THE EFFECT OF DISINFECTANTS AND ANTIVIRALS ON THE INFECTIVITY AND REPLICATION OF LARGEMOUTH BASS VIRUS

THESIS

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Brian A. Scott, B.S.

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INTRODUCTION

Largemouth Bass Virus (LMBV) is thought to be the etiological agent of several largemouth bass *Micropterus salmoides* fish kills throughout the eastern United States. The virus was first isolated from wild adult largemouth bass in the Santee-Cooper Reservoir in 1995, when more than a thousand largemouth bass had died (Plumb et al. 1996). Since its discovery, LMBV has been associated with many other fish kills throughout the United States, including Texas. Fish kills thought to be associated with LMBV can last for several months and result in the loss of more than thousands of largemouth bass (Plumb et al. 1996). Although LMBV is the primary infectious agent responsible for the fish kills, it has been found to persist within largemouth bass populations in the absence of fish kills (Hanson et al. 2001). Fish kills have only occurred between the months of June and September, which suggests that seasonal temperature may play a role in LMBV pathogenesis. Factors thought to contribute to the initiation of fish kills include stresses such as high water temperature, low dissolved oxygen, and poor handling of fish by anglers (Herr and Boston 2002). Since these stress factors are relatively uncontrollable, LMBV disease prevention is focused on containment of the virus to lakes that have been shown to contain the virus. The significant reduction in largemouth bass populations, due to a suspected detrimental effect from LMBV, has incited state agencies to initiate studies on LMBV and its geographic range.

HOST RANGE

The family *Iridoviridae* includes four genera, *Iridovirus*, *Chloriridovirus*, *Lymphocystivirus*, and *Ranavirus* (van Regenmortel et al. 2000). Members of the family have been found to infect both invertebrates and vertebrates. The genus *Iridovirus* includes invertebrate viruses such as Tipula iridescent virus and Black beetle iridescent virus. Members of the genus *Chloriridovirus* include invertebrate viruses such as Aedes taeniorhynchus iridescent virus and Mosquito iridescent virus. The genus *Lymphocystivirus* includes vertebrate viruses such as Flounder virus and Dab lymphocystis disease virus (van Regenmortel et al. 2000). LMBV is a member of the genus *Ranavirus*, which infects cold-blooded vertebrates, such as amphibians, reptiles, and fish (Willis 1990). Other members of *Ranavirus* include Frog virus 3 and Rainbow trout virus. As compared to invertebrate iridoviruses, vertebrate iridoviruses have about 20% of their genomic cytosine residues methylated (Willis and Granoff 1980). It is thought the cytosine methylation is important for DNA encapsidation and may have a function in protecting viral DNA from endonucleolytic attack (Goorha et al. 1984).

PHYSICAL CHARACTERISTICS

Restriction fragment length polymorphism analysis (RFLP), sequence determination of the major capsid protein (MCP) and DNA methyltransferase genes, and analysis of viral protein synthesis in infected cells classify LMBV as a member of the family *Iridoviridae* in the genus *Ranavirus* (Mao et al. 1999). Recently, RFLP analysis has

resulted in the identification of at least three intraspecific strains of LMBV. In cell culture, these strains are phenotypically similar based on replication rates. In live fish, however, a phenotypic difference in virulence is observed (Goldberg et al. 2003). LMBV is closely related to Doctor fish virus and Guppy fish virus, both found in ornamental fish in Southeast Asia (Mao et al. 1999). Iridoviruses possess a linear dsDNA genome approximately 150-350 kbp in length. Viruses in the genus *Ranavirus* have a 150-170 kbp genome that is compacted into a long coiled nucleoprotein core approximately 10 nm in length (van Regenmortel et al. 2000). Like *Enterobacteria phage T4* and *Enterobacteria phage P22*, the viral DNA is circularly permuted and terminally redundant (Goorha and Murti 1982). The guanine and cytosine content of the genome is 53%, and all cytosines in the 5′ to 3′ dinucleotide cytosine-guanine (CpG) are methylated by a viral encoded DNA methyl transferase. This methylation may be important for encapsidation of the viral DNA and may also protect the DNA from endonucleophytic attack (Goorha et al. 1984). Viral genes found on both strands of the genome code for approximately 54 proteins (Fields et al. 1996). Approximately 36 proteins make up the structural proteins of the viral capsid all ranging in mol wt. from 1×10^4 to 2.5×10^5. The most abundant viral encoded protein is the MCP, which comprises approximately 40% of the viral genome (van Regenmortel et al. 2000). In addition to structural proteins, various virion-associated enzymes have been found. These enzymes include a DNA-dependent RNA-polymerase II subunit, a helicase, both ssRNA and dsRNA ribonucleases, deoxyribonucleases, nucleotide phosphohydrolase, protein kinase, protein phosphatase, GTP phosphohydrolase, thymidine kinase, poly (A) polymerase, and mRNA capping enzymes (Fields et al. 1996; van Regenmortel et al. 2000).

Iridoviruses possess an icosahedral capsid approximately 120-200 nm in diameter. The capsid consists of identical capsomers arranged to form trisymmetrons and pentasymmetrons in members of the genera *Chloriridovirus* and *Iridovirus*. Capsomers are composed of an internal and external trimer, and are approximately 6-7 nm in diameter and 7-13 nm in height (van Regenmortel et al. 2000). A lipid envelope, which is derived via budding through the host outer cell membrane, surrounds the capsid. This lipid envelope is not necessary for virus infectivity (van Regenmortel et al. 2000).

REPLICATION

Studies of Frog virus 3, an iridovirus in the genus *Ranavirus*, have revealed that iridovirus entry occurs via pinocytosis with envelope uncoating occurring inside phagocytic vesicles (van Regenmortel et al. 2000). The viral DNA is released into the cell nucleus, where it is replicated into genome length molecules (Goorha 1982). This DNA may serve as a template for early stage transcription in the nucleus by a modified cellular RNA polymerase II, or it may be transported to the cytoplasm (van Regenmortel et al. 2000). A second round of DNA replication in the cytoplasm results in the formation of concatamers (Goorha 1982). A viral-encoded DNA integrase-recombinase may be involved in the recombination and the organization of the viral genome into newly formed capsids (van Regenmortel et al. 2000). This form of DNA concatamer processing follows a mechanism similar to that of *Enterobacteria phage T4*. After viral assembly in the cytoplasm new virions are released through the host cell membrane via a

budding mechanism, which provides an envelope for the viral capsid (van Regenmortel et al. 2000).

PATHOGENESIS

In nature, LMBV causes fish kills which are associated only with adult largemouth bass. A study by Plumb, J.A. involved the capture of wild adult largemouth bass and homogenization of various tissue samples. Samples were screened for LMBV via tissue culture infectious dose 50% assays (TCID50) employing the Kärber method (Mahy and Kangro 1996). Fish positive for LMBV were observed to have enlarged and inflamed swim bladders containing yellowish mucus, perhaps limiting a fish's ability to control its buoyancy (Plumb et al. 1999). Although LMBV only affects adult largemouth bass in nature, it can be lethal to both juvenile largemouth bass and juvenile striped bass in a controlled laboratory setting (Plumb and Zilberg 1999a). A study performed to demonstrate the lethal dose of LMBV revealed that virus-injected fish were slightly darker than normal, had mild abdominal distension, and had inflammation at the site of injection. Fish injected with 0.1 ml of LMBV titered at $10^{6\,2}$ TCID50/ml died as soon as 36 h post-exposure (Plumb and Zilberg 1999a).

CELL CULTURE

Numerous cell lines are permissive for the replication of LMBV including bluegill fry-2 (BF-2), fathead minnow (FHM), channel catfish ovary (CCO), epithelioma

papillosum cyprini (EPC), and chinook salmon embryo cells (CHSE-214) (Bowser and Plumb 1980; Fijan 1983; Fryer et al. 1965; Gravell and Malsberger 1965; Wolf and Quimby 1966). After a 48 h incubation at 30°C, LMBV titers (TCID50/ml) have been shown to be as high as $10^{10.5}$ in BF-2, $10^{9.5}$ in FHM, $10^{7.8}$ in CCO $10^{6.5}$ in EPC, and $10^{7.5}$ in CHSE-214 cells (Piakoski et al. 1999). After 5 d incubation at 30°C, LMBV titers are reduced only in CCO, EPC, and CHSE-214 cells to $10^{7.5}$, $10^{5.5}$, and $10^{6.3}$ TCID50/ml, respectively. LMBV demonstrates similar cytopathic effect (CPE) when compared to other iridoviruses, which begins with pyknosis, followed by rounding of cells in the monolayer, and ending with lysis and detachment of the infected cell from the growth substrate (Piakoski et al. 1999).

CONTROL AND PREVENTION

Currently, there is no known immunization or cure for LMBV disease. State agencies recommend the control of LMBV passage from infected to non-infected water habitats as the best method to prevent fish kills (Herr and Boston 2002). To achieve this goal, bass anglers are educated on the mechanisms of virus spread among water habitats. Anglers are instructed not to fish in more than one lake per day, and to let boats and equipment completely dry in the sun for 24 h before subsequent use in another lake. Bass anglers are reminded to handle fish more carefully to reduce stress (Herr and Boston 2002). This form of LMBV control is thought to slow the spread of the virus. However, in addition to drying and sunlight, more potent means of viral inactivation must be found and made readily available to bass anglers and recreational fisherman to prevent spread of the virus.

The virus has been reported to remain infectious in water for at least three to four hours, which gives it the potential to move to uninfected water habitats in live-wells of boats contaminated with the virus (Herr and Boston 2002). Since many surfaces of a boat are not accessible to sunlight and may not dry completely, the use of chemical disinfection to aid in preventing the spread of LMBV and subsequent fish kills may be of benefit. Known antiviral agents for LMBV could be used in conjunction with physical disinfection to help decontaminate virus infected fishing equipment. Chemical agents such as household bleach and ethanol, if found to be antiviral against LMBV, would make ideal agents for the disinfection of boats and fishing equipment since they are inexpensive and readily available to the public.

CHEMICAL AND PHYSICAL DISINFECTION

Formalin. - Formalin is a chemical fixative that contains 10% formaldehyde. It is used for the preservation of tissue and cell samples and for the decontamination of biological safety cabinets. It is an extremely reactive chemical that affects proteins by reacting with primary amides and amino acid side chains (Fraenkel-Conrat 1961). This property may interrupt the ability of the virus surface proteins to interact with host cells, thus preventing entry into the cell. Formalin has also been found to cross-link proteins to DNA in SV-40, thus preventing DNA replication from occurring (Permana and Snapka 1994). Since formalin is a more hazardous chemical than the bleach, ethanol, or iodine, it would not useful outside of the laboratory setting. Formalin has been found to be effective against other fish viruses such as Infectious pancreatic necrosis virus and

Egtved virus (Jorgensen 1973). Formalin, if found to be antiviral against LMBV, would be of use in preserving or preparing largemouth bass tissue samples contaminated with LMBV. This would prevent cross contamination of fish work areas.

Iodine. - Iodine is another antimicrobial that is readily available to the public, and it is currently widely used as an antiseptic to prevent bacterial infection. Like bleach and ethanol, the exact mechanism of iodine is unknown (McDonnell and Russell 1999). Iodine is only known to penetrate microorganisms attacking a key group of proteins containing sulfur containing amino acids such as cysteine and methionine (Gottardi 1991; Kruse 1970). In addition, iodine causes damage to nucleotides and fatty acids (Apostolov 1980; Gottardi 1991). Iodine was found to be antiviral against Sea bass neuropathy nodavirus (SBNV), reducing the titer from $10^{6\,125}$ to 10^{0} TCID50/ml after 5 minutes of exposure to 25 ppm I_2 (Frerichs et al. 2000). If found to be antiviral against LMBV, iodine would provide a safe and efficient way to disinfect surfaces contaminated with LMBV.

Chlorhexidine Diacetate. - Chlorhexidine diacetate is the main ingredient in some antimicrobial disinfectants used in veterinary clinics to decontaminate various surfaces. It is effective against a wide variety of animal viruses including Canine distemper virus, Equine influenza virus, Hog cholera virus, and various other animal viruses (Nolvasan, Fort Dodge, IA.). Chlorhexidine is a biguanide that has been found to affect the cell membrane of bacteria, protazoans, and yeasts, causing leakage (McDonnell and Russell 1999). In addition, high concentrations cause the precipitation of proteins and nucleic

acids in bacteria and yeast (Hugo and Longworth 1965, 1966, 1964). It is more effective against enveloped virus as opposed to non-enveloped viruses. Since chlorhexidine diacetate is not readily available to the public, it would better serve fish hatcheries or companies that deal with mass quantities of largemouth bass.

Bleach. - Bleach, with sodium hypochlorite as its active ingredient, is a widely used hard-surface disinfectant that affects a broad range of microbes. It is currently used to clean spillage of Human immunodeficiency virus (McDonnell and Russell 1999). While the true mechanism of action is unknown, sodium hypochlorite is a highly reactive oxidizing agent that destroys the functional activity of cellular proteins (Bloomfield 1996). A similar effect on LMBV proteins may serve to inactivate the virus.

Ethanol. - Not much is known about the specific mechanism of ethanol other than it causes rapid denaturation of proteins and membrane damage (Larson and Morton 1991; Morton 1983). Ethanol is widely used as a surface disinfectant in microbiological work areas. While isopropyl alcohol is more effective against bacteria, ethanol is more effective against viruses, based on concentration (McDonnell and Russell 1999). The properties of ethanol may serve to inactivate LMBV by affecting both its proteins and envelope.

Ultraviolet Light and Temperature. - Ultraviolet (UV) light has recently been shown to reduce the titer of SBNV greater than 99.9% in eight minutes (Frerichs et al. 2000). Additionally, rapid inactivation of SBNV was reported at 37°C, with no viable virus

detected after 4 days. Current research on LMBV indicates the virus is stable in largemouth bass tissue frozen at -10°C for a period of 5 months (Plumb and Zilberg 1999b). A study of the effect of temperature and UV light on the infectivity of LMBV would be helpful in developing guidelines that bass anglers, fish hatcheries, and other organizations could follow when dealing with largemouth bass in LMBV contaminated areas.

Tissue Culture Infectious Dose 50% Assay. – TCID50 assays are used to titer infectious viruses that cause visible CPE in infected monolayers. Unlike other assays such as hemagglutination and plaque assays, the titer obtained from the TCID50 assay represents the dosage of virus rather than the number of virus particles. TCID50 assays are useful when a virus induces a type of CPE that is not easily quantified, such as granuolization, syncytia formation, or cell rounding. Briefly, replicates of cell monolayers are infected with sample dilutions of the virus to be assayed. Following an incubation period, monolayers are observed microscopically for any CPE caused by the virus. The visualization of the CPE may be aided by preservation and the addition of vital dyes to the monolayer. The Spearman-Kärber method is used to calculate the 50% endpoint, resulting in a virus titer in units of TCID50/ml. The TCID50 is defined as the dilution of virus required to cause CPE in 50% of the infected cell monolayers (Mahy and Kangro 1996). The TCID50 assay may be performed in multi-well tissue culture plates fairly easily, allowing for the simultaneous assay of multiple virus samples.

ANTIVIRAL AGENTS

Pokeweed Antiviral Protein. - Pokeweed antiviral protein (PAP) is an antiviral agent that is purified from the leaves of pokeweed *Phytolacca americana*. It has a molecular weight of 29 kDa and is found to exist in several forms (Aron and Irvin 1980; Barbieri et al. 1982). The antiviral activity of PAP comes from its ability to inhibit protein synthesis within the cell, thus preventing viral protein synthesis and replication. Entry into the cell is essential for PAP to express its antiviral effects. PAP can enter the cell on its own following prolonged periods of incubation, however the process of PAP entry is enhanced in the presence of a viral infection (Irvin and Aron 1982). Upon cell entry, PAP can inactivate eukaryotic ribosomes by catalytically removing a specific adenine residue from the 60S ribosomal subunit, thus preventing viral mRNA from being translated (Aron and Irvin 1988). PAP is an effective antiviral agent against both plant and animal viruses, including Cucumber mosaic virus, Herpes simplex virus, Poliovirus, and Human immunodeficiency virus (Aron and Irvin 1980; Tomlinson et al. 1974; Ussery et al. 1977; Zarling et al. 1990). Due to its ability to inhibit both DNA and RNA viral replication through the inhibition of cell ribosomes, PAP should prove to be antiviral against LMBV.

Guanidine Hydrochloride. - Guanidine is a simple molecular compound (CH_6N_3) that exists as a positively charged ion at physiological pH. It has been used in studies involving the determination of protein molecular weight, denaturation of proteins, and nucleic acid extraction from cellular constituents (Tershak et al. 1982). It was first

recognized as an antiviral agent in the early 1960s, primarily on studies with members of the family *Picornaviridae*. The Picornaviruses are lipid-free animal viruses that contain single-stranded messenger-active RNA (Fields et al. 1996; Tershak et al. 1982). There are currently four proposed mechanisms for the antiviral activity of guanidine. One involves the rapid inhibition of initiation of viral RNA synthesis. The second involves the inhibition of release of membrane dependent plus-stranded 35S RNA from the replication complex. The third involves the interference of movement of newly synthesized membranes from the rough endoplasmic reticulum to smooth viral specific vesicles. The fourth involves the prevention of association of the procapsid with the replication complex (Koch and Koch 1998). Guanidine is more effective when added prior to the appearance of a viral RNA dependant RNA polymerase. In addition, it has been demonstrated with Poliovirus infected cells that synthesis of viral RNA becomes less sensitive to the action of guanidine as the viral growth cycle progresses (Caliguiri and Tamm 1968). Although LMBV is not an RNA-containing virus, guanidine may prove to be an effective inhibitor of LMBV replication since it may possess multiple antiviral mechanisms.

Ribavirin. - Ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) is a stable, water soluble, colorless nucleoside analog that has antiviral properties against a wide range of both DNA and RNA viruses (Markland et al. 2000; Sidwell et al. 1972). It was one the first antiviral agents found to be effective against both DNA and RNA virus (Sidwell et al. 1972). There are currently three proposed mechanisms for the antiviral activity of ribavirin (Markland et al. 2000). One involves inhibition of viral RNA

transcription and/or elongation. The second involves the inhibition of viral mRNA guanylyltransferase to cap the 5′ end of viral mRNA. The third involves the removal of intracellular GTP and dGTP via inhibition of inosine monophosphate dehydrogenase, which inhibits pathways necessary for viral DNA and RNA synthesis. Recently ribavirin has been approved for the treatment of disease caused by Respiratory syncytial virus, and in combination with IFN-α for the treatment of chronic Hepatitis C infection (Markland et al. 2000). If found to inhibit the replication of LMBV, ribavirin may be useful in the development of a therapy to treat largemouth bass populations infected with the virus.

XTT Cell Viability Assay. - XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) is a tetrazolium salt that is used for quantifying viable cells. Upon addition to metabolically active cells, XTT molecules are converted to an orange water-soluble formazan salt by cellular mitochondrial dehydrogenase activity. Formazan and XTT have different UV absorbance properties, which allow them to be quantified spectrophotometrically. Since metabolically active cells are essential for the conversion of XTT to formazan, the amount of formazan present is directly related to the amount of viable cells. Cell viability assays using XTT are quick, safe, accurate, and sensitive to low numbers of viable cells. The XTT assay has many uses, including assessing the affect of cytotoxic agents on cell viability, studying the affect of various cellular growth factors on cells, and determining the infectivity of virus particles on cell monolayers. Similar assays employ the tetrazolium salt MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is also converted to a formazan salt in the presence of metabolically

active cells. However, MTT cell viability assays require the solublization of the formazan salt, since it is non-water-soluble molecule. The XTT cell viability assay will be used to determine the effect of PAP, guanidine hydrochloride, and ribavirin on the replication of LMBV.

The objective of this research is to determine the effect of various disinfectants and antivirals on the infectivity and replication of LMBV. The effect of the chemical and physical disinfectants formalin, iodine, chlorhexidine diacetate, bleach, ethanol, UV light, and temperature on the infectivity of LMBV will be determined by TCID50 assays. The effect of PAP, guanidine hydrochloride, and ribavirin on the replication of LMBV will be determined by XTT cell viability assays. The results of this study will aid in the prevention of the spread of LMBV to uncontaminated water habitats, and the treatment of fish in infected waters.

MATERIAL AND METHODS

Cell Culture. - Bluegill Fry cells (BF-2) were supplied by A.E. Woods State Fish Hatchery, San Marcos, TX. Cells were grown in Eagle's Minimum Essential Medium (E-MEM) containing 10% fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO), 1% non-essential amino acids, 0.24% $NaHCO_3$, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma-Aldrich Chemical Co., St. Louis, MO). Cells in 75-cm^2 tissue culture flasks (Sarstedt Inc., Newton, NC) were placed in zip seal plastic bags at 30°C in a water-jacketed incubator (NuAire Inc., Plymouth, MN). Upon monolayer formation, cells were placed at room temperature and maintained in E-MEM. The BF-2 cells were passaged every one to four weeks, by enzymatic and physical methods as follows: monolayers were rinsed with Earle's Balanced Salt Solution (EBSS; Sigma-Aldrich Chemical Co., St. Louis, MO) followed by addition of 1% trypsin (Sigma-Aldrich Chemical Co., St. Louis, MO) to place the cells in suspension. Cell scrapers (Baxter Healthcare Corp., McGaw Park, IL) were also used to disassociate the cell monolayer. Fresh E-MEM was added to each flask at a volume of 50 ml, and the resulting cell solution was split 1:2. Cells were incubated at 30°C in a water-jacketed incubator for 24 h or until monolayer formation. To maintain the cells, growth medium was changed 24 h following cell passage and at least once every three to four weeks.

Cell Storage. - The BF-2 monolayers (24 h) were harvested using a modified procedure for cell passage, as described above. After the cell monolayer was disassociated from the flask, 10 ml of fresh E-MEM was added and the cell suspension was centrifuged at 200 x g for 10 min at 4°C. The supernatant was discarded and the cells were resuspended in 2 ml of E-MEM containing 10% dimethyl sulfoxide (DMSO; Fisher Scientific Co., Fair Lawn. NJ). Cell aliquots which contained 1 ml in cryogenic vials (Nalge Co., Rochester, NY) were placed directly into liquid nitrogen (-196°C; Locator 8, Barnstead/Thermolyne Corp., Dubuque, IA).

Virus. - Largemouth Bass Virus (LMBV) was obtained from A.E. Woods State Fish Hatchery, San Marcos, TX. LMBV stocks were prepared by the addition of virus to BF-2 cell monolayers in 75-cm^2 tissue culture flasks. Growth medium was removed from 24 h monolayers, and rinsed with 10 ml EBSS. LMBV at a titer of 10^6 TCID50/ml was used to infect the monolayers at an approximate multiplicity of infection (MOI) of 1-2. The infected cells were incubated for 90 min at 30°C with tilting every 10 min to allow maximum virus absorption. Following absorption of virus, unabsorbed virus was removed, the monolayer was washed with 10 ml of EBSS, and fresh E-MEM was added. The infected monolayers were incubated at 30°C for 48 h. Following incubation the infected cells were freeze-thawed 3x at -80°C. The cell lysate was removed from the flasks and centrifuged at 2,300 x g for 20 min at 4°C. The supernatant was sterile filtered (0.45 μm) and dispensed in 1.5 ml aliquots. The virus, which contained approximately 10^8 TCID50/ml, was stored at -80°C and rapidly thawed in a 35°C water bath just prior to use.

Formalin Treatment. - Aliquots of LMBV, at a titer of 10^8 TCID50/ml in 0.5 ml of E-MEM, were mixed with equal volumes of 4% and 1% formalin (Fisher Scientific Co., Fair Lawn, NJ), yielding final concentrations of 2% and 0.5% formalin, respectively. Virus-formalin mixtures were held at 4°C and 10 µl samples withdrawn following a 1, 5, 10, and 30 min exposure times. Formalin treated virus samples were immediately diluted 1:100 in E-MEM and stored at -30°C (Frerichs et al. 2000).

Iodine Treatment. - Betadine (1% available iodine, Purdue Frederick, Purdue Pharma, L.P., Stamford, CN) was supplied by Veterinary Hospital of Leon Springs, San Antonio, TX. Aliquots of LMBV, at a titer of 10^8 TCID50/ml in 0.5 ml of E-MEM, were mixed with equal volumes of 2.5% and 1.25% betadine solution, yielding final concentrations of 0.0125% and 0.00625% iodine, respectively. Virus-iodine mixtures were held at 4°C and 10 µl samples withdrawn following a 1, 5, 10, and 30 min exposure time. Iodine treated virus samples were immediately diluted 1:100 in E-MEM and stored at -30°C (Frerichs et al. 2000).

Chlorhexidine Diacetate Treatment. - Nolvasan (2% chlorhexidine diacetate, Fort Dodge Animal Health, Fort Dodge, IA) was supplied by Veterinary Hospital of Leon Springs, San Antonio, TX. Aliquots of LMBV, at a titer 10^8 TCID50/ml in 0.5 ml of E-MEM, were mixed with equal volumes of 50%, 25% and 12.5% normal use-strength Nolvasan, yielding final chlorhexidine diacetate concentrations of 1%, 0.5% and 0.25%, respectively. Virus-chlorhexidine mixtures were held at 4°C and 10 µl samples

withdrawn following a 1, 5, 10, and 30 min exposure time. Chlorhexidine diacetate treated virus samples were immediately diluted 1:100 in E-MEM and stored at -30°C.

Bleach Treatment. - Aliquots of LMBV, at a titer 10^8 TCID50/ml in 0.5 ml of E-MEM, were mixed with equal volumes of 50%, 5%, and 0.5% use-strength household bleach (Hill Country Fair, H-E-B, San Antonio, TX), to yield final concentrations of 0.07%, 0.007%, and 0.0007% sodium hypochlorite, respectively. Virus-bleach mixtures were held at 4°C and 10 µl samples withdrawn following a 1, 2, 5, 10, and 30 min exposure. Bleach treated virus samples were immediately diluted 1:100 in E-MEM and stored at -30°C.

Ethanol Treatment. - Aliquots of LMBV, at a titer 10^8 TCID50/ml in 0.5 ml of E-MEM, were mixed with equal volumes of 70%, 7%, and 0.7% ethanol (Fisher Scientific Co., Fair Lawn, NJ), yielding final ethanol concentrations of 35%, 3.5%, and 0.35% ethanol, respectively. Virus-ethanol mixtures were held at 4°C and 10 µl samples withdrawn following a 1, 2, 5, 10, and 30 min exposure. Ethanol treated virus samples were immediately diluted 1:100 in E-MEM and stored at -30°C.

Ultraviolet Light Treatment. - Aliquots of LMBV, at a titer 10^8 TCID50/ml in 1 ml of E-MEM, were placed in sterile petri dishes at a depth of 3-4 mm. Dishes were placed under a UV lamp with a wavelength of 253.7 nm at distances of 10, 20, and 30 cm. Samples (100 µl) were withdrawn at various intervals from 20 s to 2 min, immediately

diluted 1:10 in E-MEM, and stored at -30°C. The surface light intensity of the UV light at 10 cm was 2 x 10^3 ergs · sec^{-1}· cm^{-2}.

Temperature Stability. - A 1:10 dilution of LMBV, at a titer 10^8 TCID50/ml in E-MEM, was distributed in 1 ml aliquots in triplicate at 23°C, 30°C, and 37°C in controlled water baths (Precision Scientific, Chicago, IL). Samples (10 µl) were withdrawn at 24 h intervals over a 7 d period and diluted 1:100 in E-MEM. Temperature treated virus samples were immediately stored at -30°C.

Tissue Culture Infectious Dose 50% Assay. - Virus was rapidly thawed at 35°C, and serially diluted 1:10 in 96-well plates in sextuplet, at a final volume of 90 µl. Plates, which contained diluted virus samples, were placed at 4°C during preparation of the cell suspension. A BF-2 cell suspension was prepared as previously described and cell concentration was determined using a hemacytometer (Fisher Scientific Co., Fair Lawn. NJ) following a 1:5 dilution of the cell suspension in trypan blue (1:6 solution of 0.4% trypan blue in 0.85% saline; Allied Chemical, Morristown, NJ). The cell suspension was adjusted to 5.5 x 10^5 cells/ml in E-MEM and added (90 µl) to the diluted virus samples. Tissue culture plates, which contained viral dilutions and cell solution, were agitated to ensure proper distribution of cells in suspension, and incubated at 30°C for 5 d. After incubation, the monolayers were observed microscopically for cytopathic effect. The virus titer was determined using the Spearman-Kärber method (Mahy and Kangro 1996).

Cytotoxicity of Disinfectants. - To determine the effect formalin, iodine, chlorhexidine diacetate, bleach, and ethanol on the viability of the BF-2 cells, cytotoxicity assays were performed. Disinfectant concentrations used for cytotoxicity assay were tested at twice the highest concentration used in the TCID50 assay. Formalin (0.04%), iodine (0.005%), chlorhexidine diacetate (0.01%), bleach (0.0014%), and ethanol (0.7%), was serially diluted 1:2 in E-MEM with a final volume of 90 µl in a 96-well tissue culture plate. The disinfectant dilutions were placed at 4°C during the preparation of a BF-2 cell suspension, and an equal volume of cell suspension, which contained 5 x 10^5 cells/ml in E-MEM, was added to each dilution of disinfectant dilutions. Plates were agitated to allow even distribution of the cells, and incubated at 30°C for 5 d. After incubation, the cell monolayers were observed microscopically for cytotoxicity.

Antiviral Agents. - Pokeweed antiviral protein (PAP) was kindly provided by J.D. Irvin (Texas State University, San Marcos, TX). Guanidine (aminomethanamidine hydrochloride) was purchased from Sigma Chemical Co. (St. Louis, MO). Ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) was a gift from ICN Pharmaceuticals (Costa Mesa, CA). All drugs were rehydrated in E-MEM, sterile filtered (0.45 µm), and stored at -30°C.

XTT Cell Viability Assay. - The effect of PAP, guanidine hydrochloride, and ribavirin on the infectivity of LMBV was determined in XTT cell proliferation assays. Each antiviral agent (PAP 5 µmol/ml, guanidine hydrochloride 2,630 µg/ml, and ribavirin 380

μg/ml), in 100 μl aliquots, was serially diluted 1:2 in 50 μl of E-MEM in a 96-well tissue culture plate. For high multiplicity of infection (MOI) treatment, an equal volume (50 μl) of LMBV, at a titer of 10^8 TCID50/ml in E-MEM (1:10,000 dilution of $10^{9\,5}$ TCID50/ml for low MOI treatment), was added to wells which contained the dilutions of antiviral agents. The tissue culture plates containing the antiviral agent-virus mixture were placed at 4°C during BF-2 cell suspension preparation. A BF-2 cell suspension, which contained 1.2 x 10^6 cells/ml (1.0 x 10^6 cells/ml for low MOI treatment) in E-MEM, was added (100 μl) to all wells of the tissue culture plate, the plate was agitated to allow even distribution of the cells and placed at 30°C for 24 h (5 d incubation for low MOI treatment). An XTT labeling mixture was prepared aseptically in minimal light conditions by mixing 5 ml of XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent, in RPMI 1640, 1 mg/ml without phenol red, with 0.1 ml of electron coupling reagent PMS (N-methyl dibenzopyrazine methyl sulfate), 0.383 mg/ml (1.25 mM), in phosphate buffered saline (PBS; Cell Proliferation Kit II (XTT), Boehringer Mannheim, Roche Applied Science, Indianapolis, IN). The XTT labeling mixture was then added (100 μl) to all wells of the antiviral agent-virus infected tissue culture plate, and the plate was incubated at 30°C for 4 h (3 h incubation for low MOI treatment). Formazan dye formation was determined spectrophotometrically by scanning the antiviral agent-virus infected tissue culture plates in an automated microplate reader (Bio-Tek model EL311, Bio-Tek Instruments Inc., Winoosky, VT) at a wavelength of 490 nm with a reference wavelength of 650 nm. The optical densities were used to calculate percent cell viability and percent protection according to the following equations:

$$\% \text{ Cell Viability} = (Tx/Cx) \times 100$$

$$\% \text{ Protection} = \frac{(Tx-Vx)}{(Cx-Vx)} \times 100$$

Tx = absorbance (optical density) of test sample

Cx = absorbance of cell control

Vx = absorbance of virus-infected control

Cytotoxicity of Antivirals. - Cytotoxicity assays were performed to determine the effects PAP, guanidine hydrochloride, and ribavirin on the viability of the BF-2 cell monolayers. Volumes (200 µl) of each antiviral at the following concentrations, PAP (5 µmol/ml), guanidine hydrochloride (5,260 µg/ml), and ribavirin (380 µg/ml) were serially diluted 1:2 in 100 µl of E-MEM in 96-well tissue culture plates. The antiviral agent dilutions were placed at 4°C during BF-2 cell suspension preparation. A BF-2 cell suspension, which contained 1.2 x 10^6 cells/ml in E-MEM, was added (100 µl) to the tissue culture plate containing the antiviral dilutions. The plates were agitated to allow even distribution of the cells, and incubated at 30°C for 24 h. XTT labeling mixture was added (100 µl) to the tissue culture plates and the XTT assay was prepared as previously described.

Statistical Analysis. - For each disinfectant, virus inactivation data for each concentration treatment were analyzed for differences using a One-way analysis of

variance (ANOVA) and Kruskal-Wallis test. A Tukey's multiple comparison test was performed to show the differences among treatment times. For each antiviral, cell viability data for low and high MOI treatments were analyzed for differences among antiviral concentrations using an ANOVA and Kruskal-Wallis test. A Tukey's multiple comparison test was performed to show the differences. Analyses were performed using S-PLUS version 6.1.

RESULTS

Effect of Formalin on LMBV Infectivity in BF-2 Cells. - The effect of formalin on Largemouth Bass Virus (LMBV) infectivity is shown in Figure 1. No significant reduction in virus titer was observed following treatment with 1% formalin. A 30 min exposure of LMBV to 1% formalin resulted is less than 45% reduction in virus infectivity. Exposure of the virus to 4% formalin, however, resulted in slightly greater than 90% virus inactivation following a 1 min exposure, and 99% inactivation following a 10 min exposure time. The data indicates that 4% formalin is an effective disinfectant for the inactivation of LMBV.

Effect of Iodine on LMBV Infectivity in BF-2 Cells. - The effect of iodine on LMBV infectivity is shown in Figure 2. Treatment of the virus with 0.0125% iodine resulted in greater than 99% virus inactivation following a 1 min exposure. No increase in virus inactivation was observed with longer exposure times. Virus infectivity decreased greater than 99.99% following 1 min exposure to 0.025% iodine. As observed with the 0.0125% iodine treatment, no further decreases in virus titer were observed following longer exposure times. The data indicates that both concentrations of iodine used effectively inactivate LMBV.

Effect of Chlorhexidine Diacetate on LMBV Infectivity in BF-2 Cells. - The effect of chlorhexidine diacetate on LMBV infectivity is shown in Figure 3. Exposure of LMBV to either 0.5% or 1% chlorhexidine diacetate resulted in greater than 50% virus inactivation following a 30 min exposure. Exposure of LMBV to 2% chlorhexidine diacetate resulted in greater than 60% virus inactivation following a 30 min exposure. No significant reduction in virus titer was observed following a 30 min treatment with either 2%, 1%, or 0.5% chlorhexidine diacetate. The data indicates that chlorhexidine diacetate is not an effective disinfectant for LMBV contaminated surfaces.

Effect of Bleach on LMBV Infectivity in BF-2 Cells. - The effect of sodium hypochlorite on LMBV infectivity is shown in Figure 4. A 30 min exposure of LMBV to 0.0014% and 0.014% sodium hypochlorite resulted in greater than 50% and 85% virus inactivation, respectively. Exposure of the virus to 0.14% sodium hypochlorite resulted in a 99.9% reduction of infectivity after 5 min. No further changes in virus titer were observed at longer exposure times. The results suggest that 0.14% sodium hypochlorite is an effective disinfectant for the inactivation of LMBV.

Effect of Ethanol on LMBV Infectivity in BF-2 Cells. - The effect of ethanol on the infectivity of LMBV is shown in Figure 5. Exposure of LMBV to 0.7% and 7% ethanol resulted in greater than 25% and 40% virus inactivation, respectively. A 5 min exposure of the virus to 70% ethanol resulted in 99.9% virus inactivation. No further decrease in virus titer was observed at longer exposure times. The data indicates that 70% ethanol is an effective disinfectant for surfaces contaminated with LMBV.

Effect of UV Light on LMBV Infectivity in BF-2 Cells. - The effect of UV light on the infectivity of LMBV is shown in Figure 6. Virus infectivity was significantly ($p < 0.03$) reduced following a 20 sec exposure to UV light. Following a 60 sec exposure to UV light, LMBV infectivity was reduced greater than 99.99%. The rate of inactivation of LMBV was greater the closer the virus was to the source of UV light. The data indicates that UV light is an effective disinfectant for the inactivation of LMBV.

Effect of Temperature on LMBV Infectivity in BF-2 Cells. - The effect of temperature on the infectivity of LMBV is shown in Figure 7. LMBV infectivity decreased with increasing temperature. At 23°C, virus infectivity was reduced approximately 81% at the end of a 7 d exposure time. At 30°C, virus inactivation occurred more rapidly and approached 88% and 99.6% after 2 d and 7 d, respectively. At 37°C, maximum virus inactivation (99.9%) was observed at 5 d. The rate of virus inactivation at 37°C was greater than that observed at the lower temperatures. The data suggest that a 5 d exposure at 37°C results in the effective inactivation of LMBV.

Effect of Pokeweed Antiviral Protein on LMBV Replication in BF-2 Cells. - The effect of pokeweed antiviral protein (PAP) on the replication of LMBV is shown in Figure 8. At low multiplicity of infection (MOI), no significant difference in absorbance was observed between uninfected cells and infected cells with PAP. The data indicates that PAP inhibits the replication of LMBV. At high MOI, no significant difference in absorbance was observed between virus-infected cells in the absence of PAP and virus-infected cells in the presence of PAP. The data indicates that cellular mitochondrial

dehydrogenase was inactivated, which suggests PAP's antiviral activity occurred following virus-induced cell shutdown. The data in Table 1 show cell viability and protection of BF-2 monolayers by PAP. All concentrations of PAP provided greater than 80% protection to LMBV infected monolayers at low MOI, with 100% protection observed at PAP concentrations of 1.25 and 2.5 μmol/ml. The data indicates that PAP inhibits the replication of LMBV and may be effective in the treatment of LMBV infected fish.

Effect of Guanidine Hydrochloride on LMBV Replication in BF-2 Cells. - The effect of guanidine on the replication of LMBV is shown in Figure 9. At low MOI, a significant difference ($p < 0.01$) in absorbance was observed between virus-infected cells in the absence of guanidine and virus-infected cells in the presence of guanidine at concentrations of 1,315 and 2,630 μg/ml. The data indicates that guanidine partially inhibits the replication of LMBV. At high MOI, no significant differences in absorbance were observed between virus-infected cells in the absence of guanidine and virus-infected cells in the presence of guanidine. The data in Table 2 show cell viability and protection of BF-2 monolayers by guanidine. Guanidine concentrations of 1,315 and 2,630 μg/ml provided greater than 16% and 34% protection to LMBV infected monolayers, respectively. The data indicates guanidine partially inhibited the replication of LMBV. The data suggests guanidine is not as effective as PAP in the inhibition of LMBV replication.

Effect of Ribavirin on LMBV Replication in BF-2 Cells. - The effect of ribavirin on the replication of LMBV is shown in Figure 10. At low MOI, a significant difference ($p < 0.01$) in absorbance was observed between the virus-infected cells in the absence of ribavirin and virus-infected cells in the presence of ribavirin at concentrations of 1.48, 2.97, 5.94, and 23.75 μg/ml. The data indicates that ribavirin partially inhibited the replication of LMBV. At high MOI, no significant difference in absorbance was observed between virus-infected cells in the absence of ribavirin and virus-infected cells in the presence of ribavirin. The data in Table 3 show cell viability and protection of BF-2 monolayers by ribavirin. Ribavirin concentrations of 1.48 and 2.97 μg/ml provided greater than 47% and 36% protection to LMBV infected monolayers, respectively. The data suggests ribavirin is more effective than guanidine, but not as effective as PAP in the inhibition of LMBV replication.

Figure 1. – Effect of formalin on the infectivity of Largemouth bass virus. Virus concentration symbols: (●) 1% formalin treatment; (▲) 4% formalin treatment. Time = 0 represents virus titer prior to formalin treatment. Values represent a mean of three replicates.

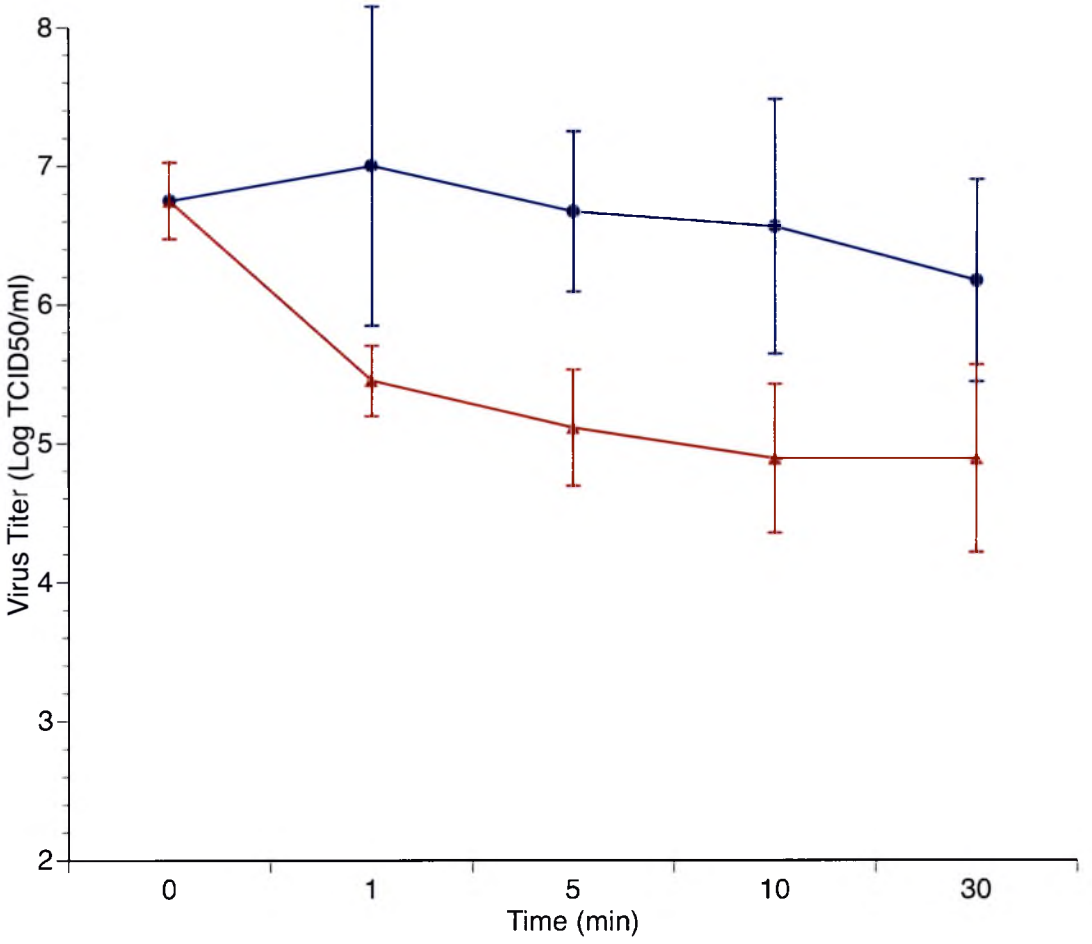
8
7
6
5
4
3
2
Virus Titer (Log TCID50/ml)
0
1
5
10
30
Time (min)

Figure 2. – Effect of iodine on the infectivity of Largemouth bass virus. Virus concentration symbols: (●) 0.0125% iodine treatment; (▲) 0.025% iodine treatment. Time = 0 represents virus titer prior to iodine treatment. Values represent a mean of three replicates.

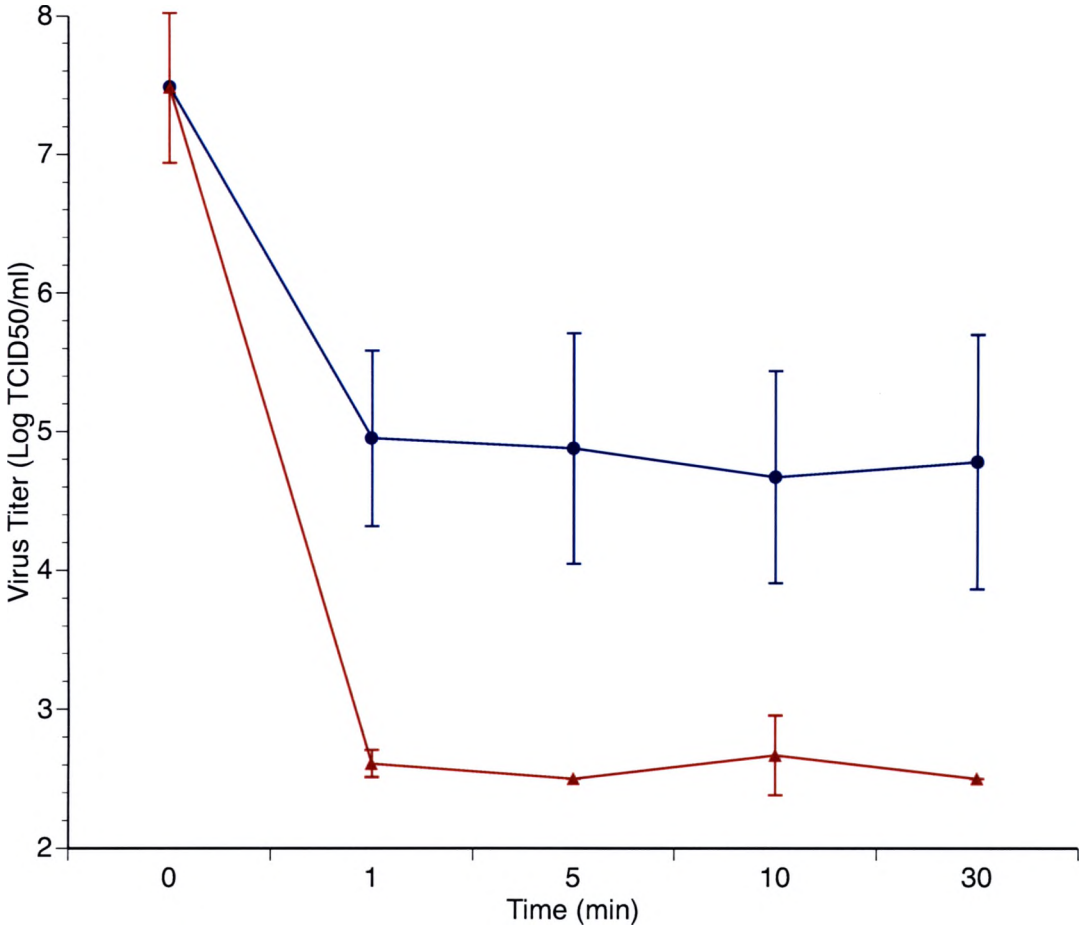
8
7
6
5
4
3
2
Virus Titer (Log TCID50/ml)
0
1
5
10
30
Time (min)

Figure 3. – Effect of chlorhexidine diacetate on the infectivity of Largemouth bass virus. Virus concentration symbols: (■) 0.5% chlorhexidine treatment; (●) 1% chlorhexidine treatment; (▲) 2% chlorhexidine treatment. Time = 0 represents virus titer prior to chlorhexidine treatment. Values represent a mean of three replicates.

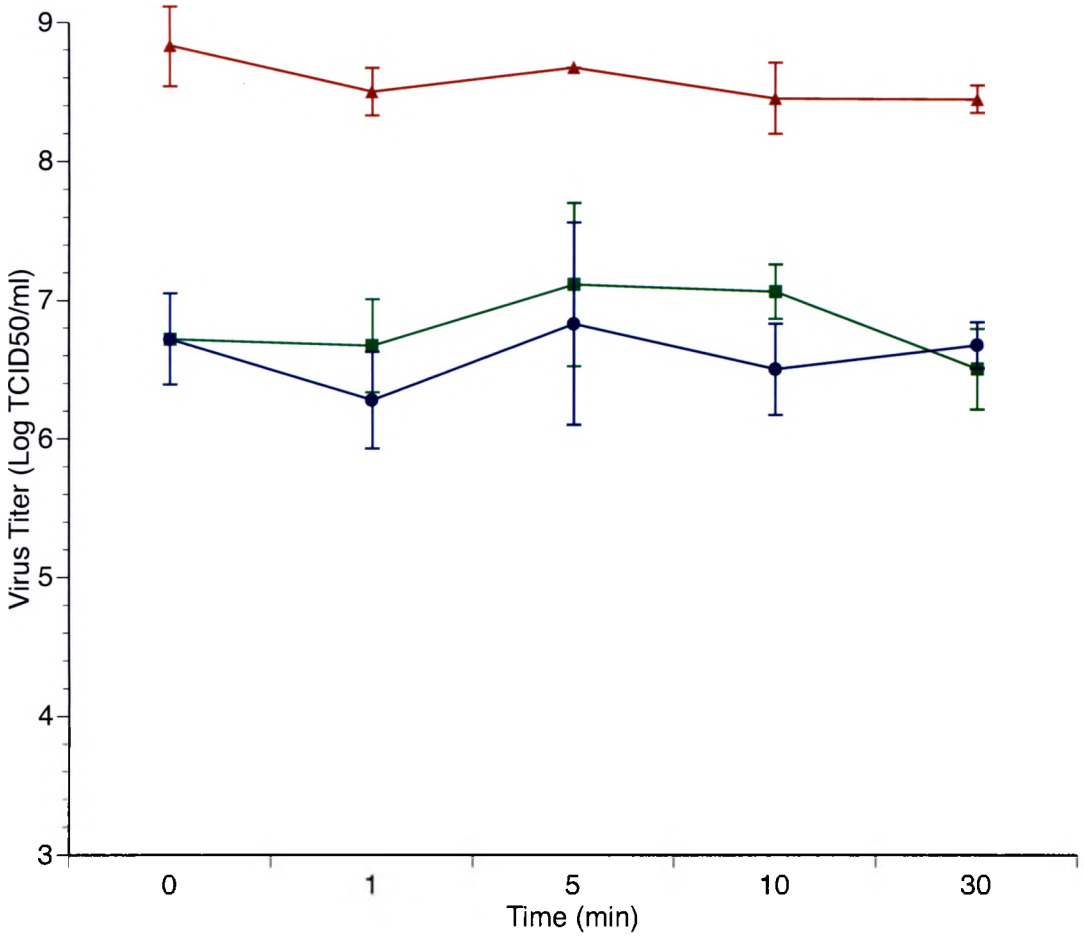
9
8
7
6
5
4
3
Virus Titer (Log TCID50/ml)
0
1
5
10
30
Time (min)

Figure 4. – Effect of bleach on the infectivity of Largemouth bass virus. Virus concentration symbols: (■) 0.0014% sodium hypochlorite treatment; (●) 0.014% sodium hypochlorite treatment; (▲) 0.14% sodium hypochlorite treatment. Time = 0 represents virus titer prior to sodium hypochlorite treatment. Values represent a mean of three replicates.

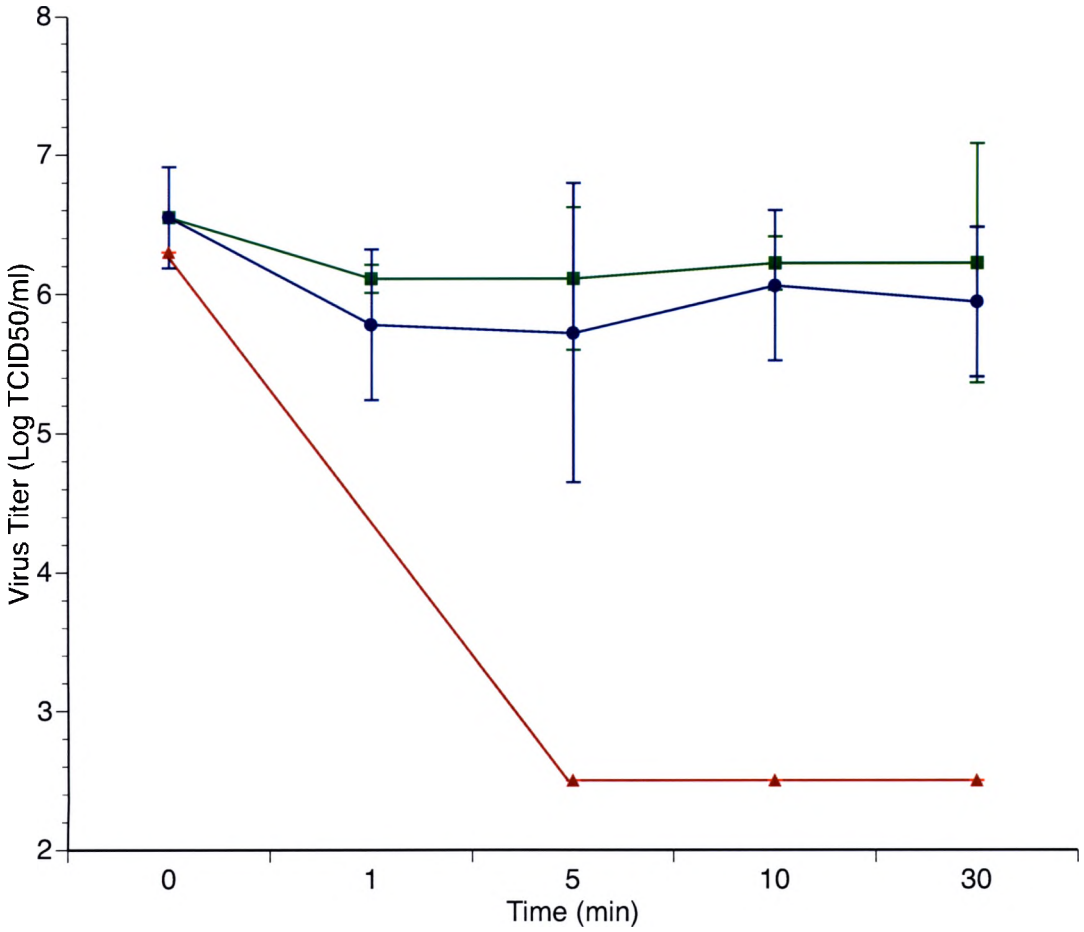
8
7
6
5
4
3
2
Virus Titer (Log TCID50/ml)
0
1
5
10
30
Time (min)

Figure 5. – Effect of ethanol on the infectivity of Largemouth bass virus. Virus concentration symbols: (■) 0.7% ethanol treatment; (●) 7% ethanol treatment; (▲) 70% ethanol treatment. Time = 0 represents virus titer prior to ethanol treatment. Values represent a mean of three replicates.

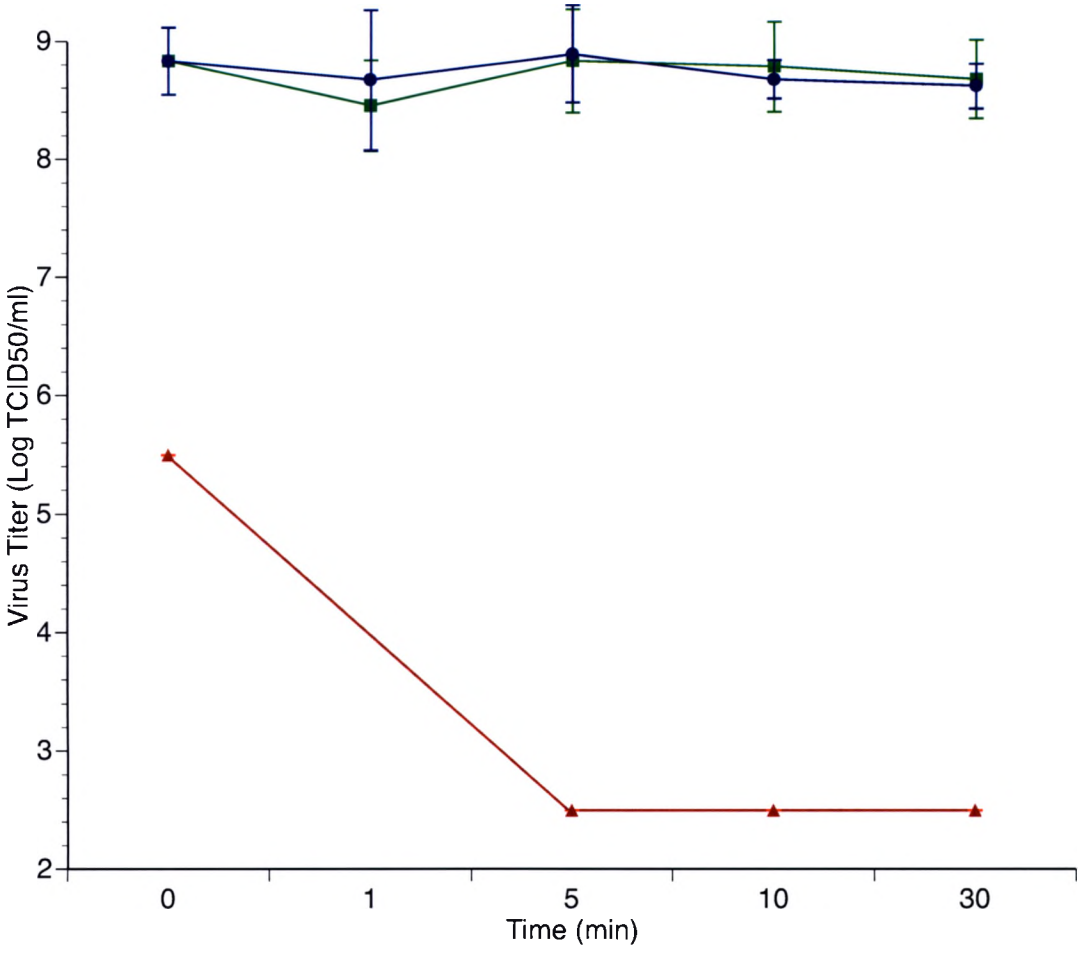
9
8
7
6
5
4
3
2
Virus Titer (Log TCID50/ml)
0
1
5
10
30
Time (min)

Figure 6. – Effect of UV light (257.3 nm) on the infectivity of Largemouth bass virus. Virus concentration symbols: (■) 10 cm treatment; (●) 20 cm treatment; (▲) 30 cm treatment. Time = 0 represents virus titer prior to UV treatment. Values represent a mean of three replicates.

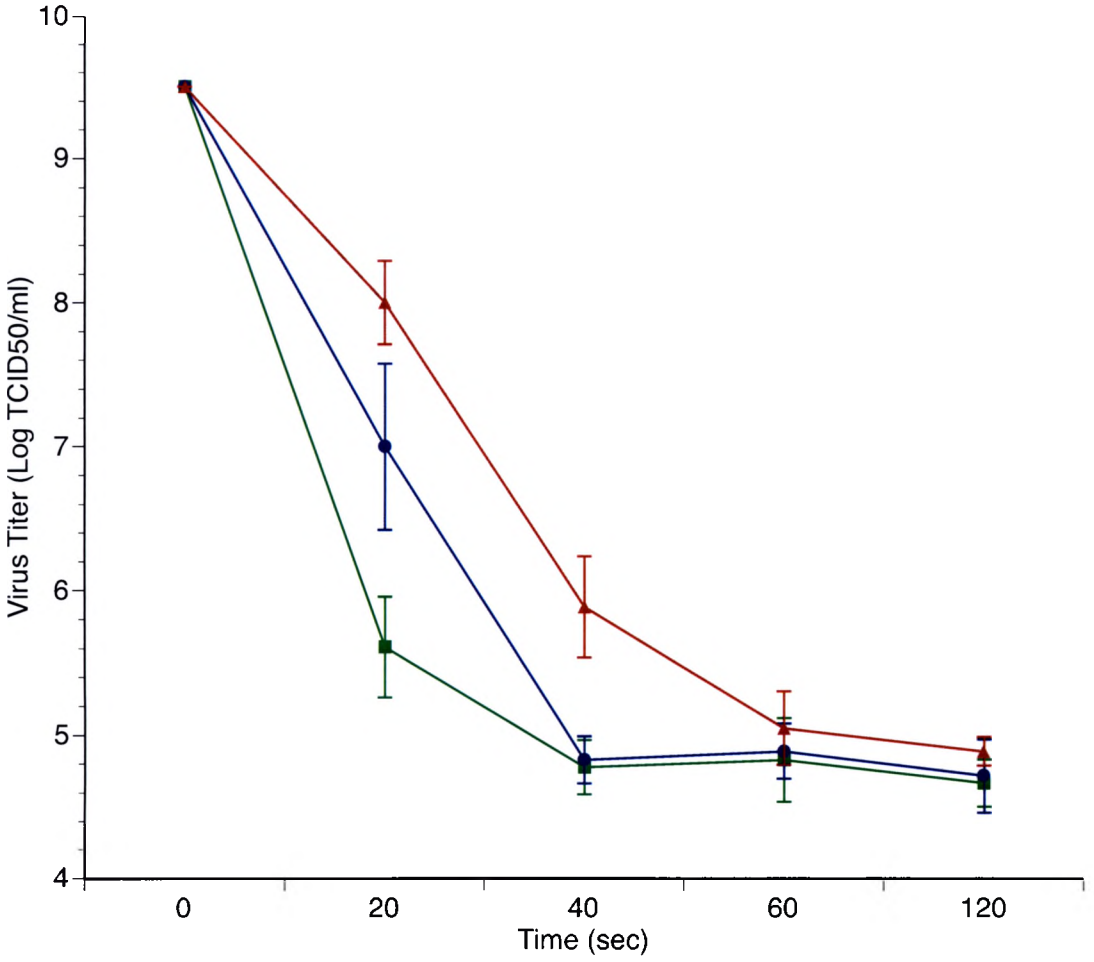
10
9
8
7
6
5
4
Virus Titer (Log TCID50/ml)
0
20
40
60
120
Time (sec)

Figure 7. – Effect of temperature on the infectivity of Largemouth bass virus. Virus concentration symbols: (■) 23°C treatment; (●) 30°C treatment; (▲) 37°C treatment. Time = 0 represents virus titer prior to temperature treatment. Values represent a mean of three replicates.

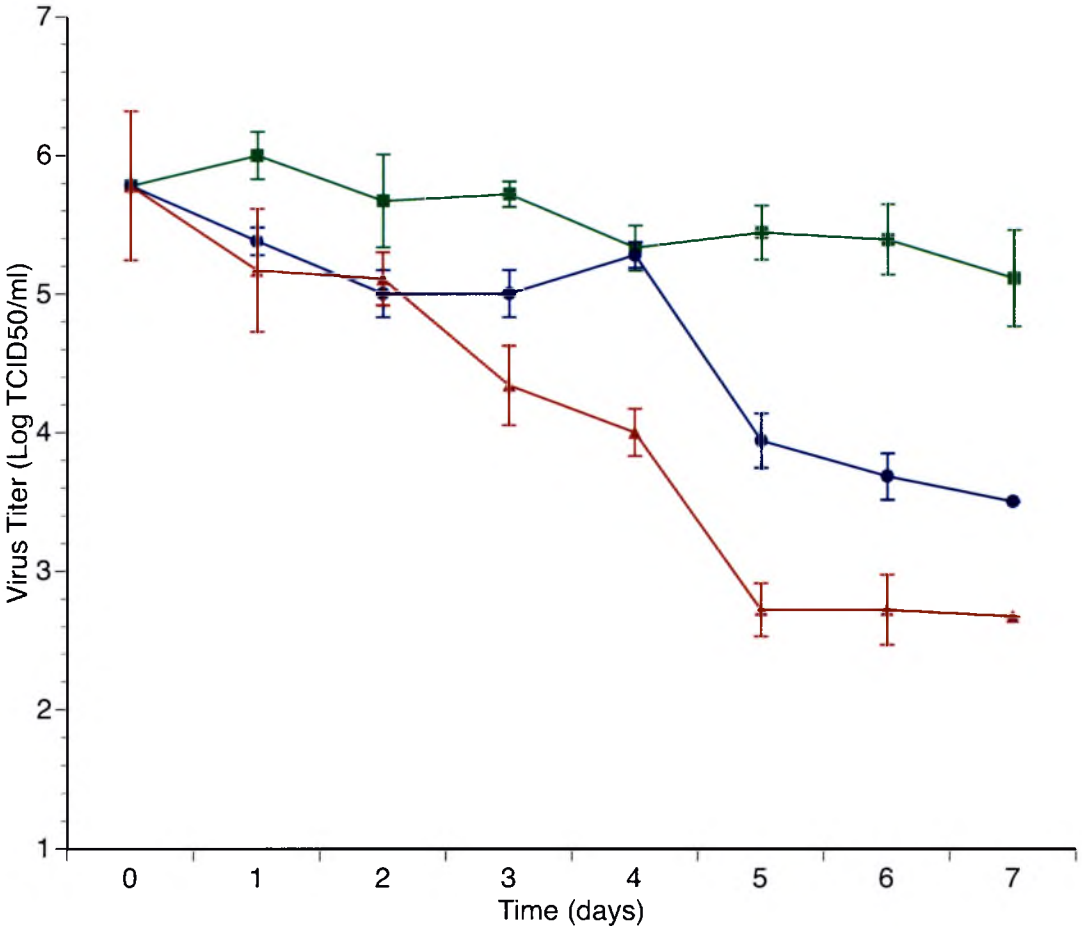
Virus Titer (Log TCID50/ml)
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
Time (days)

Figure 8. – Effect of pokeweed antiviral protein (PAP) on the replication of Largemouth bass virus. Cell viability in terms of absorbance: (●) low multiplicity of infection (MOI); (▲) high MOI. Virus controls are represented at a PAP concentration of 0 μmol/ml. Values represent a mean of eight replicates.

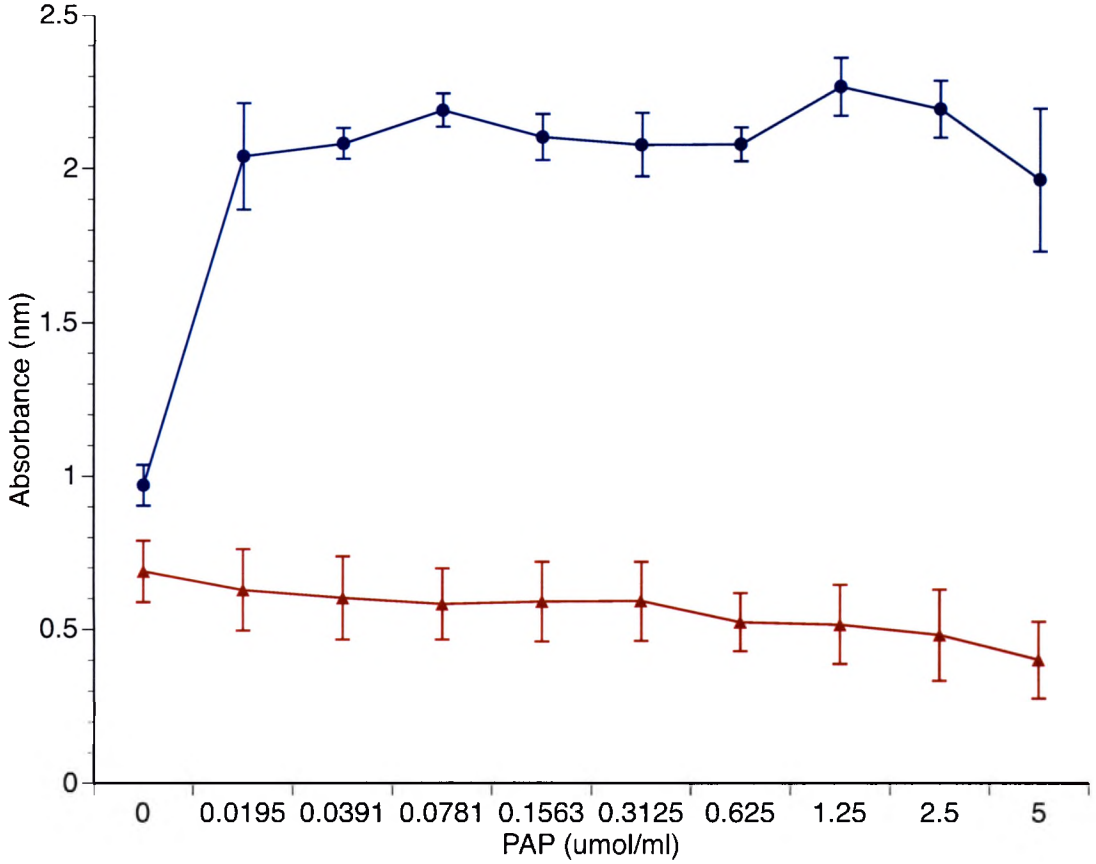
2.5
2
1.5
1
0.5
0
Absorbance (nm)
0
0.0195
0.0391
0.0781
0.1563
0.3125
0.625
1.25
2.5
5
PAP (umol/ml)

Figure 9. – Effect of guanidine hydrochloride on the replication of Largemouth bass virus. Cell viability in terms of absorbance: (●) low multiplicity of infection (MOI); (▲) high MOI. Virus controls are represented at a guanidine concentration of 0 μg/ml. Values represent a mean of eight replicates.

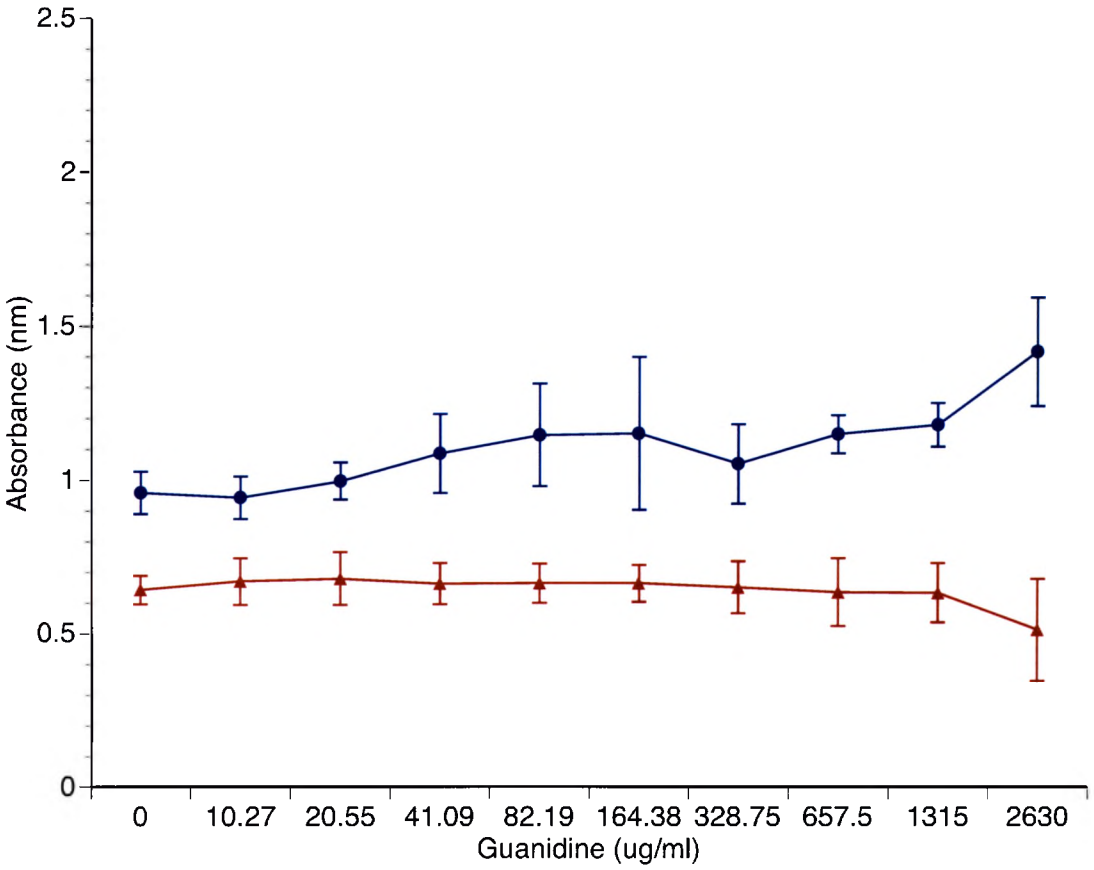
2.5
2
1.5
1
0.5
0
Absorbance (nm)
0
10.27
20.55
41.09
82.19
164.38
328.75
657.5
1315
2630
Guanidine (ug/ml)

Figure 10. – Effect of ribavirin on the replication of Largemouth bass virus. Cell viability in terms of absorbance: (●) low multiplicity of infection (MOI); (▲) high MOI. Virus controls are represented at a ribavirin concentration of 0 μg/ml. Values represent a mean of eight replicates.

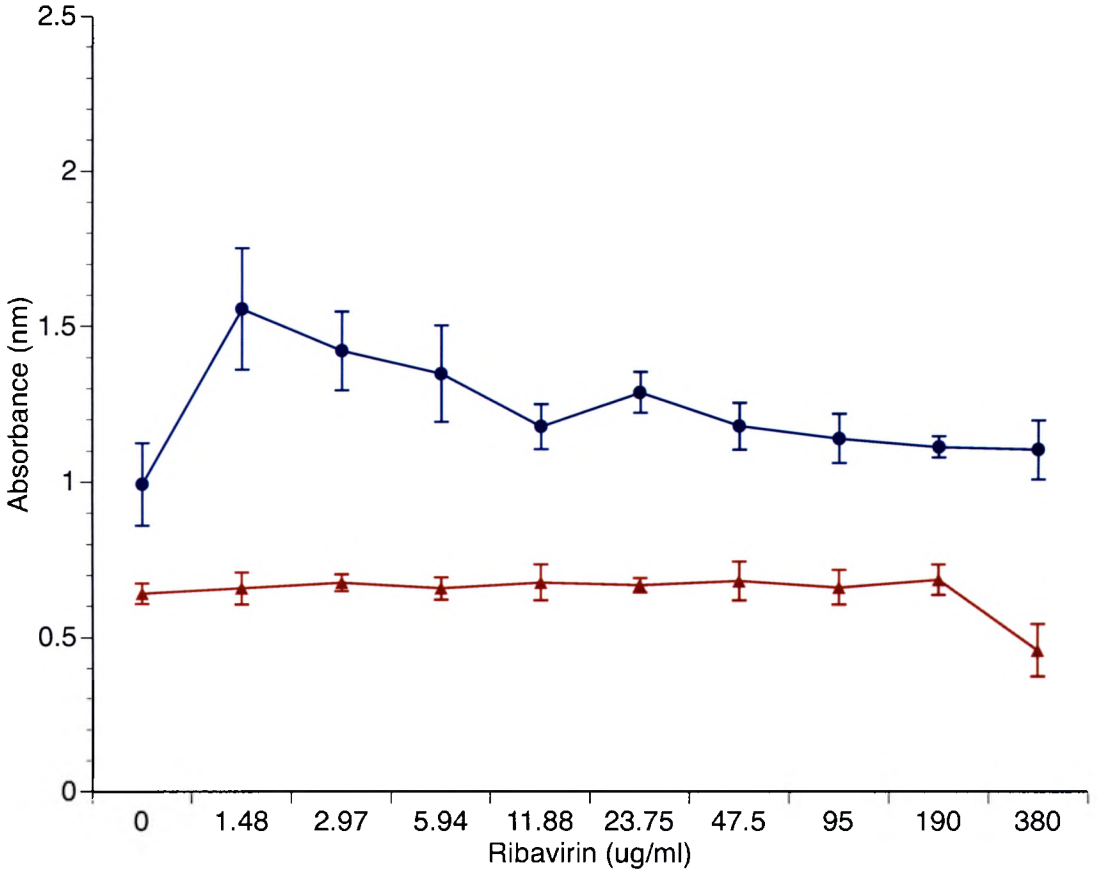
2.5
2
1.5
1
0.5
0
Absorbance (nm)
0
1.48
2.97
5.94
11.88
23.75
47.5
95
190
380
Ribavirin (ug/ml)

Table 1. - Cell viability and protection of bluegill fry cells by pokeweed antiviral protein.

	Low MOI[a]		High MOI[a]	
PAP (um/ml)	% Cell Viability	% Protection	% Cell Viability	% Protection
0	44 84	0 00	42.19	0 00
0 0195	94 27	89 61	38 49	-6 41
0 0391	96 18	93 07	36 85	-9 24
0 0781	101 19	102 15	35 70	-11.23
0 1563	97 16	94 86	36 15	-10 46
0 3125	95 98	92 72	36 23	-10 31
0 625	96 10	92 92	32 06	-17 53
1 25	104 72	108 55	31.63	-18 27
2 5	101 33	102.40	29 50	-21 96
5	90 70	83 14	24 55	-30 52

[a] Multiplicity of infection

Table 2. - Cell viability and protection of bluegill fry cells by guanidine hydrochloride.

Guanidine (ug/ml)	Low MOI[a]		High MOI[a]	
	% Cell Viability	% Protection	% Cell Viability	% Protection
0	41 77	0 00	40 60	0 00
10 27	41 09	-1 17	42 35	2 93
20 55	43 40	2 80	42 91	3 89
41 09	47 32	9 53	41 87	2 12
82 19	49 94	14 03	41 93	2 23
164 38	50 18	14 45	41 93	2 23
328 75	45 85	7 00	41.07	0 79
657 5	50 03	14 19	40.16	-0 75
1315	51 37	16 49	39 97	-1 07
2630	61 71	34 24	32 37	-13 85

[a] Multiplicity of infection

Table 3. - Cell viability and protection of bluegill fry cells by ribavirin.

Ribavirin (ug/ml)	Low MOI[a]		High MOI[a]	
	% Cell Viability	% Protection	% Cell Viability	% Protection
0	45 66	0.00	41 62	0 00
1 48	71.54	47.63	42 68	1 82
2 97	65 36	36 24	43 86	3 84
5.94	61 96	29.98	42.69	1 83
11 88	54.12	15 56	43 89	3 89
23 75	59 19	24.90	43.36	2.99
47 5	54 19	15 70	44 28	4.55
95	52.36	12.33	42 91	2.22
190	51 11	10.03	44.52	4 96
380	50.69	9.26	29.78	-20.27

[a] Multiplicity of infection

DISCUSSION

Fish kills thought to be associated with Largemouth Bass Virus (LMBV) can last for several months and result in the loss of thousands of largemouth bass (Hanson et al. 2001; Plumb et al. 1996). Fish kills may be triggered by stress factors, such as water temperature, dissolved oxygen, and handling by bass anglers. Since stress factors are relatively uncontrollable, LMBV disease prevention relies on inactivation of the virus on surfaces by drying and exposure to sunlight (Herr and Boston 2002). Chemical and physical disinfection in both industrial and recreational settings would prevent the spread of LMBV to non-infected waters. In addition, antiviral chemotherapy would control the infection of fish in contaminated waters. With the exception of chlorhexidine, all disinfectants used in this study were found to inactivate LMBV. As expected, higher concentrations of chemical disinfectants and longer exposure times to physical agents more effectively inactivated LMBV. In practice, however, increasing the concentration of disinfectant or the exposure time of the virus to the agent is not always economical or practical.

Both formalin and iodine modify amino acids side chains, which results in changes in protein shape that may lead to a partial or complete loss of function (Gottardi 1991; McDonnell and Russell 1999). Viral particles are composed of proteins essential for the attachment and penetration of the virus, and disruption of these proteins would result in

the loss of viral infectivity (Fields et al. 1996). Both formalin and iodine were found to reduce the infectivity LMBV. The inactivation of Sea bass neuropathy virus (SBNV) by formalin and iodine has been reported (Frerichs et al. 2000). Following a 30 min exposure to 2% formalin, infectivity of SBNV and LMBV were reduced from $10^{8.5}$ to 10^{7} TCID50/ml, and from $10^{6.9}$ to $10^{4.9}$ TCID50/ml, respectively. Following a five min exposure to 100 ppm and 0.0125% iodine, the titers of SBNV and LMBV were reduced from $10^{6.125}$ to 10^{2} TCID50/ml and $10^{7.45}$ to $10^{2.5}$ TCID50/ml, respectively (Frerichs et al. 2000). Formalin has been reported to inactivate other piscne viruses such as Infectious pancreatic necrosis virus, Egtved virus, and Snakehead rhabdovirus (Frerichs 1990; Jorgensen 1973). The ability of formalin and iodine to inactivate LMBV results from the denaturation of proteins essential for viral attachment and penetration. In addition to their ability to damage proteins, formalin and iodine also damage nucleic acids (McDonnell and Russell 1999). Formalin has been shown to disrupt the replication of SV-40 and bacteriophage T2 (McDonnell and Russell 1999; Permana and Snapka 1994). Iodine has been shown to damage nucleotides in myxovirus RNA (Apostolov 1980). The ability of these disinfectants to damage both viral nucleic acids and proteins act to increase their antiviral activity.

Ethanol and bleach are commonly used as disinfectants for biological spills and the decontamination of microbiological work areas (McDonnell and Russell 1999). Rapid inactivation of Human immunodeficiency virus (HIV) in suspension following exposure to 70% ethanol has been reported (van Bueren et al. 1994). Following a five min exposure to either 70% ethanol or 0.14% sodium hypochlorite (1/2 use-strength bleach as

recommended by manufacturer, Hill Country Fair, H-E-B, San Antonio, TX), LMBV infectivity was reduced 99.9%. The ability of ethanol and bleach to inactivate LMBV may be due to denaturation of capsid proteins essential for viral attachment and penetration. It has been reported that the attachment of Hepatitis B virus to hepatocytes is inhibited by ethanol, which results in the inactivation of virus infectivity (Ito et al. 2002). In addition, Poliovirus type 1 capsids were degraded following treatment with chlorine releasing agents, such as sodium hypochlorite (McDonnell and Russell 1999).

Although chlorhexidine diacetate has been found to inactivate animal viruses such as Canine distemper virus, Equine influenza virus, and Hog cholera virus, it was not found to inactivate LMBV (Nolvasan, Fort Dodge, IA.). Chlorhexidine has been shown to target lipid molecules, which would make it more effective against enveloped viruses compared to non-enveloped viruses. Non-enveloped viruses, such as rotavirus and poliovirus, have been shown not to be inactivated by chlorhexidine (McDonnell and Russell 1999). LMBV is an enveloped virus, however, the envelope may not be required for infectivity (van Regenmortel et al. 2000). Thus, damage of the LMBV envelope by chlorhexidine would not result in loss of infectivity.

State agencies recommend the control of LMBV passage from infected to non-infected water habitats as the best method to prevent fish kills (Herr and Boston 2002). To achieve this goal, bass anglers are instructed not to fish in more than one lake per day, and to let boats and equipment completely dry in the sun for 24 h before subsequent use in another lake(Herr and Boston 2002). The results of this study demonstrate a

temperature of 37°C only results in 75% inactivation of the virus in 24 h. Thus, current recommendations may not be sufficient to prevent LMBV from spreading to non-infected waters. Ultraviolet (UV) light significantly reduced the titer of LMBV *in vitro*. A similar study revealed a 99.9% inactivation of SBNV by UV light *in vitro*. The wavelength of UV light used in these studies is found in small amounts in natural sunlight (Kime 1980). Thus, exposure of infectious virus to sunlight alone may not result in the effective inactivation of LMBV. Furthermore, surfaces contaminated with LMBV may not be accessible to sunlight. It has been recommended that UV irradiation not be the only means of viral disinfection for water supplies, due to the possibility of residual virus particles producing infection *in vivo* (Frerichs et al. 2000). The results of this study indicate that the most effective disinfection procedure for LMBV would be to increase the sunlight dry time to 5 d, with the additional use of a chemical disinfectant.

Pokeweed antiviral protein (PAP) has been shown to inhibit the replication of numerous DNA and RNA viruses, including Cucumber mosaic virus, Herpes simplex virus, Poliovirus, and Human immunodeficiency virus (Aron and Irvin 1980; Tomlinson et al. 1974; Ussery et al. 1977; Zarling et al. 1990). In this study PAP, at low concentration, was shown to effectively inhibit the replication of LMBV. Furthermore, the data suggests PAP inhibited an event late in the replication cycle of the virus following host cell shutdown. Virus-infected cells undergo programmed cell death (apoptosis) to inhibit viral replication. Many viruses including African swine fever virus, a close relative of *Iridoviridae*, delay apoptosis via the production of E1B, a p53 inhibitory protein (Fields et al. 1996). The data indicates PAP inhibited LMBV

replication at a step late in the infection cycle since PAP's antiviral activity failed to prevent cell death. Treatments for parasitic and bacterial infections in fish involve the exposure of diseased fish to waters containing chemotherapeutic agents. A reduction in mortality in salmonids with bacterial gill disease was observed following treatment with Chloramine-T (Bowker and Erdahl 1997). A similar method of exposure to PAP may be an effective therapy for the treatment of largemouth bass populations infected with LMBV.

Guanidine was not as effective as PAP in the inhibition of LMBV replication. Guanidine (2,630 μg/ml) provided 34% protection to BF-2 monolayers. Guanidine has been shown to inhibit the replication of RNA-containing viruses, primarily those of the family the *Picornaviridae* (Caliguiri and Tamm 1968; Tershak et al. 1982). There are no reports of the inhibition of DNA virus replication by guanidine. A mechanism for the antiviral activity of guanidine proposes the interference of movement of newly synthesized membrane from the endoplasmic reticulum to smooth viral specific vesicles (Koch and Koch 1998). The partial inhibition of LMBV replication by guanidine suggests a role for endoplasmic reticulum in the transport of LMBV proteins and release of infectious virions.

Ribavirin was more effective than guanidine, but not as effective as PAP in inhibiting the replication of LMBV. Ribavirin (1.48 μg/ml) only partially inhibited the replication of LMBV, providing 47% protection to BF-2 monolayers. The antiviral activity of ribavirin has been shown to be host cell dependent. Studies involving the effect of

ribavirin on replication of orthopoxvirus revealed that the degree of antiviral activity is dependent upon the host cell line (Smee et al. 2001). Ribavirin inhibited the replication of orthopoxviruses at considerably lower concentrations in 3T3 cells, when compared to the inhibition observed in Vero 76 cells (Smee et al. 2001). Thus, ribavirin's antiviral activity may not be similar *in vivo* compared to its activity *in vitro*.

Chemotherapeutic agents with different mechanisms of action tend to act synergistically toward virus replication (Shigeta et al. 1997). Recently, ribavirin in combination with IFN-α, has been approved for the treatment of chronic hepatitis infection caused by Hepatitis C virus (Markland et al. 2000). A synergistic effect toward the inhibition of Newcastle disease virus replication was observed with PAP in combination with either guanidine or ribavirin (Weaver and Aron 1998). Although guanidine and ribavirin partially inhibited the replication of LMBV, combinational drug therapy with PAP may increase the effectiveness of guanidine and ribavirin.

SUMMARY

The inactivation of Largemouth bass virus (LMBV), a member of the family *Iridoviridae*, following exposure to chemical disinfectants was determined by TCID50 assay. LMBV was inactivated greater than 99% following exposure to formalin (2%), iodine (0.0125%), sodium hypochlorite (0.07%), and ethanol (35%). The effect of ultraviolet (UV) light and heat on the infectivity of the virus was also determined. Exposure of infectious virus to UV light (2×10^3 ergs $\cdot$ $sec^{-1} \cdot$ cm^{-2}) for 20 seconds, and a temperature of 37°C for 5 days resulted in greater than 99% inactivation of the virus. In addition, the antiviral agents pokeweed antiviral protein (0.078 μmol/ml), guanidine (2,630 μg/ml), and ribavirin (1.48 μg/ml) provided 100%, 34%, and 47% protection from virus infection, respectively, to BF-2 cell monolayers.

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VITA

Brian Andrew Scott was born in San Antonio, Texas, on July 29, 1977, the son of Janice Faye Grant and Willis Manuel Scott. After completing his work at Canyon High School, New Braunfels, Texas, in 1995, he entered Texas A&M University - Corpus Christi. In 1997, he transferred to Texas State University - San Marcos (formerly Southwest Texas State University). He received the degree of Bachelor of Science from Texas State University - San Marcos in December 1999. In August 2000, he entered the Graduate College of Texas State University - San Marcos. While at Texas State University - San Marcos, he worked as an instructor assistant for Introductory Microbiology, Immunology, and Virology laboratories. He has presented his research at four Texas Branch American Society for Microbiology meetings, and at a National meeting of the American Society for Microbiology.

Permanent Address: 1600 Bretzke Lane
New Braunfels, Texas 78132

This thesis was typed by Brian Andrew Scott