Journal of Virological Methods 188 (2013) 148–152

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

In situ hybridisation assay for localisation of rabbit calicivirus Australia-1 (RCV-A1) in European rabbit (*Oryctolagus cuniculus*) tissues

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Article history:
Received 4 May 2012
Received in revised form
26 September 2012
Accepted 26 November 2012
Available online 20 December 2012

Keywords:
Calicivirus
Rabbit
RCV-A1
RHDV
In situ hybridisation
Pathogenesis

A B S T R A C T

Recently, a new lagovirus enzootic in Australian wild rabbits was identified and described as rabbit calicivirus Australia-1 (RCV-A1). Unlike the closely related Rabbit Haemorrhagic Disease Virus (RHDV), which causes fulminant hepatitis and rabbit death, RCV-A1 does not appear to induce any clinical disease. RCV-A1 has been postulated to act as an imperfect natural vaccine to RHDV thus reducing RHDV-induced rabbit mortality, which is detrimental for bio-control of rabbits in Australia. This study was carried out to determine in which cells RCV-A1 replication occurs. An in situ hybridisation (ISH) protocol was developed using a RCV-A1 specific probe to localise the virus in rabbit tissues. The results were compared to those obtained with a quantitative RT-PCR assay that had previously been developed to measure RCV-A1 RNA in rabbit tissues. The histology of the tissues was also examined. ISH showed that virus replication, inferred by the presence of detectable RNA, was limited to a small number of epithelial cells towards the tip of the villi in the duodenum. Quantitative RT-PCR detected RCV-A1 RNA in jejunum, ileum and lymphoid tissue at day 3, 4 and 7 post-infection, but no hybridisation was detected in these tissues.

1. Introduction

Recently, a new lagovirus was identified and described as rabbit calicivirus Australia-1 (RCV-A1) (Strive et al., 2009). It is endemic in Australian wild rabbit populations and evolutionary analysis suggests that it was introduced to Australia with the first wild rabbits approximately 150 years ago (Jahnke et al., 2010). RCV-A1 is antigenically and genetically closely related to Rabbit Haemorrhagic Disease Virus (RHDV). Both RHDV and RCV-A1 belong to the genus *Lagovirus* within the virus family *Caliciviridae* (Green et al., 2000). However, unlike RHDV, which causes fulminant hepatitis and usually kills over 90% of infected rabbits within 72 h (Abrantes et al., 2012) RCV-A1 does not appear to induce any clinical disease. Viral RNA is found in the small intestine, lymphoid tissues and bile, and infected rabbits develop a robust antibody response to RCV-A1 which also cross-reacts in RHDV-specific serological tests (Liu et al., 2012; Strive et al., 2010).

The lagovirus genome is a positive-sense single-stranded, polyadenylated RNA molecule that encodes a poly-protein of approximately 257 kDa, which is cleaved into non-structural proteins and the capsid protein VP60 (Boniotti et al., 1994; Meyers et al., 2000). The genomes of RCV-A1 and RHDV are approximately 7.4 kbp in length and share approximately 85% identity at the amino acid level. However, the benign virus differs not only in pathogenicity, but also in tissue tropism being barely detectable in the liver (Strive et al., 2009, 2010). Previous RCV-A1 infection can protect rabbits from lethal RHDV infection, and therefore interfere with bio-control of rabbits in Australia (Cooke et al., 2002; Strive et al., 2010).

Similar non-pathogenic or mildly pathogenic caliciviruses have been demonstrated in rabbits in Europe, North America and New Zealand and inferred by serology in other localities around the world (Bergin et al., 2009; Capucci et al., 1996; Forrester et al., 2007; Le Gall-Recule et al., 2011; Marchandeau et al., 2005). Based on the early studies of Capucci et al. (1996), it is generally thought that these viruses circulate in a faecal-oral cycle with replication in the intestine, but nothing is known of the cells in which the virus replicates and whether any pathology is induced.

Previously, a quantitative reverse transcriptase-PCR (qRT-PCR) assay has been used to measure RCV-A1 in rabbit tissues (Strive et al., 2009, 2010), but this does not identify the cells in which RCV-A1 replicates. Hence, an in situ hybridisation (ISH) protocol was developed using a RCV-A1 specific probe to localise the virus in rabbit tissues that were previously demonstrated to be positive in the qRT-PCR (Strive et al., 2009, 2010). The tissues for histopathological changes due to RCV-A1 infection were also examined. Such studies are important to gain a better understanding of how such viruses can spread successfully through populations in the absence of any clinical signs despite stimulating strong immune responses.

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http://dx.doi.org/10.1016/j.jviromet.2012.11.043

2. Methods

2.1. Experimental design and tissue preparation

An homogenate was prepared from the duodenum of a rabbit that had tested PCR-positive for RCV-A1 (strain BM49, Jahnke et al., 2010) and 1 ml of this homogenate was used to infect a 12 week old rabbit. The duodenum of this animal was harvested five days post infection (d.p.i.) and a 20% homogenate in PBS was prepared. The number of genome equivalents in the homogenate was determined using quantitative RT-PCR and adjusted to 10^8 copies/ml by adding PBS. Five adult New Zealand white rabbits were orally infected with RCV-A1 using 1 ml of this inoculum. One control rabbit received 1 ml PBS via the same route. The inoculated rabbits and the control were kept in single cages with ad libitum food and water. All animals were monitored daily. One rabbit was euthanized at 3 d.p.i., two rabbits at 4 d.p.i. and two rabbits at 7 d.p.i. The control rabbit was euthanized at 4 d.p.i. For euthanasia, animals were first anaesthetised by intramuscular injection of Zoletil (40 mg/kg), followed by intracardiac injection of pentobarbitone sodium solution (162.5 mg/kg). Samples of duodenum, jejunum, ileum, gallbladder, liver, spleen, mesenteric lymph nodes, and Peyer's patches were collected at autopsy. Gut contents were left in situ so that the mucosal layer of the gut tissues remained intact. Bile was collected from the gallbladder using a syringe and needle. Tissues were divided and one piece of tissue was fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4) for 24 h, and transferred into 70% ethanol. After paraffin embedding, sections were cut at 4 μm. The other piece of tissue was stored in RNA Later (Sigma, Sydney, Australia) at −20 °C. RNA was extracted from 50 mg of tissue and extracted using the RNeasy kit (Qiagen, Melbourne, Australia) according to the instructions of the supplier. Tissue homogenisation was carried out in lysis buffer using a Mini-Beadbeater (Daintree Scientific, St Helens, Australia). All procedures involving animals were carried out according to the 'Australian Code of Practise for the Care and Use of Animals for Scientific Purposes' and were approved by the CSIRO Ecosystem Sciences Animal Ethics Committee (CESAEC # 10-13).

2.2. Quantitative reverse transcriptase-PCR

The amount of RCV-A1 in the collected rabbit tissues was determined by qRT-PCR of each sample in duplicate. All RNA quantifications were performed using primers that amplified the capsid gene (RCV-A1 realtime fw and RCV-A1 realtime rev2) (Jahnke et al., 2010), and single stranded RNA standards as described in Strive et al. (2009). The iScript One Step RT-PCR kit and a CFX Real time PCR machine (BioRad, Sydney, Australia) were used for amplification according to the instructions of the manufacturer. Each 10 μl reaction contained a final concentration of 0.25 μM of each primer. Cycle conditions used were 10 min at 50 °C, 5 min at 95 °C followed by 41 repeats of a three step cycle with 10 s at 95 °C, 30 s at 62 °C and signal acquisition for 10 s at 78 °C.

2.3. Preparation of the probes

For the detection of viral mRNA in situ, antisense and sense RNA probes were produced from the full length VP60 gene (1743 nt). The probes were transcribed in vitro from plasmid pcDNA3-RCV-A1 VP60 (Strive et al., 2009) and labelled with digoxigenin-11-UTP using a RNA labelling kit (Roche, Sydney, Australia). Before transcription, the plasmid was linearised with *Hind*III (antisense probe) or *Not*I (sense probe), transcribed from the T7 or Sp6 promotor, respectively, DNase treated, then extracted with phenol/chloroform followed by ethanol precipitation. Approximately 30 μg of each probe was produced, resuspended in 100 μl of DNase free water and stored at −80 °C. Before use, the probes were fragmented using carbonate buffer (60 mM Na_2CO_3, 40 mM $NaHCO_3$, pH 10.2) at 60 °C for 20 min, neutralised with hydrolysis buffer (3 M NaAcetate, 1% acetic acid), precipitated with 100% ethanol and resuspended in 50 μl of RNase free water.

2.4. Preparation of sections

Paraffin-embedded histological sections (4 μm) were de-waxed and rehydrated through graded ethanol (100, 90 and 70%) to PBS. Sections were postfixed with freshly prepared 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 15 min. Sections were then treated with 0.2 N HCl for 10 min to inactivate endogenous intestinal alkaline phosphatase (AP) (Kiyama and Emson, 1991). After acid treatment the sections were acetylated in 0.25% acetic anhydride containing 0.1 M triethanolamine for 5 min and treated with 0.25 mg/ml proteinase K at 37 °C for 15 min. After each step, sections were washed with PBS for 5 min and at the end of this process, dehydrated with an ethanol series (70, 90 and 100%) and air-dried.

2.5. In situ hybridisation

Dehydrated sections were hybridised with a total volume of 100 μl hybridisation buffer per section [2× SSC (0.3 M sodium chloride/0.03 M sodium citrate), 40% deionised formamide, 10% dextran sulphate, 1× Denhardt's solution, 1 mg/ml yeast t-RNA and 10 mM DTT] and 300 ng of RNA probe. Hybridisation was carried out at 60 °C overnight in a humidified chamber. The next day, post-hybridisation washes were performed with 2× SSC for 30 min, 1× SSC for 30 min and 0.1× SSC for 60 min at 37 °C. The detection process was started with a wash in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl) pH 7.5 for 10 min. Then the blocking solution (Roche, Cat # 11096176001) was added for 60 min followed by the AP-conjugated anti-digoxigenin antibody at a dilution of 1:500 in blocking solution (Roche, Cat # 11096176001) for a further 60 min. Sections were washed with maleic acid buffer and detection buffer (0.1 M Tris, 50 mM $MgCl_2$) pH 9.5 for 10 min each followed by incubation with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (NBT/BCIP) (Roche) and 1 mM levamisole in detection buffer for 2–3 h. Once the colour reaction was complete the sections were washed with distilled water, counterstained with FastRed (Sigma), dehydrated, cleared with xylene and mounted.

2.6. Pathology

For histopathology, paraffin embedded sections were cut at 4 μm and stained with haematoxylin and eosin (H&E).

2.7. Serology

Serum samples of the rabbits euthanised 7 d.p.i. were tested for RCV-A1 IgM, IgA and IgG antibodies as previously described (Liu et al., 2012).

3. Results

3.1. Quantitative RT-PCR

The qRT-PCR analyses showed consistently high viral loads in the duodenum: 1.3×10^5 to 9.4×10^6 RCV-A1 copies per mg of tissue, but only 3.2×10^3 to 1.1×10^5 RCV-A1 copies per mg of ileum (Table 1). Viral loads in the jejunum were similar to those in the duodenum at 3 and 4 d.p.i. but much lower at 7 d.p.i. Within the lymphoid tissues examined, the Peyer's patches had the highest number of copies per mg tissue with 5.1×10^5 to 3.3×10^7 whereas levels in the spleen were much lower ($3.1–6.8 \times 10^3$). The lowest