REPLICATION OF *Oryctes* NUDIVIRUS (OrNV) IN INSECT CELL LINE DSIR-HA-1179 AND ITS INFECTIVITY ON NEONATES OF RHINOCEROS BEETLE, *Oryctes rhinoceros*

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ABSTRACT

The Oryctes *nudivirus (OrNV) or previously known as* Oryctes *baculovirus is a biocontrol agent for rhinoceros beetle,* Oryctes rhinoceros. *The replication of three types of OrNV, the types A, B, and C in the insect cell line DSIR-HA-1179 was studied. The cells were grown in PS100 media containing 10% fetal bovine serum. The signs of infection in cells by all types of OrNV were clearly seen at 10 to 14 days after treatment (DAT). The infected cells experienced cytopathic effects (CPE) involving formation of high number of vesicle or globule like-structures around the cells. The density of viable cells in flasks inoculated by the nudivirus decreased over time. At 16 DAT, the viable cell density in flasks inoculated with OrNV types A, B, and C was 1.49×10^5, 0.29×10^5, and 4.78×10^5 cells ml^{-1} respectively, significantly lower (P<0.05) compared to control flask of 7.98×10^5 cells ml^{-1}. Based on the Tissue Culture Infectious Dose 50% (TCID$_{50}$), it was found that the OrNV type B was more virulent than the OrNV types A and C as it produced high infectious virus particles. The TCID$_{50}$ values for the OrNV types A and C were 3.00×10^7 infectious unit (IU ml^{-1}) and 8.03×10^5 IU ml^{-1} as compared to type B which was 7.09×10^7 IU ml^{-1}. The PCR analysis showed that all types of OrNV had successfully replicated in the cells as early as 4 DAT. Pathogenicity study on neonates using cells-propagated OrNV has again showed that the OrNV type B was more pathogenic than the other types of OrNV. The LT$_{50}$ value for the OrNV type B was 33.47 days, shorter than the OrNV type A (49.35 days) and the OrNV type C (48.58 days). The DNA profiles of OrNV extracted from dead infected neonates in the bioassay were the same as those from infected gut tissues collected from field population. Thus, it is suggested that the Malaysian OrNV types A, B and C were successfully produced in cell line DSIR-HA-1179.*

INTRODUCTION

The rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) is a notorious pest of palm trees, particularly the oil palm *Elaeis guineensis* and coconut *Cocos nucifera* planted in South-east Asia and Pacific Islands (Bedford, 2013). Infestation rendered by the adult beetles which fed on the spear tissues of the palm lead to yield reduction and delayed in palm maturity. Severe damage can cause palm death. The infestation of the beetle may expose the palm to a secondary attack by the palm weevils *Rhynchophorus* sp. (Bedford, 2013). The outbreaks of the rhinoceros beetle were commonly reported in newly replanted areas. This is mainly due to high abundance of

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decomposed oil palm biomass that acts as breeding sites of the beetles (Cik Mohd Rizuan *et al.*, 2014). In Malaysia, a survey in 2004 showed that the beetles were estimated infesting 12% of the total young planted area, and causing yield losses ranging between 20% and 25% (Chung *et al.*, 1999; Norman and Basri, 2004).

The *Oryctes rhinoceros* nudivirus (OrNV) or previously known as *Oryctes* baculovirus, is a natural pathogen of the rhinoceros beetle (Huger, 1966). The virus was discovered from diseased larvae in Malaysia. Following intensive studies covering taxonomy, structures and infectivity, the OrNV was found highly infective to larvae and adults of the rhinoceros beetle (Huger, 2005). The entry point of the nudivirus into the host is via the alimentary tract. In larvae, the virus replicates in the midgut and fat body tissues, while in the adult the virus replicates only in the midgut tissues (Huger, 2005). Subsequent to a successful pilot release study in Samoa, the OrNV was introduced into coconut growing countries within Asia and the Pacific Islands to control rhinoceros beetle infestation (Marschall, 1970; Bedford, 1980). The adult beetle was found to act as a flying vector, responsible for transmitting the virus between healthy adults. The main mode of infection in the population was via mating or feeding on virus-contaminated substrates (Huger, 2005). As the nudivirus lack occlusion bodies, it is susceptible to inactivation by unfavourable environmental factors such as high temperature, low moisture and microbial activity in breeding materials of the beetles (Wang *et al.*, 2012; Bedford, 2013).

In Malaysia, based on DNA profiles produced from digestion by endonuclease restriction enzyme *Hind*III, four different types of OrNV have been discovered (Ramle *et al.*, 2011). The OrNV type B is the most pathogenic against the rhinoceros beetle, therefore, it has the potential to be used in integrated pest management of the rhinoceros beetle with other methods such as chemical, mechanical, biological and physical control (Ramle *et al.*, 2011). Previously, the OrNV was mass produced via *in vivo* technique using live larvae or adults of rhinoceros beetle as its host media. Although this technique was practical and economical, yet it was tedious and inconsistent in producing high purity inoculum, which depends on the collection of beetles from the fields. Thus, an *in vitro* study to mass produce OrNV on cell line was initiated using a cell line DSIR-HA-1179 originated from sterilised eggs of African black beetle, *Heteronychus arator* (Crawford, 1982). As this is the only insect cell line that could support the propagation of OrNV (Crawford and Sheehan, 1985), the technology has been used to produce OrNV inoculum for virus introduction in many outbreak areas (Crawford and Sheehan, 1984; Zelazny *et al.*, 1987).

In this study, the replication of freshly isolated Malaysian OrNV types A, B and C from infected gut tissues on insect cell line DSIR-HA-1179 was evaluated. The infectivity of OrNV produced from cell culture on neonates of rhinoceros beetle was also determined.

MATERIALS AND METHOD

Source and Maintenance of Insect Cell Line

The insect cell line DSIR-HA-1179 used in the study was obtained from AgResearch, New Zealand that has been passaged for 467 times. The cells were maintained in a 25 cm^2 culture flask (Falcon) containing PS100 media with 10% of fetal bovine serum (FBS) and antibiotics. The cell cultures were kept in an incubator at 27°C and after the cells reached 80% confluent, they were then sub-cultured into a new flask. During sub-culturing process, the cells were dissociate from the flask wall by repeated aspiration of the medium to the attached cells. Subsequently, 1 ml of cell suspension was transferred into new flask containing 5 ml fresh PS100 medium supplemented with FBS and antibiotics.

Preparation of PS100 Media

The PS100 media was prepared by adding 45.7 g of Grace's Insect Culture Medium (Gibco, Invitrogen) into 1 litre Schott bottle containing 900 ml of sterilised Milli-Q water (SMqH$_2$O). The solution was mixed using a stirrer until all materials were dissolved. Then, 2.95 g of tryptose phosphate broth (Sigma, Sigma-Aldrich) and 1 ml of TC100 vitamin solutions were added into the media. The TC100 vitamin solutions were prepared according to recipe in *Table 1*. The media was then adjusted to pH 6.2 by adding 10 M sodium hydroxide (KOH) and the final volume was made up to 1 litre. To inhibit bacterial growth, 1 ml of gentamicin (Gibco, Invitrogen) was added into the media. Subsequently, the media solution was vacuum filtered through a 0.22 μm membrane filter (Corning, USA). Aliquots of 45 ml filtered media was then prepared in a 50 ml sterile tube and stored at 4°C, preferably in dark condition. Prior to usage, the PS100 media was added with 5 ml of FBS (Gibco, Invitrogen, Life Technologies) and an antibiotic 10 μl plasmocin (Invivogen) to inhibit the growth of mycoplasma.

Extraction of DNA of OrNV for PCR Analysis

The extraction of OrNV DNA from the infected gut tissues of adult beetles and the infected cell line DSIR-HA-1179 followed the method by Ramle *et al.* (2010). A total of 150 μl of supernatant from

TABLE 1. COMPOSITION OF TC100 VITAMIN STOCK FOR PS100 CELL CULTURE MEDIA

No.	Vitamin	Volume (mg litre^{-1})
1	Thiamine HCl	200
2	Calcium pantothenate	110
3	Pyridoxine HCl (vit B6)	200
4	p-aminobenzoic acid	200
5	Folic acid	200
6	Niacin (nicotinic acid)	200
7	I-inositol (iso-, myo-, meso-)	200
8	Cyanocobalmin	100
9	Riboflavin*	200
10	Biotin*	100

Note: * Dissolve in 1 N KOH first.
Make up stock to 1 litre and store frozen in 1 ml aliquots or larger.

homogenated gut tissues or cells solution were mixed with 300 μl disruption buffer (DB) which was made by mixing 50 μl 1M Tris pH 8, 10 μl 0.5M EDTA, 5 μl 10% SDS, 2.5 μl Proteinase K (20 mg ml^{-1}), and 232.5 μl SMqH$_2$O. The mixture was gently mixed and incubated at 65°C for an hour, then added with equal volume of phenol-chloroform-isoamylalcohol (PCI) (25:24:1). Mixing of solution was done by inverting the tube 100 times and followed by centrifugation at 13 000 rpm for 10 min. The clear upper solution containing the DNA samples was transferred into a new 1.5 ml centrifuge tube. The solution was then added with 10% NaAc pH 5.2 and two times the volume of 100% ethanol to precipitate the DNA before storing in -20°C for an hour. After centrifugation at 13 000 rpm, the solution was carefully discarded and the DNA pellet was air-dried before suspending in 100 μl TE buffer, containing 10 mM Tris HCl pH 7.5 and 1 mM EDTA pH 8.

Extraction of Genomic DNA of OrNV for Restriction Enzyme Analysis

The extraction of genomic OrNV DNA from the infected gut tissues was conducted using the method by Ramle *et al.* (2005). As restriction endonuclease enzyme required high amount of DNA to produce good DNA profiles, gut samples with swollen symptoms due to virus infection were grouped, macerated till homogenous and the debris was pelletised by centrifuging at 13 000 rpm for 2 min. The supernatant was then collected, filtered through a membrane filter of 0.45 μm pore size and the filtrate was centrifuged at 20 000 rpm for 3 hr. The supernatant was removed without disturbing the adhered pellet. A total of 600 μl of DB containing 100 μl 1M Tris pH 8, 20 μl 0.5 M EDTA, 10 μl 10% SDS, 5 μl Proteinase K (20 mg ml^{-1}), and 465.0 μl SMqH$_2$O was added into the tube. After incubating at 65°C for an hour, an equal volume of PCI was added into the mixture, mixed by inverting 100 times and centrifuging at 13 000 rpm for 10 min. The clear upper solution was transferred into a new tube and added with 1 μl of RNAse and then incubated at 65°C for an hour. To get high quality DNA, the previous steps starting from the addition of PCI up to centrifugation of mixture were repeated. The clear upper solution was collected and transferred into a new tube and then added with double volume of absolute alcohol and 10% of NaAc pH8 to precipitate the OrNV DNA. Precipitation of DNA was done by placing the tube at -20°C for about an hour. The OrNV DNA were pelleted by centrifuging at 13 000 rpm for 10 min and finally air-dried in a safety cabinet. The DNA was then suspended in 35 μl TE buffer, containing 10mM Tris HCl pH 7.5 and 1 mM EDTA pH 8.

Digestion of Genomic DNA by a Restriction Enzyme *Hind*III

The *Hind*III enzyme was commonly used in profiling the genomic DNA of OrNV by various workers (Ramle *et al.*, 2005; 2011; Crawford *et al.*, 1986). Prior to digestion using an endonuclease enzyme *Hind*III, the yield of each sample of the extracted genomic DNA of OrNV was analysed on 1.8% agarose gel. Appropriate volume of DNA was used for digestion process performed in a 1.5 ml centrifuge tube with a total volume of 100 μl solution. The digestion solution containing appropriate volume of OrNV DNA, 1 μl *Hind*III enzyme, 10.0 μl digestion buffer (10X), and appropriate volume of SMqH$_2$O. The tube was placed in a water bath at 37°C overnight and the digested DNA was then electrophoresed on 0.8% agarose gel for 20 hr.

PCR Analysis of OrNV

The PCR analysis was performed in a 0.2 ml PCR tube using a pair of specific OrNV primers developed by Richards *et al.* (1999) and the reaction was optimised following the method of Ramle *et al.* (2010). Briefly, a total of 26 μl PCR reaction mixture was prepared by mixing of 22 μl lysis buffer (14.63 μl of SMqH$_2$O, 1.25 μl of MgCl$_2$, 2.5 μl of 10X PCR buffer 10 mM, 0.5 μl DNTP, 1 μl each primer 15a and 15b, 0.125 μl *Taq* DNA polymerase, and 1 μl BSA 20 mg ml^{-1}) with 4 μl of OrNV DNA solution. Two control tubes were prepared, which were positive and negative controls. The positive control used confirmed OrNV DNA, while the negative control used only SMqH$_2$O. The PCR was carried out in a thermal cycler machine (Vapo Protect, Eppendorf) programmed for 30 cycles; denaturing at 94°C for 1 min, annealing at 72°C for 2 min and extension at 50°C for 1 min.

The amplified DNA samples were then run on 1.8% agarose gel prepared in 1X TAE buffer.

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The PCR products were then viewed under UV transillumination to confirm the presence of single band DNA of 945 bp.

Experiment 1: Replication of Malaysian OrNV on Cell Line DSIR-HA 1179

Source and preparation of OrNV inocula. The virus inocula were prepared from the infected gut tissues of adult beetles collected from the fields using trapping method. The OrNV type A was prepared from gut tissues collected from Munkar Estate in Johor, Malaysia and OrNV type B from Seberang Perak Estate in Perak, Malaysia and OrNV type C from Lahad Datu in Sabah, Malaysia. The adult beetles were dissected and the whole gut tissues with advanced sign of OrNV infection were collected and placed into a 1.5 ml centrifuge tube filled with 0.5 ml 50 mM Tris HCL. The tissues were then homogenised using a motorised micropestle homogeniser and followed by a short centrifugation at 13 000 rpm. The supernatant containing the OrNV particles was then collected and double filtered through 0.45 μm then 0.22 μm membrane filters. The types of OrNV were confirmed by restriction endonuclease enzyme as previously described. The concentration of each type of OrNV from each sampling locality was standardised by PCR Comparative Dilution Method.

Inoculation on cells with OrNV inocula. The experiment was conducted in the 25 cm^2 culture flask with the initial cell density of 1.0×10^5 viable cells ml^{-1}. The cell cultures used in this study have been passaged for 471 times. At the fourth day after passage, at which the cells were still at log phase, the cell culture was inoculated by adding 100 μl of respective OrNV solution. The flask for the control treatment was left un-inoculated. The cells were then incubated at 27°C in dark condition. To evaluate the successful replication of OrNV in the cell culture, the cell suspensions were sampled every four days up to 16 days after treatment (DAT). At each sampling day, three flasks were randomly selected and the density of viable cells growing in the culture was determined using a protocol as described below. Subsequently, 150 μl cell suspensions were collected and subjected to DNA extraction and PCR analysis using the methods as previously described (Richards *et al.*, 1999; Ramle *et al.*, 2010). The morphological changes of the infected cells in the culture were also observed.

Determination of viable cell density. As cell line DSIR-HA-1179 tend to aggregate, so dissociation cells method developed by Pushparajan *et al.* (2013) was applied to ensure accurate counting of cell density in the flask. The culture medium was firstly removed from the flask without affecting the adhered cells. Then, 3 ml of phosphate buffer saline (PBS) pH 7.4 (Gibco, Invitrogen) was added and then gently swirled to wash the calcium and magnesium ion remaining in the flask. The PBS was then discarded and 3 ml of new trypsin solution (TrypLETM Express, Gibco, Life Technologies) was added to cleave the protein bonding between the cells. The flask was then incubated at 37°C for 10 min. The trypsinisation process was halted by addition of 6 ml of the fresh PS100 medium into the flask. Continuous aspiration of the cell suspension may be required for resolving any clumped cells.

The viable cell density was determined using the Trypan blue Exclusion Method in which 100 μl of cell suspension was mixed with 100 μl of 0.4% Trypan blue dye (Gibco, Invitrogen) and left for 5 min. The cells were loaded onto a Neubauer Haemocytometer and the viable cells were counted. The viable cells appeared to be opaque in colour, while the non-viable cells were blue as it took up the Trypan blue dye colour on the haemocytometer (Strober, 2001).

Harvesting of OrNV from infected cell DSIR-HA-1179. The infected cells were harvested from three culture flasks for each type of OrNV at 16 DAT. The adhered and clumped cells were gently separated from the flask by aspiration. The cell suspension was then transferred into a 12 ml centrifuge tube and pelletised by centrifuging at 3000 rpm for 10 min. The supernatant was filtered through a 0.45 μm membrane filter. Samples of OrNV solution were subjected to DNA extraction for PCR analysis to confirm the replication of the virus in the cultures. The concentration of each OrNV was standardised using PCR Comparative Dilution Method as it would be used in the next experiment to determine the virus infectivity on cell line DSIR-HA-1179.

Experiment 2: Quantification of Infectious OrNV on Cell Line DSIR-HA-1179 Using the Tissue Culture Infectious Dose 50% (TCID$_{50}$)

The amount of infectious virus particles of each tested OrNV produced on cell line DSIR-HA-1179 was quantified using TCID$_{50}$ assay. The TCID$_{50}$ determine the amount of infectious virus particles produced in the cell culture and quantified as the number of infectious unit per millilitre (IU ml^{-1}). Titration was conducted using a 96-well titre plate. Prior to inoculation with the virus inocula, two drops of cell suspension collected from 100% confluent flask at passage number of 471 were added into each well of a 96-well plate. After two days of incubation at 27°C, each well was inoculated by adding 20 μl of OrNV solution which has been prepared from the respective 10-fold serial dilutions. Three titre plates were used to estim ate the TCID$_{50}$ for each type of

OrNV. The titre plates were placed inside an air-tight container and kept in an incubator at 27°C for two weeks before scoring of cells with cytopathic effect (CPE) was carried out. To maintain high moisture content inside the container, wet tissue papers were placed underneath the plate. The cells with CPE could be observed within 10 to 14 DAT. Wells with cells showing CPE were scored as positive and *vice versa*. The data was then transferred into an appropriate datasheet Microsoft Excel for estimation of $TCID_{50}$ (IU ml^{-1}) using a method developed by Reed and Muench (1938).

Experiment 3: Pathogenicity of OrNV Propagated in Cell Culture on Neonate of Rhinoceros Beetle

Rearing larvae for neonates of rhinoceros beetle. Third instar larvae (L3) of rhinoceros beetle were collected from rotting heaps of chipped oil palm trunks at Regent Estate in Melaka, Malaysia. Only larvae which appeared healthy or without any signs of fungal or virus infection were collected and placed inside a large plastic container (60 cm long × 45 cm wide × 30 cm high) half filled with rotting trunks. In the laboratory, the larvae were reared individually in plastic containers filled with rotting oil palm trunk tissues until they turned to neonates. The beetles were kept in an outdoor insectory at temperature of 25°C to 28°C. The food source for the larvae was changed biweekly. The development of larvae was observed during the replacement of new food materials and those larvae that turned to pre-pupae or pupae were separated and undisturbed until they transformed into neonates. The newly emerged neonates were collected and then transferred into a transparent box and kept for at least a week before they were used for bioassay. Prior to inoculation, the neonates were transferred into an empty container to starve for 24 hr. Starving beetles made the inoculation easier as they sucked virus solution faster (Ramle *et al.*, 2011).

Inoculation of neonates with OrNV. Four treatments, including control, were tested in this study. Three treatments involved inoculation of neonates with OrNV types A, B, and C produced from 2-week old cell culture. The experiment was conducted in a completely randomised block design (RCBD) with five replications. In each replicate, five neonates were tested. Prior to inoculation, the concentration of cell propagated OrNV was standardised using the PCR Comparative Dilution Method. The DNA used in the PCR was extracted from the cell suspensions with identified $TCID_{50}$ values determined as in Experiment 2. The concentration of OrNV inoculated onto each neonate was estimated at 1-2 × 10^4 IU ml^{-1}. Inoculation was done by placing 30 μl of OrNV solution prepared in 10% sucrose onto the mouthparts of the neonates. The inoculated neonates were then placed in a transparent plastic container, 11 cm width × 17 cm length × 7.5 cm height, in a group of 5 individuals per container. Sugar-cane was added into the container as a food source for the neonates. The containers were kept in the laboratory at temperature between 25°C to 28°C. The neonates in control were only inoculated with 30 μl of 10% sucrose solution. Data on mortality was recorded every four days until 60 DAT. The cadavers were dissected and the midgut tissues were sampled for confirmation of infection determined using PCR method as described above.

Statistical Analysis

Data on viable cell density in Experiment 1, and the values of $TCID_{50}$ in Experiment 2 were subjected to analysis of variance (ANOVA) using SAS analysis (SAS, 1997). The percentage of mortality and percentage of infection of OrNV on neonates in Experiment 3 were angularly transformed and then analysed using one-way ANOVA. The means were separated using Duncan's Multiple Range Test at P=0.05 (SAS, 1997). Probit analysis to determine the LT_{50} was performed using data on percentage of infection of OrNV confirmed by PCR, following method by Finney (1971) and Wigley and Kalmakoff (1977).

RESULTS AND DISCUSSION

The Production of Malaysian OrNV on DSIR-HA-1179 Cell Line

The successful propagation of OrNV in cell cultures was assessed based on three parameters: 1) the morphological changes of the infected cells, 2) the reduction of viable cell density, and 3) the result of PCR analysis of using OrNV specific primers 15a and 15b.

The infected cells showed typical CPE when the morphological changes of the infected cells such as the formation of high numbers of small vesicles around the cells were observed under 100X magnification of inverted microscope (*Figure 1*). Cells inoculated with types A, B, and C exhibited CPE starting from 10 DAT. The infected cells formed a round shape as the appendage structures disappeared from cells. Growth of the infected cells was usually noticeably lesser than the healthy cells (Crawford and Sheehan, 1985; Pushparajan *et al.*, 2013).

The impact of OrNV on replication of cells was also determined by assessing the reduction of viable cell density in the treated flasks as compared to the

untreated flasks. The density of the viable cells in the treated flasks was significantly lower (P<0.05) than the untreated flasks, starting from eight DAT up to 16 DAT (*Figure 2*). At 16 DAT, the OrNV type B recorded the lowest value of cell density 0.29 × 10^5 viable cells ml^{-1}, followed by the type A at 1.49 × 10^5 viable cells ml^{-1} and the type C at 4.78 × 10^5 viable cells ml^{-1}. The number of viable cells in flasks treated with all types of OrNV were significantly

lower (P<0.05) as compared to the control flask at 7.98 × 10^5 cells ml^{-1}. The reduction in the number of infected cells was due to the deterioration of cell nucleus, thus inhibiting cell division which led to cell death (Crawford and Sheehan, 1985).

Since the OrNV needs approximately two weeks to replicate in the cells and to show the infection symptoms, therefore, the impact of OrNV on replication of cells was only seen starting from
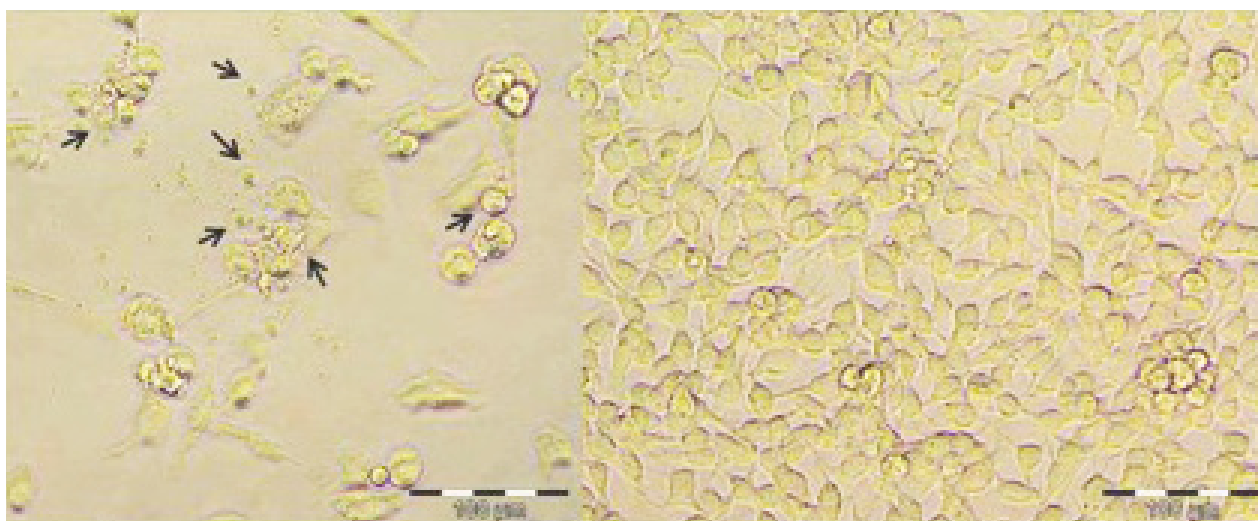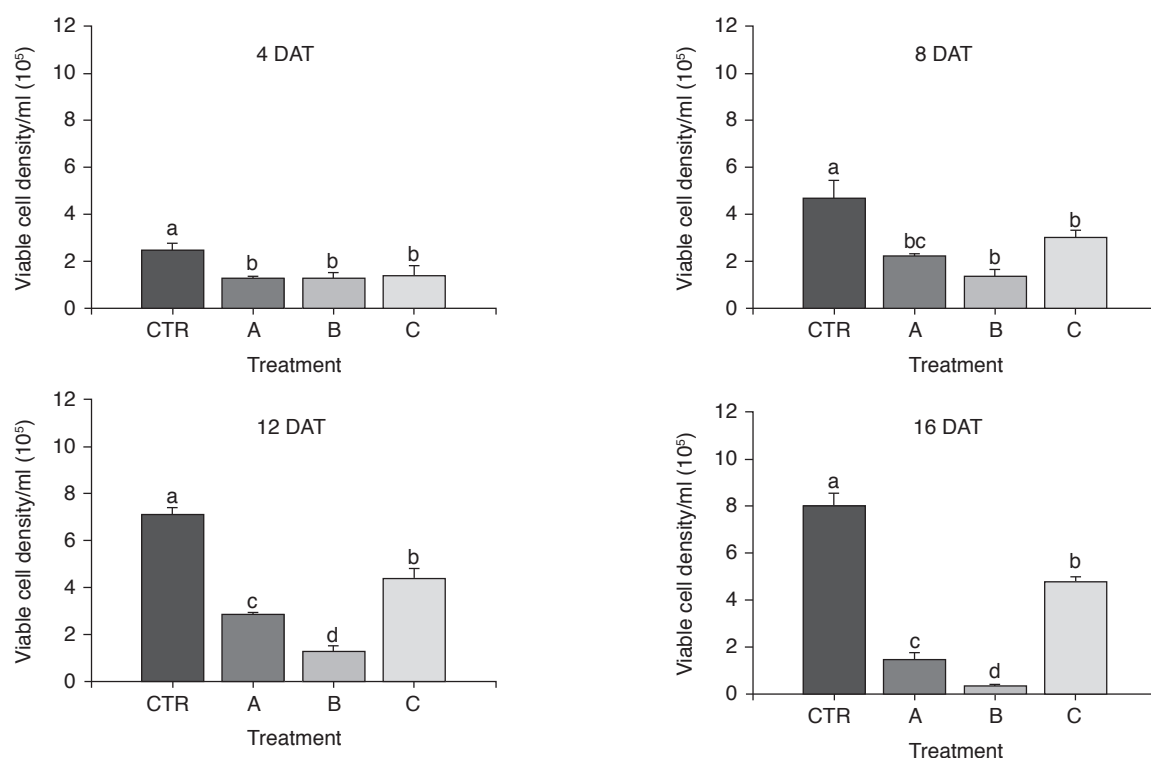

Figure 1. The morphological appearance between the infected (left) and non-infected (right) cells by Oryctes *nudivirus. Arrows showing the infected cells with cytopathic effects which form vesicles around the cells at 14 days after treatment.*

Note: DAT - days after treatment.

Figure 2. The effect of Orytes *nudivirus (OrNV) types A, B, and C on propagation of cell line DSIR-HA-1179. Bars in each graph with the same letters were not significantly different after Duncan's Multiple Range Test (DMRT) at P=0.05.*

12 DAT. Type B was found the most virulent against cells, causing significant reduction (P<0.05) on cell growth as compared to other types of virus, starting from 12 DAT onwards. Type A was ranked second and type C was the least virulent against the cell line DSIR-HA-1179.

The PCR results indicated that all types of OrNV had successfully multiplied in the cell culture as early as 4 DAT. The presence of a band at 945 bp on each sample was a clear indication that the cells were infected with the tested OrNV (*Figure 3*). The PCR method was reported to be highly sensitive in detecting the OrNV infection on rhinoceros beetle and has been used to determine the presence of virus in samples of rhinoceros beetle collected from various fields (Ramle *et al.*, 2010; 2011).

Quantification of Infectious OrNV on Cell Line DSIR-HA-1179 Using TCID$_{50}$

The amount of an infectious virus in a cell suspension is the fundamental knowledge required for large scale production of virus. The simplest

virus of 709.0×10^5 IU ml^{-1}, followed by OrNV type A of 300.0×10^5 IU ml^{-1} and OrNV type C of 8.00×10^5 IU ml^{-1} (*Table 2*). This experiment supported the findings in Experiment 1, where as a highly virulent OrNV, type B has caused the highest reduction in viable cell density. Massive replication of the virus in the nucleus of the cells has inhibited the cell division, thus increased the possibility of producing more virus particles in the other uninfected cells (Crawford and Sheehan, 1985). This situation explained the significant reduction on the numbers of viable cells inoculated with type B in Experiment 1.

This finding suggested that the OrNV type B was the most virulent on cell line DSIR-HA-1179, followed by OrNV type A and lastly OrNV type C. The range of TCID$_{50}$ recorded by OrNV types A and B was in agreement with the study carried out by Pushparajan *et al.* (2013), which also determined the TCID$_{50}$ values of OrNV in four different cell culture media. The OrNV type C was 100 times less virulent than types B and A and took longer time to establish CPE on cells.
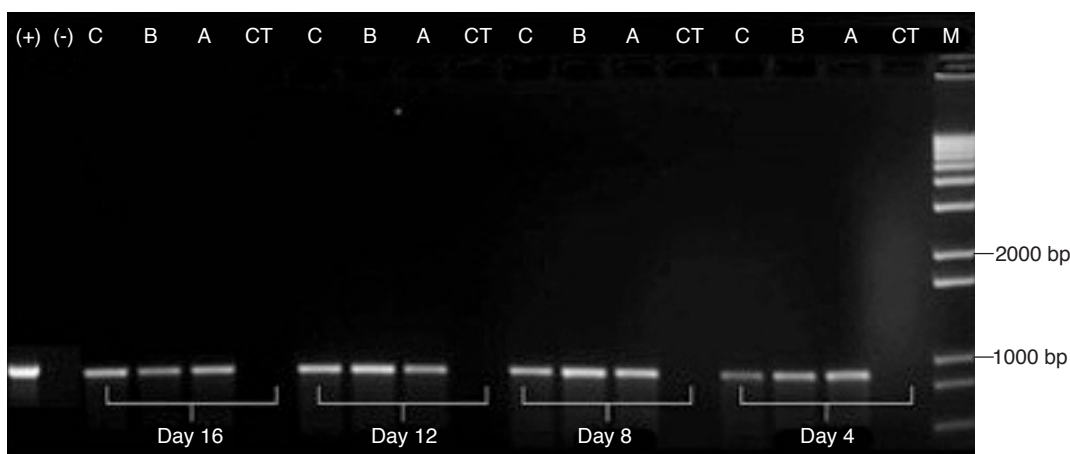

Figure 3. The polymerase chain reaction (PCR) analysis showing the presence of a single band of 945 bp on samples of cells inoculated with Orytes nudivirus (OrNV). M-1kbp Plus DNA ladder, CT- control (uninfected cells), A - OrNV type A, B - OrNV type B, C - OrNV type C, (-) - negative control, (+) - positive control.

and reliable technique to determine the amount of infectious virus particles is through the TCID$_{50}$ assay. The TCID$_{50}$ is defined as the virus concentration needed to cause lethal effect to 50% of the cells (Flint *et al.*, 2009). Higher TCID$_{50}$ values indicated higher production of infectious OrNV per titre of cell suspensions (Toong, 2016). In this study, the time required for OrNV to infect cells in plate and to show clear CPE was found between 10 to 14 DAT. At this period, cells with CPE were clearly observed, making wells with infected cell symptoms for TCID$_{50}$ analysis to be easily scored.

The titration results showed that the OrNV type B produced the highest concentration of infectious

TABLE 2. THE TCID$_{50}$ VALUES FOR *Oryctes* NUDIVIRUS (OrNV) TYPES A, B AND C AFTER TWO WEEKS OF TREATMENT IN CELL LINE DSIR-HA-1179

The TCID$_{50}$ values ($\times 10^5$ IU ml^{-1})*			
Type of OrNV	Type A	Type B	Type C
Mean**	300.0a	709.0a	8.0b

Note: *Calculation was based on Reed-Muench formula.
**Mean in row with the same letters were not significantly different after Duncan's Multiple Range Test (DMRT) at P=0.05.
TCID$_{50}$ - Tissue Culture Infectious Dose 50%.
IU ml^{-1} – infectious unit per millilitre.

Pathogenicity of OrNV Propagated on Cell Culture on Neonates of Rhinoceros Beetle

The percentages of mortality and infection of neonates inoculated with all three types of OrNV are shown in *Table 3*. Up to 60 DAT, the percentage mortality of neonates caused by all three cell-propagated OrNV increased at each data recording, however, differences between types were not significant (P>0.05). As early as 12 DAT, type A recorded the highest mortality of 12%, as compared to the OrNV types B and C, which caused 8% mortality. At this stage, all types of cell-propagated OrNV caused the same infection level of 4% on neonates. Starting from 24 DAT until 48 DAT, the OrNV type C caused the highest mortality as compared to OrNV types A and B. Nevertheless, the number of dead neonates due to infection by type B was higher than those by types A and C. At 60 DAT, the percentage mortality of neonates reached the highest level, in which both OrNV types A and B recorded the same mortality of 96% and type C recorded 92%.

Zelazny (1973) stated that the infected beetles took approximately 25 days to die as compared to the normal lifespan of adult beetles of 75 days. However in this study the infected neonates died as early as 12 DAT, then increased to the highest rate at 60 DAT. Based on the percentage of infection at 60 DAT, it was found that the OrNV type B was the most pathogenic, causing 88% infection, significantly higher (P>0.05) than type A at 68% or type C at 56% (*Table 3*). Findings of this study supported earlier works carried out by Ramle *et al*. (2011), who used OrNV freshly prepared from infected gut tissues in the bioassays. Their study found that type B is the most virulent against neonates, followed by OrNV types A and C. Even so, the percentage of infection in this study was much higher than those recorded by Ramle *et al*. (2011), who recorded 57.9% for type B, 28.8% for type A and only 3.2% for type C.

Probit analysis to determine the lethal time to kill 50% (LT_{50}) based on the dead beetles due to OrNV infection again showed that the OrNV type B was the most pathogenic against the neonates of rhinoceros beetle. The LT_{50} value for type B was 33.47 days, shorter than types A and C which required 49.35 days and 48.58 days, respectively (*Table 4*). The LT_{50} values for OrNV types B and C in this study were shorter than those results recorded by Ramle

TABLE 3. THE PERCENTAGE CUMULATIVE MORTALITY AND INFECTION OF NEONATES INOCULATED WITH CELLS-PROPAGATED *Orytes* NUDIVIRUS (OrNV) TYPES A, B AND C

Treatment	Percentage cumulative mortality and infection at days after treatment (DAT)									
	12 DAT		24 DAT		36 DAT		48 DAT		60 DAT	
	M	I	M	I	M	I	M	I	M	I
Control	0a	0a	0b	0b	0b	0b	0b	0b	0b	0c
Type A	12a	4a	28ab	16a	44a	28a	68a	44a	96a	68b
Type B	8a	a	40ab	32a	60a	52a	76a	68a	96a	88a
Type C	8a	4a	48a	28ab	72a	40a	84a	48a	92a	56b

Note:

M - mortality, I – infection.

Mean in column with the same letters were not significant different after Duncan's Multiple Range Test (DMRT) at P=0.05.

TABLE 4. PROBIT ANALYSIS OF CELLS-PROPAGATED *Orytes* NUDIVIRUS (OrNV) TYPES A, B, AND C PROPAGATED IN CELL CULTURE AGAINST THE NEONATES OF RHINOCEROS BEETLE

Parameter	Types of OrNV		
	Type A	Type B	Type C
LT_{50} (days)	49.35	33.47	48.58
Fiducial limit (days) (lower- upper)	30.87-78.89	23.82-47.01	26.64-88.61
Regression	Y= 3.17x − 0.38	Y= 3.87x − 0.90	Y=2.43x+ 0.90
Slope	3.17	3.87	2.43
Chi-square (χ^2 , df=3)	0.25**	0.17**	0.19**
Standard error slope	0.28	0.29	0.27

Note:

* Neonates of rhinoceros beetle were reared from larvae collected at the Regent Estate, Melaka, Malaysia.

** The significant regression represent the experiment at P = 0.05 (df=3, χ^2_{table} =7.8).

Y - Logarithm of days, X - Empirical probit for percentage cumulative infection.

et al. (2011), who found the LT$_{50}$ values for type B was 36.86 days and type C was more than 100 days. Results of this study could suggest that the OrNV produced *in vitro* on cell line DSIR-HA-1179 has improved the OrNV ability in infecting the neonates. This finding also supported the earlier work carried out by Jayawardena (2013).

Visual observation on the gut of infected dead beetles showed that most of them exhibited infection symptoms as described by Huger (2005) and Ramle *et al*. (2011). Infected gut tissues appeared to be swollen and whitish, while the healthy uninfected gut tissues appeared to be thin and brownish. The proliferation of OrNV occurred in the hypertrophied nuclei of the midgut epithelial cells. Sudden substantial proliferation of the epithelial cells was due to viral replication which caused the swelling of midgut lumen (Huger, 1966; Huger and Krieg, 1991). It was also observed that the virus infected neonates reduced their feeding activity as compared to a healthy ones.

The genomic DNA profiles of each tested OrNV extracted from the infected gut tissues of neonates digested by *Hind*III enzyme are as shown in *Figure 4*. The DNA profiles of each type of OrNV were found to be almost similar with those profiles produced by Ramle *et al*. (2005). For type A, the DNA profiles produced in this study are the same as by Ramle *et al*. (2005) and OrNV strain PV505 from Philippines identified by Crawford *et al*. (1985). Ramle *et al*. (2005) differentiated the type B from type A by having an additional single band at size about 15 kbp. However, our study found that there was also an insertion of a single band approximately at 2.5 kbp.

Insertion and deletion of bands from digested DNA profiles seemed to be common for viruses produced either from insect cell cultures or wild-type (Crawford *et al*., 1986; Mohan and Gopinathan, 1992). These incidences were presumed to happen due to inclusion of DNA from the host-cells during *in vitro* propagation of viruses, mutations or mixed
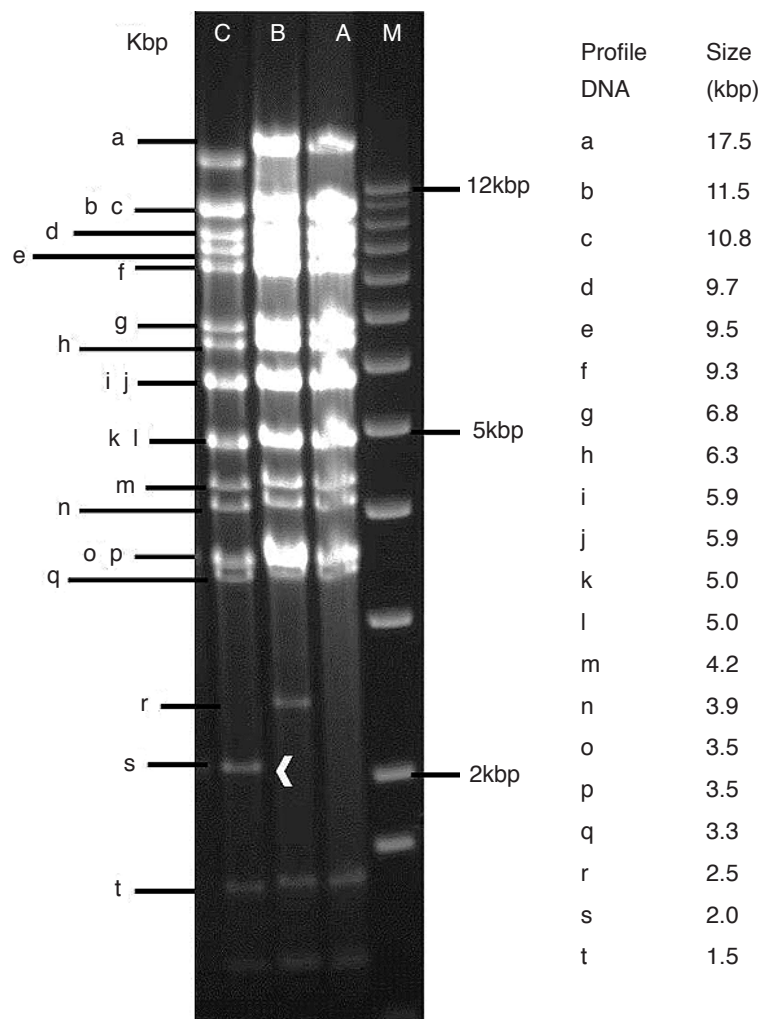

Profile DNA	Size (kbp)
a	17.5
b	11.5
c	10.8
d	9.7
e	9.5
f	9.3
g	6.8
h	6.3
i	5.9
j	5.9
k	5.0
l	5.0
m	4.2
n	3.9
o	3.5
p	3.5
q	3.3
r	2.5
s	2.0
t	1.5

Figure 4. The DNA profiles of Orytes *nudivirus (OrNV) types A, B, and C extracted from dead infected neonates and digested using* Hind*III enzyme. Arrow showing DNA fragment diffentiated the type C from types A and B. M-1 kbp Plus DNA ladder, A - type A OrNV, B - type B OrNV, C - type C OrNV.*

infections with other pathogens in wild-type isolates (McIntosh *et al.*, 1987; Smith and Crook, 1988). For type C, the DNA profiles produced were similar with the type C profiles as reported by Ramle *et al.* (2005). This type C was found restricted only in Sabah and recognisable by the presence of a single band at 2 kbp.

Based on the similarity of OrNV DNA profiles produced in this study with the DNA profiles prepared from infected gut tissues used for inoculation, and coupled with findings by Ramle *et al.* (2005), it was confirmed that the OrNV types A, B and C had successfully propagated in the insect cell line DSIR-HA-1179.

CONCLUSION

The OrNV types A, B, and C were successfully propagated on cell line DSIR-HA-1179. The infected cells showed cytopathic effects as early as 10-14 DAT. Inoculation of OrNV in cell culture affected the cell proliferation, where the cell density reduced over time. The PCR analysis showed that replication of OrNV on infected cells occurred as early as four DAT. Based on the $TCID_{50}$ values, the OrNV type B was the most pathogenic on cell line DSIR-HA-1179 as it produced more infectious virus and followed by types A and C. All OrNV produced on cell culture were pathogenic against the neonates of rhinoceros beetle. OrNV type B was the most pathogenic against neonates, causing the highest infection and the lowest LT_{50} value, and followed by the OrNV types A and C. It was also noted that the OrNV produced in cell culture was slightly more virulent than the OrNV prepared from the infected gut tissues. The similarity in DNA profiles between OrNV produced *in vitro* and from infected gut tissues, shows the successful propagation of OrNV in cell culture. The virulence of OrNV produced on cell culture was no different with the wild type OrNV.

Future research needs to be carried out especially to improve the cell culture media by reducing the use of serum which is costly. Specific primers for potential types of Malaysian OrNV need to be urgently developed, making the post-monitoring of virus introduction for control of rhinoceros beetle in field much simpler and economical.

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