POTENTIAL OF BIOFILMS TO HARBOR LARGEMOUTH BASS VIRUS (LMBV)

THESIS

Presented to the Graduate Council
of Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Master of SCIENCE

by

Shubhankar Nath, B.S.

San Marcos, Texas
April 2009

POTENTIAL OF BIOFILMS TO HARBOR LARGEMOUTH BASS VIRUS (LMBV)

Committee Members Approved:

_____
Robert J. C. McLean (Chair)

_____
Gary M. Aron

_____
Dittmar Hahn

Approved:

_____
J. Michael Willoughby
Dean of Graduate College

DEDICATION

This thesis is dedicated to my parents.

ACKNOWLEDGEMENTS

I would like to thank my advisor and most favorite professor, Dr. Robert J. C. McLean, for his guidance, support, and encouragement throughout the duration of this project. I would also like to thank Dr. Gary M. Aron for teaching me all the virology laboratory techniques whenever I needed those for this project. I would also like to thank Dr. Dittmar Hahn for his advice and helpful discussions. Special thanks should go to Gregory M. Southard for allowing me collecting biofilm samples from A. E. Wood Fish Hatchery, San Marcos, TX. Furthermore, I would also like to thanks Dr. Joseph Koke and Dr. Dana Garcia and all the members of their labs for teaching me how to use the qPCR thermocycler and different microscopes.

Further special thanks are endowed to my parents and family for their moral support throughout my education. Additionally, I would also like to extend my gratitude to all my peers and friends from the McLean lab ("the Slime Gang") and the Aron lab especially Mary, Alicia, Matt, Babur, Allana and Tommy.

This manuscript was submitted on April 21, 2009.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	base pair
˚C	degree centigrade
cat #	catalog number
CFU	colony forming unit
cm	centimeter
CPE	cytopathic effect
C_T	cycle threshold
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dpi	dots per inch
EMEM	Eagle's minimum essential medium
EPC	epithelioma papulosum caprini
Fig	figure
gm	gram
h	hour
HBSS	Hanks Balanced Salt Solution
HCl	hydrochloric acid
K	thousand times

KV	kilo volt
lb	pound
LMBV	largemouth bass virus
M	molar solution
mM	milli Molar solution
µM	micro Molar
min	minutes
mL	milli liter
µL	micro liter
NA	not applicable
ND	not detected
NIH	National Institute of Health
nm	nano meter
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PFA	paraformaldehyde
pfu	plaque forming units
PTA	phosphotungstic acid
qPCR	quantitative PCR
r^2	regression coefficient
rpm	rotation per minute
SD	standard deviation
SE	standard error

$TCID_{50}$	tissu culture infective dose 50
TEM	transmission electron microscope
TSA	Tryptic Soy Agar
v/v	volume by volume
w/v	weight by volume

ABSTRACT

POTENTIAL OF BIOFILMS TO HARBOR LARGEMOUTH BASS VIRUS (LMBV)

by

Shubhankar Nath, B. S.

Texas State University-San Marcos

April 2009

SUPERVISING PROFESSOR: ROBERT J. C. MCLEAN

Biofilms are surface-attached microbial communities encased by extracellular matrix. This mode of growth enables microbial survival during adverse environmental conditions. In the present study, we investigated whether bacterial biofilms could serve as potential reservoirs for largemouth bass virus (LMBV), a fish virus that does not infect biofilm microorganisms. LMBV, a member of family *Iridoviridae* is one of the naturally occurring fish viruses, causing fatal disease of largemouth bass (*Micropterus salmoides*). The reservoir of LMBV is currently unknown. Laboratory investigations of this phenomenon consisted of mixing various concentrations of LMBV with lab-grown biofilms of *Pseudomonas fluorescens* ATCC 13525, a bacterium commonly found in aquatic environments. Control experiments consisted of mixing LMBV with the biofilm substratum in the absence of bacteria. LMBV was detected using standard and

quantitative (real-time) PCR techniques. Virus infectivity was measured using the tissue culture infective dose ($TCID_{50}$) technique. Artificially introduced LMBV was detected in lab grown biofilms by PCR and infectivity assays. Real Time PCR was able to quantify a maximum 6.22% of total LMBV in the adjacent bulk environment incorporated within biofilms. The results also indicated that LMBV associated with lab grown biofilms is directly correlated to the LMBV concentration in the adjacent liquid environment. Real Time PCR detected 100 fold less LMBV copy number than conventional PCR. Epifluorescent microscopy of biofilms grown in presence and absence of LMBV did not reveal any structural differences in bacterial community structure caused by virus introduction. LMBV recovered from biofilms were further observed under transmission electron microscope (TEM) and tested for infectivity using the $TCID_{50}$ method. Biofilm-association did not change the tissue culture infectivity of LMBV. In a separate experiment, natural biofilm samples were collected from 4 different ponds of A. E. Wood Fish Hatchery, San Marcos, Texas; all of which were screened negative for LMBV by PCR. However, lab studies showed that natural biofilm samples could acquire the virus when placed in LMBV-spiked pond water. This study demonstrates the importance of examining microbial and ecological niches as potential reservoirs in the control of viral diseases.

I. INTRODUCTION

Largemouth bass virus (LMBV), a member of the family *Iridoviridae* (genus: *Ranavirus*) is one of more than hundred naturally occurring viruses that causes fatal disease in largemouth bass (*Micropterus salmoides*) (Herr and Brett, 2002). This virus, first found in Florida in 1991 (Grizzle et al., 2002) now has been reported from more than 15 states in the US including Texas. Though LMBV has only been reported fatal for largemouth bass, it can also infect smallmouth bass, Suwanee bass, bluegill, redbreast sunfish, spotted bass, white crappie, black crappie and can be asymptomatically carried by amphibians, reptiles, and other fish species (Herr and Brett, 2002). The source of LMBV infection in any pond or hatchery is unknown. In the present study, we hypothesized that biofilms may act as a reservoir of LMBV. Biofilms are the complex communities of microorganisms that are enclosed in an extracellular matrix of mainly polysaccharides which gives a strong protection to microbial cells against adverse environmental conditions, antibiotic therapy or host immune system.

Biofilms can be formed on any liquid-solid interfaces including dental enamel (Marsh, 2004), urinary catheters (Trautner and Darouiche, 2004), pacemakers (Marrie et al., 1982), GI tracts (Probert and Gibson, 2002), water distribution pipes (Storey and Ashbolt, 2003; Lehtola et al., 2004; Langmark et al., 2005; Skraber et al., 2007) and even plant leaves (Morris and Monier, 2003; Ramey et al., 2004). Biofilms express properties not exhibited by the same organisms growing in planktonic culture. Numerous experiments have been done to establish biofilms as a potential reservoir of other

pathogens. Fluorescence staining is a popular and well accepted method for viral enumeration (Patel et al., 2007). Storey and Ashbolt (2001 and 2003) reported the incorporation of enteric bacteriophages within biofilms of water distribution pipe at a concentration representing 1% of that present in the adjacent bulk water environment. They used polyclonal antisera against purified B_{40}-8 bacteriophages for *in situ* detection of virions in biofilms. A similar study was done by Lacroix-Gueu et al. (2005) who used fluorescence spectroscopy (FCS) for the *in situ* measurement of viral particles which had penetrated inside the extracellular matrix of mucoid biofilms. Quignon et al. (1997 and 1997) examined the 'behavior' of poliovirus-1 in biofilms. They studied the comparative tendency of poliovirus-1 to accumulate within biofilms in the presence or absence of clay or chlorine. They showed that if no clay is added, a greater amount of viruses was recovered from the biofilms than from the water flow. The incorporation of viral particles into multi species bacterial biofilms is a continuous process and can remain constant, which suggests equilibrium between viral incorporation and release (Skraber et al., 2007). Lehtola et al. (2004) described a SYBR Green I staining method to enumerate virus-like particles in bacterial biofilms when the biofilms were allowed to grow on copper and plastic pipes of a pilot drinking water distribution system. Biofilms are considered as a potential source of pathogenic viruses and a cause of public health hazards (Skraber et al., 2005).

All of these studies were based on non-PCR based detection methods. Moreover, in most of these studies, bacterial viruses (i.e., phage) were used as model viruses incorporated within biofilms, which is expected because of phage parasitism on bacteria. So it is also expected that eukaryotic viruses could be found in association with biofilms

because both are in same environment, though there is no question of parasitism. In the present study, we developed a PCR based technique to specifically detect our target virus (LMBV) in natural and lab grown biofilms. We used glass fibers, which have become a popular substance to grow biofilms on (King, 2001; Kalmokoff et al., 2006). Glass fibers interfere the least in nucleic acid extraction, and more surface area is available within a customizable shape. Grizzle et al. (2003) first described the PCR method to detect LMBV and designed the primers LMBV288F (5'-GCG GCC AAC CAG TTT AAC GCA A-3) and LMBV353R (5'-AGG ACC CTA GCT CCT GCT TGA T-3') which we used in this study for both conventional PCR and real-time quantitative PCR (qPCR). qPCR method amplifies a specific target gene and measures the fluorescence after each reaction cycle emitted by the double stranded DNA when it binds with SYBR Green. The degree of fluorescence is directly proportional to the amount of DNA present in the template. Thus it helps in absolute quantification when compared with a known copy number. We preferred the SYBR Green method because of its reaction simplicity and ease of interpretation. Getchell et al. (2007) and Goldberg et al. (2003) described a TaqMan real time PCR method to detect LMBV using different primers. Getchell et al. (2007) evaluated this method be 100 times more sensitive to detect low copy numbers of LMBV in diseased and spiked fish organs compared to conventional cell culture method.

A series of further experiments was done to visualize the structure of the biofilms and LMBV recovered from biofilms using epifluorescent, and transmission electron microscopy. Conventional cell culture techniques, i.e., plaque assay and $TCID_{50}$, were used to evaluate the retention of infectivity of the recovered LMBV from biofilms.

The main objective of this study was to investigate whether or not biofilms can serve as a reservoir for largemouth bass virus. In continuation, we also tried to determine whether biofilm association afecteded LMBV infectivity.

II. MATERIALS AND METHODS

Viral and Bacterial Stock. LMBV stocks with the same titer (or copy number) were stored at -80˚C in 1.8 mL cryovials. LMBV stock was prepared in tissue culture medium without added antibiotic to eliminate their effect on the viability of the biofilms. LMBV was thawed slowly at room temperature for 15 min. *Pseudomonas fluorescens* ATCC 13525, a naturally found bacterial strain was used to grow biofilms in vitro. Biofilms were grown on 0.42 grams of glass fibers in most cases unless mentioned separately. Prior to use the glass fibers were cleaned by incubating overnight with 1M HCl solution followed by rinsing in deionized water several times and sterilized at 121˚C for 15 min.

Plaque Assay of Stock LMBV. Plaque assays, for viral enumeration were performed as described by McClenahan et al. (2005) with the following modifications. A confluent monolayer of EPC cells was prepared in 24-well flat bottom tissue culture plates (Sarstedt, Inc. Newton, NC) for 2 days in E-MEM (Eagle Minimum Essential Medium, Sigma, St. Louis MO) supplemented with 10% (v/v) fetal bovine serum (Sigma), 1% (w/v) L-glutamine-penicillin-streptomycin (Sigma), 1% (w/v) non-essential amino acid (Sigma), and 3.2% (w/v) $NaHCO_3$ into a final volume of 500 mL in E-MEM. For the viral assay, the culture medium was removed and the EPC monolayer was washed twice with HBSS (Hank's Balanced Salt Solution, Sigma). A ten-fold dilution of stock LMBV was made in EMEM and 0.1 mL was added to the EPC monolayer in triplicate. The

tissue culture plate was then incubated at 30°C for 40 min with continuous shaking (110 rpm) to allow maximum absorption of virus (McClenahan et al., 2005). After adsorption, 2mL of 2% methyl cellulose-2X E-MEM mixture was added to each well and the monolayer was incubated at 30°C in an atmosphere containing 5% CO_2. Monolayers were observed for cytopathic effects after 5 days of incubation.

Preparation of Methyl cellulose. 4 grams of methyl cellulose was taken into a 500 mL glass bottle and 100 mL of sterile deionized milipore water was added to it very slowly while a stir bar was applied to prepare the suspension. The suspension including the stir bar in it was then autoclaved at 121˚C, 15 lb pressure for 30 min with slow exhaust. The bottle was then kept in room temperature with continuous stirring by the bar inside. When the temperature became near 30˚C, the suspension turned to a thick white jelly with numerous air bubbles within it. At that point, 100mL of 2X concentrated MEM media was added slowly and mixed thoroughly by stirring. 2X concentrated MEM was prepared by mixing MEM powder (Sigma) with all the other components at twice concentration. The methyl cellulose-MEM media was then kept refrigerated with stir bar in it until use.

Counting Plaques. The plate was checked for the cytopathic effects every day and on 5^{th} day of incubation countable plaques were visible. The overlay of methyl cellulose media was removed using a broken tip pipette and the cells were fixed by adding 3 mL of methanol to each well for 15 min at room temperature. Methanol was removed and 1mL crystal violet stain (0.5%) was added to each well and kept for 20 min. Crystal violet stain was prepared by mixing 0.5 g of crystal violet with 20 mL of methanol making the final volume to 100 mL by adding deionized water. The plate was rinsed in tap water and

left to air-dry. The number of plaques was counted under inverted light microscope using a green filter at a total magnification of 200X.

DNA Extraction, PCR and qPCR of Stock LMBV and Standard Curve. Though unpurified cell culture supernatant could be used as template for PCR (McClenahan et al., 2005), we preferred DNA purification method for this project. 10-fold serial dilution of stock LMBV was done in PBS . Qiagen DNAeasy Blood and Tissue Kit (cat. # 69506) was used for DNA extraction with little modification of the protocol listed in 'isolation of total DNA from cultured animal cells'. We directly used 200 µL of sample to be mixed with 20 µL of proteinase K and 200 µL of buffer AL without any centrifugation. We assumed that centrifugation at lower rpm would not be able to form any pellet of viral particles. Rest of the protocol has been followed according to the kit manual. Extracted DNA was kept in -20˚C freezer until used for PCR or qPCR. A negative control (sterile millipore water) was also performed using the same protocol.

The purpose of doing PCR and qPCR separately was to compare the detection limit of these two protocols. The primers used for the PCR and qPCR have been mentioned earlier. Each 25 µL PCR reaction mix included 2.5 µL of 10X PCR buffer, 3.0 µL of 25 mM $MgCl_2$, 2.0 µL of 10 mM dNTP blend (2.5 mM each), 0.625 µL of both the primers (20 µM), 0.125 µL of AmpliTaq GOLD Polymerase (5 U/ µL), 1.25 µL of template DNA and rest 14.88 µL of sterile deionized water. The thermocycler was set at 95˚C for 10 min for polymerase activation; 20 cycles for 15 sec at 94˚C, 30 sec at 66˚C, 30 sec at 72˚C; and more 10 cycles for 15 sec at 94˚C, 30 sec at 55˚C, and 30 sec at 72˚C; finally 5 min at 72˚C and the product is stored at 4˚C. Different laboratory desks, pipettes, and tips were used for DNA extraction and making master mix to reduce the chances of

contamination. All the materials required for DNA extraction and PCR or qPCR were autoclaved before every assay. The PCR products were run in 4% agarose gel and stained with ethidium bromide to visualize the bands.

The same extracted DNA was used for the real time qPCR. We useded EXPRESS One-Step SYBR® GreenER™ qRT-PCR Universal Kit manufactured by Invitrogen™ (cat. # 11794-200) and Eppendorf 'Mastercycler® ep realplex' thermocycler. A standard reaction size of 20 µL per tube contained 10 µL of EXPRESS SYBR GreenER qPCR SuperMix Universal, 0.4 µL of each 10 µM forward (LMBV288F) and reverse(LMBV535R) primer, 5 µL of template DNA, and 4.2 µL of DEPC treated water. SuperScript was not required for amplifying genomic DNA. Three replicates of each DNA template samples were taken under consideration (starting from 10^{-1} to 10^{-7}). 'No template' controls included 15 µL of master mix and 5 µL of sterile water. Master Mix was prepared in excess for 2 more reactions to reduce any shortage due to pipetting errors. 0.2 mL PCR tube strips and optically clear flat cap strips (8 tubes and caps per strip) manufactured by Bio-Link Scientific, LLC (cat. # BL3008ST and BL3008FC respectively) were used for the reactions. Special care was taken to make sure that all the tubes were sealed with caps properly not to allow any evaporation and all components are at the bottom of the tube after proper mixing. The total set up was prepared on ice. Using the software associated with the instrument, a template for reaction temperature (Fig. 1) was saved and used for further assays. Base line, noise band, and threshold were set to default values by the software itself. By the end of total reaction process, the data and melting curve were analyzed. The cycle threshold (C_T) value, which is the number of cycle when a fluorescence signal reaches the threshold value, for each dilution of

stock LMBV was plotted in Microsoft® Excel 2007 and analyzed to get a standard curve (Fig. 4).

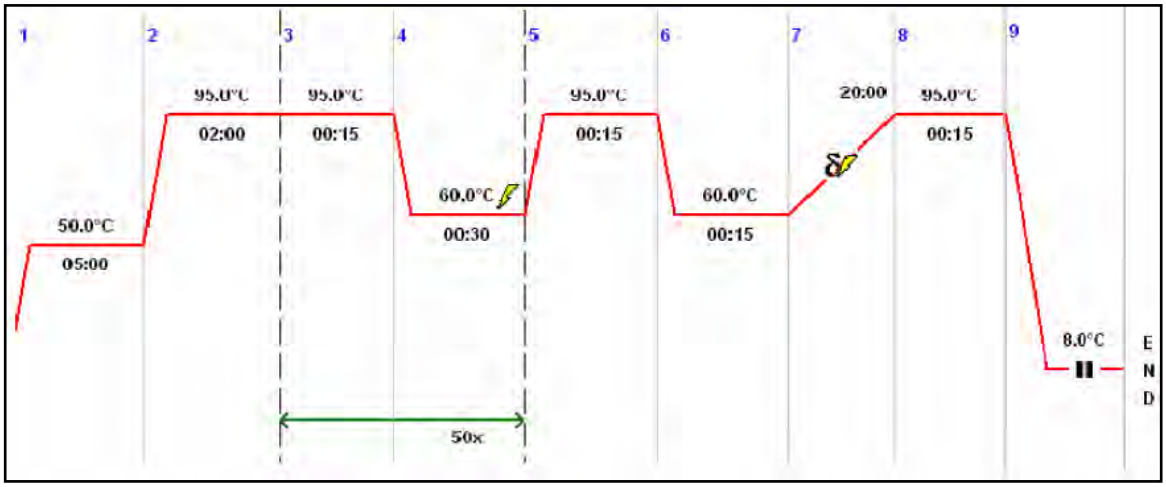

Fig. 1. Temperature Template for qPCR. This figure shows the temperature specifications that were used for real time polymerase chain reaction. A total of 50 cycles were run and by the end of each cycle the fluorescence was measured for each samples. Polymerase chain reaction was done in two main steps: holding the reagents at 95°C for 15 sec (denaturation) followed by at 60°C for 30 sec (annealing and extension). Step 7 shows the melting curve analysis, where temperature is increasing from 60°C to 95°C with a continuous measuring of fluoresce.

Experiment with Lab Grown Biofilms. Fiber glass, used as a biofilm substratum (Oosthuizen et al., 2002), was prepared by acid washing (1M HCl) overnight, rinsing in deionized H_2O and autoclaving. Bacterial concentrations within biofilms were evaluated using a previously described sonication and dilution plating protocol on R2A agar (Difco) (Bates et al., 2006). Bacterial cultures were incubated at 25°C. For lab-grown biofilm assay, *Pseudomonas fluorescens* ATCC 13525, a biofilm-forming organism, commonly encountered in freshwater environments (Caldwell and Lawrence, 1986), was grown in replicate in 50 mL tryptic soy broth (Difco) overnight at 25°C with continuous shaking at 110 rpm. To each flask, we added 0.42 g fiberglass as biofilm colonization

substrate. At the same time, 10-fold dilutions of LMBV cultures were added to the bacterial cultures. Two control conditions were set with fiberglass - one without any bacteria (biofilm control) and another without any LMBV (virus control). After 2 days of incubation, all the fiber glass samples were rinsed separately for three times, to remove unattached or loosely attached bacteria, then suspended in 10 mL sterile PBS in a scintillation vial. Viable count was determined after sonicating for 8 min following dilution plating method (Bates et al., 2006). An aliquot of 200 µL of the sonicated material was also used for DNA extraction (described earlier) for qPCR analysis. All lab biofilm experiments were conducted in triplicate.

CFU Counting. Conventional method of counting CFU was applied in this experiment. 1 mL of sonicated material was used for 10-fold dilution in sterile deionized water followed by platting on tryptic soy agar (TSA). All the dilution plates were incubated at 25˚C for 3 days to get countable colonies.

DNA Extraction and qPCR. The same DNA extraction and qPCR protocol were used as stated earlier. The lab grown biofilm samples were kept in triplicate with each dilution of LMBV and triplicate DNA template samples were taken for qPCR from each replicate of lab grown biofilm samples. In this way, each dilution of LMBV yielded 9 outcomes.

Experiments with Natural Biofilms. Natural biofilm samples were collected from four different ponds of a local fish hatchery by scraping materials from the edge of cement boundaries with sterile metal scrappers and placing the materials into 10 mL phosphate buffered saline (PBS). Pond water samples (500 mL) were also collected from each pond. All the materials were kept on ice and analyzed for biofilm cell density by sonication and

dilution plating (Bates et al., 2006), and LMBV by qPCR, within 1 h of collection. Biofilm and pond water samples all tested negative for LMBV by PCR and qPCR. To test the potential for natural biofilms to harbor LMBV, bacteria within pond water samples were allowed to colonize glass fibers and form biofilms as described above. These biofilm samples were split so that some were kept as 'control' (i.e., without any spiked stock LMBV) and others as 'test' (i.e., with spiked stock LMBV). For the 'test' samples, biofilms were allowed to grow on 0.4 g glass fibers in presence of 180 µL of stock LMBV mixed with pond water to a final volume of 25 mL; whereas for the 'control' samples biofilms were allowed to grow only in presence of 25 mL of pond water. A 'no biofilm control' was kept which included 0.4 g glass fibers in 180 µL of stock LMBV mixed with sterile water to a final volume of 25 mL. One 'negative control' was also kept which included same amount of glass fibers in 25 mL of sterile water. All the beakers containing these cultures were incubated at room temperature (23°C) for 2 days with continuous shaking (110 rpm). After 2 days, biofilms samples were processed for bacterial and LMBV enumeration as described elsewhere.

Microscopy. To determine any structural differences in biofilms, *P. fluorescens* broth culture was incubated with clean glass cover slips in presence and absence of LMBV at room temperature for 48 hrs with continuous shaking (110 rpm). After incubation the reverse side of the cover slip was scrapped and cleaned with sterile cotton swab. The biofilm specimen on cover slip was fixed overnight in 4% paraformaldehyde (PFA)/phosphate buffered solution (PBS) followed by staining with 1 µM SYTO® stain for 15 min in complete darkness. The cover slip was then washed twice with PBS for 1 min each and then air dried. Image was taken using an Olympus BH2 epifluorescent

microscope containing an Olympus Q color-3 camera and edited with NIH Image J and Adobe Photoshop CS software. The other specifications are mentioned below the corresponding images.

For transmission electron microscopy (TEM), LMBV particles were isolated from lab grown biofilms by filtering (0.2 µM HT Tuffryn® Membrane, Life Sciences) the sonicated materials. High titer of virus was used for this purpose to have more possibility of finding them. One drop of virus filtrate was kept for 5 min on formvar coated 300 mesh copper grids for viral adsorption followed by removal of extra viral suspension using a clean Whatman filter paper. 2% phosphotungstic acid (PTA) was added on the grids for another 15 min. Excess amount of PTA was removed in the same manner as stated before. After air drying, the grid was viewed using a JEOL 1200 Transmission Electron Microscopy at 120 KV and 50 K magnification. Film was processed using standard processing technique and negatives were scanned at 2400 dpi using an Epson 2450 scanner and images were edited using NIH ImageJ and Adobe Photoshop 7.0 software.

Cell Culture infectivity tests. To evaluate the retention of infectivity of LMBV isolated from biofilms, a screening test was done primarily. The 2 days old EPC cells grown in a 75 cm flask were washed in HBSS. Then 3 mL of biofilm filtrate material was inoculated into the flask which was then incubated at 25°C incubator for 40 min with intermittent shaking in every 3-4 minutes for optimum viral absorption. After this incubation period, the filtrated media was replaced by 20 mL EMEM and the flask was kept at 25°C for 3 days and observed following everyday for CPE or viral plaques.

The results of above experiment prompted us to conduct $TCID_{50}$ assay for the quantitative analysis of retention of viral infectivity. Biofilms of *P. fluorescens* were allowed to grow on 0.65 gms of cleaned glass fibers in presence of two 10- fold dilutions of stock LMBV in addition to one virus control and one biofilm control. Other growth conditions remained same as the previous study. Biofilm samples were processed as described before followed by CFU count, DNA extraction for qPCR, and $TCID_{50}$ assay. Day old monolayer of EPC cells were prepared in 96 wells plates which were used for this assay. Either 2-fold or 10-fold dilutions of filtrated sonicated biofilm materials in sterile PBS were prepared and 100µL of which was added to each corresponding wells. A total of 10 replicates of each dilution and a total of 6 serial dilutions were tested for each viral suspension in this assay (see Fig. 2). The plates were incubated at 25°C for 3 days and observed for CPE. $TCID_{50}$ was calculated following Reed and Muench method (adapted from "Virology: a laboratory manual", Academic Prem, Inc., 1992). $TCID_{50}$ of stock LMBV and isolated LMBV from biofilm growth media were also determined using the same method. We also run real time qPCR from the extracted DNA followed by running agarose gel to authenticate true positive results and to compare with melting curve results.

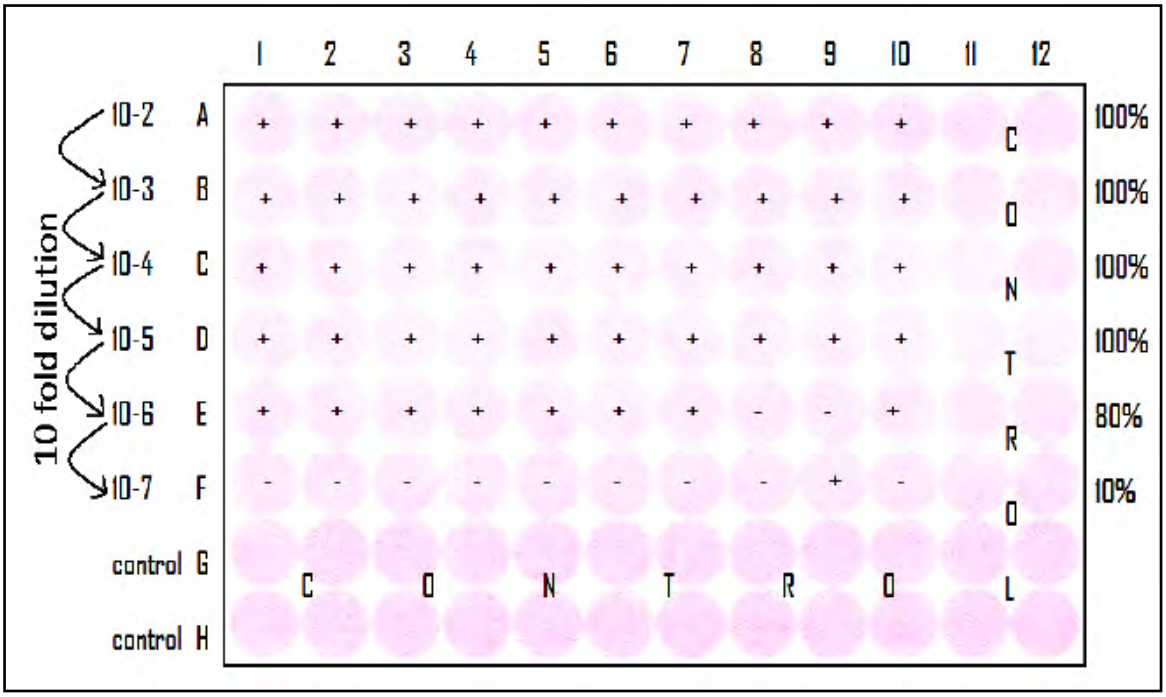

Fig. 2. $TCID_{50}$ assay of Stock LMBV. Confluent monolayer of EPC cells were grown in 96 wells plate for one day in presence of 100 µL of EMEM media in each well. 100 µL of each 10 fold dilution of virus was added to each corresponding well. Row G and H and Column 11 and 12 were kept as control which contained EPC cells in 100 µL of EMEM media and 100 µL of PBS. Plate was observed for cytopathic effect on every following day and $TCID_{50}$ was calculated following Reed and Muench method. Here, '+' sign indicates presence of at least one plaque, whereas '-' sign indicates no plaque. The right most column shows the percentage of infectivity for each dilution.

We also investigated whether sonication has any adverse effect on viral infectivity. Stock LMBV vials were thawed from -80°C to room temperature and then sonicated at 15 min intervals for 1 hr. The temperature of the water of the bath sonicator was maintained at approximately room temperature by changing the water frequently. All the sonicated stock samples were then tested for $TCID_{50}$ as previously described.

III. RESULTS

Plaque Assay of LMBV Stock. After 5 days, the total number of plaque forming units (pfu) calculated was 4.1X 10^7 per 0.1 mL of stock. Wells containing countable numbers of plaques were considered for this estimation. No contamination was found in the control wells.

PCR and qPCR of LMBV Stock. Extracted DNA from serially diluted LMBV stock was used for conventional PCR. Only to the 10^{-4} dilution, amplification products were visible (Fig. 3) after agarose gel electrophoresis. This was just to compare the sensitivity of PCR and qPCR.

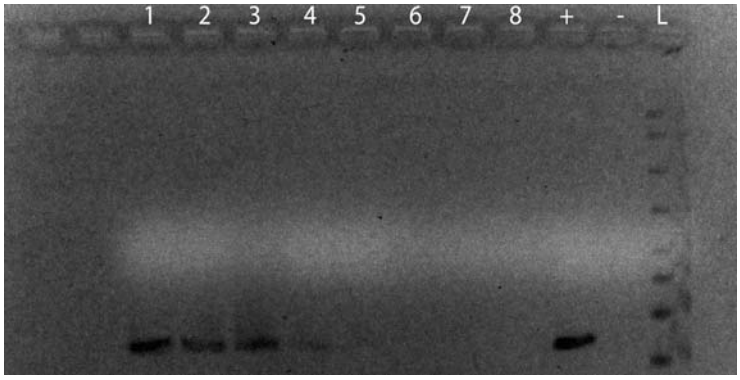

Fig. 3. PCR detects stock LMBV while serially diluted. A serial dilution of stock LMBV was made in EMEM from 10^{-1} to 10^{-8} and after polymerase chain reaction they were run on agarose gel from 1 to 8 respectively. A decreasing pixel intensity of bands indicates decreasing level of LMBV DNA in template. A very faint band is also visible in 4^{th} row.

qPCR was useful to detect upto 10^{-6} dilution of LMBV stock (Fig. 5). Table 1 showed the mean C_T values from qPCR at different dilution of LMBV stock. The absolute value of viral titer of stock was measured by plaque assay. C_T represents the number of cycle at which the fluorescence reaches the threshold level. Here the threshold value was set default by the Eppendorf software itself. False negative amplifications were found after 33 cycles.

Table: 1. Quantitative comparison of LMBV stock at different dilution derived from real-time PCR and plaque assay. C_T value represents the number of cycle at which fluorescence due to SYBR Green binding to genomic DNA exceeds the threshold value. A lower C_T value indicates higher the input copy number of largemouth bass virus in real-time PCR.

Dilution	Copy Number of LMBV (from plaque assay)	C_T Value ± SE (from real-time PCR)
10^{-1}	4100000	14.86 ± 0.081
10^{-2}	410000	18.38 ± 0.098
10^{-3}	41000	22.09 ± 0.052
10^{-4}	4100	25.80 ± 0.150
10^{-5}	410	30.32 ± 0.283
10^{-6}	41	32.76 ± 0.433

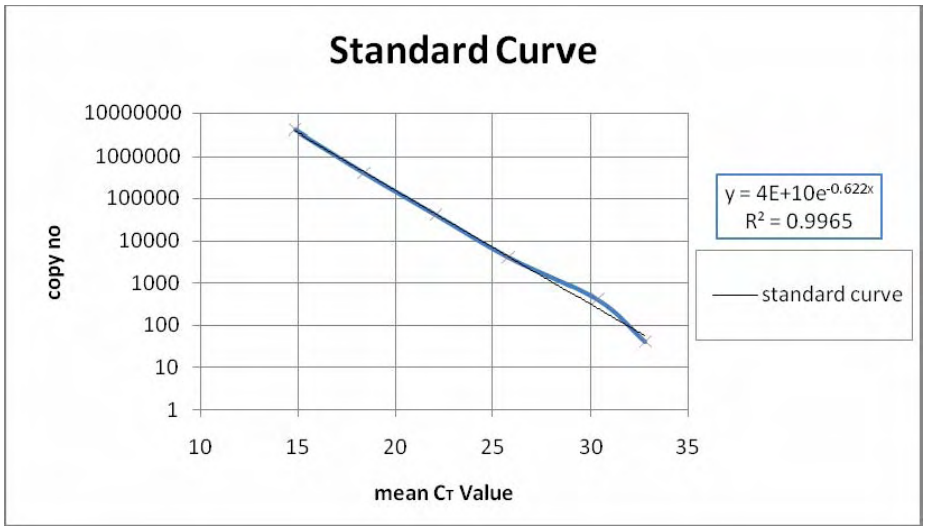

Fig: 4. Standard Curve. A standard curve has been derived by plotting the mean CT value (x-axis) and LMBV copy number (Y-axis) in spreadsheet to get XY scattered diagram using Microsoft® Excel 2007. The mean CT values have been derived from real-time qPCR assay in comparison to absolute quantification of LMBV copy number derived from plaque assay. These data have shown a statistically strong (r^2=0.9965) and significant (P <0.0001) correlation.

The mean C_T values obtained from the qPCR were plotted in Microsoft Excel spread sheet against the absolute copy number obtained by plaque assay. An exponential trend line was derived with correlation coefficient (r^2=0.9965) using the software (Fig. 4). The results indicate strong statistical significance (P< 0.0001). The trend line is referred as the standard curve which accompanied a standard equation (y = $4E+10e^{-0.622x}$). This equation has been used for the absolute quantification for the further biofilms experiments. The specificity of the qPCR amplification was further checked by running the products in agarose gel electrophoresis. A definite series of bands was visible at the site of 248 bp indicating the specificity of the reaction (figure not shown). A further study of melting curve revealed the specificity of qPCR amplifications.

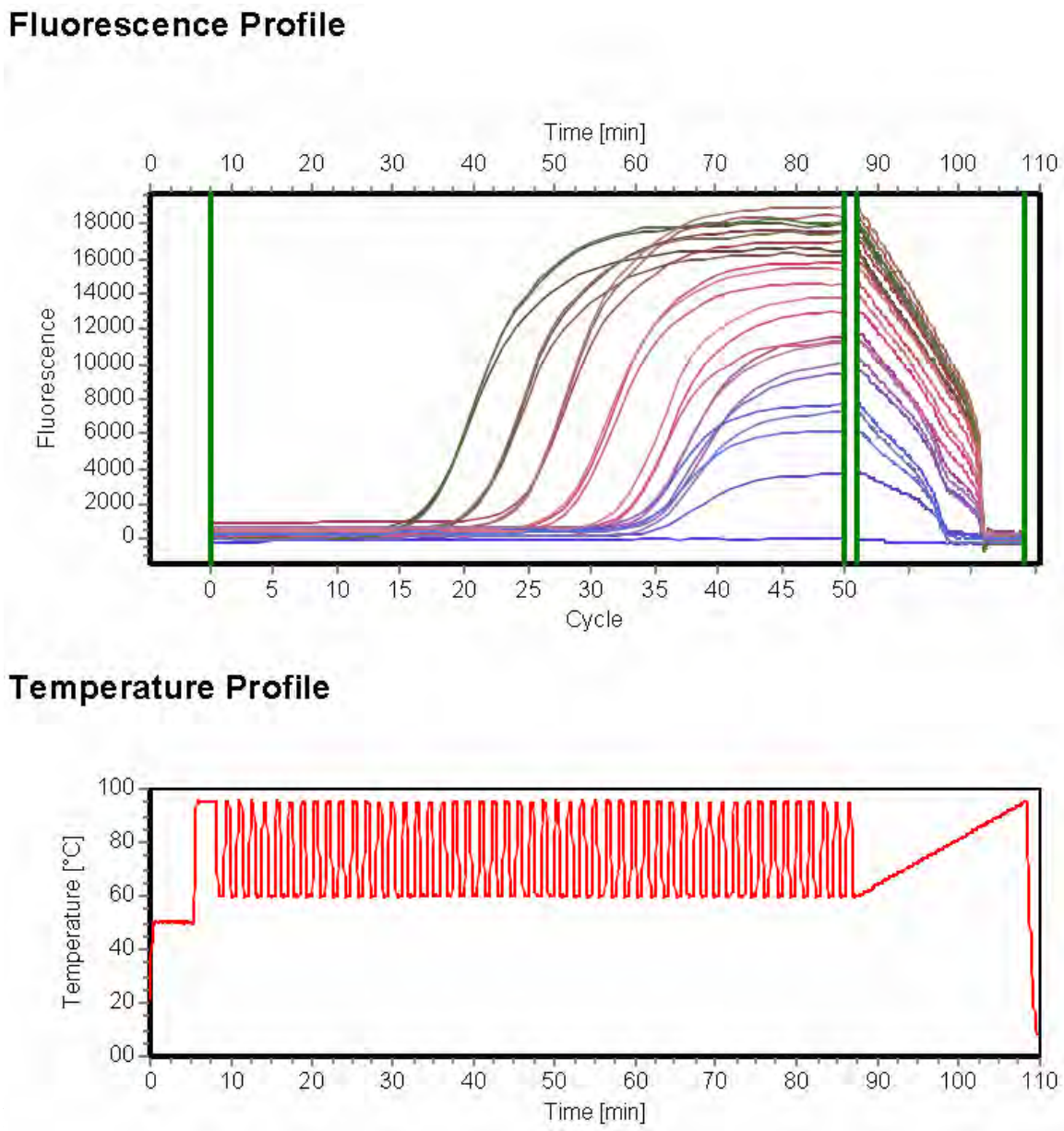

Fig. 5. qPCR Fluorescence Profile of serially diluted stock LMBV. (Top) Fluorescence signals were measured after each cycle of polymerase chain reaction which were plotted by the software itself. Stock LMBV was serially diluted and extracted DNA was run for amplification. An initial higher amount of template DNA produces more signals and thus quickly reaches the threshold value of fluorescence which is indicated as C_T. (Below) Temperature changes have been plotted graphically by the software.

Lab Grown Biofilms. LMBV were detected within lab grown biofilms using conventional PCR (Fig. 6). Lab grown biofilms of *P.fluorescens* were found to harbor LMBV which was directly related to the viral concentration in the adjacent bulk micro

environment (Fig. 8). Nine sets of data were derived from the each sample set incubated with different viral concentration. The negative control samples also gave some fluorescence (Fig. 7) which was identified as non specific amplification after analyzing the melting curve. The false negative results had a C_T value or more than 32 which were further confirmed by running 4% agarose gel. The amount of biofilms was measured in terms of total CFU count on TSA plates. LMBV found in association with biofilms was at a maximum of 6.22% of total viruses in the bulk adjacent environment (Table: 2).

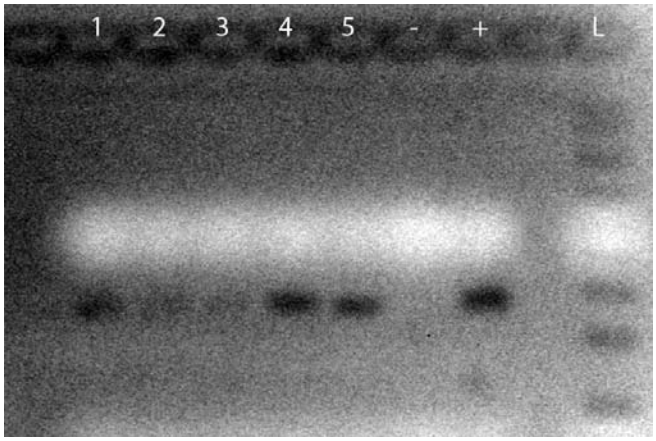

Fig. 6. PCR detects LMBV in lab grown biofilms. Primary screening was done using conventional PCR to detect whether or not biofilms can harbor LMBV. The gel image shows decreasing level of LMBV in serially sensed off water (row 1-3) where as there is a certain increase in LMBV within biofilms (row 4-5).

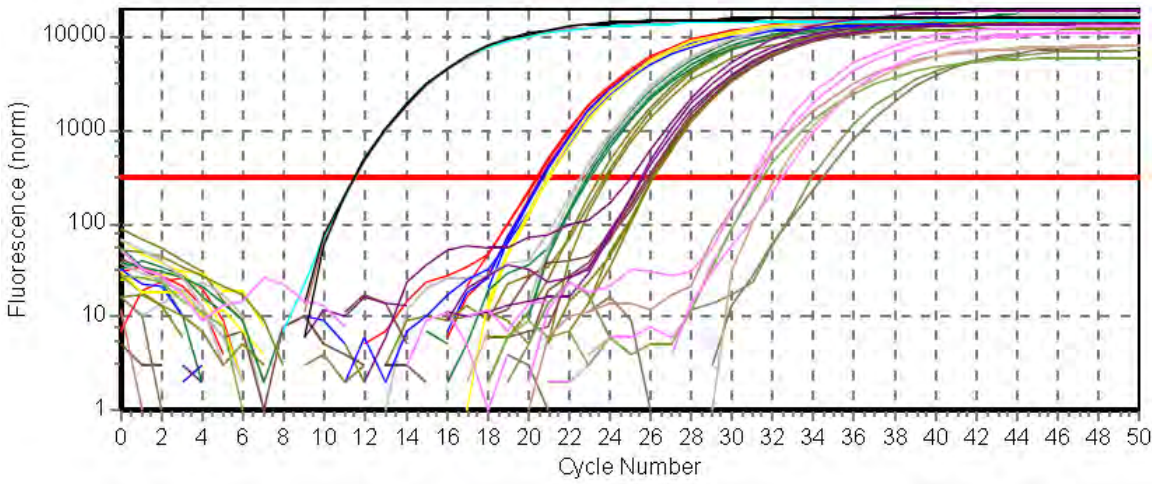

Fig. 7. qPCR Fluorescence Profile of LMBV isolated from lab grown biofilms. This figure shows fluorescence profile of LMBV DNA after extracting from lab grown biofilms. A positive sample was run which shows minimum C_T value. Signals from 9 replicates of each 3 different dilutions of bulk LMBV incorporated within biofilms have been plotted here. The non-specific amplification also shows some false positive results which were eliminated by melting curve analysis and running 4% agarose gel.

Table: 2. Comparison of Lab grown biofilms when incubated with different dilution of LMBV. The table shows the tendency of LMBV to get into the biofilms at a higher number when viral concentration is more in adjacent micro environment. 'No LMBV' control showed non-specific amplification and gave some fluorescence during real-time PCR. A very negligible percentage of total LMBV got adsorbed to glass fibers in comparison to biofilms harboring LMBV.

SAMPLE NO	Amount of biofilms (mean CFU± SD)	LMBV concentration	Mean CT ± SE	LMBV harbored	% total LMBV
	(x 10^8)	(per 50mL)			
1	1.1 ± 0.2	$3.7X10^8$	20.76 ± 0.14	$5.2X10^6$	1.41%
2	2.3 ± 0.8	$3.7X10^7$	23.12 ± 0.31	$1.2X10^6$	3.24%
3	2.4 ± 1.2	$3.7X10^6$	25.79 ± 0.23	$2.3X10^5$	6.22%
C_1(No LMBV)	5.3	NA	33.3 ± 0.9	ND	ND
C_2(No Biofilms)	0	$2.1X10^7$	31.56 ± 0.46	$6.3X10^3$	0.03%

ND: Not Detected
NA: Not Applicable

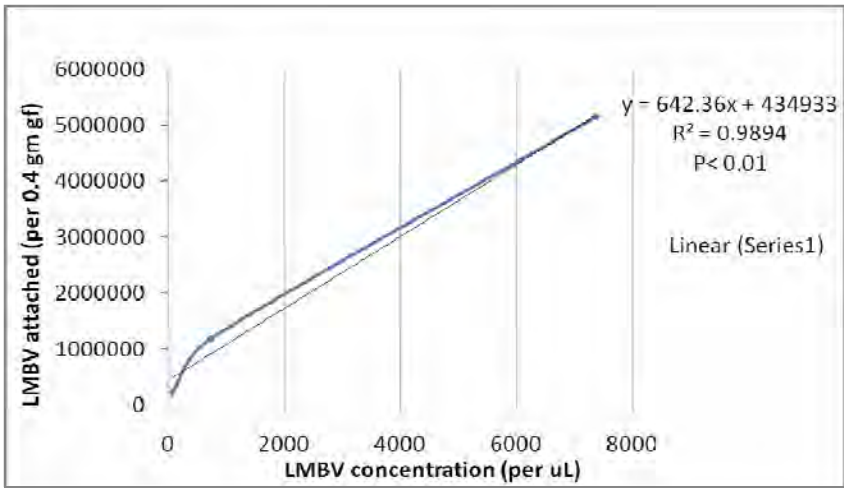

Fig. 8. Correlation between LMBV concentration inside and outside biofilms. Lab grown biofilms showing correlation between LMBV concentration in the adjacent environment and LMBV harbored within biofilms. Correlation coefficient (r^2= 0.9894) shows statistical significance (P<0.01).

Natural Biofilms. All the natural biofilm and water samples from the A. E. Wood Fish Hatchery tested negative for LMBV by PCR. But LMBV were found in association with all the biofilms when glass fibers were incubated with LMBV spiked different pond water to grow biofilms on it. Table 3 describes the summery of the findings. Pond water itself contained less concentration of bacteria and thus fewer amounts of biofilms (CFU ~ 10^5) was obtained from each corresponding pond water samples in comparison to lab grown biofilms. False negative results were noticed when the C_T values were more than 33 which indicate that at the point of sample collection water of all the four ponds of A. E. Wood Fish Hatchery were free of any LMBV infection. False positive signals were considered as non-specific amplification of DNA, contamination or primer-dimer after melting curve analysis (Fig. 9) and running agarose gel.

Table: 3. Detection of LMBV by real-time PCR from the biofilms when grown with LMBV spiked natural pond water from A. E. Wood Fish Hatchery. All the non-spiked biofilm samples were detected as negative for LMBV, still giving some non-specific amplification and C_T value. Melting curve analysis suggested that LMBV was not detected (ND) from any control sample. However, glass fiber control having no biofilms on it could harbor some viral particles.

	SAMPLE NO	Sample Description	mean CFU	LMBV con	Mean CT ± SE	LMBV harbored	% total LMBV
			($X10^5$)			($X10^5$)	
POND 10	1	test	2.6		26.96 ± 0.13	1.1	0.15%
	2	control	3.5		34.08 ± 0.02	ND	ND
				$7.4X10^7$			
POND 11	3	test	72		25.14 ± 0.06	3.4	0.46%
	4	control	153	per	36.62 ± 0.26	ND	ND
POND 20	5	test	42	25 mL	28.78 ± 0.18	0.36	0.05%
	6	control	21		33.28 ± 0.23	ND	ND
				In			
POND 30	7	test	57		26.26 ± 0.16	1.7	0.23%
	8	control	27	All	33.04 ± 0.30	ND	ND
	9	No biofilms	0	cases	30.79 ± 0.37	0.1	0.01%
	10	Sterile Water	0		33 ± 0.08	ND	ND

ND: Not Detected

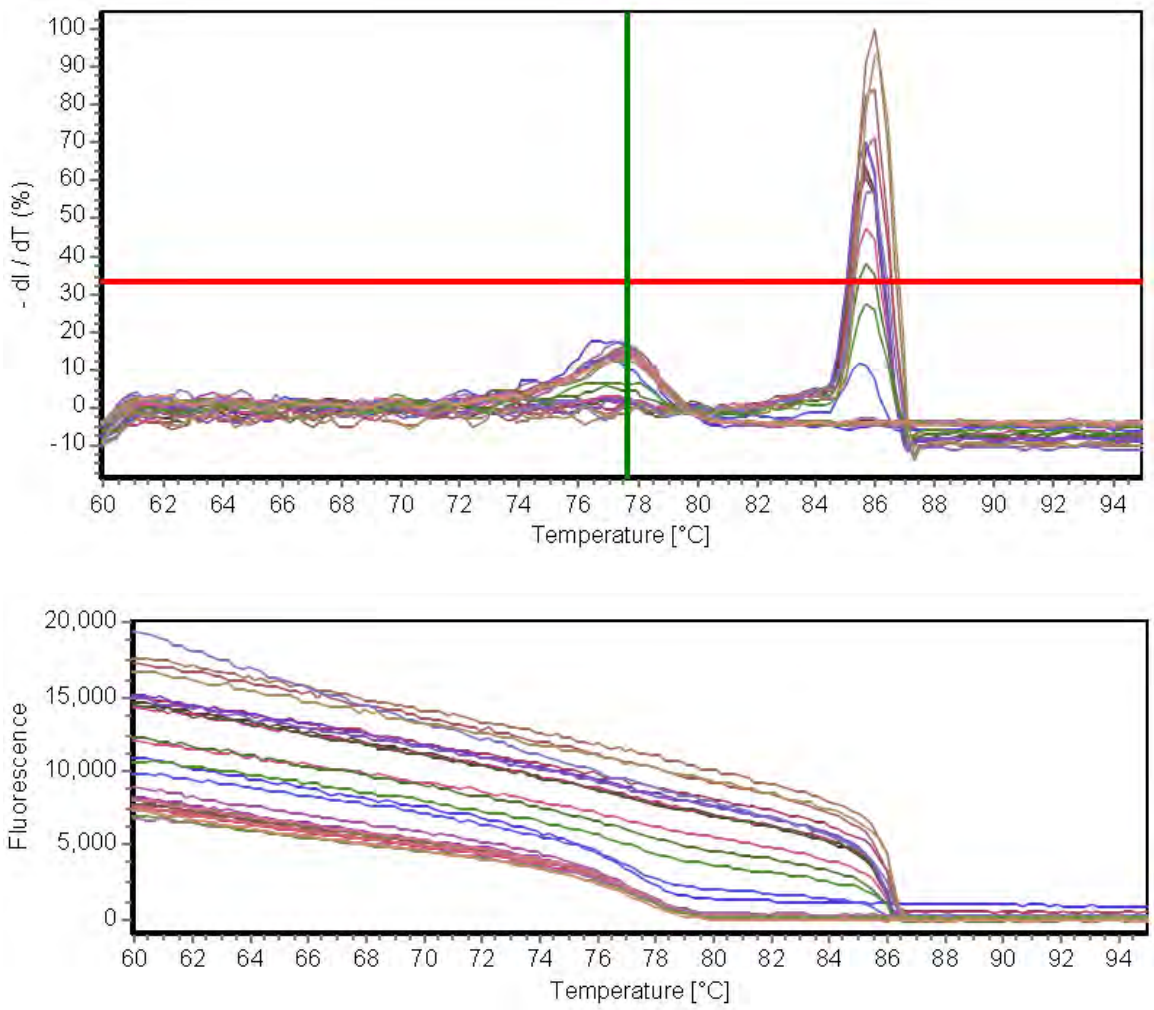

Fig. 9. Melting Curve Analysis. Melting curve analysis reveals the non-specific amplification in polymerase chain reaction which shows a different melting point of DNA giving a platue shape.

Microscopy. The images from epifluorescent microscopy (Fig. 10) of lab grown control and test biofilms showed no structural differences. TEM images (Fig. 11) showed possible icosahedral structure of LMBV.

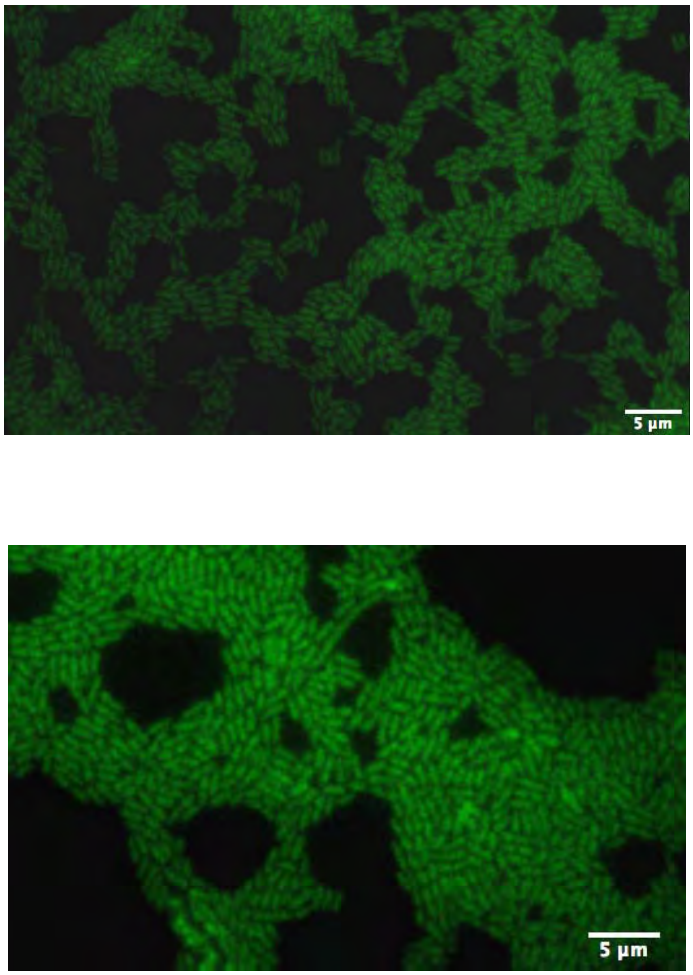

Fig. 10. Epifluorescent microscopy of lab grown biofilms in presence (top) and absence (bottom) of LMBV. *P. fluorescens* biofilms were grown on glass coverslips. The reverse side of the cover slip was scrapped and cleaned with sterile cotton swab. The biofilm specimen on cover slip was fixed overnight in 4% paraformaldehyde (PFA)/phosphate buffered solution (PBS) followed by staining with 1 µM SYTO® stain for 15 min in complete darkness. The cover slip was then washed twice with PBS for 1 min each and then air dried. Image was taken using an Olympus BH2 epifluorescent microscope containing an Olympus Q color-3 camera and edited with NIH ImageJ and Adobe Photoshop 7.0 software.

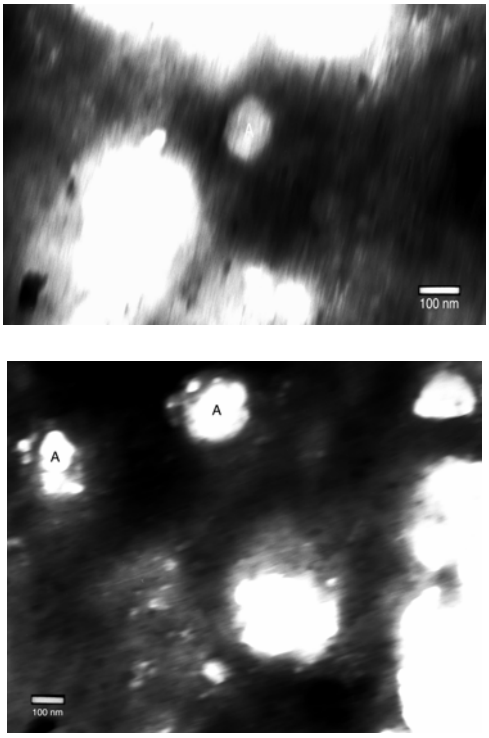

Fig. 11. Transmission Electron Microscopy of LMBV isolated from lab grown biofilms. LMBV particles were isolated from lab grown biofilms by filtering (0.2 µM HT Tuffryn® Membrane, Life Sciences) the sonicated materials. High titer of virus was used for this purpose to have more possibility of finding them. One drop of virus filtrate was kept for 5 min on formvar coated 300 mesh copper grids for viral adsorption followed by removal of extra viral suspension using a clean Whatman filter paper. 2% phosphotungstic acid (PTA) was added on the grids for another 15 min. Excess amount of PTA was removed in the same manner as stated before. After air drying, the grid was viewed using a JEOL 1200 Transmission Electron Microscopy at 120 KV and 50 K magnification. Film was processed using standard processing technique and negatives were scanned at 2400 dpi using an Epson 2450 scanner and images were edited using NIH ImageJ and Adobe Photoshop 7.0 software.

Cell culture infectivity test. After incubation for 3 days, the monolayers showed numerous plaques (Fig.12) under inverted light microscope at a total magnification of 200X. This result indicates that the viruses isolated from biofilms are still infective which leads to further investigation towards $TCID_{50}$ assay for quantitative analysis.

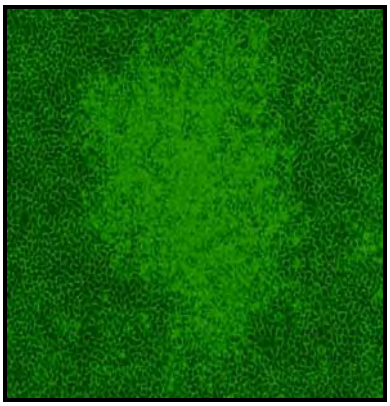

Fig. 12. Infectivity test of LMBV isolated from biofilms. 75 cm flask with confluent monolayer of Epithelioma Papulosum Caprini (EPC) cells were inoculated with sonicated filtered biofilm materials which showed cytopathic effect (CPE) after a incubation period of 3 days. This result concludes that the isolated viruses from biofilms are still infective. The image has been taken using a camera fitted with inverted light binocular microscope with a green filter and finally edited to grayscale using Microsoft® Word 2007 software.

The Reed and Muench method was used to calculate the 50% endpoint titer of virus. By accumulating the infected and non-infected test units over the whole dilution range, the effective test population is enlarged beyond the actual number of test units on either side of 50% endpoint. Below is the calculation for estimating $TCID_{50}$ of stock LMBV (also see Fig. 2):

$$\text{proportionate distance} = \frac{(\%\text{positive above } 50\%)-50\%}{(\%\text{positive above } 50\%) - (\%\text{positive below } 50\%)}$$

$$= \frac{80\%-50\%}{80\%-10\%}$$

$$= 30/70$$

$$= 0.43$$

Now, $\log ID_{50}$= (log dilution above 50%) + (proportionate distance X log dilution factor)

$$= (-6) + (0.43 \times -1.0) = -6.43$$

So, $ID_{50} = 10^{-6.43}$

This is the end-point dilution, namely the dilution that will infect 50% of the test units inoculated. The reciprocal of this number yields the titer in terms of infectious dose per unit volume. As the inoculums added to an individual test unit was 0.1 mL or 100 µL, the titer of the virus suspension (stock LMBV) would therefore be:

$10^{6.43}$ $TCID_{50}$/0.1 mL = 10 X $10^{6.43}$ $TCID_{50}$/mL = $10^{7.43}$ $TCID_{50}$/mL

$TCID_{50}$ of all the other isolated LMBV suspensions were determined using the same protocol. The results have been summarized in Table 4 below.

Table 4. Summary of viral infectivity results. Viruses were isolated either from biofilms or from biofilm growth media in presence or absence of bacteria. All the viral suspensions were prepared using 0.45 µm filter (Fisherbrand, cat. # 09-719B). $TCID_{50}$ has been determined following the same method mentioned earlier. The data within parentheses indicate the expected $TCID_{50}$ values compared to the $TCID_{50}$ at the beginning of the assay. 'Test sample 1' contained 10 times more LMBV than 'test sample 2'.

Category of LMBV suspension	$TCID_{50}$/mL (assay starting value)	Absolute LMBV copy no/mL (assay starting value)
Isolated from biofilms	$10^{2.3}$	4.4 X 10^5
media in absence of any bacteria, ('biofilm control')	$10^{3.67}$ ($10^{5.73}$)	9.5 X 10^4 ($10^{6.34}$)
media in presence of bacteria, ('test sample 1')	$10^{5.63}$ ($10^{6.73}$)	3.7 X 10^5 ($10^{7.34}$)
media in presence of bacteria, ('test sample 2')	$10^{3.71}$ ($10^{5.73}$)	1.0 X 10^5 ($10^{6.34}$)

The stock LMBV was also tested for effect of sonication on viral infectivity. $TCID_{50}$ was determined for each sonication period (i.e., 0 min, 15 min, 30 min, and 45 min) following the same protocol stated before. The summery of $TCID_{50}$ data has been described in Fig. 13 below. Sonication for 1 h did not yield any results.

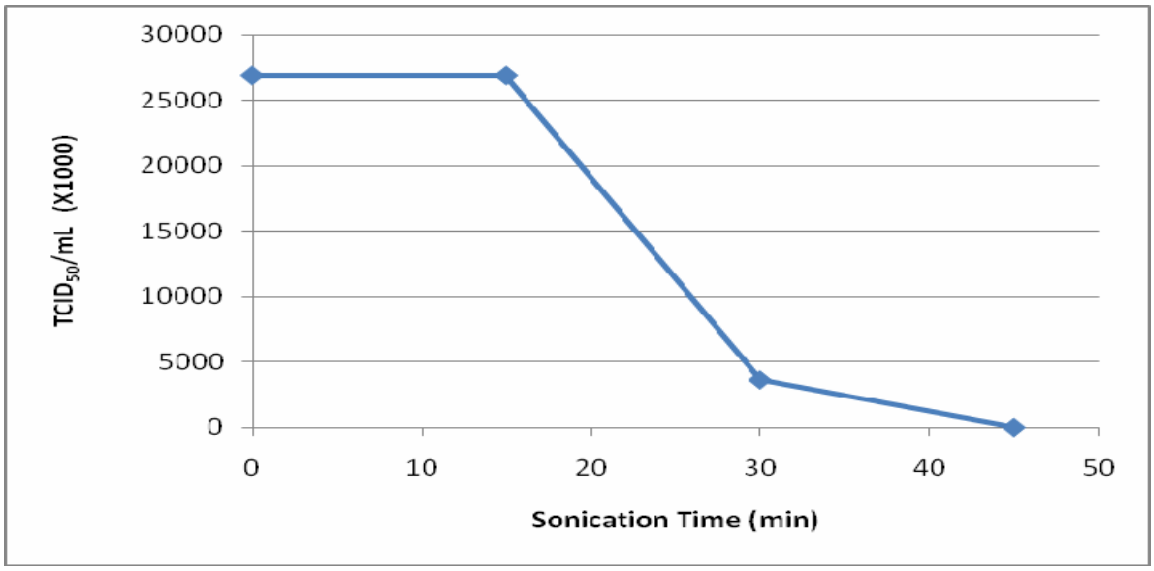

Fig. 13. Effect of sonication on viral infectivity. Stock LMBV samples were sonicated for 0 min, 15 min, 30 min, 45 min and 1 h and then all of them were used for determining $TCID_{50}$. Sonication for 1 h did not show any $TCID_{50}$ and hence was not considered for this graph. The results indicate that optimum sonication for upto a maximum of 15 min could be used for any related experiment.

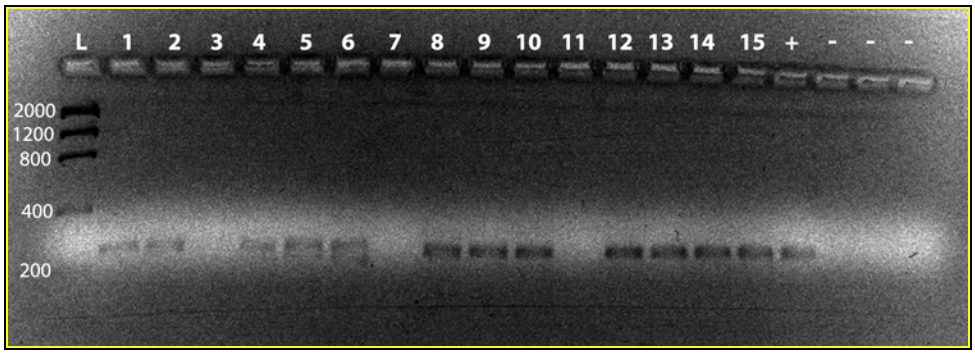

Fig: 14. Agarose gel confirms specific amplification of LMBV DNA template by qPCR. Real Time qPCR products were further tested for authentication for specific amplification of DNA template which exactly matches the conclusion from melting curve analysis (see supplemental data). The true positive results showed visible bands at 248 bp region.

The absolute LMBV copy number was determined following real time qPCR method. A comparative study of $TCID_{50}$ and viral copy number has been shown in Table 4. The qPCR products were further tested for reaction specificity by running 4% agarose gel electrophoresis. The gel image (Fig. 14) exactly matches the melting curve analysis data from qPCR (see supplemental data) which validates the sensitivity and specificity of real time instrument we used.

IV. DISCUSSION

The study describes a technique to identify and quantify LMBV within bacterial biofilms. The results indicate that both natural and laboratory grown biofilms are able to act as a reservoir for LMBV. Perhaps natural biofilms can harbor multy types of viruses regardless of their acquiescence to mono-species or multi-species bacterial biofilms. This might be due to the very small size of a virus in comparison to a bacterium. Extracellular polymatrix, a sticky substance may act as a trap for this type of phenomenon. The possibility of passive adsorption of LMBV to any solid surface is much less than the trapping of LMBV within biofilms. Our results show, lab grown biofilms harbored at a maximum of 6.2% of the total LMBV in the adjacent environment, whereas a 'no biofilms' control (i.e., only glass fibers) was shown to carry LMBV at a much lower percentage (0.03%). The question may arise that sonication is not as effective torelase for adsorbed viral particles. *Giardia* and *Cryptosporidium* cysts which are larger than LMBV were found in association with waste water biofilms which signifies that biofilms can entrap microorganisms with a size up to 15 µM in stagnant or running water (Skraber et al., 2007). Storey and Ashbolt (2001) proved the presence of two model phage viruses (MS-2 and B_{40}-8) both sizing between 23 to 30 nm within biofilms in a model distribution system. In the present study, we showed the existence of a comparatively larger virus LMBV (average size 132 nm from facet to facet and 145 nm from corner to corner) in association with biofilms (Plumb et al., 1996). This emphasizes the importance of reevaluating the detection and eradication protocol for LMBV control and other

similar type of viruses. It is quite possible that LMBV is transmitted from one reservoir to another in association with biofilms growing on fishing equipment, boats, and trailers. It has been shown that biofilms have great resistance against commonly used disinfectants and thus could protect LMBV harbored within biofilms (McDonnell and Russell, 1999). Future studies can explore whether or not biofilms can protect LMBV in the presence of different disinfectants used singly or in combination. Standard methods to detect viral infectivity is $TCID_{50}$ and plaque assay. Real time PCR can quantify viral DNA copies but it does not imply that the DNA fragments are from infectious virus. So obtaining a numeric value of viral genome by real-time PCR is likely higher than by conventional plaque assay. Getchell et al. (2007) showed that the real-time PCR assay could detect approximately 100 times more LMBV genome copies than by plaque assay. In our study, we found no structural differences between laboratory grown biofilms of *P. fluorescens* in the presence and absence of LMBV. This result may indicate that there is no biological interaction between the biofilm bacteria and the viruses. Biofilm just acted like a trap, a sticky substance which had nothing to do with largemouth bass viruses. Another future scope for research may include the comparative quantitative study of biofilm structure in absence and presence of different concentration of LMBV. Confocal images of biofilms structure can be quantitatively analyzed using a novel computer software COMSTAT based mainly on the pixel quality of the images (Heydorn et al., 2000 and Heydorn et al., 2000). Transmission electron microscopy would also be helpful to study multilayer defense mechanism of biofilms, if any, for protecting LMBV in presence of strong disinfectants. All the natural water and biofilm samples from A. E. Wood Fish Hatchery, where LMBV outbreaks were reported eight years ago were

detected negative for LMBV (Herr and Boston, 2002). This may be due to inactivation of LMBV from those ponds due to long term exposure to sunlight or any other treatment by the hatchery management or the viral load is beyond the detection limit of real-time protocol. The inactivation of this virus due to temperature, pH, etc has not yet been studied in detail although Scott and Aron (2003) reported LMBV is not inactivated at 37˚C for up to 24 hrs. In the current research, we reported that the largemouth bass viruses isolated and recovered from biofilms were still infective after residing within biofilms for a period of 24 hrs. No significant loss of infectivity of recovered LMBV was determined. A following experiment indicates that long term sonication (more than 15 min) showed a decreased infectivity in terms of $TCID_{50}$ value. But surprisingly sonication for 15 min yielded more $TCID_{50}$ than that of stock LMBV. This may suggest that the viruses remain in clump form and thus produce fewer plaques. Long term sonication most possibly destroys the envelop structure of the virus which can explain our result of getting less to no infectivity of largemouth bass virus after 1 h of sonication. An extensive study must be conducted to know the exact nature and transmission pathway of this type of economically important viruses. Our study indicates the importance of considering biofilm as a potential reservoir for pathogenic viruses, and possibly a stronger shield for those viruses.

V. CONCLUSIONS

The results of this study demonstrated that biofilms have the potential to act as a reservoir for LMBV. LMBV attachment to laboratory grown biofilms directly correlated to LMBV concentration in the adjacent bulk environment. Though natural biofilms tested negative for LMBV, they still have the potential to harbor LMBV. Our results showed that real time qPCR is more sensitive than conventional PCR for LMBV detection. Biofilm association had no significant influence on LMBV infectivity. Epifluorescent microscopy revealed that LMBV introduction did not cause any structural changes to *Pseudomonas fluorescens* biofilms. This study demonstrates the importance of examining microbial and ecological niches as potential reservoirs in the control of viral diseases.

REFERENCES

Bates, C. L., Forstner, M. R. J., Barnes, M. B., Whiteley, M., McLean, R. J. C., 2006. Heterotrophic limestone-adherent biofilm isolates from the Edwards Aquifer, Texas. Southwestern Associ. Natural. **51:** 299-309.

Burleson, F. G., Chambers, T. M., Wiedbrauk, D. L., 1992. Virology: a laboratory manual. Academic Press, Inc., page: 54-55.

Caldwell, D. E., Lawrence, J. R., 1986. Growth kinetics of *Pseudomonas fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. **12:** 299-312.

Getchell, R. G., Groocock, G. H., Schumacher, V. L., Grimmett, S. G., Wooster, G. A., Bowser, P. R., 2007. Quantitative polymerase chain reaction assay for largemouth bass virus. J. Aquat. Anim. Health **19:** 226-233.

Goldberg, T. L., Coleman, D. A., Grant, E. C., Inendino, K. R., Philipp, D. P., 2003. Strain variation in an emerging iridovirus of warm-water fishes. J. Virol. **77:** 8812-8818.

Grizzle, J. M., Altinok, I., Fraser, W. A., Francis-Floyd, R., 2002. First isolation of largemouth bass virus. Dis. Aquat. Organ. **50:** 233-235.

Grizzle, J. M., Altinok, I., Noyes, A. D., 2003. PCR method for detection of largemouth bass virus. Dis. Aquat. Organ. **54:** 29-33.

Herr, V., Boston, B., 2002. Largemouth Bass Virus III Session Transcripts, State Updates & Presentation Slides. (online source:)

Heydorn, A., Ersboll, B. K., Hentzer, M., Parsek, M. R., Givskov, M., Molin, S., 2000. Experimental reproducibility in flow-chamber biofilms. Microbiology **146:** 2409-2415.

Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B. K., Molin, S., 2000. Quantification of biofilms structures by the novel computer program COMSTAT. Microbiology **146:** 2395-2407.

Kalmokoff, M., Lanthier, P., Tremblay, T., Foss, M., Lau, P. C., Sanders, G., Austin, J., Kelly, J., Szymanski,C. M., 2006. Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. J. Bacteriol. **188:** 4312-4320.

King, R. K., 2001. The Presence of bacterial pathogens in recirculating aquaculture system biofilms and their response to different sanitizers. PhD Dissertation. Virginia Polytechnique Institute and State University.

Lacroix-Gueu, P., Briandet, R., Leveque-Fort, S., Bellon-Fontaine, M., Fortaine-Aupart, M., 2005. In situ measurements of viral particles diffusion inside mucoid biofilms. C. R. Biologies **328:** 1065-1072.

Langmark, J., Storey, M. V., Ashbolt, N. J., Stenstrom, T. A., 2005. Accumulation and fate of microorganisms and microspheres in biofilms formed in a pilot-scale water distribution system. Appl. Environ. Microbiol. **71:** 706-712.

Lehtola, M. J., Miettinen, I. T., Keinanen, M. M., Kekki, T. K., Laine, O., Hirvonen, A., Vartiainen, T., Martikainen, P. J., 2004. Microbiology, chemistry and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. Water Research **38:** 3769-3779.

Marrie, T. J., Nelligan, J., Costerton, J. W., 1982. A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. Circulation **66:** 1339-1341.

Marsh, P. D., 2004. Dental plaque as a microbial biofilm. Caries Res. **38:** 204-211.

McClenahan, S. D., Beck, B. H., Grizzle, J. M., 2005. Evaluation of cell culture methods for detection of largemouth bass virus. J. Aquat. Anim. Health **17:** 365-372.

McClenahan, S. D., Grizzle, J. M., Schneider, J. E., 2005. Evaluation of unpurified cell culture supernatant as template for the polymerase chain reaction (PCR) with largemouth bass virus. J. Aquat. Anim. Health **17:** 191-196.

McDonnell, G., Russell, A. D., 1999. Antiseptics and disinfectants: action, and resistance. Clin. Microbiol. Rev. **12:** 147-179.

Morris, C. E., Monier, J. M., 2003. The ecological significance of biofilm formation by plant-associated bacteria. Annu. Rev. Phytopathol. **41:** 429-453.

Oosthuizen, M. C., Steyn, B., Theron, J., Cosette, P., Lindsay, D., Holy, A., Brozel, V., 2002. Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. Appl. Environ. Microbiol. **68:** 2770-2780.

Plumb, J. A., Grizzle, J. M., Young, H. E., Noyes, A. D., 1996. An iridovirus isolated from wild largemouth bass. J. Aquat. Anim. Health **8:** 265-270.

Probert, H. M., Gibson, G. R., 2002. Bacterial biofilms in the human gastrointestinal tract. Curr. Iss. Intest. Microbiol. **3:** 23-27.

Quignon, F., Kiene, L., Levi, Y., Sardin, M., Schwartzbrod, L., 1997. Virus behavior within a distribution system. Wat. Sci. Tech. **35:** 311-318.

Quignon, F., Sardin, M., Kiene, L., Schwartzbrod, L., 1997. Poliovirus-1 inactivation and interaction with biofilm: a pilot –scale study. Appl. Environ. Microbiol. **63:** 978-982.

Ramey, B., Koutsoudis, M., Bodman, S. B. von, Fuqua, C., 2004. Biofilm formation in plant-microbe associations. Curr. Opin. Microbiol. **7:** 602-609.

Scott, B., 2003. Effect of disinfectants and antivirals on the infectivity and replication of largemouth bass virus. MS Thesis, Texas State University-San Marcos,TX.

Skraber, S., Helmi, K., Willame, R., Ferreol, M., Gantzer, C., Hoffmann, L., Cauchie, H. M., 2007. Occurrence and persistence of bacterial and viral faecal indicators in wastewater biofilms. Wat. Sc. Technol. **55:** 377-385.

Skraber, S., Schijven, J., Gantzer, C., Husman, A. M. de R., 2005. Pathogenic viruses in drinkning-water biofilms: a public health risk? Biofilms **2:** 1-13.

Storey, M. V., Ashbolt, N. J., 2003. Enteric virions and microbial biofilms-a secondary source of public health concern? Wat. Sci. Tech. **48:** 97-104.

Storey, M. V., Ashbolt, N. J., 2001. Persistence of two model enteric viruses (B40-8 and MS-2 bacteriophages) in water distribution pipe biofilms. Wat. Sci. Tech. **43:** 133-138.

Trautner, B. W., Darouiche, R. O., 2004. Role of biofilm in catheter-associated urinary tract infection. Am. J. Infect. Control **32:** 177-183.

SUPPLEMENTAL DATA

Table A. Melting curve analysis for Viral infectivity test. All the samples below showed some fluorescence in qPCR amplification plot (fig not shown) but the melting curve reveals those non-specific amplification as false positive results. Here, zero value for 'No.Tm SYBR' indicates false positive results which were further tested running a 4% agarose gel (see fig. 14).

Melting Curve SYBR

Pos	Name	No. Tm SYBR	Tm x (°C) SYBR	Tm y (°C) SYBR	Mean SYBR	Dev. SYBR
A3	1	1	86.2		86.3	0.1
A4	1	1	86.4		86.3	0.1
A5	1	1	86.4		86.3	0.1
A6	2	1	86.3		86.4	0.1
A7	2	1	86.3		86.4	0.1
A8	2	1	86.5		86.4	0.1
A9	3	0				
A10	3	0				
B3	3	0				
B4	4	1	86.0		86.0	0.0
B5	4	1	86.0		86.0	0.0
B6	4	1	85.9		86.0	0.0
B7	5	1	86.0		86.2	0.1
B8	5	1	86.3		86.2	0.1
B9	5	1	86.2		86.2	0.1
B10	6	1	86.0		85.9	0.2
C3	6	1	85.6		85.9	0.2
C4	6	1	85.9		85.9	0.2
C5	7	0				
C6	7	0				
C7	7	0				
C8	8	1	86.0		85.9	0.1
C9	8	1	86.0		85.9	0.1
C10	8	1	85.7		85.9	0.1
D3	9	1	85.6		85.7	0.1
D4	9	1	85.7		85.7	0.1
D5	9	1	85.9		85.7	0.1

Cont..

Pos	Name	No. Tm SYBR	Tm x (°C) SYBR	Tm y (°C) SYBR	Mean SYBR	Dev. SYBR
D6	10	1	85.6		85.7	0.0
D7	10	1	85.7		85.7	0.0
D8	10	1	85.7		85.7	0.0
D9	11	0				
D10	11	0				
E3	11	0				
E4	12	1	85.7		85.7	0.1
E5	12	1	85.7		85.7	0.1
E6	12	1	85.7		85.7	0.1
E7	13	1	85.7		85.7	0.1
E8	13	1	85.8		85.7	0.1
E9	13	1	85.7		85.7	0.1
E10	14	1	85.6		85.6	0.1
F3	14	1	85.6		85.6	0.1
F4	14	1	85.7		85.6	0.1
F5	15	1	85.8		85.7	0.1
F6	15	1	85.7		85.7	0.1
F7	15	1	85.6		85.7	0.1
F8	16	1	85.9		85.8	0.2
F9	16	1	85.9		85.8	0.2
F10	16	1	85.6		85.8	0.2
G3	17	0				
G4	17	0				
G5	17	0				
G6	NO SYBR	0				
G7	NO TEMPLATE	0				
G8	ONLY WATER	0				
G9	BLANK	0				
G10	DEPC WATER	0				

VITA

Shubhankar Nath was born in Berhampore, West Bengal, India, on 6th October, 1984 to Dipak Kumar Nath and Shikha Nath. After graduating from high school, he entered the West Bengal University of Animal and Fishery Sciences (India) majoring in Veterinary Medicine and Animal Husbandry. As a veterinarian, he travelled different remote villages, safari parks and zoos throughout India during several internship programs. Following graduation in the year of 2007, he moved to the US to pursue his M.S. in Biology at Texas State University-San Marcos under the direction of Dr. Robert (Bob) McLean. As a graduate student, he worked as an instructional assistant for Microbiology course and also as a summer research assistant. Shubhankar has been accepted into the PhD program in Microbiology at the University of Texas at Austin where he will be exploring microbial pathogenesis of bacteria.

Permanent Address:

Vill: Mathpara, Satigachha
PO: Anulia, Ranaghat
Dist: Nadia, West Bengal
India

eMail: snath.vet2000@gmail.com

This thesis was typed by Shubhankar Nath.