TRANSMISSION OF TICK-BORNE BUNYAVIRUSES BY COFEEDING IXODID TICKS

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Summary. – Palma and Bhanja bunyaviruses replicated in *Dermacentor marginatus, D. reticulatus, Rhipicephalus sanguineus, R. appendiculatus* and *Ixodes ricinus* ticks after parenteral inoculation and appropriate incubation and feeding. Palma virus was transmitted to *D. marginatus* and *D. reticulatus* males, *D. reticulatus* and *R. sanguineus* females, and *R. appendiculatus* nymphs while cofeeding with infected ticks on laboratory mice. Bhanja virus was transmitted to *D. marginatus* males and *R. appendiculatus* nymphs. Laboratory mice developed low levels of viraemia detectable only by intracranial (i.c.) inoculation of newborne laboratory mice.

Key words: Palma virus; Bhanja virus; bunyaviruses; cofeeding infection

Introduction

Out of a large *Bunyaviridae* family containing about 350 distinct viruses in 5 genera most viruses are transmitted by mosquitoes but few by ticks, and some do not have any identified arthropod vector. Viruses of Bhanja antigenic group (Group 1 of seven groups of bunyaviruses unassigned to any genus; Murphy *et al.*, (1995)) belonging to the family *Bunyaviridae* and so far unassigned to any genus are transmitted by ticks. These viruses have been isolated in regions as diverse as India, various parts of Africa, former USSR and Europe (Karabatsos, 1985). In Europe, Bhanja group viruses have been isolated in Italy (Verani *et al.*, 1970), Bulgaria (Pavlov *et al.*, 1978), former Yugoslavia (Vesenjak-Hirjan *et al.*, 1973) and Slovakia (Hubálek *et al.*, 1988). A virus isolated in Portugal and designated Palma was proposed a member of *Bunyaviridae* family, Bhanja group (Filipe *et al.*, 1994).

Various tick species have been incriminated as vectors of these viruses, however, there was so far no published experimental evidence on their transmission by ticks. In the present study, we addressed a question whether Bhanja and Palma viruses, the former known from India, Slovakia, and other parts of Europe, and the latter from Portugal, can be biologically transmitted by various ixodid ticks using laboratory mice as hosts. Among the tested tick species were species native for Slovakia as well as for Portugal. As the virulence of Bhanja group viruses is so far not well characterised, we tested the level of viraemia in laboratory mice at various time intervals after virus application by two routes. We compared the usuall way of experimental infection by virus inoculation with the more natural way by a tick bite. An additional reason for assesing viraemia was its controversial role in arbovirus transmission in nature (Nuttall *et al.*, 1994).

Materials and Methods

Viruses. Bhanja virus used in the experiments was isolated from the pool of 15 *D. marginatus* male ticks collected from sheep in Kecovo, southern Slovakia (Hubálek *et al.*, 1988). As a stock vi-

Abbreviations: i.c.= intracranial; i.p.= intraperitoneal; p.i. = post infection.

rus, 10% mouse brain suspension in the 6th mouse passage of a titre of $10^{6.5}$ LD_{50}/0.01 ml has been used for tick inoculation. The LD_{50} relates to i.c. infection of mice. Palma virus was originally isolated from *Haemaphysalis punctata* male ticks collected from cattle in southern Portugal (Filipe *et al.*, 1994). The virus was used in its 5th i.c. mouse passage and the stock suspension titered to $10^{6.5}$ LD_{50}/0.01 ml. Undiluted stock virus suspensions were used for tick inoculation and their 1:10 dilutions for intraperitoneal (i.p.) inoculation of laboratory mice.

Ticks. A laboratory colony of *R. sanguineus* ticks has been established at the Centre for Vector and Infectious Diseases Research, National Institute of Health, Águas de Moura, Portugal in May 1996 from unfed adults collected from dogs in Águas de Moura. The colony has been maintained by feeding ticks at all developmental stages on white New Zealand rabbits kept at ambient temperature and 85 – 90% relative humidity. A colony of *R. appendiculatus* ticks has been maintained in comparable conditions at the Institute of Zoology, Bratislava. *D. marginatus* ticks were field collected at Pinheiro (southern Portugal), and *D. reticulatus* and *I. ricinus* ticks were collected in the surroundings of Bratislava, Slovakia.

Virus infection of ticks and transmission experiments. Female ticks as donors for the transmission experiments were inoculated with 0.002 ml of stock virus into the membrane between coxa and trochanter by means of a microcapillary. Ticks were incubated for at least 10 days before use in transmission experiments. Various combinations of infected tick donors and uninfected recipients were used. Tick donors as well as recipients were placed in transparent plastic retaining chambers on the back of adult laboratory mice (weight 20 – 25 g). The ticks were allowed to feed either to repletion (larvae and nymphs) or for various time intervals (adults). Donor ticks were tested immediately after removing them from mice for the virus presence and recipient ticks were incubated for 10 days at the ambient temperature before further processing.

Virus assay. Ticks were homogenised individually in a microtissue grinder in Eagle's Minimal Essential Medium supplemented with 10% foetal bovine serum and antibiotics. Blood samples were obtained from *sinus orbitalis* of anaesthetised laboratory mice 1 – 6 days post commencement of tick feeding or post i.p. inoculation with 0.5 ml of diluted stock virus. As Vero E6 and PS cell cultures were demonstrated not to be sensitive enough for the detection of the Bhanja group viruses in very low concentrations, an i.c. inoculation of 0.01 ml of examined samples into newborn (1 to 3-day-old) laboratory mice was used as the detection system. Each sample was inoculated into the litter of 6-10 mice and observed for the signs of illness or death for 3 weeks. The specificity of the observed signs was confirmed by virus identification in the dissected brain tissues of sacrificed diseased mice. In selected donor ticks, virus titration was performed by i.c. inoculation of mice.

Results

Viraemia in laboratory mice

Viraemia caused by Palma or Bhanja viruses in laboratory adult mice was low, regularly not exceeding the level of detection by i.c. inoculation of newborne laboratory mice and never above the titre of $10^{1.0}$ LD_{50}/0.01 ml. Viraemia of two days duration was observed after i.p. virus inoculation, occuring earlier with Bhanja virus than Palma virus. Irregular viraemia occuring for several days was observed after an infectious Palma or Bhanja virus tick bite (Table 1).

Table 1. Viraemia in laboratory mice after i.p. inoculation of Palma and Bhanja viruses and after an infectious *D. reticulatus* tick bite

Day	Palma virus					Bhanja virus				
	i.p.	i.p.	tick	tick	tick	i.p.	i.p.	tick	tick	tick
1	–	–	+	–	–	–	–	–	–	+
2	–	–	–	+	–	+	+	+	+	–
3	–	–	+	–	–	+	+	–	+	+
4	+	+	+	+	+	–	–	+	–	+
5	–	+	+	–	–	–	–	–	–	+
6	–	–		+	–	–	–	–	–	–

(+) = virus detected in the blood, titre $\geq 10^{1.0}$ LD_{50}/0.01 ml.
(–) = virus not detected by i.c. inoculation of 1– to 3-day-old mice.

Donor ticks

Three different ixodid tick species were used as the donors of infection of laboratory mice. In total, 27 *D. marginatus*, 14 *D. reticulatus*, and 12 *I. ricinus* ticks were tested for virus transmission in groups of 2-4 ticks on 24 mice. A Palma or Bhanja virus transmission to the host was observed on 21 mice (87.5% transmission) either by detection of virus in the blood of mice they were feeding upon (Table 1) or by virus transmission to the cofeeding ticks (Tables 2 and 3), i.e. at least 21 out of 51 tested ticks (41%) transmitted the virus. However, all tested donor ticks (26) inclusive those feeding on mice where no transmission was detected (Table 2, mouse No. 4, Table 3, mice Nos. 1 and 5) contained the virus in the titre of $10^{1.0}$ – $10^{2.5}$ LD_{50}/0.01 ml. No differences regards the infection and transmission were observed between different tick species used as donors (data not shown).

Cofeeding transmission

A Palma virus transmission from infected to uninfected ticks feeding together was tested on 12 mice and was accomplished on 11 of them (92%). Three developmental stages and four tick species were tested as recipients of infection. In total, 28 of 77 recipients (36%) acquired the infection. The most efficient recipients were *D. reticulatus* females. As many as 64% of them acquired the virus. *D. marginatus* males were infected in 39% and *R. appendiculatus* nymphs in 32% (Table 2).

A Bhanja virus transmission during cofeeding was tested on 6 mice and was succesfully accomplished on 4 of

Table 2. Transmission efficiency of Palma virus between cofeeding donor and recipient ticks

Mouse No.	Donor ticks infected/fed (sp.)	Day p.i.	Recipient ticks infected/fed		
			Females (sp.)	Males (sp.)	Nymphs (sp.)
1	3/3 (DM)	3	0/4 (RS)	1/3 (DM)	–
2	4/4 (DM)	3	2/4 (RS)	2/4 (DM)	–
3	3/3 (DM)	4	3/6 (RS)	3/4 (DM)	–
4	4/4 (DM)	4	–	0/4 (DM)	–
5	2/2 (DM)	6	0/5 (RS)	1/3 (DM)	–
6	2/2 (DR)	6	1/4 (DR)	–	–
7	2/2 (DR)	6	2/3 (DR)	1/1 (DR)	–
8	2/2 (DR)	6	3/4 (DR)	0/2 (DR)	–
9	2/2 (DR)	6	2/4 (DR)	0/1 (DR)	–
10	2/2 (IR)	6	–	–	3/7 (RA)
11	2/2 (IR)	6	–	–	2/6 (RA)
12	2/2 (IR)	6	–	–	3/12 (RA)

DM = *Dermacentor marginatus*, DR = *D. reticulatus*, RS = *Rhipicephalus sanguineus*, RA = *R. appendiculatus*, IR = *Ixodes ricinus*.

Table 3. Efficiency of transmission of Bhanja virus between cofeeding donor and recipient ticks

Mouse No.	Donor ticks infected/fed (sp.)	Day p.i.	Recipient ticks infected/fed		
			Females (sp.)	Males (sp.)	Nymphs (sp.)
1	4/4 (DM)	4	0/1 (RS)	0/4 (DM)	–
2	4/4 (DM)	6	0/5 (RS)	1/3 (DM)	–
3	3/3 (DM)	6	–	1/4 (DM)	–
4	2/2 (IR)	6	–	–	3/6 (RA)
5	2/2 (IR)	6	–	–	0/2 (RA)
6	2/2 (IR)	6	–	–	2/8 (RA)

DM = *Dermacentor marginatus*, DR = *D. reticulatus*, RS = *Rhipicephalus sanguineus*, RA = *R. appendiculatus*, IR = *Ixodes ricinus*.

them (67%). In total, 33 recipient ticks were used and 7 of them became infected while feeding with donor ticks. *R. sanguineus* females did not acquire the infection, however, only a limited number of ticks (6) were feeding on them and were subsequently tested. The virus transmission to *D. marginatus* males and *R. appendiculatus* nymphs was successful in 18% and 31%, respectively. With the limited data available, the transmission efficiency of Bhanja virus seems lower in comparison with Palma virus.

Discussion

Bhanja virus was isolated in Slovakia from *D. marginatus* male ticks (Hubálek *et al.*, 1988) and Palma virus was isolated in Portugal from *Haemaphysalis punctata* male ticks (Filipe *et al.*, 1994). *H. punctata* ticks were not available for our experiments, however, the other tick species used were competent vectors in laboratory conditions using laboratory mice as host animals. Interestingly, both the mentioned isolates were obtained from tick males and the latter acquired infection frequently in our experiments while cofeeding with infected females.

For the survival of any arbovirus in nature an efficient transmission from an infected to uninfected vector must be ensured. To accomplish the transmission, virus has to pass through a vertebrate host. It has been believed that host viraemia is crucial for a vector to acquire a viral infection (WHO, 1985). The first evidence for the "non-viraemic" way of virus transmission was obtained with Thogoto virus (Jones *et al.*, 1987). Subsequently, the same was demostrated also for tick-borne encephalitis virus (Alekseev *et al.*, 1991; Labuda *et al.*, 1993a) and its validity for virus survival in nature was proved in experiments on natural hosts (Labuda *et al.*, 1993b).

Natural hosts of the Bhanja group bunyaviruses are so far not well defined and may include e.g. goats, cattle and other domestic animals (Filipe *et al.*, 1994). A very low viraemia has been demonstrated even in virus sensitive animals such as laboratory mice. It is unlikely that the viraemia would be higher in natural hosts. The non-viraemic transmission of Palma and Bhanja viruses detected in our experiments by use of various donor and recipient tick species demostrates a possible way of survival of these bunyaviruses in nature. In addition, the importance of the tick cofeeding for the maintenance of tick-borne arbovirus transmission cycles in nature is stressed again.

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