Optimization of the polymerase chain reaction (PCR) method for the detection of Oryctes rhinoceros virus

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OPTIMIZATION OF THE POLYMERASE CHAIN REACTION (PCR) METHOD FOR THE DETECTION OF *Oryctes rhinoceros* VIRUS

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ABSTRACT

Optimization of the polymerase chain reaction (PCR) method for the rapid detection of Oryctes rhinoceros *virus (OrV) was studied. The virus DNA was extracted from the gut tissues by a robust method. Using a pair of specific primers, Primer 15a and 15b, infection was confirmed when the PCR product produced a single 945 bp DNA band. The optimized concentrations of the PCR components were at 2.0 mM $MgCl_2$, 1.0 mM 10X PCR buffer, 0.2 mM Primer 15a and 15b, 0.5 U Taq-DNA polymerase and 0.4 mg bovine serum albumin (BSA). All tested virus DNA concentrations at 0.085, 0.170 and 0.255 $\mu g\ \mu l^{-1}$ were suitable for virus detection. Addition of BSA (20 mg ml^{-1}) at 0.4 mg in the reaction increased the PCR sensitivity. The method is capable of detecting OrV infection from DNA diluted one million times or equivalent to a virus DNA concentration as low as 2.23 pg μl^{-1}. The PCR detected 83.2% adult beetles from pheromone traps as being infected by OrV, 13.6% higher ($P<0.05$) than the results based on observations on the gut morphological appearance (69.6%). A typical OrV infection symptom is a swollen gut filled with milky fluid. Of the 839 guts with this symptom, 97.6% were diagnosed to be infected which was not significantly different ($P>0.05$) compared to the method based on gut morphological appearance. The PCR was also capable in detecting virus at an early infection stage and in dead adults with decayed tissues. Of the 307 adults that appeared to be healthy, 36.1% of them were found to be infected. As much as 61.6% of dead adults with decayed tissues (N = 428) were diagnosed to be infected by the OrV. The method can be used in further research studies relating to OrV for the management of the rhinoceros beetle.*

Keywords: *Oryctes rhinoceros* virus, PCR, oil palm pests, rhinoceros beetle.

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INTRODUCTION

The rhinoceros beetle, *Oryctes rhinoceros* (L.), was previously a major pest of coconut, but has now adapted to attacking oil palm (*Elaeis guineensis*) in many tropical countries (Wood, 1968; Bedford, 1980; Norman and Mohd Basri, 1997). In Malaysia, an extensive replanting programme that prohibits the burning of oil palm residues has created abundant breeding sites for the beetle, which has led to an increase in the beetle population (Liau and Ahmad, 1991). Damage by the adult beetles reduces oil palm yield to 25% in the first two years after the attacks (Liau and Ahmad, 1991). The *O. rhinoceros* virus (OrV) is a natural enemy of the beetle and was first discovered in Malaysia in 1963 (Huger, 1966). The virus was successfully introduced to control the rhinoceros beetle in coconut in the Pacific Islands (Marschall, 1970; Hammes, 1978; Gorick, 1980; Young and Longworth, 1981), the Philippines (Zelazny and

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Alfiler, 1991), Indonesia and Maldives (Zelazny *et al*., 1992). Remarkable reductions in beetle population and palm damage were observed within one to two years after the virus was introduced (Bedford, 1986).

Previously, detection of OrV infection in the field was done by staining the gut of the beetle with 3% Giemsa (Zelazny, 1978) and by an indirect sandwich ELISA method (Young and Longworth, 1981). Currently, sets of DNA primers that specifically amplify OrV DNA have been developed (Richards *et al*., 1999). Using the polymerase chain reaction (PCR) method, detection of OrV in *Oryctes* beetle is rapid and simple as compared to the previous methods. The OrV DNA extracted from the gut tissues of *O. rhinoceros*, especially of the larvae, often contains organic materials or soil, which inhibit the PCR amplification (Juen and Traugott, 2006; Lotti and Zambonelli, 2006). Therefore, optimization of the PCR components is needed, before the method can be routinely used for OrV detection. Furthermore, the optimization study could make the PCR more repeatable, sensitive and specific (Wangsomboondee and Ristaino, 2002; Alexandrino *et al*., 2004; Ortiz *et al*., 2005).

This study reports the optimization of the PCR components using a pair of DNA-specific primers developed by Richards *et al.* (1999). The optimized PCR was then evaluated to diagnose OrV infection in adults of *O. rhinoceros* collected from various plantations in Malaysia.

MATERIALS AND METHODS

Extraction of Gut Tissues from Adults of *O. rhinoceros*

Gut tissues were extracted from *O. rhinoceros* adults collected at Pekan Estate, Kluang, Johor. First, the head of the beetle was cut and removed, followed by removal of the elytra and wings. Both sides of the abdomen were carefully cut from the anterior to the posterior region. Using forceps, the upper portion of abdomen was gently peeled until the gut was exposed. Prior to gut extraction, the morphological appearance of the gut tissues was observed and recorded. Gut tissues with advanced OrV infection are swollen and full with a whitish milky content (Zelazny *et al*., 1987). All the gut tissues were removed and placed inside a 1.5-ml tube filled with 500 μl sterilized millique water (sMqH_2O). Five sets of gut tissues were used to extract the OrV genome by the method that follows.

Extraction of OrV Genomic DNA

Gut tissues were homogenized in a 1.5-ml tube using a motorized micropestle until a cloudy solution was formed. The tube was added with sMqH_2O to reach 1.5 ml in volume, the contents were thoroughly mixed and spun at 13 000 rpm for 5 min. The supernatant was collected and filtered through a filter membrane of 0.45 μm pore size into a 12.5-ml centrifuge tube. This tube was then spun at 30 000 rpm at 10ºC for 2 hr to sediment the virus particles. Extraction of the OrV genome was performed by adding 600 μl disruption buffer containing 100 μl 1.0 M Tris at pH 8.0, 20.0 μl 1.0 M EDTA, 10.0 μl 10% SDS, 5.0 μl Proteinase K (20 mg ml^{-1}) and 464.6 μl sMqH_2O. The mixture was thoroughly mixed for a few minutes until a cloudy solution was formed, then transferred into a new 2.0-ml tube and incubated overnight in a water bath at 37ºC. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added, mixed by inverting the tube a hundred times, and spun at 13 000 rpm for 10 min. A total of 400-500 μl aqueous solution was collected and transferred into a new 1.5-ml tube, added with 1.0 μl RNAse (20 mg ml^{-1}) and incubated in a water bath at 37ºC for 6 hr. Precipitation of the OrV DNA was performed by adding a 10% volume of 3M NaAc at pH 5.2 and two volumes of absolute ethanol, and keeping the tube at -20ºC for 1 hr. To pelletize the DNA, the tube was spun at 13 000 rpm for 10 min. The DNA pellet was then dried at room temperature for about 30 min and dissolved in 100 μl TE buffer (10 mM Tris-HCl at pH 7.5, 1 mM EDTA at pH 8.0). The concentration and quality of the OrV DNA were quantified using a spectrophotometer at absorbances of A_{280}, A_{260} and A_{350}. The OrV DNA concentration was found to be 1.70 μg μl^{-1} and the quality (P) was 1.71. The DNA concentration was then diluted to 0.085 μg μl^{-1} and stored at -20ºC.

Optimization of the PCR Components

Optimization of the PCR components was performed in a 25-μl volume reaction. A pair of 20 mers specific primers developed by Richards *et al*. (1999) was used. The primer sequences and expected amplification product are listed in *Table 1*.

TABLE 1. SEQUENCES AND EXPECTED AMPLIFICATION PRODUCT OF POLYMERASE CHAIN REACTION

	Sequence	Product
Primer 15a	5′ - ATT ACT TCG TAG AGG CAA TC - 3′	945 bp
Primer 15b	5′ - CAT GAT CGA TTC GTC TGT GG - 3′	-

Each component was tested at three concentrations: low, medium and high (*Table 2*). Every concentration of each component was mixed with three concentrations of the other components, making a total of nine reactions for each component. *Table 3* shows an example of the optimization of the $MgCl_2$ concentration.

Amplification was performed in a thermal cycler machine (GeneAmp 9600, Perkin Elmer) programmed at 30 cycles; denaturing at 94ºC for 1 min, annealing at 72ºC for 2 min and extension at 50ºC for 1 min. Amplification of each test concentration was repeated twice. The PCR product was electrophoresed on 2.0% (w/v) agarose gel prepared in 1x TAE buffer. The gel was then stained in ethidium bromide (5 mg ml^{-1}), rinsed in distilled water and observed under UV light. The DNA band was photographed using a Polaroid DS34 camera with 665 film. Successful PCR amplifications of OrV DNA were confirmed when a single DNA band at 945 bp appeared on the agarose gel.

TABLE 2. CONCENTRATION OF EACH POLYMERASE CHAIN REACTION (PCR) COMPONENT TESTED IN THE EXPERIMENT

PCR component	Concentration of each component in 25 μl		
	Low	Medium	High
1. $MgCl_2$ (50 mM)	1.5 mM	2.0 mM	2.5 mM
2. PCR 10X buffer (10 mM)	1.0 mM	2.0 mM	3.0 mM
3. dNTPs (10 mM)	0.1 mM	0.2 mM	0.3 mM
4. Primer 15a (10 mM)	0.2 mM	0.4 mM	0.6 mM
5. Primer 15b (10 mM)	0.2 mM	0.4 mM	0.6 mM
6. Taq-DNA polymerase (5 U μl^{-1})	0.5 U	0.75 U	1.0 U
7. BSA (20 mg ml^{-1})	0.4 mg	0.8 mg	1.6 mg
8. Virus DNA (0.085 μg μl^{-1})	0.085 μg	0.170 μg	0.255 μg

TABLE 3. OPTIMIZATION OF $MgCl_2$ CONCENTRATION FOR DETECTION OF *O. rhinoceros* VIRUS BY POLYMERASE CHAIN REACTION

Component	Concentration								
	1	2	3	4	5	6	7	8	9
$MgCl_2$ (mM)	**1.5**	**1.5**	**1.5**	**2.0**	**2.0**	**2.0**	**2.5**	**2.5**	**2.5**
10X buffer (mM)	1	2	3	1	2	3	1	2	3
dNTPs (mM)	0.1	0.2	0.3	0.1	0.2	0.3	0.1	0.2	0.3
Primer 15a (mM)	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6
Primer 15b (mM)	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6
BSA (mg ml^{-1})	0.4	0.8	1.2	0.4	0.8	1.2	0.4	0.8	1.2
Taq DNA (U μl^{-1})	0.5	0.75	1.0	0.5	0.75	1.0	0.5	0.75	1.0
Virus DNA (μg μl^{-1})	0.085	0.17	0.255	0.085	0.170	0.255	0.085	0.17	0.255

TABLE 4. LOCALITY, CONCENTRATION AND QUALITY OF VIRUS DNA USED IN THE SENSITIVITY EXPERIMENT

	Replication 1	Replication 2
Location of sample	Paloh Estate, Kluang, Johor	MAB Estate, Sepang, Selangor
DNA concentration	0.63 $\mu g\ \mu l^{-1}$	2.23 $\mu g\ \mu l^{-1}$
DNA quality	1.43	1.44

PCR Sensitivity Study

The OrV genomic DNA was extracted from adults collected in two plantations. Each plantation represented one replication. For Replication 1 (R1), adults were sampled from the Paloh Estate in Kluang, Johor, while for Replication 2 (R2), adults were sampled from Malaysia Airports Bhd (MAB) Estate in Sepang, Selangor. The OrV genome was extracted and quantified following the method described previously. The concentration and quality of DNA are listed in *Table 4*. For each replication, the extracted DNA was diluted 10-fold down to a million times. The presence of OrV DNA at each dilution was amplified using the optimized components of PCR. Two control reactions, the positive and the negative, were included in the amplifications. The positive control used the OrV DNA that was previously confirmed by PCR and the negative control used only the $sMqH_2O$.

Effects of Bovine Serum Albumin on PCR Amplification

Two tests were conducted to determine the effects of bovine serum albumin (BSA) on PCR amplification. The first test was performed with BSA, while the second was without BSA. The OrV genomic DNA was extracted from infected adults collected from Estate Kemayan Sdn Bhd following the method described previously. The OrV DNA was then diluted 10-fold down to a million times. The presence of OrV in each dilution was determined using the optimized PCR components. The tests used 4 μl DNA in each reaction, and each reaction was repeated twice. Two controls, positive and negative, were used for each test.

Diagnosis of *O. rhinoceros* Virus in Adult Beetles by PCR

O. rhinoceros beetles were collected from various oil palm plantations in Peninsula Malaysia and Sabah. Adult samples were mostly collected at the plantations using traps supplied with a synthetic pheromone, ethyl 4-methyloctanoate. The captures were grouped (20 to 50 adults) and placed in a box of dimensions 30 cm (length) x 25 cm (width) x 20 cm (height), filled with rotting oil palm tissues, and then sent to the laboratory. At some estates, the adults were collected by placing the pheromone traps for one to five days in the field, depending on the capture results. To ensure that the adults survived during transportation, a cutting of 15-20 cm sugarcane was placed in the box as a food source for the beetles. In the laboratory, the adults were dissected and the gut tissues were extracted. Observations on the gut morphological appearance were recorded and categorized as follows:

Category 1. Live infected adults – guts swollen and full with whitish milky content following the description by Zelazny (1978), Zelazny *et al.* (1992) and Dhileepan (1994) (*Figure 1a*).

Category 2. Live and healthy adults – guts thin and full with brownish fluid (*Figure 1b*).

Category 3. Dead adults – gut tissues rotted and decayed.

For live infected and healthy adults, about 1.0 cm long of gut tissues was excised and placed in a 1.5-ml tube containing 150 μl $sMqH_2O$. For the dead adults, samples were collected from the rotted tissues. The tissue samples were kept in a deep freezer at -30ºC before OrV DNA extraction was conducted. The level of OrV infection diagnosed by the PCR and based on infection symptoms in the gut was analysed by a t-test at P=0.05 (SAS System, 1997).

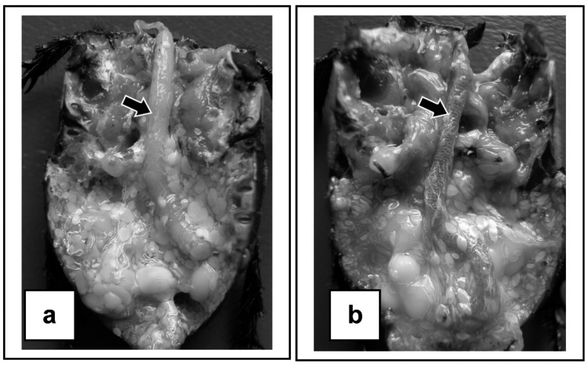


Figure 1. Morphological appearence of gut tissues of O. rhinoceros *adults. a) Gut with advanced OrV infection symptoms – swollen and full of whitish milky content (arrow), and b) gut of healthy adult – thin and full of brownish fluid (arrow).*

Extraction of Virus DNA for PCR Amplification

The gut tissues were homogenized using a motorized micropestle for 1-2 min, then the tubes were spun at 13 000 rpm for 2-3 min. A total of 150 µl supernatant was transferred into a new 1.5-ml tube and mixed with 300 µl disruption buffer containing 50 µl 1 M Tris at pH 8.0, 10 µl 1 M EDTA, 5 µl 10% SDS, 2.5 µl Proteinase K (20 mg ml^{-1}) and 232.3 µl $sMqH_2O$. The mixture was gently mixed, incubated in a water bath at 65ºC for 3 hr (or overnight at 55ºC), and then added with 450 µl of a solution of pheno l:chloroform:isoamylalchohol (25:24:1). After the mixture was the mixed and spun at 13 000 rpm for 10 min, the aqueous solution (400 µl) was collected and transferred into a new 1.5-ml tube. To precipitate the DNA, 50 µl 3M NaAc at pH5.2 and 900 µl absolute ethanol were added into the tube and kept at -20ºC for an hour. The tube was spun again at 13 000 rpm for 15 min, then the DNA pellet was dried at room temperature and finally dissolved in 100 µl TE buffer (10 mM Tris-HCl at pH7.5, 1 mM EDTA at pH 8.0).

The presence of OrV was diagnosed using the optimized PCR components as described previously. In every test, two control reactions, positive and negative, were included in the amplifications. The PCR products were run in 2.0% agarose gel prepared in 1x TAE buffer, stained in ethidium bromide (5 mg ml^{-1}) and photographed using a Polaroid film No. 665.

RESULTS AND DISCUSSION

Optimization of PCR Components

At the concentrations of 1.5, 2.0 and 2.5 mM $MgCl_2$, the PCR successfully amplified the OrV DNA, except for reactions 3, 6 and 9 (*Figure 2a*). These three reactions contained the highest concentrations of the other components. Therefore, failure in these three reactions was because of the high concentrations of the other components, but not the $MgCl_2$ concentration itself. Based on this finding, it was suggested that the optimum concentration of $MgCl_2$ was between 1.5 and 2.5 mM. For the 10X PCR buffer, all reactions (1 to 6) at 1.0 and 2.0 mM produced the band. At the highest concentration of 3.0 mM, only reaction 9 produced the band (*Figure 2b*). This suggests that the optimum concentration for the 10X PCR buffer was between 1.0 and 2.0 mM.

For dNTPs, of nine reactions tested, only four successfully amplified the OrV DNA. Two successful reactions were recorded at the 0.1 mM dNTPs, with the concentrations of the other components being low and medium (*Figure 2c*). Another two reactions were observed at 0.2 and 0.3 mM, at the low concentrations of the other components. The optimum concentration of dNTPs was suggested at 0.1 mM. Although at 0.2 and 0.3 mM dNTPs could amplify the OrV DNA, it must be mixed with low concentrations of the other components.

Results for the PCR products at the different concentrations of primers, enzyme Taq-DNA polymerase and virus DNA samples are shown in *Figures 2d*, *2e* and *2f*, respectively. The results show that the PCR successfully amplified the OrV DNA at low and medium concentrations of Primers 15a and 15b (0.2 and 0.4 mM), Taq-DNA polymerase (0.5 and 0.75 U μl^{-1}) and virus DNA (0.085 and 0.170 µg μl^{-1}). No amplification was recorded at the highest concentrations of these three PCR components as shown in reactions 3, 5 and 9. Failure of these reactions was due to the highest concentrations of the PCR components, especially the dNTPs. This was supported by the fact that the concentration of dNTPs was critical in ensuring the successful amplification of OrV DNA. *Table 5* shows that of the nine reactions with dNTPs, five failed to amplify, as compared to only three reactions for the other PCR components.

Table 5 also shows that three sets of reactions, reactions 1, 2 and 4, produced the band for all the PCR components. Based on this, reaction 4, which contained 0.2 mM dNTPs, 2.0 mM $MgCl_2$, and other components at low concentrations, was chosen for the diagnosis of infection by OrV in the next study. The concentrations of the PCR components for reaction 4 are listed in *Table 6*.

In developing a new mixture of PCR, or changing either the dNTPs or primer concentrations, the concentration of $MgCl_2$ should first be optimized (Sambrook *et al.*, 1989). As the dNTPs was the only source of a phosphate group that was used for producing a new copy of DNA by the enzyme DNA polymerase, any change in its concentration would affect the availability of ion Mg^{++}. Here, the optimal concentration of PCR was at reaction 4, with the concentration of $MgCl_2$ at 2.0 mM, still within the range suggested by Sambrook *et al.* (1989), who found that the concentration of $MgCl_2$ was between 0.5 and 5.0 mM. This reaction 4 also demonstrated that the optimum concentration of dNTPs was at 0.2 mM, also within the range suggested by Sambrook *et al.* (1989). Many workers have been using the same concentrations of $MgCl_2$ and dNTPs as in this study, when diagnosing either virus or bacteria infecting plants and humans (Dasgupta *et al.*, 1996; Nunan and Lightner, 1997; Romaine *et al.*, 2002; Rinttila *et al.*, 2004).

Sensitivity of PCR

The DNA extracted from the insects that were breeding in rotting oil palm materials or in the soil (such as the larvae of *O. rhinoceros*) could possibly be contaminated with soil which inhibits amplification of PCR. To avoid this, BSA has been commonly used

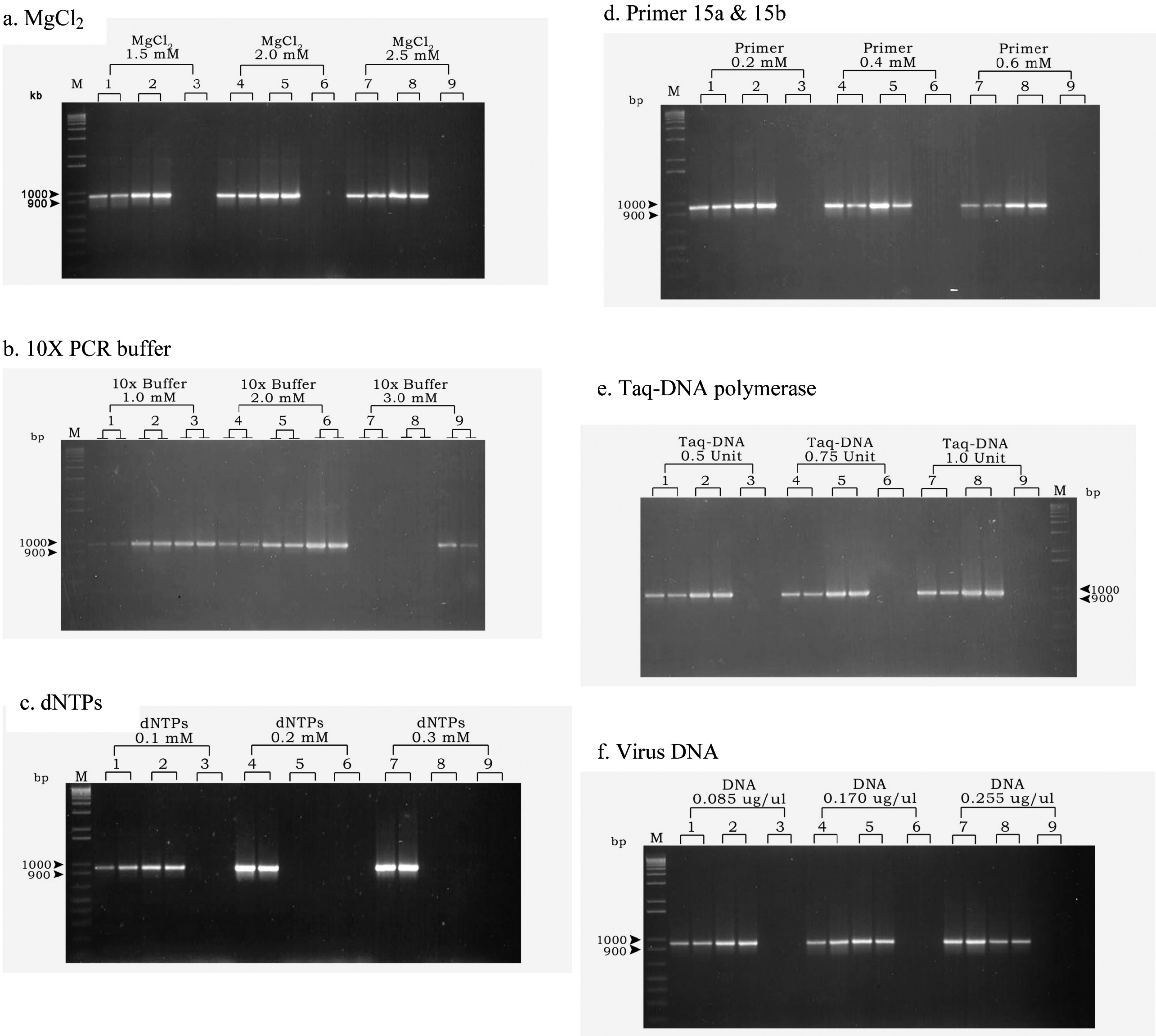


Figure 2. Amplification Oryctes rhinoceros *virus DNA using different concentrations of polymerase chain reaction components. a) $MgCl_2$, b) 10X PCR buffer, c) dNTPs, d) primer 15a and 15b, e) Taq-DNA polymerase enzyme, and f) virus DNA. M = 1 kb DNA ladder.*

TABLE 5. OPTIMIZATION OF POLYMERASE CHAIN REACTION IN DIAGNOSING *Oryctes rhinoceros* VIRUS INFECTION IN ADULT RHINOCEROS BEETLES

Component	Reactions								
	1	2	3	4	5	6	7	8	9
$MgCl_2$ (50 mM)	+	+	-	+	+	-	+	+	-
PCR 10X buffer (10 mM)	+	+	+	+	+	+	-	-	+
dNTPs (10 mM)	+	+	-	+	-	-	+	-	-
Primer 15a (10 mM)	+	+	-	+	+	-	+	+	-
Primer 15b (10 mM)	+	+	-	+	+	-	+	+	-
Taq DNA polymerase (5 U μl^{-1})	+	+	-	+	+	-	+	+	-
DNA sample (0.085 μg μl^{-1})	+	+	-	+	+	-	+	+	-

Note:
+ PCR produced a band at the tested concentration of the component.
- PCR failure, no band appeared on the agarose gel.

TABLE 6. OPTIMIZED CONCENTRATION OF POLYMERASE CHAIN REACTION (PCR) COMPONENTS FOR DETECTION OF *Oryctes rhinoceros* VIRUS INFECTION ON RHINOCEROS BEETLES

No.	Component	Optimum concentration	Volume of component (μl)
1	$MgCl_2$ (50 mM)	2.0 mM	1.0
2	10X PCR buffer (10 mM)	1.0 mM	2.5
3	dNTPs (10 mM)	0.2 mM	0.5
4	Primer 15a (10 mM)	0.2 mM	0.5
5	Primer 15b (10 mM)	0.2 mM	0.5
6	Taq-DNA polymerase (5 U)	0.5 U	0.1
7	BSA (20 mg ml^{-1})	0.4 mg	0.5
8	DNA sample (0.085 μg)	0.085 μg	1.0
9	Sterilized millique water ($sMqH_2O$)	-	18.4
Total volume			25.0

by various researchers, especially to detect DNA of bacteria or mycorrhiza that are isolated from soil (Felske *et al*., 1996; MgGregor *et al*., 1996; Lotti and Zambonelli, 2006; Castrillo *et al*., 2007). In this study, adding BSA increased the PCR sensitivity in detecting the OrV. *Figure 3* shows that the PCR could detect the OrV from DNA diluted one million times. The band in Replication 2 can be seen clearly. The band in Replication 1 was faint and possibly cannot be recognized by those who are inexperienced in molecular work. Therefore, based on the data from Replication 2, it is suggested that the optimized PCR can detect the presence of OrV from a concentration of DNA as low as 2.23 pg μl^{-1}.

Figure 4 shows that the addition of BSA not only increased the PCR sensitivity, but it also overcame the inhibitory effect caused by contaminated DNA. With BSA at 0.4 mg, the PCR successfully amplified the virus DNA diluted 10^6 times. However, without BSA, it only detected OrV DNA at 10^5 times dilution. It was also noticed that the addition of BSA in the PCR reaction facilitated the detection of the virus in higher DNA quantities, 10 times higher than without BSA. Other studies show that BSA has been used as an additional component in the PCR for the detection of old microorganisms from herbarium leaf samples, that commonly produce low DNA yield (Savolainen *et al*., 1995) and from a root insect pest, *Amphimallon solstitiale*, that was extracted from the gut of its predator *Poecilus versicolor* (Juen and Traugott, 2006). As BSA has proven to be able to avoid the inhibitory effect of PCR, it will be routinely used in the reactions for detecting OrV infection, especially from the larvae of the beetle.

Infection Level of *O. rhinoceros* Virus in Adults

The PCR products that successfully amplified the OrV from field-collected adults of the beetle are shown in *Figure 5*. Infection by OrV was confirmed when a single 945 bp DNA band appeared on the agaros gel. *Table 7* shows estimates of the infection levels by OrV in adults collected from several states in Malaysia. Of a total of 1146 adults captured in the pheromone traps, 839 adults fell into Category 1, in which the gut tissues were swollen and filled with a milky content. By the PCR method, 97.6% (823) of the adults with these symptoms were found to be infected, and this result was not significantly different ($F=1.04$, df: 33,33, $P>0.05$) from the detection based on gut visual appearance (*Figure 6a*). With this finding, it was concluded that swollen gut tissues with a whitish milky content are typical symptoms of advanced OrV infection in adults. Results of this study support observations by previous workers (Zelazny, 1978; Zelazny *et al*., 1992; Dhileepan, 1994).

Out of the 1146 adults, 307 adults were in Category 2, consisting of adults with no signs of virus infection. The adults in this category had thin guts filled with a brownish digestive fluid. At this stage, any infection by OrV could not be seen. However, PCR detected about 46.6% of the adults as being infected by the virus, which was significantly higher than the detection based on gut morphological appearance ($F=4.03$, df: 33,33, $P<0.01$) (*Figure 6b*). This finding was expected as the PCR method could detect OrV at a very early stage of infection, even when the gut was not yet showing any signs of infection. Although early OrV infection can be diagnosed by staining with 3% Giemsa (Zelazny,

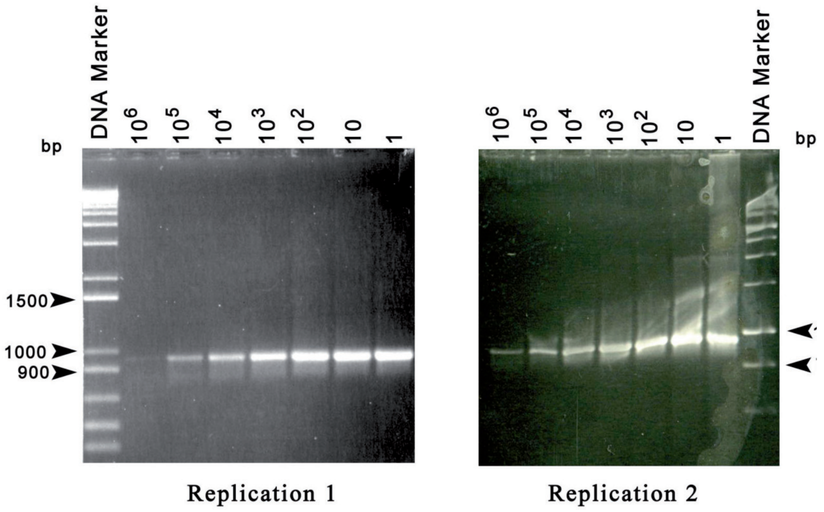


Figure 3. Polymerase chain reaction amplifications using a series of diluted Oryctes rhinoceros *virus DNA.*

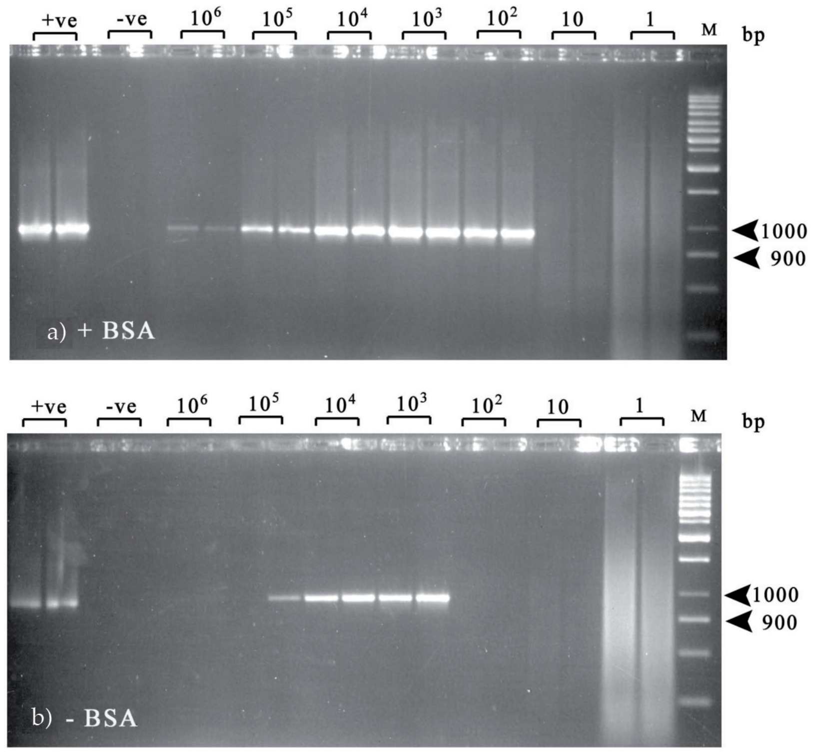
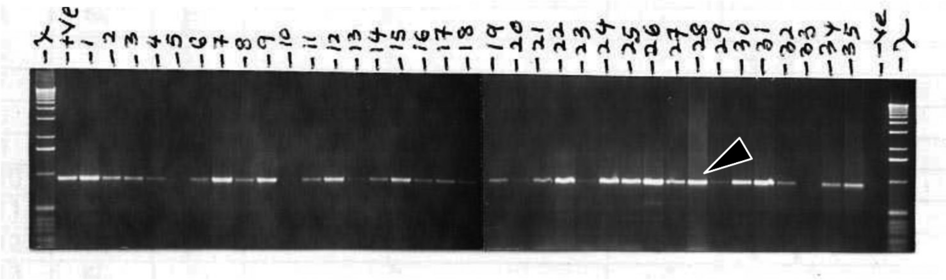


Figure 4. Polymerase chain reaction amplifications using a series of diluted Oryctes rhinoceros *virus DNA. a) Reaction with addition of bovine serum albumin (BSA), and b) reaction without BSA. +ve= positive control; -ve = negative control; M = DNA marker.*

a) Sedenak Estate, Johor



b) Kok Foh Estate, Negeri Sembilan

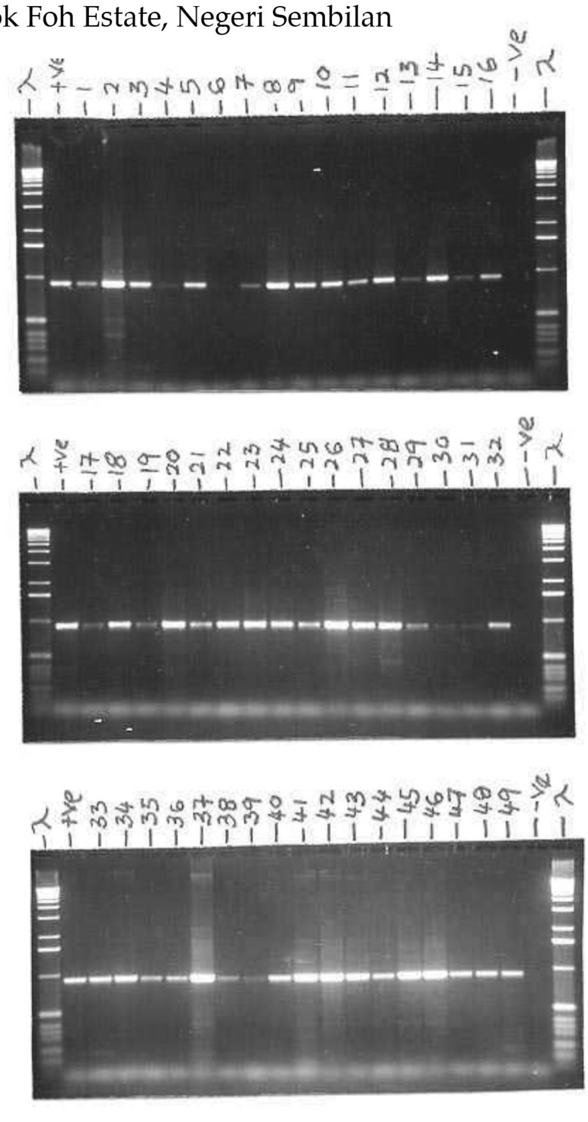


Figure 5. Examples of polymerase chain reaction products for diagnosing Oryctes rhinoceros *virus infection in* rhinoceros *beetles. a) Samples from Sedenak Estate, Johor, and b) samples from Kok Foh Estate, Negeri Sembilan. An adult was confirmed infected by* Oryctes rhinoceros *when a single DNA band of 945 bp (arrow) appeared on the agaroe gel. λ = DNA Marker; +ve = positive control; -ve = negative control. Numbers in the top row indicate the sample number used in the tests.*

TABLE 7. INFECTION LEVEL BY *Oryctes rhinoceros* VIRUS DIAGNOSED BY POLYMERASE CHAIN REACTION (PCR) AND BASED ON GUT MORPHOLOGICAL APPEARANCE IN *Oryctes rhinoceros* ADULTS COLLECTED AT VARIOUS LOCALITIES IN PENINSULA MALAYSIA AND SABAH

Locality	No. of adults tested (N)	Adults with infection symptoms		Adults without infection symptoms			Infection based on symptoms (%)	Infection based on PCR	
			+ PCR		+ PCR	- PCR			
		(n)	(n)	(n)	(n)	(n)		(n)	(%)
Kedah	12	9	9	3	2	1	75.0	11	91.7
Johor	354	267	260	88	46	42	73.6	306	85.1
Perak	263	189	185	74	41	34	67.7	226	80.9
Selangor	176	159	157	17	9	7	88.6	165	94.1
Negeri Sembilan	124	95	104	29	21	8	77.5	115	94
Pahang	40	12	11	28	10	18	30.0	21	52.5
Terengganu	9	9	9	0	0	0	100.0	9	100.0
Sabah	166	99	98	65	11	54	53.1	109	59.2
Total	1146	839	823	307	143	164	-	963	-
Average (% infection)	-	-	-	-	-	-	69.6	-	83.2

Note:
+ PCR = PCR test produced a single DNA band on the agarose gel.
- PCR = PCR test did not produce any band on the agarose gel.

1978) or by the indirect sandwich ELISA (Young and Longworth, 1981) methods, these methods are time-consuming as they need further validation by bioassay studies. Overall, the current study showed that the PCR could diagnose 83.2% of the live adults as being infected, significantly higher ($P=4.52$, df: 33,33, $P<0.05$) than the method based on the visual appearance of gut tissue (69.6%) (*Figure 6c*).

Another advantage of the PCR method was that it could also detect the presence of OrV in the decayed tissues of dead adults. Of the 428 cadavers studied, 61.6% of them were infected (*Table 8*). In the field, such large numbers of dead infected adults harbouring OrV could act as a source of virus inoculum, which is responsible for virus transmission to living adults or larvae. However, the remaining 38.4% dead adults were without OrV particles. These cadavers probably died as a result of abiotic causes such as high temperature, drowning and starvation. This was possible as the adults were trapped in a bucket that was placed in the field for several days before being collected.

A high rate of infection of the adults collected from pheromone traps by OrV was common (*Table 7*). Of the eight states sampled, only two (Pahang and Sabah) yielded samples which were diagnosed with OrV infection less than 60%. Samples from the remaining states were diagnosed with high OrV infection, ranging from 80 to 100%. The higher levels of OrV infection could be the result of three factors. First, the PCR method was highly sensitive and was capable of detecting early virus infection in adults that appeared healthy. This was supported by the fact that 46.6% of the adults without any infection symptoms were found to be actually infected by the virus (*Figure 6b*).

Second, cross contamination among the adults may have occurred to those which were placed in the same box during transportation from the plantations to the laboratory. It is well-known that virus infection begins in the cell nucleus, which later replicates in the hypertrophied cells before accumulating in the gut lumen (Huger, 1966; Huger and Krieg, 1991). The infected beetle adults had become a virus reservoir, releasing virus particles as much as 0.3 mg a day in their faeces (Monsarrat and Veyrunes, 1976), which then contaminated the substrates in the boxes. Thus, high virus transmission probably occurred among adult beetles in the same box, via direct contact with the contaminated substrates or through mating (Zelazny, 1976).

Third, it was possible that the pheromone traps attracted more infected adults than healthy ones. Although the reason for this is still uncertain, it was

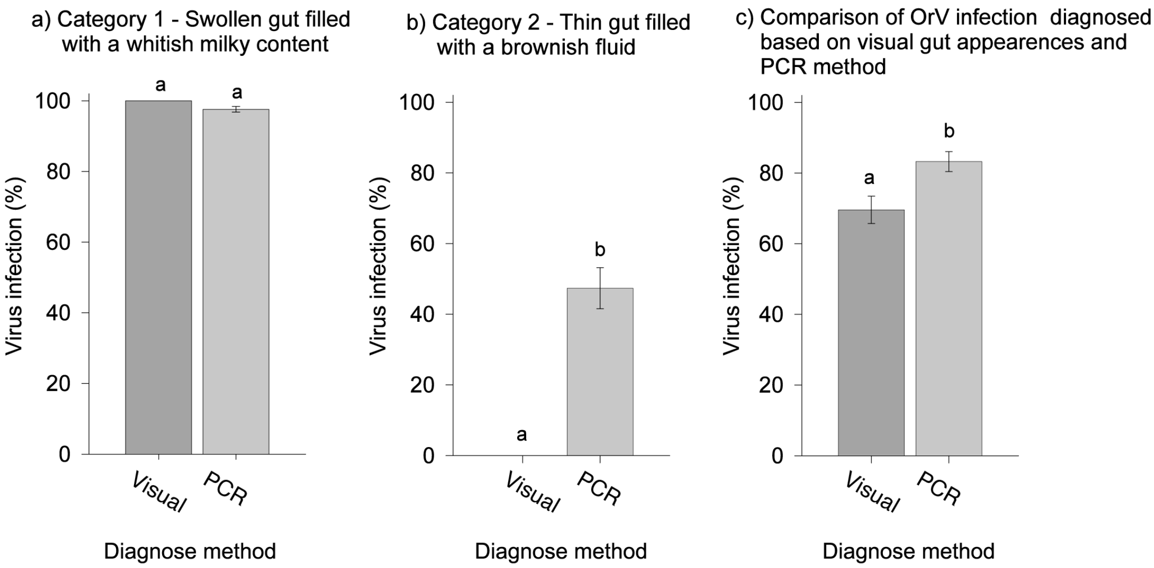


Figure 6. Infection levels by Oryctes rhinoceros *virus (ORV) in* Oryctes *adults with symptoms, healthy adults and dead adults with decayed internal gut tissues as diagnosed by polymerase chain reaction (PCR) method. Bars with the same letter are not significantly different by t- test at P=0.05.*

TABLE 8. INFECTION BY *Oryctes rhinoceros* VIRUS AS DIAGNOSED BY POLYMERASE CHAIN REACTION (PCR) IN DEAD *Oryctes* ADULTS COLLECTED FROM VARIOUS LOCALITIES IN MALAYSIA

Origin of samples	No. of samples tested (N)	Virus infection as diagnosed by PCR	
		(n)	(%)
Nam Heng Complex, Johor	18	6	33.3
Kulai Besar Estate, Johor	61	28	45.9
Jendarata Estate, Perak	15	5	33.3
Bukit Paloh Estate, Johor	37	21	56.8
Sungai Rayat Estate, Johor	177	147	83.1
Koh Foh Estate, N. Sembilan	47	36	76.6
Lam Soon Estate, Pahang	64	55	85.9
Ulu Jempol Estate, Pahang	9	7	77.8
Total	428	305	-
Mean ± SE	53.50 ± 19.11	38.12 ± 16.71	61.58 ± 7.81

Note: SE = standard error.

suspected that virus infection may have changed the behaviour of the adults. It is well-known that OrV infection reduces adult activities, especially in search of food and in preparing their breeding sites (Zelazny and Alfiler, 1991). This will make it much easier to attract them to the pheromone traps, as compared to the healthy adults, which may be more active looking for food and suitable places for laying their eggs.

Although the PCR method is more expensive than the conventional diagnostic method, the former has proven to be more sensitive, accurate and repeatable. Therefore, it is commonly used in many industries. For example, PCR is widely used in medicine, especially to detect the human immunodeficiency (HIV) virus in human patients (Gibson *et al.*, 1993; Colimon *et al.*, 1996), and the foot and mouth disease virus in cattle (House and Meyer, 1993). In the aquaculture industry, PCR has been used to diagnose the presence of the virus which causes the white spot disease in shrimps (Chang *et al.*, 1993; Nunan and Lightner, 1997). In agriculture, the method is commonly used to diagnose virus infection in *Helicoperva armigera*, an insect pest of cotton, tomato, corn and tobacco (Christian *et al.*, 2001), and to detect the plant viruses of several crops such as barley, grape, lemon and rice (Canning *et al.*, 1996; Dasgupta *et al.*, 1996; Valsesia *el al.*, 2005).

CONCLUSION

The components of PCR were successfully optimized. The reaction consisted of 0.2 mM $MgCl_2$, 1.0 mM 10X PCR buffer, 0.2 mM Primer 15a and 15b, 0.2 mM dNTPs and 0.5 unit Enzyme Taq-DNA polymerase. Addition of BSA between 0.4 mg and 1.2 mg in the reaction increased the PCR sensitivity and acted as an anti-inhibitory agent of PCR. The method was sensitive, capable of detecting virus DNA from as low as one million times dilution, that is equivalent to a DNA concentration as low as 2.23 pg μl^{-1}. Virus infection was confirmed when the PCR amplified a single DNA fragment at 945 bp, which appeared as a single band on the agarose gel.

Using a robust DNA extraction method, detection of OrV in adults by PCR is simple. Furthermore, the method detected 83.2% OrV infection in adults, 13.6% higher than the method based on gut morphological appearance, which only detected 69.6%. A swollen gut with a whitish milky content is the common virus infection symptom in adult beetles. The PCR method showed that 97.6% of the samples with this symptom were confirmed to be infected by the virus. The PCR could diagnose the presence of OrV at an early stage of infection, and also detect the virus in the dead adults with rotting internal tissues. Of the 307 apparently healthy adults, 46.6% or 143 adults were in fact infected, and, of the 428 cadavers tested, 61.6% of them were confirmed to be infected by OrV.

This study demonstrated that PCR can be used for the rapid detection of OrV infection in the rhinoceros beetle. Therefore, the method is recommended for use in any work that is related to the application of the *O. rhinoceros* virus in the management of the *O. rhinoceros* beetle.

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