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Cloning and Comparative Characterization of Nucleopolyhedroviruses Isolated from African Bollworm, *Helicoverpa armigera*, (Lepidoptera: Noctudiae) in Different Geographic Regions

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A total of 162 clones were isolated by plaque assay with Hz-AM1 cells from uncloned field populations of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) collected from Kenya, South Africa, Zimbabwe and Thailand. Restriction endonuclease (REN) analysis showed that 25 out of the 162 clones exhibited unique REN patterns, which should be characterized as variants of HearNPV. Five clones, NNg1, NS2, NMa1, NZ3 and NT1, were randomly selected from the 25 clones and characterized in both Hz-AM1 cells and *H. armigera* larvae, together with the clone G4 which was isolated in China and sequenced previously. In Hz-AM1 cells, clones NNg1, NMa1, NZ3 and NS2 produced more budded virions than clones G4 and NT1, whereas a higher amount of polyhedrin was produced in clones NNg1 and G4. The dose-mortality analysis revealed that clone NNg1 expressed the highest insecticidal activity against third instar *H. armigera* larvae, showing a 50% lethal dose (LD_{50}) value of 10 occlusion bodies (OBs)/larva and a 50% lethal time (LT_{50}) value of 4.0 days following peroral infection with OBs from infected larvae. Clones NS2, NMa1 and NZ3 had LD_{50} values of 32, 35 and 146 OBs/larva and LT_{50} values of 5.1, 4.4 and 4.8 days, respectively, indicating that these three clones had insecticidal activity somewhat lower than that of clone NNg1. In contrast, clones NT1 and G4 had markedly lower insecticidal activities compared with the four other clones, recording LD_{50} values of 826 and 3115 OBs/larva and LT_{50} values of 5.8 and 8.3 days, respectively. These results indicate that clone NNg1 is the most promising candidate for biological control programs of *H. armigera* larvae among the six clones characterized in this study.

Key words: *Helicoverpa armigera*, African bollworm, nucleopolyhedrovirus, HearNPV, variant, insecticidal activity, REN analysis, plaque assay, Hz-AM1 cell, bioassay

INTRODUCTION

The African bollworm, *Helicoverpa armigera* (Hübner), is a major pest which causes extensive economic losses to over 60 cultivated crops and 67 wild plant species belonging to 47 families throughout the tropics and sub-tropics (Zalucki *et al.*, 1986, 1994; King, 1994). The importance of *H. armigera* as a pest is due to its polyphagy, high fecundity and short generation time, high mobility, preference for harvestable fruiting parts of its host plants and its propensity to develop resistance to chemical pesticides (Cherry *et al.*, 2003).

Low economic damage thresholds in high valued crops like cotton, citrus, pulses, tobacco, vegetables and flowers require a high level of control of *H. armigera* that leads to exclusive reliance on chemical pesticides in almost all the cases where its larvae are reported to feed on the crops in Africa (Cherry *et al.*, 2003). Because of increasing problems with resistance of larvae to chemical insecticides and other biological control agents such as *Bacillus thuringiensis* (Gould, 1998; Gunning *et al.*, 2005), alternative control strategies are being explored with the aim of developing safer and more effective control agents.

The usefulness of nucleopolyhedroviruses (NPVs) in control of the *H. armigera* larvae has been recognized and shown to be a viable alternative to chemical pesticides because of its safety for the environment, beneficial arthropods and non-target organisms (Allen and Ignoffo, 1969; Roome, 1975; Teakle *et al.*, 1985; Figueiredo *et al.*, 1999; Moore *et al.*, 2004; Ogembo *et al.*, 2005). Naturally occurring *H. armigera* NPVs (HearNPVs) have been isolated from the US, Brazil, Japan, Australia, Thailand, Poland, Israel, the Netherlands, China, India, Portugal, Spain, South Africa, Botswana and Kenya (Hunter-Fujita *et al.*, 1998). In some of these regions, HearNPVs cause epizootic infections almost every year (van den Berg, 1993). There is focused interest in characterizing the biochemical and biological properties of these isolates to select strains with higher virulence and genetic stability.

Comparative studies of some of the HearNPV isolates have shown differences in their insecticidal activities, but

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in many instances the wild isolates have been used as the biological control agent without cloning. Clonal viral isolates have the advantage over uncloned viral isolates in monitoring possible genetic alterations, which may occur in the viruses during mass production and/or following the release into the field. Also, characterization of cloned viral isolates with different insecticidal activities from the same host must help in elucidating possible mechanisms underlying the expression of insecticidal activities of the viruses at the molecular level. To date, there is no report on the cloned African HearNPV isolates, despite much data indicating their high insecticidal activity to *H. armigera* larvae (Roome, 1975; Whitlock, 1977; Moore *et al.*, 2004; Ogembo *et al.*, 2005).

In this study, we isolate a total of 162 clones from uncloned HearNPV populations collected from Kenya, South Africa, Zimbabwe and Thailand, and show by restriction endonuclease (REN) analysis that these clones consist of 25 distinct HearNPV variants, which are different from the sequenced Chinese HearNPV clone G4 (Chen *et al.*, 2001). Based on bioassays employing third instar *H. armigera*, we also show that the African clone NNg1 expresses the highest insecticidal activity among the six clones characterized in this study. Three other African clones, NS2, NMa1 and NZ3, exhibit significantly higher insecticidal activities than clones G4 and NT1, which were isolated in China and Thailand, respectively. These results demonstrate that strain selection of the viruses from wild populations is useful to develop NPVs desirable for biopesticides, and indicate that the African HearNPV clones isolated in this study, clone NNg1 in particular, represent good candidates for biological control programs of *H. armigera* larvae.

MATERIALS AND METHODS

Viruses and cell culture

HearNPV stocks used in these experiments are shown in Table 1. Stock NS was a gift from Dr. Sean Moore of Citrus Research International, Pretoria, South Africa (Whitlock 1974). Stock NZ was derived from laboratory *H. armigera* culture stock infected accidentally with HearNPV at the University of Zimbabwe (Ogembo, 2002). Stocks NNg, NMa, NKi, NSi, NNa, NJu, NSa and NVo were collected from a field survey conducted in Kenya by the International Centre of Insect Physiology and Ecology (ICIPE) from 1999 to 2000 in various field crops around the country (Baya, 2000). Stock NT was collected in Thailand by Dr. Sudawan Chaeychomsri. Clone G4, a sequenced Chinese HearNPV clone, (Chen *et al.*, 2001) was obtained from Prof. Just Vlak, Wageningen Agricultural University, the Netherlands.

The continuous cell line BCIRL-Hz-AM1 (Hz-AM1: McIntosh and Ignoffo, 1981) from *Helicoverpa zea* used in these experiments was maintained in TC100 medium (JRH Biosciences) supplemented with 10% fetal bovine serum at 28°C as described previously (Lua and Reid, 2000).

Cloning of HearNPV

To prepare BV inocula for cell culture, semi-purified occlusion bodies (OBs) of HearNPV stocks from Kenya, South Africa, Zimbabwe and Thailand were inoculated perorally to the second instar *H. armigera* larvae. At 6 days post-inoculation, dead larvae were collected and homogenized in 800 µl of deionized water containing 20 µl of 1 M phenylthiourea. The homogenate was centrifuged at 3000 rpm for 2 min and the supernatant was filter-sterilized through a 450-nm cellulose acetate membrane (DISMIC-25 cs; Advantec Toyo, Osaka, Japan). The sterile supernatant was used to amplify the respective uncloned HearNPVs in Hz-AM1 cells, and the culture media harvested from the infected Hz-AM1 cells were used as the inocula for the cloning of HearNPVs by plaque purification.

Cloning of HearNPV was performed by plaque purification procedure with Hz-AM1 cells according to the method described previously (Laviña *et al.*, 2001; Kamiya *et al.*, 2004). Each plaque from the first round of plaque purification (tentative clone) was picked by Pasteur-pipette, suspended in 1 ml of TC100 medium and used for the infection of Hz-AM1 cells. Viral DNA was isolated from the infected Hz-AM1 cells and subjected to restriction endonuclease (REN) analysis to examine possible genetic variations. After the REN analysis, the tentative clones with unique genotypes were subjected to two additional rounds of plaque purification and HearNPVs from the final plaques were again examined by REN analysis to confirm their genomic identity with unique genotypes.

Viral DNA isolation and restriction endonuclease (REN) analysis

Viral DNA was isolated by phenol-chloroform (1:1) from the HearNPV-infected Hz-AM1 cells as described previously (Stewart and Possee, 1993; Laviña *et al.*, 2001; Laviña-Caoili *et al.*, 2001; Kamiya *et al.*, 2004). The REN analysis of viral DNA was carried out as described previously (Laviña *et al.*, 2001; Laviña-Caoili *et al.*, 2001; Kamiya *et al.*, 2004). Each viral DNA (2 µg) was digested at 37°C for 4 h with 3 µl of *Eco*RI, *Bgl*II, *Xba*I or *Hin*dIII in 40 µl of reaction mixture. After digestion, DNA fragments were ethanol-precipitated, dissolved in 10 µl of TE-8 buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and separated on a 0.5% agarose gel (Agarose H; Nippon Gene, Osaka, Japan). The gel was stained with ethidium bromide and DNA bands were visualized in UV light

(Epi-Light UV FA1100; Aishin Cosmos, Kariya, Japan). For the estimation of DNA fragment sizes of the newly cloned HearNPVs, genomic DNA of sequenced HearNPV clone G4 was digested with respective restriction endonucleases, and resultant DNA fragments of known molecular sizes were used as molecular size standards. In some experiments, λDNA *Hin*dIII-digest and λDNA *Hin*dIII-*Eco*RI double-digest (Nippon Gene, Osaka, Japan) were also used as molecular size standards.

Sample preparation for budded virion (BV) titration, slot-blot hybridization analysis and immunoblot analysis

Samples for BV titration, slot-blot hybridization analysis of viral DNA and for immunoblot analysis of polyhedrin were prepared from the cell culture in a single flask at each time postinfection (pi) as described previously (Laviña *et al.*, 2001). Hz-AM1 cells (1×10^6) in 12.5-cm^2 flasks were infected with each of the HearNPV clones at a multiplicity of infection (MOI) of 1 plaque-forming unit (PFU) per cell, and cultured in 2.5 ml of TC100 medium. At different times pi, 500 µl aliquots of the culture media were harvested and stored at −80°C for BV titration. Infected cells were scrapped into the culture media and 50 µl aliquots of cell suspensions were removed into microcentrifuge tubes for slot-blot hybridization analysis. The remaining cell suspensions were centrifuged at 3000 rpm for 5 min at 4°C and the resulting pellets were lysed in 60 µl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) 2-mercaptoethanol) for SDS-polyacrylamide gel electrophoresis for immunoblot analysis.

Slot-blot hybridization analysis

Slot-blot hybridization analysis of viral DNA was carried out as described previously (Ikeda and Kobayashi, 1999; Shirata *et al.*, 1999; Laviña *et al.*, 2001). A portion of viral DNA polymerase gene (ca. 1 kbp) was used as the probe. The probe was amplified by PCR using HearNPV clone NS2 DNA as the template and two synthetic oligonucleotides (5'-ACGATACGATTTGAGTCC GG-3' and 3'-CGCATAAACAACGGCATGCT-5') as paired primers and labeled by Fluorescein Gene Images (Amersham Biosciences). The Fluorescein-labelled probe hybridized was detected by Gene Images CDP-Star detection module (Amersham Biosciences), and chemiluminescence was monitored and analyzed by Lumi-Imager F1 Work Station (Boehringer Mannheim). A known quantity of HearNPV DNA (4, 20, 100 and 500 ng) was blotted onto the same Hybond N+ nylon membrane as the reference of viral DNA amounts.

Immunoblot analysis

Proteins from infected cells were resolved on SDS-polyacrylamide gels and processed for immunoblot analysis as described previously (Shirata *et al.*, 1999; Laviña *et al.*, 2001; Kamiya *et al.*, 2004). The HearNPV polyhedrin was probed with anti-BmNPV polyhedrin antiserum as the primary antibody (Kobayashi *et al.*, 1990) and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Zymed Laboratories, San Francisco, CA) as the secondary antibody. Detection of signals was carried out using Konica Immunostain HRP-1000 (Konica, Osaka, Japan). Dr. Western (Oriental Yeast, Tokyo, Japan) was used as the molecular weight marker.

Budded virion (BV) titration

BVs in the culture media of infected cells were titrated by plaque assay as described previously (Shirata *et al.*, 1999; Laviña *et al.*, 2001).

Preparation of occlusion bodies (OBs) for bioassay

OBs used for bioassay were prepared *in vivo* in third instar *H. armigera* larvae by inoculating perorally with the OBs previously produced in Hz-AM1 cells infected with each of the selected HearNPV clones. After inoculation, the larvae were maintained on an uncontaminated artificial diet (Southland Inc. Ltd, New Jersey) and dead larvae were collected immediately after death. The cadavers were homogenized in deionized water and centrifuged at 3000 rpm for 15 min at 4°C. The pellet was suspended in OB washing buffer (10 mM Tris-HCl, pH 8, 0.02% Tween 80), filtered through two layers of gauze, and centrifuged at 600 rpm for 5 min at 4°C. The OBs in the supernatant were further purified by the same differential centrifugation. The OB suspension obtained was treated for 60 sec with homogenizer and stored at 4°C until used for bioassay. Concentration of OBs was determined using a Thomas hemocytometer under a light microscope.

Insect and bioassay of selected HearNPV clones

The *H. armigera* eggs were obtained from a laboratory culture from Gifu Prefectural Institute of Bioindustrial Technology, Japan. The larvae were reared on an artificial diet in a growth chamber at 25 ± 2°C, 60% relative humidity and a photoperiod of 12:12 h (light and darkness). The larvae for bioassay were standardized by the head capsule width size and body weight as described previously (Ogembo *et al.*, 2005).

The droplet-feeding bioassay method (Hughes and Wood, 1981; Hughes *et al.*, 1986) was used to inoculate *H. armigera* larvae. The newly ecdysed third instar larvae were starved for 16 h and inoculated with 8 µl of droplets containing OBs in 10% sucrose solution with blue dye. A series of 10-fold dilutions of OBs ranging from 1.8×10^2 to 1.8×10^7 OBs/ml was prepared to allow for the design

of definitive bioassay. After ingestion of OBs, the larvae were transferred to 24-well tissue culture plates (C-D International, Pitman, NJ) and reared individually on an uncontaminated artificial diet at 25°C. The inoculated larvae were examined daily until death or pupation. For definitive bioassay, larvae were fed on 2×10^2, 1×10^3, 5×10^3, 2.5×10^4 and 1.25×10^5 OBs/ml. The data was analysed for 50% lethal dose (LD_{50}) and 50% lethal time (LT_{50}) by the Probit method (Finney, 1978).

RESULTS

Cloning of HearNPVs and REN analysis of genomic DNA of the cloned HearNPVs

HearNPV isolates from Kenya, South Africa, Zimbabwe and Thailand were cloned using the standard plaque purification procedure. The Kenyan isolates were obtained from insects collected from different ecological regions and they were treated separately as such. A total of 162 plaques were isolated from the 11 HearNPV stocks used in this study (Table 1). The viruses from each of the plaques from the first round of plaque purification were infected to Hz-AM1 cells, and viral DNAs were extracted from the infected cells at 72 h pi. The viral DNAs were subjected to REN analysis using *Eco*RI, yielding 25 tentative clones with unique REN patterns (data not shown). The 25 tentative unique clones were subjected to two additional rounds of plaque purification, and REN patterns of viral DNAs for the clones obtained in the final round of plaque purification were examined again with *Eco*RI. The REN patterns proved that all of the 25 tentative clones from the first round of plaque purification exhibited distinct HearNPV genotypes. These 25 clones were named in Table 1 for future reference. On the basis of the REN analysis, the HearNPV isolates from some geographic regions (Malindi (NMa), Simba Hills (NSi), Juja (NJu), Samburu (NSa) and Thailand (NT)) consisted of only a single genotype, whereas those from other geographic regions contained two or more unique clones (Table 1).

Five clones NS2, NNg1, NMa1, NZ3, and NT1 were randomly selected from the 25 clones for *in vitro* and *in vivo* studies and for more comprehensive REN analysis and genomic size estimation using endonucleases *Bgl*II, *Xba*I and *Hin*dIII (Fig. 1; Table 2). Comparative REN analyses with *Bgl*II, *Xba*I and *Hin*dIII yielded one or more unique bands for all the selected clones, which could be used as restriction fragment length polymorphism (RFLP) markers to distinguish the clones from each other. When digested with *Bgl*II, NS2 and NMa1 clones yielded no unique fragment (Fig. 1A; Table 2). Clone NNg1 could be identified by the absence of 6.9 kbp fragment which was present in all other clones, whereas fragments of 11.5 kbp and 3.2 kbp could be used as RFLP markers for NZ3. Clone NT1 could be distinguished by the presence of 8.8 kbp fragment and the absence of 15.0 kbp fragment. The Chinese clone G4 used as a reference clone had two unique fragments of 5.8 and 5.0 kbp, but it lacked the fragment of 10.7 kbp present in all five other clones (Fig. 1A; Table 2).

When digested with *Xba*I, unique fragments of 4.4, 3.4, 3.35 and 12.4 kbp were yielded for clones NS2, NNg1, NZ3, and NT1, respectively. NMa1 clone did not have any unique DNA fragments (Fig. 1B; Table 1). On the other hand, the absence of 3.2, 9.1, 5.9, and 7.2 kbp fragments was unique for clones NS2, NMa1, NZ3 and NT1, respectively. Clone G4 had two unique fragments of 2.5 and 1.3 kbp but did not possess 1.0 kbp fragment present in all other clones (Fig. 1B; Table 1).

Table 1. *Helicoverpa armigera* nucleopolyhedrovirus stocks and clones isolated in this study

Stock		No. of isolated clones	No. of clones with unique REN pattern	Location where the isolates were obtained
No.	Name			
1	NS	20	3 (NS1, 2, 3)[1]	South Africa[2]
2	NNg	16	4 (NNg1, 2, 3, 4)	Kenya (Nguruman)
3	NMa	24	1 (NMa1)	Kenya (Malindi)
4	NKi	12	5 (NKi1, 2, 3, 4, 5)	Kenya (Kibwezi)
5	NSi	13	1 (NSi1)	Kenya (Simba Hills)
6	NNa	14	4 (NNa1, 2, 3, 4)	Kenya (Nairobi)
7	NJu	11	1 (NJu1)	Kenya (Juja)
8	NSa	10	1[3]	Kenya (Samburu)
9	NVo	18	2 (NVo1, 2)	Kenya (Voi)
10	NZ	12	3 (NZ1, 2, 3)	Zimbabwe
11	NT	12	1 (NT1)	Thailand
Total		162[4]	25[5]	

[1]Designation of clones with unique REN pattern.
[2]First reported by Whitlock (1974).
[3]This clone exhibited REN pattern identical to that of NJu1.
[4]Total number of isolated clones.
[5]Total number of clones with unique REN patterns.

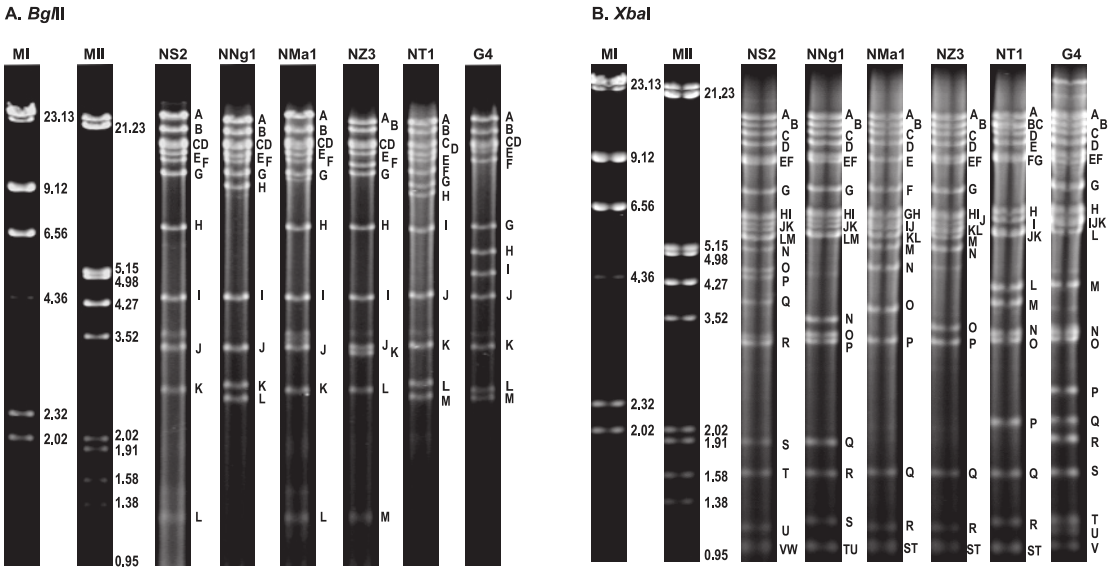

Fig. 1. Restriction endonuclease fragmentation patterns of genomic DNA of the six selected HearNPV clones. Viral DNA was extracted from Hz-AM1 cells infected with HearNPV clones NS2, NNg1, NMa1, NZ3, NT1 and G4 and digested with *Bgl*II (A) and *Xba*I (B). Resultant DNA fragments were analyzed on 0.5% agarose gels and stained with ethidium bromide. MI and MII indicate molecular size markers that are prepared from λ phage DNA by digestion with *Hin*dIII and *Hin*dIII plus *Eco*RI, respectively.

When digested with *Hin*dIII, 7.7 kbp fragment was exclusively detected in clone NNg1 whereas fragments of 3.0 and 1.9 kbp were unique for NMa1 and NZ3, respectively (Table 1). The absence of 3.3 kbp fragment was unique to NT1 clone, whereas clone NS2 did not yield any unique fragments.

The average genome sizes of these clones estimated by summation of the DNA fragments generated upon digestion with each of *Bgl*II, *Xba*I and *Hin*dIII were found to vary in range between 126.4 kbp for clone NMa1 and 132.7 kbp for clone NS2 (Table 2). The approximate average genomic sizes of clones NNg1, NZ3, NT1 and G4 were 130.1, 127.1, 129.7, and 129.5 kbp, respectively, under these conditions.

Cytopathology

Light microscopic examination showed that the growth of Hz-AM1 cells was arrested upon infection with any of the six HearNPV clones. Morphological alterations could be detected at 24 h pi and became clearly visible with the increase in time pi (Fig. 2). The infected Hz-AM1 cells became spherical and increased in size. These infected cells, however, were tightly attached onto the bottom of the culture flasks until 48 h pi, but at 72 h pi about 50% of cells containing OBs were suspended into the culture medium. OB formation was first observed at 24 h pi in Hz-AM1 cells infected with clone NNg1. For the other clones, the formation of OBs was delayed to 48 h pi, and at this time less than 50% of the cells had OBs. The infection progressed over time until around 80% of the cells produced OBs for all the clones at 96 h pi. Careful observation revealed that clone NZ3 produced fewer OBs per cell at 96 h pi, compared with other clones, although about 90% of the infected cells had OBs (Fig. 2).

Budded virus yield

Hz-AM1 cells were infected with each of the six clones at an MOI of 1, and BVs in the culture medium were titered at 0, 24, 48, 72 and 96 h pi (Fig. 3). The BV titer increased much faster with a steep slope until 24 h pi and then increased gradually reaching a peak at 48 h pi, thereafter forming a plateau at 72 h pi for clones NS2, NNg1, NMa1 and NZ3. At 48 h pi, there were no significant differences in the BV titers among clones NS2, NNg1, NMa1 and NZ3, but the titers of these four clones were significantly higher than those of clones G4 and NT1. BV titers of clones G4 and NT1 continued to increase with time untill 96 pi. The overall BV titers of clones NT1 and G4 were significantly lower than those of four other clones assayed.

Viral DNA production

Viral DNA accumulation in HearNPV-infected Hz-AM1 cells was examined by slot-blot hybridization analysis (Fig. 4). The viral DNA was first detected at 24 h pi in all the clones and increased reaching a plateau at 72 h pi. At 24 h pi, clones NNg1 and NS2 had the highest and the second highest amounts of viral DNA, respectively, among the clones analyzed, suggesting that these two clones extensively synthesized the viral DNA during the first 24 h of infection. Following these two clones, clones G4, NMa1 and NZ3 produced viral DNA at a similar

Table 2. Restriction fragments of different HearNPV clones generated by restriction endonucleases *Bgl*II, *Xba*I and *Hin*dIII

Size (kbp)	*Bgl*II						Size (kbp)	*Xba*I						Size (kbp)	*Hin*dIII					
	NS2	NNg1	NMa1	NZ3	NT1	G4		NS2	NNg1	NMa1	NZ3	NT1	G4		NS2	NNg1	NMa1	NZ3	NT1	G4
25.5	A	-	A	-	-	A	14.2	A	A	A	A	A	A	22.6	A	A	A	A	A	A
23.7	-	A	-	A	A	-	13.0	B	B	B	B	B	B	17.6	-	-	-	-	B	B
18.7	B	B	B	B	B	B	12.4	-	-	-	-	C	-	17.1	B	-	B	B	-	-
15.3	C	C	C	C	C	C	11.9	C	C	C	C	D	C	14.5	-	B	-	-	C	C
15.0	D	D	D	D	-	D	10.6	D	D	D	D	E	D	13.5	C	-	C	C	-	-
13.3	E	E	E	E	D	E	9.3	E	E	E	E	F	E	13.0	D	C	D	D	D	D
12.4	F	F	F	-	E	F	9.1	F	F	-	F	G	F	11.0	E	D	E	E	E	E
11.5	-	-	-	F	-	-	7.2	G	G	F	G	-	G	10.8	F	E	-	F	F	-
10.7	G	G	G	G	F	-	6.2	H	H	G	H	H	H	10.7	-	F	-	-	-	F
9.4	-	H	-	-	G	-	6.1	I	I	H	I	-	-	10.4	G	G	F	G	G	G
8.8	-	-	-	-	H	-	5.9	J	J	I	-	I	I	10.0	H	H	G	H	H	H
6.9	H	-	H	H	I	G	5.8	-	-	-	J	-	J	8.2	I	-	H	-	-	-
5.8	-	-	-	-	-	H	5.7	K	K	J	K	J	K	7.7	-	I	-	-	-	-
5.0	-	-	-	-	-	I	5.5	L	L	K	L	K	L	7.5	J	J	I	I	I	I
4.3	I	I	I	I	J	J	5.4	M	M	L	M	-	-	6.7	-	K	-	-	J	J
3.3	J	J	J	J	K	K	4.8	N	-	M	N	-	-	4.0	-	-	J	-	K	-
3.2	-	-	-	K	-	-	4.6	O	-	N	-	-	-	3.3	K	L	K	J	-	K
2.7	-	K	-	-	L	-	4.4	P	-	-	-	-	-	3.0	-	-	L	-	-	-
2.6	K	-	K	L	-	L	4.0	-	-	-	-	L	M	2.6	L	M	M	K	L	L
2.5	-	L	-	-	M	M	3.6	Q	-	O	-	M	-	1.9	-	-	-	L	-	-
1.3	L	-	L	M	-	-	3.4	-	N	-	-	-	-	1.5	M	N	N	M	M	M
							3.35	-	-	-	O	-	-							
							3.3	-	-	-	-	N	N							
							3.2	-	O	P	P	O	O							
							3.1	R	P	-	-	-	-							
							2.5	-	-	-	-	-	P							
							2.1	-	-	-	-	P	Q							
							1.9	S	Q	-	-	-	R							
							1.6	T	R	Q	Q	Q	S							
							1.3	-	-	-	-	-	T							
							1.2	-	S	-	-	R	-							
							1.17	U	-	R	R	-	U							
							1.1	V	T	S	S	S	V							
							1.0	W	U	T	T	T	-							
Total (kbp)	129.3	131.3	129.3	129.8	132.0	130.6	Total (kbp)	137.4	126.6	122.1	126.2	124.9	126.6	Total (kbp)	131.5	132.3	127.7	125.2	132.2	131.4

level until 24 h pi. Clone NT1 produced the least amount of viral DNA during the same period of time.

Polyhedrin production

Polyhedrin accumulation in the Hz-AM1 cells infected with the six clones of HearNPV was examined by immunoblot analysis with an antiserum against BmNPV polyhedrin (Fig. 5). The polyhedrin was first detected at 24 h pi and gradually increased over time for all the six clones examined. Accumulation of polyhedrin was higher in clones NNg1 and G4 than in other clones examined throughout the experiments. Polyhedrin production in the cells infected with clones NT1 and NZ3 was very low at 24 and 48 h pi, but increased thereafter over time until 96 h pi. Molecular masses of the polyhedrin estimated on SDS-polyacrylamide gels were approximately 32 kDa for all the six clones.

Insecticidal activity of selected HearNPV clones

The insecticidal activities of the selected HearNPV clones were determined for third instar *H. armigera* larvae in terms of LD_{50} and LT_{50}. The calculated dose-mortality regression lines and the associated statistics are shown in Table 3. The mortality increased with dosage for all the clones. Clone NNg1 had the lowest LD_{50} value of 10 OBs/larva, suggesting that it was highly pathogenic to *H. armigera* larvae (Table 3). The LD_{50} values of clones NMa1, NS2, and NZ3 were 32, 35 and 146 OBs/larva, respectively, with overlapping confidence limits, indicating that their insecticidal activities were not significantly different. Clones G4 and NT1 had LD_{50} values of 3115 and 826 OBs/larva, respectively, which were significantly

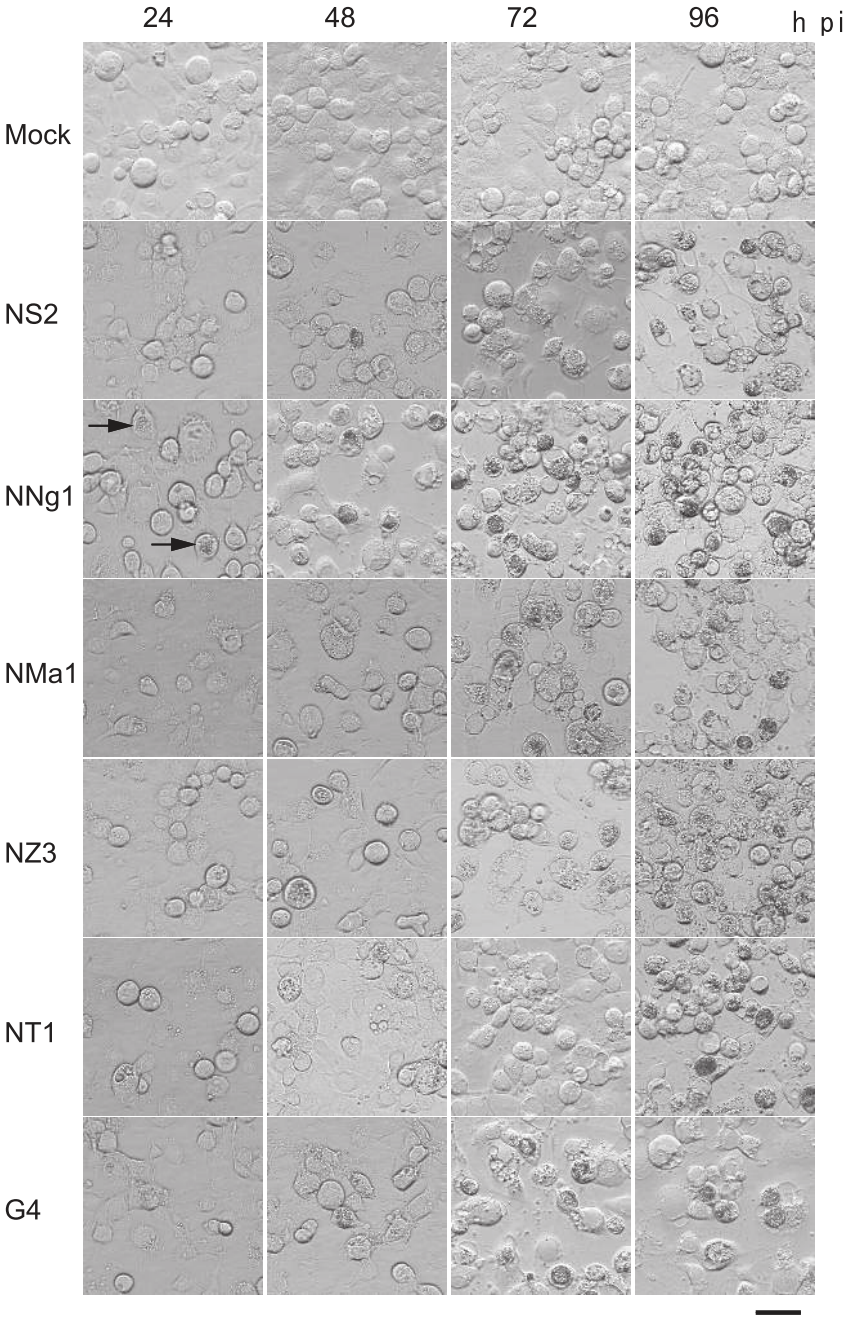

Fig. 2. Cytopathology in Hz-AM1 cells infected with different HearNPV clones. Hz-AM1 cells were infected with HearNPV clones NS2, NNg1, NMa1, NZ3, NT1 and G4 at an MOI of 1 PFU per cell and incubated at 28°C. Cytopathology of infected cells was examined every 24 h until 96 h pi under a microscope. The arrows in the NNg1-infected cells at 24 h pi indicate the cells containing polyhedra. Mock-infected Hz-AM1 cells are also presented for comparison. The bar indicates 40 μm.

higher than those of the other clones.

The LT_{50} was calculated using only the larvae inoculated with 5×10^3 OBs/ml for all the clones (Table 4), since lower doses did not yield 50% mortality for clones G4 and NT1 and the higher dosages caused rapid and high mortality. Clone NNg1 killed 50% of the infected larvae at 4.0 days post-inoculation, while clone G4 took 8.3 days to kill 50% of the infected larvae. There were overlapping LT_{50} values for clones NZ3, NS2, NMa1 and NT1, indicating no significant difference in their killing speed.

DISCUSSION

In this study, we cloned HearNPVs from 11 uncloned geographical HearNPV isolates, which included one each from South Africa, Zimbabwe and Thailand and eight from Kenya, and characterized the cloned HearNPVs in cell cultures and insect hosts. Through three rounds of

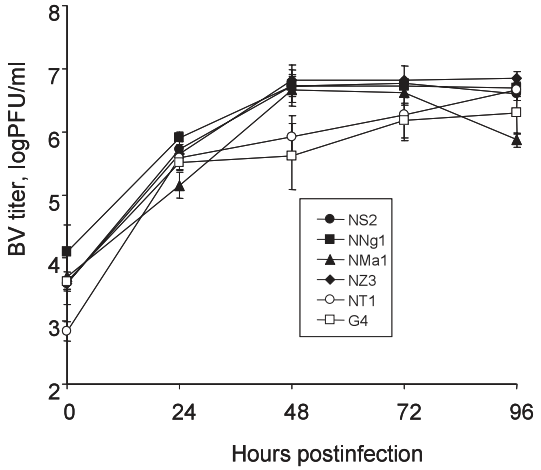

Fig. 3. Budded virus yields of the six selected HearNPV clones in Hz-AM1 cells. Cells were seeded at a density of 1×10^6 in each 12.5-cm^2 flask and infected with each of the six clones at an input MOI of 1 PFU/cell. The infected cells were washed twice with TC100 medium after 1 h of adsorption period at room temperature, and cultured in 3 ml of fresh TC100 medium at 28°C. Culture medium was harvested at 0, 24, 48, 72 and 96 h pi and examined for virus titers by standard plaque assay in Hz-AM1 cells. Each virus dilution was done in triplicate. The bars represent the standard errors.

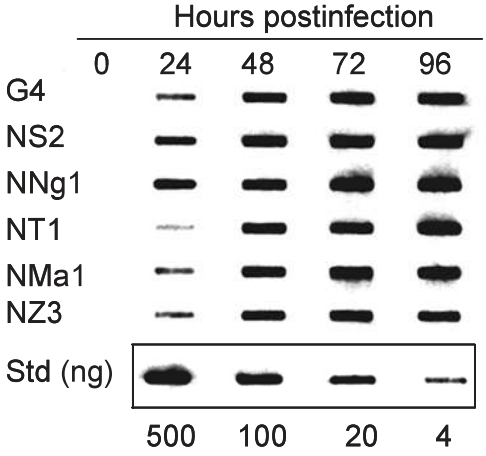

Fig. 4. Slot-blot hybridization analysis of viral DNA accumulation in Hz-AM1 cells infected with six selected HearNPV clones. DNAs from the infected cells were denatured in supersaturated NaI and blotted onto a Hybond N+ nylon membrane. Viral DNAs were hybridized with the probe prepared from a portion of DNA polymerase gene of HearNPV clone NS2 and labeled with Fluorescein by Gene Images random-prime labeling module. The probe hybridized was visualized by the CDP-Star detection module. Serial dilutions of HearNPV clone NS2 DNA (500, 100, 20, and 4 ng) were included for quantitative comparison. For viral infection, see the legend to Fig. 3.

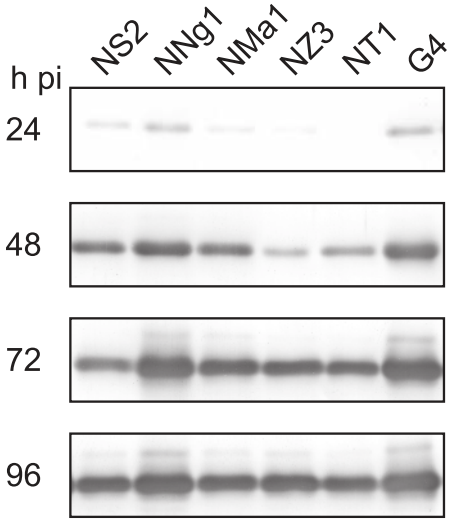

Fig. 5. Immunoblot analysis of polyhedrin protein production in Hz-AM1 cells. Polypeptides from infected cells were resolved on SDS-polyacrylamide gels and processed for immunoblot analysis with anti-BmNPV polyhedrin as a primary antibody. Polypeptides equivalent to 8×10^4 of infected cells at infection were analyzed in each lane. Dr. Western molecular weight marker was used to estimate the size of the polyhedrin protein. For viral infection, see the legend to Fig. 3.

plaque purification in Hz-AM1 cells, we isolated a total of 162 clones, which consisted of 25 distinct clones displaying unique REN patterns. The overall REN patterns of the 25 clones, however, were similar to each other, sharing many bands co-migrating on agarose gels. Examination of REN patterns on agarose gels also showed no clones with severe genomic deletions which occurred in several NPVs from field insect populations or during propagation in cell cultures (Muñoz *et al.*, 1998; Pijlman *et al.*, 2001; Kamiya *et al.*, 2004, Simon *et al.*, 2004), and average genomic sizes of five randomly selected clones in this study were estimated by summation of the sizes of DNA fragments generated by *Bgl*II, *Xba*I and *Hin*dIII to be in a range between 126.4 and 132.7 kbp, comparable to 131,403 and 130,759 bp for Chinese clones G4 and C1, respectively, which were estimated by genomic sequencing (Chen *et al.*, 2001; Zhang *et al.*, 2005). These results indicate that HearNPV clones isolated in this study should be considered variants of the HearNPV. The occurrence of genotypic variants in distinct geographical isolates is common for both NPVs and GVs (Lee and Miller, 1978; Maruniak *et al.*, 1984; Smith and Crook, 1988; Muñoz *et al.*, 1998, 1999).

Genotypic variants of baculoviruses identified by REN analysis have been shown to exhibit different biological activities in both cell cultures and insect hosts (Gettig and McCarthy, 1982; Lynn *et al.*, 1993; Figueiredo *et al.*, 1999; Kamiya *et al.*, 2004; Cory *et al.*, 2005). Our results with selected HearNPV clones in Hz-AM1 cells showed that there were differences among the clones in the parameters concerning productivity of viruses, including viral DNA, BVs and polyhedrin, and that the parameters in each clone were not completely consistent among viral DNA, BVs and polyhedrin. For BVs, our results showed that the four African clones NNg1, NS2, NMa1 and NZ3, yielded significantly more progeny virions than clones G4

Table 3. Dose-mortality responses and LD_{50} values of different HearNPV clones against third instar *Helicoverpa armigera* larvae[1]

Clone	n[2]	Regression line	Slope	χ^2	d.f.	LD_{50} (OBs/larva)	95% Confidence limits	
							Lower	Upper
NS2	190	y = 0.77x − 1.20	0.66	2.2	3	35	25	140
NNg1	190	y = 0.32x + 0.23	0.55	2.8	3	10	6	19
NMa1	188	y = 0.65x − 0.98	0.59	2.6	3	32	26	151
NZ3	183	y = 0.55x − 1.22	0.46	2.2	3	146	92	494
NT1	192	y = 1.17x − 3.40	0.57	3.6	3	826	453	1358
G4	190	y = 0.43x − 2.32	0.43	3.8	3	3115	1384	6555

[1] Newly ecdysed third instar *H. armigera* larvae were perorally inoculated with OBs at different concentrations. After the inoculation, mortality from NPV was examined daily until 10 days post-inoculation. Mortality data were analyzed by Probit method (Finney, 1978). OBs used for inoculation were produced in *H. armigera* larvae.
[2] The number of insect larvae used in bioassays.

Table 4. LT_{50} (in days) of third instar *Helicoverpa armigera* larvae infected with 5×10^3 OBs/ml of different HearNPV clones

Clone	n[1]	Regression line	χ^2	LT_{50} (days)	95% Confidence limits	
					Lower	Upper
NS2	135	y = 5.9x − 4.2	4.75	5.1	4.7	5.5
NNg1	135	y = 8.4x + 5.2	5.44	4.0	3.9	4.3
NMa1	135	y = 4.8x − 3.1	17.34	4.4	3.7	5.1
NZ3	135	y = 4.2x − 2.9	10.29	4.8	4.3	5.3
NT1	135	y = 5.1x − 3.9	2.41	5.8	5.4	6.3
G4	135	y = 3.3x − 3.0	6.89	8.3	7.4	9.9

[1]The number of insect larvae used in bioassays.

and NT1. On the other hand, a larger amount of polyhedrin was produced in clones NNg1 and G4 compared with those in the rest of the clones, and clone NT1 produced a very low amount of polyhedrin. Thus, together with the fact that clone NNg1 is the earliest among the clones characterized to produce OBs in the infected Hz-AM1 cells, it appears that overall productivity in Hz-AM1 cells is highest in clone NNg1 and lowest in clone NT1. Interestingly, clone G4 that produced a large amount of polyhedrin yielded the least amount of BVs among the six clones characterized.

Our results from dose-mortality analysis showed that insecticidal activity against third instar *H. armigera* larvae differed significantly between some of the six selected HearNPV clones. Clone NNg1 expressed the highest insecticidal activity, giving an LD_{50} value of 10 OBs/larva following peroral infection with OBs prepared from infected larvae. Other African clones, NS2, NMa1 and NZ3, had LD_{50} values of 32, 35 and 146 OBs/larva, respectively, indicating that insecticidal activities of these clones were somewhat lower than that of clone NNg1. In contrast, clones NT1 and G4 had significantly lower insecticidal activities compared with those of four other African clones, recording LD_{50} values of 826 and 3115 OBs/larva, respectively. Based on LT_{50} values obtained in the bioassay in third instar *H. armigera* larvae following peroral infection at a concentration of 5×10^3 OBs/ml, clone NNg1 again showed the highest insecticidal activity, giving the lowest LT_{50} value of 4.0 days. Other African clones NMa1, NZ3 and NS2 showed LT_{50} values of 4.4, 4.8 and 5.1 days, respectively, which were significantly lower than 5.8 and 8.3 days for clones NT1 and G4, respectively. These results indicate that clone NNg1 has the highest insecticidal activities and is the best candidate for biological control of *H. armigera* larvae among the six clones characterized in this study.

Our results with third instar *H. armigera* larvae showed that insecticidal activity in terms of LD_{50} and LT_{50} values was significantly lower in clone G4 than those in African clones NS2, NNg1, NMa1 and NZ3. These results agree with the previous results, which showed that virulence of clone G4 is significantly lower than that of clone C1 (Zhang *et al.*, 2005). The exact causes underlying the lower insecticidal activity of clone G4 are unknown. It is unlikely that absence of viral genes promoting peroral infection of insect larvae, including *p74*, *pif* (*per os* infectivity factor) and *pif 2* genes (Kuzio *et al.*, 1989; Heldens *et al.*, 1996; Faulkner *et al.*, 1997; Kikhno *et al.*, 2002) are involved in the lower insecticidal activity of clone G4, since these genes with a high degree of homology are conserved in clone G4, as well as clones C1 and NNg1 (Chen *et al.*, 2001; Zhang *et al.*, 2005; Ogembo *et al.*, unpublished). Our visual examination under a microscope showed that the sizes of OBs used for the bioassay were not signifi-

cantly different between clone G4 and other clones, suggesting that difference in the numbers of virions, as predicted from OB sizes, was not a possible factor that accounted for the lower insecticidal activity of clone G4, although whether population densities of the virions in the OBs differ between clone G4 and other clones remains to be explored. Based on our results in Hz-AM1 cells, a possible explanation for the lower insecticidal activity of G4 clone is its lower productivity of BVs which play a crucial role in spreading the infection throughout the infected insects (Blissard, 1996). In support of this, our data also showed that clone NT1 which yielded a low titre of BVs exhibited low insecticidal activity. Further studies are needed to identify possible factors involved in the observed low insecticidal activity of clone G4 of HearNPV.

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