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Experimental Study of the Mechanical Transmission of Rabbit Hemorrhagic Disease Virus (RHDV2/b) by *Aedes albopictus* (Diptera: Culicidae) and *Phlebotomus papatasi* (Diptera: Psychodidae)

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Abstract

Rabbit hemorrhagic disease (RHD) is caused by a lagovirus mainly affecting European rabbits (*Oryctolagus cuniculus*), although other European and North American lagomorph species are also susceptible to fatal infection by the new viral variant RHDV2/b. In the present work, direct mechanical transmission of the rabbit hemorrhagic disease virus (RHDV2/b variant) by the hematophagous Diptera *Aedes albopictus* (Skuse) (Diptera: Culicidae) and the sand fly *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae) was tested. For each species, six and three laboratory rabbits were exposed to bites of dipterous females partially fed on RHDV2/b viral suspension 2 h and 24 h prior to exposure, respectively. The rabbits were then monitored for clinical changes and mortality for 35 d, and seroconversion was assessed by indirect ELISA. No rabbit died or showed clinical signs of disease, and seroconversion was recorded in two rabbits challenged with *P. papatasi* females fed the viral suspension 2 h prior to exposure. The number of RHDV2/b RNA copies/female was higher in *Ae. albopictus* than in *P. papatasi* but the decrease over time of RNA load in *Ae. albopictus* was greater than that in *P. papatasi*. The results of this study suggest the inability of *Ae. albopictus* to serve as a direct mechanical vector of RHDV2/b, but sand flies could play a role in the local transmission of RHD.

Key words: Diptera, Lagovirus, Rabbit hemorrhagic disease, viral transmission

Rabbit hemorrhagic disease (RHD) is caused by a lagovirus (Rabbit hemorrhagic disease virus, RHDV) belonging to the *Caliciviridae* family. The disease is a constant threat to domestic and wild European rabbits (*Oryctolagus cuniculus*) and is characterized by acute necrotizing hepatitis, which causes high mortality rates (Abrantes et al. 2012). In 2010, a new RHDV variant (hereafter referred to as RHDV2/b) was identified in France (Le Gall-Reculé et al. 2011), and this variant quickly replaced the previous RHDV circulating in most countries (Dalton et al. 2014, Lopes et al. 2014, Ramsey et al. 2020). This variant, now spread worldwide, is able to infect several species of hares in Europe. Recently it has been reported that North American *Sylvilagus* and *Lepus* species appear to be susceptible to fatal RHDV2/b infection as well, demonstrating a wider spectrum of hosts than previous RHD viruses (reviewed in Le Pendu et al. 2017, Lankton et al. 2021).

Transmission of RHDV can take place by both direct and indirect mechanical transmission by several fly species belonging to the Calliphoridae and Sarcophagidae families (Gehrmann and Kretzschma 1991, Asgari et al. 1998, Barrat et al. 1998, McColl et al. 2002). No RHDV has been detected in adult flies that developed from maggots on RHDV-infected rabbit carcasses (Asgari et al. 1998) indicating that transstadial transmission does not occur. Although there is only one study on biological transmission of RHDV by insects (Asgari et al 1998), it is generally assumed that RHDV does not replicate in insects and therefore the transmission to a vertebrate host is mechanical.

In addition to fly-borne mechanical transmission, the role of other hematophagous Diptera species as potential mechanical vectors in the epidemiology of RHD has also been described, although it has rarely been tested. RHDV has been detected in the mosquitoes *Ochlerotatus (Aedes) postspiraculosus* (Dobrotworsky) (Diptera: Culicidae) and *Aedes notoscriptus* (Skuse) (Diptera: Culicidae) collected in Australia (Cooke 2001, McColl et al. 2002). The technical report of Lenghaus et al. (1994) is the only study reporting the ability of a mosquito, *Culex annulirostris* (Skuse) (Diptera: Culicidae), to infect susceptible rabbits under laboratory conditions.

The present study aimed to evaluate the potential direct mechanical transmission of the RHDV2/b variant by the mosquito *Aedes albopictus* (Skuse) (Diptera: Culicidae) and the sand fly *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae), which use capillary feeding (solenophagy) and pool feeding (telmophagy) blood-sucking methods, respectively. In addition to their availability in laboratory colonies, these species of dipterans were selected because they usually feed on rabbits and because of their propensity to take several meals from different hosts over a short period of time that can increase their efficiency as mechanical vectors of viruses (Garret-Jones and Grab 1964, Niebylski et al. 1994, Carn 1996, Chelbi et al. 2008, Pereira-dos-Santos et al. 2020).

Materials and Methods

Virus

The RHDV2/b isolate used in this study (GenBank accession number MG022138 for the *VP60* capsid protein) was already characterized by Calvete et al. (2018), and was shown to cause an average mortality of 43% in laboratory rabbits. A feeding media of 20% liver homogenate in PBS was prepared from the liver of a laboratory rabbit that died from acute RHD caused by this virus isolate. The tissue suspension was centrifuged, and the supernatant (0.22 μm pore size filtered) was diluted 100-fold in PBS, yielding a viral suspension that was aliquoted and frozen until use.

Mechanical Transmission Test Procedures

Females of *Ae. albopictus* (5-7 d old) and *P. papatasi* (3-5 d old) were obtained from long-established laboratory colonies reared in the Agrifood Institute of Aragon (IA2). Approximately 400 females of *Ae. albopictus* were distributed in four holding 30 × 30 × 30 cm Bugdorm cages (BioQuip) (100 females per cage). The colony was supplied with cotton soaked in a 10% common white sugar (sucrose) solution ad libitum; however, to stimulate the blood-sucking behavior of the selected females, they were starved for 18 h before experimental infection by depriving them of the sucrose solution.

As shown in the workflow diagram (Fig.1), the mechanical transmission test was started by allowing the females to feed on the RHDV2/b viral suspension through a chick skin membrane provided by an artificial feeder device set at 37. 5 ± 0. 5°C (Hemotek Inc., Blackburn, UK) placed on the top of the cages. Individual females were allowed to feed for a short time (15–30 sec), and as they became partially engorged, they were transferred to separate cages and distributed in 15 batches, with 15 females per batch. Two hours after the start of feeding and once the batches were established, females from three batches (positive controls at 2 h) were killed (by freezing), immersed in RNAlater, stored at 4°C for two days, and subsequently maintained at –30°C until analysis, whereas six other batches were transferred to a climate chamber at 26°C and a relative humidity of 70% for 24 h. The remaining six batches were transferred to a BSL2 experimental facility in which the rabbit challenge was performed under negative pressure and filtered air. The challenge was performed on six 4-mo-old, non-RHD-vaccinated, New Zealand laboratory rabbits reared in individual cages 2 m apart. The rabbits were anesthetized with ketamine/xylazine (35/5 mg/kg given intramuscularly), and one ear
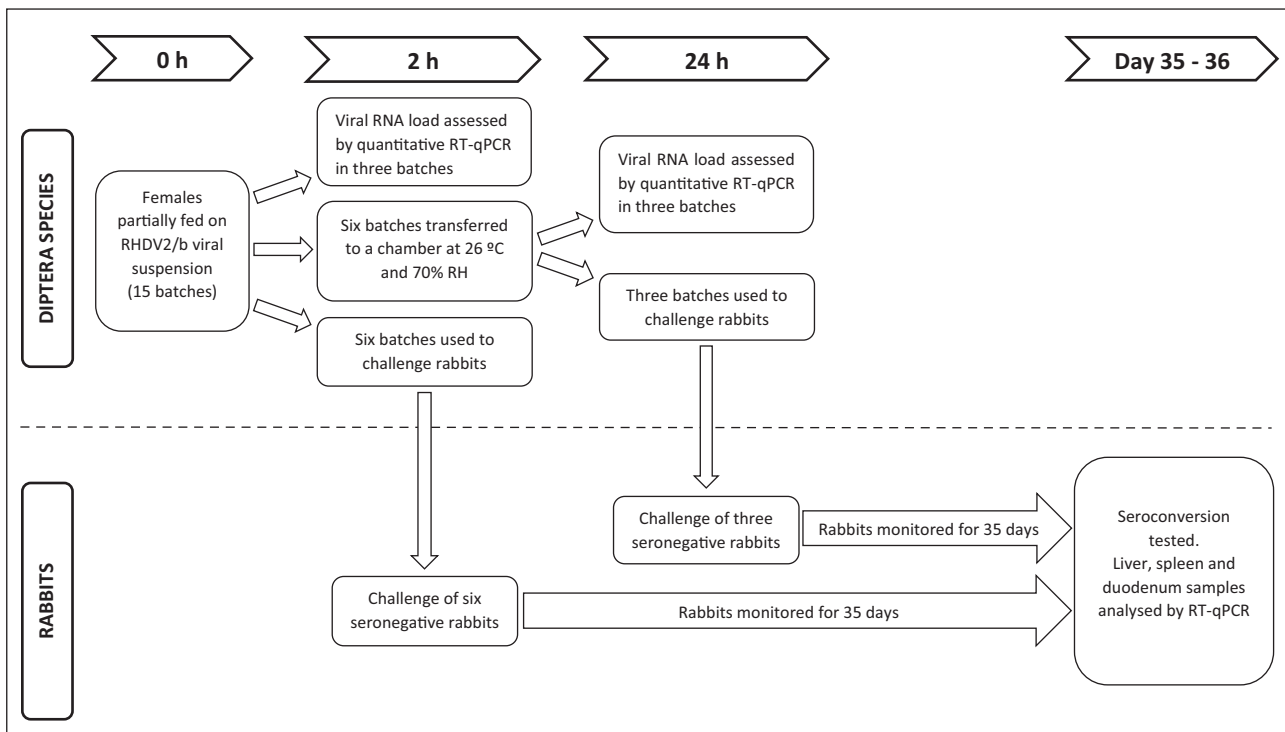

Fig. 1. Workflow diagram depicting the mechanical transmission test procedures used in this study

of each rabbit was introduced in the holding cage of each batch through the sleeve for 45 min, allowing females to feed until they were engorged.

The same procedure was followed for the six batches maintained in the climate chamber 24 h later. Females from three batches were killed and conserved in the same way (positive control at 24 h), whereas females from the remaining three batches were used to test mechanical transmission to three other rabbits located in other pressurized rooms, following the same procedure as before. These short time frames (2–24 h) were chosen because some viruses are inactivated rapidly on mouthparts of bloodsucking insects (Carn 1996). Given the lack of information on RHDV viability on insect mouthparts, these periods aimed to evaluate mechanical transmission in the short time, before RHDV presumably lost all viability.

Before the challenge (day −1), a blood sample was taken from the marginal ear vein of each rabbit to confirm that it was seronegative against RHD with a commercial indirect ELISA kit (Ingezim Rabbit. INGENASA Lab., Madrid, Spain). After the challenge, the rabbits were monitored for clinical changes and mortality at least 4 times daily for 35 d. On day 35, a new blood serum sample was taken from each rabbit to monitor seroconversion using the same commercial indirect ELISA kit. The surviving rabbits were then humanely euthanized, necropsied, and examined for macroscopic lesions. Samples of the liver, spleen, and duodenum were collected and analyzed to detect viral RHDV2/b RNA by quantitative reverse-transcription PCR (RT-qPCR).

A mechanical transmission test of *P. papatasi* was performed following the experimental design described above. However, due to the smaller size of sand flies and the difficulty in handling individual specimens, the assay started with a higher number of females (~ 1000), and the number of partially engorged females per batch ranged from 30 to 70. To facilitate their handling, partially engorged females were transferred to cylindrical 200 ml polymethylpentene jars. The base of these jars was cut away, and both the base and the original lid were replaced with a cotton mesh cloth on both ends of the jar. In this way, the rabbit ear could be easily introduced to the jar through one end to allow *P. papatasi* females to feed.

All animal experiments were performed in compliance with the provisions of Spanish national and European laws (Law 32/2007, modified 6/2013, and RD 53/2013) and were approved by the CITA Ethical Committee for Animal Experimentation (protocol 2017-5).

Determination of the RHDV2/b RNA Load

The RHDV2/b RNA load was assessed by quantitative reverse-transcription PCR (RT-qPCR) analysis of positive control batches at 2 and 24 h to compare viral RNA clearance over time in both species. Females were dissected by removing the abdomen using flame-disinfected entomological needles. The rest of the body of each insect (head and thorax) was washed in clean RNAlater, and the RNA load was determined for each batch from heads and thoraxes from all individuals pooled together. As the number of females varied among batches for *P. papatasi*, the RNA load was divided by the number of females in each batch, obtaining an estimate of the number of RNA copies (in the head and thorax) per female and batch for both species. Moreover, the RHDV2/b RNA load (number of copies/μl) was also determined in three 500 μl aliquots of an immediately-thawed viral suspension (viral suspension control at 2 h) and in three other aliquots maintained for 24 h (viral suspension control at 24 h) in the same climate chamber as Diptera females to control for RNA viral degradation under those conditions. Additionally, three unfed female batches of each species taken directly from colonies were analyzed

as negative controls, confirming the absence of positive RT-qPCR results in unfed individuals.

RT-qPCR

A specific RT-qPCR was implemented. Total RNA was extracted from 25 mg of each rabbit tissue and pooled females using TRIzol Reagent (Sigma Life Science, Madrid, Spain) according to the manufacturer's instructions. RT-qPCR was performed using the ABI Prism 7500 platform (Applied Biosystem, Madrid, Spain). Amplification was carried out in a 25 μl reaction volume using the verso OneStep RT-qPCR Kit (Fisher Scientific, Madrid, Spain) according to the manufacturer's recommendations. The primers and probes for the RHDV2/b variant were the same as those described in Calvete et al. (2018) for duplex qPCR, with optimal concentrations of 1 mM and 0.2 mM for each primer and probe, respectively. The thermal cycling conditions included one cycle at 50°C for 15 min for reverse transcription, one cycle at 95°C for 15 min for *Taq* polymerase activation, and 50 cycles of cDNA amplification (95°C for 15 s and 60°C for 1 min). All samples were amplified in duplicate in the same run. Fluorescence was measured during each extension step. The negative controls contained RNA from liver tissue of healthy domestic rabbits ($n = 25$) or RHDV-infected rabbits ($n = 5$). The specificity of the OneStep RT-qPCR assay for the detection of RHDV2/b was evaluated using RNA extracted from previously infected rabbits that tested positive via duplex qPCR ($n = 50$) (Calvete et al. 2018).

To estimate the absolute quantification of the viral load by a standard curve, an 840-bp fragment (amplified with the primers 5'-TCCAGCAAGACCGTTGACTCG-3' and 5'-AGGCATAAGTGCCGATGAGT-3'), which included the 200-bp fragment amplified by the OneStep RT-qPCR Kit, from an RHDV2/b variant field strain (GenBank accession number MT9403487) was amplified and cloned into the pCR2.1 TA vector using One Shot TOP10, which was then transfected into chemically competent *Escherichia coli* (Invitrogen, Fisher Scientific, Madrid, Spain). Colonies were selected by blue/white screening, expanded in 50 ml Luria broth supplemented with kanamycin, and harvested using a GeneElute Plasmid Midiprep kit (Sigma, Madrid, Spain). To confirm the presence of inserts, the plasmid constructs were Sanger sequenced using universal M13 primers by STAB VIDA (STAB VIDA, Caparica, Portugal). Linearization of the plasmid construct with HindIII (Fisher Scientific, Madrid, Spain) was confirmed by agarose gel electrophoresis. The linearized plasmid was excised, purified with a QIAquick Kit (Qiagen, Werfen, Spain), and quantified with a Qubit 2.0 Fluorometer (Fisher Scientific, Madrid, Spain). In vitro transcription was performed using a MAXIscript T7 Kit (Invitrogen, Fisher Scientific, Madrid, Spain) according to the manufacturer's instructions. In vitro-transcribed RNA was treated with RNase-free DNase I (Invitrogen, Fisher Scientific, Madrid, Spain) and purified with an RNeasy Mini Kit. The RNA concentration was determined using a Qubit 2.0 Fluorometer, and in vitro-transcribed RNA was used to generate standard curves for absolute quantification. The number of viral RNA molecules in the standard sample was calculated using the following formula:

RNA copy number in the amplification reaction = amount of RNA (g) in the reaction/[Molecular mass of the transcribed RNA/6.022×10^{23}]. The molecular mass of one transcribed RNA molecule was calculated (970 nucleotides)

The efficiency of PCR amplification was calculated with the equation $E = 10^{-1/slope} - 1$. The mean Cq values, standard deviation (SD), and percent coefficient of variation (%CV) were calculated

independently for each cDNA dilution. The inter-assay variability was evaluated in three independent runs performed on three different days. The mean, standard deviation, and coefficient of variation were calculated from all the Cq values obtained for each dilution in each run. The range (minimum and maximum values) of each parameter was determined.

Statistical Analysis

The variation of the number of copies of RHDV2/b RNA in the viral suspension and dipterous females that occurred over time after the viral suspension was thawed and females were allowed to feed on it was determined by fitting an ANOVA model to the $\log_{10}$-transformed value of the number of copies of viral RNA estimated for each viral suspension aliquot and female batch positive controls. As predictors, the type of control (the viral suspension as the model base level), the time elapsed (2 h as the base level), and their interaction were included in the model.

Results

The robustness of the specifically developed RT-qPCR was confirmed by the consistency of the data from three independent regression analysis experiments. The efficiencies ranged from 1.94 to 1.96, and the correlation of 0.99 reflected the good linearity of the standard curve from dilutions of 10^{-1} (5.84×10^9 copies) to 10^{-9} (58.4 copies). The lowest limit of detection of serially diluted RNA was 10^{-9}. Mean Cq values from 16.52 to 16.64 (%CV ranging from 0.15 to 0.52) for the 10^{-1} RNA dilution to 43.8–44.19 (%CV ranging from 0.63 to 1.21) for the 10^{-9} dilution were obtained. The percent coefficients of variation did not exceed 1.21% for any of the three independent experiments or dilutions.

The ANOVA model fit to the RHDV2/b RNA load ($R^2 = 99.06\%$; $n = 18$; $F = 253.89$; $P < 0.001$) showed that the mean number of viral RNA copies/female was higher in *Ae. albopictus* than in *P. papatasi* (parameter ± SE: 1.93 ± 0.11; $t = 17.20$; $P < 0.001$) and that the number of RNA copies decreased over time (Table 1) in both the viral suspension and insects (parameter ± SE: -0.43 ± 0.08; $t = -5.48$; $P < 0.001$), but at different speed. The decrease over time in *P. papatasi* females (0.72 log-units) was not significantly different than the decrease in the viral suspension (0.23 log-units) (parameter ± SE: 0.08 ± 0.11; $t = 0.68$; $P = 0.509$); however the decrease over time in *Ae. albopictus* (1.67 log-units) was greater than that in *P. papatasi* (parameter ± SE: -0.40 ± 0.11; $t = -3.56$; $P = 0.004$) (Table 1).

No rabbit died or showed clinical signs of disease until day 35 after challenge. Serological analyses confirmed the seronegativity of all rabbits on days 0 and 35, with absorbance (OD) values ranging

Table 1. Mean (± SD) of the $\log_{10}$ RHDV2/b RNA copies determined in the viral suspension and in females of *Aedes albopictus* and *Phlebotomus papatasi* at 2 h and 24 h after the viral suspension was thawed and females were allowed to feed on it

	Time elapsed	
	2 h	24 h
Viral suspension	9.04 ± 0.01	8.81 ± 0.07
Ae. albopictus	3.84 ± 0.26	2.17 ± 0.52
P. papatasi	3.43 ± 0.17	2.71 ± 0.55

RNA copies were calculated per µl of the viral suspension and per Diptera female (from the pooled head and thorax).

from 0.06 to 0.09, except for two rabbits that showed clear seroconversion on day 35, with OD values of 0.96 and 1.52, which are typically reached by infected rabbits that have developed active immunity after RHDV2/b replication (Calvete et al. 2018). The two rabbits were challenged with batches comprising 31 and 59 females of *P. papatasi* that were partially fed on the viral suspension 2 h prior to exposure.

At necropsy, no macroscopic lesions were observed in rabbits, and no RHDV2/b RNA was detected in any sample, except in the duodenum of one of the rabbits that showed clear seroconversion, in which viral RNA was detected at a high quantification cycle number (44.7 Cq) that was out of the calibration range of RT-qPCR.

Discussion

The estimated mean concentration of RHDV2/b RNA copies in the viral suspension at the moment females were allowed to feed on it were 1.09×10^9 and 6.52×10^8 copies/µl at defrosting and 24 h after defrosting, respectively. These values were in agreement with the range of the viral RNA levels estimated from the blood of RHDV2/b-infected rabbits (Dalton et al. 2018), confirming that the viral suspension was adequate to simulate virus availability from the blood of an infected rabbit.

Gehrmann and Kretzschmar (1991) found that 10–100 virus particles was the minimum dose required to induce RHD in rabbits. Taking into account the RNA virus loads estimated in the head and thorax (and presumably in the upper digestive tract) of *P. papatasi* and *Ae. albopictus* females after partial feeding, greater success of mechanical transmission of RHDV2/b would be expected, especially in the case of *Ae. albopictus*, which uses the capillary feeding (solenophagy) blood-sucking method and since *Aedes* spp. can mechanically transmit other viruses, such as myxoma virus, Shope fibroma virus, and rabbit papilloma viruses (Fenner and Ratcliffe 1965, Carn 1996). Nevertheless, in agreement with the results of the present study, Fortuna et al. (2021) demonstrated that *Ae. albopictus* was unable to mechanically transmit SARS-CoV-2 virus, suggesting that mosquitoes that are partially engorged are unable to mechanically release the virus immediately after biting an uninfected host. Additionally, the faster estimated clearance of RHDV2/b RNA in *Ae. albopictus* suggests a higher sensitivity of the virus to the biochemical environment present in the mouthparts of this species, favoring its rapid inactivation and degradation (Carn 1996).

In the case of *P. papatasi*, the serological results confirmed the effective infection of two out of the six rabbits challenged 2 h after sand fly females partially fed on the viral suspension. The absence of mortality was not surprising given the relatively low mean mortality rate estimated for the strain used (Calvete et al. 2018). This result demonstrates the capability of this species to mechanically transmit the RHDV2/b virus, at least in the short term, since none of the three rabbits challenged 24 h after sand fly females partially fed on the viral suspension became infected. Nevertheless, given the reduced and different sample sizes, the probabilities of becoming infected at 2 and 24 h were hardly comparable. Similarly, the probability of becoming infected may be directly related to the number of sand flies feeding on a rabbit. However, this finding was not observed in the present study since seroconversion was detected in two rabbits challenged with high and low numbers of sand flies. Likely, the low sample size also precluded the visualization of this relationship.

Sand flies are pool feeders, which involves the excavation of a pit with the mouthparts to imbibe the blood that accumulates at the site of feeding. This requires a large amount of saliva to

maintain the blood flow, which enables the release of viral particles into the bloodstream and tissues of the host. Nevertheless, Asgari et al. (1998) demonstrated that RHDV lasted for up to 7 h on the legs of flies (Diptera: Calliphoridae) after they fed on infected liver. Therefore, although sand flies were fed through a membrane, contamination of their bodies (and legs) with the viral suspension flowing through the membrane cannot be dismissed, and consequently, it cannot be ruled out that mechanical transmission may have taken place in this way.

In conclusion, the results of this study suggest the inability of the mosquito *Ae. albopictus* to serve as a direct mechanical vector of RHDV2/b under laboratory conditions. However, the results of this study show that *P. papatasi* sand flies have a limited capacity to mechanically transmit the virus directly from an infected rabbit to a susceptible rabbit. This capacity indicates that RHDV2/b transmission by these insects might be merely incidental in the wild and of negligible epidemiological importance. However, given that the feeding preferences of both *P. papatasi* and other *Phlebotomus* spp. species include rabbits, and use rabbit burrows as breeding and daytime resting places (Chelbi et al. 2008, Lucientes et al. 2016, González et al. 2017), the results suggest that sand flies could play some role in the local transmission of RHD.

These conclusions, however, should be treated with caution because of the limited design of the present study. Therefore, it is desirable that further studies using higher number of animals, different timing conditions, and especially, different RHDV2/b strains, will be performed in order to evaluate more precisely the potential direct mechanical transmission of the RHDV2/b by these species.

Acknowledgments

Data Availability

All data relevant to the study are included in the article. Complementary data are available on reasonable request.

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