3. Evaluation of PCR methodology for grasserie disease detection

To determine whether PCR is applicable for grasserie disease detection, preliminary tests were performed using BmNPV artificially inoculated samples.

3.1 Detection of BmNPV in silkworm eggs

When the specific primers were used to amplify fragment of BmNPV *polh*, PCR amplified products of the expected size (424 bp) were observed in the gel. As illustrated on the gel, PCR products of the Thai BmNPV *polh* had the same size of the clone of polyhedrin gene in pGEM®-T vector (positive control) while there was no amplified product when using PCR reaction mixture without DNA template (negative control). Eggs laid by artificially infected silkworm moths were used to illustrate the grasserie disease detection by PCR. The results revealed that genomic BmNPV DNA could be detected when using the sample as low as one egg. Increasing the number of eggs for DNA extraction provided higher concentration of DNA template resulting in high intensity of DNA band visualized on the gel (Figure 21). PCR products were specific to the virus used as the DNA template and no nonspecific sequences were observed. Strong intensity of PCR product bands were clearly visualized on the gel when using DNA extracted from 5 to 20 eggs. Thus, for accurate interpretation, 20 silkworm eggs were used to represent one sample of egg for all experiments in this study.

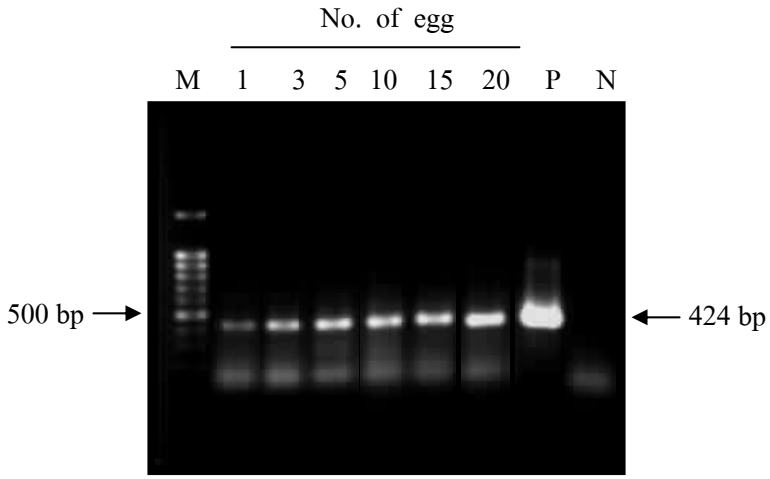


Figure 21 PCR products of DNA extracted from 1, 3, 5, 10, 15, 20 eggs laid by BmNPV-artificially infected silkworm moths

M=DNA marker (100 bp+1.5 kb)

P= positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no DNA template)

The colour and the characteristic of eggs laid by BmNPV- infected silkworm moth were not different from those laid by the healthy one. However, number of eggs laid by BmNPV-infected moth was about 40% less than those from healthy moth. Healthy female moth laid an average of 453 eggs while infected moth laid an average of 269 eggs (Table 3). This result was similar to the study of Khurad *et al*. (2004) who indicated that the number of eggs from healthy moth (580 eggs) was 40% more than the number of eggs from infected moth (360 eggs).

Table 3 Number of eggs laid by healthy and artificially BmNPV-infected silkworm moths, *Bombyx mori*

Couple of moths	No. of eggs laid by healthy moth	No. of eggs laid by BmNPV-infected moth
1	494	298
2	523	308
3	473	313
4	553	241
5	385	217
6	481	338
7	400	306
8	423	250
9	407	174
10	395	248
total	**4534**	**2693**
%	**100**	**59.38**

This study provided evidence that PCR is an efficient tool for detection of grasserie disease in silkworm. The detection could be made as early as in egg stage and only one egg can be used for the detection. In the past, scientists believed that only pebrine disease of silkworm is transmitted through eggs laid by infected silkmoth. Hence, most of the reports on disease detection in silkworm egg were dealing with pebrine disease and rarely with viral diseases. Attathom *et al.* (1994) reported that DNA probe was applicable for the detection of BmNPV in silkworm eggs of which the parents were artificially infected with BmNPV. PCR method was previously tested for *Lymantria dispar* NPV (LdNPV) detection on the surface of an egg. Samples of gypsy moth's egg were obtained by artificially contaminated surface of the eggs with LdNPV (Burand *et al*., 1992). In this study egg samples were those oviposited by mother moths infected with BmNPV during the larval stage. The existing of BmNPV in the eggs could suggest

that BmNPV may be transmitted vertically from parents to offsping. In recent study on BmNPV of silkworm, the result of PCR amplification of BmNPV DNA isolated from the viral-infected parents and the F1 offspring had confirmed that the viral infection was vertically transmitted to the progeny, however, the authors did not detect viral infection in egg (Khurad *et al.*, 2004). Ikuno *et al*. (2004) could detect BmNPV in infected eggs of silkworm using PCR with primers specific to polyhedrin gene. However, when compared with this study, their method comprised of many steps for DNA extraction. For example, eggs were washed with sodium hypochoride, crushed, and ground in STE-buffer, DNA was extracted from 40 mg of egg sample (approximately 11 eggs) by means of incubation with SDS and proteinaseK and followed by purification using DNA purification kit. However, in this study, DNA was extracted by grinding the eggs in solution II following the treatment with solution III, chloroform:isoamlyl alcohol, isopropanol and finally the DNA was washed by 70% alcohol.

3. 2 Detection of BmNPV in artificially inoculated silkworm larvae

The appropriate number of the first instar larvae for DNA extraction and subsequent PCR amplification was determined. The expected PCR products of 424 bp were obtained and there were no different of band clearness and intensity when using DNA template extracted either from 1, 2, 3 or 4 larvae (Figure 22). This result suggested that only one larva of the first instar provided adequate DNA template to be amplified by PCR method. DNAs extracted from individual artificially inoculated larva of the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} instar provided PCR products of expected size. No PCR products were observed when using DNA extracted from healthy larvae (Figure 23).

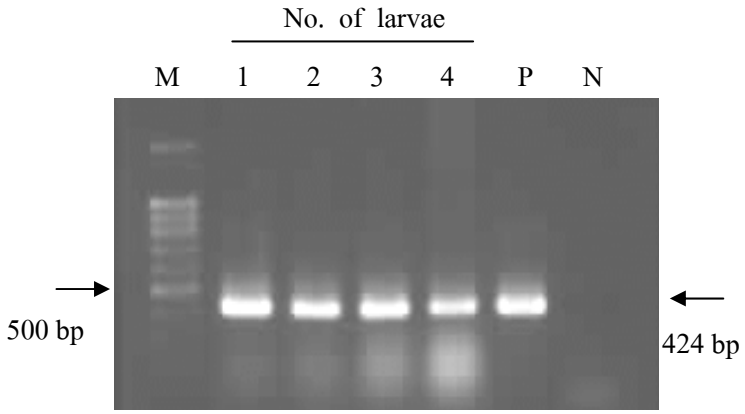


Figure 22 PCR products of DNA extracted from various numbers of BmNPV-artificially infected first instar larvae

M=DNA marker (100 bp+1.5 kb)

P=positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no DNA template)

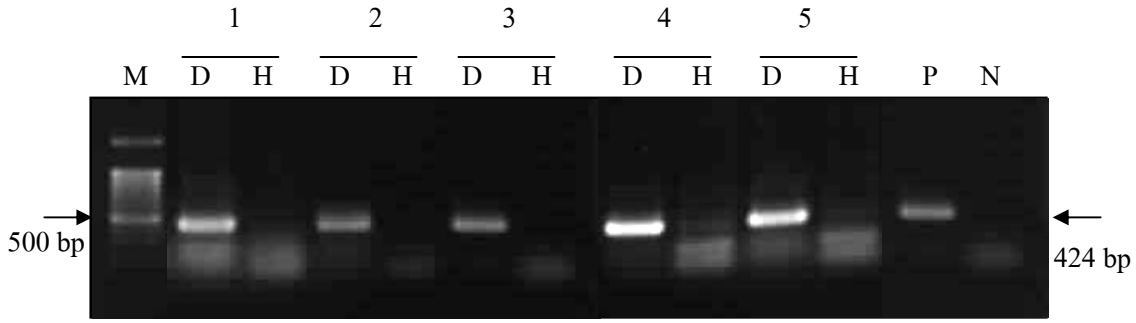


Figure 23 PCR products of DNA extracted from various instars of BmNPV-artificially infected larvae. Numbers on the top row indicated instar of the larvae.

M=DNA marker (100 bp+1.5 kb)

P=positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no DNA template)

H=healthy larva D=diseased larva

Silkworm, *Bombyx mori* undergoes four moults, thus there were five instars during the larval development (Figure 24). Symptoms of grasserie disease as normally shown in late instar of BmNPV infected larvae were illustrated in Figure 25. The symptoms could hardly be noticed during the early larval instar, except that the larvae were slightly sluggish. Disease symptoms started with the colour of larval body changed from white to yellow (Figure 25 a). As the disease progress, the body became swollen (Figure 25 b) and the colour of intersegmental region turned brown (Figure 25 c). In the later stage of infection, the skin ruptured, turbid haemolymph containing innumerable polyhedral bodies released and the larvae died (Figure 25 d). Frequently, characteristic symptom of NPV infection was observed. The larvae became restless and crawled aimlessly along the rims of rearing tray. Diseased larvae lost the clasping power of the abdominal legs except the caudal legs by which it hanged with the head downwards (Figure 25 e). Subsequently, they fell on the ground and died. Death took place after infection in about 3-5 days in the young larvae and 5-7 days in the late instar larvae.

Using PCR, BmNPV could be detected in the larval stage as early as the first instar larva. In all larval instar, only one individual infected larva provided adequate BmNPV DNA to use as PCR template. This result was similar to the study of Ikuno *et al*. (2004) who reported that PCR with complementary primers to the *polh* region could be used to detect BmNPV in haemolymph of all larval instars. Previous reports revealed methods for the detection of BmNPV in all larval instars including indirect ELISA (Vanapruk *et al.*, 1992) and DNA probe (Attathom *et al.*, 1994). However, these two methods were complicated and used rather expensive chemicals. In addition, some other techniques such as monoclonal antibody, 2-3 second instar larvae were needed for BmNPV detection (Kalsi *et al.*, 1998) and for sandwich ELISA, 5-6 second instar larvae were required for the detection (Shamim *et al.*, 1994).

Figure 24 The healthy mulberry silkworm, *Bombyx mori* larvae

a) first instar b) second instar

c) third instar d) fourth instar

e) fifth instar

Figure 25 Symptoms of grasserie disease of silkworm, *Bombyx mori* caused by BmNPV

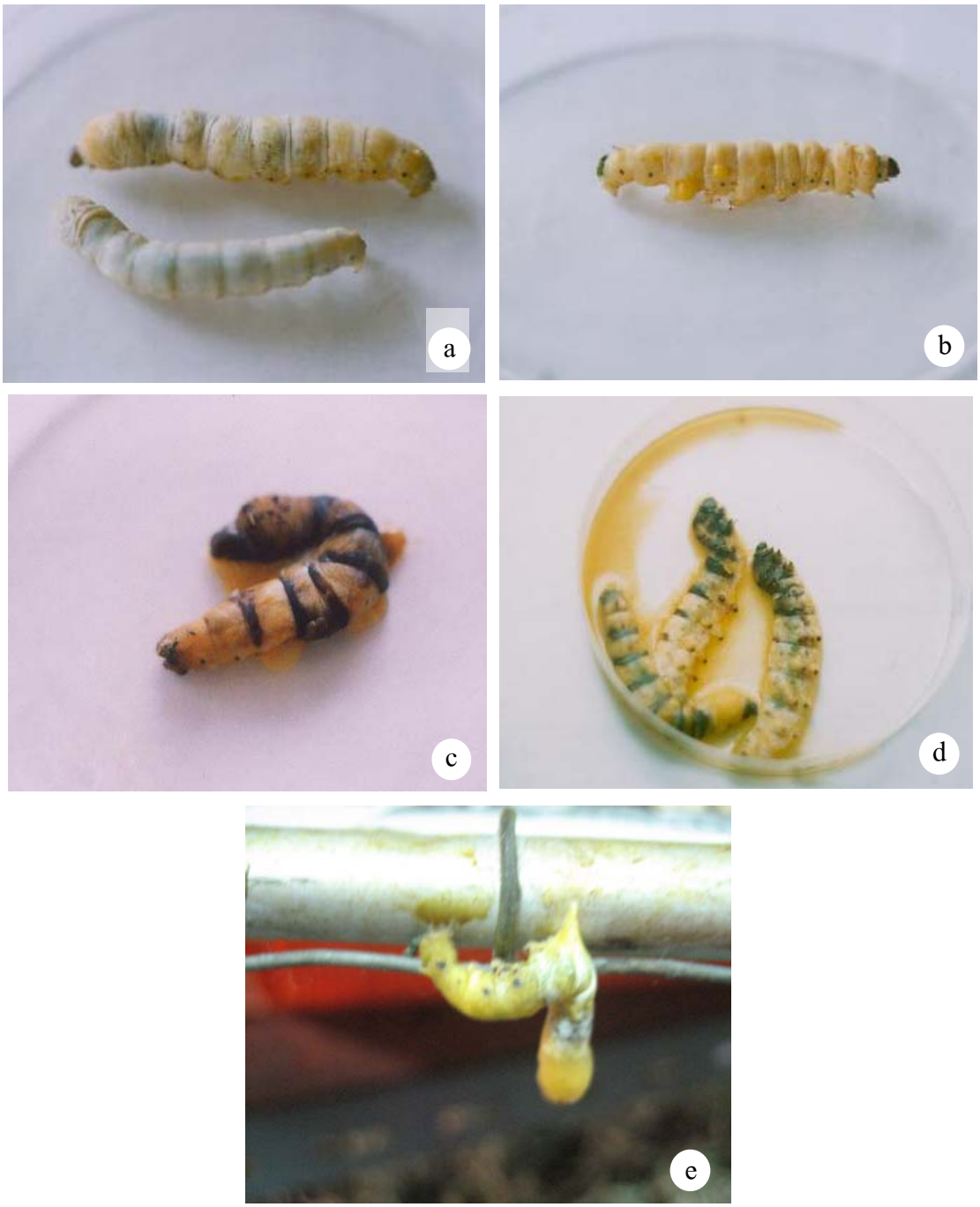
a) Healthy larva (top) compared with BmNPV infected larva (bottom) which appeared yellowish in body colour.

b) Larva showed robust body with swollen segments

c) Intersegmental area turned brown and the body became fluffy

d) Dead larvae marked by skin rupture and exudation of tubid haemolymph contained numerous polyhedra of the virus

e) Larva showed characteristic symptom of NPV infection. The larva hanged dead with head downwards.


a
b
c
d
e

3.3 Detection of BmNPV in silkworm pupae

With the developed PCR method, BmNPV could be detected in both infected male and female pupa while no BmNPV DNA was detected in healthy pupa (Figure 26). PCR products of expected size (424 bp) were observed in infected male and female pupae.

There were a few reports on BmNPV detection in pupal stage. In practical, grasseries detection at pupal stage is useless because if this disease occurred in pupal stage, it was too late to manage silkworm rearing. The only anticipated benefit is that one can eliminate infected pupae and keep, perhaps clean silkworm colony for next generation. Previously, Vanapruk*s* (1991) reported that the indirect ELISA could be used to detect BmNPV in the pupal stage after inoculation the fourth instar larvae with the virus at 10^6 PIB/ml. In addition, Attathom *et al*., (1994) reported the use of digoxiginin labeled probes to detect BmNPV DNA in infected pupae.

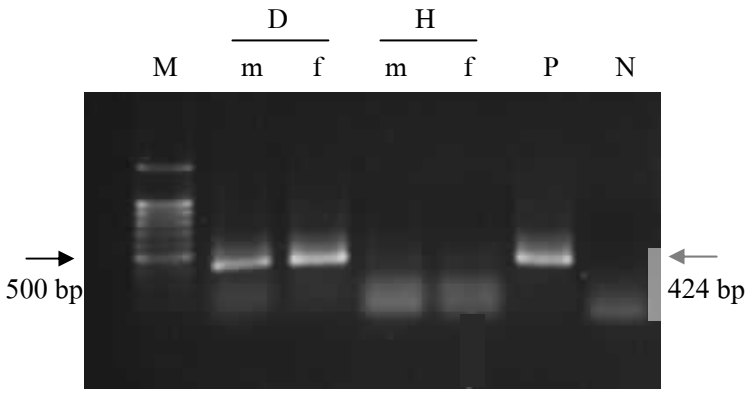


Figure 26 PCR products of DNA extracted from BmNPV-artificially infected male and female pupae

M=DNA marker (100 bp+1.5 kb)

P=positive control (pGEM® -T vector harboring polyhedrin gene)

N=negative control (no DNA template)

H=healthy pupa; D=diseased pupa

f= female pupa; m=male pupa

The yellow cocoons and healthy pupae of local silkworm race were shown in Figure 27 a and b. When the silkworms were artificially inoculated with BmNPV at their fifth larval instar, there were many symptoms appeared during the final moult and pupation. For example, some larvae could not change into pupal form and die within the old cuticle (4%) (Figure 27 c). However, some larvae changed into pupa but could not spin fibres (9%) (Figure 27 d), and some could not change into pupal form but spinned abnormal cocoon and finally died inside the cocoon (30%) (Figure 27 e). Several of the infected pupae showed abnormal pupal shape (22%) (Figure 27 g). Only 35% of the infected pupae appeared as normal pupae (Table 4).

Table 4 Percentage of several abnormal symptoms of pupae of silkworm, *Bombyx mori* artificially infected with nucleopolyhedrovirus.

Symptom	Percentage
1. normal pupa	35
2. larva cannot change into pupal form	4
3. larva can change into pupal form but cannot spin fibres	9
4. larva cannot change into pupal form but can spin fibres	30
5. abnormal pupa	22
total	**100**

The essential hormone involves in moulting process in insect is ecdysone. BmNPV was known to harbor *egt* gene encoded for EGT protein which was produced in insect cell during the viral infection. This protein transfers the sugar moiety from a UDP-sugar to ecdysone. The function of EGT protein is to block the moulting of insect host during infection (O'Reilly *et al*, 1992). Therefore, the deformed shape of BmNPV infected pupae, could occur from the action of the existing *egt* gene in BmNPV genome to inhibit normal development of the silkworm. In addition, NPV had some effect on pupal size as reported by Myers *et al.* (2000) who described that pupae of gypsy moth infected with *Lymantria dispar* nucleopolyhedrovirus (LdNPV) were smaller than the healthy pupae.

Figure 27 Symptoms of grasserie disease of silkworm, *Bomby mori* caused by nucleopolyhedrovirus as appeared in the pupal stage

a) Yellow cocoon of healthy pupae

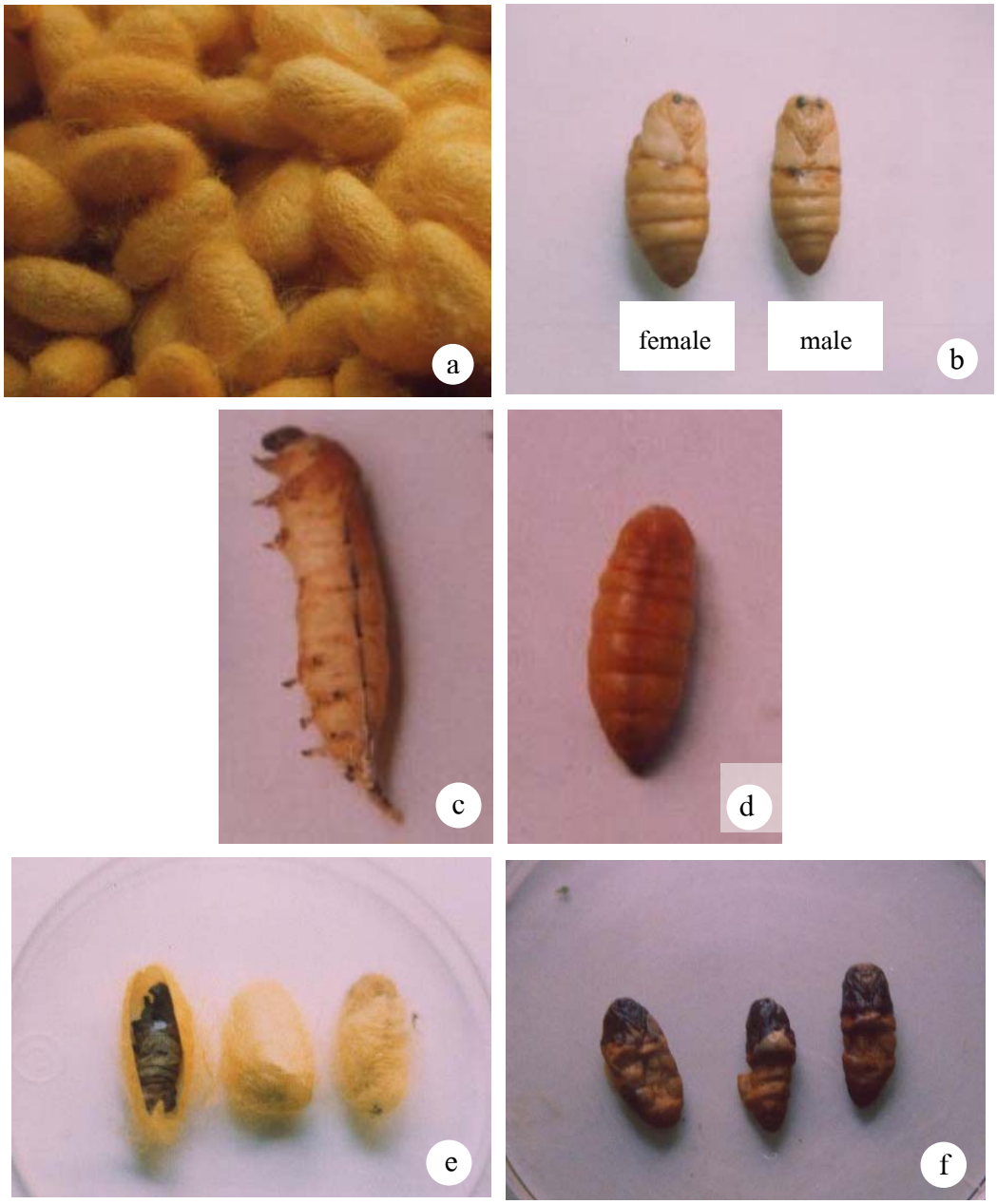
b) Healthy male and female pupae.

c) Larva cannot change into pupal form

d) Larva can change into pupal form but cannot spin fibres

e) Larva cannot change into pupal form but can spin fibres

f) Abnormal pupae


a
female
male
b
c
d
e
f

3.4 Detection of BmNPV in silkworm moths

Detection of grasserrie disease in silkworm moths by PCR gave similar result as found in pupae. DNA extracted from diseased male and female moths were amplified using primers specific to Thai *polh* gene, and PCR products of expected size were obtained. DNA from healthy samples gave no PCR products (Figure 28).

The healthy moths emerged by rupturing one end of the cocoon after which male and female moths started to mate. Normally, female is bigger than male (Figure 29 a). After mating, the female moth laid group of eggs that glued on paper (Figure 29 b). The BmNPV-infected moths were in irregular shape as compared to the healthy moths. Some moths could not emerge from the pupae (Figure 29 c) while the others had shortened or distorted wings (Figure 29 d). Some BmNPV infected moths were larger than the healthy moths. This is due to the fact that BmNPV infected nuclei of several insect cells and tissues. Numerous polyhedra were produced in the nuclei which caused the cells to undergo hypertrophy and hence the insect body swollen (Boucias and Rohrmann, 1998).

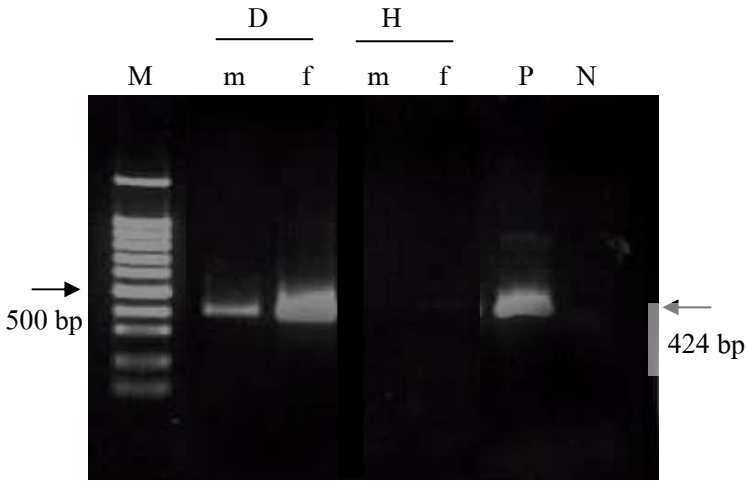


Figure 28 PCR products of DNA extracted from BmNPV-artificially inoculated male and female moths

M= DNA marker(100 bp+1.5kbp)

P= positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no DNA template)

H=healthy moth D=diseased moth

f= female moth m=male moth

Figure 29 Healthy and BmNPV-infected silkworm moths, *Bombyx mori*

a) Male and female healthy moths

b) Female moth lays group of eggs that glue on paper

c) Moth cannot emerge from pupal shell

d) Moth with abnormal body and wings

In this study, BmNPV-infected male and female silkworm moths were allowed to mate and laid their eggs. After that, BmNPV detection in the parent moths and their eggs by PCR was performed. From 30 samples, BmNPV was detected in 30% of the male moth, 10% of the female moth and 37% of the egg. BmNPV was detected less in male and female moths than in egg. In this study the whole body of moth was crushed and DNA was extracted and used as DNA template for PCR detection. Concentration of the viral DNA per volume of the solution may be lower than that obtained from eggs. For viral detection in moth, it may be necessary to detect the virus in each tissue or organ separately. Attention can be focused on tissues or organs that had records as sites of viral infection. The result of this study was similar to previous report by Vanapruk *et al.* (1992) who, using ELISA, detected BmNPV in all stages of silkworm except the adult moths which were inoculated with the virus at their 4^{th} larval instar. This may be due to the level of the virus in the sample was too low to be detected. However, Attathom *et al.* (1994) was able to detect BmNPV in adult silkworm moth using dot blot hybridization.

This evaluation study showed that grasserie disease of silkworm could be detected by PCR. Detection could be made in all developmental stages and in both sexes of silkworm. Therefore, PCR method was further used to demonstrate its efficacy in detecting grasserie disease in collected samples of silkworm from the rearing houses.