CRB diagnostics for *Oryctes* nudivirus infection from *Oryctes rhinoceros* tissue (Guam)

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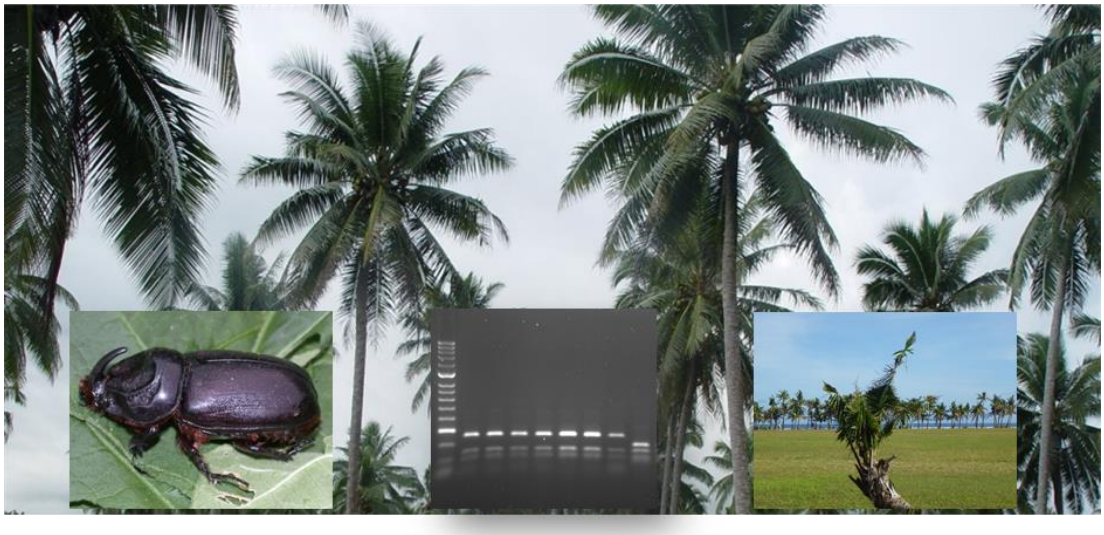

Report for University of Guam (Guam)
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1. EXECUTIVE SUMMARY

The coconut rhinoceros beetle (CRB; Oryctes rhinoceros) is a major pest of coconut and oil palm, but the discovery and release of Oryctes rhinoceros nudivirus (OrNV) in the 1960s and 70s suppressed the pest such that no new invasions of uninfested islands by CRB were reported for over 30 years after implementation of the biocontrol programme. Surprisingly, a highly damaging outbreak was reported from Guam (2007), which could not be controlled by OrNV. Subsequently, new invasions have been reported from Port Moresby, Papua New Guinea (2009); O'ahu, Hawai'i (2013); and Honiara, Solomon Islands (2015). Genetic evidence has shown that all of these outbreaks have been caused by a previously unrecognized haplotype, CRB-G, which appears to be tolerant to OrNV isolates commonly released in the Pacific region for managing CRB populations (Marshall et al., 2017). Entomologists working on this problem agree that the most feasible solution is establishment of biological control using an isolate of OrNV that is highly pathogenic to CRB-G.

The University of Guam and AgResearch are collaborating in an effort to identify an OrNV isolate that is able to infect CRB-G. The aim of the current work was to begin testing new candidate *Oryctes* nudivirus isolates that may be effective in managing outbreak populations of CRB-G. In one line of experimentation, a series of host-OrNV dosing passages was carried out in an attempt to select for a putative low abundance mutation that may have been present to in a mixed virus solution. Unfortunately, evidence for OrNV infection was not able to be demonstrated. However, previous work identified a candidate adult CRB-G specimen (Dug42) from Philippines as being infected with OrNV (based on visual observation, DNA detection of virus, and pathohistological analysis). We were able to purify OrNV from this gut tissue to obtained the OrNV isolate Dug42. OrNV-Dug42 has been propagated in the permissive cell line DSIR-Ha-1179 and aliquots sent to University of Guam so that a pathogen challenge assay could be set up against Guam collected CRB-G.

Recommendations:

- Based on discussion with the Guam-based team, further refinement of passaging methodology is recommended if further 'witch's brew' selection experiments are to be attempted.
- OrNV-Dug42 has been propagated in the permissive cell line DSIR-Ha-1179 and aliquots sent to University of Guam so that a pathogen challenge assay could be set up against Guam collected CRB-G. Further investigation is required to determine if OrNV-Dug42 is able to cause infection and/or mortality in CRB-G.
- It is still important to continue searching for alternative OrNV isolates (and other potential biocontrol agents) in regions known to harbor CRB-G populations, particularly where CRB caused palm damage is not being reported as an issue.

2. BACKGROUND

The coconut rhinoceros beetle (CRB; *Oryctes rhinoceros*) is a major pest of coconut and oil palm, but the discovery and release of *Oryctes rhinoceros* nudivirus (OrNV) in the 1960s and 70s suppressed the pest such that no new invasions of uninfested islands by CRB were reported for over 30 years after implementation of the biocontrol programme. Surprisingly, a highly damaging outbreak was reported from Guam (2007), which could not be controlled by OrNV. Subsequently, new invasions have been reported from Port Moresby, Papua New Guinea (2009); O'ahu, Hawai'i (2013); and Honiara, Solomon Islands (2015). Genetic evidence has shown that all of these outbreaks have been caused by a previously unrecognized haplotype, CRB-G, which appears to be tolerant to OrNV isolates commonly released in the Pacific region for managing CRB populations (Marshall et al., 2017).

Entomologists working on this problem agree that the most feasible solution is establishment of biological control using an isolate of OrNV that is highly pathogenic to CRB-G. The objective of this collaborative project between the University of Guam and AgResearch (New Zealand) was to begin testing new candidate *Oryctes* nudivirus isolates that may be effective in managing outbreak populations of CRB-G. Two approaches are currently being pursued: 1) a series of CRB-G-OrNV dosing passages was carried out in an attempt to select for a putative low abundance mutation that may be present within a mixed virus solution (a 'witch's brew' experiment); and 2) a traditional pathogen challenge bioassay using new OrNV isolates against CRB-G adults to identify effective candidate OrNV against CRB-G.

3. METHODS

The molecular DNA methods used for this work were based on those previously described in Marshall et al. 2017.

3.1 Genomic DNA extraction from *O. rhinoceros* gut tissue

DNA was extracted from the gut tissue supplied using the ZR Genomic DNA Tissue MiniPrep (Zymo Research) column system following manufacturer instructions. DNA elution was carried out using 100 µl of elution buffer from the appropriate kit, and aliquots of eluted DNA samples were subsequently used for further analyses.

3.2 PCR detection of *O. rhinoceros* nudivirus infected *O. rhinoceros* beetles

O. rhinoceros gut tissue dissected from moribund or dead bioassay specimens had DNA extracted as described above. The PCR protocol for detection of OrNV was based on that described in Richards et al. (1999), and has been subsequently modified by using undiluted and diluted DNA template (from undiluted down to 1 in 5 0000) to better distinguish infection from mere presence due to dosing with OrNV for the pathogen challenge assay. The primer pairs used to amplify a 945 base pair (bp) fragment of the OrNV genome were OrNV15a (5'-ATTACGTCGTAGAGGCAATC-3') and OrNV15b (5'-ATGATCGATTCGTCTATGG-3') (Richards et al., 1999). Each 25 µl PCR reaction contained 0.2 µl Taq DNA Polymerase (Thermo Fischer Scientific), 2.5 µl 10x PCR buffer, 2 µl $MgCl_2$ (25 mM),2 µl dNTP mixture (10 mM), 0.5 µl OrNV15a (10 µM), 0.5 µl OrNV15b (10 µM), 2 µl diluted DNA (reactions of 1 in 100, and 5 000), and 15.3 µl water. PCR amplifications were performed in a C2100 (BioRad) thermocycler with a cycling profile of 35 cycles of 94°C denaturation (30 s), 50°C annealing (45 s), 72°C extension (1 min) with an initial denaturation of 3 min at 94°C and a final extension of 5 min at 72°C. An 8 µl aliquot of each PCR reaction was separated by

agarose gel electrophoresis (1%, 0.5xTBE) alongside a 100 bp ladder, stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). Detection of OrNV PCR product in the 1 in 5 000 dilution was considered here as indicative of OrNV infection, while no detection within undiluted DNA template is consider completely free of OrNV presence.

3.3 Isolation and propagation of live OrNV from infected tissue

Isolation of OrNV from infected CRB gut tissue proceeded as follows. Dissected gut tissue (putatively containing live OrNV) was initially macerated using a microhomogenizer a 2.0 ml tube containing 50 mM Tris pH 8.0 buffer. The macerate was clarified by pelleting the cell debris and passing the crude supernatant material sequentially through a series of sterile disc filtration units (0.8, 0.45, and 0.2 µm pore sizes).

Propagation of virus was carried out using the DSIR-Ha-1179 cell line, which were maintained using standard insect cell culture techniques as previously described (see Crawford, 1982; Crawford and Sheehan, 1985; Lynn, 2002), but with a few minor changes. Briefly, the Ha1179 cells were routinely grown at 27 °C as an adherent culture in surface treated tissue culture flasks containing PS100 medium made up as follows: Graces' Insect medium (Invitrogen or Sigma), 2.95 g/L tryptose phosphate broth (Sigma), TC100 vitamins (Sigma), adjusted to pH 6.2 with KOH, and supplemented with 10% fetal bovine serum (Invitrogen). For cell line maintenance, once cells reached 80-90% confluence (~10-14 days), cells were gently aspirated off the bottom of the flask using a transfer pipette and sub-cultured at a 1:5 ratio of cells to fresh medium. Production of OrNV essentially followed the method previously described (Crawford and Sheehan, 1985) for infection. Cells were grown to approximately 25% confluence in 25 cm^2 tissue culture flasks, infection was initiated by directly adding an aliquot of OrNV directly to flasks containing cells with medium. The infected flasks were gently rocked to mix contents, followed by stationary incubation at 27 °C for 14 days. Virus was harvested from infected cells by dislodging cells from the bottom of the flask (via transfer pipette), centrifugation (10 min at 100 *g*) to pellet the cell material, and the virus containing supernatant collected and subsequently stored at 4 °C. The virus titre for a given sample was measured by using of a tissue culture infectious dose 50 (TCID50) assay, which uses a 50% endpoint dilution technique (in 96-well plates) and statistical analysis (based on the Reed-Muench or Spearman-Kärber formulae) (Finney, 1952; Reed and Muench, 1938) to calculate the infectious per millilitre (IU/ml).

4. RESULTS AND DISCUSSION

4.1 OrNV detection

Following genomic DNA extraction of 27 tissue samples from the 'witch's brew' experiment, a dilution PCR analysis of DNA extracted from *O. rhinoceros* tissue was used to detect the incidence of OrNV infection from the *O. rhinoceros* gut tissue specimens from the 'witch's brew' specimens provided for analysis. The results are summarized in Table 1, with Figure 2 showing the original agarose gel images. OrNV amplicons were not detected any of the 1 in 5000 DNA dilutions; only specimens GU2-1 and 2-2 at a 1 in 100 dilution displayed an OrNV.

Generally, true OrNV infection displays distinct and easily distinguished amplification at the 1 in 5000 dilution and very robust at the 1 in 100 dilution. As no positive PCR reactions were detected at the 1 in 5000 dilution it is unlikely that active virus infection was established from the original 'witches brew' experiment. Further discussion with the Guam-based team highlighted the observation that the material obtained from successive passaging became

highly viscous and also contained *Metarhizium majus* fungal spores. Based on this, further refinement of the passaging methodology is recommended if further 'witch's brew' selection experiments are to be attempted.

Table 1: Summary of OrNV detection results from subset of *Oryctes rhinoceros* gut tissue received by AgResearch from the University of Guam.

Tube Label	OrNV Detected	Comment
GU1-1	No	No OrNV infection
GU1-2	No	No OrNV infection
GU-3	No	No OrNV infection
GU-6	No	No OrNV infection
GU-9	No	No OrNV infection
GU-12	No	No OrNV infection
GU-15	No	No OrNV infection
GU-18	No	No OrNV infection
GU-21	No	No OrNV infection
GU-24	No	No OrNV infection
GU-27	No	No OrNV infection
GU-30	No	No OrNV infection
GU-33	No	No OrNV infection
GU-36	No	No OrNV infection
GU-39	No	No OrNV infection
GU-42	No	No OrNV infection
GU-45	No	No OrNV infection
GU-48	No	No OrNV infection
GU2-1	Yes (only 1:100)	Potential OrNV infection
GU2-2	Yes (only 1:100)	Potential OrNV infection
GU2-3	No	No OrNV infection
GU2-3	No	No OrNV infection
GU2-4	No	No OrNV infection
GU2-5	No	No OrNV infection
GU2-6	No	No OrNV infection
GU4-1	No	No OrNV infection
GU4-2	No	No OrNV infection

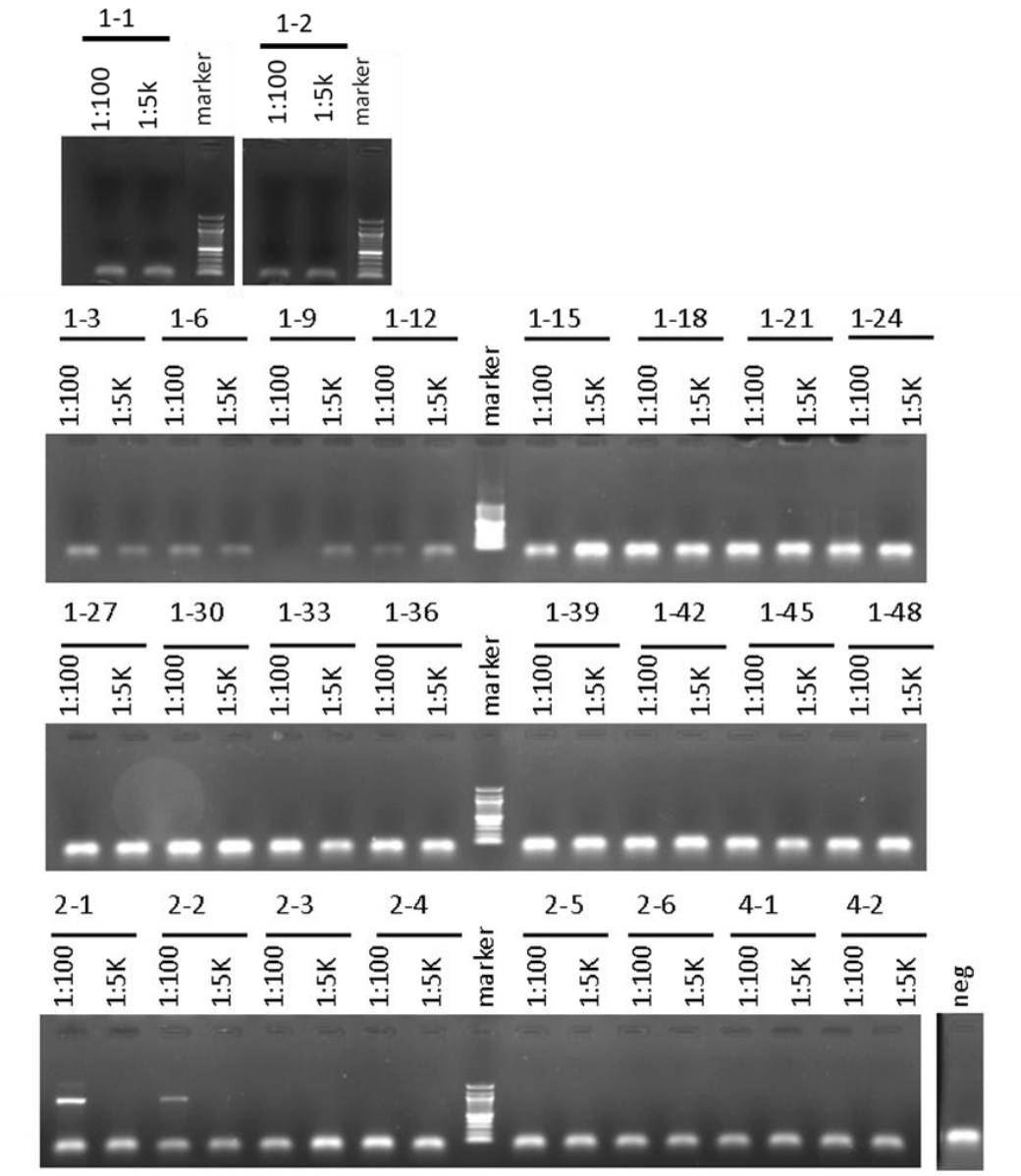

Figure 2: Agarose gel PCR assay results for OrNV detection.
Presence of a 945 base pair PCR product indicates OrNV DNA is present. Obvious detection of OrNV PCR product in the 1 in 5000 (1:5K) dilution is considered as indicative of OrNV infection. The marker (M) used has DNA 'ladder rungs' every 100 base pairs.

4.2 Isolation and propagation of OrNV-Dug42

From a previously identified OrNV infected adult CRB-G specimen (Dug42), *Oryctes* nudivirus was purified from the gut tissue and designated as OrNV isolate Dug42 (OrNV-Dug42). Further propagation was carried out using the permissive cell line DSIR-Ha-1179, whereby the characteristic cytopathic effects were observed and virus was readily detected from this material. Ten 1 ml aliquots of OrNV-Dug42 titred at ~1.0 x 10^6 infectious units/mL were sent to University of Guam for further testing within a pathogen challenge assay against Guam collected CRB-G adults.

5. CONCLUSIONS AND RECOMMENDATIONS

- The PCR haplotyping results indicated that all specimens analyzed were CRB-G.
- Data from the 'witch's brew' experiment for establishing of OrNV infection suggested no virus was present. Although two OrNV PCR positive result (GU-2-1 and 2-2) were observed at the 1 in 100 dilution point. They were not observed at the 1 in 5000 threshold, which has been previously validated to distinguish true infection from spurious contamination.
- Discussion with the Guam-based team highlighted the observation from the 'witch's brew experiment' that the material collected from successive passaging became highly viscous and also contained *Metarhizium majus* fungal spores. Based on this, further refinement of passaging methodology is recommended if further 'witch's brew' selection experiments are to be attempted.
- OrNV-Dug42 has been propagated in the permissive cell line DSIR-Ha-1179 and aliquots sent to University of Guam so that a pathogen challenge assay could be set up against Guam collected CRB-G. Further investigation is required to determine if OrNV-Dug42 is able to cause infection and/or mortality in CRB-G. For future investigations, additional collection and preservation of tissue for histological analysis may assist with interpretation of results from pathogen challenge experiments.
- It is still important to continue searching for alternative OrNV isolates (and other potential biocontrol agents) in regions known to harbor CRB-G populations, particularly where CRB caused palm damage is not being reported as an issue.

6. ACKNOWLEDGEMENTS

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