DETECTION OF BACULOVIRUS INFECTION IN RHINOCEROS BEETLE (*ORYCTES RHINOCEROS*) AND THE PURIFICATION AND IDENTIFICATION OF VIRUS STRAINS

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SUMMARY

1) A dot blot assay has been introduced at the Coconut Research Institute in Manado, which can be used routinely to determine whether a beetle is infected with *Oryctes* baculovirus when symptoms are ambiguous. The method still needs some further modifications so that samples of midgut can be applied directly to the nitrocellulose membrane in the field. This would mean only the membrane needs to be brought or sent back to the laboratory. The method should also be compared with a bioassay to determine its sensitivity.

2) A genetically engineered, recombinant *Oryctes* baculovirus strain (Rec 26) has been tested in the laboratory in Manado. The advantage of releasing this strain during control trials in the field would be that the above dot blot assay can be easily modified to react specifically with this particular strain. The recombinant strain showed similar biological characteristics to its parent strain (PV 505). Comparisons of host range, and growth characteristics in both cell culture and in the beetles themselves have shown no significant differences between the two strains. *Oryctes* baculovirus has already been extensively safety tested. The foreign gene inserted into the recombinant virus is not expressed by the virus but if it were it would produce a polyhedrin protein from another baculovirus *Autographa californica* nuclear polyhedrosis virus that has also been safety tested and has been granted an experimental field release permit by the US Environmental Protection Agency. The consultant considers the recombinant strain to be safe for field experiments. A suitable release site would be Marinsaw, North Sulawesi where considerable rhinoceros beetle damage is occurring during a replanting program and where there is excellent co-operation from the plantation manager in collecting beetles for analysis. Such a trial would allow for the first time to monitor properly a virus release program in a region containing endemic virus.

3) It is recommended that a sponsor be found to support studies to determine whether resistance to Oryctes baculovirus has developed in the outbreak rhinoceros beetle populations of South Sulawesi, East Java, and other parts of Indonesia. This research should be carried out in a non-coconut growing country so that there is no risk of virus resistant beetles spreading to new areas. New Zealand, which has extensive experience in research on rhinoceros beetles and the *Oryctes* baculovirus, would be a very suitable country. The consultant hopes that present moves by the Indonesian Government to submit an official request for such aid to the Government of New Zealand will be successful.

4) Because of the possibility that rhinoceros beetle outbreaks in some parts of Indonesia (e.g. East Java and South Sulawesi) are due to the presence of less pathogenic virus strains rather than beetle resistance, the release of proven pathogenic strains such as X2B and BU27 would be also beneficial. This work already begun by the Project in collaboration with Dinas Perkebunan should be given a high priority.

INTRODUCTION

Indonesia frequently experiences serious outbreaks by the coconut palm rhinoceros beetle (*Oryctes rhinoceros*). Although this pest is considered one of the most serious constraint to coconut farmers in all of South-East Asia, Indonesia seems to have more problems with *Oryctes* than any other country in the region. For example, surveys conducted by the Integrated Coconut Pest Control Project in 1987 showed very high *Oryctes rhinoceros* populations in South Sulawesi and East Java. (see Annual Project Report, 1987). These high populations were associated with a very low incidence of infections by the *Oryctes* baculovirus, a naturally occurring lethal disease that is the major biocontrol agent of this pest. There appears to be little difference in the coconut growing practices and *Oryctes* breeding conditions of South Sulawesi and East Java compared to other parts of Indonesia or the Philippines, which don't have high beetle populations. It would therefore appear that the outbreak populations of South Sulawesi and East Java are either resistant to baculovirus infections or the baculovirus strains found in these regions have become less pathogenic.

Determining the percentage of virus infected beetles in the population is an important part of assessing field control trials. Different types of assays have been developed. The simplest has been to diagnose baculovirus infections in rhinoceros beetles by dissecting the insect and examining the beetle midgut for symptoms. In an uninfected beetle the midgut is thin, approx. 1.5 mm in diameter. It has a brown colour and when opened the gut contents are a clear brown fluid similar in appearance to tea without milk. In infected beetles however the gut is swollen (up to 3 mm in diameter) and has a white exterior. The contents are milky when the midgut is opened up. An alternative, more reliable and more sensitive method is to feed the remains of the beetle to known healthy larvae and observe the larvae for virus-infections and subsequent death. However, this bioassay procedure not only requires 4-5 weeks but also a constant supply of healthy larvae.

Problems have been encountered by the project when the guts of beetles from Sulawesi and East Java were examined. Often beetles have swollen midguts that remain brown with clear contents or are thin but contain milky contents. This has created difficulties for the research program of the project and was the impetus for establishing a non-radioactive dot blot assay for the detection of *Oryctes* baculovirus. The assay will provide an independent test for those beetles with doubtful symptoms. Further a baculovirus strain (Rec 26) is available which can be specifically detected with the dot blot assay. This would facilitate the evaluation of field trials on adding virus-infected beetles to areas where the baculovirus already exists. (It seems that the virus occurs naturally in all parts of Indonesia where *O. rhinoceros* is found). If strain Rec 26 is used in such a control trial the dot blot test would not only be able to determine all baculovirus infections in sampled beetles but also specifically those which are infected with strain Rec 26.

While setting up the dot-blot assay was the major task of the present consultancy other aspects and techniques connected to research on the *Oryctes* baculovirus were covered:

1) A method for purifying the baculovirus from midguts using controlled pore glass chromatography.

2) Filtration of purified baculovirus to produce an aseptic stable inoculum for field use.

3) Using the purified baculovirus to extract the DNA and determine the virus strain by restriction endonuclease analysis.

4) Comparing the infectivity of the recombinant baculovirus strain (Rec 26) with that of its parent strain.

EXPERIMENTS CONDUCTED

The following three experiments were conducted during the consultancy:

1. Examine the gut symptoms developing after inoculating beetles with different dosages of baculovirus and compare the symptoms with the results of the dot-blot assay.

2. Attempt to detect excretion of virus by virus-infected beetles with the dot-blot assay. If this is possible it might lead to a useful comparison of different baculovirus strains under laboratory conditions. The amount of virus excreted might vary between different strains, and the more virus is excreted by the beetle, the better the virus will probably spread to other beetles and larvae.

3. Comparing the infectivity of three strains of the baculovirus. One of the three strains (Rec 26) is a genetically altered strain for which a specific dot blot assay can be used.

The experimental methods and results are described in detail in Appendix 1 (page 130). Appendices 2-5 give detailed instructions on how to conduct the dot-blot assay, prepare the probe for the dot-blot assay, purify the virus and its DNA, and prepare sterile virus filtrate.

DIAGNOSIS OF *ORYCTES* BACULOVIRUS INFECTION

As mentioned in the introduction, diagnosis of *Oryctes* baculovirus infections in beetles has been giving problems because intermediate or ambiguous gut symptoms are often found in beetles.

There is a highly sensitive bioassay method available for the detection of *Oryctes* baculovirus infection. This involves growing 2 first- or second instar rhinoceros beetle larvae in the presence of the suspected virus infected material for 4 weeks. If during that time the larvae become infected it indicates that the material contained virus. While very sensitive and reliable, this methods has

serious logistical drawbacks. Large numbers of disease-free larvae are required which are difficult to produce given the insect is so long-lived and has low fecundity. Also the length of time that the assay takes (4 weeks) can be a problem if the diagnosis is needed urgently.

The dot-blot assay method introduced to the laboratory provides a diagnosis within 2 days and does not have the requirement for a large rhinoceros beetle rearing facility. A commercially available kit was used to label the DNA used as a probe and to detect its binding. Many thousands of beetles could be assayed using the one kit, bringing the materials cost down to less than 10 cents US per diagnosis.

The dot-blot assay which has been used extensively by molecular biologists for the past five years involves the detection of DNA sequences specific to *Oryctes* baculovirus. This is done by immobilizing the virus DNA on a piece of nitrocellulose membrane in a single stranded form. A piece of *Oryctes* baculovirus DNA which has been cloned in a plasmid and labelled with a substance called digoxygenin is also made single stranded and added to the filter.

The digoxygenin labelled single stranded DNA from *Oryctes* baculovirus, called the labelled probe, will recognize the same DNA sequences in the DNA from the virus that is immobilized on the filter and bind to them. The binding of the labelled probe to this DNA can then be detected by first adding an antibody to which an enzyme called alkaline phosphatase has been bound. The antibody recognizes and binds to the digoxygenin of the labelled probe. Substrate for the alkaline phosphatase is then added which will turn the filter blue where alkaline phosphatase is present. Therefore a blue colour on the filter at the spot where the midgut homogenate was placed indicates that DNA sequences of *Oryctes* baculovirus are present and that the beetle was infected.

It is important to use a cloned *Oryctes* baculovirus fragment rather than purified virus DNA because small impurities of *Oryctes* DNA would give unspecific colour reactions.

The dot-blot assay was tested in a number of ways :

1) A range of field collected beetles were dissected and carefully analyzed for symptoms of infection. These results were recorded, then the midgut was homogenized and a small sample of this homogenate was spotted on the nitrocellulose membrane for assay by dot-blot.

2) All the beetles from the above experiments No. 1 and 3 were also examined in a similar manner to the above.

3) Fecal material was also spotted on to the membrane from experiment 2 and assayed by dot-blot.

With a small number of infections (approx 2 % of midguts analyzed) midguts showing clear symptoms of infections gave a positive result and those with no symptoms a negative result (Fig. 1). Most midguts showing doubtful symptoms of infection gave a positive result indicating that these partial symptoms probably represent early stages of the infection process.

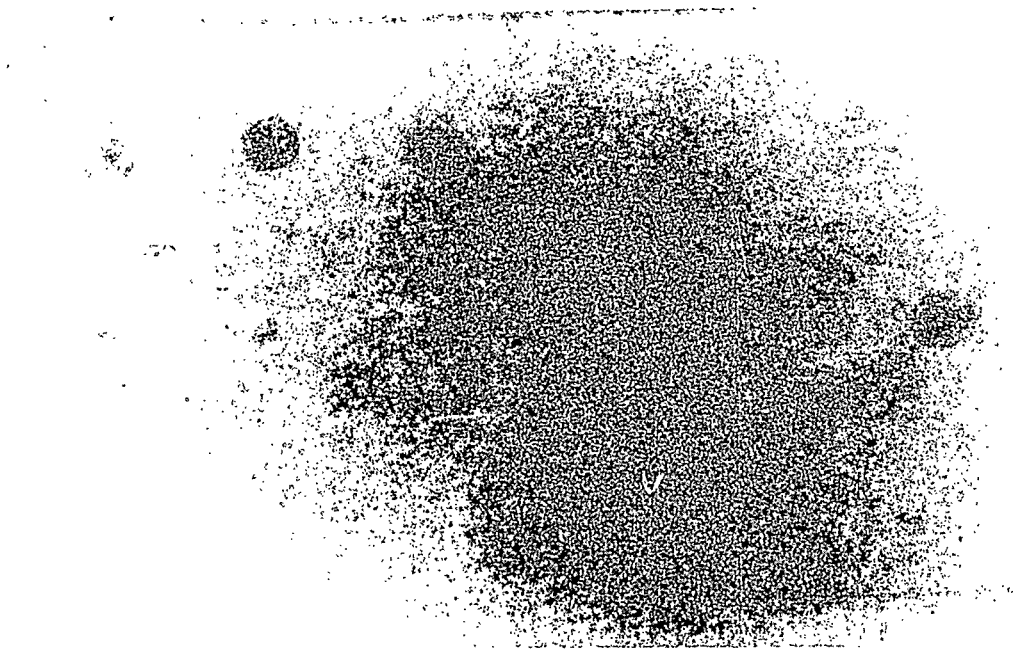

Fig. 1. Result of a dot blot test with gut extracts from 13 beetles, 4 of them gave a reaction positive for the presence of the baculovirus.

The results of experiment no. 2 (see Appendix 1,page 131) showed that the dot blot assay is very suitable for detecting baculovirus in feces of rhinoceros beetles. Although, it should be possible to obtain quantitative information with the assay, the trial indicated that the amount of virus excreted by an infected beetle can vary considerably from one day to another. For accurate comparisons of different virus strains it will therefore be necessary to test samples pooled over a number of days.

There are a number of improvement that could be made to the method, recognizing however that same compromise must be made between the ease of use of the method and the clarity of the result. The following tests should be conducted in the future.

1) To improve the ease of use and save having to homogenate each midgut it would be worth trying to take a small piece of midgut and squash it directly on to the nitrocellulose membrane.

2) To try and reduce interference from proteins and lipids, pretreatment of the spot or the homogenate with SDS should he tried. This should be done prior to making the DNA single stranded with the ALKALAI SALT SOLUTION.

3) The dot-blot assay should be further tested by comparing it with the bioassay, when sufficient numbers of larvae are available. As the dot-blot assay showed most doubtful symptoms to be associated with the presence of the virus, the bioassay test should be used to confirm this.

FIELD USE OF THE RECOMBINANT STRAIN OF *ORYCTES* BACULOVIRUS (REC 26)

In areas such as Indonesia where *Oryctes* baculovirus is endemic it is very difficult to monitor the effectiveness of any viral release programs. This is because there is no easy method for distinguishing the released virus strain from that already present. A recently engineered recombinant *Oryctes* baculovirus solves this problem. This recombinant virus (Crawford, 1988) contains a piece of foreign DNA inserted into the genome which can be specifically probed by using the same dot-blot assay mentioned above. Thus the dot-blot assay provides a simple method that will positively distinguish the recombinant from all other *Oryctes* baculovirus strains.

The piece of foreign DNA inserted into the recombinant *Oryctes* baculovirus is the polyhedrin gene from another baculovirus, *Autographa californica* nuclear polyhedrosis virus. The protein coded for by this gene called polyhedrin, has been shown to be non-toxic to vertebrates. In fact this virus which infects *Trichoplusia ni* (the cabbage looper) is commonly found on cabbages grown in North America. It is likely that humans have been ingesting this protein, with no ill effects, for as long as cabbages have been consumed in North America. This virus has been granted an experimental field release permit by the U.S. Environmental Protection Agency.

Tests have also shown that this piece of DNA is silent, i.e. no messenger RNA is made from the foreign DNA and no protein is produced. In other words the foreign DNA is only an additional piece of inactive DNA.

Studies at the DSIR, Mt Albert Research Centre in New Zealand have shown that the recombinant virus has exactly the same growth characteristics as its parent strain when grown in tissue culture. Also no differences in host range were observed. The recombinant and parent strains were both infectious for the scarabs, *Costelytra zealandica*, and *Pericoptus sp.* but not infectious for *Tenebric molitor, Galleria mellonella*, or *Mythimna separata.*

It was important however to also make these comparisons in the beetles themselves. Experiments performed during this consultancy showed that the growth characteristics of the parent and recombinant strains in adult beetles were similar (See experiment 3 - Appendix 1, page 132).

In summary, therefore, all the data presented above indicates that the inserted gene will not present any hazard to humans or other vertebrates and that its insertion has not affected any of the biological characteristics of *Oryctes* baculovirus itself. The consultant is certain that this recombinant can be safely used to monitor virus release programs.

Unfortunately, no suitable probe for detecting specifically the Rec 26 virus strain could be made during the consultancy. This was due to a defect in the DNA sample brought from DSIR. A new sample will be sent to the Project in the near future.

METHODS FOR PURIFYING *ORYCTES* BACULOVIRUS AND ANALYZING STRAIN DIFFERENCES

The method of purifying *Oryctes* baculovirus using a controlled pore glass column was first developed in the Philippines (Zelazny et al., 1985). This method was demonstrated during the consultancy to the counterpart staff.

Virus purification has two uses: First the purified virus can be passed through a 0.45 µm filter to remove contaminating bacteria to make a stable virus inoculum that is ideal for field release of the virus (Zelazny et al., 1987). A new way of storing the sterile baculovirus preparation in disposable plastic pipettes (Fig. 2) was tried and this is expected to have a number of advantages over using glass vials. Second DNA can be extracted from the purified virus and analyzed to determine the strain of the virus. A number of *Oryctes* baculovirus strains have been characterized and are recognizable by their restriction enzyme profiles (Crawford et al., 1986). Equipment and methods for restriction enzyme analysis of *Oryctes* baculovirus DNA were also set up and demonstrated. This will enable the group to look for genetic differences in geographical isolates from beetle population throughout Indonesia.

An opportunity to demonstrate the usefulness of these techniques came when virus infected beetles were found in the *Oryctes* rearing colony. Using restriction enzyme analysis of virus DNA from these beetle, it was possible to show that infection was due to a local isolate and therefore the source of the infection was from the field rather than the virus strains being worked with in the laboratory.

REFERENCES

Crawford, A.M. (1988) An *Oryctes* baculovirus recombinant: insertion of the polyhedrin gene from *Autographa californica* nuclear polyhedrosis virus into *Oryctes* baculovirus. J. Gen. Virol. (in press).

Crawford, A.M., Zelazny, B. and Alfiler A.R. (1986). Genotypic variation in geographical isolates of *Oryctes* baculovirus. J. Gen. Virol. 67,949-952.

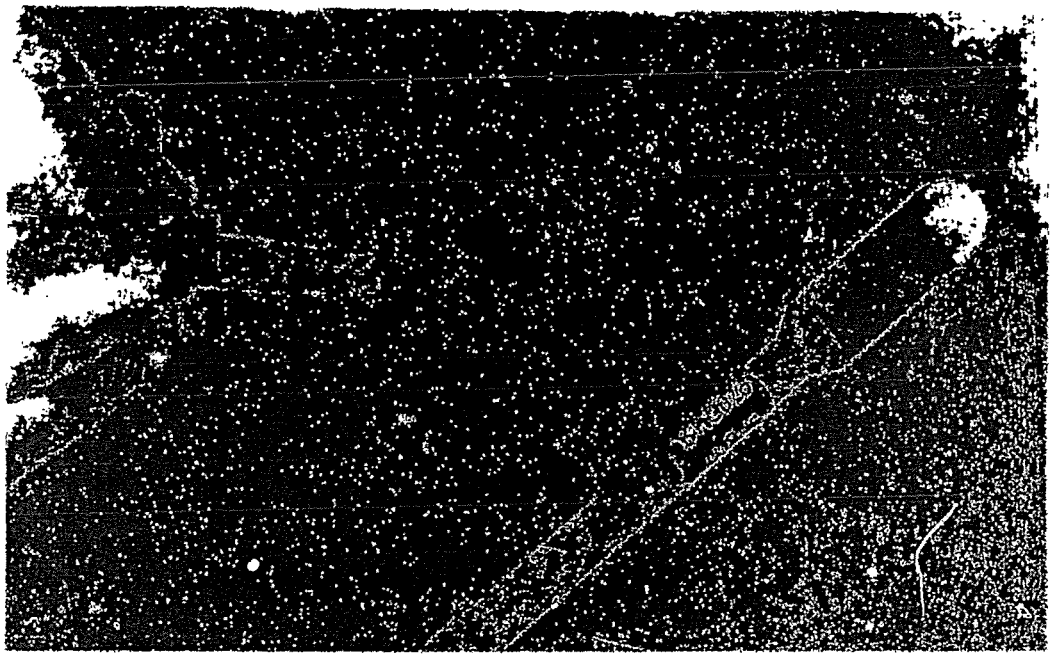

Fig. 2. Inoculation of a rhinoceros beetle with baculovirus stored under sterile conditions in disposable pipettes.

Zelazny, B., Alfiler A., and Mohamed, N.A. (1985) Glass permeation chromatography for purification of the baculovirus of *Oryctes rhinoceros* (Coleoptera: Scarabaeidae). J. Econ. Entomol. 78,992-994.

Zelazny, B., Alfiler, A., and Crawford, A.M. (1987). Preparation of a baculovirus for use by coconut farmers to control rhinoceros beetle (*Oryctes rhinoceros*). FAO Plant Prot. Bull. 35: 36-42.

EXPERIMENTS PERFORMED DURING THE CONSULTANCY

Experiment 1

Aim: Correlation of symptoms with the presence or absence of a virus infection as determined by dot blots.

Methods: Two freshly dissected beetle midguts showing advanced symptoms of infection (white, swollen) with the Bogor strain of *Oryctes* baculovirus were homogenized in 5ml of TE buffer containing 0.5g of sucrose. All materials were kept on ice or in the refrigerator throughout the experiment

The following dilutions (in TE buffer containing 10% sucrose) were made 1:100, 1:1,000, 1:10,000, These inocula. were fed to beetles by placing a 5µl drop on the mouthparts and leaving for 5 minutes followed by a 5µl drop of 10% sucrose in TE Buffer. After allowing approximately 5 minutes for each drop to be imbibed by the beetles they were placed in individual plastic containers half filled with a mixture of sawdust and cowdung.

The following numbers of beetles were infected:

concentrated inocula	20 beetles
1:100 dilution	10 beetles
1:1,000 dilution	10 beetles
1:10,000 dilution	10 beetles
uninoculated control	10 beetles

At 1 week following infection 5 beetles inoculated with the concentrated virus were dissected and the midgut examined.

At 10 days after infection the remaining beetles were examined.

All beetles were dissected, their midguts examined and the symptoms noted. The midguts were then homogenized in 1 ml TE Buffer. A 5 µl sample of this homogenate was diluted with 15 µl TE Buffer, then 2.5 µl was spotted on to a nitrocellulose membrane for analysis by dot blot.

Result:

A total of 48 beetles were analyzed by both midgut symptoms and the dot blot assay.

Symptoms	Total beetles	Positive dot-blot	Negative dot-blot
Clearly positive	26	26	0
Doubtful	11	9	2
Decayed midgut	4	2	2
Negative	7	3	4

Correlation of positive symptoms with the dot-blot assay is good, however most midguts with doubtful symptoms and some with no symptoms also give a positive dot-blot result suggesting that the dot-blot assay will pick up virus infection before symptoms become obvious.

Experiment 2

Aim: To determine if a virus infection can be detected by a dot-blot assay of fecal material.

Methods: The following infections were set up using the same inocula as described in experiment 1

concentrated inocula	5 beetles
1:100 dilution	5 beetles
1:1,000 dilution	5 beetles
1:10,000 dilution	5 beetles
uninoculated control	5 beetles

Beetles were kept individually in cowdung/sawdust medium for 1 week. They were then taken from the medium and washed in tap water prior to being placed over 1 ml of TE Buffer for 24 hours to collect fecal material that was excreted into the buffer.

The buffer containing the virus was spotted on to a nitrocellulose membrane and assayed by dot blots as described in Appendix 2 (page 134).

Results:

After 1 week, 3 beetles showed evidence of virus in their faeces. This number increased after two weeks. While the experiment clearly demonstrated that virus can be detected in the faeces by the dot-blot assay, the experimental design did not allow any conclusion to be drawn about the totals excreted by the beetles, because of the sporadic nature of defecation in beetles.

If all fecal material is kept and assayed it should be possible to quantify the virus excreted by beetles and make comparisons between different strains of virus. Infection with a strain that makes the beetle excrete large quantities of virus would improve the transmission rate between beetles.

Experiment 3

Aim: To compare the infectivity, by injection, of three strains of *Oryctes* baculovirus.

Methods: Each of the virus strains, Rec 26, PV505, and X2B, were diluted 1:10, 1:1,000, and 1:100,000 in TE Buffer containing 1% BSA. The diluent was sterilized by filtration (0.22 µm.)

Each dilution was then injected into 5 beetles. Each insect was injected with 25 µl of inoculum into the flight muscle (through the middle of the right or left metasternum)

The undiluted inocula all contained $2x10^7$ infectious units per ml ($TCID_{50}$ IU). There were 10 control beetles.

Beetles were kept for 2 weeks then dissected. The midgut symptoms were analyzed and a dot blot assay made with the homogenized midgut.

Results :

Two of the controls showed clearly positive symptoms and were positive by dot blot assay. One further control midgut which showed no symptoms was positive by the dot blot test. It was later discovered that a virus infection had become established in two containers of beetles from the rearing facility some of these beetles were used in this experiment.

1) Analysis of Symptoms

	Number showing clear symptoms			
Dilution	X2B	PV505	REC 26	TOTAL
1 : 10	4 (N=5)	1 (N=4)	2 (N=4)	7 (N=13) 54 %
1 : 1,000	3 (N=5)	4 (N=4)	2 (N=4)	9 (N=13) 69 %
1 : 100,000	4 (N=5)	3 (N=5)	3 (N=3)	10 (N=13) 77 %
Total	11 (N=15) 73 %	8 (N=13) 62 %	7 (N=11) 64 %	

2) Analysis of dot blot

	Number showing clear symptoms			
Dilution	X2B	PV505	REC 26	TOTAL
1 : 10	5 (N=5)	4 (N=4)	4 (N=4)	13 (N=13) 100 %
1 : 1,000	5 (N=5)	4 (N=4)	4 (N=4)	13 (N=13) 100 %
1 : 100,000	5 (N=5)	4 (N=5)	3 (N=3)	11 (N=12) 92 %
Total	15 (N=15) 100 %	12 (N=13) 93 %	11 (N=11) 100 %	

PROCEDURE FOR DOT-BLOT ASSAY FOR *ORYCTES* BACULOVIRUS INFECTION

1] Make appropriate spots on the nitrocellulose membrane (NCM) and air dry. Store in an air tight container containing a few grains of Silica Gel.

2] Place the NCM on top of a piece of filter paper saturated with ALKALAI SALT SOLUTION. Leave at room temperature for 15 minutes.

3] Carefully remove the NCM from the filter paper and place on top of a second piece of filter paper saturated with 1M TRIS pH 7.4. Leave at room temperature for 5 minutes.

4] Carefully remove the NCM from the filter paper and place on a dry piece of paper and dry in the oven at 80°C for 30 minutes.

5] Wash NCM 2x 30 minutes with MEMBRANE WASHING SOLUTION at 65°C with shaking. At the end of the first wash remove any insect tissue still stuck to the filter by gently scraping with the tip of a finger.

6] Incubate NCM for 30 minutes in HYBRIDIZATION SOLUTION at 65°C with shaking.

7] Place 5µl of labelled DNA (prepared as described in APPENDIX 3) in an Eppendorf tube with 50 µl of HYBRIDIZATION SOLUTION and incubate in a boiling water bath for 10 minutes then IMMEDIATELY add to the NCM already shaking in the HYBRIDIZATION SOLUTION.

8] Leave shaking at 65 C overnight to allow the labelled DNA to hybridize to the spots on the NCM.

9] Wash the membrane 2x 15 minutes at 65°C with shaking in the MEMBRANE WASHING SOLUTION.

10] NB: FROM THIS POINT ON ALL INCUBATIONS ARE AT ROOM TEMPERATURE !!!

11] Incubate 5 minutes with 50 ml BUFFER 1 with shaking.

12] Incubate 30 minutes with 50 ml BUFFER 2 with shaking.

13] Incubate 5 minutes with 20 ml BUFFER 1 with shaking.

14] Incubate 30 minutes with 10ml BUFFER 1 containing 2 µl of VIAL 8 (anti-digoxigenin alkaline phosphatase conjugate)

15] Remove NCM and place in a clean container.

16] Incubate 2x 15 minutes with 50 ml BUFFER 1 with shaking.

17] Incubate 5 minutes with 50 ml BUFFER 3 with shaking.

18] Remove the NCM and place in a plastic bag. Add to the bag 10 ml BUFFER 3 containing 45 µl VIAL 9 + 35 µl VIAL 10. This solution must be made IMMEDIATELY prior to use.Fold the top of the bag over or seal with a plastic bag sealer to prevent the liquid leaking out, then lay flat on the bench and cover with a light proof box or bucket. It has also been found that this reaction can be performed in a glass petri dish covered with a light proof box.

19] Check after about 10 minutes to determine whether colour has developed. If nothing has happened in this time then leave for a further 60 minutes. If still nothing has happened leave overnight.

20] When satisfactory colour has developed stop the reaction by removing the NCM from the plastic bag and washing in 50 ml of TE BUFFER for 10 minutes with shaking.

21] The colour will slowly fade on drying however a permanent record of the results can be made by photocopying the NCM.

SOLUTIONS USED IN THIS PROCEDURE:

1] ALKALAI SALT SOLUTION:(0.5M NaOH,1.5M NaCl)

To make 100 ml:	2 g	NaOH
	8.7 g	NaCl

dissolve in distilled water to a final volume of 100 ml.

2] MEMBRANE WASHING SOLUTION:

To make 1 litre:	100 ml	20X TNE BUFFER
	4 ml	25% SDS
	896 ml	distilled water

3] HYBRIDISATION BUFFER:

To make 400 ml:	20 g	Dextran sulphate.
	80 ml	20X TNE BUFFER
	8 ml	50X DENHARDTS SOLUTION
	4 ml	DNA SOLUTION
	4 ml	25% SDS
	300 ml	distilled water

- store at -20°C, lasts indefinitely.

4] BUFFER 1:(0.1M TRIS pH 7.5 + 0.15M NaCl)
To make 1 litre: 12.11 g TRIS
8.76 g NaCl
- dissolve in approximately 900 ml of distilled water then add concentrated hydrochloric acid (HCl) until the pH is 7.5,
- add additional water to bring the final volume to 1 litre,
- store at room temperature,lasts 2 months.

5] BUFFER 2:(0.1M TRIS pH 7.5 + 0.15M NaCl + 0.5% Blocking Agent from the Labelling Kit.)
To make 500 ml: 2.5g Blocking Agent
- dissolve in 500 ml BUFFER 1. To dissolve will require heating at 65°C for about 1 hour with occasional stirring,
- store at 4°C, lasts for 6 months.

6] BUFFER 3:(0.1M TRIS pH 9.5 + 0.1M NaCl + 0.05M $MgCl_2$)
To make 500 ml: 6.05 g TRIS
2.92 g NaCl
10.16 g $MgCl_2$
- dissolve in 450 ml of distilled water then add concentrated HCl until the pH is 9.5,
- add additional water to bring the final volume to 1 litre,
- store at room temperature,lasts 2 months.

7] TE BUFFER:(0.01 M TRIS pH 7.4 + 0.001M EDTA)
To make 1 litre: 10 ml 1M TRIS Buffer pH 7.4
5 ml 0.2M EDTA
985 ml distilled water
- store at room temperature, lasts 2 months.

8] 1M TRIS BUFFER pH 7.4:
To make 1 litre: 121.1 g TRIS
- dissolve in 900 ml distilled water,
- add concentrated HCl until the pH is 7.4,
- add extra water to bring the final volume to 1 litre,
- store at 4°C, lasts 1 year.

9] 20X TNE BUFFER:
To make 1 litre: 175.2 g NaCl
50 ml 1M TRIS pH 7.4
10 ml 0.2M EDTA
- dissolve NaCl in approximately 800 ml distilled water,
- add the two solutions and bring to a final volume of 1 litre with additional distilled water,
- store at room temperature, lasts 6 months.

10] 25% SDS:

To make 100 ml: 25 g SDS (Sodium Dodecyl Sulphate)

- dissolve in 100 ml distilled water,
- store at room temperature,lasts indefinitely.

11] DNA SOLUTION:

To make 25 ml: 0.05 g DNA

- dissolve in 25 ml TE BUFFER at 65°C (will only dissolve slowly),
- shear the DNA by passing through a small needle (eg 26 gauge) forcefully,
- place in a boiling water bath for 10 minutes,
- store at -20°C, lasts indefinitely.

12] 50X DENHARDTS SOLUTION:

To make 100 ml: 1 g PVP (polyvinyl pyrolidone)
1 g BSA (Bovine serum albumin)
1 g Ficoll

- dissolve in 100 ml distilled water,
- store at -20°C, lasts indefinitely.

13] 0.2M EDTA:

To make 100 ml: 5.84 g EDTA
1.6 g NaOH

- dissolve in 100 ml distilled water, if the EDTA refuses to dissolve add drop wise some 1M NaOH until it dissolves but do not exceed pH 8.5,
- store at 4°C, lasts 1 year.

MAKING NON-RADIOACTIVE DNA PROBES USING THE BOERINGER KIT

1] Take approximately 2 µg of plasmid DNA (either pOrVHindN for normal Oryctes baculovirus diagnosis or pAcBamF for recombinant diagnosis) and cut with an appropriate restriction endonuclease.

2] Place in a boiling water bath for 10 minutes then remove and immediately place it on ice and as soon as it is cools (about 1 minute) remove 5 µl and place in a tube containing 2 µl VIAL 5 + 2 µl VIAL 6 and 10 µl TE BUFFER which is also on ice.

3] Take this tube to the freezer and add 1 µl of Klenow enzyme (VIAL 7). Mix the contents and incubate for two hours at 37°C.

4] Store the probe ready for use at -20°C.

PURIFYING *ORYCTES* BACULOVIRUS DNA

1] Grind up approx 5 heavily infected midguts in 4 ml VIRUS EXTRACTION BUFFER, making certain they are well homogenized.

2] Centrifuge at 5000 rpm for 10 minutes and collect the supernatant, leaving behind for discard the pellet and any lipid layer that forms above the supernatant.

3] Add the supernatant to the top of a controlled pore glass column (1.5 x 80 cm) using TE BUFFER as eluant. Consult Barton (J.Gen Virol 35: 77-87) for details of how to set up the column.

4] Allow 65 ml of eluant to be collected in a measuring cylinder then begin collecting 5 ml fractions in numbered test tubes.

5] Collect about 24 fractions and identify those containing virus by their pearly white/blue colour. This virus can then either be used to make Virus Inocula (see APPENDIX 5) or for DNA purification as follows:

6] Pool the fractions containing virus and centrifuge at 18000 rpm for 1 hour. Collect the pelleted virus in a total volume of 0.5 ml TE BUFFER. Add 20 µl of 25% SDS and incubate at 65 C for 10 minutes. Remove from the water and if available add Proteinase K at 500 µg/ml, (from a stock solution at 10 mg/ml -store frozen) Incubate overnight at 50°C. If Proteinase K is not available leave out this step. It will mean that your yield of virus DNA from the phenol extractions will probably be reduced.

7] Add 0.5 ml of buffer saturated phenol and vortex for 2 minutes. Spin in a microfuge for 3 minutes or in the Europa 24M for 5 minutes at 8000 rpm at 20°C to separate the phases. Use polypropylene centrifuge tubes for all the extractions.

8] Collect the top phase (aqueous phase), being careful to leave behind the white material at the interface of the two solutions.

9] Re-extract the interface material by adding 0.5 ml TE BUFFER to the phenol and interface material and vortexing for a further 2 minutes. The phases are then separated by centrifugation as described above.

10] After centrifugation collect the top phase and combine with the previous top phase. Extract this material 3X with an equal volume of a 50:50:2 mixture of PHENOL:CHLOROFORM: ISO-AMYL-ALCOHOL. The extractions are carried out in exactly the same way as for the phenol extractions above.

11] After all the extractions are complete determine the volume of the final top phase collected and add half the volume of 7.5M Ammonium acetate, then add two volumes of absolute ethanol and leave for 30 minutes at -20 C to allow the DNA to precipitate.

12] Centrifuge at 10,000 rpm for 10 minutes to collect the precipitated DNA

13] Wash the DNA pellet with 70% ethanol and collect by centrifugation.

14] If the pellet cannot be dried under vacuum resuspend the still wet pellet in TE BUFFER as a small amount of ethanol should not affect the restriction enzyme digestions but air drying the pellets often makes them difficult to resuspend.

APPENDIX 5

PREPARING STABILIZED VIRUS INOCULA FOR FIELD USE

1] Collect the virus containing fractions from the controlled pore glass column as described in APPENDIX 4 and add 0.5 g Sucrose (refined white sugar is OK) for every 10ml of virus solution.

2] Dissolve the sucrose and in a sterile environment (eg a laminar flow hood) pass the solution through a 0.45 µm nitrocellulose filter into a sterile container.

3] Using sterile plastic pasteur pipettes take up approximately 0.5 ml then place the tip of the pipette in a flame until it begins to burn. Remove the pipette from the flame and press the tip together with a spatule to extinguish the flame and seal the end.

4] This pipette now contains sterile inoculum that can be stored indefinitely at 4°C and will survive for a month at tropical temperatures. To use; the tip of the pipette is cut off and the pipette is used to place a drop of virus inoculum on the beetle's mouthparts.