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A comparison of techniques for detecting *Invertebrate iridescent virus 6*

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Abstract

The aim of this study was to compare the sensitivity and precision of various methods for the detection and quantification of *Invertebrate iridescent virus 6* (IIV-6) (*Iridoviridae*) isolated from a the stem-boring moth *Chilo suppressalis*, and to apply these techniques to the detection of covert infections in the wax moth, *Galleria mellonella*. The relationship between the virus concentration and absorbance at 260 nm was linear over the range of 1.6×10^9–5.6×10^{10} particles/ml. $TCID_{50}$ assays using 12 different cell lines indicated that two *Drosophila* lines, DL2 and DR1, had the highest susceptibility whereas cell lines from *Aedes albopictus* and *Plutella xylostella* were four orders of magnitude less sensitive. $TCID_{50}$ values for IIV-6 in *Spodoptera frugiperda* Sf9 cells gave the particle–infectivity ratios of 15–64 virus particles/IU. An insect bioassay involved injecting doses of 1–100 IIV-6 particles into the third instar *G. mellonella* larvae. The prevalence of patent infection was 20–26% at a dose of 1 particle per larva rising to 86–92% at 10 particles and 100% at doses of 50 or 100 particles. Of the insects that survived to adulthood, between 5.8 and 75% caused patent infections when injected into *G. mellonella* larvae, indicating that they were covertly infected. A PCR technique resulted in 95% detection at 1000 virus particles per insect. Of the insects that proved positive for covert infection by insect bioassay, 41% also proved positive by PCR analysis. It is concluded that the *G. mellonella* bioassay is highly reliable for detection of doses of 10 particles or more and for determining the relative activity of IIV-6 preparations at doses as low as 1 particle per insect. PCR had a slightly lower sensitivity followed by the insect cell culture assay. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Detection of inapparent infections; Insect bioassay; PCR; Cell culture assay

1. Introduction

Invertebrate iridescent viruses (IIVs) of the genus *Iridovirus* (family *Iridoviridae*) are icosahedral particles of 120–130 nm diameter with an internal lipid layer and a large dsDNA genome. IIVs typically infect invertebrates, especially insects, in damp and aquatic habitats (Williams et al., 2000). The viruses acquired their name because of the remarkable iridescent hues shown by

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patently infected individuals prior to their death. The iridescence results from the accumulation of a superabundance of IIV particles that form paracrystalline arrays in the cytoplasm of infected host cells. This causes interference between incident light and reflected light, resulting in the iridescent phenomenon. However, abundant covert IIV infections have also been reported from several host insect species (Williams, 1995; Marina et al., 1999; Tonka and Weiser, 2000). Covertly infected insects appear healthy and the infection is not lethal.

The natural transmission routes of IIVs are poorly understood although cannibalism, predation and vectoring by parasites are assumed to be important in a number of IIV–host systems. Vertical transmission has not been demonstrated for viruses in the *Iridovirus* genus (Williams, 1998; Mullens et al., 1999).

Several techniques have been used to detect the presence of IIVs including latex agglutination (Carter, 1973), ELISA (Ward and Kalmakoff, 1991), plaque assay (Czuba et al., 1994) or PCR (Williams, 1993). One of the most sensitive techniques involves an insect bioassay in which IIV suspensions are injected into larvae of the wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) (Day and Gilbert, 1967). The absolute concentration of IIV suspensions can be estimated from direct counting of virus suspensions mixed with a known concentration of latex spheres, using a scanning electron microscope (SEM). However, direct observation is not appropriate for the quantification of IIV particles in invertebrate hosts as it is time consuming and inaccurate. The inaccuracies arise from the low density of IIV particles in covertly infected hosts (Tonka and Weiser, 2000) and the loss of virus that occurs during the purification procedures required for SEM observation.

Day and Mercer (1964) reported that 100% of final instar *G. mellonella* larvae became patently infected by injection of a dose of 1.7–6.9 particles of a coleopteran IIV isolate (IIV-2). Similarly, Marina et al. (2000) reported that the ID_{50} value for *Invertebrate iridescent virus 6* (IIV-6) was 1.9–4.0 particles injected into the third instar *G. mellonella* larvae compared with 160 particles for an IIV isolate from *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). However, the precision of this insect bioassay is uncertain as it fails to take into account the possibility of covert infections of *G. mellonella* larvae that have been injected with IIV particles but which fail to develop iridescence. This would result in a subestimation of the presence of IIV particles in a test suspension.

The aim of this study was to compare the sensitivity and precision of various methods for the detection and quantification of IIV-6 and to apply these techniques to the detection of covert infections of insects. We selected the most representative system available for this study: IIV-6 is the type virus species of the *Iridovirus* genus and was originally isolated from the rice pest, *Chilo suppressalis* (Lepidoptera: Pyralidae) in Japan (Fukaya and Nasu, 1966). Larvae of *G. mellonella* are used as the standard insect host for the production and study of IIVs (Williams, 1998).

2. Materials and methods

2.1. Quantification of virus

The IIV-6 isolate used in this study originated from the virus collection of CSIRO Entomology, Canberra, Australia and has been studied by Webby and Kalmakoff (1998), Marina et al. (1999) and Marina et al. (2000). IIV-6 was produced by injection into *G. mellonella* larvae. When patently infected, individual larvae were homogenized in sterile distilled water and the resulting mixture purified by sequential steps of differential centrifugation followed by centrifugation through 35% (w/v) sucrose and two washes in sterile distilled water. The final suspension was diluted in 68% (vol/vol) isopropanol mixed with polystyrene beads of 460 nm diameter (Aldrich Chemical Company, USA). The concentration of each virus stock suspension was then determined by scanning electron microscopic (SEM) observation of virus preparations dried onto polylycine coated glass coverslips. A minimum of eight fields of vision from each of the five replicate virus–polystyrene mixtures were counted for each virus preparation, representing >1000 virus particles

in all cases. Virus suspensions were stored at 4 °C and used within 3–5 days of purification.

To determine the relationship between the virus concentration and absorbance, quantified virus suspensions were diluted between 50-fold and 1200-fold in a volume of 1 ml sterile distilled water and measured for absorbance at 260 nm in a Beckman spectrophotometer. This procedure was performed on six independent IIV-6 preparations each purified from a single patently infected *G. mellonella* larva and resuspended in a volume of 1 ml sterile distilled water.

2.2. Cell culture assay

Studies were carried out with 12 insect cell lines to ascertain the most suitable line(s) for routine quantification of infectious titers of IIV-6. Cell lines used in the current study were obtained from Dr Art McIntosh (Biol. Contr. Insect Res. Lab., Columbia, USA), with the exception of *Drosophila* line 2 (DL2) (Schneider, 1972) and *Drosophila* line 1 (DR1) (Schneider, 1972) cells, that were obtained from Dr Paul Scotti (Horticulture Research, Auckland, NZ). *Aedes albopictus* (Singh, 1967), *Aedes aegypti* (Peleg, 1968), DR1 and DL2 cells were maintained in Schneider's media (Sigma Cell Culture, St Louis, USA) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml). *Anticarsia gemmatalis* (BCIRL-AG-AM) (McIntosh and Ignoffo, 1989), *Helicoverpa zea* (BCIRL-Hz-AM1) (McIntosh and Ignoffo, 1981), *Helicoverpa armigera*, *Heliothis virescens*, *Pieris rapae* (PR-5) (Quhou and McIntosh, unpublished), *Plutella xylostella* (BCIRL-PX2-HNV3) (Chen et al., 1983) *Spodoptera frugiperda* line 21 (Sf21) (Vaughn et al., 1977) and *Trichoplusia ni* (TN-CL1) (McIntosh and Rechtoris, 1974) cells were maintained in Media 199 (Sigma Cell Culture, St Louis, Missouri, USA) as modified by McIntosh and Rechtoris (1974) (TC199MK) and supplemented with 10% FCS, 2 mM glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml). *Spodoptera frugiperda* line 9 (Sf9) (Summers and Smith, 1987) cells were maintained in Grace's Media (Sigma Cell Culture, St Louis, Missouri, USA) supplemented with 10% FCS, 2 mM glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml). All cell lines were maintained at 26 ± 1 °C and were subcultured at weekly intervals. The infectivity of a preparation of IIV-6 was estimated by the end-point dilution in each of the above cell lines.

Subsequently, three preparations of IIV-6 were purified from the patently infected *G. mellonella* larvae and resuspended in a final volume of 1 ml of sterile distilled water. Particle concentration was determined by SEM counting and the infectivity of each suspension was estimated by end-point dilution in Sf9 cells.

2.3. Insect bioassay

Dilutions of quantified virus suspensions were made immediately prior to the bioassay using sterile distilled water in siliconized microcentrifuge tubes. Groups of 50 third instar *G. mellonella* larvae from a laboratory culture were injected with doses of 1, 2.5, 5, 10, 50 or 100 IIV-6 particles in a mean volume of 8.3 μl using a manual microinjector fitted with a 1 ml syringe (Burkard Co., UK). Control larvae were injected with sterile water. Following injection, *G. mellonella* larvae were placed individually in 30 ml plastic cups half filled with the semisynthetic diet (modified from Hunter and Boraston, 1979) and held at 25 ± 1 °C. Individual rearing of insects eliminated the possibility of virus transmission during the post-inoculation rearing period.

Inoculated insects were checked visually for iridescence at 8 and 14 days post-injection. Patently infected larvae and pupae were scored and discarded; patent IIV infection causes pupal malformation and patches of translucent cuticle in the area between the abdomen and the ventral thorax through which the iridescent color of the insect can be viewed. Apparently, normal pupae were surface disinfected in 1% hypochlorite solution for 5 min, then rinsed in tap water and placed in a clean plastic cup until the emergence of adult. The bioassay was performed at three separate occasions.

To detect the presence of virus in covertly infected insects, adult survivors of the bioassays were killed by freezing and then placed individu-

ally on a clean sheet of paper, sexed, and cut in half longitudinally using a razor blade. Precautions were taken to avoid contamination between insect samples. Body halves were placed in separate sterile 1.5 ml microcentrifuge tubes. One tube was labeled and stored at −20 °C. The other half of the insect body was homogenized in 500 μl of sterile distilled water using a 1 ml pipette tip. The homogenate was partially purified by centrifugation at $525 \times g$ for 5 min. The supernatant was transferred to a sterile 1 ml syringe and 8.3 μl volumes were injected into a group of 30 healthy third instar *G. mellonella* larvae maintained on semisynthetic diet at 25 °C following Marina et al. (1999). The occurrence of patent infections in these larvae was determined at 10–12 days post-injection.

2.4. PCR detection of IIV-6

The frozen half of the insect sample was thawed, homogenized in 500 μl sterile distilled water and heated to 100 °C for 5 min to deactivate the nucleases. After the homogenate had cooled, 50 μl 10% sodium dodecyl sulphate (SDS) was added and the suspension mixed thoroughly. Insect debris was then removed by centrifugation at $600 \times g$ for 5 min. The supernatant was transferred to a fresh tube, and 2 μl of proteinase K (10 mg/ml) was added. The mixture was incubated at 37 °C for 30 min and then centrifuged at 16 000*g* for 5 min. DNA was purified from the supernatant by phenol–chloroform treatment and dialyzed overnight at 4 °C in three changes of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0) (Sambrook et al., 1989).

A 1 μl sample of this DNA was taken for use in a PCR reaction. The conditions of the reaction and the primers used were identical to those described by Webby and Kalmakoff (1998) except that 40 amplification cycles were performed for each reaction. The primers were targeted at conserved regions of the major capsid protein (MCP) gene located 508 bp apart. The presence of PCR product was determined by electrophoresis of a 10 μl volume of the reaction mixture in 0.8% agarose under TBE buffer (90 mM Tris–borate pH 8.3, 2.5 mM EDTA) and visualized on a UV transiluminator (302 nm) following staining with ethidium bromide. Amplification products were cleaned using PCR product quick spin columns following the manufacturer recommended procedures (Roche Inc.). A 13 μl volume of the eluted solution was diluted with an equal volume of sterile distilled water and incubated with 10 units *Alu*I (Roche Inc.) to detect the presence of an *Alu*I restriction site at positions 1234–1237 in the IIV-6 MCP gene sequence (Webby and Kalmakoff, 1998). Treatment by *Alu*I resulted in fragments of 298 and 210 bp as confirmed by electrophoresis in 1.2% agarose as described above.

The sensitivity of the PCR reaction was calibrated by adding 0, 100, 500, 1000, 2500 and 5000 IIV-6 particles to samples of uninfected *G. mellonella* adults prior to the extraction and purification steps described above. This procedure was replicated four times and performed on five occasions.

2.5. Statistical analysis

The dose–infection relationship was determined by logit analysis using a binomial error structure in GLIM and the Fieller macro present in the program (Numerical Algorithms Group, Oxford, UK). The prevalence of covert infections and other binomial distributed data were also analyzed in GLIM using binomial errors. GLIM presents the results of such analyses in terms of χ^2 values (Crawley, 1993). Error estimates of means of binomial data are asymmetrical. Where necessary, small degrees of overdispersion were corrected using the Williams correction macro present in this program (Collett, 1991) or by scaling the error distribution and calculating the significance values in terms of F statistics (Crawley, 1993). Fifty percent tissue culture infectious doses ($TCID_{50}$) were calculated using the methodology outlined by Reed and Muench (1938) using a program written in Excel (v. 5.0, Microsoft Corporation) by Dr Paul Scotti (Horticulture Research Institute, Auckland, NZ).

3. Results

3.1. Quantification of virus

Direct SEM counts on the numbers of virus particles and polystyrene beads in mixtures derived from purified IIV-6 suspensions proved to be repeatable and of low variability, confirming it to be a highly appropriate method for the quantification of iridescent virus (Day and Gilbert, 1967; Marina et al., 2000). The SEM counts were used to calculate the dilutions necessary to give the different doses used in the insect bioassay described below. The relationship between the virus concentration and absorbance at 260 nm was linear over the range of 1.6×10^9–5.6×10^{10} particles/ml representing 0.08–1.36 Abs_{260} units, respectively (Fig. 1). There was little variation about the regression line over this range.

3.2. Cell culture assay

The two mosquito-derived lines (*A. albopictus* and *A. aegypti*) and the lepidopteran line from *P. xylostella*, proved to be the most insensitive of the cell lines tested and were 3–5 orders of magnitude less sensitive than the other lines (Table 1). The most sensitive of the lines tested were the two *Drosophila* lines, DL2 and DR1. Many of the lepidopteran lines tested namely, *H. zea* (BCIRL-HZ-AM3), *H. virescens* (BCIRL-HV-AM1), *P. rapae* (PR-5) and the two *S. frugiperda* lines (Sf9 and Sf21) were similarly susceptible but around 10-fold less or so than the *Drosophila* lines. The lepidopteran lines derived from *A. gemmatalis* (BCIRL-AG-AM) and *T. ni* (TN-CL1) showed intermediate levels of sensitivity.

$TCID_{50}$ values for IIV-6 in Sf9 cells were 1.82×10^9, 4.38×10^9 and 8.75×10^9 IU/ml for suspensions containing 1.17×10^{11}, 1.15×10^{11} and 1.35×10^{11} particles/ml, respectively. Particle to infectivity ratios for the three independent preparations ranged from 15.4–64.3 virus particles/IU, indicating that between 15 and 64 virus particles could be detected in the Sf9 cell culture assay system.

Table 1
Relative susceptibility of 12 insect cell lines to IIV-6

Cell line	Titer (IU/ml)	Range of 95% confidence interval
Drosophila melanogaster DR1	1.39×10^9	3.24×10^8–5.95×10^9
Drosophila melanogaster DL2	4.38×10^8	1.02×10^8–1.87×10^9
Pieris rapae PR-5	8.75×10^7	2.04×10^7–3.74×10^8
Spodoptera frugiperda Sf21	7.12×10^7	1.66×10^7–3.05×10^8
Spodoptera frugiperda Sf9	6.43×10^7	1.51×10^7–2.74×10^8
Heliothis virescens BCIRL-HV-AM1	5.39×10^7	1.26×10^7–2.29×10^8
Helicoverpa zea BCIRL-HZ-AM3	1.82×10^7	5.94×10^6–7.76×10^7
Trichoplusia ni TN-CL1	6.43×10^5	1.51×10^5–2.74×10^6
Anticarsia gemmatalis BCIRL-AG-AM	4.38×10^5	1.03×10^5–1.87×10^6
Aedes aegypti	8.75×10^4	2.05×10^4–3.73×10^5
Plutella xylostella BCIRL-PX2-HNV3	4.38×10^4	1.03×10^4–1.87×10^5
Aedes albopictus	3.62×10^4	8.49×10^3–1.54×10^5

A standard preparation of IIV-6 was titrated by end-point dilution in each of the cell lines and titers estimated from the $TCID_{50}$ value.

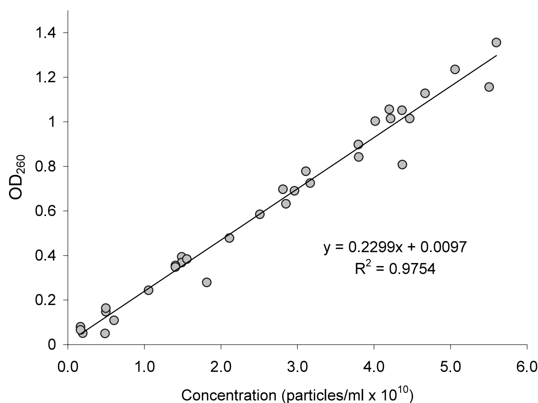

Fig. 1. Relationship between virus concentration and absorbance at 260 nm for IIV-6.

3.3. Insect bioassay

All insects became patently infected and failed to reach adulthood at a dose of 50 and 100 particles and as such, these doses were not considered in the analyses described below. A significant linear relationship was detected between the logarithm of the virus dose and the proportion patent infection of *G. mellonella* larvae ($\chi^2 = 238$, d.f. = 1, $P < 0.001$) (Fig. 2a). The prevalence of patent infection was 20–26% at a dose of 1 particle per larva rising to 86–92% at 10 particles per insect. The ID_{50} dose for patent infections was calculated as 2.4 particles per larva (range of 95% confidence limits: 2.1–2.8 particles). Similarly, the percentage of individuals that survived until adulthood in each replicate declined from 70–78% at a dose of 1 particle to 6–10% at 10 particles per larva (Fig. 2b).

Of the 242 insects that survived to adulthood, seven were patently infected and were not considered further. However, of the 235 apparently healthy adult surviving insects, between 5.8 and 75% caused patent infections when injected into *G. mellonella* larvae, indicating the presence of a covert infection. The prevalence of covert infections was highly variable between replicates and was not related to the dose of inoculum to which the survivor had been exposed (Fig. 2c) ($\chi^2 = 0.41$, d.f. = 1, $P = 0.67$). Considering patent infections and covert infections detected by insect bioassay, the overall prevalence of infection increased significantly with dose from 61.3 to 98.6% at 1 and 10 particles per insect, respectively ($\chi^2 = 68.6$, d.f. = 1, $P < 0.001$) (Fig. 2d). Likewise, the degree of error between apparent and real prevalence of infection decreased significantly from 37.2 to 6.9% between 1 and 10 particles per larva, respectively ($\chi^2 = 46.7$, d.f. = 1, $P < 0.001$) (Table 2).

The average percent patent infection of *G. mellonella* larvae injected with the homogenate of survivors of an inoculum challenge was 44.3% ($n = 1651$). As the survivors of virus inocula were homogenized in 500 µl of water and 8.3 µl of the resulting semipurified suspension was injected into *G. mellonella* larvae, and assuming that an average of 2.1 particles are required to produce 44% patent infection (calculated from the dose–infection regression), a minimum of approximately 125 particles must have been present in the homogenate from half the insect body, or approximately 250 particles per insect. As larvae were inoculated with no more than 10 virus particles, this represents an evidence for virus replication in the absence of patent disease. None of the control insects became patently infected or produced patent infections when adults were homogenized and injected into *G. mellonella* larvae.

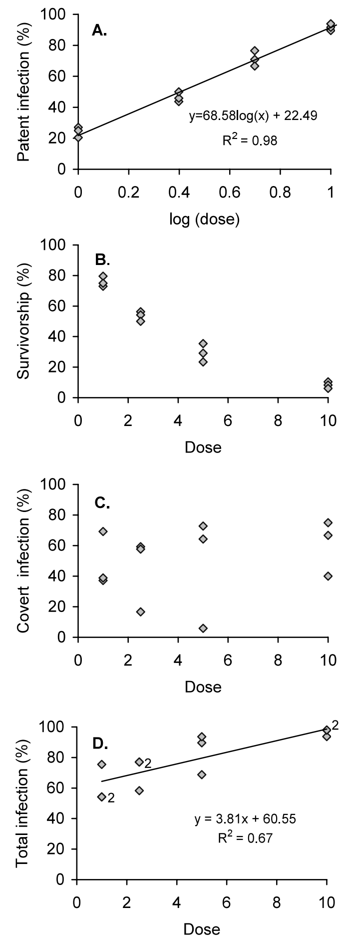

Fig. 2. Relationship between virus dose and (A) percent patent infection; (B) percent survival; (C) percent covert infection of adults; and (D) total infection (patent infection of larvae + covert infection of adult survivors) observed in *G. mellonella* injected with IIV-6 in the third larval instar.

Table 2
Prevalence of patent and covert infections in *G. mellonella* larvae injected with doses of 1–100 particles of IIV-6, showing the difference between apparent (patent) infection and the true prevalence of infection (patent + covert infection) at each dose

Dose	Number patent infections	Number covert infections	Total infected insects	*N*	Apparent percent infection	True percent infection	Difference
1	35	54	89	145	24.1	61.3	37.2
2.5	67	35	102	144	46.5	70.8	24.3
5	102	18	120	143	71.3	83.9	12.6
10	133	10	143	145	91.7	98.6	6.9
50	150	0	150	150	100	100	0
100	150	0	150	150	100	100	0

3.4. PCR detection of IIV-6

Calibration of the PCR technique indicated that detection was highly consistent at 1000 particles per insect, resulting in 19/20 positive samples (95% detection), but was never detected at 500 or 100 particles per insect (0/20 positives at each concentration). All samples of 2500 and 5000 particles per insect proved positive for an amplicon of the predicted size and identity. Given that the volume of the insect homogenate and phenol–chloroform extract was 500 μl, of which 1 μl was used in each PCR reaction, a detection threshold of 1000 particles per insect corresponds to two virus genome copies per reaction.

As only half the insect body was used for PCR, the whole insect would have had to contain approximately 2000 particles to give a positive PCR result. As the maximum dose of inoculum injected into these insects was 10 particles, this represents additional evidence of virus replication in the absence of patent disease. None of the control insects proved positive by PCR.

Of the 117 insects that proved positive for covert infection by insect bioassay, 112 were subjected to PCR analysis and 46 (41%) resulted in an amplicon of the predicted size (Table 3). In all cases, the identity of the product was confirmed by treatment with *Alu*I resulting in fragments of 298 and 210 bp. Of the 123 insects that proved negative for covert infection by insect bioassay, 46 were subjected to PCR analysis but only five resulted in an amplicon when subjected to PCR. As before, the identity of this amplicon was confirmed by *Alu*I treatment (data not shown).

4. Discussion

There is an increasing evidence that covert IIV infections may be common in nature (Williams, 1993, 1995; Tonka and Weiser, 2000) and although not fatal, such infections may result in sublethal disease (Marina et al., 1999). Quantifying the prevalence and severity of covert infections is therefore important in understanding the impact of these viruses on host invertebrate populations. A sensitive, accurate and reproducible assay system is therefore of utmost importance in being able to study covert iridescent virus infections and their effects on the host.

Of the 12 insect cell lines tested, sensitivity to IIV-6 detection ranged across almost five orders of magnitude (Table 1). The ability of IIV-6 to replicate in the different cells lines tested was not a function of the origin of the cells; IIV-6 was as capable of replicating and causing CPE in dipteran-derived cell lines as it was in lepidopteran-derived lines. Such findings are in agreement with the earlier studies that have demonstrated the catholic replicative requirements of the majority of IIVs (Poinar et al., 1980; Czuba et al., 1994) and of IIV-6 in particular (Ohba, 1975; Ohba and Aizawa, 1979; Charpentier et al., 1986).

To test the sensitivity of a cell culture assay for the detection and quantification of IIV-6, three independent preparations of IIV-6 were assayed in Sf9 cells. While Sf9 was not the most sensitive cell line tested for the detection of IIV6, the more sensitive cell lines from *Drosophila melanogaster* are susceptible to several other insect pathogenic viruses, including *Cricket paralysis virus* (Scotti, 1976) and *Junonia coenia densovirus* (P. Christian, unpublished data), both of which are also capable of replicating in *G. mellonella*. As our interest in initiating the current study was to determine the most sensitive method for detecting covert infections in the *G. mellonella*–IIV-6 system, it seemed prudent to avoid using a cell line that would readily detect other potential contaminants of such a system. Moreover, Sf9 cells had been demonstrated previously to be suitable for assay of IIV-1 both biologically, in the replication of the virus, and physically, in the formation of suitable cell monolayers and the production of discrete virus-induced plaques (Czuba et al., 1994).

The cell culture based assay proved to be reasonably sensitive to the detection of IIV-6 and was capable of detecting between 15 and 64 virus particles. However, the assay is limited by the need to purify extensively the virus prior to the cell culture assay. Virus preparations of low purity gave a high particle–infectivity ratio, i.e. the sensitivity of this assay is affected by the purity of the virus sample. Suitable purification procedures also result in loss of virus from the experimental preparation. For example, filtration of the sample through a 0.45 μm syringe filter results in approximately a 10-fold loss of the virus titer (data not shown).

In contrast, the insect bioassay using *G. mellonella* was shown to have the highest sensitivity of any of the methods tested. Patent infection of *G. mellonella* larvae was a highly reliable indicator of the presence of IIV-6 at doses of 10 particles or more because doses of >10 particles were likely to result in 100% patent infection of *G. mellonella* larvae. There was also a very low degree of variation in the relationship between virus doses of less than 10 particles and the prevalence of patent infections, indicating that patent infection can be employed as a relative indicator of IIV-6 activity at doses of 1–10 particles per larva. At doses less than 10 particles, the prevalence of covert infections was variable and not dose-dependent, but for the purposes of detection and quantification of IIV-6 this is not of consequence owing to the clear linear relationship between the log virus dose and percent patent infection. Moreover, detecting covert IIV infection in samples of homogenized insects may only require a single step of low-speed centrifugation to sediment insect debris prior to injecting the homogenate into *G. mellonella* larvae (Marina et al., 1999).

The total infection of *G. mellonella* was 61% at a dose of 1 particle, indicating that the overall prevalence of infection at very low doses probably depends more on the probability of receiving a

Table 3
PCR detection of covert IIV-6 infection in *G. mellonella* adults that survived different doses of IIV-6

PCR applied to insects that had been bioassayed previously				
Dose	Number positive/total tested (% positive)			
	Insects classified as covertly infected by the bioassay		Insects classified as not infected by the bioassay	
1	26/48	(54.2)	2/15	(13.3)
2.5	13/34	(38.2)	0/12	(0.0)
5	5/23	(21.7)	3/14	(21.4)
10	2/7	(28.6)	0/5	(0.0)
Overall total	46/112	(41.1)	5/46	(10.9)

Experimental insects were classified earlier as covertly infected or not by bioassay in *G. mellonella*. There were no survivors in the 50 or 100 particle doses; therefore testing for covert infection by either method was not possible.

single virus particle in the applied inoculum, described in theoretical terms by the Poisson distribution, than in factors related to the activity of the virus preparation per se.

There was generally good agreement between PCR and insect bioassay methods for detecting covert infections, although the insect bioassay has a higher sensitivity, probably because of the dilution steps inherent in the DNA extraction and purification procedures. Analysis of samples spiked with known quantities of IIV particles indicated that reliable detection was possible with 1000 particles per insect sample. Given that the volume of the insect homogenate and phenol–chloroform extract was 500 μl of which 1 μl was used in each PCR reaction, this was equivalent to two virus genome copies per reaction. The detection of *Wolbachia* DNA sequences in insects and mites were reported recently as being improved by the use of a DNA polymerase mixture (Taq and Pwo) designed for the amplification of long sequences (Jeyaprakash and Hoy, 2000). The use of such a polymerase mixture may have improved the detection of IIV sequences, but was not tested in the present study, principally owing to the small PCR product (0.5 kb) amplified from the MCP gene region.

It is concluded that the presence of covert infections in *G. mellonella* larvae does not reduce the accuracy of this method for quantifying IIVs or as a technique for qualitatively detecting the presence of IIV particles in covertly infected insects. The *G. mellonella* bioassay is highly reliable for the detection of doses of 10 particles or more per insect that are likely to result in 100% patent infection. This technique is also appropriate for determining the relative activity of IIV-6 preparations at doses as low as 1 particle per insect.

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