Video Article

Propagation of *Homalodisca coagulata virus-01* via *Homalodisca vitripennis* Cell Culture

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Abstract

The glassy-winged sharpshooter (*Homalodisca vitripennis*) is a highly vagile and polyphagous insect found throughout the southwestern United States. These insects are the predominant vectors of *Xylella fastidiosa (X. fastidiosa),* a xylem-limited bacterium that is the causal agent of Pierce's disease (PD) of grapevine. Pierce's disease is economically damaging; thus, *H. vitripennis* have become a target for pathogen management strategies. A dicistrovirus identified as *Homalodisca coagulata virus-01* (HoCV-01) has been associated with an increased mortality in *H. vitripennis* populations. Because a host cell is required for HoCV-01 replication, cell culture provides a uniform environment for targeted replication that is logistically and economically valuable for biopesticide production. In this study, a system for large-scale propagation of *H. vitripennis* cells via tissue culture was developed, providing a viral replication mechanism. HoCV-01 was extracted from whole body insects and used to inoculate cultured *H. vitripennis* cells at varying levels. The culture medium was removed every 24 hr for 168 hr, RNA extracted and analyzed with qRT-PCR. Cells were stained with trypan blue and counted to quantify cell survivability using light microscopy. Whole virus particles were extracted up to 96 hr after infection, which was the time point determined to be before total cell culture collapse occurred. Cells were also subjected to fluorescent staining and viewed using confocal microscopy to investigate viral activity on F-actin attachment and nuclei integrity. The conclusion of this study is that *H. vitripennis* cells are capable of being cultured and used for mass production of HoCV-01 at a suitable level to allow production of a biopesticide.

Video Link

The video component of this article can be found at https://www.jove.com/video/51953/

Introduction

The glassy-winged sharpshooter (*Homalodisca vitripennis* Germar 1821) has been identified as the predominant vector of *Xylella fastidiosa* (*X. fastidiosa*), the causal agent of Pierce's disease of grapevine (PD) in North America[1]. Insect population management has quickly become the focus of research to combat this devastating problem to the viticulture industry in California and across the southern United States. A positive-sense, single-stranded RNA virus belonging to the family *Dicistroviridae*, *Homalodisca coagulata virus-01* (HoCV-01*)* has been identified in wild *H. vitripennis* populations and shown to increase mortality in those populations[2-4], while lowering the insect's resistance to insecticides.

Development of methods to effectively rear infected *H. vitripennis* to adulthood in a laboratory setting have been difficult because *H. vitripennis* have different stage-specific nutritional needs that require a variety of host plants[5-8]. Specific facilities are required to rear live *H. vitripennis* in the United States; therefore, cell culture is more economical and a viable alternative, as well as increasingly vital for HoCV-01 detection and replication[2,9]. While basic methods for establishing cell cultures of *H. vitripennis* are described, these methods have not yet been utilized for commercial production of biological control agents, such as viruses[2].

The overall goal of the following procedures is to produce a high concentration of HoCV-01 suitable for utilization as a biological control agent. Viral replication requires a living cell, which is why successfully cultivating and optimizing *H. vitripennis* cultures is vital to the progress of producing profitable levels of virus.

Protocol

1. Cell Culture

NOTE: *Homalodisca vitripennis* cell lines established by the Dr. Wayne Hunter laboratory at the USDA Agricultural Research Service (Ft. Pierce, FL USA) were used to initiate a lab stock composed of mixed cell stages including initial fibroblasts and monolayers.

1. Perform the following procedures in a sterile laboratory environment maintained at a temperature range of 20-24 °C with 25 cm^2 culture flasks.
2. Cultivate and maintain cultures in 25 cm^2 tissue culture flasks using H2G+ Leafhopper medium, a modified WH2 honeybee media[10] (**Table 1**).
3. Incubate culture flasks at 24 °C with ~53% humidity.
4. Use an inverted microscope at a total magnification (TM) of 100X to monitor culture growth, *e.g.,* check for any abnormal cellular growth, monitor for any potential contaminants, and check growth progress across the culture surface.
5. Perform a complete medium change (~4 ml culture medium per flask) every 7 - 10 days without disturbing the culture surface.
6. Pass cultures when the culture surface is approximately 80% covered by cell growth (confluent) using 0.25% trypsin containing ethylenediaminetetraacetic acid (EDTA) to dissociate cells. Add ~2 - 3 ml of trypsin to each culture flask and expose culture(s) to the enzyme for short periods of time (5 - 10 min) to achieve complete cell dissociation.
7. Add an equal amount of fresh medium to the culture flask(s) (~2 - 3 ml) to stop enzyme activity and transfer the whole solution to a conical tube for centrifugation.
8. Pellet cells by centrifugation at 4 °C for 6 min at 350 x g.
9. Remove the supernatant without disturbing the cell pellet and add ~8 ml of fresh medium to each tube. Gently homogenize cells using a pipette.
10. Split cultures at a 1:2 ratio for 25 cm^2 flasks (~4 ml of cell solution per flask) and leave freshly passed cultures undisturbed for 48 hr to allow cells in suspension to attach securely to the surface of the flask.
    NOTE: Cultivation of cells in 48-well sterile tissue culture plates with a growth surface of 1 cm^2 is also possible and they can be maintained in the same manner as culture flasks with a reduction in volume of culture medium to ~250 µl.

2. Whole Virus Extraction

1. Homogenize whole bodies of virus positive *H. vitripennis* in phosphate buffer, pH ~7.2, with 0.02% sodium diethyldithiocarbamate trihydrate (DETCA) by vortexing for ~10 sec intervals until there are no more large clumps of tissue present. Extract virus via superspeed centrifugation at 124,500 x g for 4 hr at 4 °C or 22,000 x g for 16 hr at 4 °C. If a precipitate forms at the top, remove it with a sterile cotton swab[11].
2. Discard the supernatant.
3. Collect the pellet and dissolve with 5 ml of 10 mM phosphate buffer (no DETCA), pH ~7.2, containing 0.4% Na-deoxycholic acid and 4% polyethylene glycol hexadecyl ether (Brij 52).
    1. To mix the pellet well, remove the pellet from the side of the tube and crush up until in solution if necessary.
    2. Add more 10 mM phosphate buffer in ~5 ml increments to help the pellet go into solution if needed and combine into 2 tubes[11].
4. Centrifuge the solution at 300 x g for 15 min[11].
5. Remove the supernatant and pass the solution through a 0.45 µm filter, and collect the filtrate in large collection tube[11].
6. Transfer filtrate to a dialysis membrane with a molecular weight cut off (MWCO) of 3.5 kD, using small amounts of 10 mM phosphate buffer (pH 7) containing no DETCA if needed[11].
7. Place the dialysis membrane in a large beaker filled with ddH_2O at 4 °C. Change the ddH_2O every hour for 5 - 6 hr, until a white precipitate forms in the membrane[11].
8. Collect the purified virus from within the membrane and subject the 100% virus solution to a 10-fold dilution series by adding 10 µl of solution to 90 µl of ddH_2O. Subsequently add 10 µl of the dilution to another 90 µl of ddH_2O until reaching a dilution of 1:100,000.
9. Store virus solution at -80 °C.

3. Viral Replication

1. Seed cells in 48-well culture plates and grow all rows until cell growth is 80% confluent (approximately 72 hr post-pass if cells are growing at an average rate).
2. Inoculate each row of cells with the serial diluted virus once cell growth reaches confluency, *i.e.,* one row of a 1:10 dilution, one row of a 1:100 dilution, *etc.*, except for the top row. Use the top row as a control and add 10 µl of ddH_2O to each well as a volume control.
    1. In order to establish a starting baseline cell concentration for comparison with experimental cell counts, dissociate and count the first well of each row prior to the initial inoculation of any wells in the cultures with virus.
3. Monitor culture plates every 24 hr after viral inoculation for any color change in the medium indicating a pH change and for any changes in cell morphology.
4. Image one column of the test plate at each time point, using an inverted microscope at 100X TM.
5. Remove all medium from the column that was imaged at each 24 hr time point and store it short-term at -20 °C for RNA extraction and viral quantification or long term at -80 °C.
6. Dissociate cells from the wells after removing the medium as previously described in steps **1.6 – 1.9**, using only ~250 µl of 0.25% trypsin EDTA and fresh medium to stop enzyme activity and ~250 µl of fresh medium to re-suspend the cell pellet.
7. Add 10 µl of 0.4% trypan blue stain to each tube containing cells to perform cell counts after cell dissociation for each 24 hr period over one week. Allow stain to sit for 10 min before performing cell counts.
    1. Perform cell counts within 1 hr of exposure to stain or viable cells will begin to uptake stain as well as non-viable cells.
8. Slowly add 10 µl of the stained cell solution to each side of a standard hemocytometer using a micropipette. Allow the solution to be taken up by capillary action to avoid air bubbles.
9. Count the number of viable (non-stained) cells on both sides of the hemocytometer (the equivalent of 4 counts of the 16 squares in the hemocytometer). Perform cell counts for each well of the column that was removed.
10. Average the cell counts from each column and use the following formula to obtain the total cell count number: (average number of cells / 4) x dilution factor = number of cells x 10^4 ml. This is the cell density or number of viable cells in the culture.

4. Virus Extraction from Cell Culture

1. Remove treated *H. vitripennis* cells from culture flasks as previously described in steps **1.6 – 1.8**, using only ~250 µl of 0.25% trypsin EDTA and fresh medium to stop enzyme activity.
2. Discard the supernatant.
3. Extract virus from culture cells following the protocol previously described in steps **2.3 – 2.8**.

5. RNA Extraction

1. Extract RNA from medium samples collected during each one-week virus trial using a guanidinium thiocyanate-phenol-chloroform extraction designed for liquid samples per the manufacturer's protocol.
2. Store extracted samples at -80 °C.

6. RT-PCR

1. Establish viral standards for RT-PCR by running traditional PCR using the primer pair HoCV RT-PCR primer 1 (forward 5′-GCTCCCCGGCTTTGCTGGTT-3′, reverse 5′-ACGACGGATCTGCGTGCCAA-3′) with virus isolate from whole body *H. vitripennis*.
2. Subject PCR product to gel electrophoresis for 60 min at 120 V in a 2% agarose gel containing 0.1% ethidium bromide.
3. Excise the bands from the gel and purify using a gel extraction kit as per the manufacturer's protocol.
4. Pool all excised and gel purified product and further purify by basic ethanol precipitation. Elute the precipitation product in approximately 30 µl of Tris EDTA (TE) to increase the overall sample concentration.
5. Quantify the level of cDNA in pooled samples via spectrophotometry using 260/230 wavelength settings for nucleic acid detection.
6. Perform a ten-fold serial dilution series of the purified sample ranging from 57 ng/µl to 57 ag/µl (10^{-18}). Perform the serial dilution similar to the one described in **2.8** with adjustments made for the difference in concentration requirements.
7. Determine detection limits of the dilution series by performing qRT-PCR using a qRT-PCR kit with reliable quantification of low-abundance transcripts.
   NOTE: Viral concentrations lower than 5 x 10^{-3} copies are not detectable.
8. Extract RNA from experimental samples as described previously in step **5.1** and quantify using spectrophotometry.
9. Normalize all the extracted samples to 5 ng/µl using nuclease free water.
10. Perform qRT-PCR on all samples, in duplicates, as 25 µl reactions using a one-step qRT-PCR kit with the ability to sense low copy numbers as follows: 50 °C hold for 10 min; 95 °C hold for 5 min; 30 cycles of 95 °C for 10 sec, 60 °C for 30 sec; melt from 50 - 99 °C for 5 sec on each step.
    1. Make the master mix so that each reaction mixture contains 12.5 µl of 1x master mix, 1.0 µl (0.3 µM) of forward primer, 1.0 µl (0.3 µM) of reverse primer, 0.25 µl of reverse transcriptase and variable amounts of template based on standardization values.
    2. Bring the total reaction volume to 25 µl with RNase free water.
    3. Include five standard concentrations in each PCR run with the following copy numbers: 5 x 10^{-10}, 5 x 10^{-8}, 5 x 10^{-6}, 5 x 10^{-4}, and 5 x 10^{-2} copies. Set the threshold for each run to just below a fluorescence of $10x^{-2.5}$ to reduce noise during early acquisition at the beginning of each run.

7. Confocal Microscopy

1. Grow *Homalodisca vitripennis* cells in a twelve well plate containing glass coverslips measuring 18 mm in diameter in each well.
2. Inoculate one column on the plate every 24 hr for a period of four days, once a monolayer is achieved. Ensure that each column contains a control well, a low viral dilution (1:10) well and a high viral dilution (1:100,000) well. Use virus solutions obtained from step **2.8**.
3. Remove all media on the fifth day and wash cells twice with 1x PBS (pH 7.4).
4. Fix the cells with cold 4% paraformaldehyde at 4 °C for 30 min.
5. Add 500 µl of 1x Phosphate buffered saline (PBS) to the cells and wash them for 10 min at RT on a rocker at low speed. Wash the cells three times.
6. Add 500 µl of 0.1% Triton X-100 to the cells to permeabilize them. Let sit for 10 min at RT.
7. Wash cells again as previously described in step 7.5.
8. Add 500 µl of a 5% bovine serum albumin (BSA) solution to block cells at RT. Let sit for 2 hr and then remove.
9. Dilute stock Rhodamine red-conjugated phalloidin (RCP) 1:250 in 1x PBS containing 5% BSA and add 250 µl of the dilution to each well to stain for F-actin.
10. Cover plate in aluminum foil to prevent the dye from bleaching and incubate cells at 4 °C O/N.
11. Remove the RCP after 12 hr of incubation and replace it with 250 µl of 4′,6-diamidino-2-phenylindole (DAPI) (250 µg/ml) diluted in 1x PBS containing 5% BSA, to stain the nuclei of the cells.
12. Incubate the cells containing DAPI at RT for 1 hr.
13. Wash cells three times with 1x PBS as previously described in step 7.5.
14. Gently remove the coverslips from the wells and mount to microscope slides using a mounting media with an anti-fade reagent.
15. Allow the slides to dry in lightproof boxes until viewed under the confocal microscope. View prepared slides as soon as possible as dyes can fade rapidly.
16. Image stained cells using a confocal system equipped with a microscope containing a 63X (oil) plan-apochromate lens.
    1. Set the laser wavelengths to 543 ± 10 nm excitation and 575 ± 10 nm emission for Rhoadmine red-conjucated phalloidin, and 369 ± 10 nm excitation and 450 ± 30 nm emission for DAPI.
    2. Use identical gain and off-set settings for the detector to obtain all images.

3. Process images using appropriate software for image processing and sorting and import desired images into an image managing software.

Representative Results

Cell attachment and growth was seen within 48 hr of passage in both small and large culture flasks, from primary cultures and continued passages. Fibroblast growth and development was also observed within this time frame. When newly seeded flasks were disturbed before 48 hr, there was a visible decline in cell attachment, leading to slower growing cultures and sometimes no attachment or growth at all. Cells were approximately 80% confluent within one week of passing and formed a monolayer in 10-14 days (**Figure 1**). Primary cultures that were cultivated at the initiation of this study, have survived 30+ cell passages without any morphological deterioration or overall cell viability decline.

Cell attachment and growth was seen within 48 hr of passage from flasks to plates. Monolayer formation was achieved in a shorter time period, approximately 5-6 days, as it is a smaller growth surface. Infected cultures photographed at 100X under light microscopy showed signs of declining cell shape integrity. At 48 hr post-infection, what appear to be large holes were present on the surface of cells. Across culture flasks, cells shrunk and detached from the culture surface approximately 72-96 hr after being infected with non-diluted HoCV-01 (**Figure 2**).

Mean live cell counts for control and experimental samples were calculated and plotted to depict differences in abundance of live cells between viral loads over time (**Figure 3**). A consistent increase in live cells for the control group is present, indicating healthy cells. Comparatively, all treatment groups had a marked decline in the number of live cells present over time. The higher viral treatment groups indicate a much more marked decline in culture health with a major drop in live cells between 48-72 hr, while the lower viral groups slowly decline until dropping off around 144 hr. A two-way ANOVA with Bonferroni post-hoc analysis was used to test the differences in cell culture kill rates by HoCV-01 based on live cell counts in each treatment group compared to each time point in the study, as well as between groups. The two-way analysis of variance had a significant main effect of the time factor, F (7, 432) = 82.5, $p < 0.0001$, suggesting that the length of time cultures were exposed to a treatment affected culture longevity. The effect of the type of treatment cultures received was significant as well, F (5, 432) = 170.6, $p < 0.0001$, indicating that the amount of viral load a culture initially receives affects culture survival. A significant effect in the interaction between the time factor and treatment factor is also present, F (35, 432) = 17.63, $p < 0.0001$, underlining that the higher the viral load received the shorter amount of time needed to reduce culture fitness and conversely the lower the viral load, the longer period of time required for the same effect. Bonferroni post-hoc tests illustrate a significant difference between treatment and control groups, indicating a notable dose response.

Survival probability of cells inoculated with higher viral treatments was lower as tested by Kaplan-Meier curves (**Figure 4**), correlating to the conclusions drawn from the mean live cell count analyses. There was a 100% survival rate for control or non-infected cells. Cells exposed to the high viral treatments had a marked decline in survival probability over time, while lower viral treatments have a greater probability of survival until the 144 hr, then a decline in survival probability is present. Cox proportional hazards model analysis was not significant, treatment coeff = 0.8812 (95% CI [0.76, 1.02]), $p > 0.05$. While not significant, the data suggests that cells exposed to virus are 88% more likely to exhibit lower survival rates over time.

For each qRT-PCR run, the higher viral standards ramped up earlier than lower viral standards and experimental samples. From each run, replicate Ct values were analyzed using a two-way ANOVA to test for differences in the abundance of HoCV-01 RNA present in experimental samples and compare values to multiple control values. The two-way analysis of variance showed no significant main effect of the time factor, F (7, 289) = 0.38, $p > 0.05$, or in the interaction between time and treatment groups, F (63, 289) = 0.14, $p > 0.05$. A significant main effect between treatment groups, F (9, 289) = 135.7, $p < 0.0001$, indicating that amount of virus initially introduced to cell culture affects the amount of viral RNA detected was observed. Bonferroni post-hoc tests were run and show significant interactions between treatment groups and control measures, but no significant interactions between treatment groups alone, indicating no measureable dose response.

Differences in cell morphology of healthy and HoCV-01 infected *H. vitripennis* cells at 24 and 72 hr were seen under fluorescence. The decline of number of nuclei present as well as the misshapen appearance of F-actin in the cells exposed to HoCV-01 as compared with controls indicate that the virus has a major impact on culture health. Cells exposed to the higher 1:10 viral load showed greater distress than the cells exposed to the lower viral treatment. Control cells appear more abundant and to have normal morphology between the two time points (**Figure 5**).

Grace's Insect medium (supplemented, 1x)	210 ml
0.06M L-histidine monohydrate solution (pH = 6.5)	290 ml
Medium 199 (10x)	10 ml
Medium 1066 (1x)	17 ml
Hank's Balanced Salts (1x)	33 ml
L-Glutamine (100x)	1.5 ml
MEM, amino acid mix (50x)	1.5 ml
1 M MgCl solution	6 ml
Pen-Strep (w/ Glutamine)	2.5 ml/500 ml
Nystatin	1.0 ml/500 ml
Gentamycin	1.5 ml/500 ml
Dextrose	1.8 g
Fetal Bovine Serum	10% of final volume

Table 1. H2G+ leafhopper medium components.

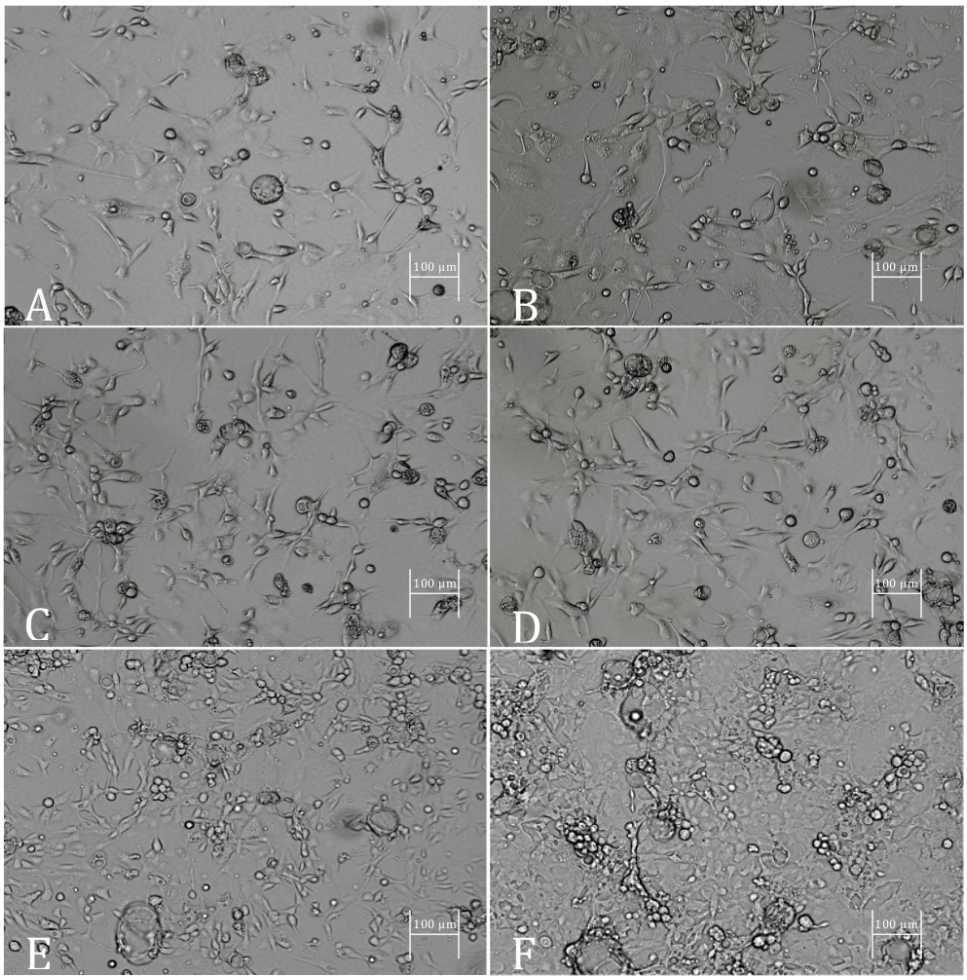

Figure 1. Images of *H. vitripennis* cell growth *in vitro* captured at 100X TM. (A) Cells two days (48 hr) post-passage exhibiting attachment and fibroblast development. **(B-E)** Cells four, six, eight and ten days post-passage continuing to grow across culture surface. **(F)** Monolayer formation occurring ~10-14 days. Scale bars: 100 µm. Please click here to view a larger version of this figure.

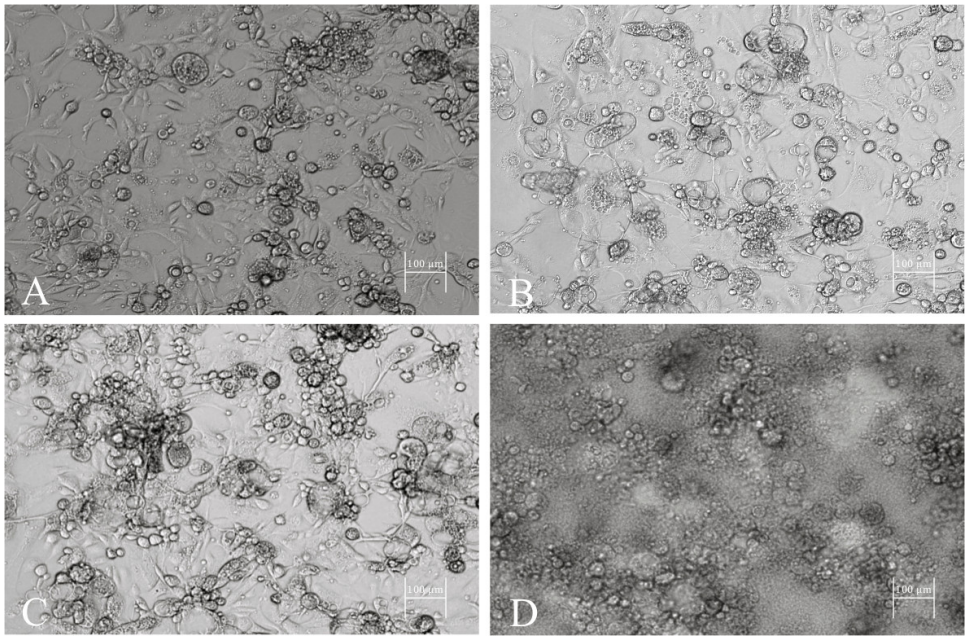

Figure 2. Infected *H. vitripennis* cells imaged at 100X TM to capture morphological changes. (A) Fibroblast growth prior to inoculation. **(B)** Cells 24 hr post-infection. **(C)** Cells 48 hr post infection. **(D)** 96 hr post infection cells have mostly detached from the culture surface and medium has become cloudy. Scale bars: 100 µm. Please click here to view a larger version of this figure.

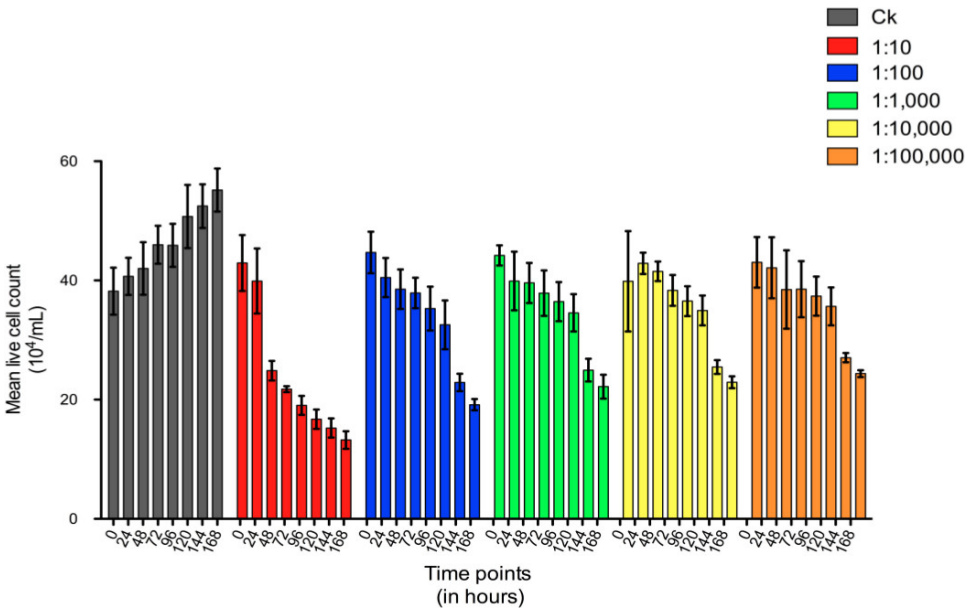

Figure 3. Bar chart showing mean live cell counts for experimental samples. Mean live cell counts were calculated for experimental samples by viral load received for each day during the experimental period and are shown here with standard deviation bars. Mean cell counts show a significant decrease in live cells ~72 hr post-infection with high viral loads and significant decreases in live cell counts at ~144 hr post-infection with lower viral loads. Please click here to view a larger version of this figure.

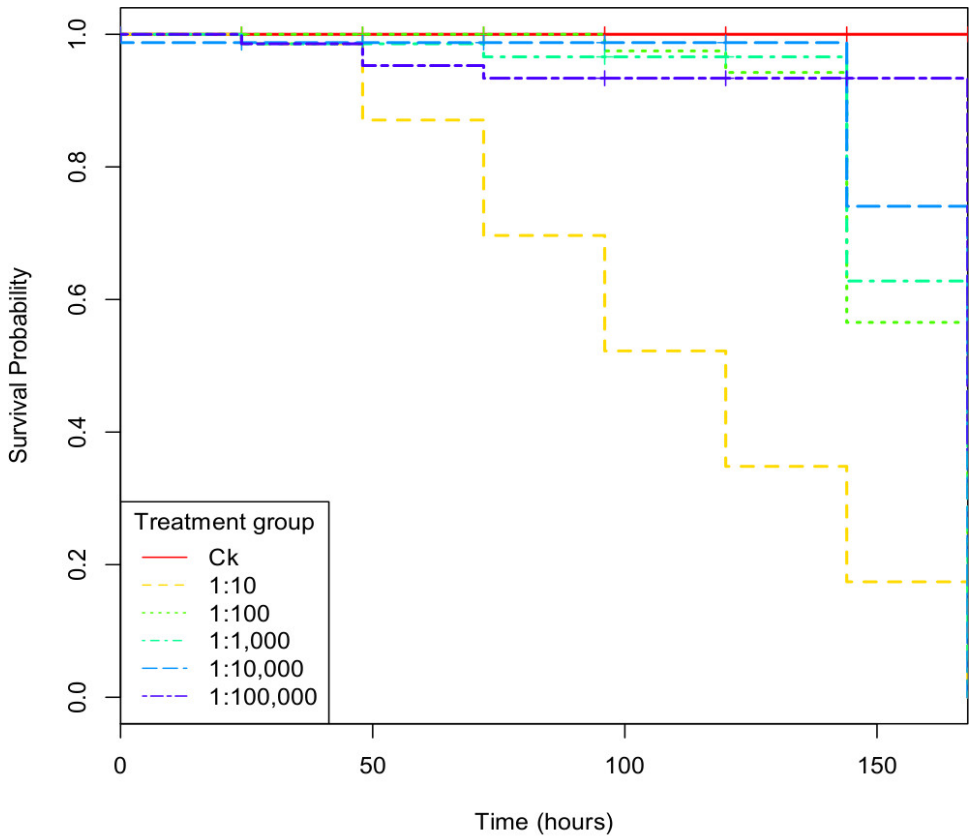

Figure 4. Kaplan-Meier survival analysis of five different HoCV-01 treatments compared to a non-infected control in *H. vitripennis* cell cultures. Control cultures maintained a 100% survival rate compared to the five treatment groups. The lowest survival probability was seen in the high treatment group and all treatment groups head towards zero survival probability at 168 hr when n = 10. Please click here to view a larger version of this figure.

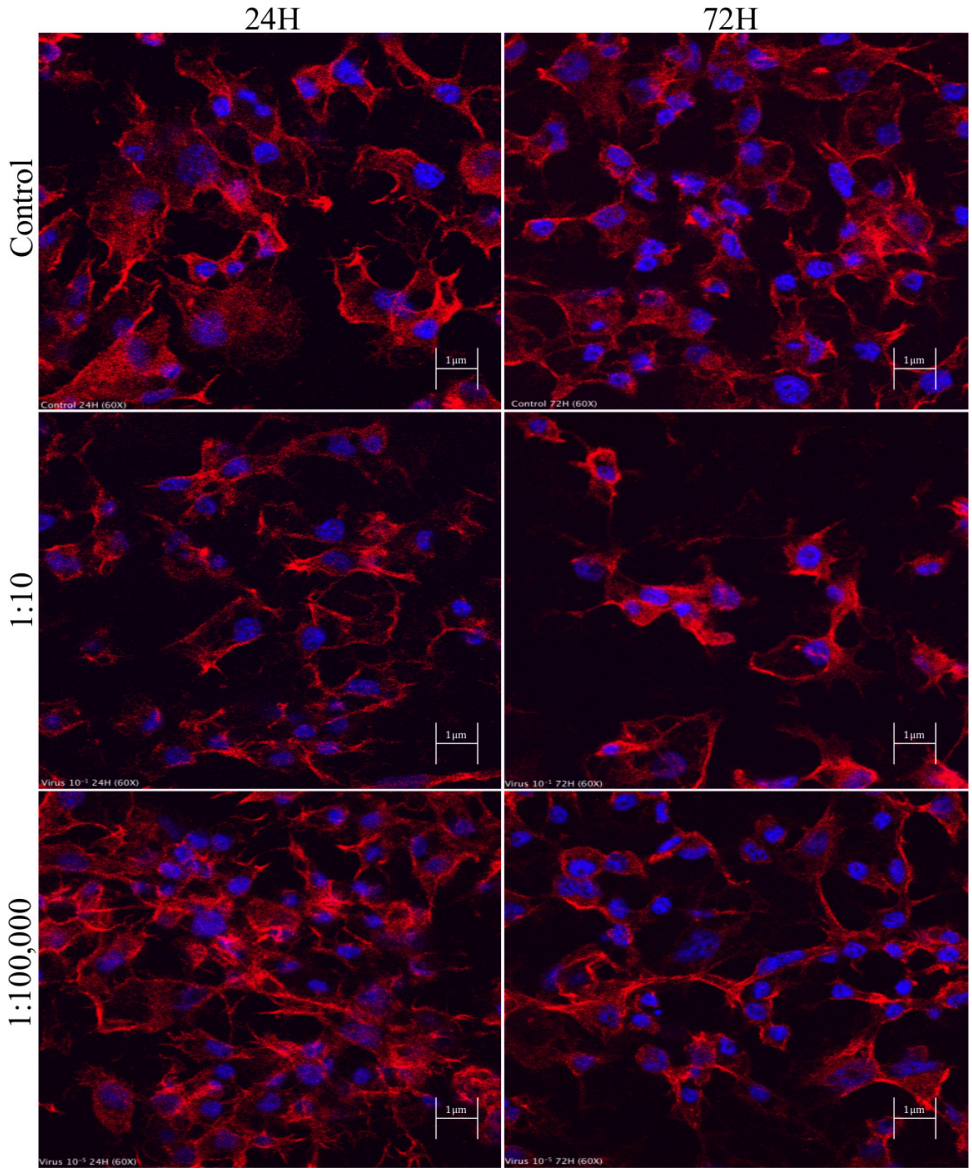

Figure 5. Confocal images of control and infected *H.vitripennis* cells. *Homalodisca vitripennis* cells were infected with serial diluted HoCV-01 in 1:10 and 1:100,000 concentrations at 24 hr intervals. Cells were treated with rhodamine phalloidin and DAPI stains to visualize F-actin and nuclei within the cultures. Confocal images were captured at 63X and show a break down in cell morphology at 72 hr at both low and high viral dilutions. Scale bars: 1 µm. Please click here to view a larger version of this figure.

Discussion

Rising concerns regarding the influx of invasive agricultural species have lead to an increased demand for new methodologies to defend against emerging pests and pathogens. A focus of disease prevention and management involves the management of pathogen vectors and was the primary target of this study. Economics play a vital role in the decision to produce this type of biopesticide to manage pathogen vectors in agriculture because the practical application needs to be large quantities over large areas but at a low cost[12]. The practice of utilizing cell culture for research and development has become increasingly common and as such, the impacts of this study are significant. Identifying economically feasible integrated pest management (IPM) strategies is key to continued successful agricultural production worldwide and the findings in this study contribute to progress in improving IPM strategies and reducing the occurrence of PD of grapevine.

Primary *H. vitripennis* cell cultures were propagated and maintained for over a year without any visible morphological deterioration. Passage numbers can drastically affect the results of *in vitro* studies in mammalian cells by changing cell metabolism and growth characteristics[13]. As cells replicate *in vivo* and *in vitro,* telomeres shorten with each round of cell replication and eventually reach a critical limit where telomeres become too short and induce cellular senescence[14]. When severe telomere shortening occurs, genetic instability and finally crisis, or massive cell death occurs[15]. Because of this phenomenon, cultures rarely survive beyond 50 subcultivations or one year, deemed the Hayflick Limit, based on the following criteria: retention of sex chromatin, histotypical differentiation, inadaptability to suspend culture, non-malignant characteristics *in vivo,* finite limit of cultivation, similar cell morphology to primary tissue, increased acid production compared to cell lines, retention of Coxsackie A9 receptor substance and ease with which strains could be developed[16]. The potential complications of passage

numbers were not observed during the duration of this study indicating that this method of cell line propagation is capable of continual production over extended periods of time. Because utilization of cell culture over live insect rearing or other expensive and complicated production methods is rapidly becoming commonplace across many disciplines, the longevity of this type of cell line has many practical applications. If maintained properly, a single primary cell line could be used for multiple rounds of virus production.

The two major factors in successful long-term maintenance of these cells were disturbance time for freshly seeded cultures and proper medium preparation. Cultures that remained untouched for the first 48 hr after passage showed a marked increase in cross-flask growth compared to those that were moved within that initial window. When left undisturbed, the rapid replication of cells achieved monolayers in as little as ten days post-passage in culture flasks. Medium preparation was as vital during the study as disturbance time as far as general culture health was concerned. Even with antibiotics present in the medium, bacterial contamination was still an important factor to consider when preparing medium. By allowing aliquots of medium to remain at RT for several days before use in cultures, the likelihood of a devastating series of culture collapses because of bacterial contamination was reduced to a nearly non-existent factor. Antibiotics, such as gentamicin and streptomycin, are commonly used in cell culture medium to combat bacteria found within the cells and any outside contamination. Both of these antibiotics have been linked to a depression of cell growth in mammalian cultures and to a decrease in the use of aseptic techniques and concern for increasing the likelihood of developing antibiotic resistant strains of bacteria[17,18]. Antibiotics should not be used excessively; however, their use is necessary for preventing cell culture contamination issues.

The implications of these factors are such that up-scaling production of cells is a viable option for quick mass production of biopesticide materials with minor steps to ensure quality of cell cultures. Bioreactors emerged in the 1950's and 1960's, and have since evolved to provide efficient means of producing billions of cells in an exceptionally short amount of time[19]. Many types of bioreactors exist that could be utilized to dramatically increase the number of *H. vitripennis* cells produced at one time and the process of developing this type of production system would require the development of a method to treat cells to prevent shearing from growth surfaces in bioreactors.

Successful continued growth of cell lines is crucial to HoCV-01 replication and once achieved, can be used to address the issue of how much virus is needed for quantifiable *in vitro* replication and how long should the virus be allowed to remain within cultures. A clear correlation was found between amount of initial viral load received by cells and the duration of time virus particles were permitted to incubate within cells. The higher the viral load received, the lower the time requirement for cell death, indicating rapid viral replication. However, across all treatments cell numbers declined to below the threshold value of 25×10^4 cells/ml at approximately 144 hr post-infection, demonstrating an overarching cell culture survivability threshold. The results illustrate that large amounts of virus can be produced quickly if larger amounts of virus are readily available for initial infection, or that increased amounts of virus can be produced over a longer period of time with lower initial dosage. Variability in the relationship between concentration and time factors allows some flexibility in production options for larger-scale studies with an optimal viral extraction time of 72-96 hr post-infection.

Using cell cultures for viral studies is dependent on the ability to detect the target virus and quantify the results of the study. A reliable method for this is to use PCR to check for the presence of viral RNA sequences within experimental samples. The analysis of Ct values from PCR data in this study does not give a clear answer to what the optimal extraction time of virus would be; however, lack of a definitive extraction time is not indicative of an inability to replicate HoCV-01 *in vitro,* but of the sensitive nature of viral studies. Cell counts using trypan blue do lend to a clearer view of optimal extraction times but are by no means a definitive answer. Cell death data illustrates that high viral loads lead to highly decreased cell survivability after 72 hr, indicating that viral extraction between 48 hr and 72 hr post-infection may be ideal to reduce cellular breakdown of viral particles as the cells in the culture begin to die exponentially. Extraction times at lower initial viral loads are more ambiguous, but with dramatic decreases in cell survivability after 144 hr, it can be speculated that optimal extraction time would be 24 - 48 hr prior to that time point. Determination of optimal viral extraction time is vital to effectual production of biopesticides for use against *H. vitripennis* infestations and this study has taken an important step towards determination of those times.

Microscopy is a key tool for cell culture analysis and this study is the first one to use confocal microscopy with *H. vitripennis* cells. Imaging protein attachment increase or decline and abundance of cell nuclei is the first step towards more detailed studies into the intracellular activity of HoCV-01 *in vitro*. Throughout this study, cell cultures were maintained with no visible morphological deteriorations. After each subsequent cell passage, normal fibroblast growth was observed, followed by the formation of a monolayer. Cells remained uniform in shape and size. However, when infected cultures were imaged with confocal microscopy, cell morphology declines were observed, especially at the 72 hr time point, with both high and low initial viral loads. Small hole-like structures could be seen on cell surfaces, possibly due to cell death and expelling of intracellular material. Cells across the flask surface shriveled and eventually began to detach from the surface. The implications from this first use of higher resolution microscopy correlate to the results seen in the cell survivability analysis and gives rise to other possible uses for increased viral studies. Advanced microscopy techniques could be utilized to its maximum capabilities if antibody development for HoCV-01 was conducted. Antibodies for the virus would not only allow visualization of intercellular workings of the viral particles but could also help determine proliferation rates and even more precise extractions times.

The process of scaling up production methods developed in this study to produce an effective biological control agent to a point where large biomasses of cells are harvested for virus and then applied to fields is mostly a matter of cost. Initial costs of building up large scale systems is high and many factors have to be considered, such as: profitability, cell and virus productivity, cell culture medium costs, application rate, production scale and batch production costs[12]. Despite initial cost, the potential pay offs are worth the investment. By utilizing cell culture techniques, down stream purification issues are greatly reduced because the possibility of gut microbe and other viruses found within a whole body insect are vastly reduced, as well as facility costs for live insect maintenance and rearing.

With cell culture already being utilized for production of proteins, biopesticides and other pharmaceuticals, the economic value for this area of research is increasing. For large-scale production of biopesticides in agriculture, it would be beneficial to try a similar study to the one completed here but using a larger cell growth system. Bioreactors and the new methodology of 3D matrix cell growing systems allow for larger volume production of cells and, in the same respect, larger volumes of viral production[20]. While the initial cost of building up large-scale systems is high, the payoff in the amount of product able to be produced has the potential to be even greater. An area of study that would greatly lend to furthering transmission studies in large-scale systems described above is antibody design. Antibody design has been used increasingly in viral

studies for diseases like HIV and Hepatitis C. While it is a time consuming and detailed process, for HoCV-01, it would allow for visualization of viral activity *in vitro* with confocal microscopy and could lead to other areas of investigation.

Disclosures

The authors have nothing to disclose.

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