ceased exporting fish after the isolation of SVCV in 2003. Since the goldfish were part of a stock of fish imported into the

UK from a supplier in Italy we can only assume that the virus was introduced into the Italian suppliers holding facility prior to the discovery of SVCV in Tianjin in 2003. Alternatively, the Italian supplier may have received fish from sources that had contact with the carp culture sites in Tianjin prior to the discovery of SVCV, and the infection has remained undetected on these contact sites.

One hundred percent nucleotide identity was shared between G067 isolated from goldfish imported from China in 2004 and G144 3.1 from koi carp imported into the UK from Italy in the same year, H243 which was isolated from common carp in the UK in 2005, and the G gene sequence of isolate 464 (AY842485) recovered from koi carp in China. The latter sequence was submitted to GenBank in 2004, suggesting that the virus was identified between the first discovery of SVCV in June 2003 and the sequence submission date of December 2004. This date is consistent with the exportation of infected fish to the UK and Italy during 2004, and the timing also allows for the transfer of infected fish to the UK from Italy in the same year. At present there is no documentation to link the common carp in this case with a fish import.

During the period from 1997 to 2006 there were 32 separate isolations of SVCV in the UK, USA and Canada. Sequence analysis has revealed strong genetic relationships between 14 of the 26 viruses isolated in the UK during this period, and viruses with origins in China. In some cases the nucleotide sequences were identical, suggesting a direct link between the infected fish. In at least one case where the fish were sampled during routine import checks the infection can be traced to China, but in other cases, particularly where fish

movement records show that fish from more than one country were held on the same site, it is more difficult to establish a direct link to the Chinese ornamental fish industry. The isolation of the group Ia virus from fish imported from Italy complicates matters further and additional investigations are required to establish whether the Italian suppliers were acting as a holding facility for fish destined for the European market. Nonetheless, the sequence divergence supports the introduction of the virus into the UK and the USA on a number of separate occasions from common ancestral sources.

Histological samples from a wild common carp kill in the Pentenwell Flowage Wisconsin River, USA in 1989, produced RT-PCR products that indicated the presence of a SVCV virus of European genotype (Subgenogroup Id) (R.M.Le Deuff, S. Marcquesnski, P.F. Dixon unpubl. data). Johnson et al. (1999) also reported on a European genotype SVCV from penaeid shrimp in Hawaii. This demonstrates that different strains (besides genotype Ia) of SVCV exist in the US, suggesting further independent introductions of this virus.

Biosecurity plays a very important role in prevention and spread of pathogens such as SVCV. Knowledge of likely geographical disease-risk sources coupled with health certifications demonstrating freedom of disease from SVCV risk areas would be important in preventing new SVCV introductions.

The USDA APHIS have been unable to recover SVCV from their volunteer SVCV susceptible broodstock surveillance survey which covered 30 sates and was initiated in 2002 (O. Miller unpubl. data). However, if there were evidence of endemic SVCV in North America it would most likely be significantly distinct from

European and Asian isolates and indemnification and depopulation policies would need to be revised.

The UK regulates the importation of fish, and the only hosts of SVCV that can be legally imported are ornamental fish such as goldfish, koi carp etc. There has been SVCV surveillance in the UK since the first major outbreak in 1988. There is evidence that some of the isolations resulted from the illegal importation of fish.

Currently, the majority of international trade of SVCV-susceptible fish consists of imports into the European Union and USA. Asia represents over 90% to the world aquaculture production (Bondad-Reantaso et al. 2005). Identifying trace country of origin from point source outbreaks could avert further introductions and spread of SVCV. Thus, the recent USDA APHIS implementation in the autumn of 2006 of the SVC import protocols, as other countries require, could bring some degree of balance to introductions of transboundary diseases (*Federal Register* 2006).

The recent expansion of the European Community to include several eastern European countries, in which carp culture is a significant part of the aquaculture industry, could see a significant shift in the carp trading patterns, particularly, imports of coldwater cyprinid species such as koi carp and grass carp into the UK.

Phylogenetic analysis with bootstrapping is an additional tool that epidemiologists can use for making geographical topology inferences about likely trace sources of outbreak strains. The coding region of the P gene (930 bp) of SVCV used in this study was well suited for molecular epidemiological studies to

determine nucleotide similarities among outbreak strains and genetic relatedness between American and non-American isolates of SVCV. Combining the results of the P-gene analysis with G-gene sequences could prove an invaluable tool in the surveillance and control of SVCV as the international trade in SVCV-susceptible fish increases in the future.

Acknowledgements:

We sincerely thank A. E. Goodwin, K. Snekvik, J. Thompson, J. Warg, and B. Souter for providing us with the SVCV isolates. The authors also are most grateful to G. Kurath, Western Fishery Research Center, Seattle, WA, J. Thorne, R. Anholt and B. Wiegmann of North Carolina State University for their assistance in the preparation of this manuscript. This study was funded by USDA Grant no. 52533.

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3. RECOMMENDATIONS TO PREVENT INTRODUCTION AND CONTROL THE SPREAD OF SVCV FROM INTERNATIONAL AND DOMESTIC SHIPMENTS OF SVC SUSCEPTIBLE FISH

Abridged version submitted for publication in *Global Aquaculture Alliance Magazin*e.

SUMMARY

"As aquaculture expands and new species are farmed, disease will continue to emerge and affect both wild and farmed fish adversely." Alexander Murray (Murray et al. 2005).

Pruder (2004) states "Biosecurity is and will remain an absolutely essential part of intensive animal production systems." A good biosecurity program results in control of disease transmission by eliminating pathogen introduction onto farm(s) and dissemination between farms or between ponds on any farm. Each method of possible pathogen movement to new susceptible animals is addressed by a biosecurity plan and prevention is implemented by education and appropriate actions of employees. A biosecurity plan seeks to control: 1. people carrying pathogens on person including visitors and employees, 2. inspection and quarantine of incoming fish, 3. contaminated inanimate objects like vehicles, nets, and other equipment, 4. wild animals that may act as vectors for the pathogen, and 5. water source. Daily operations of a farm must focus on these controls to prevent disease incidence, or if disease has already occurred, its spread. No biosecurity system is perfect, but the closer daily activities approximate the written plan, the better the disease control will be. This chapter will discuss establishing a biosecurity plan and identifying biosecurity measures through the use of a disease risk self-assessment tool found in Appendix I. The major objective of this section is the control and prevention of industry disruptive

and economical devastating infectious diseases of ornamental fish (like spring viremia of carp and koi herpes virus) and review the best practice biosecurity measures necessary to include in such a plan.

INTRODUCTION

Ornamental fish production includes species grown for water gardens (nishikigoi -koi),aquarium (tropical fish) and bait (cyprinids) such as minnows and goldfish. In global aquaculture, cyprinids are the most important taxonomic family by quantity and production with $16.3 billion in 2004 (Bondad-Reantaso et al. 2005). They mainly are produced as baitfish, food fish and ornamental fish (koi and goldfish). In the United States (US) the production of baitfish, koi carp and goldfish predominate with the total ornamental industry value estimated to be $175 million (FAO 2006).

The 2005 US Census of Aquaculture (NASS 2005) reported farm-level sales of approximately $1.1 billion. Of that figure, $51.3 million and $38 million were from sales of ornamental goldfish, koi and baitfish. The 2005 census indicates the total value of farm sales from these species was $49.6 million exceeding, the tropical fish farm sales of $34.4 million during that same period. The 2005 aquaculture census responses indicated 358 ornamental fish farms and 257 baitfish farms. The ornamental fish farms produced their highest sale value from ornamental koi carp, ornamental goldfish, and tropical fish species. The baitfish farms produced

their highest sale value from fathead minnows, feeder goldfish, and golden shiner species (NASS 2005).

Although the number of ornamental fish farms increased by 13, the sales value decreased $17.7 million compared to the 1998 Census of Aquaculture. Baitfish sales value increased over the same period of time. There were 18 less baitfish farms in 2005 with a slight increase of $0.5 million in sales value. Tropical fish had a loss of $22.7 million in value compared to the 1998 Census survey (Table 4).

Table 4. 2005 number of farms and value of sales for ornamental fish and baitfish in the US.

Type of fish	Number of farms	Value of sales ($ million)
	US total	
Ornamental fish[a]	358 (345)[b]	51.3 (69.0)[b]
Ornamental koi carp	193	6.6
Ornamental goldfish	92	9.7
Tropical fish	158 (192)[b]	34.4 (57.1)[b]
Other ornamental fish	22	561
Bait Fish[a]	257 (275)[b]	38.0 (37.5.)[b]
Fathead minnow	160	9.8
Golden shiner	76	17.1 (18.1)[b]
Feeder goldfish	40	6.3(9.3)[b]
Other baitfish	39	1.1

Source: USDA, NASS, 2005 Census of Aquaculture

[a]*Number of farms may not add up to total due to additional counting of farms producing multiple species.*

[b] *1998 USDA NASS Census of Aquaculture farms and sale values*

The above species are from the Cyprinidae family. Cyprinds are susceptible to a number of infectious diseases such as those caused by koi herpes virus (KHV) disease and spring viremia of carp (SVC) (Hoole et al. 2001, Ahne at al. 2002, OIE 2007). We suggest that there may be a link between the 5 US cases of SVCV from 2002-2004 and the resulting US 2005 Census sales decreased and farm increase data for ornamental fish. That link being the three detected foreign introductions of SVCV from China (Miller et. al. 2007), not to mention those undetected or undiagnosed that are related to KHV and SVCV (two of these introductions were in farms of commercial ornamental fish breeders. One commercial breeder was a large hatchery operation shipping to approximately 45 States).

United States imports of ornamental fish has increased consecutively from 2003-2005 to a value of $46.1 million, while U.S. exports after rising for 4 years (2001-2004) fell sharply to $5.7.millon (ERS 2006). As the US continues to increase the importation of ornamental fish, the impact of disease on production losses due to morbidity or mortality at a national and multinational level must be considered. Inadequate or poorly implemented biosecurity measures have led to significant losses due to transboundary aquatic animal diseases worldwide e.g., Japan – (KHV), $16.4 M; Ecuador-whitespot disease (WSD), $280.5M; USA-SVCV, $11.7M; and USA-infectious salmon anemia (ISA), $8.3M (Bondad-Reantaso et al. 2005). Rising global intensification and commercialization of aquaculture have increased the need for improved aquaculture biosecurity.

According to the World Organization of Animal Health there have been five outbreaks of SVC and one outbreak of KHV in the US and eight outbreaks of SVC and two outbreaks of KHV internationally (OIE 2004a). The OIE reports indicated SVC in koi and goldfish breeder farms in Virginia and North Carolina (July 2002) and Missouri (June 2004), in a backyard pond of koi and goldfish in Washington (June 2004), and a public lake experienced a 10 ton die-off of feral common carp in Wisconsin (August 2002). However, on June 28, 2007, APHIS confirmed SVCV in the upper Mississippi river between Onalaska and Genoa Wisconsin and Dresbach, Minnesota from feral common carp samples submitted by the US Fish and Wildlife Service who were investigating a carp kill that occurred on May 7, 2007 (USFWS 2007-www.fws.gov/midwest/news/release07-68.html ,Startribune 2007-www.startribune.com/531/v-print/story/1277118.html).

International OIE disease reports (CEFAS 2006) list SVC outbreaks in over 30 countries with rising first time reports in Spain (1991), Switzerland (2001), US (2002), Denmark (2002), Moscow Province (2003), China (2004), and Canada (2006). Major outbreaks of KHV have been reported by OIE in Japan in 2004 and in the US- KHV was diagnosed in 12 states in 2004 (R. Hedrick pers. comm.).

Given the necessity of commerce and the volume of national and international trade in the ornamental fish industry and increased live ornamental product importation into the US, biosecurity on an ornamental fish farm should be a first line of defense to a production farm's economic sustainability. However, many

fish farmers are not convinced about the cost benefit nor effectiveness of certain biosecurity measures (Delabbio et al. 2005). In addition, they may not be aware of the potential economic importance of having a good, well documented biosecurity plan, and strict on-farm compliance.

A well written and executed biosecurity plan protects the producer, wholesaler, and retailer from severe and catastrophic losses (OATA 2006, Bondad-Reantaso et al. 2005, FAO 2006). It protects the producer and retailer from losses associated with the introduction and spread of diseased fish and enables the company to gain a reputation for producing and selling high quality fish by acknowledging that their product is value added and can be relied upon for repeat sales. A sound well implemented biosecurity plan can enhance the reputation of wholesalers as vendors of healthy fish resulting and help ensure repeat sales. This report outlines measures for developing a producer driven biosecurity plan

DISEASE RISK ASSESSMENT TOOL

Ornamental fish farmers may not believe that they have any biosecurity issues or that their farm is adequately protected in this area. Having a tool to provide an assessment of the farm's biosecurity could prove useful to confirm their belief or reveal unknown weaknesses (Delabbio et al. 2005).

Pruder (2004) states:"the poultry industry is a viable model for aquaculture development." He believes, as others, that aquaculture remains far behind the poultry industry in its understanding of diseases/pathogens and efficient methods of biosecurity. The following producer self-assessment tool (see Appendix I) is provided to help producers identify biosecurity risks and identify needed changes in aquaculture practices. The form used by major poultry breeder production facilities (Shane et al. 1995), was revised for fish farm breeder production. The six sections of the tool identify Critical Control Areas of Risks (CCAR), contained within the sections, for risk assessment that allows the farmer to determine high and low areas of risk and then use the completed report for risk management and risk communication. The six sections of the tool identified for farm risk analysis is adapted from the OIE International Animal Health Code's (2007) four components of risk analysis: hazard identification, risk assessment, risk management, and risk communication. Contained within the risk assessment component are three other assessments types. They are: release, exposure and consequence. These three assessments require the skills of fish pathologists and veterinary epidemiologists and others trained in similar disciplines (OIE 2007). The OIE principles of risk assessment have been applied in considering the day to day management decisions, exposure of fish stocks and movement of stock, equipment and personnel within and between farms (Rodgers 2000). The similarity between poultry and aquaculture breeder production has allowed the retention of most of the original quantifying numbers (weighted according to risk) from the poultry industry (Shane et al. 1995). In section I, Farm management ,

(Appendix I) each factor (e.g., are entry doors locked?) has four numbers (each in parentheses) that indicate a management decision that is known by experience to either increase or decrease the probability of the introduction or spread of disease. The higher number reflects the greater risk (on an arbitrary scale of 0 to 25) as the lowest number is considered the least risk. Sections II, III, and IV have numbers (in parentheses) weighted according to risk based on answers to daily management operations (Shane et al.1995). While section V addresses risk communication regarding possible disease dissemination, it encourages documentation of farms and surface waters that are within ½ mile. A weight of 2 is given for each farm and body of surface water in a radius of the above distance. After adding the totals for each section, one can determine whether there are low or high sections of risk in a facility. Adding all the sections together gives one a grand total that determines if the facility is low or high risk.

The six sections of the tool identified for risk assessment along with the range of risk assessment scores are:

(I) Farm management practices, (28-204)

(II) Vector control, (0-11)

(III) Cleaning and disinfection procedures, (9-18)

(IV) Personnel, (0-120)

(V) Disease dissemination, (0-8) and

(VI) Farm identifiers (0-125).

The higher the number, the greater the risk, with a grand total score of 486 being the highest risk facility for the potential introduction of SVCV, and a facility with a grand total score of 28 being the least likely to have SVCV become a fish health and economic problem.

An in-depth list of factors to consider for each of these sections was developed from APHIS Veterinary Services based upon information published by the Australian Veterinary Authority (AVA) for disinfection of agricultural facilities (AVA 2000), OIE guidelines for aquatic animals (OIE 2007), information from US ornamental fish producers and a two-day meeting of experts from ornamental, tropical and baitfish industries along with local, State and feral authorities that established the APHIS VS SVC Technical Committee (SVCTC 2003 unpubl. data). The SVCTC 2003 is responsible for the majority of the data provided in Appendix II. [A binder produced by APHIS for this meeting contained the reference materials mentioned in this chapter along with additional information that was not published.]

DISEASE REDUCTION

When using the assessment tool and developing a biosecurity plan, the fish farmer should think about the daily production operations and how to reduce potential pathogen introductions and their spread to facilities and grounds (farm to farm and pond to pond), and from equipment and personnel (Rodgers 2000). Since ornamental fish production among breeders involves a high degree of culling for breed selection, co-mingling of stocks should be restricted as well as placement of these mixed stocks into various pond and farm sites (APHIS SVCTC 2003 unpubl. data). The fish farmer should complete and review the assessment form for areas where the scores were high. A discussion on some ways to reduce high assessment scores follow.

CRITICAL CONTROL AREAS OF RISKS (CCAR)

Within each of the six topics of the disease risk assessment tool there are various CCAR that must be identified to minimize disease introductions and disease spread. The main zones wherein these CCAR are located include facilities and grounds, and equipment and personnel (SVCTC 2003 unpubl. data, Pillay and Kutty 2005). The farmer should also use the items within the following tables as a model to develop their own basic biosecurity plan.

Table 5. Facility and Grounds[2]

- Post a sign at each entrance to every property that notifies people of the biosecurity requirements. Suggested wording: "STOP, BIOSECURE AREA, DO NOT ENTER, Call _____ to schedule an appointment."
- Keep a log of persons entering and exiting each property. This should include everyone, even employees who have a regular, predictable schedule on the property. The log book should be monitored weekly to insure proper recording of dates, name of the visitors, where visitors are arriving from, and purpose of the visits.
- Maintain premises in a clean manner. Keep grass and other growth cut short to discourage rodents and other wildlife.
- Promptly collect and remove all debris to discourage rodents.
- Provide for control of rodents and other pests.
- Fence facility and use locked gates at every entrance.
- Fence or enclose the water supply.
- Periodically check water quality and maintain in good condition.
- Keep water supply free of wild (feral) fish or use double screens to keep them out of your facility.
- Cover ponds with bird netting where practical.
- Replace dirt runs with cement is desirable
- Do not allow free public access to sensitive areas.
- Escort all visitors.

[2] Tables 5 -7 based on recommendations from APHIS SVCTC 2003 meeting.

Table 6. Equipment

- Proper equipment disinfection requires thorough removal of mud and debris prior to disinfection.
- Maintain a separate set of equipment such as nets, waders, crowders, etc. for each farm or site. Do not use same equipment between sites. Mark or color code equipment to insure that it is not moved between sites.
- Store all equipment in a clean, well maintained area away from pests.
- Clean and disinfect equipment between ponds, daily or immediately after each use. Retire equipment made of wood or other porous materials as it cannot be properly and adequately disinfected.
- Require any public equipment, such as vehicles, waders, boots, nets, etc., be disinfected as they enter and leave premises.
- Properly disinfect all nets prior to their re-use.
- Use dedicated equipment for handling mortalities and diseased fish. This equipment should not be used for other purposes and should be marked to avoid confusion.
- Properly disinfect everything that comes in contact with dead, moribund, or sick fish.
- Do not drive unnecessary vehicles onto premises. Use dedicated vehicles and equipment on each site as much as possible.

Table 6. (continued)

- Park personal vehicles at the entrance, on a paved or graveled area, away from the ponds, and use the dedicated vehicles.
- Do not return to personal vehicles until you have changed outer garments and thoroughly washed your hands.
- Vehicles and equipment that must be shared between sites should be cleaned and disinfected upon entering and leaving each site.
- Vehicles designated to move equipment should have clean and dirty areas set aside (dirty areas are to be separate from the passenger compartment) and equipment hauled in the dirty areas of the vehicle should be disinfected as per guidelines below.

Personnel

Train all employees and especially new employees on proper biosecurity procedures identified in the plan and maintain a log of this training (http://www.fsrrn.net/modules/content/index.php?id=54). Require refresher courses at least annually. Train employees in their duties and provide refresher courses and cross-training to assure accurate completion on a continual basis. This training will be especially helpful if migrant workers are hired and English is not their primary language (APHIS.SVCTC 2003 unpubl.)

Table 7. Personnel

- Site specific personnel offer the greatest assurance of stopping any disease pathogen transmission.
- Provide workers with appropriate clean outer garments, gloves, boots, etc.
- Provide a showering/changing area where employees can remove street clothing and change into dedicated clothing for the premises or freshly laundered clothing. In this area, post biosecurity reminders. Require that employees shower or change and wash hands each time they enter the premises, prior to beginning work. In the absence of a shower/changing area on site, personnel reporting to start the day's work should have clean clothing, boots, gloves, be showered as per personnel guidelines below. Upon daily start and frequently during the day, hands should be washed with disinfectant hand soap and warm water.
- In situations where site specific personnel are not possible, personnel must consider the pathogen being carried on clothing, waders, boots, and other personal items. A change of clothes and/or disinfection of these items and equipment are needed before their use in another operation.
- It is desirable for personnel to shower (warm water shower and antibacterial soap) and disinfect all personal items prior to leaving a farm.

Table 7. (continued)

- When a vehicle is used between sites, contact of clothing and skin with possibly contaminated areas of the vehicle must be considered in a plan to assure the absence of pathogens when entering a new farm or site.
- Cleaning and disinfecting of clothing, waders, boots, and person is particularly important for persons contacting dead fish, moribund fish, or fish with clinical signs in the performance of daily duties.
- Personnel should use only properly disinfected equipment during the workday.
- Personnel leaving the premise at the end of the day should wash hands with antibacterial soap and warm water, properly clean and disinfect boots and waders, remove clothing for laundering by bagging in plastic sacks and placing in dirty area of transport vehicle, shower using methods below, clean fingernails, clear respiratory passages by blowing nose, clearing throat, and expectorating into sink with running water. Personnel should avoid contact with any fish outside the property.

By incorporating the above listed CCARs and implementing the additional CCARs from the categories that follow (e.g., isolation and quarantine, clean and disinfect, animal health certificates, employee training and dead fish disposal),

the fish farmer will have a daily recordable and documented check-off method of disease reduction to further validate the biosecurity plan. Explanation of these additional categories is expanded upon below from recommendations of the APHIS SVCTC 2003.

Isolation and Quarantine

Having a separate area isolated from exposure to other fish and facilities goes a long way in preventing cross-contamination and co-mingling during quarantine and treatment of incoming fish. This is especially important prior to introducing new fish onto the farm (OATA 2006).

When introducing new fish: wild fish, fish from other farms, or fish returned to the farm by a distributor should all be considered potential sources of disease. New fish should be placed in the isolated area, and inspected for susceptible diseases before they are brought onto the farm. Minnows (feeder-fish) returned to producers from dealers should be kept separate from established fish stocks (OATA 2006).

Producers of ornamental fish species should quarantine new fish in ponds or facilities separated from the rest of the farm by the greatest practical distance and as far away from existing broodfish stocks as possible (OATA 2006). The duration of the quarantine should be at least several weeks and involve the full

range of spring or fall temperature fluctuations (quarantining fish in the winter for a disease that only occurs in warm water is not useful).

Incoming fish should not be quarantined for less than 2 weeks because it may take that amount of time for diagnostic virus isolation test results to be obtained (OATA 2006). Holding the fish for 4 weeks would be best, as this would allow time for clinical signs of other pathogens, e.g., parasites, to appear if present. Therefore, the farmer benefits from producing fish of high quality as a result of healthy fish introductions into the ponds. A 30-day hold or quarantine period could be considered as a "value added" item applied toward the sale of production fish at harvest time.

Contaminated water*:* The safest (most pathogen-free) water for fish production is well water (OIE 2004b, 2007). However, concerns about declining water tables and pumping costs have raised interest in re-using water and in the use of water pumped from rivers. Water recirculated within a farm from pond to pond is not likely to be the source of new pathogens but may enable existing pathogens to build up larger populations. River water may contain new pathogens not present on the farm and is the least desirable source of water for fish production. If river water must be used, it should be pumped through the finest filter practical and aged in fish-free ponds before use (Goodwin 2004). Additional water treatments should also be considered to render the surface water specific-

pathogen free e.g., ozone, and sand filtration followed by ultraviolet light (Figure 14) (OATA 2006, Pillay and Kutty 2005).

Clean and Disinfect (C&D)

Treatment surfaces must be thoroughly cleaned and removed of all organic debris for chemicals to be effective disinfectants. The use of the correct and proper chemicals that can actually kill the infectious organism, and some knowledge of the disease could be vitally important (AVA2000, OIE 2006, Pillay and Kutty 2005). It is extremely important to follow the manufacturer's directions concerning concentrations, shelf-life, storage and temperature (AVA 2000). Table 8 lists disinfectants applicable to some microorganisms pathogenic to aquatic animals, indications and their methods of use is provided,[taken from (OIE 2006) and (Torgersen and Hasten 1995)]. Ahne (1986) provides specific disinfection measures for SVCV.

Table 8. Disinfection and method of use.

Processes	Indications	Method of use*	Comments
Physical			
Desiccation, sunlight	Fish pathogens on earthen bottoms	Dry for 3 months at an average temperature of 18°C	Drying period can be reduced by the use of a chemical disinfectant
Dry heat	Fish pathogens on concrete, stone, iron, ceramic surfaces	Flame-blower, blow-lamp	
Damp heat	Fish pathogens in transportation vehicle tanks	Steam at 100°C or more for 5 minutes	
Ultra-violet rays UV-C (254 nm)	Viruses and bacteria	10 mJ/cm^2	Minimum lethal dose
Ultra-violet rays UV-C (254 nm)	Infectious pancreatic necrosis (IPN) and nodavirus (VNN/VER [viral nervous necrosis/viral encephalopathy and retinopathy]) in water	125-200 mJ/cm^2	
Chemical			
Acetic acid	Infectious salmon anemia (ISA)	0.04-0.13%	
Quartenary ammonia	Virus, bacteria, hands, plastic surfaces	0.1-1 g/litre for 1-15 minutes	IPN virus resistant
Calcium oxide[a]	Fish pathogens on dried earth-base	0.5 kg/m^2 for 4 weeks	Replace in water and empty disinfected pools keeping the effluents at pH <8.5
Calcium hypochlorite[a]	Bacteria and viruses on all clean surfaces and in water	30 mg available chlorine/litre. Leave to inactivate for several days or neutralise with sodium thiosulfate after 3 hours	Can be neutralised with sodium thiosulfate. See special recommendations

Table 8. (continued**)**

Processes	Indications	Method of use*	Comments
Calcium cyanamide[a]	Spores on earthen bottoms	3000 kg/ha on dry surfaces; leave in contact for 1 month	
Chloramine T	Destroys ISA	1% for 5 minutes	
Chloramine T	Destroys IPN	1% for 30 minutes	
Chlorine dioxide	ISA	100 ppm for 5 minutes	In water of low organic loading
Formic acid	Ensile fish waste	pH <4 after at least 24 hours	Destroys bacterial fish pathogens and ISA but not IPN
Formalin	Fish pathogens in sealed premises	Released from formogenic substances, generally trioxymethylene. Comply with instructions	Nodavirus resistant
Hydrogen peroxide	ISA virus	0.02-0.06%	
Iodine (iodophors)	Bacteria, viruses on nets, boots and clothing	200 mg iodine/litre for a few seconds	See special recommendations
Iodine (iodophors)	Hands, smooth surfaces	>200 mg iodine/litre for a few seconds	

Table 8. (continued)

Processes	Indications	Method of use*	Comments
Chemical			
Ozone	Sterilisation of water, fish pathogens	0.2-1 mg/litre for 3 minutes	Costly and very toxic for fish and humans
Ozone in seawater	Surfaces, equipment	0.5-1 mg/litre TRO (total residual oxidant) for 30-60 minutes	
Poroxy compounds, e.g. Virkon	IPN virus SVCV, VHS	1% for 1 minute	
Peracetic acid	ISA virus	0.08-0.25%	
Sodium hydroxide[a]	Fish pathogens on resistant surfaces with cracks	Mixture: Sodium hydroxide, 100 g Teepol®, 10 g Calcium hydroxide, 500 g Water, 10 litres Spray, 1 litre/10 m^2 Leave for 48 hours	The most active disinfectant $Ca(Ca(OH)_2$ stains the surfaces treated; Teepol® is a tensio-active agent.
Sodium hypochlorite[a]	Bacteria and viruses on all clean surfaces and in water	30 mg available chlorine/litre. Leave to inactivate for a few days or neutralise with Na thiosulfate after 3 hours	
Sodium hypochlorite[a]	Nets, boots and clothing	200 mg to 1 g available chlorine/litre for several minutes. Leave to inactivate for a few days or neutralise with Na thiosulfate after 3 hours	
Sodium hypochlorite[a]	Hands	Rinse with clean water or neutralise with Na thiosulfate	

Source: OIE (Office International des Epizooties).2003.Manual of Diagnostic Tests for Aquatic Animals, 4th edition. Office International des Epizooties, Paris, France.47-48 pp.

[a] Dangerous - See precautions indicated in general recommendations

* The concentrations indicated are those for the active substance. NB: The chemicals must be approved for the prescribed use and used according to the manufacturer's specifications

Clean and disinfect equipment and outer garments, boots, gloves, etc. between ponds. Provide and use disinfecting foot baths and hand washes (Morley 2005). These should be properly maintained. Information available at:www.biosecuritycenter.org/article/bootDisinfect. Ideally there are two foot baths. One should have plain water or a detergent/water solution where the boots are scrubbed, and one with disinfectant solution that would immerse the boots. Placing hand washing facilities directly over the foot bath encourages longer standing in the foot bath (CEFAS 2006).

Drying kills many fish pathogens and is therefore a useful disinfection tool when moving equipment from pond to pond. When practical, trucks, seines, and other equipment that have been used for fish from another facility or from the wild should be rinsed (warm soapy water is even better) and allowed to dry before re-use. This is especially critical for seines and trucks that have been used to harvest or transport sick fish. As a mechanism to transfer disease from farm to farm, a wet mucus-laden seine is almost as dangerous as the transfer of infected fish (Goodwin 2004).

Farm vehicles: Farm vehicles and their contents can contribute to the dissemination of pathogens between farms. Floor mats must be removed and scrubbed with detergent and disinfected. Virkon Aquatic is listed as virucidal against rhabdoviuses like SVCV, VHSV, and IHNV. (http://www.syndel.com/virkon_aquatic/virkon_aquatic_efficacy.html)

Vehicles should be parked on a tarp or other suitable substrate. Encrusted dirt should be removed from wheels, wheel wells, and undercarriages using brushes (APHIS SVCTC 2003 unpubl. data, Pillay and Kutty 2005). Dirt and debris should be collected, disinfected, and disposed of (OIE 2006). Powerful jets of water should not be used to remove encrusted dirt in order to minimize the potential dispersal of dirt. All organic matter should be removed from all transport vessels (fish tanks, trash containers, truck beds, etc.), collected, disinfected, and disposed in plastic bags and incinerated These areas can then be treated with Virkon Aquatic (http://www.syndel.com/virkon_aquatic/virkon_aquatic_brochure.pdf.) (OIE 2006). Ensure that surfaces are wet with disinfectant for at least 10 minutes before being allowed to air dry. Cleaning and disinfection personnel should park personal vehicles off-site (AVA 2000).

To summarize C&D:

- Chose the correct chemical that should be effective against the pathogen
- Use the chemical according to manufacturers directions
- Allow adequate fallow time between restocking
- Understand the biological properties of the pathogen (e.g., the temperature dependency of SVCV and the fish immune system will alert you to the proper seasons and temperatures to test and quarantine for SVC).

Health Certificates (from an approved lab [NVSL 2004])

There are many farms that would still be operating and economically sustainable if they had not bought fish of unknown health status and added them into their

farms (e.g., the NC and MO commercial broodstock operations infected by SVCV) (Miller et al. 2007).

If there are concerns about diseases that could jeopardize the entire farm's operation, like SVC, then at a minimum, new fish should not be introduced to the farm operation without knowing if the new fish have diseases of concern. All incoming fish should be properly inspected and from health approved sources. For countries listed as free of a disease with OIE, and especially developing countries, importation of fish must be accompanied by health certification based upon testing outlined in the OIE sampling and surveillance plan (OIE 2006). For domestic shipments where a health certificate may not be available have one of the approved labs provide a laboratory report indicating the status of SVCV.

Employee Training

How many times has a farm, with a good biosecure facility, become infected with disease because of poor personnel practices? This is by far the most common point of introduction of fish disease and its spread (Subasinghe 2005, FAO 2006). A facilities biosecurity plan should outline annual and regular orientation training for new and current employees, and describe proper disinfection techniques of equipment, clothing and facilities. It should also bring awareness of personnel and management practices that undermine the entire farm's operations (Hardy-Smith 2006).

Dead Fish Disposal

Quick and adequate removal of dead and dying fish is an absolute must in preventing the spread of disease through a farm, pond, hatchery or holding facility (Pillay and Kutty 2005). Not only should they be removed, but also it is essential that dying fish be taken to a diagnostic laboratory to determine the cause of death. Having an unknown disease on a farm could lead to improper treatment attempts and unnecessary cleaning /disinfection expenses that may not prevent the spread of the disease and result in loss of more fish (APHIS SVCTC 20003 unpubl.data).

Good Biosecurity Plan

In summary, a well written and executed biosecurity plan protects the producer, wholesaler and retailer from severe and catastrophic losses (Shane et al. 1995,AVA 2000, Pillay and Kutty 2005). It protects the producer and retailer from losses associated with the introduction and spread of diseased fish and enables the company to gain a reputation for producing and /or selling high quality fish by acknowledging that their product is value added and can be relied upon for repeat sales. It protects the wholesaler by gaining a reputation for distributing healthy fish resulting in repeat sales.

Using the information provided in this chapter, a model biosecurity plan can be drafted by the fish farmer to incorporate into the farm's operations. The details in

this chapter provide a basic biosecurity model for ornamental fish producers that incorporates the CCARs previously mentioned.

USDA SVC PROGRAM

The United States Department of Agriculture, Animal and Plant Health Inspection Service (APHIS) may be able to provide financial assistance to producers of farm-raised fish if their sick fish are diagnosed with SVC, a viral foreign aquatic animal disease of common carp, koi carp, and goldfish (NARA 2004). The Animal and Plant Health Inspection Service may be able to assist the farmer in the development of an action plan that describes how to depopulate and dispose of sick and dying fish and provide guidance in preparing a biosecurity plan that demonstrates how to clean and disinfect equipment and personnel for farms infected with SVC. In addition, APHIS may provide indemnity up to 50% of the appraised fair market value for the depopulated fish and up to 100% of the cost for cleaning and disinfection provided the producer can meet and agree to certain conditions (NARA 2004).

The Animal and Plant Health Inspection Service published two notices in the *Federal Register*, Vol. 69 No. 95 Rules and Regulations amending 9 Code of Federal Regulations Parts 53 and 71 that provide payment of indemnity and pay the cost for cleaning and disinfection of the farm to owners of fish destroyed because of SVC and to prevent the movement of fish infected with or exposed to SVC, except for those being transported for slaughter, respectively. The

comment period closed on July 16, 2004. One can find more details on payment of indemnity for SVC and requirements for eligibility in the *Federal Register,* which was published on May 17, 2004 (NARA 2004).

Acknowledgements. Special thanks to Rosemary Sifford, Don Rush, Jill Rolland and Kay Wheeler, A .E. Goodwin and the SVCV Technical Committee for their assistance in the development of this manuscript.

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4. CONCLUSION AND FUTURE DIRECTION

Conclusions

An epidemiological study was conducted to examine the diversity of SVCV nucleotide sequences of the phosphoprotein gene in comparison with the glycoprotein gene. Three phylogenetic analyses: (1) confirmed the P gene's ability to detect G gene established genogroups Ia to Id), (2) assessed the genetic diversity of the P gene to discriminate two previously unidentified subgroups within genogroup Ia, and (3) determined the genetic relationship among 35 spring viremia of carp virus (SVCV) genogroup Ia isolates based on the nucleotide sequence of the P gene and G gene sequences where the P gene provided higher bootstrap support values. The higher P gene diversity allowed us to conclude that at least two independent introductions of SVCV occurred in the US and Canada since 2002 and that both of these introductions were from SVC virus strains that have a genetic link to China.

During the period from 1997-2006 there were 32 separate isolations of SVCV in the UK, USA and Canada. Sequence analysis revealed strong genetic relationships between 14 of the 26 viruses isolated in the UK during this period, and viruses with origins in China. In some cases the nucleotide sequences were identical, suggesting a direct link between the infected fish. In at least one case where the fish were sampled during routine import checks the infection can be traced to China, but in other cases, particularly where fish movement records show that fish from more than one country were held on the same site, it is more difficult to establish a direct link to the Chinese ornamental industry. At least

three introductions of SVCV into the US were either directly from China or received by a third party based on epidemiologic investigation. Molecular epidemiology allows further source tracing of outbreak strains based on genetic relationships. It is not surprising that Asia is a source of a number of imported pathogens since the FAO (2006) World Aquaculture report indicating that 90% of world aquaculture production comes from Asia with 70% of that total from P.R. China (Bondad-Reantaso et al. 2005).

Biosecurity plays a very important role in prevention and spread of disease. A through understanding of likely geographical disease-risk sources coupled with health certifications demonstrating freedom of disease from SVCV risk areas would be important in preventing new SVCV introductions. Identifying trace country of origin from point source outbreaks could avert further introductions and spread of SVCV.

Given the necessity of commerce and the volume of national and international trade in the ornamental fish industry and increased importation of live ornamental product into the US, biosecurity on an ornamental fish farm should be a first line of defense to a production farm's economic sustainability. However, many fish farmers may not be convinced about the cost benefit nor effectiveness of certain biosecurity measures (Delabbio et al. 2005) or may not be aware of the economic importance of having a good biosecurity plan that is routinely practiced on the farm and of the importance of having such a plan in a written format.

Ornamental fish farmers may not believe that they have any biosecurity issues or that their farm is adequately protected in this area. Having a tool to provide an assessment of the farm's biosecurity could prove useful to confirm their belief or reveal unknown weaknesses. The disease risk self-assessment tool (see Appendix I) is provided to help fish farmers identify what level of biosecurity risk a farm may actually incur.

Future Directions

The work presented in this dissertation provided the basis for further applications in molecular epidemiology using the P gene of SVCV. This includes phylogenetic analysis of the P gene in comparison with the G gene especially in making biogeographical inferences to SVCV outbreaks. The higher diversity of the P gene sequence compared to the G gene, particularly in genogroup Ia viruses, provided the information that allowed genogroup Ia viruses to be subdivided into two separate groups (Miller et al. 2007). These phylogenies, when property rooted with an outgroup, can provide lines of descent (common ancestry) to distinguish between outbreak strains or further demonstrate their relatedness.

This work provides applications beyond outbreaks of SVCV. It is clear that from the rapid evolution of RNA viruses and the heterogeneity of the RNA virus genomes, future RNA viral disease outbreaks including newly emerging viruses are enviable.

Of the seven viral fish diseases listed by OIE, three are rhabdoviruses, (four are RNA and three are DNA).

The dissertation also highlights areas for consideration of future biosecurity measures in the US. Federal import protocols and import quarantine requirements should be equivalent to the exporting country. At present, many countries have higher import standards for aquatic animals than the US. Also, developing countries that may not have appropriate animal health controls and sanitary measures would not be encouraged to do so as long as US import requirements remain in their current state.

At the farm level, producers should take into consideration the biosecurity measures (see chapter 3) from the pilot study APHIS conducted in 2002 to 2006 that resulted in a SVC-free aquaculture facility according to OIE (2007) guidelines. The restocking of SVCV-free broodstock (per health certificate and lot testing) and use of sand filtration with UV disinfection of surface waters to fill ponds that don't have well water are applications for future use (Figure 14). For the steps to become an OIE SVC-free aquaculture facility see Figure 15.

Figure 14. Sand filtration and UV control units. Pictures by B. Vinci.

1. *Farm tested twice annually for 2 yrs with samples of 150 fish per test*
2. *Wild fish tested annually, 150 fish for 2 yrs*
3. *SVC –free water source*
4. *Not connected to a watercourse*
5. *After 2yrs, maintain twice yearly inspections with samples of 30 fish*

Figure 15. Steps for a farm to become an OIE SVC-free facility.

Since there is no treatment for SVCV, without adequate vaccine protection against SVCV, significant disease outbreaks will continue. Vaccine design and development could prove critical to the economic sustainability of producers, importers and retailers. A highly effective vaccination for SVCV could prove to be a significant management tool in much the same way that the recently commercially approved DNA vaccine against IHNV vaccine will greatly enhance control of IHNV. Future directions could be using the SVCV clones produced with this research for gene expression and DNA vaccine development.

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5. APPENDICES

Appendix I

Risk Assessment Disease Tool for Ornamental Fish Suppliers

(Numbers besides answers are weighted according to risk. For total score, add the numbers next to your answers for sections I-VI)

I. Farm Management Practices (Question/Observation)	**(Circle one) Score Response**
A. Are entry doors locked?	(24) Never (12) Sometimes (<25%) (6) Most of the time (>50%) (3) Always
B. Are foot baths being used?	(24) Never (12) Sometimes (6) Most of the time (3) Always
C. Does farm labor wear footwear provided specifically for the farm?	(24) Never (12) Sometimes (6) Most of the time (3) Always
D. Dead fish disposal system	(4) Rendering (3) Composing (2) Pit (1) Incinerator
E. Evidence of rodent and vermin control	(16) Poor (8) Fair (2) Good (1) Excellent
F. Efficiency of fly control in hatchery.	(4) Poor (3) Fair (2) Good (1) Excellent
G. Frequency of unauthorized visitors.	(24) Often (12) Sometimes (6) Rarely (3) Never

H. General neatness of work rooms and service areas.	(4) Poor (3) Fair (2) Good (1) Excellent
I. General appearance of area surrounding pond & hatchery including weed control and storage and placement of equipment and other items.	(16) Poor (8) Fair (4) Good (2) Excellent
J. Quarantine & isolation of incoming fish.	(16) Never (8) Sometimes (4) Rarely (2) Minimum of 14 days
K. Type of water source?	(24) – River (12) – Lake (6) –First use spring (3) –Well/borehole, UV, ozone treated surface water
L. How long has farm labor been employed on farm?	(24) <2 months (12) 2-<6 months (6) 6 months-<1 year (3) >1 ½ years

Score below 30 (excellent farm management biosecurity) = Total I _____

II. Vector control **(Circle one)**

A. Does the farm have bird depredation control program? No (1) Yes (0)

B. Are dogs used for bird control or sheep/cows for weed control? No (0) Yes (1)

C. Are dogs changed between farms? No (2) Yes (0)

D. Are pets allowed inside work rooms and hatchery? No (0) Yes (1)

E. Is there proper drainage? (can ponds overflow into other ponds during heavy rainfall?) No (2) Yes (0)

F. Does the contractor frequent a specific location where other growers or service personnel from different companies associate? If yes, where? _____ No (0) Yes (4)

Score = Total II _____

III. Cleaning and disinfection Procedures

A. Is contractor responsible for farm/pond clean-out and decontamination? If not, who is hired? _____ No (2) Yes (0)

B. Is equipment cleaned and disinfected? No (4) Yes (0)

C. Pond water drained? No (1) Yes (0)

D. Is pond bottom disinfected with lime before the new fish arrives? Was pond allowed to fallow? No (3) Yes (0)

E. Are dead fish completely removed from pond before the new fish arrives? No (2) Yes (0)

F. Are the nets washed and disinfected thoroughly? No (2) Yes (0)

G. Are designated nets used at each site? No (1) Yes (0)

H. Are wading boots cleaned or changed during clean-out seining? No (1) Yes (0)

I. Are trucks and personnel clothing cleaned thoroughly between farms? No (2) Yes (0)

Score = Total III_____

IV. Personnel	**(Circle one)**	
A. Does the farm use seasonal or migrant labor?	No (0)	Yes (20)
B. Does the contractor provide boots and coveralls for laborers?	No (5)	Yes (0)
C. Do laborers own fish or exotic birds?	No (0)	Yes (25)
D. Do laborers work for other fish farms?	No (0)	Yes (25)
E. Do laborers associate with friends or relatives that work on other fish farms?	No (0)	Yes (10)
F. Are laborers instructed in biosecurity procedures?	No (25)	Yes (0)
G. Do laborers live on the premises?	No (10)	Yes (0)

Score = Total IV _____

V. – Disease Dissemination

A. Number of farms/natural waters within ½ mile, 2pts. Per farm

Farm Name:	Natural Shared Water Name:
1.	1.
2.	2.
3.	3.
4.	4.

Score = Total V _____

VI. Farm Identifier

Record Name and Number on Grid Line:

Other Fish Farms	
Within ¼ mile	25 pts. ¼ mile
Between ¼ and ½ mile	10 pts. ½ mile

Owners' names and road numbers.

1.

2.

3.

4.

5.

Score = Total VI _____

Score = Grand Total _____

Note on scoring: Scores are proportional to the risk of infection.

Appendix II

Guidelines for the framework for development of an aquaculture biosecurity plan.

(I) **Farm management practices**

1. Visitors who have had on site contact at other fish farms within the past 24 hours should not be allowed access to the premises.

2. Reduce the number of visitors by asking people such as utility companies and vendors to talk with you over the phone or meet off-site to get the information they need.

3. Require all visitors to wear disposable boots and/or other appropriate garments to prevent contamination.

4. Stock all facilities at the optimum level. This helps prevent overcrowding as fish grow. This can avoid other stressful situations which may precipitate a disease outbreak.

5. Take fish off feed for 1-2 days before and after handling, grading, shipping, etc.

6. Assure all incoming fish are from properly inspected and from health approved sources. For countries listed as free of a disease with OIE, importation of fish must still be accompanied by health certification based upon testing outlined in the OIE sampling and surveillance plan (OIE 2006).

7. Attend to youngest fish first and handle diseased fish last.

8. Give proper amounts of feed to reduce waste and optimize growth.

9. Feed by hand to attain more even distribution of feed and to better observe fish for health and behavior changes.

10. Remove and properly dispose of mortalities on a daily basis. Bury with quick lime or burn. Prevent access by birds, insects and other pests to the carcasses at all times.

11. Keep records of the number of daily mortalities. This may indicate the beginning of a disease outbreak.

12. Personnel handling diseased fish or mortalities should not return to other duties until they have properly cleaned and disinfected outer garments, gloves, etc. and washed hands.

13. Use appropriate vaccines where possible.

14. Provide and use disinfecting foot baths and hand washes. These should be properly maintained. Hand washes should provide a waterless antibacterial gel or antibacterial soap and running water. Foot baths should include a brush so that visible contaminants can be scrubbed off and should be deep enough that the foot bottom of the boot can be immersed. New foot baths should be prepared daily or when visibly contaminated.

15. Provide a secure and appropriately maintained storage area for feed. Protect feed from birds, pests, and excessive heat.

16. Clean raceways, runs, screens, and hatchery areas daily.

17. Provide double screens at the lower end of every run, raceway, or pond. Place grates inside ends of drain overflow pipes to prevent entry of animal vectors like turtles from rivers and lakes.

18. Disinfect runs and raceways between each lot of fish, if possible.

19. Maintain quiescent zones and sediment settling areas free of fish.

20. Establish annual biosecurity audits by an outside party.

21. Broodstock and broodstock candidates should be stocked, raised, and/or held only in specially designated broodstock ponds. They should not be intermixed with fish to be harvested.

22. Allow a pond as much down time as possible (at least 2 or 3 weeks) between groups of fish.

23. Animals should be maintained and closely observed in a “quarantine” tank for at least 30 days prior to being introduced into a pond with other animals.

(II) **Vector control**

1. The most important animal vector for fish diseases are birds. Turtles, otters and snakes should also be taken into account if they are a problem on the farm. Several species of fish-eating birds carry life stages of parasitic metacercaria (“grubs”) that infest fish.

2. A bird control program that uses the most effective legal means to discourage birds from visiting farm ponds should be used (see your extension agent or the animal control specialists from USDA-APHIS Wildlife Services).

3. The same parasites that travel in birds frequently also have snails in their life cycles so chemical or biological control of snail populations is beneficial in species where grubs are a problem.

4. There is also some evidence that birds may be able to transmit bacteria or viruses through their droppings.

5. Birds may also drop fish that they have removed from one body of water into another (Goodwin 2004).

(III) **Cleaning and disinfection procedures**

1. Vehicles moving between farms: All mud and debris removed from wheel wells and disinfected with Virkon S as per manufacturer instructions (OIE 2004b). Vehicle exterior and interior surfaces cleaned.

2. Porous equipment like nets and seines: Disinfection as per OIE guidelines Manual of Diagnostic tests for Aquatic Animals, chapter 1.1.5 with sodium hypochlorite (bleach) at 200 mg available chlorine/liter for several minutes (OIE 2004b).

3. Non-porous equipment with all mud and organic debris removed: sodium hypochlorite (bleach) at 30 mg available chlorine/liter for 3 hours (OIE 2004b).

4. Waders and boots**:** remove all mud and organic material by washing, scrub with soapy water solution, rinse, and apply sodium hypochlorite (bleach) at 200 mg available chlorine/liter for several minutes or Virkon S as per manufacturer's instructions(OIE 2004b).

5. Washable clothing: spray with sodium hypochlorite (bleach) at 200 mg available chlorine/liter and let sit for several minutes (OIE 2004b). Launder routinely.

6. Skin and hair: Commercial soap and warm water shower.

(IV) **Personnel** (see personnel section under Critical Control Areas of Risks (CCAR)

(V) **Disease dissemination**

Fish on any one premise, being the carrier of potential disease agents, should be site specific and maintained on that site. Movement between sites would only be possible following appropriate testing to determine freedom from disease. Spring viremia of carp virus testing is limited to water temperatures between 51 and 64 degrees Fahrenheit on a constant basis (for periods of time of a month minimum) as is found in winter, early spring and late fall to minimize false negatives (APHIS 2003). At other times fish should be maintained site specific to prevent possible movement of virus. When it is necessary to move animals from one site to another they should be moved in leak-proof containers. Water from one site should not be introduced to another site with the animals.

1. When populations of fish stop feeding, behave strangely, or suffer significant mortality, samples should be submitted to one of the twelve USDA APHIS National Veterinary Services Laboratories (NVSL) approved disease diagnostic laboratories (NVSL 2004). These labs assist APHIS by providing diagnostic test results used to issue and endorse health certificates for international exportation .

2. Until a diagnosis is determined, sick fish should be quarantined and any movement of fish, water, or equipment from the pond should be prevented (Goodwin 2004).

3. If an exotic disease is diagnosed, the infected fish should be treated or eradicated to prevent the spread of the disease (Goodwin 2004). If SVC is diagnosed see comments under heading “USDA SVC Program.”

(VI) **Farm identifiers**

Just as it is important to know how to minimize disease risks on the farm, It is also important to know of possible sources of disease risk from the outside or at least other outside sources that could be at risk because of the fish farmer’s operation. This section is apart of risk communication that simply asks that the fish farmer take time to inventory the location of other fish farm operations in the area and be aware of the species they produce. It would be helpful if other fish farmers in the same vicinity were notified should an adjacent fish farm experience a disease outbreak or severe mortalities from unknown sources.