EXPERIMENTAL INFECTION OF *HYALOMMA MARGINATUM* TICKS WITH WEST NILE VIRUS

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Summary. – To define the possible role of *Hyalomma marginatum* ticks in the transmission of West Nile virus (WNV) in Portugal an experimental infection was established. Ticks were fed on viremic rabbits previously infected with WNV. In different developmental stage of *H. marginatum* virus isolation and detection of viral antigen and viral RNA were attempted. The oral infection rates were 3%, 33% and 75% for engorged larvae, nymphs and females after oviposition, respectively. Transstadial transmission rates for nymphs exposed to virus as larvae, for adults exposed as larvae, and for adults exposed as nymphs were 33%, 11% and 46%, respectively. No evidence of transovarial transmission was obtained. Ticks in the stages of nymphs and adults were able to transmit the infection to uninfected hosts. This study demonstrated that *H. marginatum* could be involved in the natural circulation of WNV in Portugal.

Key words: West Nile virus; ticks; Hyalomma marginatum; experimental infection; Portugal

Introduction

WNV is an arbovirus (the species *West Nile virus*, the genus *Flavivirus*, the family *Flaviviridae*) belonging to the Japanese encephalitis virus group (Fauquet *et al*., 2005). Its isolation has been reported from Africa, Asia, and the Middle East, and outbreaks caused by this virus in humans and animals have occurred in Europe, South Africa, Israel and North America (Komar, 2000). Recognized since the 1960's in Europe, WNV has been considered a remerging mosquito-borne disease in old world after the recent occurrence in Romania (Tsai *et al*., 1998), Czech Republic (Halouzka *et al*., 1998), and Russia (Platonov *et al*., 2001). However, this agent achieved its biggest public impact in 1999, after the first WNV epidemic in the United States.

In Portugal, the first serological indication for the presence of WNV came from epidemiological studies done in human and animal populations during the 1960's. In 1969, the virus was isolated from *Anopheles maculipennis* mosquitoes in Roxo's Dam, Alentejo, South Portugal (Filipe, 1971, 1972). Thirty years later, in 1998, the second isolation of WNV was done from the same mosquitoe species collected in the Tejo Estuary, Central Portugal (Fernandes, 1998). More recently, a WNV isolation was recorded in Algarve, South Portugal (Connell *et al*., 2004).

Although WNV is considered a mosquito-borne flavivirus, it has been already isolated from several species of *Ixodid* and *Argasid* ticks (Schmidt and Said, 1964; Lvov *et al*., 1975; Hoogstraal *et al*., 1976; Hubálek and Halouzka, 1999). This fact has been attributed to a possible mechanism of persistence of the virus in endemic foci during periods of vector inactivity (e.g. winter) (Reeves *et al*., 1994; Nasci *et al*., 2000). In order to better define the possible role of *Ixodid* ticks in the natural circulation of WNV in Portugal we carried out and examined an experimental infection of *H. marginatum* ticks with this virus.

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Abbreviations: IFA = immunofluorescence assay; WNV = West Nile virus

Materials and Methods

Virus. A Portuguese (Roxo) strain of WNV, isolated from *Anopheles maculipennis* mosquitoes in Portugal in 1969 (Filipe, 1971,

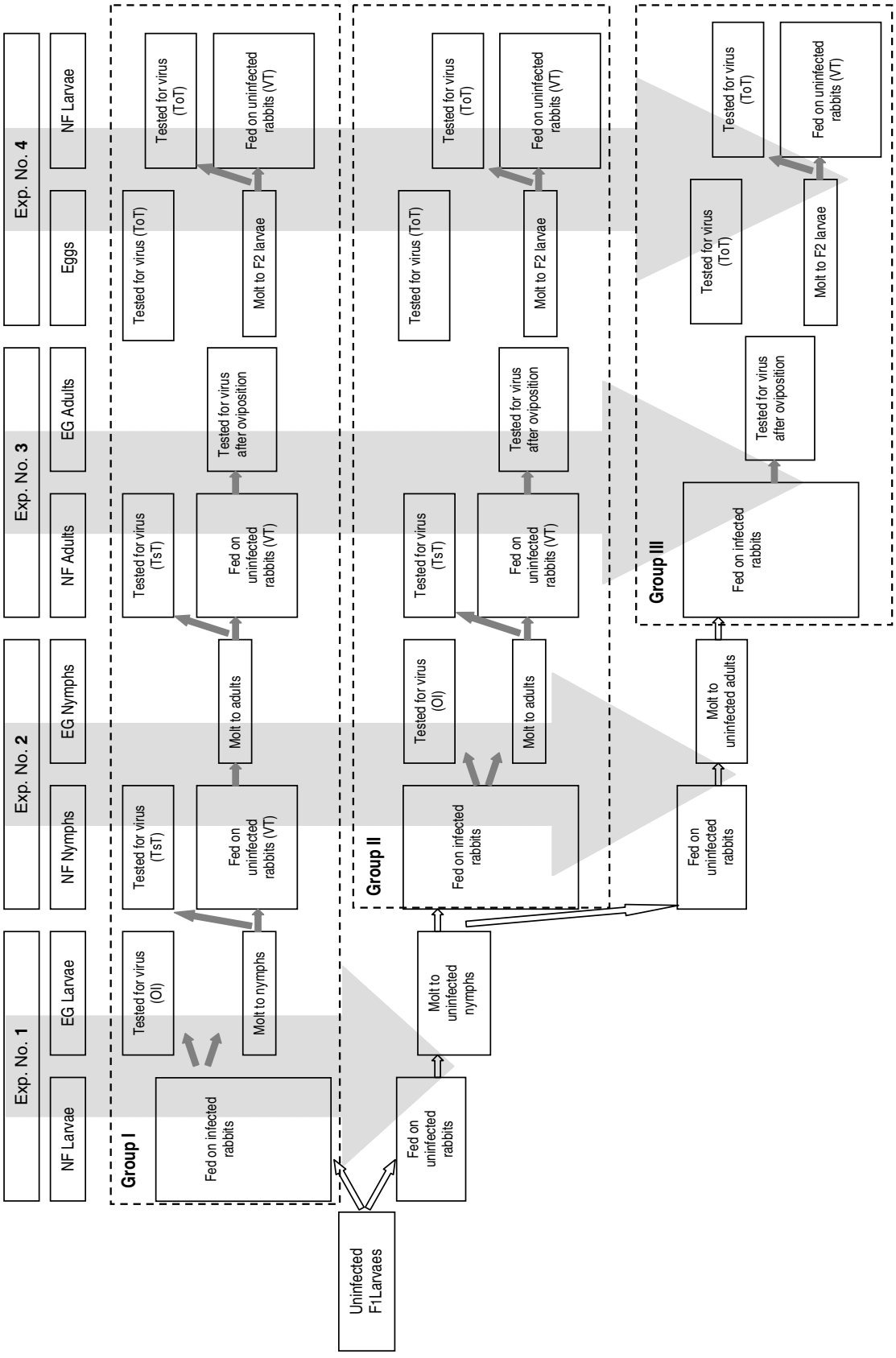

Fig. 1

Scheme of transmission experiments with WNV and *H. marginatum* ticks

NF = not fed; EG = engorged; OI = oral infection; TsT = transstadial transmission; VT = viral transmission; ToT = transovarial transmission. Groups I, II and III consisted of ticks originally exposed to the virus as larvae, nymphs and adults, respectively.

1972), was used. The virus underwent 3 intracerebral (i.c.) passages in suckling mice and its PFU titer was assayed in Vero E6 cells (Dulbecco, 1952).

Ticks. H. marginatum ticks were obtained from the laboratory colony PoTi 56.99 maintained in this Institute. All three-tick stages were fed on naive rabbits (*Oryctolagus cuniculus,* Hyla strain); during free-living phases, the ticks were held in container tubes at 24°C and 70–80% relative humidity (RH) (Santos-Silva and Filipe, 1998). The tick colony was checked for WNV by RT-PCR and virus isolation.

Vertebrate host. Infected and uninfected rabbits (*Oryctolagus cuniculus,* Hyla strain) were used as hosts to feed uninfected and potentially infected ticks, respectively. Rabbits were inoculated subcutaneously with 1.6 x 10^6 PFU in 1 ml. The animals' care and manipulation were done in accordance with the European animal welfare act from 1986 (86/609/CEE). Ticks were placed on rabbit ear in standard manner (Santos-Silva and Filipe, 1998).

Experimental design. Four transmission experimental series were performed, each with a different developmental stage of ticks, starting with the F_1 larvae and ending up with the F_2 larvae (Fig. 1). Ticks in three developmental stages (larvae, nymphs, and adults) representing groups I, II and III, respectively, were exposed to the virus. To determine virus transmission from host to tick (oral infection) and *vice versa* (viral transmission), uninfected and potentially infected ticks were allowed to fed on virus-infected and uninfected hosts, respectively. Two hundred larvae, 50 nymphs, and 20 adults (10 males and 10 females) were fed per rabbit. Immature ticks were placed on rabbits on days 4 (larvae) and 2 (nymphs) p.i. Concerning adults the inoculation of rabbits was postponed until ticks kept feeding. The rabbits were checked daily until all ticks dropped off. Fed ticks were maintained at 24°C in 70–80% RH until tested for the virus. Once the oviposition ended, the eggs were grouped into pools of approximately 50 pieces and stored under the same conditions as above. The rabbits were bled before the inoculation or the exposure to potentially infected ticks, post-inoculation, and after tick drop off. To determine oral infection, virus isolation and RT-PCR from engorged larvae (transmission experiment 1), engorged nymphs (transmission experiment 2), and fed adults (transmission experiment 3) were performed. To allow for blood meal digestion ticks were kept alive for 15 days after drop off before being stored at -70°C. Females after the oviposition were stored under the same conditions. To assess transstadial transmission, F_1 nymphs and adults were frozen at -70°C approximately four weeks after molting and tested for the virus. To determine tick-to-host transmission, F_1 nymphs and adults were fed on uninfected rabbits that were subsequently tested for WNV antibodies by indirect immunofluorescence assay (IFA). To verify transovarial transmission, eggs and F_2 larvae, approximately 4 weeks after hatching, were stored at -70°C and subjected to virus isolation and RT-PCR. The remaining F_2 larvae were fed on uninfected rabbits that were tested for WNV antibodies by IFA.

Virus isolation. Ticks (pools and individuals) were homogenized in sterile PBS containing 7.5% BSA and antibiotics. The suspensions were centrifuged at 800 x g for 30 mins at 4°C and stored at -70°C until assayed. Suckling mice were inoculated i.c. with the suspensions and checked daily for 14 days for signs of illness.

RT-PCR. Total RNA was extracted from tick suspensions with the RNeasy Mini kit (Qiagen). RT-PCR amplified a region of the envelope gene of WNV (Berthet *et al*., 1997) using the Gene Amp RNA PCR kit (Perkin Elmer) according to the manufacturer's instructions. The primers WN240 (5'-GAGGTTCTTCACTCCAT-3') and WN132 (5'-GAACATCAAGTATGAGG-3') were used. PCR consisted of initial denaturation at 95°C for 105 secs and 35 cycles of 95°C/15 secs, 49°C/30secs, and 72°C/2 mins, followed by final extension at 72°C for 7 secs. The PCR products were analyzed by electrophoresis in 1.5% agarose gel in the presence of ethidium bromide.

IFA. Rabbit blood samples were tested for WNV antibodies by an indirect IFA as described previously (Lanciotti *et al*., 1999). Briefly, Teflon-coated slides with WNV-infected Vero E6 cells were incubated with serial 2-fold dilutions of the sera at room temperature for 1 hr. FITC-labeled pig anti-rabbit IgG, IgA and IgM (Dako, Denmark) were added at the 1:40 dilution at room temperature for 1 hr. A serum with a titer ≥64 was considered positive.

Results

In all experiments, the rabbits before tick placement and virus inoculation were negative for serum WNV antibodies. Control rabbits remained negative for both the virus and serum WNV antibodies during the experiment. In virus isolation assay, control mice did not show any signs of sickness.

Host-to-tick transmission

All three stages of *H. marginatum* became infected with the virus after feeding on viremic host. Infection rates were calculated as the number of positive engorged ticks/total number of ticks tested. Infection prevalence in larvae pools was determined according to $p = 1–(1–s/r)^{1/m}$, p = infection prevalence, s = positive pools, r = tested pools, and m = number of ticks/pool (Farrington, 1992). Infection rates for engorged larvae and nymphs and post-oviposition females were 3%, 33%, and 75%, respectively (Table 1).

Table 1. Infection rates in *H. marginatum* ticks after feeding on WNV-infected rabbits

Exp. No.	Developmental stage	Infection rate (%) (positives/tested)[a]
1	Larvae	3*
2	Nymphs	33 (4/12)
3	Adults (males and females)	75 (6/8)

[a]Tested by RT-PCR.

*Infection prevalence calculated for pools according to $p = 1–(1–s/r)^{1/m}$, p = infection prevalence, s = positive pools, r = tested pools, and m = number of ticks per pool.

Transstadial transmission

Transstadial transmission of the virus from potentially infected larvae to nymphs (group I) and from potentially infected nymphs to adults (groups I and II) was demonstrated (Table 2). The transmission rate was calculated as the number of molted positive ticks/total number of ticks tested.

Table 2. Transstadial transmission rates in molted *H. marginatum* ticks

Exp. No.	Group	Developmental stage	Transmission (%) (positives/tested)[a]
2	I	Nymph	33 (2/6)
3	I	Male	11 (2/18)
		Female	11 (1/9)
3	II	Male	29 (2/7)
		Female	67 (4/6)

[a]Tested by RT-PCR.

Tick-to-host transmission

Infected nymphs and females were able to transmit the virus to uninfected host if ticks at the stages of larvae or nymphs were exposed to the virus. Uninfected rabbits that were exposed to potentially infected F_1 nymphs (group I) and females (group I and II) developed WNV antibody titers. The group II F_2 larvae were evidently not able of infecting rabbits as a seroconversion in the latters was not observed (Table 3).

Table 3. WNV antibodies in rabbits after feeding infected *H. marginatum* ticks

Exp. No.	Group	Developmental stage	No. of ticks fed	No. of rabbits with seroconversion	WNV antibody titer
2	I	F1 Nymph	30	1	128
3	I	F1 Female	7	1	64
3	II	F1 Female	3	1	512
4	II	F2 Larvae	100	0	–

Transovarial transmission

No evidence for transovarial transmission was obtained. In all three tested groups F_2 larvae were not able to infect the host after feeding. On the other hand, if eggs and hatched larvae were inoculated into mice, the virus could not be isolated and the RT-PCR was negative too.

Discussion

This study is the first report of WNV transmission by *Hyalomma marginatum* ticks. Although other studies on the virus transmission by ticks have been reported (Hurlbut, 1956; Whitman and Aitken, 1960; Vermeil *et al*., 1960; Abassy *et al*., 1993; Anderson *et al*., 2003; Lawrie *et al*., 2004), none has tested particularly *H. marginatum*. This tick specie was chosen on the basis of the facts that it occurs frequently in Portugal, its adult forms parasitize large mammals, and immature forms are frequently associated with the birds (Caeiro, 1999; Silva *et al*., 2001), which are natural hosts of WNV. Moreover, the virus isolation from *H. marginatum* has already been reported from other countries (Hoogstraal *et al*., 1976; Zeller and Murgue, 2001). In this study, ticks in all stages became infected after feeding on viremic host, but the infection rates increased with the development stage. This is consistent with the fact that larvae ingest less blood and for shorter periods of time compared to nymphs or adults. Transstadial transmission was demonstrated in *H. marginatum* through the virus detection in nymphs and adults originally infected as larvae and nymphs, respectively. The occurrence of transstadial transmission is an an important fact as it may represent the way of the virus maintenance in the vector population. The transstadial transmission rates in *H. marginatum* were the highest for adults exposed to the virus as nymphs and the lowest for nymphs exposed to the virus as larvae.

The ticks infected as larvae were not able to transmit the virus as efficiently as the ticks infected as nymphs. This could be also explained by shorter attachment time and smaller blood volume ingested by larvae. No evidence for transovarial virus transmission was obtained: neither hosts that served as feeding support for F_2 larvae developed antibodies to the virus nor F_2 larvae gave positive RT-PCR. Transovarial transmission constitutes a way to ensure the continuous maintenance of the virus in the ticks' population. Nevertheless, the rate of transmission for flavivirusses is usually less than 1% (Labuda *et al*., 1993). The virus isolation attempts were all negative. This fact could be due to low concentration of viral particles. Similar results have been reported by others authors (Nuttall *et al*., 1994).

Giving the longevity and capacity to survive for long periods without feeding, ticks can maintain the virus till its transmission. The persistence of viruses in these vectors is an advantage for their survival during unfavorable conditions, namely in temperate climate regions. This study revealed that ticks could be infected with WNV through

feeding on infected hosts and could transmit the infection transstadially and to hosts during feeding. Based in these transmission experiments, *H. marginatum* could play a role as potential reservoir of WNV in Portugal.

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