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EXPERIMENTAL TRANSMISSION OF CHANDIPURA VIRUS BY *PHLEBOTOMUS ARGENTIPES* (DIPTERA: PSYCHODIDAE)

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Abstract. Experiments were carried out to demonstrate the susceptibity and transmission potential of *Phlebotomus argentipes* (Annandale & Brunetti) for Chandipura virus (CHPV). In India, *P. argentipes* is one of the predominant species found in many areas endemic for CHPV. Although its laboratory colonization is difficult, we have demonstrated that 65% of *P. argentipes* were susceptible to CHPV infection by the oral route. Transmission experiments were carried out by intra-thoracic inoculation because of re-feeding problems with this species. After incubation for 24 hours, efficient transmission of CHPV to mice was observed. The estimated minimum transmission rate among the inoculated flies was 32%. CHPV in sand flies as well as in mice was detected and confirmed by immunofluorescent antibody assay and reverse transcription–polymerase chain reaction, respectively. The susceptibility of *P. argentipes* to CHPV and its potential to transmit the virus by bite has importance in epidemiology of CHPV.

INTRODUCTION

Chandipura virus (CHPV, family Rhabdoviridae, genus *Vesiculovirus*) was isolated originally from human serum during an outbreak of febrile illness in Nagpur, Maharashtra, India in 1965.[1,2] Recently, an outbreak of febrile illness with encephalopathy occurred in Andhra Paradesh, India, and CHPV isolated from a human cerebrospinal fluid sample.[3] In India, CHPV has been isolated from phlebotomine sand flies collected at Aurangabad, Maharashtra.[4] Transovarial transmission of CHPV in the laboratory has been reported for *Phlebotomus papatasi* (Scopoli).[5] Recently, CHPV has been detected in sergentomine sand flies.[6] Therefore, sand flies are considered to be main vector of CHPV.

Vector competence refers to the intrinsic permissiveness of an arthropod vector for infection, replication, and transmission of a virus.[7] When an arthropod vector takes a viremic blood meal, the virus has to cross two physiologic barriers: 1) midgut barriers for viral infection and dissemination into the hemocoel, and 2) salivary gland barriers for viral secretion and transmission by bite.

Among *Phebotomine* sand flies, *P. argentipes* is one of the predominant species in several regions of India, especially in the CHPV-epidemic areas of Warangal and Chotta Udaypur, Gujarat, India.[8] The purpose of our study was to demonstrate the susceptibility and transmission potential of *P. argentipes* for CHPV.

MATERIALS AND METHODS

Collection and rearing of sand flies. Wild sand flies were collected in cattle sheds and human dwellings by hand aspirator. The next morning, flies were placed in a cloth feeding cage. Two mice (5–6 days old) were then introduced in the cage. After feeding, blood-engorged flies were removed from the cage and were placed individually in specially designed plastic jars with plaster of paris at the bottom and were maintained at 28–30°C. After oviposition, females were removed from the jar and were used for subsequent identification up to the species level by using the key of Lewis.[9] Emerging larvae from the deposited eggs were fed with larval food comprising of sand, cow dung, and goat liver powder in the ratio of 55:40:5. The emerging adult females of *P. argentipes* (F_1) were used for susceptibility and transmission experiments.

Oral infection and viral dissemination. The F_1 females were removed from the jars and placed in a cloth feeding cage and starved for 24 hours. In three separate trials, two viremic mice (2–3 days old) were introduced into the cage. These mice were inoculated intraperitoneally 24 hours earlier with approximately 102 plaque-forming units (PFU) of CHPV (National Institute of Virology strain no. 653514.). After exposure to sand flies for three hours, mice were removed from the feeding cage and bled. Blood was diluted 1:10 in phosphate-buffered saline solution and stored in freezer at −70°C for subsequent virus titration. All visibly blood-engorged flies were removed from the cages and put in small plastic jars, which were then covered with muslin cloth and set in a pan. These sand flies were provided daily with glucose as the sources of carbohydrate and housed in an insectary maintained between 28°C and 30°C at a relative humidity of 95% and with 12 hours of light per day for 4–5 days. After extrinsic incubation, these sand flies were collected in test tubes, anesthetized at −70°C, and head squashes were prepared. The bodies were placed in sterile screw-capped vials and stored at −70°C for further virus detection.

Viral transmission by bite. The re-feeding of the flies was the major problem faced in transmission experiments. Despite repeated efforts, no re-feeding on mice was observed. Thus, for further experimentation, we inoculated *P. argentipes* females intrathoracically with CHPV. Transmission of CHPV was demonstrated in three separate trials by exposing two newborn Swiss-Albino mice (2–3 days of age) to inoculated sand flies (n = 10) 24 hours after inoculation. Mice were exposed in a feeding cage overnight. At the end of this period, these sand flies were killed and stored at −70°C for virus detection. The exposed mice were then returned to their mother and examined daily for infection. The sick and dying mice were killed and brains were harvested and triturated. The resultant suspension was used for viral RNA detection by reverse transcription–polymerase chain reaction (RT-PCR).

Virus detection and confirmation. The disseminated CHPV was detected in brain cells of the fed sand flies after oral

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feeding and extrinsic incubation by using an immunofluorescent antibody assay following the procedure of Kuberski and Rosen[10] and further confirmed by RT-PCR following the methods of Geevarghese and others[3] and Rao and others.[6] Head squashes of the *P. argentipes* were prepared on slides and processed to detect viral antigen using hyperimmune serum raised against the CHPV virus in mice. The bodies of these flies were triturated in 1 mL of sterile 0.75% bovine albumin phosphate saline. The resulting suspension was then inoculated intraperitoneally into mice. The harvested brains of the sick and dying mice were processed by RT-PCR to detect the viral RNA by using CHPV G protein–specific primers. The CHPV transmitted by bite was detected in the brains of the sick mice by using RT-PCR.

RESULTS

Sand flies collection and rearing. A total of 178 (97 female and 81 male) sand flies were collected, of which 74 (48 females and 26 males) were identified as *P. argentipes*. Feeding and egg laying were recorded in 20–25% of the flies. A total of 390 eggs were collected and kept for hatching in six different plastic jars. A total of 148 (68 females and 80 males) adults emerged with female-to-male ratio of 1:1.17.

Oral infection and viral dissemination. Three groups of 10 females were fed on viremic mice (titer = approximately 106 PFU). Feeding was observed in 40–70% of the females. After feeding, these females were examined after 4–5 days. Among the 17 fed females, 11 (65%) had disseminated virus in brain cells (Table 1).

Viral transmission by bite. Three groups of 10 female flies each were inoculated intrathoracically and kept overnight in three different cages, with each cage containing a pair of baby mice. Fly feeding rates in the three groups were 60%, 60%, and 70% (mean = 63%, Table 1). Viral dissemination rates in the three groups of flies were 100%, 100%, and 80% (mean = 93%, Table 1). Because pairs of mice were each exposed to 10 inoculated flies, it was not possible to demonstrate transmission caused by individual flies. Instead, minimal transmission rates were calculated by dividing the number of transmission events (i.e., number of mice becoming ill after infection with CHPV) by the number of potential transmitters (i.e., number of blood-fed sand flies in the cages). Minimum transmission rates in the three groups were 33%, 33%, and 29% (mean = 32%, Table 1).

DISCUSSION

The major constraints faced during colonization of this species were high mortality after oviposition and no females could survive longer than 8–9 days. Approximately 25% of the wild-caught females and 60–70% of the F_1 *Ph. argentipes* females could feed during the initial exposure to mice but not on hamsters. No wild-caught or F_1 generation females of this species would re-feed on these host on re-exposure. Our present studies confirm earlier reports that the *Phlebotomine* sand flies are difficult to colonize in laboratory conditions.[11,12]

We showed that CHPV can multiply in *P. argentipes* and can be transmitted by bite. This observation is significant because *Phlebotomine* sand flies have a broad host range that includes humans and a wide range of mammals.[13] An interesting feature of this study was that *P. argentipes* was able to transmit CHPV to newborn mice by bite.

Our study showed that *P. argentipes* is susceptible to CHPV infection and the virus is disseminated to salivary gland by overcoming the midgut barrier and midgut escape barrier within 4–5 days and is then transmitted to other vertebrate hosts by crossing salivary gland barrier in the next 24 hours. This indicates that the extrinsic incubation period of CHPV in *P. argentipes* is approximately 5–6 days. In *P. papatasi* and some mosquito species, it was shown that CHPV has an extremely short extrinsic incubation period.[5,14] Although we were are not able to identify the precise incubation period because of the re-feeding problem, it appears that CHPV has short extrinsic incubation period that may favor rapid transmission of CHPV.

To ensure efficient transmission of the virus by the vector species, rapid virus multiplication in vectors and its proximity to human habitation would seem to be essential. In our experiments, we have shown that the CHPV can multiply in *P. argentipes* and that this species can transmit CHPV by bite. Distribution data for sand flies showed that *P. argentipes* is one of the predominant species found in several parts of India endemic for CHPV.[8] It has also been reported that this species rests in cattle sheds found near areas of human habitation and fed mostly on bovines.[8] There were also reports of *Phlebotomine* sand flies re-feeding and some can re-feed as often as every two days.[15]

Our inability to demonstrate CHPV transmission by the orally infected *P. argentipes* probably resulted from the inability of *P. argentipes* to re-feed. However, the ability of *P. argentipes* to acquire CHPV from a viremic host and transmit it has immense epidemiologic importance.

TABLE 1

Experimental infection rate and transmission of Chandipura virus (CHPV) by *Phlebotomus argentipes* sand flies*

Method of infection	Total no. of flies feeding/total no. of flies tested	Viral dissemination rate	Minimum transmission rate†
Orally exposed sand flies	17/30	65%	Not determined
Inoculated sand flies	19/30	93%	32%

† Total number of CHPV-positive mice/total number of inoculated flies that engorged.

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