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Short Communication

Culex pipiens affected by joint infection of a mosquito iridescent virus and *Strelkovimermis spiculatus*

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ABSTRACT

Dual infections with a mosquito iridescent virus (MIV) and the mermithid nematode, *Strelkovimermis spiculatus* were recorded in natural *Culex pipiens* populations around La Plata city, Argentina. *S. spiculatus* was detected in 82% of samples that were positive for MIV infection. Dissected larvae of *Cx. pipiens* with patent MIV infection presented 42% infection with *S. spiculatus*. Larvae of *Cx. pipiens* exposed to MIV and *S. spiculatus* under laboratory conditions produced a high joint infection rate (82.5%) while no infection was recorded on larvae exposed to virus suspension only. Field and laboratory results suggest a strong association between *S. spiculatus* and MIV in natural populations of *Cx. pipiens*, in which *S. spiculatus* could be a mode of entry for the virus into the mosquito hemocele.

1. Introduction

Culex pipiens (L.) is an important vector of human and domestic animal diseases in Argentina, such as St. Louis encephalitis virus (Monath et al., 1985; Diaz et al., 2006), *Dirofilaria inmitis* (Vezzani et al., 2006) and West Nile Virus (Morales et al., 2006). This species breeds in stagnant water with high levels of organic matter, such as man-made containers (Almiron and Brewer, 1996; Garcia et al., 2002) and drainage ditches (Campos et al., 1993) in urban and suburban areas of Argentina.

During a survey for pathogens of *Cx. pipiens* larvae in suburban areas of La Plata city, Argentina, we observed immature specimens of *Cx. pipiens* with mixed infections that involved a mosquito iridescent virus (MIV) and a parasitoid of mosquito larvae, *Strelkovimermis spiculatus* Poinar and Camino. This neotropical mermithid species was first isolated from larvae of the flood-water mosquito *Ochlerotatus albifasciatus* (Macquart) in La Plata, Argentina (Poinar and Camino, 1986) but later it was also isolated from other mosquito species including *Cx. pipiens* (García and Camino, 1990). MIV belongs to the Iridoviridae, a double stranded DNA virus family that infects both vertebrates and invertebrates. Mosquito iridescent viruses (MIVs) have been reported in several species of Culicidae around the world (Williams, 2008). In Argentina, MIV was first detected in *Cx. pipiens* populations breeding in habitats distributed around La Plata city in 2010 (Muttis et al., 2012). A similar association between Diptera, Nematoda and Iridoviridae has been previously reported by Mullens et al. (1999).

In this study we explore the association of both pathogens in natural *Cx. pipiens* populations and the possible role of the parasitoid *S. spiculatus* in the MIV transmission pathway.

2. Materials and methods

2.1. Field studies

Samples of mosquito larvae were taken from several drainage ditches located in suburban areas of La Plata, Buenos Aires Province, Argentina, from September 2010 to January 2013 using a 300 ml dipper. The larvae were transported to the laboratory in plastic containers with water from the same sites. Mosquitoes were identified using taxonomic keys (Darsie and Mitchell, 1985). Virus and nematode prevalence was calculated for each sample. In order to determine the percentage of nematode parasitism, from 20 to 150 fourth instar larvae per collection were individually placed in multiwall plates for nematode emergence. These parasites were identified according to their original descriptions (Poinar and Camino, 1986) 10 days later, when the post-parasites became adults (Camino and Reboredo, 1994). All the mosquito larvae collected in each sample were observed under stereomicroscope on black background in order to detect the turquoise iridescence usually observed in iridoviridae infection. MIV prevalence was calculated from the total number of larvae collected in each sample. To estimate this number for the large samples, larvae were concentrated in 2 l of water, and homogenized using a shaker. After that, a 300-ml subsample was extracted, and the number of larvae was counted to calculate the final larval number for the 2 l volume.

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MIV was confirmed in patently infected larvae from several collections by PCR assays as described by Muttis et al. (2012).

In order to determine if the presence of virus from field larvae samples is associated to the presence of nematode from the same samples Fisher's exact test were used as appropriate according to the sample sizes. The proportion of MIV infected larvae from field samples was correlated with the proportion of nematode infected larvae from the same samples by the use of Pearsońs coefficient.

In order to record the presence of nematodes inside larvae with iridescence, forty-five MIV infected larvae (15 larvae each of instars 2, 3 and 4) from the same field samples were dissected.

2.2. Laboratory studies

To investigate the horizontal transmission pathway of MIV into mosquito larvae, we performed the following assay: one hundred healthy second instar *Cx. pipiens* larvae were placed in a plastic cup containing 100 ml of dechlorinated water. These mosquito larvae were exposed to *S. spiculatus* juveniles ($n = 500$) along with three MIV field-infected third instar larvae previously killed at −20 °C and cut into small pieces. After 24 h of exposure, the larvae were placed in trays with 2 l of dechlorinated water and fed with finely ground rabbit chow. They were kept at 26 ± 1 °C, with a 12:12 light–dark photoperiod. The treated larvae were observed under a stereomicroscope for symptoms of infection 72 h post-exposure, because this is the time required to detect iridescence as according to observations from preliminary assays. MIV prevalence was calculated as the fraction of infected larvae with virus out of the number of live larvae × 100. Three controls were used (containers with healthy larvae only, healthy larvae and MIV, and healthy larvae with nematodes). In the control trial, nematode prevalence was recorded approximately 7 days after exposure, when the nematodes emerge from mosquito hosts at 26 °C. The assay was performed twice. Healthy *Cx. pipiens* larvae and juveniles of *S. spiculatus* were obtained from colonies maintained at the Centro de Estudios Parasiltológicos y de Vectores (CEPAVE). The basic methodology for rearing *S. spiculatus* in the laboratory involved the procedures previously described by Camino and Reboredo (1996).

3. Results

3.1. Field studies

Immature *Cx. pipiens* stages were collected from September 2010 to January 2013 from four drainage ditches. Mosquito larvae infected with MIV were observed in 17 samples ($n = 24$). The prevalence of this virus ranged from 0.09% to 31.6% (Table 1). *S. spiculatus* was detected in 82% ($n = 17$) of samples positive for MIV infection and in 42.8% ($n = 7$) of samples negative for this virus.

Nematode prevalence ranged from 0.1% to 94.5%. Infections by MIV were recorded from first to fourth instar *Cx. pipiens* larvae during all seasons in the four drainage ditches sampled (sites 1–4, Table 1). Only two infected pupae were recorded in two different samples. Infected larvae and pupae usually died a few days after collection.

The presence of MIV and *S. spiculatus* infections was significantly associated ($p = 0.021$) in field-collected samples. The proportion of MIV infected larvae in field samples was positively correlated with the proportion of nematode infected larvae ($r = 0.59$, $p < 0.01$).

In field-dissected larvae with symptoms of MIV infection, the prevalence of *S. spiculatus* infection was of 47% in second instar larvae ($n = 15$), 47% in third instar larvae ($n = 15$) and 33% in fourth instar larvae ($n = 15$). In some fourth instar larvae with this virus, the nematodes were observed inside the larvae as last-stage juveniles (Fig. 1).

Table 1
Field sampling. Nematode and MIV prevalence (%) in immature *Culex pipiens* sampled in four different drainage ditches (site) in La Plata, Argentine from September 2010 to January 2013.

Date	Site	MIV prevalence (n)	Nematode prevalence (n)
09-21-10	1	2.00 (203)	85 (20)
10-20-10	1	1.18 (706)	4.2 (20)
03-16-11	2	0 (1539)	0 (75)
03-16-11	4	0 (250)	0 (75)
04-13-11	2	0.16 (1873)	0 (50)
04-28-11	2	0.10 (4900)	0 (50)
05-10-11	2	0.09 (2287)	2 (50)
05-26-11	2	0 (1167)	5 (100)
06-13-11	2	0 (883)	0 (50)
06-27-11	2	0 (6)	17 (6)
07-01-11	1	31.6 (38)	73 (26)
07-05-11	1	9.60 (104)	89 (80)
07-12-11	2	0 (4)	25 (4)
07-13-11	1	10.6 (142)	94.5 (128)
11-17-11	2	0.20 (3879)	6 (148)
12-01-11	2	0 (6500)	0 (100)
03-08-12	3	6.66 (3000)	0.1 (100)
03-16-12	3	5.50 (2294)	0.2 (100)
04-10-12	3	5.10 (3260)	15.3 (72)
04-11-12	4	4.40 (250)	12 (50)
11-14-12	3	7 (287)	25 (60)
11-21-12	3	0.25 (1191)	2.08 (48)
01-04-13	3	6.95 (953)	1.31 (76)
01-25-13	3	0.5 (778)	0 (75)

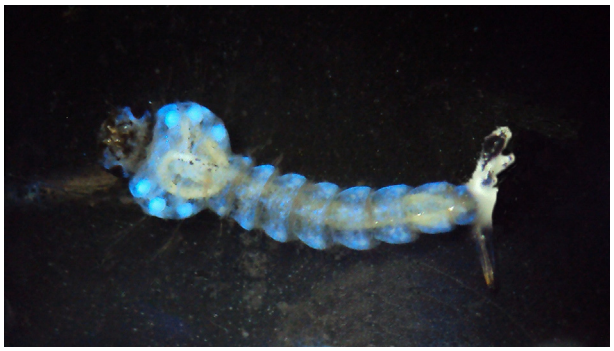

Fig. 1. Field-collected fourth instar *Culex pipiens* larvae showing MIV infection and late juvenile of *Strelkovimermis spiculatus* inside of its body.

3.2. Laboratory studies

When healthy *Cx. pipiens* larvae were exposed to MIV (controls with healthy larvae and MIV) no symptoms of infection were recorded. Moreover, we did not obtain infections by MIV in mosquito larvae in any of the controls. However, patent infection by MIV was observed in mosquito larvae exposed to both the virus and the nematode [82.5 ± 13% (mean ± SD), ($n = 182$)]. The nematodes were generally observed as early juvenile stages inside these MIV-infected larvae. Although nematode prevalence in this test was not recorded, the nematode prevalence in controls was 81 ± 9% (mean ± SD) ($n = 153$). Mortality in treated and control assays was less than 10%.

4. Discussion

In this study we recorded mixed infections with MIV and *S. spiculatus* in natural populations of *Cx. pipiens* immature individuals. Experimental transmission assays showed that larvae of *Cx. pipiens*

simultaneously exposed to MIV and juveniles of *S. spiculatus* presented a high viral infection rate, while no infection was recorded in larvae exposed to virus suspensions only. Interestingly, the percentage of viral infection was similar to the nematode prevalence recorded in the control. Field and laboratory results suggest a strong association between *S. spiculatus* and MIV in *Cx. pipiens* natural populations, in which this nematode could help MIV to enter mosquito larvae during host penetration by the nematode.

Under laboratory conditions, the nematodes were generally observed as early juvenile stages inside MIV-infected larvae, while in some field MIV-infective larvae, late juvenile stages of *S. spiculatus* were recorded indicating that the nematode would be able to develop to the post-parasite stage in some circumstances and emerge from larvae.

Dissection of MIV field-infected larvae showed that less than fifty percent of these larvae had nematodes. This suggests that there may be other ways of virus entry into the host. Cannibalism, injures, nematode penetration, and hymenopteran endoparasitoid oviposition have been proposed as possible mechanisms of horizontal transmission for iridescent viruses (Williams, 2008). Other possible explanation could be related to the immune response from mosquito host, given that some mosquito species have shown ability to block the normal development of preparasite nematodes (Poinar et al., 1979), which would result in non-detection of *S. spiculatus* within mosquito larvae but still allowing the MIV to access the host.

Related findings have been reported in other pathogen–host systems. Mullens et al. (1999) reported an association among *Culicoides variipennis sonorensis* (Diptera), *Heleidomermis magnapapula* (Nematoda) and Iridoviridae in natural breeding sites. The presence of *Corethrellonema grandispiculosum* (Nematoda) and Iridoviridae infecting *Corethrella brakeleyi* (Diptera) in the same field samples was also mentioned by Chapman et al. (1971). Moreover, in a terrestrial system Poinar et al. (1980) observed isopods infected with both iridescent virus and a mermithid parasite, with the mermithid also infected by the iridovirus. In any case, the natural life cycle of these viruses is still unclear, and future studies would be necessary to elucidate the full range of pathways involved in MIV transmission.

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References

Almiron, W.R., Brewer, M.M., 1996. Classification of inmature stage habitats of Culicidae (Diptera) collected in Córdoba, Argentina. Mem. Inst. Oswaldo Cruz. 91, 1–9.

Camino, N.B., Reboredo, G.R., 1994. Biología de *Strelkovimermis spiculatus* Poinar y Camino, 1986 (Nematoda: Mermithidae) parásito de mosquitos (Díptera: Culicidae) en condiciones de laboratório. Neotrópica 40 (103–104), 45–48.

Camino, N.B., Reboredo, G.R., 1996. Producción de *Strelkovimermis spiculatus* Poinar y Camino 1986 (Nematoda: Mermithidae). Neotrópica 42, 47–50.

Campos, R.E., Macia, A., Garcia, J.J., 1993. Fluctuaciones estacionales de culícidos (Diptera) y sus enemigos naturales en zonas urbanas de los alrededores de La Plata, Provincia de Buenos Aires. Neotrópica 39, 55–66.

Chapman, H.C., Clark, T.B., Anthony, D.W., Glenn Jr., F.E., 1971. An Iridescent virus from Larvae of *Corethrella brakeleyi* (Diptera: Chaoboridae) in Louisiana. J. Invertebr. Pathol. 18 (2), 284–286.

Darsie, R.F., Mitchell, C.J., 1985. Mosquitoes of Argentina. Part I and II. Keys for identification of adult females and fourth stage larvae in English and Spanish (Diptera, Culicidae). Mosq. Syst. 17, 163–334.

Diaz, L.A., Almiron, W.R., Farias, A., Vazquez, A., Sanchez-Seco, M.P., Alquilar, J., Spinsanti, L., Konigheim, B., Visintin, A., Garciá, J., Morales, M.A., Tenorio, A., Contigiani, M., 2006. Genotype III Saint Louis encephalitis virus outbreak, Argentina, 2005. Emerg. Infect. Dis. 12 (11), 1752–1754.

García, J.J., Camino, N.B., 1990. Primera cita para la Argentina de infecciones naturales en larvas de Culex pipiens (L.) (Diptera: Culicidae). Neotrópica 36, 83–86.

Garcia, J.J., Micieli, M.V., Achinelly, M.F., Marti, G., 2002. Establecimiento de una población de Aedes aegypti L. en La Plata, Argentina, In: Salomón, D.O. (Ed.), Actualizaciones en Artropodología Sanitaria Argentina. Serie: Enfermedades Transmisibles, RAVE, Ciudad Autónoma de Buenos Aires, Argentina. pp. 149–153.

Monath, T.P., Sabattini, M.S., Pauli, R., Daffner, J.F., Mitchell, C.J., Bowen, G.S., Cropp, C.B., 1985. Arbovirus investigation in Argentina, 1977–1980. IV. Serologic Surveys and Sentinel Equine Program. Am. J. Trop. Med. Hyg. 34 (5), 966–975.

Morales, M.A., Barrandeguy, M., Fabbri, C., Garcia, J.B., Vissani, A., 2006. West Nile virus isolation from equines in Argentina, 2006. Emerg. Infect. Dis. 12, 1559–1561.

Mullens, B.A., Velten, R.K., Federichi, B.A., 1999. Iridescent virus Infection in *Culicoides variipennis sonoriensis* and Interaction with the Mermithid Parasite *Heleidomermis magnapapula*. J. Invertebr. Pathol. 73, pp. 321–233.

Muttis, E., Miele, S.A.B., Belaich, M.N., Micieli, M.V., Becnel, J.J., Ghiringhelli, P.D., García, J.J., 2012. First record of a mosquito iridescent virus in *Culex pipiens* L. (Diptera: Culicidae). Arch. Virol. 157 (8), 157–1571.

Poinar, G.O., Camino, N.B., 1986. Strelkovimermis spiculatus n.sp. (Mermithidae: Nematoda) parasitizing *Aedes albifasciatus* Mac. (Culicidae: Diptera) in Argentina. J. Nematol. 18, 317–319.

Poinar, G.O., Hess, R.T., Cole, A., 1980. Replication of an Iridovirus in a Nematode (Mermithidae). Intervirology 14, 316–320.

Poinar, G.O., Roberta, T.H., Petersen, J.J., 1979. Immune responses of mosquitoes against *Romanomermis culicivorax* (Mermithida: Nematoda). J Nematol. 11 (1), 110–116.

Vezzani, D., Eiras, D.F., Wisnivesky, C., 2006. Dirofilariasis in Argentina: historical review and first report of *Dirofilaria immitis* in a natural mosquito population. Vet. Parasitol. 136, 259–273.

Williams, T., 2008. Natural invertebrate hosts of iridoviruses (Iridoviridae). Neotropl. Entomol. 37 (6), 615–632.