Research paper

Experimental transmission of rabbit haemorrhagic disease virus (RHDV) from rabbit to wild mice (Mus spretus and Apodemus sylvaticus) under laboratory conditions

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1. Introduction

Rabbit haemorrhagic disease (RHD) is a highly lethal and contagious viral disease that produces haemorrhagic lesions in liver and lungs of domestic and wild rabbits (Oryctolagus cuniculus). This study investigates the transmission of RHDV from infected rabbits to mice, based on the detection of viral RNA. Sixteen wild mice (Mus spretus, n = 12 and Apodemus sylvaticus, n = 4) were put in contact with nine rabbits inoculated with RHDV. No mice died following exposure to RHDV-infected rabbits or developed macroscopic haemorrhagic lesions.

This situation worsened in 2010 following the emergence of a new variant of RHDV (RHDVb or RHDV2) in France (Le Gall-Reculé et al., 2011), which spread quickly throughout Europe causing high mortality rates in Spain (Delibes-Mateos et al., 2014). This variant differs from the classical RHDV strains because it affects young rabbits less than 30 days old (Dalton et al., 2012).

The exact origin and evolution of the RHDV remains unknown. Although the first record comes from China, several studies have suggested that RHDV originated in Europe, where the virus had been circulating long before the first epidemic was reported in 1984 (Kerr et al., 2009; Alda et al., 2010; Kinnear and Linde, 2010). More recent analyses suggest the date of origin the period between 1970 and 1981, shortly before the first epidemic that took place in 1984 (Eden et al., 2015).

Two major hypotheses are currently debated to explain the origin of RHDV pathogenicity. The first one proposes that pathogenic strains have evolved from non-pathogenic viruses circulating in European leporids, while the second one suggests that pathogenic RHDV might...
have an origin in species sympatric with the European rabbit, and have been able to jump between species (reviewed in Esteves et al., 2015). Similarly, little is known about the key elements of RHDV epidemiology, such as aspects associated with its propagation and sources of infection. Potential factors influencing the spread of the disease includes the activity of arthropods (Gould et al., 1997) and predatory mammals and birds (Mitro and Krauss, 1993), and the role of warrens in harbouring the virus after epizootic outbreaks (Cooke, 1996; Cooke et al., 2000; Calvete et al., 2002). The main sources of infection are believed to be direct transmission from infected rabbits (Xu and Chen, 1989), young rabbits less than 20 days old that manage to survive the disease (Rosell et al., 1989), rabbits with persistent infection (Lölliger and Eskens, 1991), and flies (Asgari et al., 1998). Additionally, other variables that affect natural transmission mechanisms include environmental conditions (Cooke, 2002), the presence of carcasses of animals that have died from infection (McColl et al., 2002), and interaction with outbreaks of myxoma virus (Mutze et al., 2002).

So far there have been no reports of RHDV affecting wild or domestic species other than lagomorphs (Nowotny et al., 1997), although a long list of vertebrate species develops an antibody response after inoculation (Buddle et al., 1997), and oral challenge with RHDV (Leighton et al., 1990), although no experimental studies have been conducted recognizing strain RHD-4764-183-ET (23/09/97) (G1) supplied by Laboratorios Hipra S.A. An infective dose of 1000 DLC/50 in 0.6 ml was administered to each rabbit. The nine rabbits and 16 mice (four A. sylvaticus and 12 M. spretus) were immediately placed together in the experimentation room (contact room). At the same time, two positive control mice were inoculated subcutaneously with 0.1 ml of the same virus suspension and placed in the positive control room; two untreated negative control mice were placed in the negative control room.

During the contact phase, the experimentation room was kept closed except during the removal of rabbit carcasses. The dead rabbits were necropsied and examined for the presence of macroscopic lesions consistent with RHD. Rabbit carcasses were collected and placed in individual, labeled sterile containers. Twice a day, 30-minutes of direct observation was carried out to check for clinical signs and monitoring progress of the experiment. After the four-day contact period, mice were placed in separate sterile containers properly labeled and transferred to the observation room. Four mice (M1 to M4) were euthanized by cervical dislocation. The negative and positive control group mice were euthanized seven days after the start of the experiment. To detect the presence of viral RNA, liver samples and faecal pellets in the intestinal tract were collected from all rodents and rabbits. Liver and faecal pellets were chosen because the first site of RHDV replication is in the crypts of Lieberkühn, within the intestine (Gregg et al., 1991), and most of the subsequent viral replication occurs in the liver (Marcato et al., 1991). Sterile laboratory materials and sterile techniques were used to avoid cross-contamination of samples (Merchán et al., 2011). Pellets from the gut of animals were carefully collected to avoid contamination with blood, gut tissue and gut contents. All samples were stored at −20 °C until analysis (Wang et al., 2008).

During the second phase (observation phase), the remaining mice (M5 to M16) were maintained in the observation room for several days: Mice M5 to M8 were euthanized three days after the contact period (7 days after the start of the experiment), Mice M9 to M12 were euthanized after ten days post-contact (14 days after the start of the experiment), and M13 to M16 mice were killed 64 days post-contact.
(68 days after the start of the experiment). Liver and faecal samples were collected from all specimens.

2.4. Molecular laboratory methods

Viral RNA was extracted from all the liver and faecal samples. Approximately 125 mg of tissue were homogenized in 1.25 ml sterile PBS buffer and centrifuged at 2500 × g for 15 min. One hundred microliters of the 10% (w/v) homogenate was extracted using the TriPure reagent (Roche), following the manufacturer's instructions. A fragment of the capsid protein encoding gene VP60 was amplified in a nested PCR using primers RHDV1, RHDV2, RHDV3 and RHDV4 (Moss et al., 2002), following the same conditions described by Alda et al. (2010).

RNA was extracted simultaneously from all samples collected in each phase of the experiment and amplified at the same time including negative controls to check for contamination. RHDV reference strain RHD-4764-183-ET was amplified in a nested PCR using primers RHDV1, RHDV2, RHDV3 and RHDV4 (Moss et al., 2002), following the same conditions described by Alda et al. (2010).

In all cases, we obtained 543 bp-long sequences that span positions 831–1373 from the complete coding region of the VP60 protein gene. The reference sequence obtained from the inoculum was deposited in GenBank under accession number KJ841711.

3. Results

3.1. Contact phase

The first clinical signs associated with RHD appeared in a lethargic rabbit 48 h post-inoculation. This rabbit and four others were found dead within 72 h. The remaining four rabbits died within 96 h. Thus 100% of the rabbits had died by the end of the first phase of the experiment, and all presented with lesions consistent with RHD. All samples of liver and faeces from rabbits were positive by RT-PCR, and all the sequences were identical to the strain inoculated.

None of the rodents, including the control groups, showed either behavioral changes or clinical signs of disease during the first contact phase of the experiment. Although no lesions or macroscopic alterations were observed in the samples extracted after the contact phase, viral RNA was detected in faeces of three of the four mice euthanized, and in two livers (Table 1). Whenever viral RNA was detected in the liver, they were also detected in the faeces. In one individual, viral RNA was detected only in faecal samples, and in one mouse viral RNA was not detected in either faeces or liver. In the positive control mice, viral RNA was detected only in faecal samples, and in one mouse viral RNA was not detected in either faeces or liver. In the positive control mice, viral RNA was detected in liver and faeces, whereas negative control mice did not show evidence of RHDV-RNA in any sample (Table 1). All the virus sequences obtained from mouse livers and faeces (n = 16, including positive controls) were identical to the virus strain inoculated into the rabbits, which belongs to the GI.1d genogroup, and differs only in one base pair from the Triptis strain, from Germany (Forrester et al., 2008).

3.2. Observation phase

Fig. 1. Schematic representation of the experimental design and timeline.
was also identical to the sequence of the virus strain inoculated into the rabbits.

4. Discussion

This study shows, for the first time, experimental evidence of RHDV transmission following direct contact between infected rabbits and two wild mouse species, *A. sylvaticus* and *M. spretus*. Although much has been documented about the main transmission mechanisms of the disease among rabbits and the importance of rabbit carcasses as a source of infection in natural conditions (Ohlinger et al., 1993; McColl et al., 2002), few studies have demonstrated cross-species transmission of rabbit lagovirus (Lopes et al., 2014). Experimental inoculation of RHDV in 31 domestic, feral (including *Mus musculus*), and Australian native mammal and bird species failed to show any evidence of RHDV infection, or presence of its genome (Lenghaus et al., 1994; Collins et al., 1995; Gould et al., 1997).

Although RHDV is considered highly species-specific (Lenghaus et al., 2000; Cooke and Fenner, 2002), this study shows that when infected rabbits are brought into contact with wild mice under controlled laboratory conditions, virus transmission can occur from rabbits to rodents and persist in the latter. The presence of RHDV in mouse liver could indicate active viral replication, however there was no evidence of lesions consistent with RHD, as observed in rabbits, and no evidence of viability of the detected or excreted viral RNA. The virus genome was detected in the faeces of three of the four rodents tested immediately post-contact with the infection source, in all of those tested ten days post-contact and in one of four mice tested 64 days post-contact. This may indicate that *M. spretus* (and possibly *A. sylvaticus*) is capable of hosting viral RNA for at least 64 days.

Although based on our results it seems that RHDV is capable of remaining in mice hosts for a long time with no clinical symptoms, little is known about many aspects of the effect the virus has on these rodents, such as the type of immune response (cellular or humoral), the existence of immunopathology or immune adherence phenomena, and the existence of specific cell receptors or other components required for replication of this virus, which are necessary conditions for infection (Pastoret et al., 1990).

The more frequent presence of RHDV RNA in mice faeces than in livers could suggest that the intestine is the organ where the virus is located after entering through the oral route, as in the case of RCV (Marchandeau et al., 2005), or that virus genetic material in rabbit faeces, urine or fomites has been consumed by mice, and is transiting through their gastrointestinal tracts. These results open up new lines of study to shed light on RHDV pathogenesis in wild mice and its possible cross-species origin (Fenner and Fantini, 1999). New experiments with qPCR will be necessary to see if there is substantial virus titre in the mice, and testing for mouse-rabbit transmission. In fact, some researchers have alluded to the potential for mutation of RHDV, which could enable it to cross species barriers and cause disease (Smith, 1999), and to how colonization of new but related host species may represent the principle mode of macro-evolution in RNA viruses (Kitchen et al., 2011).

The oral route is considered a major way for RHDV transmission in the field (Parkes et al., 2001), so it is likely that in this experiment, where an environment with a high viral load contaminates water and food, the route of entry in the rodents might be the same. Furthermore, considering that virus particles in rabbit faeces can remain infectious for 1–2 weeks (Henning et al., 2005), the ingestion of contaminated rabbit faeces (Ohlinger et al., 1993) may greatly enhance transmission in the wild. In consequence, rodent consumption of rabbit faeces when sharing the same habitat (Valverde, 1967), combined with the ubiquitous nature of mice, which commonly use rabbit warrens for shelter (Delibes-Mateos et al., 2008), could support their role as sources of infection and aid in RHDV transmission.

Based on our new evidence, the possibility that rodents play a role in RHDV epidemiology cannot be ruled out. Therefore, this experiment represents a forward step towards providing a convincing argument for role. Currently, persistence of RHDV is mostly attributed to rabbit kits carrying the virus, to the persistence of virus inside of warrens (Cooke et al., 2000; Cooke, 2002), to circulation among rabbit subpopulations (Schwensow et al., 2014), or to insect vectors (Asgari et al., 1998). If rodents are acting as RHDV hosts, then this could influence the density-dependent dynamics of the virus itself (Fa et al., 2001; Calvete, 2006), and its epidemiological impact. The population dynamics of these non-specific hosts may therefore determine the introduction, survival or disappearance of the virus at a given site, as suggested by Mills et al. (1992) and Porcasi et al. (2005). However, more in-depth studies are needed to determine if the virus is replicating in mice, and if viral RNA shed by mice is infectious, which could confirm the potential of rodents to spread the virus to rabbits. Coexistence of rodents and rabbits may encourage cross-species transmission of the virus, with epidemiological consequences that have yet to be determined, both on the impact of RHD and on rabbit survival.

**Ethical standards**

The practices applied in this study comply with the laws of Spain.

**Conflict of interest**

We have no conflicts of interest.

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