ASSESSMENT OF A VIRUS SAMPLING AND DETECTION METHOD FOR WATER TESTING USING *Pepino mosaic virus*

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Bachelor of Science

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2013

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2016

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DETECTION METHOD FOR WATER TESTING USING

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Date of Degree: DECEMBER, 2016

Title of Study: WATERBORNE PLANT VIRUSES SAMPLING AND DETECTION IN AQUEOUS ENVIRONMENT

Major Field: PLANT PATHOLOGY

Abstract: The aim of this research is to study water sampling as a critical step for detection of unwanted waterborne plant pathogenic viruses that can seriously damage agriculture and the environment. Water sources, such as reservoirs, lakes, farm ponds, tanks, and irrigation systems are vulnerable to inadvertent (most frequent) and intentional (rare), but equally relevant, contamination by microbial pathogens. Pathogen detection in large bodies of water is complex because volume, dilution, and water dynamics. This project assesses the development and adaption of a method for water sampling and detection of waterborne plant viruses of concern for water-security. Rapid detection and monitoring of high consequence plant pathogens in water systems present a significant challenge where viruses occur at low titers. This project seeks statistical validation of a water sampling model that uses q-PCR as the method to detected *Potexvirus,* a group of plant viruses reported to have water borne species such as *Pepino mosaic virus* selected as model virus for this study. To determine the minimum amount of virus detectable in water, sensitivity tests were conducted using ELISA and qPCR. A range of pH were tested during virus filtration using self-made glass-wool filters. Virus capturing based on the isoelectric point affinity between the virus capsid proteins with glass wool was hypothesized. This research supports water-biosecurity decision-making, and will be useful for survey analysis, prevention, detection, and mitigation of unwanted waterborne plant viruses.

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CHAPTER I

INTRODUCTION AND OBJECTIVES

Introduction

Waterborne plant virus epidemiology and ecology is not well understood due in part to the following: 1-) Lack of detection methods, difficulties on establishing standards to assess the concentration of viruses in water; 2-) Effects caused by the dynamic of water, environmental conditions and physical-chemical properties of water such as pH. It is known that viruses from more than seven genera of plant viruses are reported to be found in aqueous environment (20). Hydroponics system and irrigation are two important pathway and reservoirs for rapid spreading of water-transmitted pathogens such as fungus spores, oomycetes, bacteria and viruses. Greenhouse production of tomatoes (*Solanum lycopersicum* L.) is an important cultivated plant and an example of intensive irrigation where *Pepino mosaic virus* (PepMV) occurs. *Pepino mosaic virus* is a virus in the genus *Potexvirus* from the family *Alphaflexiviridae* (37). Plants can get infected from PepMV from mechanical transmission, and cause economic losses to the tomato industry. The symptoms induced by PepMV in tomatoes plants are systemic mosaic, vein clearing and yellowing (38). Recent studies using PepMV and tomatoes growing in hydroponic systems showed PepMV is released from roots to water and remain infectious in water after 3 weeks at water maintained at 20±°C (21) Molecular techniques are the most sensitive method for detecting viruses in water. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and virus concentration

procedures are reported techniques available for detection of viruses in water (21). The need for precise detection of waterborne plant viruses has reinforced the relevance of developing sensitive molecular tools and sampling procedures. There is a need for developing an economic and easy to use detection method for capturing and monitoring viruses in irrigation water and hydroponic systems, because, the existing tools are either too expensive or too complex (32). Glass wool filter has been used worldwide to concentrate virus that are known to persist in water, and other pathogens that can be found in water. The construction of a filter using glass wool as the main capturing material is economic. This type of filters are easy to transport and handle in the field a submergible pump, flowmeters and hoses are needed. Large filters allows filtration of volumes of water, up to 1000 liters (32). Capturing viruses in glass wool filters is reported to be pH dependent and the efficiency of the capturing might vary upon to the targeted virus. In this case it is important to know the isoelectric point (IEP or pI) of the specific virus desired to be captured. Isoelectric point is the pH that the virion has no electrical charge can also be an indicator for sorption process (22).

Hypotheses

There are three main hypotheses in this project.

H1: The pH of water may influence the capture of plant virus such as PepMV.

H2: The isoelectric point of viruses can be used as reference for PepMV capture.

H3: The higher the volume of sampled water is more effective for virus capturing and detection.

Objectives

The main purpose of this research is to adapt and develop a water filtration system able to capture waterborne plant viruses in agricultural settings to improve detection of this plants pathogens that cause diseases in crops.

The **first objective** of this project involve the development of a waterborne filtration system requiring low investment, also easy to transport out to field locations and of reliable use.

The **second objective** consist on developing a waterborne virus filtration system to test in a 5 liter water system to find the best pH of water pH, glass wool pH and elution pH. Three pH ranges were tested in this objective: pH 3-4, pH 6-7 and pH 9-10.

The **third objective** aims to confirm that the larger the volume filtered water is more reliable during virus capturing.

CHAPTER II

Review of Literature

I – Global role of water and agricultural use.

Shortages of fresh water worldwide impact dramatically the economy and politics of any country. Also, regions where access to fresh water scarce are at risk of riots, and unrests because populations cannot tolerate to struggle for water which is needed for basic daily tasks (10). Although being considered one of the most important resource for human's life it is also constantly threatened by human's activities (35).

Water bioterrorism is currently an important threat for agriculture because intentional introductions of unwanted pathogens are known to cause economic impact. (28). Likewise, unintentional introduction of pathogens and pests to a new country or geographic region is also considered a threat because this type of introduction may occur at a high rate due to high volumes of international trade and globalization. Moreover, new incursions of pests triggers costly responses and sometimes eradication is simply not possible to achieve (28). Biosecurity policies are created to regulate trading and track and respond to both intentional and unintentional introductions to a country seeking

prevention of unwanted organisms to spread. This way biosecurity regulations aim to prevent economics problems and trading sanctions (28).

As the world population is constantly growing and a high demand for food and water is predicted, an increase in agricultural products is also needed to fulfill nutritional requirements of the world population (34). It is estimated that by 2050 the world population will reach 10 to 14 billion (13). In order to keep high standards for good agricultural practices it is important to have sufficient water availability. Agriculture worldwide relies on water resources to obtain high yields. In the United States of America the use of water for agricultural purposes constitute approximately 80% of the total use of the water in the nation (33). Additionally, most of the important crops cultivated worldwide for human consumption, nutrition and/or animal feed are irrigated. Therefore, improper water management can put at risk irrigation of crops and also sustainable agriculture (13).

II – Waterborne viruses

Water can be naturally accidentally or intentionally polluted with viruses which can be transported in water as suspended as colloids. One of the major difficulties identifying waterborne viruses is sampling. Water is a very dynamic natural resource which is prone to seasonal changes. Moreover, the diversity of aquatic environments pose numerous challenges and create a disadvantage in selecting sampling method (32).

Plant viruses are frequently found infecting their host plants or detected within their insect vectors, and also in natural environments or agricultural water sources. Plant virus detection has been reported in aqueous environments where a number of virus species were reported to persist. However, the scientific literature does not describe a high confidence sampling method universally used for efficient detection of plant waterborne viruses. The development and assessment of such method is also needed for virus discovery (21).

Viruses' species from at least seven genera of plant viruses are reported found in aqueous environment, and are commonly called plant waterborne viruses. These plant virus genera are: *Carmoviruses*, *Cucumoviruses*, *Necroviruses*, *Potexviruses*, *Tobamoviruses* and *Tombusviruses* (20). Most of these viruses maintain their infectivity after recovering from environmental water samples. Natural infection of plants may occurs through the roots without an intermediated vector and a broad range of hosts might be also infected. Plants infected with waterborne viruses can subsequently release virions into lakes, ponds, streams and rivers causing an increasing and faster dissemination of a particular type of virus worsen rapidly compared to other natural conditions (21).

Therefore, plant waterborne viruses can easily spread and re-circulate within agricultural fields and are of concerns regarding surface irrigation system, and hydroponic systems. Several viral diseases that affect hydroponic production are reported (21). Among plant diseases found in hydroponic systems the plant virus *Pepino mosaic virus* (PepMV) represent an example of a disease causing virus for different cultivars of tomatoes. Not to mention, viruses in hydroponic system may also spread through leaf and/or root contact between adjacent plants, and also by aerial vectors, tools, clothes and hands contaminated during crop handling (21).

Waterborne viruses represent a problem in agricultural settings and has been also a significant problem for public health. An example of waterborne viruses playing an important role in plant health that illustrate the challenges of virus recovery from water are the enteric viruses (3) Enteric viruses are reported to represent a serious problem to drinking water and are of concern for public health (29).

III – *Pepino mosaic virus (*PepMV) as a waterborne virus model

Pepino mosaic virus (PepMV) is a virus in the genera *Potexvirus*. PepMV is mechanically transmitted it does not require the help of a vector. This virus had caused significant problems to the tomato industry in tomatoes cultivated in greenhouses (23).

The first report of PepMV causing disease in tomato was from the Netherlands and the United Kingdom in 1999, and after that the virus rapidly spread across Europe (12).

There are non-conclusive studies regard insect transmission.Bumble bees, *Olpidium virulentus* (soil-borne fungus) and white flies are reported to be associated or functioning as vectors for PepMV. *Potexviruses* are generally considered to lack vectors but are highly mechanical transmitted and can be easily transmitted to healthy plants during cultivation (1).

Water and seed transmission are also two alternative pathways through which long distance spreading of PepMV is confirmed (5). PepMV is reported to occur in different countries (Austria, Bulgaria, Croatia, Cyprus, Czech Republic, Greece, Italy, Finland, France, Germany, Hungary, Netherlands, Norway, Poland, Slovakia, Spain, Sweden, Switzerland, Turkey, United Kingdom, South Africa, Syria, United States, Chile, Equator and Peru) (36).Five different strains are currently reported: Peruvian (PE) strain, EU-tomato (EU-tom) strain, US1/Ch1 strain, Chile-2 (Ch2) strain, and PES strain (5).

Common *Potexvirus* symptoms in plants are mosaic which can differ in various degrees of stunting and also reduced yields (1). PepMV causes a variety of symptoms in tomatoes and in several other solanaceous crops. It also induce symptomsin indicator plants like *Datura stramonium, Nicotiana benthamiana, Physalis floridana, S. melongena* (eggplant) and *S. tuberosum* (potato). The symptoms vary from a yellow mosaic in young leaves of pepino (*Solanum muricatum*) to mild mosaic or symptomless in wild potatoes (16). In tomatoes the symptoms varies depending on the variety which can display mosaic, mottling, bubbling,

distortion, and spiky or nettle-like heads. Besides the leaves, the fruits can also be infected showing discolored yellow spots, stripes or marbling (9).

IV – Plant Virus detection

Size and morphology of plant viruses are different and are challenging to work with regarding other pathogens the virus chemical constitution, pathogenesis, dissemination and symptomatology among hosts. Moreover viruses are not visible by naked eyes and therefore cannot be seen and visually detected using the methods traditionally used when working with other pathogens (1).

The use of electron microscopy can be helpful for rapid detection of virus presence and characterization of the virus morphology to include size and shape. Virus purification and serological methods combined with electron microscopy are considered a definitive proof for virus presence in an infected plant host. Polymerase chain reaction (PCR) has been also used as the most sensitive method for virus detection and identification for the past 10 years (1).

Precise detection and diagnostics methods to detect pathogens introduced to new locations are to be preferable based on biochemical and molecular biology to be widely used in biosecurity and microbial forensics. These disciplines require the samples to follow widely accepted standard operational procedures (SOP) in order to obtain a reliable analysis and results. PCR is a consistently and well-known validated method used to assess either presence or absence of a targeted microorganism. This method relies in the amplification of a specific sequence of the questioned pathogen and targets a specific and unique DNA segments that leads to a more specific assays and discriminatory result (28).

Plant virus once present in their host cannot be removed through chemical methods. The best strategy for plants integrated management is prevention of new infections and development of resistant cultivars. Seeds and seedling monitoring using sensitive detections methods such as

quantitative PCR can reduce the risk of spreading viruses in agriculture, and hydroponic systems. Alternative methods recommended for minimizing the risk of virus infection include monitoring irrigation water sources and recycled nutrient solutions. Hygiene measures e.i. disinfection of tools and workers clothing are also recommended (21).

Detection and assessment of waterborne pathogens is done using methodologies previously reported for contaminated water. However, these methods are either too specific for a group of microorganism or high costly (32).

Many studies to better elucidate the persistence of water borne virus were performed using enteric viruses, such as Adenovirus (HAdV). HAdV is known to be a very stable virus in water in different environmental stress conditions, such as temperature, radiation, pH variation and degree of chlorine action (6).

V – Virus adsorption and desorption from charged surfaces

The variables that influence virus transport in water and water-subsurface are poorly understood (8). It is known that there are three possible ways a virus can be attracted to a surface which are the electrostatic attraction and repulsion, van der Waals forces and hydrophobic effects (11).

The attraction or adsorption of a virus to a specific surface can happen to different kind of materials. The use of electropositive membranes and cartridges is reported, as well as electronegative membranes. Also, gauze pads, glass powder and microporous materials are used in viruses' adsorption processing (19).

Viruses' particles are negative charged when found in natural environments and it is known to have the ability of being adsorbed by different matrices using hydrophobic and electrostatic interactions (17).

Positive charged filters are the most common used for virus adsorption because it is simple and also a trustable mean to capture and detect virus from aqueous environment (15). Electronegative filters requires an adjustment to a very low water pH in order to favor the adsorption of viruses and bacteriophages, however, the acidification of water and also the addition of multivalent cations can decrease the adsorption efficiency (15).

It is required by the U.S. Environment Protection Agency the use of filters 1MDS for viruses concentration purposes, but the cost for this kind of material are relatively high for routine analysis (17). 1MDS filters are electropositively charged that can be made from different materials but always designed to capture the viruses through difference of charges (25).

In aqueous environment the attraction of the virus suspension to a positive charged fiber packet material works better at a neutral pH of 7 and also that has a low efficiency in water with pH higher than 8. When using glass wool as the filter surface it is known that if the environment pH is higher than 7.5 there is a need to adjust the pH to a more neutral pH of 7 (19).

Waterborne viruses has a large range of isoelectric point among all of them and also can have different relations and behavior for adsorption and desorption from different surfaces. For this reason is important to assess the virus types individually with different types of water (19).

There are at least five different mechanisms to explain virus binding as it shows in the figure 1. The figure 1 shows noncovalent interactions that can be all related. One of the interactions presented in the figure 1 is related to the pH. The cation exchange, cation bridge and double layer comprehension are based on the same kind of interaction (26).

The efficiency of the used filters to adsorb viruses in acidic pH can happen when viruses are in contact with cations and salt solutions (31). This can explain two interactions represented in the following figure which are the cation exchange and cation bridge, respectively.

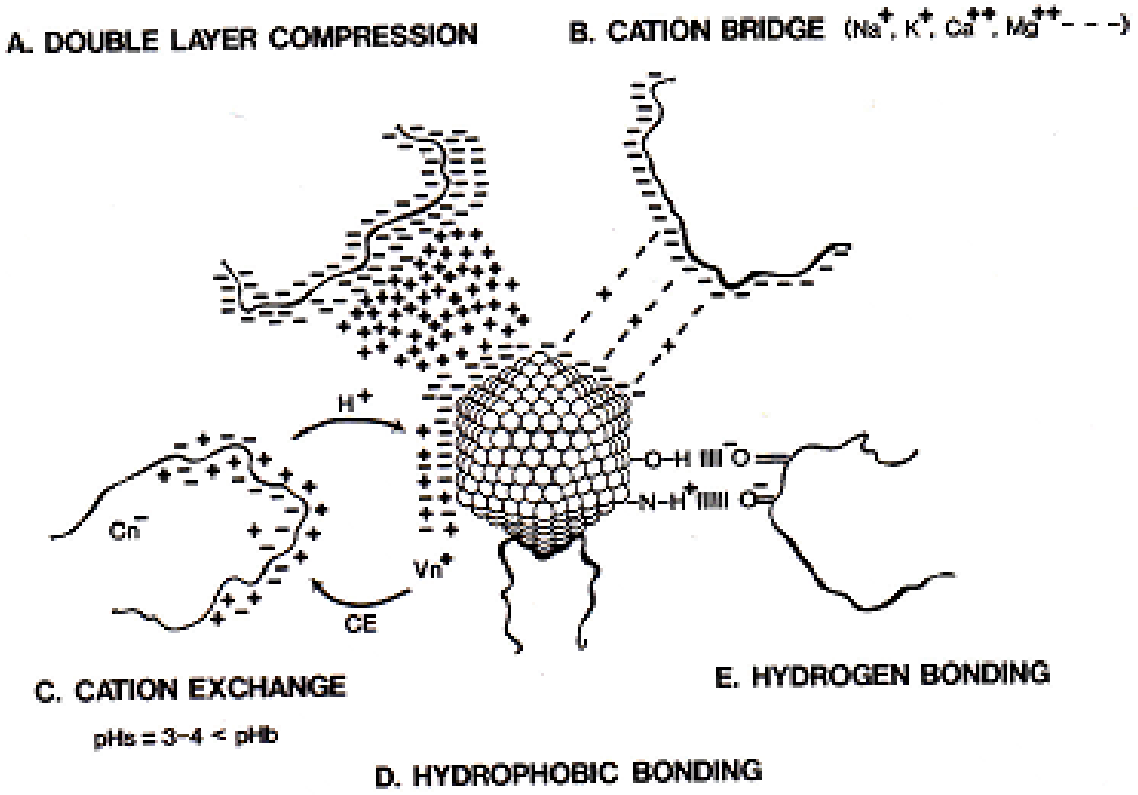


Fig. 1. Possible mechanisms of virus binding by Rao & Melnick, 1987.

All studied possible virus binding mechanisms consider the virus exists in solid environments. There are a number of variables that can change the stability of the viruses as they change or vary, which makes these mechanisms to be poorly understood (26). After viruses are attracted to surfaces from environmental samples procedures to elute and concentrate viruses from trapping surfaces are used.

The process of desorption of viruses from solid surfaces is usually done adding a high protein solution combined to an amino acids which will decrease the bonding attraction of viruses to the surface (25).The most common procedure used for virus' flocculation uses beef extract (protein) and glycine (amino acid). Beef extract, however, may have sorbents that can compete with viruses for a flocculation surface (27). The concentration of the beef extract solution might varies from 3% to 15% depending on the water source from where the sample is collected from

(4). The pH of the beef extract solution also plays a role depending on the sample. Using tap water samples, it was found having a higher recovery of viruses using beef extract at low pH (18).

PEG (polyethylene glycol) is also another method for viruses concentration from aqueous environmental samples, but requires the use of centrifugation or ultracentrifugation (14). PEG (polyethylene glycol) is a polymer that is soluble in water and is able to precipitated proteins. PEG (polyethylene glycol) has as an advantage to have low interference on viruses characteristics (7).

Viruses capsid proteins have putative isoelectric points (pI). The pI determines that at a particular pH the net charge of the virus as colloid is equal to zero. Likewise all other bio colloids also have a dependence of the pH when found in a polar media (water) (22). It has been suggested that isoelectric points may be a physical-chemical property to be considered for virus adsorption from environmental samples (8).

Guan, Schulze-Makuch, Schaffer, & Pillai, 2003 suggested that there is a critical pH, usually 0.5 below the isoelectric point of the virus, in which the virus is charged with the opposite charge of a porous medium. Michen & Graule, 2009 state that the isoelectric point is a virus property to take into consideration when studying viruses sorption to different surfaces, however it is important to notice that within the same virus specie the isoelectric point can be widely different and from where the importance of studying each virus.

CHAPTER III

EXPERIMENTAL DESIGN AND METHODOLOGY

Growth and maintenance of tomatoes plants

Tomato plants (*S. lycopersicum* L.) variety Oregon Spring were grown in the lab in a growth chamber with temperature of 22°C, RH (Relative humidity) 50% and 12 hours of light.

The seedlings were grown in small soil pellets and transferred after two weeks to pots with a soil mix (Miracle Gro potting mix®).

The tomato plants were grown both, use as a source of virus, and also not infected plants were used as a preference health control.

Source of infected plants and storage of plant tissue

Two milliliters of 1M buffer PBS (Phosphate-Buffered Saline) neutral pH was added in a bottle containing the sap of *Pepino Mosaic Virus*. In a mortar the sap was mixed with carborundum, a mild abrasive that will open small wounds in the leaves from where the virus gains entry to infect the plant tissue. The sap with carborundum was applied to the leaves using a pestle. Three weeks after inoculation plants exhibited symptoms. After harvesting, both healthy and infected plants were cut and stored in a -80°C freezer.

Common procedures:

Pepino mosaic virus was maintained on tomatoes plants infected with PepMV reference positive control from AGDIA. Symptomatic plants (Figure 2) were tested for the presence of virus by lateral flow immunoassay (Figure 2), ELISA (Figure 3) and RT-PCR (Figure 4).

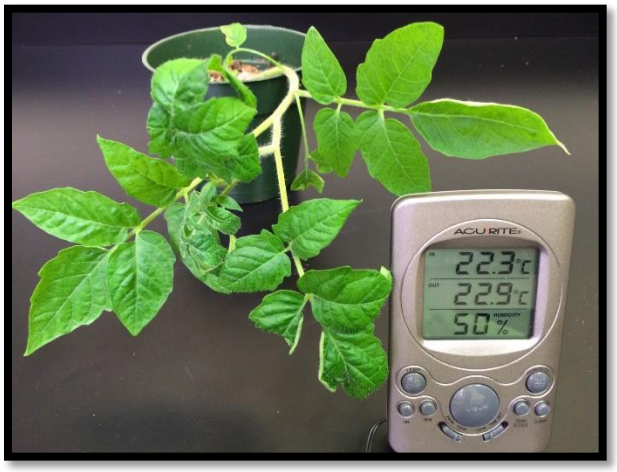


Fig. 2. Tomatoes plants with *Pepino mosaic virus* symptoms.

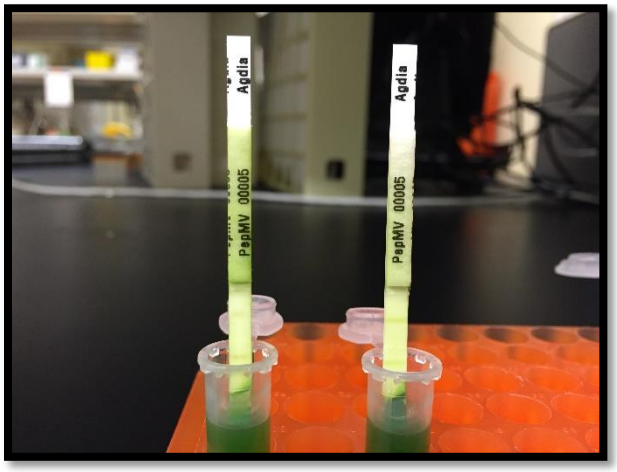


Fig. 3. Immunostrip assay with tomatoes plants infected with *Pepino mosaic virus*

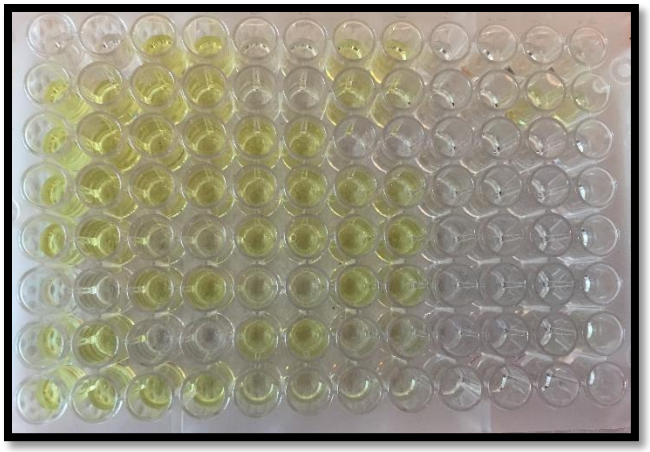


Fig. 4. DAS-ELISA plate with tomatoes plants infected with *Pepino mosaic virus.*

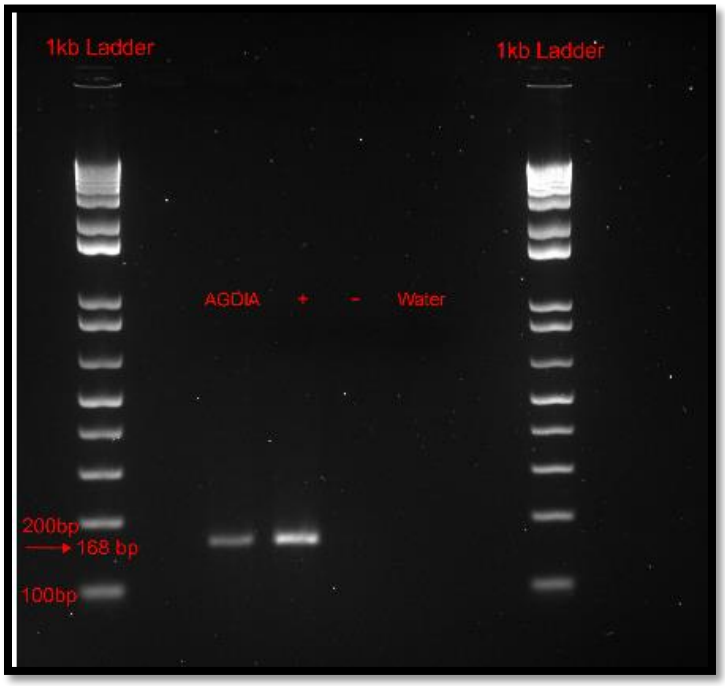


Fig. 5. RT-PCR with tomatoes plants infected with *Pepino mosaic virus.*

Lateral flow immunoassay

This is a rapid assay that uses strips to test of the presence of a specific virus. This assay was used as a first tool to test of the presence or absence in inoculated plants. Before doing the

inoculation of the plants with *Pepino Mosaic Virus* random leaves from plant were collected and chopped in order to run an immunostrip test using the kit from AGDIA. As the results showed negative for *Pepino Mosaic Virus* I proceed with the inoculation.

ELISA DAS (Double Antigen Sandwich)

Elisa is a serological test that consist of detecting a target protein using antibodies and/or antigens. ELISA is a rapid quantification method for a large number of samples in research. The ELISA used in this experiment is known as the double antibody sandwich ELISA because of the method to create a double layer with the antibody and the antigen where the target is recognized.

ELISA experiments were conducted to test the healthy plants to be used as a negative control. Also to test the inoculated plants to be used as a positive control and also as a source of virus as well to compare the virus concentration of two different sources of virus: The positive control bottle from AGDIA for *Pepino mosaic virus* and the plants that has the virus inoculated under laboratory condition.

Buffers used: GBE 1X (General buffer extraction), PBST (Phosphate buffered saline tween-20) 1X, ECI 1X, PNP 1X, Carbonate coating 1X, PBS (Phosphate buffered saline) pH 7.

Glass wool preparation

Virus assembly and disassembly are known to being a pH dependent. Considering this we have used different pH filters for each experiment. In order to adjust the glass wool pH we followed the following procedure for each required pH:

6-7 pH: A piece of glass wool was placed in a container and saturated with RO (reverse osmosis) water. It was soaked for 15 minutes. After this period of time the RO water was drained and the glass wool piece was saturated with 1M HCl pH 3 buffer and soaked for 15 minutes. The HCl was drained and the glass wool was rinsed until a neutral pH was achieved. Then the glass

wool was covered again with PBS (Phosphate-buffered saline) buffer at an acid pH and soaked for 15 minutes again. The buffer was drained and the glass wool piece was washed with RO water until a 6-7 pH was achieved.

3-4 pH: A piece of glass wool was placed in a container and saturated with RO water. It was soaked for 15 minutes. After this period of time the RO water was drained and the glass wool piece was saturated with 1M HCl pH 3 buffer and soaked for 15 minutes. The HCl was drained and the glass wool pH was checked making sure the right pH (3-4) was achieved.

9-10 pH: A piece of glass wool was placed in a container and saturated with RO water. It was soaked for 15 minutes. After this period of time the RO water was drained and the glass wool piece was saturated with PBS (Phosphate-buffered saline) 1M Ph 9.5 and soaked for 15 minutes again. The buffer was drained and the glass wool pH was checked making sure the right pH (9-10) was achieved.

Virus recovery:

Elution

Every time after the filtration process using the system developed in the experiment I (water filtration system) the glass wool filter went through an elution process which consists on wash the virus captures that was supposed to be captured and trapped in the glass wool. The elution process was done using a specific buffer for each situation. Therefore the volume of the buffers used in the elution step was always 100 mL of the specific buffer. The glass wool was taken inside of an autoclaved bicker and the buffer was added to it and let it soaked for 15 minutes. After that, using the hands to smash the glass wool and get the most of liquid out of it. After this step the liquid was transferred to containers and stored at -80°C. In order to prevent pieces of glass wool to be stored together with the liquid transferred to the final container, the

water passed through a cheese clog retaining the small pieces that possibly could be in the liquid. The samples were stored r in a -20°C and processed the following day.

PEG (Polyethylene glycol) addition and centrifugation

This step consists in recover a pellet from the water sample stored at -20°C. The water sample was thawed and poured into an Erlenmeyer with 7% PEG and was left stirring for two hours. The volume of the sample was measured using 25 ml pipettes and after that the amount of PEG was calculated. After stirring, the tubes were centrifuged for 30 minutes at 10,000 rpm at 4°C.

The supernatant was removed, and the tubes were left upside down on a paper towel in order to allow the pellets to dry. One tube was selected then washed with 450 ul of RLT buffer in order to dislodge pellet from the tube walls. The resulting solution was removed and placed into the next tube and washed in the same way. This process was repeated until the last tube was washed and the final solution containing all of the pellets was placed in a new tube to be used in the RNA extraction.

RNA Extraction

RNA was extracted from three sources: 1-water samples; 2-AGDIA positive control and 3-infected plant tissue with *Pepino mosaic virus.* A different procedure was used to extract the RNA from the different sources.

- **RNA extraction from water samples**

Water samples were centrifuged for 30 minutes at 10,000 rpm at 4°C. After centrifugation the tubes were washed with a RLT buffer and the final volume is transferred to an ependorf tube. Depending on the volume is added to the RLT mixture a certain amount of ß-mercaptoethanol. For each 100 ul of RLT 10 ul of ß-mercaptoethanol. From this step the protocol

followed was from the Plant Extraction RNeasy Mini Kit from QIAGEM or tryzol as per the manufactures protocol.

- **RNA extraction from AGDIA positive control**

RNA was extracted from lyophilized positive control for *Pepino mosaic virus* by adding 500 ul of RLT buffer to a bottle containing virus, and gently mixing. Then, 10ul of ß-mercaptoethanol was added, then a protocol was followed as directed by a kit "Plant Extraction RNeasy Mini Kit from QIAGEN".

- **RNA extraction from plant tissue**

To one milligram of thawed tissue from the tomatoes leaves infected with *Pepino mosaic virus* or from health tomatoes tissue liquid nitrogen was added, and then a protocol was followed as directed by a kit "Plant Extraction RNeasy Mini Kit from QIAGEN".

c-DNA

Pepino Mosaic Virus is a virus from the genera *Potexviruses* consisting of a coat protein and a single strand RNA as a part of its genetic information. However, the molecular tool used to detect waterborne viruses in this project requires DNA for amplification (PCR – Polymerase Chain Reaction). c-DNA is the process to reverse the RNA strand into a DNA single strand using specific enzymes and primers in a determined temperature. In this case a two-step c-DNA was conducted. In the first step was added dNTPs 10Mm, random primers, water, and the respective RNA. Then the mix was incubated at 70°C for five minutes, and then cooled on ice for 1 minute. At this point the RNA strand is relaxed and opens in order to be more susceptible for primers to bind forming the DNA structure. The second step consists of adding of a buffer 5X, and a transcriptase enzyme to transform the RNA into a DNA strand. Then, the mix containing the

enzymes was incubate for 90 minutes at 37°C to complete the denaturation of the RNA. The concentration of c-DNA was measured in the NANODROP.

RT-PCR for *Pepino Mosaic Virus*

In this study, a modified RT-PCR protocol was used for a previous designed primer set of *Pepino mosaic virus.*

The protocol used is presented in the following tables:

Reagents	Volume of reaction(ul)
Nuclease Free Water	0.6
GoTaq 2X MM	10
Magnesium Chloride 50 Mm	0.8
Potex F 5uM	2.8
Potex R 5uM	2.8
cDNA	3
Total Volume	20

Table 1. RT-PCR protocol for *Pepino mosaic virus*

97°	2 minutes
95° 20 seconds 47° 30 seconds 72° 30 seconds	**35 times**
73°	3 minutes

Table 2. RT-PCR cycle for *Pepino mosaic virus*

q-PCR for *Pepino Mosaic Virus*

Another tool used in the experiments for both identification and quantification of virus (*Pepino mosaic virus)* was the real time, also known as quantitative PCR (qPCR).

Using the same set of primers for the RT-PCR the protocol followed for this project is presented in tables 3 and 4.

Reagents	Volume of reaction(ul)
Nuclease Free Water	2
LC Green	2
Potex F 10uM	2
Potex R 10uM	2
HotStart MM	10
cDNA	2
Total Volume	20

Table 3. q-PCR protocol for *Pepino mosaic virus*

97°	2 minutes
95° 20 seconds 47° 30 seconds 72° 30 seconds	**35 times**
73°	3 minutes

Table 4. q-PCR cycle for *Pepino mosaic virus*

Gel electrophoresis

Precise identification of the virus was accomplished by gel electrophoresis after the completion of the RT-PCR. The preparation for this step was proceed adding 3 % Agarose gel and heat in the microwave with TAE buffer. 10ul ul of syber safe was added in the agarose gel after the heating before pouring in the gel trail. The gel was run for 90 minutes at 90 volts, and read using the Lab Image program.

Assessment of the filtration system

Filtration Description

This filtration system was built with focus on having an inexpensive and portable tool that easily can be taken to field locations. A filtration system protocol described by (32) was adapted using glass wool cartridge to work in a field situation.

PVC pipes, caps and tap threads were used to assemble the parts where the glass wool filter will be inserted. Prevention of leakage from the system was accomplished by the use of a nylon tape on the threaded caps. The connectors and the filter cartridge are connected to a hose that is plugged into a water pump that goes inside of the water source that pull the water towards the system.

Preliminary Experiments

As mentioned in chapter II, virus adsorption are known to being a pH dependent which means that virus capture is reliant on filter pH, water pH and elution step pH. For this reason in this experiment we test a specific pH for the glass wool, water pH and elution pH which was chose to be near neutral (6-7) for the filter and water and basic for the elution (9-10) based on the protocol standardized from (32).

The filtration system in field situations was tested by sampling water from Theta Pond at Oklahoma State University, Campus Stillwater, OK, where 120 liters of water was sampled.

Filtration system in field situation needs a power generator for the pump to push the water in the filter. The portable power generator that was used works on gasoline.

Prior to filtration water pH was adjusted to a neutral pH since that was the standard we were following for this experiment. pH measurements were performed by a pH meter MW 801& MW 802 (Milwaukee,WI).

One hundred twenty liters (120 liters) was sampled from the pond, and water was adjusted to a near neutral pH (6-7). After filtration the filter was processed in the laboratory for elution of the virus concentration in water and RT-qPCR.

The filtration system was fitted with a pre-filter that had membranes for retaining soils and solid particles that eventually could be pushed into the filtration system from the water source.

The glass wool cartridges were carried to the field inside of a cooler with ice bags to maintain a low temperature (around 4°C). After the filtration both glass wool cartridge openings (inflow and outflow) were covered with para film tapes in order to prevent leakage from the sampled water. The filtration system was easily assembled and disassembled.

Effect of pH on virus filtration

Artificial Positive Control

The c-DNA of the virus extracted from leaves infected in the laboratory was inserted in a plasmid following the protocol TOPO-TA Cloning Kit – One Shot Cells from Invitrogen by life technologies.

The plasmid was diluted from 1 nanogram to 1fantogram and a q-PCR was performed following the protocol described in the procedure section.

pH assessment experiment

In this experiment different pH during virus capturing using a pre-adjusted filtration system using glass wool to trap the virus. The pH will be tested in the water from where the virus will be capture, the glass wool filter pH and the elution pH where the virus are released into a buffer solution. The experiment used pH range 3-4, 6-7 and 9-10. The buffers used was 1M HCl for acid pH and 1M PBS (Phosphate-buffered Saline) with a neutral pH and also with a high pH.

The filtration system developed in Experiment number I will be used in this experiment in small proportions in a laboratory situation. The three different pH was tested in the glass wool filter, in water and in the elution process.

Three plastic containers with capacity of 20 liters were filled with 7.5 gallons of RO water. The containers were spiked with leaves previously tested positive for *Pepino mosaic virus.* The water in each container was infested with *Pepino mosaic virus* blending 1 grams of infected with 1 liter of RO water stirring for two hours. The containers had different pH range: 3-4, 6-7 and 9-10. The pH was changed using 1M PBS buffer at 6.5 pH and 9 pH and HCl at 4 pH.

Each container had the water filter with three times with the same glass wool pH, however each glass wool was eluted with a different pH. In each experiment 2 liters were filtrated.

After the filtration each glass wool was transferred to a container (Backer) in order to have them washed with the designed buffer.

	Container 1	Container 2	Container 3
Water pH	**6-7**	**9-10**	**3-4**
Filter pH	**6-7** **9-10** **3-4**	**6-7** **9-10** **3-4**	**6-7** **9-10** **3-4**
Elution pH	**2L each:** **6-7** **9-10** **3-4**	**2L each:** **6-7** **9-10** **3-4**	**2L each:** **6-7** **9-10** **3-4**

Table 5. Description of pH used to study the effect of pH on virus recovery.

The following steps consisted on virus recovery from Elution, PEG, centrifugation, RNA extraction from water samples, c-DNA and q-PCR to identify and quantify the virus if present.

Effect of sample size on virus detection

Using the same combination of pH from experiment I, 80 and 120 liters of water sample was tested in a glass house experiment using tanks with capacity of 120 to reaffirm if the amount of water sampled has a direct effect of the amount of viruses recovered.

The tank was filled with 120 liters of RO water and the first time all of the water was filtered and the second time only 80 liters of the water was filtered.

The infection was proceed in the same way of the bucket experiment. The same amount of plant tissue (1g) was blended with 1 liter of RO water and the mixed in the tank.

Statistically Methodology

To determine the statically significance of factors: water pH, glass wool pH and elution pH regarding virus recovery from the filtration cartridge, a statistically analysis was performed using ANOVA (Analysis of Variance). ANOVA calculates the significance value (p-value) using the mean of the studied factors

CHAPTER IV

RESULTS AND FINDINGS

Development of a water filtration system to work in the field

A low cost filtration system that captures viruses was built as shown in the diagram below.

Diagram provided by Jon Daniels.

Fig.6. Diagram of the developed water filtration system

The filtration system was taken to the Theta Pond located at OSU, and 120 liter of water was filtered and processed in the laboratory according to the methodology presented in chapter III.

Fig.7. Use of the water filtration system in the field – Theta Pond at OSU.

Fig.8. Water pH adjustment using HCl – Theta Pond at OSU.

Following the elution of the virus from the glass wool the virus was concentrated with PEG and centrifugation. Followed by RNA extraction and RT-PCR for Potexviruses (Figure 9).

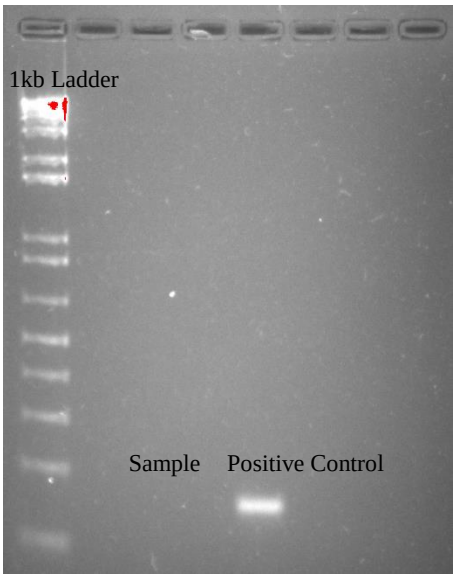


Fig.9. Gel of RT-PCR for *Pepino mosaic virus* from Theta Pond sample.

Artificial positive control

The plasmid was built following the protocol presented in chapter III. The following picture shows the qPCR comparing the plasmid amplification with PepMV with tomato tissue infected with PepMV and AGDIA positive control. (Figure 10A, 10B and 10C).

0.1 ng
0.01 ng
0.001 ng
0.0001 ng

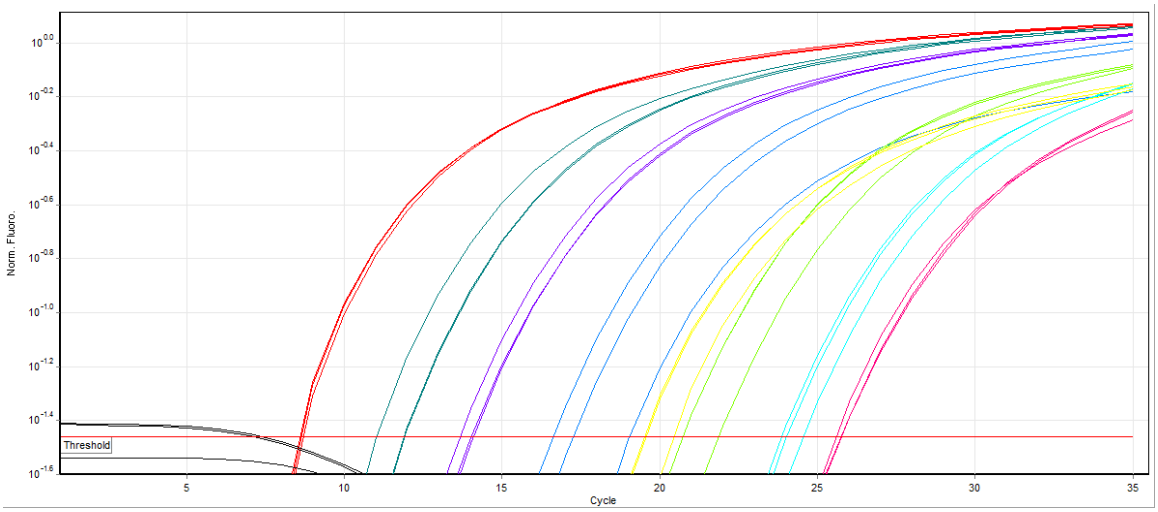


Fig.10A. Quantification of PepMV in laboratory infected tomato leaves. qPCR amplification of PepMV were from plasmids and infected tomato tissue. This graph shows a plasmid serial dilution from 1 ng to 1 fg which are indicated with numbers 1 to 6. Curve seven is the amplification of the reference positive control from AGDIA. Curve eight is the amplification of PepMV from laboratory infected tomato leaves. See DNA concentration values in table 10C.

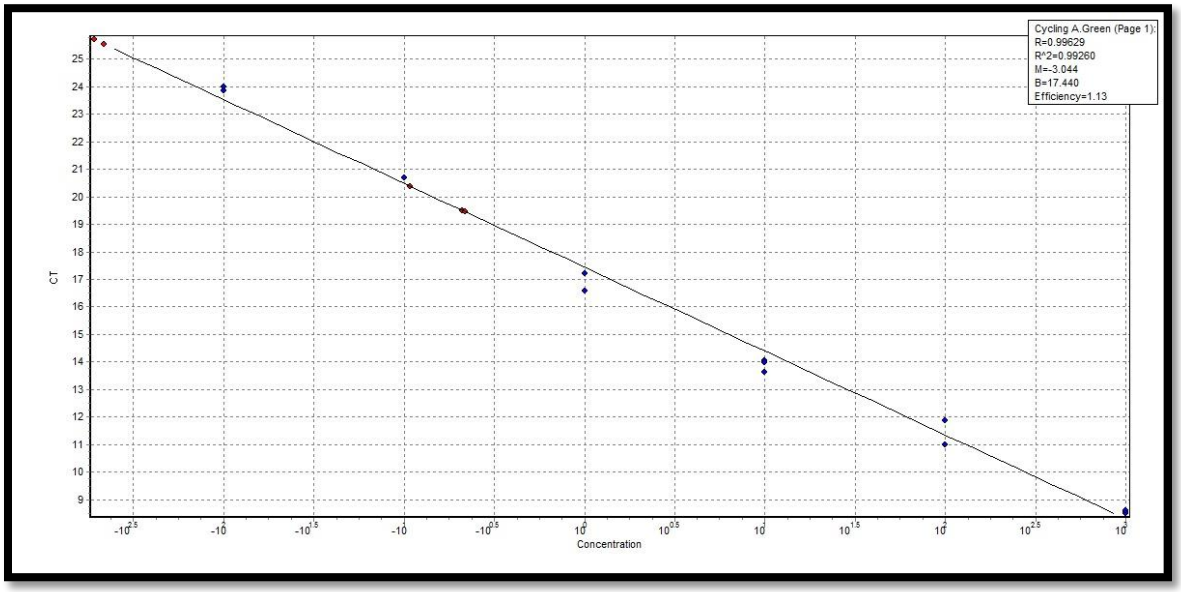


Fig.10B. Standard Curve of plasmids carrying the PepMV target and PepMV infected tomato leaves. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. $R^2 = 0.99260$ reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Given Conc (pg/ul)	Calc Conc (pg/ul)	% Var	Rep. Ct	Rep. Ct Std. Dev.
1	-9	Standard	8.53	1,000.00	845.746073	15.40%	8.55	0.06
1	-9	Standard	8.5	1,000.00	864.88067	13.50%		
1	-9	Standard	8.61	1,000.00	796.921147	20.30%		
2	-8	Standard	10.99	100	131.395155	31.40%	11.58	0.51
2	-8	Standard	11.89	100	66.74999	33.30%		
2	-8	Standard	11.86	100	67.9661	32.00%		
3	-7	Standard	14.05	10	12.955113	29.60%	13.89	0.23
3	-7	Standard	13.63	10	17.923026	79.20%		
3	-7	Standard	13.99	10	13.611911	36.10%		
4	-6	Standard	16.58	1	1.91559	91.60%	16.9	0.45
4	-6	Standard	17.22	1	1.182982	18.30%		
4	-5	Standard	20.7	0.1	0.085223	14.80%	20.7	0
5	-5	Standard	20.7	0.1	0.085156	14.80%		
5	-4	Standard	23.83	0.01	0.007943	20.60%	23.92	0.12
5	-4	Standard	24	0.01	0.006971	30.30%		
6	agdia	Unknown	25.54		0.002185		25.65	0.1
6	agdia	Unknown	25.71		0.00192			
6	agdia	Unknown	25.71		0.001923			
7	infected	Unknown	19.5		0.210604		19.78	0.53
7	infected	Unknown	20.38		0.107811			
7	infected	Unknown	19.45		0.218348			
8	NTC	NTC						
8	NTC	NTC						
8	NTC	NTC						

Fig.10C. Plasmids and infected tomato leaves qPCR quantification of PepMV. qPCR amplification of PepMV were from plasmids and infected tomato tissue. See DNA concentration in tomato leaves is 0.35 pg/ul average and 0.0033 pg/ul in the reference positive control from AGDIA, which correspond to 19.4-20.38 and 25.54-25.71 Ct values.

pH assessment

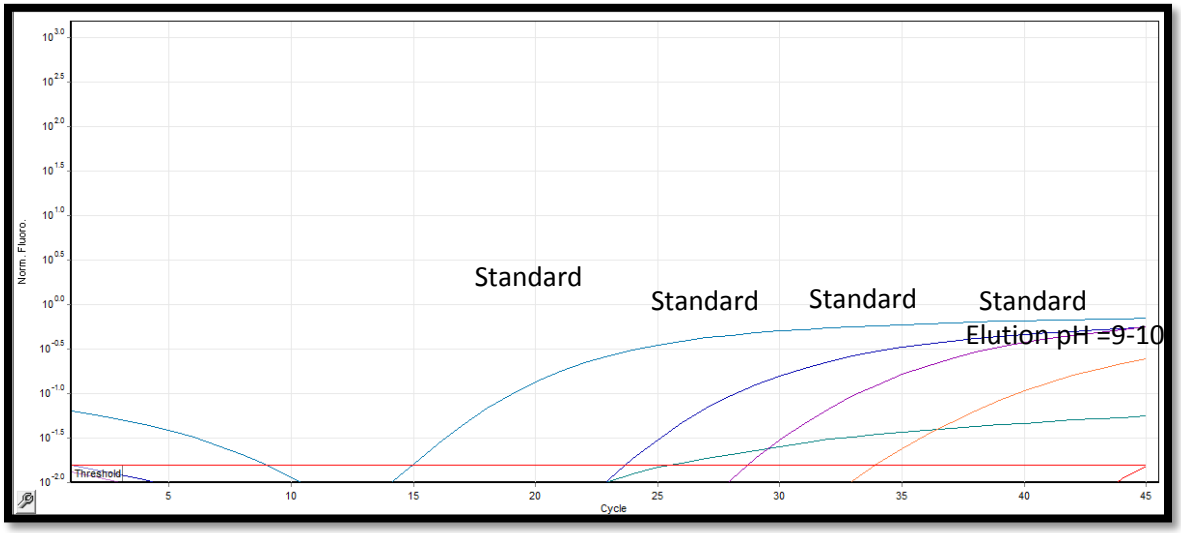
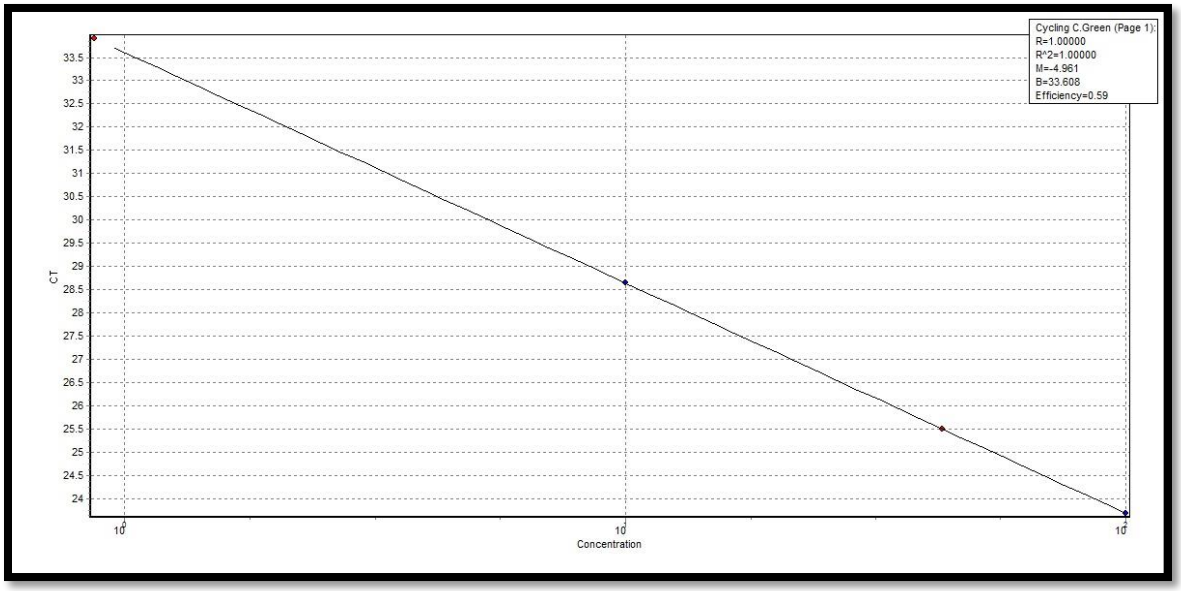


Fig.11A. Quantification of PepMV from the filtration system: pH 6-7 in the glass wool, pH 6-7 in the water and pH 9-10 in the elution.



*F*ig.11B. Standard Curve of figure 11A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. $R^2 = 1$ reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (pg/ul)	Calc Conc (pg/ul)	% Var
1	Acid Elution	Unknown					
1	Acid Elution	Unknown					
2	Basic Elution	Unknown					
2	Basic Elution	Unknown	25.51			42.924	
3	Neutral Elution	Unknown					
3	Neutral Elution	Unknown					
4	infected	sitive Cont	33.91			0.87	
5	NTC	NTC					
6	Plasmids	Standard		NEG (Multi Ct)	1,000.00		
7	Plasmids	Standard	23.69		100	100	0.00%
8	Plasmids	Standard	28.65		10	10	0.00%
9	Plasmids	Standard			1		

Fig. 11C. qPCR amplification of PepMV from figure 11A. DNA concentration from 3-4 pH elution was 42.924 pg/ul which correspond to a 25.51 Ct value.

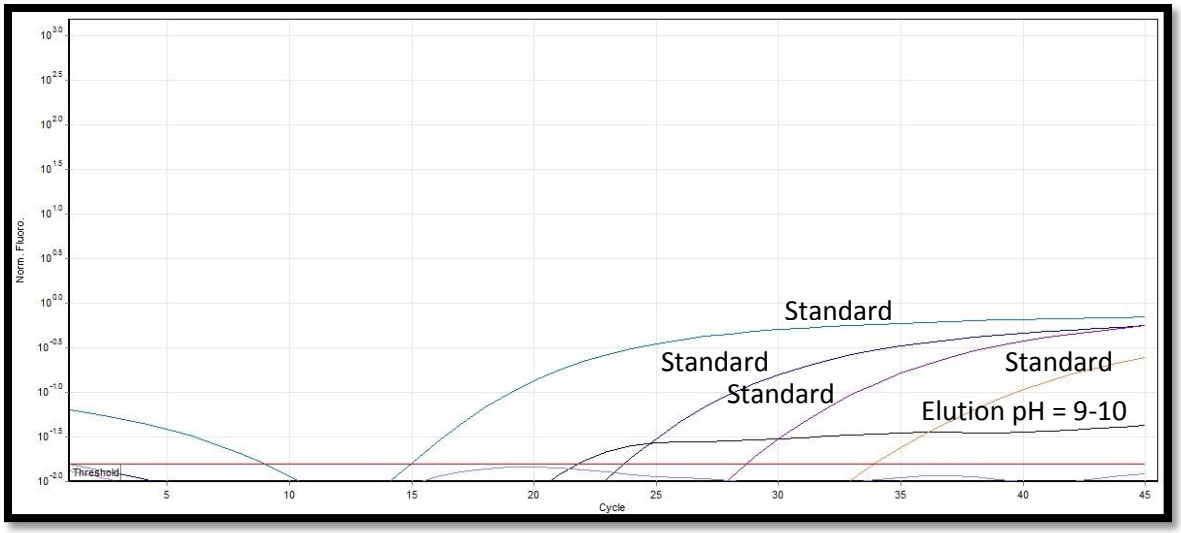


Fig.12A. Quantification of PepMV from the filtration system: pH 6-7 in the glass wool, pH 9-10 in the water and pH 9-10 in the elution.

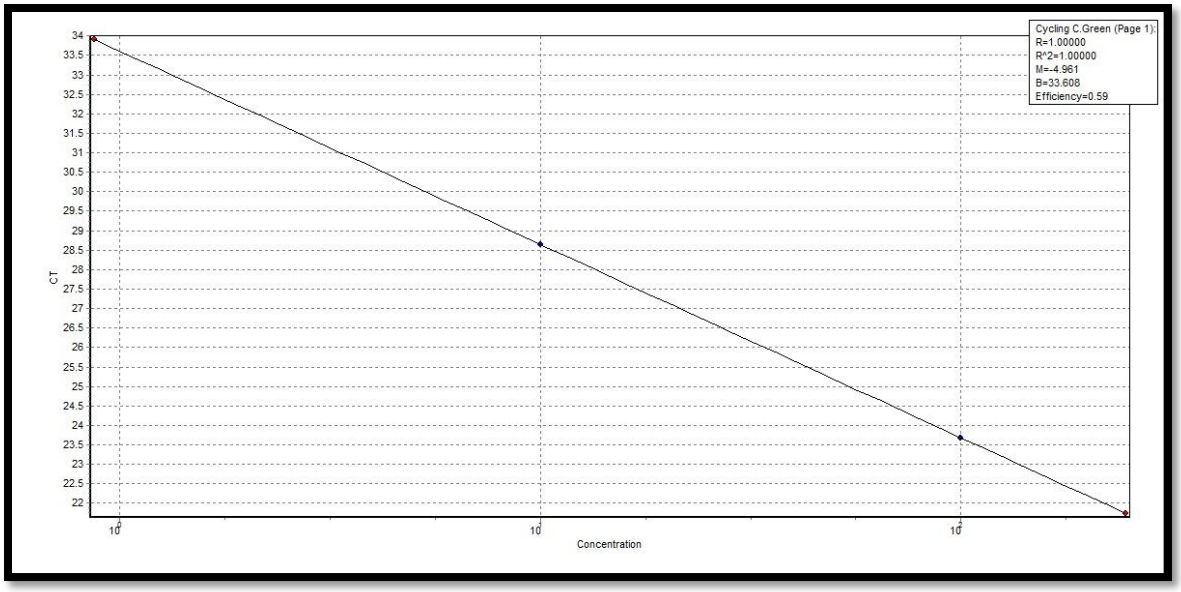


Fig.12B. Standard Curve of figure 12A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. $R^2 = 1$ reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Con	Calc Conc	% Var
1	Acid Elution	Unknown					
1	Acid Elution	Unknown					
2	Basic Elution	Unknown					
2	Basic Elution	Unknown	21.74			247.028	
3	Neutral Elution	Unknown					
3	Neutral Elution	Unknown					
4	infected	Positive Control	33.91			0.87	
5	NTC	NTC					
6	Plasmids	Standard		NEG (Multi Ct)	1,000.00		
7	Plasmids	Standard	23.69		100	100	0.00%
8	Plasmids	Standard	28.65		10	10	0.00%
9	Plasmids	Standard			1		

Fig. 12C. qPCR amplification of PepMV from figure 12A. DNA concentration from 3-4 pH elution was 247.028 pg/ul which correspond to a 21.74 Ct value.

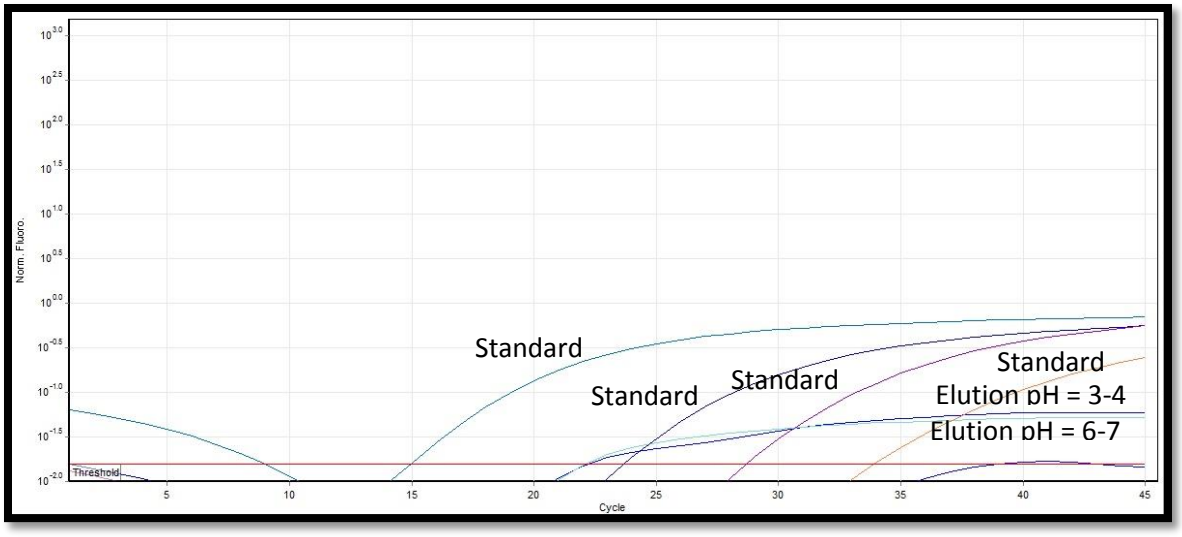


Fig.13A. Quantification of PepMV from the filtration system: pH 6-7 in the glass wool, pH 3-4 in the water and pH 6-7 and 3-4 in the elution.

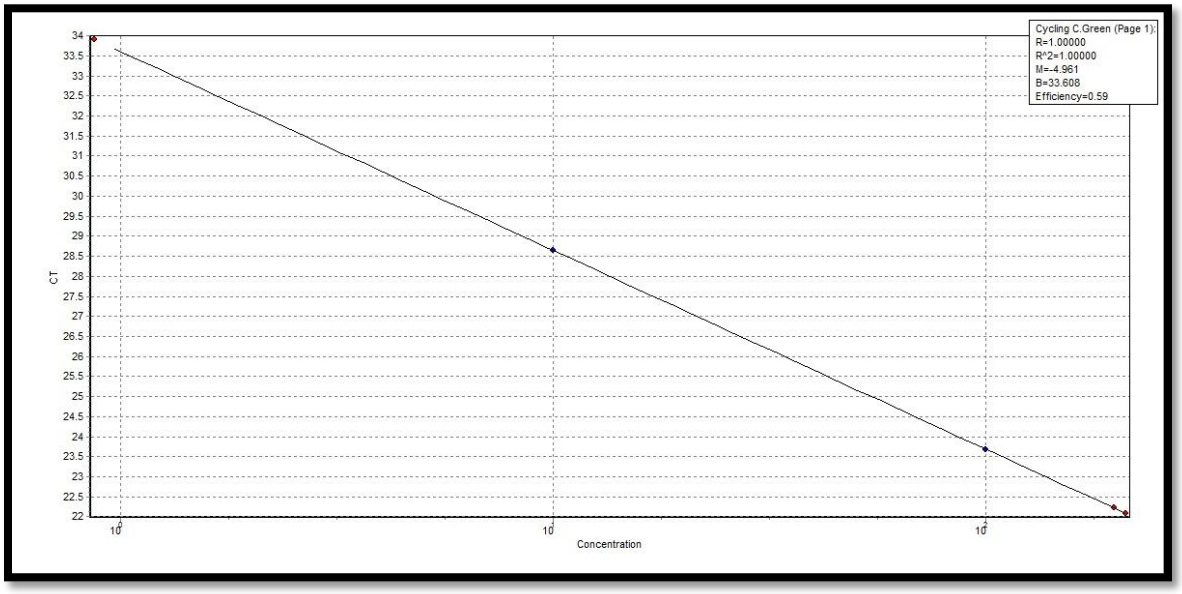


Fig.13B. Standard Curve of figure 13A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. R2 = 1 reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (pg/ul)	Calc Conc (pg/ul)	% Var
1	Acid Elution	Unknown		NEG (Multi Ct)			
1	Acid Elution	Unknown	22.22			197.38	
2	Basic Elution	Unknown					
2	Basic Elution	Unknown					
3	Neutral Elution	Unknown					
3	Neutral Elution	Unknown	22.08			210.335	
4	infected	Positive Control	33.91			0.87	
5	NTC	NTC					
6	Plasmids	Standard		NEG (Multi Ct)	1,000.00		
7	Plasmids	Standard	23.69		100	100	0.00%
8	Plasmids	Standard	28.65		10	10	0.00%
9	Plasmids	Standard			1		

Fig. 13C. qPCR amplification of PepMV from figure 13A. DNA concentration from 3-4 pH elution was 197.38 pg/ul which correspond to a 22.22 Ct value and DNA concentration from 6-7 pH elution was 210.335 pg/ul which correspond to a 22.08 Ct value.

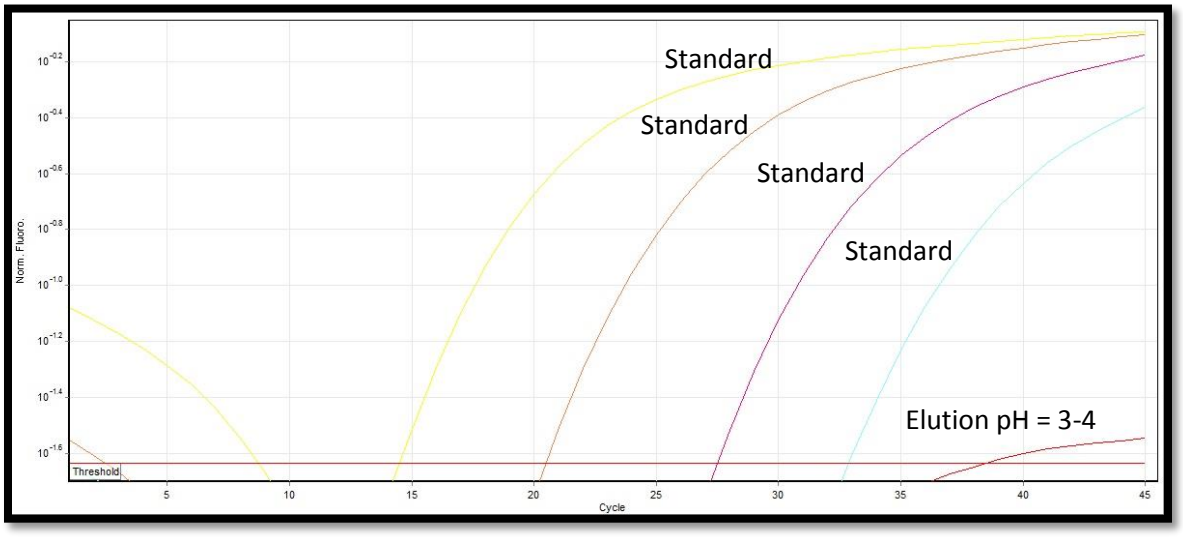


Fig. 14A. Quantification of PepMV from the filtration system: pH 9-10 in the glass wool, pH 6-7 in the water and pH 3-4 in the elution.

Fig. 14B. Standard Curve of figure 14A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. R2 = 1 reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (pg/ul)	Calc Conc (pg/ul)	% Var
1	Acid Elution	Unknown					
1	Acid Elution	Unknown	38.5			0.09	
2	Basic Elution	Unknown					
2	Basic Elution	Unknown					
3	Neutral Elution	Unknown					
3	Neutral Elution	Unknown					
4	plasmids	Standard		NEG (Multi Ct)	1,000.00		
5	plasmids	Standard		NEG (Multi Ct)	100		
6	plasmids	Standard	27.49		10	10	0.00%
7	plasmids	Standard	32.88		1	1	0.00%
8	-	Negative Control					
9	NTC	NTC					

Fig.14C. qPCR amplification of PepMV from figure 14A. DNA concentration from 3-4 pH elution was 0.09 pg/ul which correspond to a 38.5 Ct value.

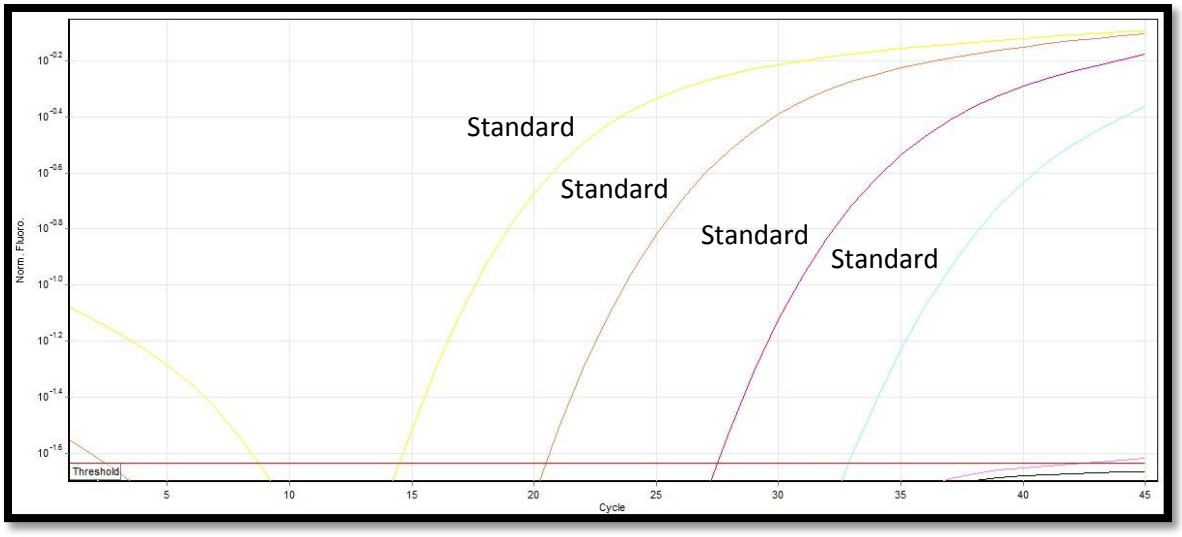


Fig.15A. Quantification of PepMV from the filtration system: pH 9-10 in the glass wool, pH 9-10 in the water. There were no recovery from any pH used for elution.

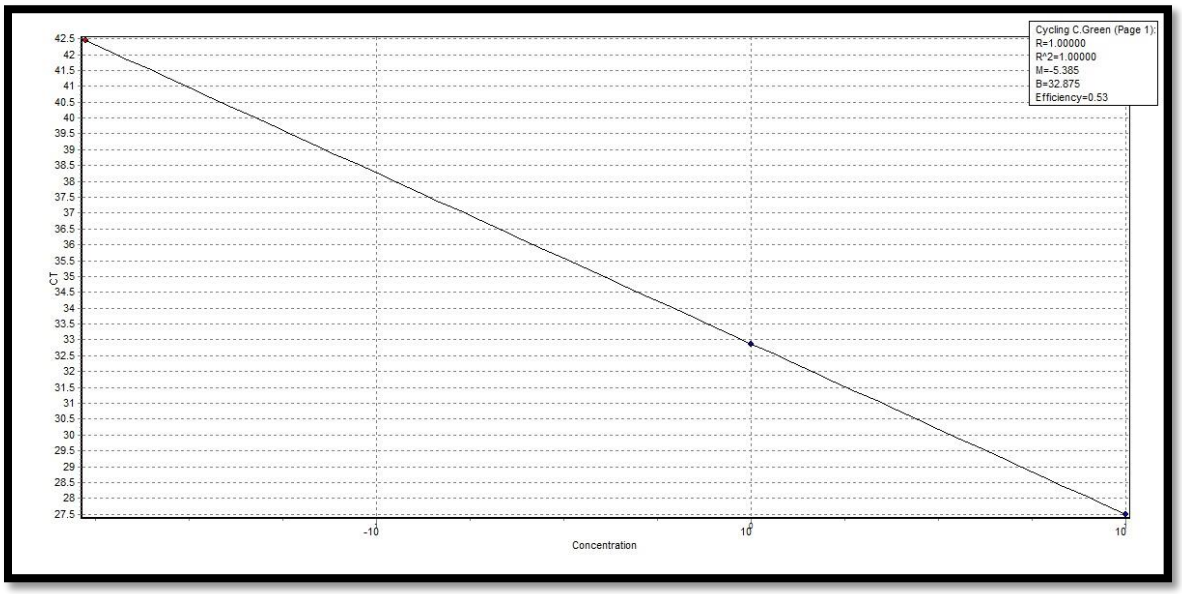


Fig. 15B. Standard Curve of figure 15A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. R2 = 1 reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (pg/ul)	Calc Conc (pg/ul)	% Var
1	Acid Elution	Unknown					
1	Acid Elution	Unknown	42.44			0.017	
2	Basic Elution	Unknown					
2	Basic Elution	Unknown					
3	Neutral Elution	Unknown					
3	Neutral Elution	Unknown					
4	plasmids	Standard		NEG (Multi Ct)	1,000.00		
5	plasmids	Standard		NEG (Multi Ct)	100		
6	plasmids	Standard	27.49		10	10	0.00%
7	plasmids	Standard	32.88		1	1	0.00%
8	-	Negative Control					
9	NTC	NTC					

Fig. 15C. qPCR amplification of PepMV from figure 15A. DNA concentration from 3-4 pH elution was 0.017 pg/ul which correspond to a 42.44 Ct value.

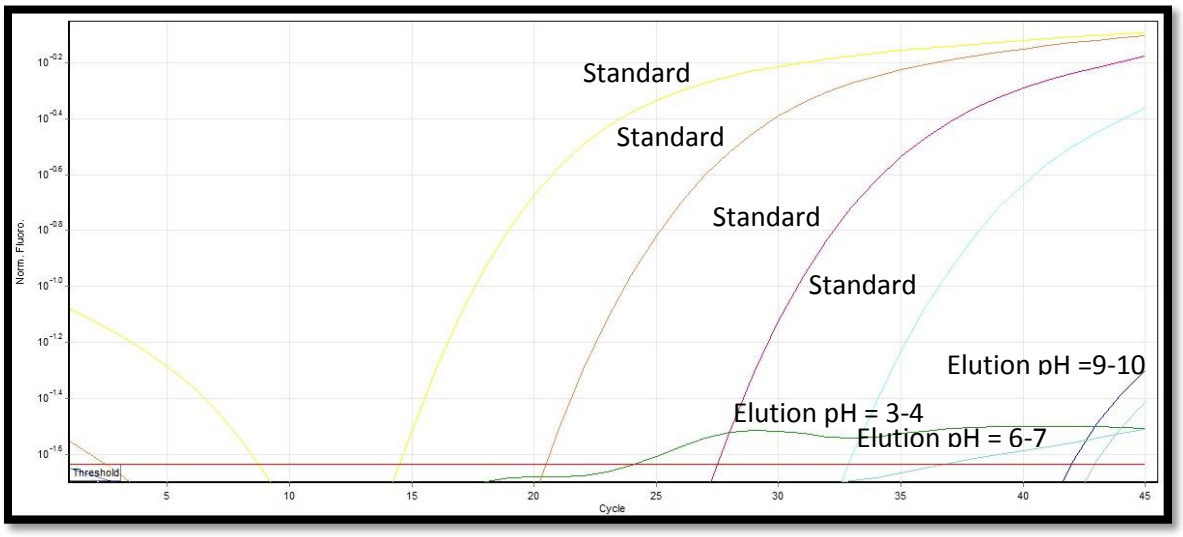


Fig. 16A. Quantification of PepMV from the filtration system: pH 9-10 in the glass wool, pH 3-4 in the water and pH 3-4, 6-7 and 9-10 pH in the elution.

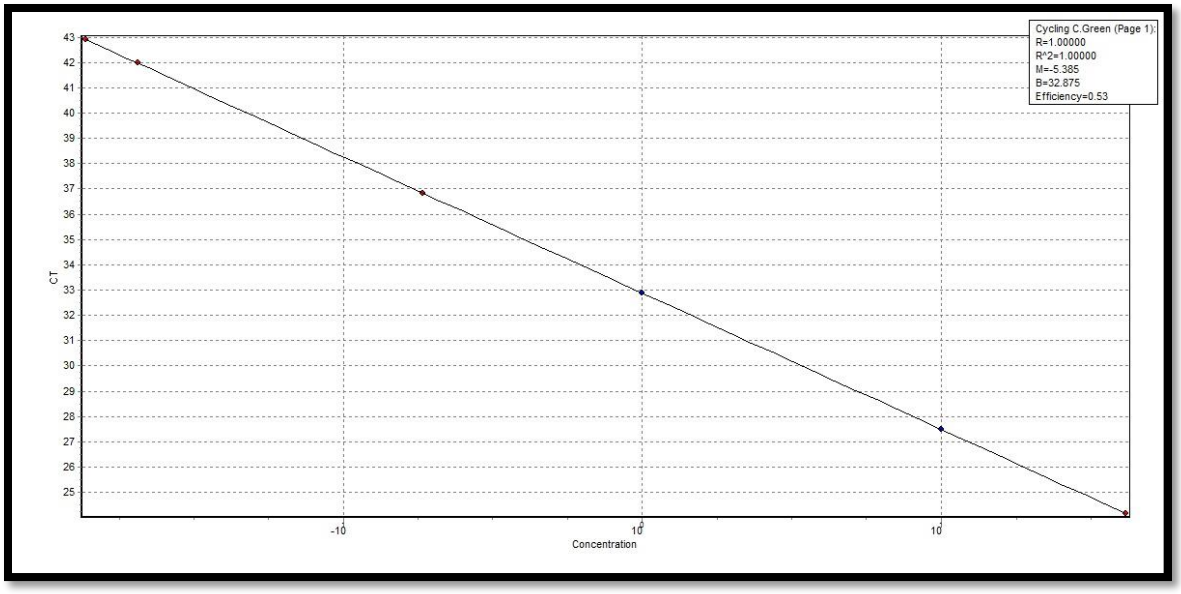


Fig. 16B. Standard Curve of figure 16A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. R2 = 1 reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (pg/ul)	Calc Conc (pg/ul)	% Var
1	2a	Unknown	41.98			0.02	
1	2a	Unknown					
2	2b	Unknown					
2	2b	Unknown	24.16			41.551	
3	2n	Unknown	36.84			0.184	
3	2n	Unknown	42.91			0.014	
4	plasmids	Standard		NEG (Multi Ct)	1,000.00		
5	plasmids	Standard		NEG (Multi Ct)	100		
6	plasmids	Standard	27.49		10	10	0.00%
7	plasmids	Standard	32.88		1	1	0.00%
8	-	Negative Control					
9	NTC	NTC					

Fig. 16C. qPCR amplification of PepMV from figure 16A. DNA concentration from 3-4 pH elution was 0.02 pg/ul which correspond to a 41.98 Ct value, DNA concentration from 9-10 pH elution was 41.551 pg/ul which correspond to a 24.16 Ct value and DNA concentration from 6-7 pH elution was 0.184 pg/ul which correspond to a 42.91 Ct value.

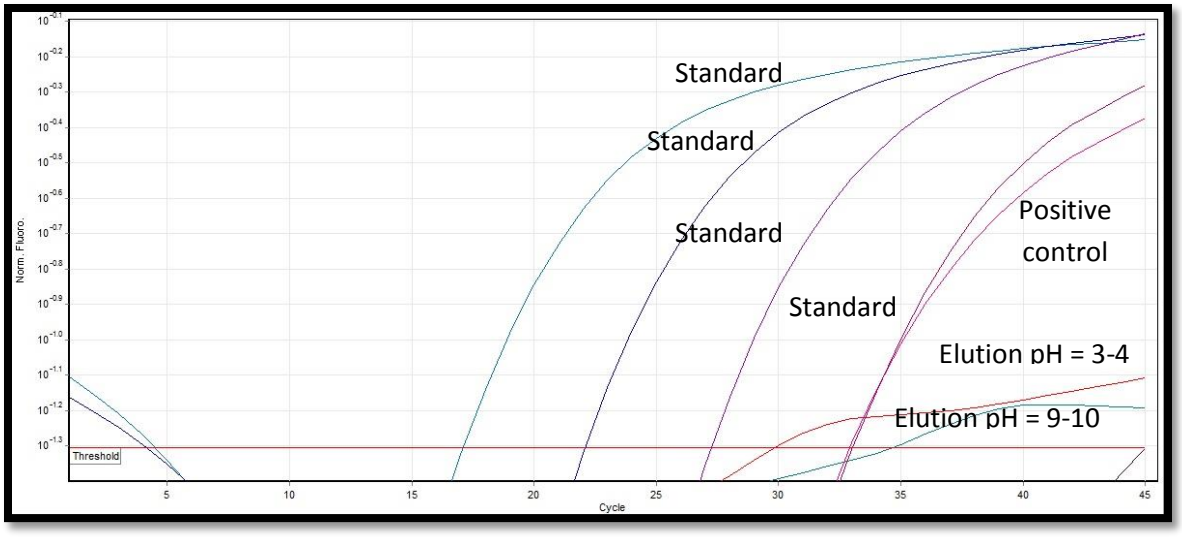


Fig. 17A. Quantification of PepMV from the filtration system: pH 3-4 in the glass wool, pH 6-7 in the water and pH 3-4 and 9-10 in the elution.

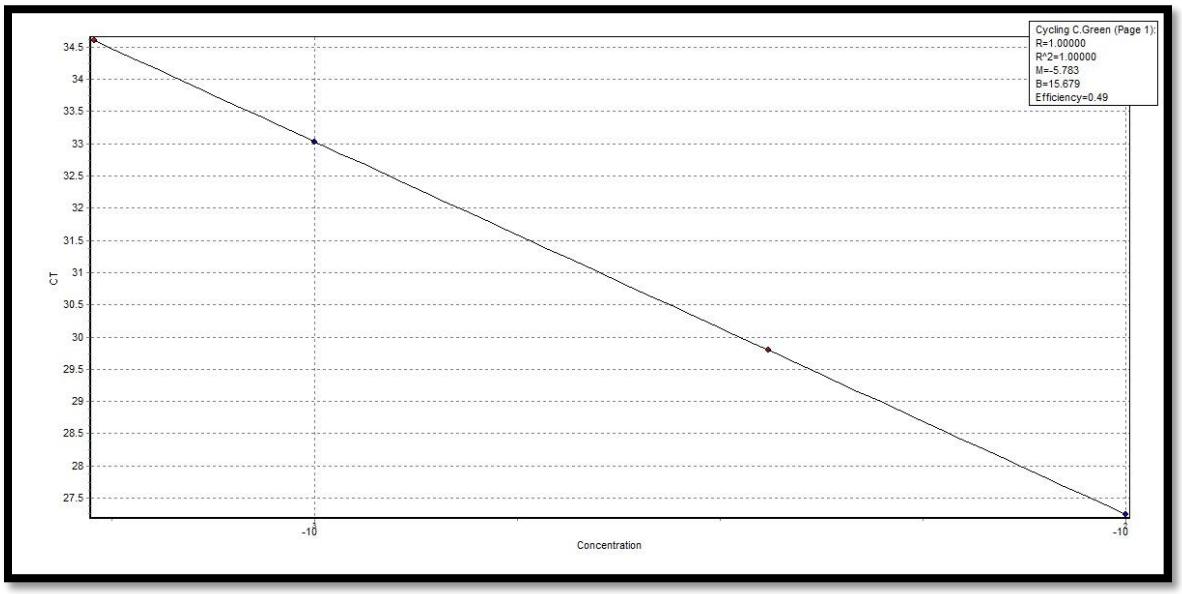


Fig. 17B. Standard Curve of figure 17A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. R2 = 1 reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (ng/ul)	Calc Conc (ng/ul)	% Var
1	Acid Elution	Unknown					
1	Acid Elution	Unknown	29.8			0.004	
2	Basic Elution	Unknown	34.6			0.001	
2	Basic Elution	Unknown					
3	Neutral Elution	Unknown					
3	Neutral Elution	Unknown					
4	-	Negative Control					
5	NTC	NTC					
6	Plasmids	Standard		NEG (Multi Ct)	1		
7	Plasmids	Standard		NEG (Multi Ct)	0.1		
8	Plasmids	Standard	27.25		0.01	0.01	0.00%
9	Plasmids	Standard	33.03		0.001	0.001	0.00%

Fig. 17C. qPCR amplification of PepMV from figure 17A. DNA concentration from 3-4 pH elution was 0.004 pg/ul which correspond to a 29.8 Ct value. DNA concentration from 9-10 pH elution was 0.001 pg/ul which correspond to a 34.6 Ct value

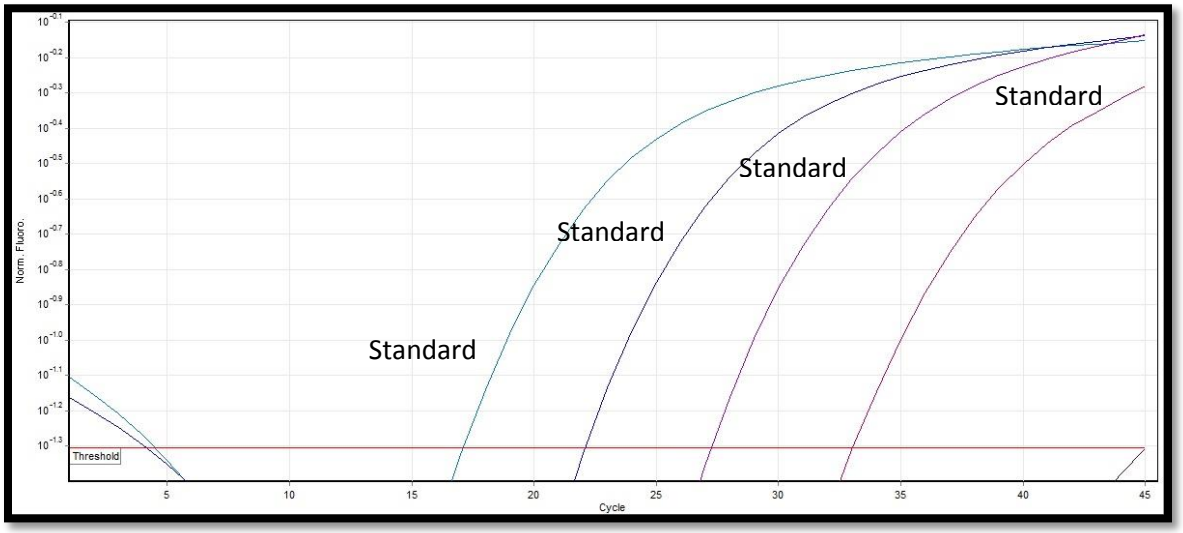


Fig. 18A. Quantification of PepMV from the filtration system: pH 3-4 in the glass wool, pH 9-10 in the water. There were no recovery.

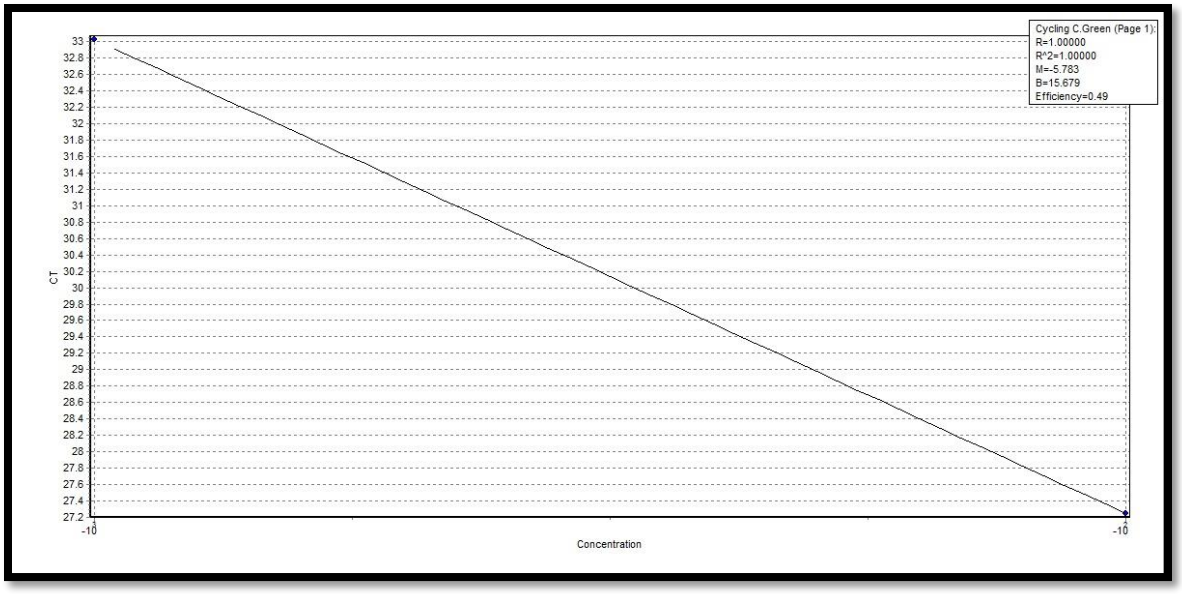


Fig. 18B. Standard Curve of figure 18A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. R2 = 1 reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (ng/ul)	Calc Conc (ng/ul)	% Var
1	Acid Elution	Unknown					
1	Acid Elution	Unknown					
2	Basic Elution	Unknown					
2	Basic Elution	Unknown					
3	Neutral Elution	Unknown					
3	Neutral Elution	Unknown					
4	-	Negative Control					
5	NTC	NTC					
6	Plasmids	Standard		NEG (Multi Ct)	1		
7	Plasmids	Standard		NEG (Multi Ct)	0.1		
8	Plasmids	Standard	27.25		0.01	0.01	0.00%
9	Plasmids	Standard	33.03		0.001	0.001	0.00%

Fig. 18C. qPCR amplification of PepMV from figure 18A.

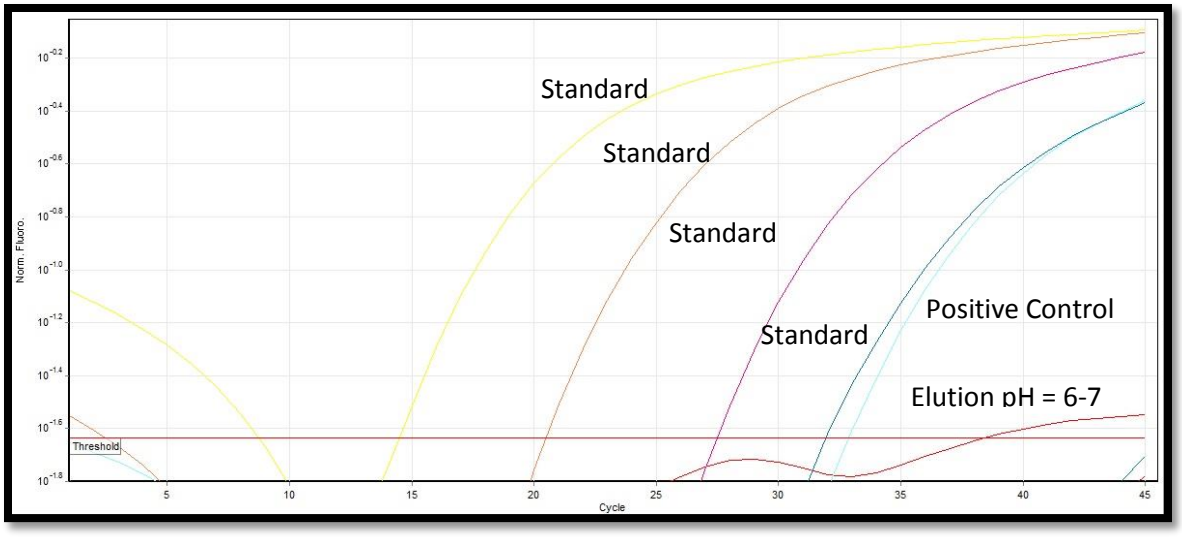


Fig. 19A. Quantification of PepMV from the filtration system: pH 3-4 in the glass wool, pH 3-4 in the water and pH 6-7 in the elution.

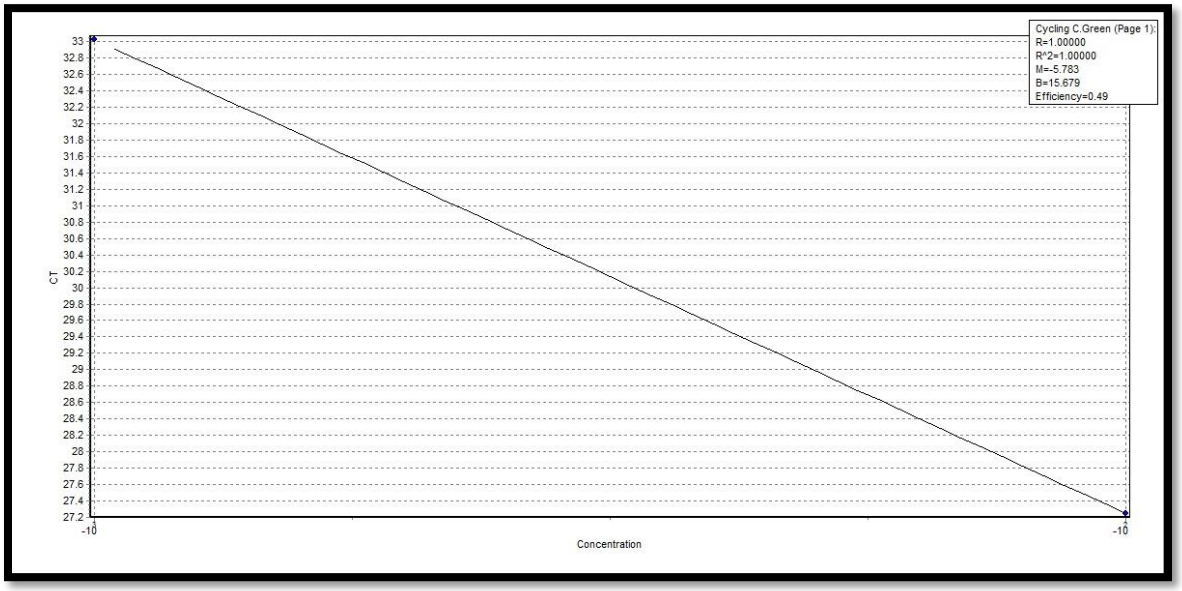


Fig. 19B. Standard Curve of figure 19A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. R2 = 1 reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (ng/ul)	Calc Conc (ng/ul)	% Var
1	Acid Elution	Unknown	30.3			0.003	
1	Acid Elution	Unknown					
2	Basic Elution	Unknown					
2	Basic Elution	Unknown					
3	Neutral Elution	Unknown	27.75			0.008	
3	Neutral Elution	Unknown					
4	-	Negative Control					
5	NTC	NTC					
6	Plasmids	Standard		NEG (Multi Ct)	1		
7	Plasmids	Standard		NEG (Multi Ct)	0.1		
8	Plasmids	Standard	27.25		0.01	0.01	0.00%
9	Plasmids	Standard	33.03		0.001	0.001	0.00%

Fig. 19C. qPCR amplification of PepMV from figure 19A. DNA concentration from 3-4 pH elution was 0.003 pg/ul which correspond to a 30.3 Ct value. DNA concentration from 3-4 pH elution was 0.008 pg/ul which correspond to a 27.75 Ct value.

Statistical Analysis for a multifactorial experiment

When determining which factor (filter pH,water pH or elution pH) had the highest impacted on recovering viruses from the filtration system , it was determined that the pH of filter was the only factor to significantly increase virus recovery from water ($p<0.05$) (Figure 20 below)

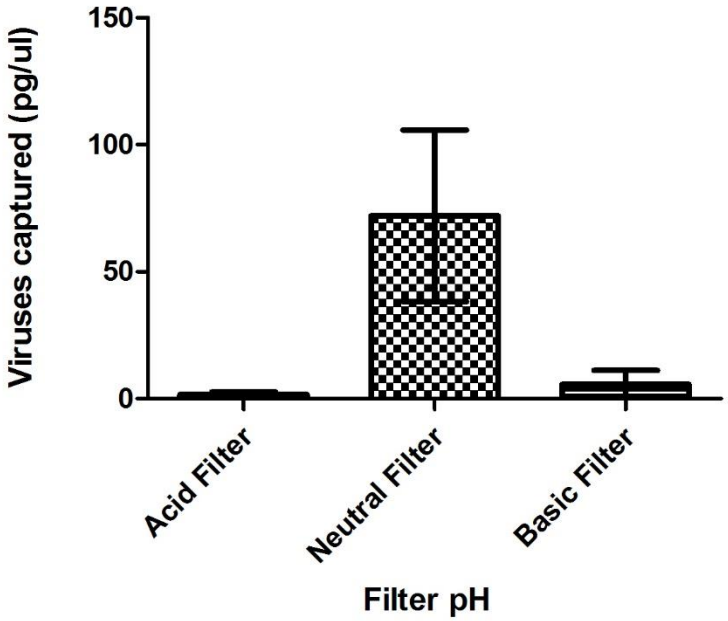


Fig.20. Filter pH impact on virus recovering from water samples with different pH range (3-4, 6-7 and 9-10) and eluted in different pH range (3-4, 6-7 and 9-10).

Elution pH and water pH did not show significant impact ($p<0.05$) on virus recovering as show in figures 21 and 22.

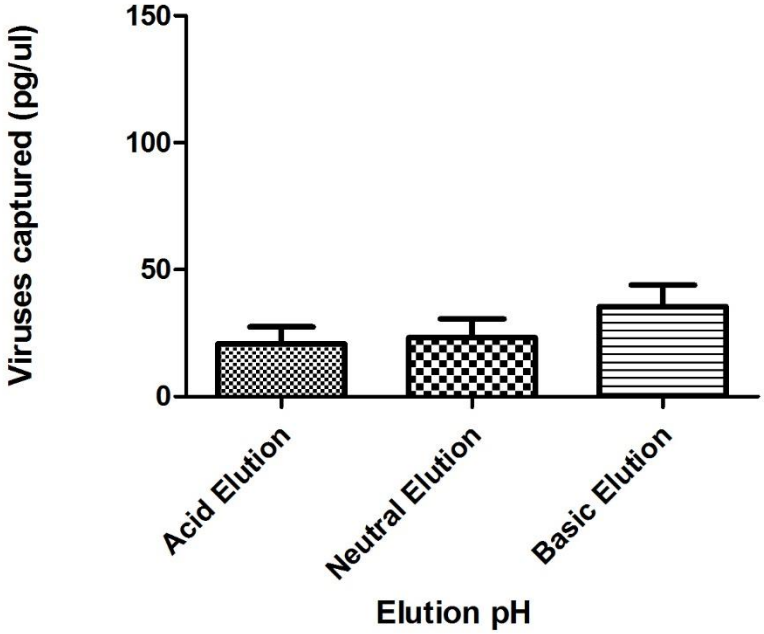


Fig.21. Elution pH impact on virus recovery from water samples with different pH range (3-4, 6-7 and 9-10) and glass wool with different pH range (3-4, 6-7 and 9-10).

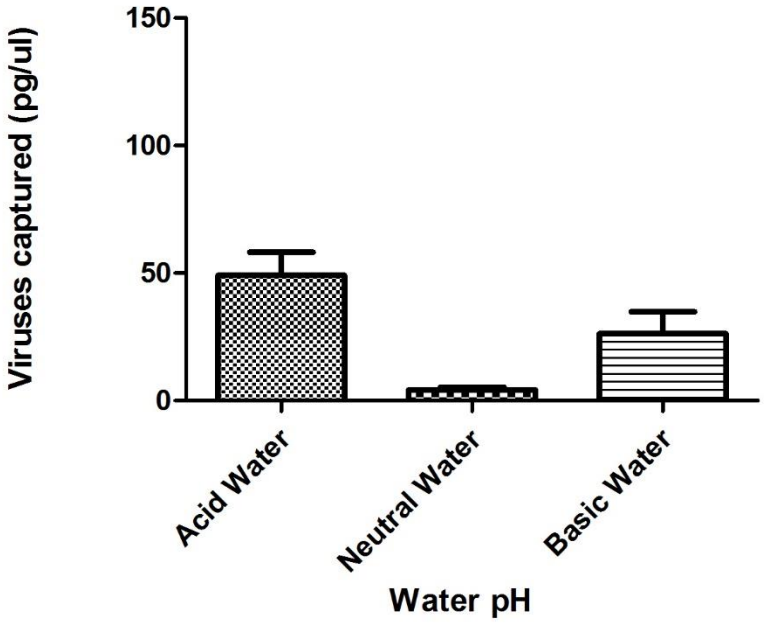


Fig.22. Water pH impact on virus recovery from water samples with different pH range in the elution process (3-4, 6-7 and 9-10) and glass wool with different pH range (3-4, 6-7 and 9-10).

Objective III: Effect of sample size over virus detection

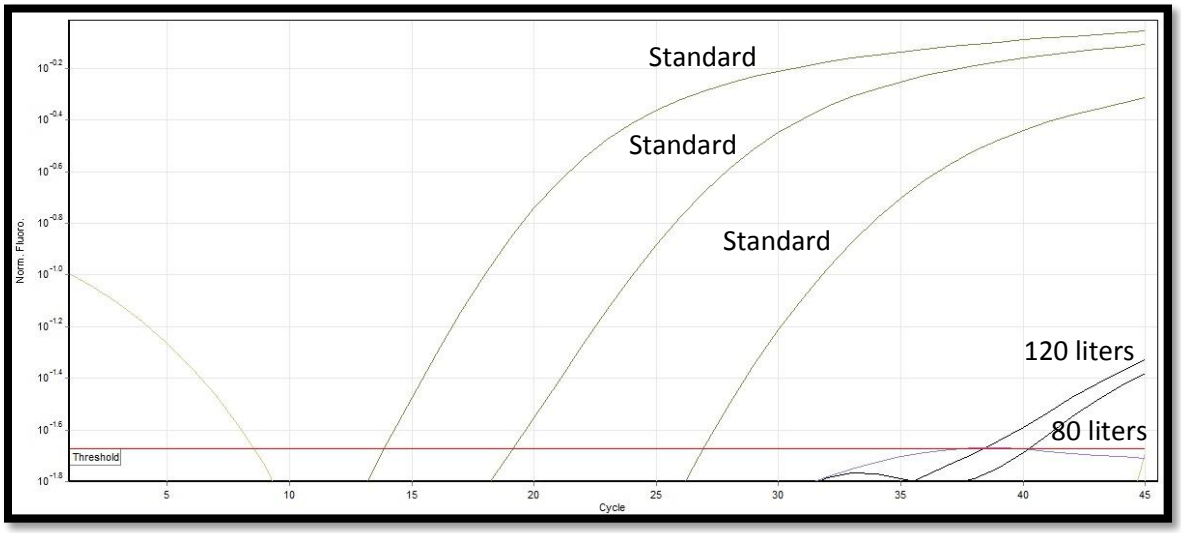


Fig. 23A. Quantification of PepMV after filtration of 80 and 120 L of water seeded with tomato leaves infected with PepMV.

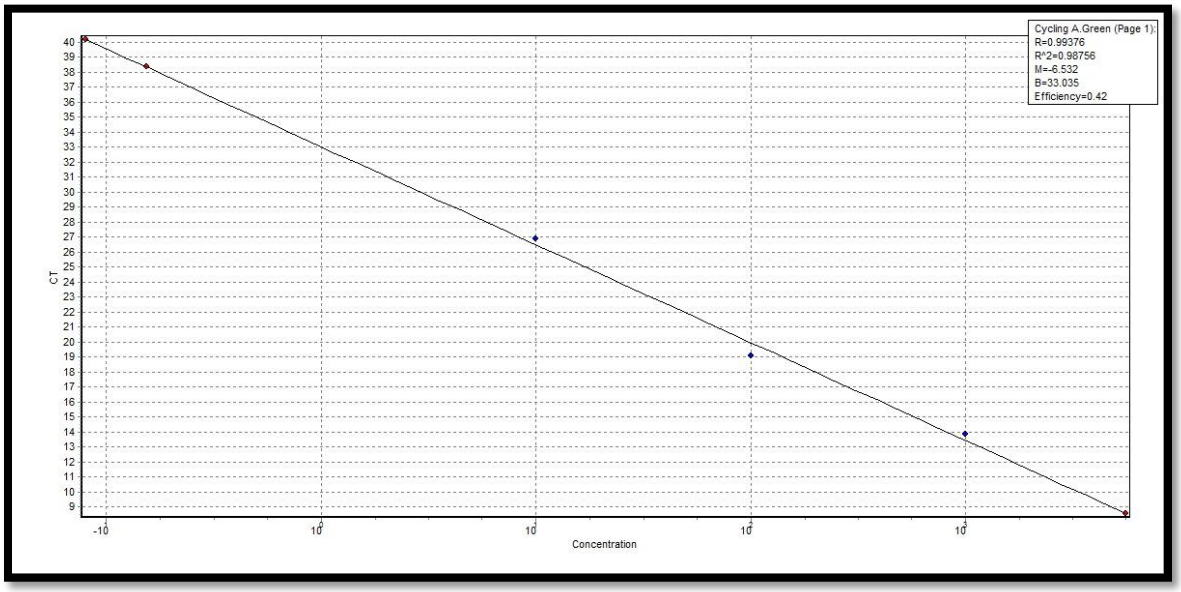


Fig. 23B. Standard Curve of figure 23A. The standard curve shows threshold cycle (Ct) on the y-axis and the DNA concentration on the x-axis. Slope, y-intercept, and correlation coefficient values shows the performance of this reaction. R2 = 0.99376 reflects the linearity of the standard curve. M measures the reaction efficiency, equivalent to -3.32 for 100% efficiency.

No.	Name	Type	Ct	Ct Comment	Given Conc (pg/ul)	Calc Conc (pg/ul)	% Var
1	positive control	Positive Control	8.58			5,547.95	
2	80 liters	Unknown	40.19			0.08	
3	120 liters	Unknown	38.36			0.153	
4		Standard	13.86		1,000.00	861.412	13.90%
5		Standard	19.12		100	134.765	34.80%
6		Standard	26.93		10	8.614	13.90%
7		Standard			1		
8	NTC	NTC		NEG (Multi Ct)			

Fig. 23C. qPCR amplification of PepMV from figure 23A. DNA concentration from 3-4 pH elution was 0.003 pg/ul which correspond to a 30.3 Ct value. DNA concentration from 3-4 pH elution was 0.008 pg/ul which correspond to a 27.75 Ct value.

CHAPTER V

DISCUSSION AND CONCLUSIONS

The purpose of this study was to adapt and to develop a transportable filtration system capable of working in a field setting at a low cost with ease assembly and disassembly. The filtration system is adaptable to work with portable power generators for application. However, the generator power is one of the pieces of the filtration system that in the future it should be considered to be replaced to another source of energy. Because first is a heavy piece of equipment to transport and can be a difficulty depending on the topography. The second reason is that power generator that we are using requires gasoline and can be an extra expense to the system. The qPCR and electrophoresis gel results showed negative results for Potexviruses. However would be important to validate the field experiment using primers for different waterborne viruses in the future.

The second objective addressed the assessment of pH on PepMV capture. An important point was to observe if the isoelectric point of the studied virus, PepMV, was a possible way to use for waterborne virus capture. As discussed in chapter II, virus isoelectric point might be useful for recovering specific viruses from water. In the case of PepMV the isoelectric point is 6.7, which means that an ideal pH range for capturing this virus it was supposed to be in the acid-neutral range.

From the results is possible to observe that the best water pH for capturing the virus from water was the pH range 9-10, however the pH used inside of the filter to was a neutral pH. And using elution from 9-10 pH.

When comparing the different pH used in the glass wool it is observed that when the glass wool was prepared using a neutral buffer pH it was when the recovery of the viruses

happened in almost every time the elution was done. While when the glass wool was prepared using 9-10 pH or acid pH 3-4 the virus recovery was very little when recovered but most of the times was not recovered. For this reason it is possible to start a better understanding about the role of the filter pH during the capture of the viruses and not only focus only in the water pH as the only source where the isoelectric point can play a role. However, during the water filtration, the pH within the cartridge is probably changing according to the pH of the water that is passing through.

The second repetition of experiment that studied the effect of pH in the water, in the glass wool and in the elution was done following the same steps as mentioned in the material and methods section. However, the eluted volume after water filtration was stored in -80°C for a month before starting being processed. The results were different if compared to the first repetition where the eluted volume were processed in the day after the filtration and store in -20°C. The long storage at lower temperatures might have caused the disruption of the RNA virus from its proteins and then during the PCR reaction the titer of virus (RNA) was very low and could not be amplified. Also it highlights the importance of having fresh samples when working on pathogens recovery from water.

In the contrary, the tissue infected with PepMV and stored at -80°C can be maintained infectious up to 2 months. The titer of the virus in the tissue decreased with time but it is still able to be captured and detected using molecular methods after 2 months.

The qPCR analysis is not only able to detect viruses in very low amount but also it can gives us the efficiency of the reactions after the PCR reaction. The efficiency of a qPCR reaction relies on the standards choice. The importance of choosing a good material to use as a standard is that will minimize the difference between the standard and samples when the PCR reaction is run (39). Saying that it is important to highlight that my samples were water samples infected with

tomatoes tissues previously infected with the virus PepMV. Also the samples were subjected to a virus concentration process where PEG was used, which is a high salty chemical compound and considering the fact that in the water, both environmental water sample and RO water from the lab contain other chemicals substances that are considered PCR inhibitors and can decrease the efficiency of the reaction (29). Concluding then that the low efficiency of the reactions is due to these previous points. In order to try to minimize the chemical interference from the virus concentration process and also to try to take out as much water contaminants as possible it would be necessary a virus purification assay before running the PCR.

The qPCR results with the plasmids show that in 0.1 gram of tissue used in the RNA extraction the amount of virus was about to 0.35 pg. Considering that in the infections made for the objectives II and III was used 1 gram of infected tissue the maximum recovered amount was supposed to be about 350 pg of viruses. And analyzing the results there was not any recovered over than 250 pg of viruses.

The third objective was to see and confirm that different volumes of water filtered through the filter it would have higher chances to recover more viruses. As shown in the qPCR graph the when filtering 120 liters of water the virus recovered was higher than when filtering 80 liters of water.

Considering the results from the three objectives it is important to point the importance of doing the experiments focusing in also in different plant waterborne virus. Also because it will be important to test the isoelectric point of viruses from different genera in water capturing.

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