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NOTE

Effect of Temperature, pH, Ion Concentration, and Chloroform Treatment on the Stability of *Invertebrate Iridescent Virus 6*

Invertebrate iridescent viruses (IIVs) (family: *Iridoviridae*) are large icosahedral DNA viruses that infect arthropods in damp or aquatic habitats. Viruses of this family possess an internal lipid envelope situated between the core and capsid shell which is believed to be of importance in the physical stability of these viruses in aquatic environments. IIVs are considered to be chloroform- and ether-resistant (Murphy *et al.,* 1995), although the evidence for this is not quantitative (Williams, 1998). This study focused on the effect of temperature, pH, ion concentration, and chloroform treatment on the persistence of infectivity of the type species of the *Iridovirus* genus, *Invertebrate iridescent virus 6* (IIV-6) from *Chilo suppressalis* (Lepidoptera: Pyralidae). For the chloroform study, an additional IIV from the velvetbean caterpillar, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) (AgIIV), was included (Kinard *et al.,* 1995).

IIV-6 and AgIIV were grown by injection into *Galleria mellonella* (Lepidoptera: Pyralidae). When larvae developed the characteristic iridescent coloration they were individually homogenized in sterile distilled water and the homogenate subjected to two cycles of centrifugation at 600*g* for 5 min. The supernatant was respun at 9500*g* for 10 min. The resulting pellet was resuspended in 300 μl sterile water and layered onto 30% (w/v) sucrose and spun at 13,000*g* for 30 min. The virus was washed twice and resuspended in 500 μl sterile water. For each experiment, the purified virus from 4–10 *G. mellonella* larvae was pooled and mixed thoroughly prior to virus quantification. The identity of each virus was confirmed by examination of the *Hin*dIII and *Eco*RI restriction endonuclease profiles (Williams, 1994).

The concentration of each stock suspension of virus was quantified by the direct counting method adapted from Day and Mercer (1964) using latex spheres of 460 nm diameter (Sigma Chemical Co.) observed by SEM. Counts were made from five fields of vision for each of five replicate samples of the virus–latex mixture.

The stock suspension of IIV-6 was bioassayed for infectivity by injection of dilutions (10^{-1} to 10^{-11}) of virus in 0.8% aureomycin into third instar *G. mellonella* larvae. A volume of 10.5 ± 0.1 μl (mean ± SE) of each dilution was injected into each of at least 20 larvae that were subsequently reared on semisynthetic diet at 25 ± 1°C and were checked for patent infection at intervals up to 15 days thereafter. This was used as the infectivity value at day zero. The stock suspension was diluted 10-fold and allocated to one of three temperatures: 4°C, 25°C, or environmental temperatures (achieved by placing Eppendorf tubes 50 cm below the water surface in a small, artificial pond of average temperature 27.1 ± 0.2°C (mean ± SE)). After 5, 10, 20, 30, 40, and 50 days, each sample was bioassayed in *G. mellonella* as described above. Logit analysis of the number of larvae that developed patent infections was used to calculate the infective dose (ID_{50}) of virus in each treatment at each time point. ID_{50} data were then subjected to least squares regression analysis; extreme outlying points at the 50-day sample were excluded from the regression. The experiment was replicated three times.

To test of the sensitivity of IIV-6 to pH, a stock suspension of virus was counted and bioassayed for infectivity in *G. mellonella* as described above. The virus was then diluted 10-fold and incubated in buffer at one of the following pH values: 2.29, 4.01, 7.05, 9.07, or 11.05. The buffers used were the McIlvaines phosphate/citrate buffer for acidic values and the Sorensen–Walbum glycine/NaCl/NaOH buffer for alkaline values (Silverton and Anderson, 1961). A single virus sample was kept in distilled water as a control. After an incubation period of 20 days at 25°C, the infectivity of each virus sample was determined by *G. mellonella* bioassay. In this test, a single dose of 8.5 particles was injected into each larva that corresponded to the ID_{50} value of the suspension at the start of the test. The test of sensitivity to ions was identical to that for pH except that experimental treatments consisted of NaCl, $CaCl_2$ or $MgCl_2$ at one of two concentrations: 0.02 or 0.1 M. The infectivity of each virus sample was determined by insect bioassay at a dose of 6.5 particles/larva (2.4 times the ID_{50} at the start of the experiment). Both experiments were replicated three times.

To test the sensitivity of IIV-6 to chloroform, volumes of 350 μl of stock suspension of virus were transferred to two Eppendorf tubes, with an additional tube containing 350 μl of sterile water acting as a control. An equal volume of chloroform was added to one virus tube and the water control. The tubes were shaken manually for 10

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min at room temperature and then subjected to centrifugation at 600*g* for 5 min to separate the phases. The upper aqueous phase was transferred to a clean Eppendorf and held in open tubes at 25°C for 24 h to allow all chloroform residues to evaporate. Serial dilutions were then made using a 0.8% aureomycin solution. Larvae of *G. mellonella* were injected with 8.4 ± 0.1 µl of each virus dilution (mean ± SE) using a calibrated microapplicator (Burkard Manufacturing Ltd., UK). Tubes containing chloroform-treated or untreated virus were held at 4°C for 35 days and were then bioassayed once more for infectivity. The ID_{50} value of treated and untreated virus at the beginning and end of the experiment was calculated as described above followed by an analysis of variance. The trial was replicated four times. An identical experiment was performed using *Anticarsia gemmatalis* IV.

The mean ID_{50} of the IIV-6 preparations at the start of the experiment was 3.98 particles. At all temperatures, there was a steady decrease in the infectivity of virus over time (Fig. 1a). The rate of inactivation appeared to increase with increasing temperature, but also became more variable such that the effect of temperature was statistically not significant ($F_{2,40} = 2.64$, $P = 0.083$, NS). The temperature-related increase in variability is apparent when considering the confidence intervals of the gradient of the regression line for each temperature (Fig. 1b). Fifty days after the start of the experiment, the infectivity of the virus preparations had fallen by approximately an order of magnitude.

The persistence of infectivity in IIV-6 was sensitive to pH (Fig. 2a). The effect of pH was significant in two of the three replicates (Fisher's exact test: Rep. 1 $P = 0.0590$; Rep. 2 $P = 0.0180$; Rep. 3 $P = 0.0069$). After 20 days at 25 ± 1°C, injection of the ID_{50} dose resulted in 16.7% patent infection. The samples of virus held under identical conditions in buffers of different pH showed reduced persistence of infectivity at pHs other than 7.0 (Fig. 2a). No patent infections were observed following injection of the ID_{50} dose of virus samples subject to extreme pH (2.3 or 11.0) for 20 days.

Following 20 days incubation of the stock suspension in distilled water (control), injection of an average of 6.5 particles resulted in 17.6% patent infection of *G. mellonella* larvae. This dose represented 2.4 times the ID_{50} at the start of the experiment. The presence of Na^+, Ca^{2+}, or Mg^{2+} ions at concentrations of 0.02 or 0.1 M had no significant effect on the persistence of infectivity of IIV-6 over a period of 20 days (Fig. 2b). The prevalence of patent infection varied from 4.8 to 20.0% (Fisher's exact tests for each treatment combination: $P = 0.421$–0.056 depending on ion and concentration, all NS).

Treatment of IIV-6 with chloroform produced a significant decrease in infectivity ($F_{1,12} = 630.9$, $P < 0.001$).

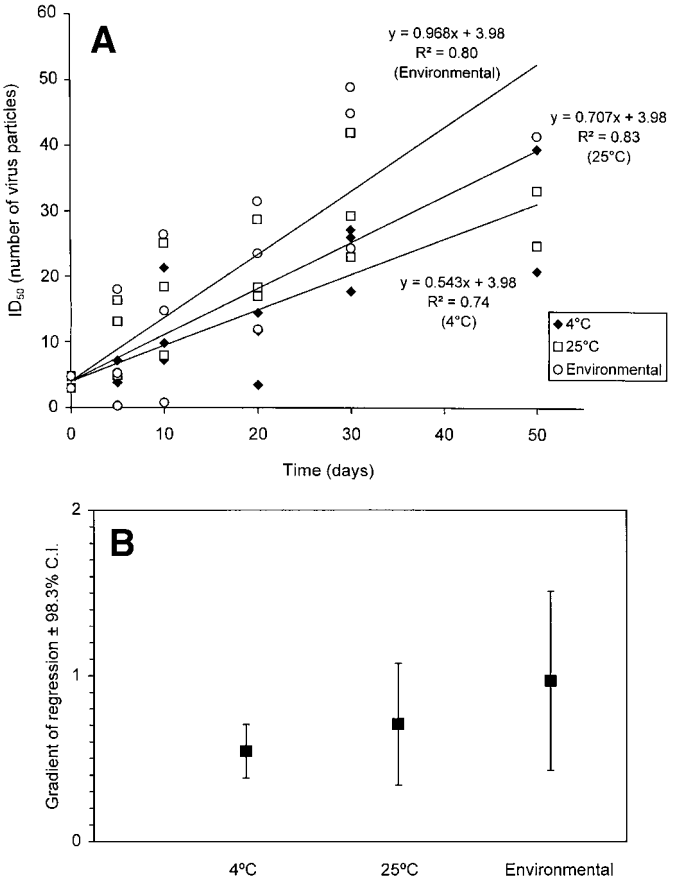

FIG. 1. (A) Change of infectivity of *Invertebrate iridescent virus 6* over a period of 50 days at different temperature regimes. Each line indicates the least squares regression of ID_{50} of the virus by insect bioassay calculated at each time point. (B) Gradients of each fitted regression with the 98.3% confidence intervals (Bonferroni correction) showing increase in rate of inactivation and increase in variability at high temperatures.

The ID_{50} value increased from a mean of 1.9 particles in untreated virus to 344 particles for chloroform-treated virus (Fig. 3). Virus from both treatments showed a significant reduction in infectivity with time ($F_{1,12} = 81.4$, $P < 0.001$); after 35 days at 4°C, the ID_{50} value of untreated virus was 9.4 particles compared to 3939 particles for chloroform-treated virus.

IIV from *Anticarsia gemmatalis* (AgIIV) was more than an order of magnitude less infective to *G. mellonella* than IIV-6. Chloroform treatment significantly reduced the infectivity of AgIIV at the start of the test from an ID_{50} of 159.5 particles (range of 95% C.I. 136.8–182.3) to 2908 particles (range of 95% C.I. 263.5–5553) following chloroform treatment ($t = 3.307$, 3 *df*, $P = 0.02$). However, by 35 days posttreatment, the infectivity of AgIIV had fallen to such a degree that the lowest dilution tested (10^{-6}) caused no more than 6.6% patent infection in *G. mellonella,* and the ID_{50} value could not be reliably calculated.

The results of this study demonstrate the high stability of IIV-6 in an aquatic environment, even at the elevated temperatures of a tropical region. pH was also influential in the persistence of infectivity of IIV-6 over a 20-day period. These results also emphasize the very high sensitivity of the *G. mellonella* bioassay which has been used to detect the presence of covert IIV infections in natural and laboratory insect populations (Williams, 1995; Marina *et al.,* 1999). It is possible that IIVs injected into *G. mellonella* may cause an inapparent infection in that host although we have no evidence that this has occurred in any of the tests we have presented here. The only alternative method for quantifying the infectivity of IIV preparations is the use of cell culture assays, although this is also prone to the possibility of inapparent infections and is not a sensitive assay requiring some 10^5 particles per plaque (Czuba *et al.,* 1994). The characteristic of solvent insensitivity of IIVs has already been questioned (Williams, 1998). The present study revealed a clear loss of infectivity in two chloroform-treated IIVs which may be due to elimination of the lipid layer or possibly due to the denaturing of one or more protein components of the virion (Cerutti and Devauchelle, 1990). Clearly, this basic taxonomic feature of the *Iridovirus* genus requires clarification.

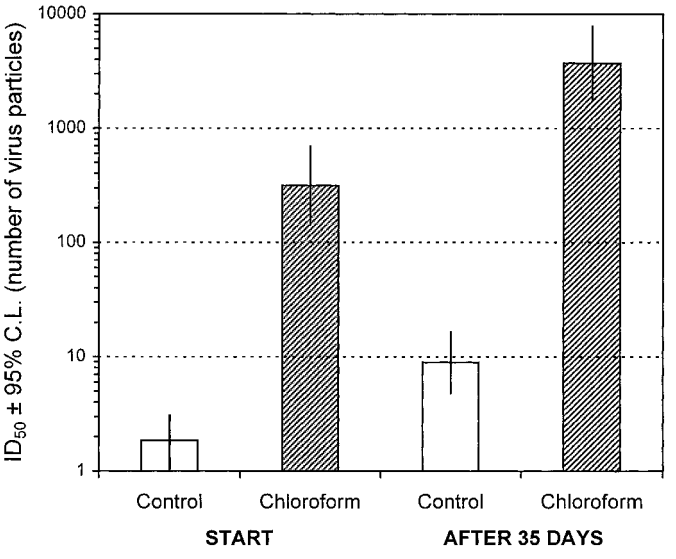

FIG. 3. Effect of chloroform treatment on the infectivity of *Invertebrate iridescent virus 6* at 24 h after treatment (start) and after 35 days incubation at 4°C.

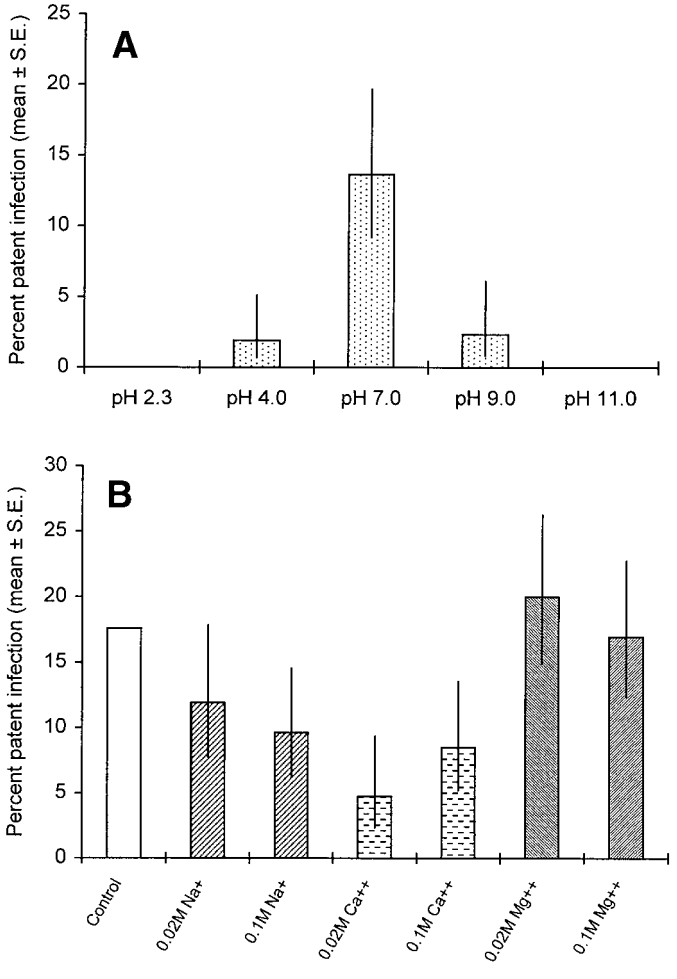

FIG. 2. Infectivity of *Invertebrate iridescent virus 6* remaining after 20 days incubation with (A) buffers of different pH or (B) ions at concentrations of 0.02 and 0.1 M. Infectivity was determined by insect bioassay of a single dose of virus.

Key Words: Iridoviridae; Iridovirus; rate of inactivation; insect bioassay; *Galleria mellonella; Anticarsia gemmatalis* IIV; physical stability; chloroform sensitivity.

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