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Universal Primer for Early and Rapid Detection of Nucleopolyhedroviruses of Multiple Species Using Polymerase Chain Reaction

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ABSTRACT

A technique using the polymerase chain reaction (PCR) was developed for detection of the nucleopolyhedrovirus (NPV) polyhedrin gene. 152 nucleotide sequences of polyhedrin gene were compared in pairwise and multiple alignment sequences. Eleven highly conserved DNA sequences within the coding region of the polyhedrin gene were identified. Two candidate regions were targeted for amplification and consequently one pair of degenerate PCR primers was designed to produce fragments of about 355 bp. The NPVs tested by this technique were *Autographa californica* (*Ac*MNPV), *Bombyx mori* NPV (*Bm*NPV), *Hyphantria cunea* NPV (*Hc*NPV), *Lymantria dispar* NPV (*Ld*NPV), *Spodoptera exigua* NPV (*Se*NPV), *S. litura* NPV (*Sl*NPV), *Spodoptera littoralis* NPV (*Spli*NPV) and nine local NPV isolates. Furthermore, three randomly chosen PCR products were cloned and sequenced. The sequencing data showed that the three PCR products were fragments of polyhedrin gene. Conclusively, this technique would be useful in monitoring the environmental fate, distribution of NPVs, release of the wild type and recombinant NPVs and quality control studies of baculoviral insecticides as well.

Keywords: Nucleopolyhedroviruses, baculovirus, PCR, polyhedrin gene

INTRODUCTIION

Baculoviruses have a large circular double-stranded DNA genome ranging from approximately 80 to 180 kb in size (Blissard and Rohrmann, 1990). They are considered to be the largest and most broadly studied insect viruses because they are of great interest and utility to large cross-sections of agricultural and biomedical research community. They have been thoroughly investigated due to its potential as insect control agent (Wood and Robert, 1991), and as a vector expressing various heterologous genes (Summers and Smith, 1987, Choi *et al*., 1999). Although Murphy *et al.* (1995) have reported baculovirus infections in over 600 insect species in the order of Lepidoptera, Hymenoptera, Diptera, Coleoptera, Neuroptera, Trichopera and Thysanura, as well as in the Crustaceae order Decapoda (shrimps), it is recently confirmed that only those derived from orders Lepidoptera, Hymenoptera, and Diptera are members of the family Baculoviridae (ICTV, 2009). Those from Orthoptera were classified as pox viruses, isolates from Coleoptera are so far not assigned (ICTV, 2009). Neuropterans were infected under laboratory conditions and larval death from the virus infection was not documented. Shrimp viruses have now been classified as *Nimaviridae* in the genus *Whispovirus* and are no longer baculoviruses (Marks *et al*., 2005). The commercialization and release of recombinant viruses in the environment created the concern that they might cause ecological disturbances, such as displacement of native microorganisms, adverse effects on non-target organisms and the horizontal transfer of DNA into non-target organisms (Leung *et al*., 1994). For the above mentioned reasons, many authors were interested in developing an accurate and easy

diagnostic method for early and rapid detection of NPV infections (Wang *et al*., 2000, Christian *et al*., 2001, Moraes and Maruniak, 2001, Woo, 2001, Lange *et al*., 2004, Jehle *et al*., 2006, Murillo *et al*., 2006).

Several methods have been employed to detect wild type or recombinant nucleopolyhedrovirus (NPV), such as microscopic diagnosis (Traverner MP, Connor, 1992), serological techniques (Brown *et al*., 1982, Naser and Miltenburger, 1983, Webb and Shelton, 1990), radioimmunoassay techniques (Smith and Summers, 1981, Knell *et al*., 1983), and DNA dot blot hybridization assays (Ward *et al*., 1987, Keating *et al*., 1989). The use of these techniques has been limited because they are either tedious and unreliable, or because they utilize radioactive materials.

Polyhedrin is the major component of polyhedra and has often been studied. After the first report about localization of the polyhedrin gene in *Ac*NPV (Vlak and Smith, 1982), Iddekinge *et al.* (1983) determined its nucleotide sequences. At the last count, there was published data on the complete genome sequence of some 41 or more NPVs. Polyhedrin is a protein of about 245 to 250 amino acids, and appears to be the most highly conserved NPV protein. These characteristics lead to the use of polyhedrin sequences as the base of NPV phylogenetic studies (Zanotto *et al*., 1993).

The polymerase chain reaction (PCR) is a highly sensitive technique, which amplifies target DNA sequences and does not employ radioactive material. PCR has been extensively used to detect many organisms such as animal, human, plant, and various pathogens. Webb *et al.* (1991) reported the use of PCR to screen baculovirus expression vector recombinants in cell cultures. Burand *et al.* (1992) was able to detect baculovirus DNA sequences from viral occlusion bodies contaminating the surface of gypsy moth eggs.

The aim of the present study was to design degenerate primer set to detect multiple NPVs using PCR technique. The ability to detect NPV polyhedrin will be a useful tool in studies seeking to rapidly elucidate a polyhedrin gene structure, to monitor the release of the wild type as well as genetically engineered NPVs, and to isolate NPVs in the natural environment.

MATERIALS AND METHODS

Polyhedrin sequence data

All polyhedrin sequences of nucleopolyhedrovirus (NPV) available in March 2010 from GenBank, EMBL, and DDBJ were downloaded. 152 nucleotide sequences were aligned using ClustalX software. Neighbour joining tree was examined using ClustalX and no divergent sequences were identified. In addition, the alignment was manually corrected by shifting sequences in places, for some sequences possessed large spans of unique deletions or insertions which threw off the alignment algorithm.

Selection of highly conserved genome regions for primer design

The term "conserved genomic regions" used here is defined as genome regions that have most frequently presented nucleotide sequences. To identify the highly conserved regions eligible for primer design, pairwise scan for the sequences was done and base by base alignment output file was produced using Mega4 and/ or ClustalX softwares. The most frequently presented base in the same coordinate for all sequences of the alignment was detected. The output (FASTA file) was then analyzed by ClustalX software to select candidate conserved regions for primer design. A candidate region was defined as a site within the polyhedrin open reading frame (*orf*) that had 17+ bases from the

3' end and with a base frequency of 0.80+. Candidate conserved regions were identified by calculating redundancy scores and the average dominant base counts. Average dominant base counts were calculated by summing the number of occurrences of the most common base at each position in a window length of 20 bases and averaging those counts across all positions in the window.

Primer design

The distance between conserved regions was taken into account when selecting conserved sites as was the potential for using mixed bases or deoxyinosines, to enhance bonding at variable positions. Standard nucleotides were preferred close to the 3' termini of the oligonucleotides. The different parameters of primer design (length and sequence, GC content, Tm,.....etc.) were taken into consideration. One set of degenerate primers common for the whole group was designed. Primers were designed to amplify 355 bp within the polyhedrin gene. The accession numbers of polyhedrin nucleotide sequences used in this study are listed in Table (1).

Table 1: Nucleopolyhedroviruses and sources of sequence information for sequences used in the present study.

EF418027	AY706678	AF068188	AF499687	GQ923762	AY519234	AY706717	AY706693
AY706686	AY706685	DQ238113	FJ182057	GQ923751	AY706679	AY706718	AJ277555
DQ231336	EU698891	M14885	AY706703	GQ923760	AB465491	AY442260	X55658
DQ231339	AY706709	AY729808	AY706702	GQ923758	FJ901339	AY706714	AY519243
AY779044	AY519249	EU201037	AY706701	GQ923757	DQ345451	AF037262	AY706708
DQ059424	AY706682	U91940	AY519258	GQ923756	M21887	AY706715	AY706704
U75359	ACU40834	AY519222	AY519213	GQ923761	M13056	AY706716	AY589504
M30925	AY706700	FJ157293	AF016916	X94437	K01149	AB326102	AY519240
DQ483053	AY706684	U67258	AY706710	GQ392064	AF300872	AF068189	AY519237
X63614	AY519255	U67257	AY706683	AY706707	AB465488	AY549963	AY126617
AY519216	AF118850	U67256	GQ475265	U30302	AY706712	AY549964	U61732
DQ399596	AY706691	U67255	AY519204	AB308406	AF093405	AY552474	FJ517140
AY706719	AY519210	AF157012	GQ923759	AY706705	EU401915	EF585249	
AF169480	AY519228	A25670	J04333	EU047914	EU401917	AY706688	
AY706720	AF232690	U97657	GQ923755	AY706706	EU401916	AY706699	
AY706687	DQ014543	FJ157291	GQ923754	AY706698	AF019882	AY706690	
AB062454	DQ014542	FJ157295	GQ923753	AY706696	AB300384	AY706692	
DQ231347	AY971675	FJ157294	GQ923752	AY706697	DQ350142	AY519219	
AY706711	AY895150	FJ157292	GQ923750	AY706681	D01017	AY706694	
AY706680	M32433	M23176	GQ923749	AY706713	AY600451	DQ231342	

Viruses, cell lines and insects

The nucleopolyheroviruses tested in this study were as follows: *Autographa californica* (*Ac*MNPV), *Bombyx mori* NPV (*Bm*NPV), *Hyphantria cunea* NPV (*Hc*NPV), *Lymantria dispar* NPV (*Ld*NPV), *Spodoptera exigua* NPV (*Se*NPV), *S. litura* NPV (*Sl*NPV), *Spodoptera littoralis* NPV (*Spli*NPV) and nine local NPV isolates. The *Ac*MNPV, *Bm*NPV and *Hc*NPV were propagated in *Sf*9 cells maintained at 27°C in a TC-100 medium (Gibco-BRL, USA) that was supplemented with 10% fetal bovine serum (Gibco-BRL, USA). The *Se*NPV, *Sl*NPV, *Spli*NPV, *Ld*NPV and local isolates were propagated in *S. littoralis* and *L. dispar* larvae. Routine cell culture maintenance and virus production procedures were carried out according to O'Reilly *et al.* (1992). Insect colonization and maintenance of the cotton leafworm, *Spodoptera littoralis*, was done in the insectary of Department of Entomology, Faculty of Science, Cairo University under highly controlled conditions from 1995 to date. The colony was maintained in the laboratory according to Seufi (2008). These insects were used for viral propagation and purification assays.

Virus DNA purification

Virus DNA was extracted from purified, semipurified polyhedra collected from infected cells and insect larvae. Total genomic DNA was also extracted from insect larvae. The virus isolates were successfully propagated and purified following the method described by Lacey *et al.* (2002). To extract virus DNA, purified or semipurified polyhedra were resuspended in a 0.1 M sodium carbonate solution (0.1 M Na2CO3, 0.17 M NaCl, 0.01 M EDTA, pH 10.9), and incubated at 37°C overnight with a final concentration of 0.5 mg/ ml of proteinase K (Sigma, USA) and 1% of SDS. A further extraction with phenol

and chloroform: isoamylalcohol (24:1) was performed and the DNA was ethanol-precipitated. The DNA was resuspended in a TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0).

PCR amplification

PCR amplification was performed according to Saiki *et al.* (1988) with minor modifications. Total DNA was extracted from the NPV isolates and the DNA segment was amplified using two primers designed based on conserved nucleotide sequences of 152 different polyhedrin sequences. Sequence of the forward and reverse primers used in this study, their length, GC content and base counts were shown in Table (3 and 4). Total reaction volume was 50 µl which contained 1× PCR buffer (Promega), 1.5 mM MgCl2, 200 µM dNTPs, 2.5 U *Taq* DNA polymerase (Promega), 100 ng of each primer and 30 ng of template DNA. The amplification program used was 3 min at 94°C (hot start), 1 min at 94°C, 2 min at 55°C and 2 min at 72°C for 35 cycles followed by one cycle of 72°C for 7 min. PCR amplification was carried out in a DNA thermal cycler (Model 380 A, Applied Biosystems, CA, USA).

RESULTS

Selection of Candidate Conserved Region for Primer Design

Alignment of the 152 NPV polyhedrin sequences were used as a guide to enable identification of conserved sequences of the gene to be used in the design of degenerate oligonucleotide primers for PCR. No potentially useful conserved sites were identified in the first complete multiple alignment, utilizing all available sequences in GenBank, EMBL, and DDBJ databases. However, once divergent sequences were removed, eleven conserved regions were identified. Two candidate regions (from 154 to 172 and 465 to 505 relative to the *Cotesia marginiventris* NPV polyhedrin gene (Acc# EF418027)) with relatively low levels of degeneracy were selected to design primers (Table 2).

Table 2: Number, length and location of the identified conserved regions in the 152 polyhedrin gene sequences used in this study. Locations were determined in relation to the *Cotesia marginiventris* NPV polyhedrin gene (Acc# EF418027). The candidate conserved regions were determined using pairwise and multiple sequence alignments.

Conserved region	Location	Length (bP)
1	154 -175*	22
2	222-242	21
3	251-277	27
4	279-297	19
5	300-347	48
6	371-409	39
7	410-446	37
8	465-505*	41
9	508-534	27
10	585-620	36
11	626-650	25

*The chosen candidate conserved regions for primer design.

One set of degenerate PCR primers (*Polh*152F and *Polh*152R) was designed from these regions.

Primer Selection

The *Polh*152F and *Polh*152R primers were designed to amplify 355 bp within the polyhedrin gene (based on 152 polyhedrin sequences). The sequences of The *Polh*152F and *Polh*152R primers and the base count of the respective viral DNAs are shown in Tables (3 and 4). Degenerate sites were considered when low base by base frequency was produced by multiple alignment in the candidate regions. The primers were selected on the basis of having relatively low levels of degeneracy. The bases represented in lower than 5% in the base count were not considered in designing the primers (Tables 3 and 4). To compensate for the primer multiplicity, a slightly higher primer concentration (50 pmole per 50 µl reaction) was used in the PCR.

Experimental verification

Two degenerate primers were designed to anneal within the *orf* of polyhedrin gene (*Polh*152F and *Polh*152R). The degenerate PCR primer set successfully amplified the expected polyhedrin DNA fragment (355 bp) from the *Ac*MNPV, *Bm*NPV, *Hc*NPV, *Ld*NPV, *Se*NPV, *Sl*NPV, *Spli*NPV as well as from nine local NPV isolates.

Table 3: Sequence and base counts of the forward primer based on the first candidate conserved region of 152 polyhedrin sequences. Base count less than 5% was neglected in primer design.

Base count	Forward primer (5'---3')																				
	V	G	G	D	C	C	B	G	G	B	A	A	R	A	A	Y	C	A	R	A	A	K
G	117	150	147	25	1		44	147	147	8		3	36		2	2	3	2	15		2	38
C	13		4	4	148	147	67	4		101	1	1	1	2		99	145	3		1		1
A	16	2	1	92		1	7	1	1	2	151	148	115	148	149		2	144	137	150	149	113
T	6			31	3	4	36		3	41				2	1	51	2	3		1	1	

V= G, A or C, D= A, T or G, B= C, G or T, R= A or G, Y= C or T.

Table 4: Sequence and base counts of the reverse primer based on the eighth candidate conserved region of 152 polyhedrin sequences. Base count less than 5% was neglected in primer design.

Base count	Reverse primer (5'---3')																				
	C	A	D	C	C	R	Y	Y	R	Y	M	N	Y	K	Y	T	T	R	S	C	B	A
G		1	94	5	1	126	2	1	127			9	2	36		1		99	45	2	19	1
C	148			144	151	1	140	141	2	125	124	65	37	1	77	1	3	4	100	140	105	
A	2	151	42	1		24	2	1	18	6	25	10	2					45	3	6	5	149
T	2		16	2		1	8	9	5	21	3	68	111	115	75	150	149	4	4	4	23	2

D= A, T or G, B= C, G or T, R= A or G, Y= C or T, K= G or T, M= C or A, S= C or G, N= A, C, T or G.

Non-specific amplification products were not observed for tested NPVs. In total all of the nine NPV species tested were amplified, seven species and two local isolates have been tested with these primers (Fig. 1). Three randomly chosen PCR products were cloned into *p*GEM-T vector and sequenced. The sequencing results showed that the three PCR products were fragments of polyhedrin gene (data not shown).

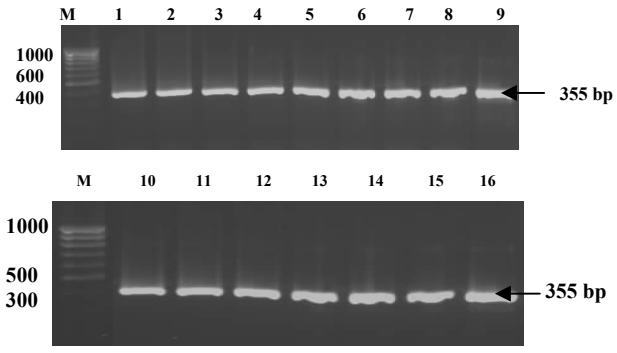

Fig. 1: Representative PCR results showing the amplification of ~355 bp fragment in the polyhedrin gene coding region of 16 NPV isolates. M: 100 bp ladder DNA marker, lanes 1: *Ac*MNPV, 2: *Sl*NPV, 3: *Se*NPV, 4: *Ld*NPV, 5: *Hc*NPV, 6: *Bm*NPV and 7: *Spli*NPV. Lanes 8-16: nine NPV local isolates. The size of the bands is shown in bp.

DISCUSSION AND CONCLUSION

Polyhedrin gene of NPVs encodes for the matrix protein of the virus occlusion body and is one of the most conserved baculovirus genes (Jehle, 2004). This gene was proved to be the most suitable gene in baculoviruses for developing generic amplification technique (Woo, 2001). Seufi (2008) characterized a highly conserved polyhedrin region of 405 bp molecular size. He reported that alignment results of this conserved region with the other published sequences produced significant alignment with 111 baculovirus isolates. The percentage of homology ranged between 99% for *Spli*NPV (Acc# D01017) and 78% for *Plusia orichalcea* NPV (Acc# AF019882). At the level of amino acid sequence, the percentage homology of ranged between 100% for *S. littoralis* polyhedrins (Acc# AAC33752 and AAR04375) and 81% for *Attacus ricini* polyhedrin (Acc# P31036). In addition, lepidopteran polyhedrin genes show about a 50% amino acid identity with granulovirus granulins, and a 40% identity with hymenopteran NPV polyhedrin (Rohrmann, 1992). These high similarities could enhance the strategy to design universal primers for rapid and early detection of NPV-infection. The advantage of such approach is that it utilized all NPV polyhedrin sequences available in the international databases (152 sequences) and simple public software programs to select optimal candidate regions in genomic sequences

for amplification. Such approach is unlikely to produce significant bias towards any one species, especially when there is no bias in the multiple sequence alignment which the approach was based on. These primers make it possible to efficiently amplify DNA from many NPV species and allow further search for unknown NPV species.

Many published reports that investigated polyhedrin gene depended primarily on a Southern hybridization using probes of the polyhedrin gene of other previously identified viruses. However, this technique is efficient only if the similarity between polyhedrin genes of the target NPV and probe NPV is high. Therefore, many limitations will arise when the study based mainly on Southern technique. One major limitation is that this technique requires multiple probes of various NPVs for detection of baculovirus. Also, traditional serological methods based on neutralization and fixed cell ELISA have proven effective for identifying baculoviruses (Brown *et al*., 1982). However, difficulties in interpreting antigenic cross reactivity or failure to identify relatively close antigenic relationships were common complains in this technology. Moreover, serology is time consuming, requires highly experienced personnel and is less precise than nucleotide sequence determination.

Generally, the use of PCR technology for virus detection, identification and characterization is a basic tool in many virological laboratories (Moraes and Maruniak, 1997, Moraes *et al*., 1999, Murillo *et al*., 2006). Indeed, a good set of primers for nucleopolyhedrovirus detection is a powerful tool for large nucleopolyhedrovirus sample screening. PCR technique is preferred because it is easy, fast, sensitive and reliable. In addition, it does not utilize radioactive materials. Although attempts to detect nucleopolyhedroviruses from soil and insects have been made using PCR techniques, it was limited to narrow NPV species (Webb *et al*., 1991, Moraes and Maruniak, 1997, Moraes *et al*., 1999). Woo (2001) designed a pair of degenerate primers (based on conserved amino acid sequence of 26 different NPVs) to detect multiple NPVs using PCR. One major problem with degenerate primers is that the concentration of some permutations in the mixture is so small that amplification is effectively inhibited (due to their great multiplicity). It was believed that the redundancy of *Polh*152F and *Polh*152R was insufficient to cause this problem. The capacity of *Polh*152F and *Polh*152R primers potentially to amplify all NPVs made them an invaluable diagnostic and taxonomic tool for virology. The ability of these primers to amplify DNA from local isolates of NPV may demonstrate their capacity to define novel NPV species.

In conclusion, the PCR primer set employed in this study was chosen from highly conserved sequences within the polyhedrin-coding region. Therefore, the possibility of amplification of multiple nucleopolyhedroviruses was more enhanced. The present study introduced a highly sensitive method for multiple nucleopolyhedrovirus detection. Higher sensitivity and cost-efficiency enabled the researcher to identify the structure of the polyhedrin gene rapidly. The amplification of highly specific and abundant products obtained in this study suggests that this method might be useful to detect nucleopolyhedroviruses with low amounts of DNA in the environment. Conclusively, the method described in this paper is universal, powerful, and could be used in the future to study the environmental fate of wild type or genetically modified recombinant NPVs. It may be usefull in quality control studies of baculoviral insecticides as well.

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ARABIC SUMMARY

تصميم زوج من البادئات العالمية للكشف المبكر والسريع عن أنواع متعددة من الباكيولوفيروسات باستخدام تفاعل البلمرة المتسلسل

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استهدفت هذه الدراسة تصميم بادئات عالمية للكشف المبكر والسريع عن أنواع متعددة من الباكيولوفيروسات باستخدام تفاعل البلمرة المتسلسل. وقد تم تطوير تقنية تفاعل البلمرة المتسلسل (PCR) للكشف المبكر و السريع عن أنواع متعددة من الباكيولوفيروسات اعتمادا على تتابعات القواعد النيتروجينية (النيوكليوتيدات) لجين البوليهيدرين. وتمت مقارنة ١٥٢ تتابعا لجينات البوليهيدرين المنشورة في قواعد البيانات العالمية باستخدام برامج كمبيوتر متخصصة. وتم تحديد أحد عشر منطقة جينية تمثل تتابعات نيوكلبوتيدية شديدة التشابه فى منطقة الترميز من جينات البوليهيدرين موضع الدراسة. وبناء عليه فقد تم اختيار اثنتين من المناطق الجينية المرشحة لتصميم زوج من البادئات لإنتاج قطعة من الدنا طولها حوالى ٣٥٥ زوجا من القواعد النيتروجينية. وقد تم اختبار سبعة أنواع مختلفة من الباكيولوفيروسات المعرفة عالميا ، بالإضافة إلى تسعة عزلات محلية جارى تعريفها. و علاوة على ذلك، فقد تم اختيار ثلاثة من نواتج تفاعل البلمرة المتسلسل للكشف عما إذا كانت تتابعاتها النيتروجينية تشبه جين البوليهيدرين أم لا ، وأظهرت النتائج أن التتابعات المختارة هى أجزاء من تتابعات جين البوليهيدرين موضع الاختبار. و قد خلصت الدراسة إلى أن هذه التقنية ستكون مفيدة في رصد انتشار و توزيع الباكيلوفيروسات فى الطبيعة ، وذلك فى حالات إطلاق هذه الفيرروسات ، سواء أنوعها البرية أو تلك المهندسة وراثيا ، كما يكن الاستفادة منها فى اختبارات الجودة للمبيدات الحيوية الفيروسة.