Identification of the viral haemorrhagic disease virus of rabbits as a calicivirus

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Summary: Liver tissue from rabbits which had died of viral haemorrhagic disease (VHD) was used to identify the causative agent of the disease. After extraction of liver homogenates and density gradient ultracentrifugation, viral particles with buoyant densities between 1.32 to 1.38 g/ml and estimated sedimentation coefficients between 100 S and 175 S were obtained. The isolated virions were determined to have a diameter of 40 nm. The particles morphologically resembled those of the Caliciviridae family, had positive reactions both in ELISA and haemagglutination assays, and were infective for rabbits. By immunoblotting, a major structural protein with a molecular weight of 60 kDa was identified. RNA of high purity and of approximately 8 kb was isolated from virions. Labelled cDNA of virion RNA detected two RNAs of 8 kb and 2 kb in Northern blots of liver RNA from animals infected with the VHD virus (VHDV). Finally, isolated virion RNA injected directly into the liver of rabbits produced a disease with clinical symptoms and pathological findings typical of VHD. A calicivirus originally designated "rabbit haemorrhagic disease virus (RHDV)" was thus identified as the causative agent of VHD. The agent causing the European brown hare syndrome (EBHS) is also a calicivirus (EBHSV). VHDV and EBHSV are different members of the Caliciviridae family; their relationship can be clearly demonstrated by using different approaches of investigation.

KEYWORDS: Caliciviruses - European brown hare syndrome - Rabbit haemorrhagic disease - Viral haemorrhagic disease of rabbits.

INTRODUCTION

Viral haemorrhagic disease (VHD) of rabbits (term adopted by the OIE) or rabbit haemorrhagic disease (term used frequently in international journals) was first described in the People's Republic of China in 1984 (20). The disease, also called haemorrhagic septicaemia or infectious necrotic hepatitis, caused severe losses throughout Eastern Europe, many countries of Western Europe and in other parts of the world. The disease is characterised by high morbidity and a mortality rate between 40-90% in adult animals (1, 4, 26), whereas infected rabbits below the age of approximately two months usually survive (45). A parvo-like virus (17, 44), a picornavirus (45) and a calicivirus (29, 30, 39) have been connected with the disease. Identification of the infectious agent was made more difficult by the failure to propagate the virus in any established cell culture system (26, 39). Thus, the causative agent had to be isolated from infected animals. After experimental infection, adult rabbits usually die within 48-72 h, showing characteristic pathological lesions, such

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as a pale, fragile liver, often with accentuation of the lobular markings and sometimes interspersed with haemorrhages, an enlarged congested spleen, reddish speckled kidneys, and lungs with haemorrhagic lesions of varying degrees (2, 28). Specific diagnosis can be obtained, since homogenates from some organs contain high titres of haemagglutinating activity with human erythrocytes (45) and also react in enzyme-linked immunosorbent assay (ELISA) (29, 34) as well as in immunofluorescence (26, 34). VHDV-antigen was consistently found in the liver, spleen, lungs and kidneys of rabbits with peracute and acute VHD. Sometimes VHD virus (VHDV) was detected in the trachea, heart muscle, thymus, brain and blood (26, 28, 38, 46). Liver tissue was used for purification of the virus, as this organ exhibited the highest titres of viral antigens (8, 12, 16, 29).

PHYSICO-CHEMICAL PROPERTIES

Supernatants from clarified homogenised liver tissue were centrifuged through a cushion of sucrose and the resuspended pellet was extracted with Freon-113. Density gradient centrifugation in cesium chloride solution resulted in a single visible band with determined buoyant densities ranging from 1.32 to 1.38 g/ml (12, 16, 29, 39, 45). Sucrose density gradient centrifugation yielded three bands with estimated sedimentation coefficients of 175 S, 136 S and 100 S (29). Similar sedimentation coefficients with values of 150 S to 200 S were reported by others (8, 12, 16). These values correlate well with those described for caliciviruses (36). Heterogeneous densities resulting in distinct bands have also been published for other caliciviruses (27) and may be due to loss of RNA (37), different pH values (35) or storage conditions (21). The presence of VHDV in the bands described was confirmed by haemagglutination (HA) assays, ELISA and animal experiments. VHDV was found to resist 0.4% formaldehyde for 1 h at 37°C or 12 h at 4°C, but was sensitive for 3 h at 37°C or for 3 days at room temperature (39). It proved stable at pH 3 for 60 min, but was sensitive to temperatures above 50°C (45).

MORPHOLOGY

Material obtained after density gradient centrifugation was negatively stained in suspension with 1% uranyl acetate, analysed by electron microscopy (EM) with a Zeiss electron microscope (model 109) and the magnification determined with a cross grating (2,160 lines/mm). Virus particles of uniform size were detected. Preparations of 175 S particles showed the highest degree of homogeneity and purity (Fig. 1). The virions were 40 nm in diameter and displayed a clearly structured surface, consisting of regularly arranged cup-shaped depressions (29). These findings are consistent with other reports, describing icosahedral, nonenveloped particles (8, 39). Whereas all the electron micrographs showed calici-like or caliciviral particles, the size of the virions varied in different reports from 29 to 40 nm in diameter (8, 12, 16, 39, 40, 41, 42). Smid et al. (39) were able to prepare in vitro immunocomplexes with convalescent serum which could be demonstrated by EM. The morphology and size of VHDV agree with data reported for caliciviruses (36).
To detect virus-specific proteins in the livers of VHD-affected animals, highly purified virus preparations were examined by SDS-polyacrylamide gel electrophoresis and Western blotting with anti-VHDV sera. A prominent protein with an apparent molecular weight (MW) of 60 kDa (Fig. 2, lane 1) was seen after Coomassie blue-staining. Western blotting with anti-VHDV serum demonstrated an antigen with apparently identical MW, which was the only protein detected in purified virions (Fig. 2, lanes 3, 4). This 60 kDa band was not observed in control preparations derived from uninfected livers (Fig. 2, lanes 2, 5 and 6).

Thus, a single virus-specific protein could be detected in preparations shown to contain viral particles which cause VHD and which resemble caliciviruses (29). A protein of approximately 60-61 kDa was also detected by others as the main polypeptide recognised in purified VHDV preparations with convalescent sera (8, 29, 34). Calicivirus particles are composed of 180 copies of a single structural protein of 60-71 kDa (7, 37). Proteins with apparent molecular weights of 24-25 kDa and 35-44 kDa were found in less purified VHDV samples (8, 28). The clarified supernatant obtained after liver homogenisation contained, in addition, apparently nonstructural
Demonstration of viral proteins

Highly purified virions (lanes 1, 3 and 4) and respective preparations from healthy rabbits (lanes 2, 5 and 6) were subjected to SDS-PAGE (lanes 1 and 2) and Western blotting (lanes 3, 4, 5 and 6).

Convalescent serum from the Federal Republic of Germany (lane 3) and an anti-VHDV serum from the People's Republic of China (lane 4) were used for detection of viral proteins.

Numbers indicate the size in kDa of the SDS-PAGE Standards.

proteins of approximately 120 kDa, 85-87 kDa, 52-56 kDa, 29 kDa and less than 20 kDa (28, 30, 34). Nonstructural proteins with molecular weights similar to the putative ones described above for VHDV were found for the San Miguel sea lion virus and other caliciviruses (3, 14, 15).

Monoclonal antibodies (MAbs) directed against VHDV showed specific reactivity in different assays, such as ELISA, immunofluorescence and haemagglutination inhibition assay. The few MAbs which work in Western blot experiments either exclusively detected the 60 kDa protein or, in less purified samples, also detected proteins of 35-38 kDa (34; Capucci, personal communication). The MAb 1H8 (8) detects the 60 kDa protein by Western blotting (Ohlinger, unpublished data) and apparently neutralises VHDV in vivo (see “Discussion”). An analysis of the MAbs which were negative by Western blotting was carried out by immunoprecipitation of intact VHDV with such MAbs followed by Western blotting with reactive MAbs. Again, the 60 kDa protein was detected indicating that these MAbs recognised the viral particles and thus probably also the non-denatured 60 kDa protein (unpublished results).
IMMUNOLOGICAL RELATIONSHIPS

Convalescent sera and MAbs against VHDV showed no significant reactivity with other viruses, such as the feline calicivirus (FCV) (26, 28), the swine vesicular disease virus (31) or the porcine parvovirus (31; Ronsholt, personal communication; Ohlinger, unpublished data). Although anti-VHDV antibodies were not found in pigs and fowl (38), low anti-VHDV titres could be detected in sera from unaffected rabbits, and in wild rabbits and hares suspected of EBHS (38). Identical results were obtained with serum samples from rabbits taken at a time when the disease was still unknown. Such rabbit sera reacted with the 60 kDa VHDV protein (28, 34) and were protective against VHDV infection, indicating the existence of a nonpathogenic, or less pathogenic, rabbit calicivirus with an immunological relationship to VHDV.

MAbs directed against VHDV were used in competition-ELISA to monitor antibody titres in affected and unaffected rabbits and in diseased hares. Different MAbs showed an identical pattern of reactivity with VHD-infected or -immunised rabbits and unaffected rabbits with low VHDV-antibody titres; however, only a few MAbs led to detection of antibody titres in hares suspected of EBHS. VHDV and EBHSV may therefore represent different viruses which share certain epitopes.

INVESTIGATION OF ANIMALS WITH VHD OR EBHS

Post-mortem examinations of rabbits with VHD and of hares diagnosed to be positive for EBHS led to similar histological, ultrastructural and immunohistological findings. It was therefore supposed that both diseases were caused by similar viruses (22). Furthermore, Lavazza et al. (19) found viral particles resembling VHDV in 10 out of 13 livers of hares with EBHS. The infectious nature of EBHS was shown by Eskens et al. (13), who succeeded in reproducing the disease by experimental infection of hares. The clinical symptoms and pathological lesions were shown to be similar to VHD in rabbits. After inoculation of rabbits with material from EBHS-positive hares only Morisse et al. (23) and Matthes et al. (personal communication) described a disease similar to VHD, whereas others failed to induce disease between species (Ohlinger and Ronsholt, unpublished data). The infection of one hare with VHDV resulted only in high anti-VHDV titres (18).

Differences between VHDV and EBHSV (EBHSV) were detected by ELISA and Western blotting using MAbs directed against VHDV. Only a few MAbs, raised by Capucci et al. against VHDV (8), reacted with EBHSV (Capucci, personal communication). These MAbs recognised all EBHS-suspicious probes after histopathological examination (manuscript in preparation) and also detected a single 60 kDa protein in material from diseased hares. The organ distribution of EBHSV in hares is similar to that of VHDV in rabbits (unpublished data). No cross-reactivity between EBHSV and VHDV was found with the anti-VHDV MAb 1H8, which shows in vivo neutralising activity against VHDV (Capucci and Ronsholt, personal communications). Hence, there is solid evidence that a calicivirus which differs from VHDV can be isolated from hares with EBHS.
THE GENOME OF VHDV

The findings outlined thus far are consistent with the idea that members of the Caliciviridae family represent the causative agents of VHD as well as EBHS. To satisfy molecular biologists, however, data about the viral nucleic acid, presumably RNA, had to be obtained. The guanidine isothiocyanate method was employed to isolate RNA from virions and from total cellular liver RNA (11, 29). This procedure leads to selective enrichment of RNA by pelleting it through a cesium chloride cushion and removes DNA as well as proteins. RNA was first isolated from purified 175 S particles. The size of the viral RNA was analysed by agarose gel electrophoresis (Fig. 3). The viral RNA was calculated to be about 8 kb (Fig. 3, lane 3), a size similar to that described for RNA from a feline calicivirus (FCV) (25).

To demonstrate viral RNA in preparations from livers, radioactively-labelled first strand cDNA was used as a hybridisation probe. RNA prepared from density gradient-purified virions served as the starting material for cDNA synthesis. In liver RNA extracted from infected animals, the resulting probe recognised a molecule of approximately 2 kb (Fig. 4, lane 3). After prolonged exposure, a smear in the size range of approximately 8 to 0.2 kb (Fig. 4, lane 3a) and resembling the signal obtained with genomic viral RNA could be detected (Fig. 4, lanes 1 and 1a). In contrast, no specific signal could be found with RNA from the liver of an uninfected animal (Fig. 4, lanes 2 and 2a). In the FCV system, an RNA of 2.4 kb has been shown to represent the major subgenomic mRNA (25).

Finally, viral RNA was injected into liver to assay the infectivity of virion RNA. Three rabbits negative for anti-VHDV antibodies were injected intrahepatically with RNA isolated from purified calicivirus particles. Three days after injection, two of the three animals died with pathological findings typical of VHD. Organs obtained from these animals were investigated further. In homogenates of liver and spleen, HA titres of > 1:4096 were recorded. Supernatants of clarified homogenised liver, spleen, lung and kidney tissues were positive in ELISA. In Western blot analyses, the single 60 kDa protein was again demonstrated. Furthermore, virions which were isolated from liver tissue after density gradient centrifugation and analysed by EM showed the typical morphology of a calicivirus. The virus particles showed characteristics identical to those from which the RNA was prepared (29).

According to hybridisation experiments with a VHDV-derived probe, the EBHS agent also represents a member of the Caliciviridae family. However, the stringency applied for hybridisation clearly shows that both VHDV and EBHSV, respectively, are different (unpublished results). This correlates well with the data obtained with MAbs reactive with either VHDV alone or with both viruses, VHDV and EBHSV.

DISCUSSION

With regard to identification of the causative agent of VHD, physico-chemical properties and morphology of isolated virions pointed towards a new member of the Caliciviridae family. Viral particles purified from liver gave positive reactions in ELISA, Western blotting and HA and were infective after injection into rabbits (29).
Demonstration of viral genomic RNA

RNA was separated electrophoretically in a 1% agarose gel and stained with acridine orange. Lane 1: 1 µg of RNA ladder (BRL, GB); lane 2: 5 µg of yeast tRNA; lane 3 approximately 0.5 µg of RNA extracted from sucrose density gradient purified virions (175 S) supplemented with 5 µg of yeast tRNA as carrier. Numbers indicate the size in kb of the RNA ladder.
Demonstration of viral RNA by hybridisation

The figure shows the same Northern blot after two different exposure times (1 h for lanes 1, 2 and 3 and 12 h for lanes 1a, 2a and 3a, respectively). Radioactively-labelled first strand cDNA synthesised from RNA extracted from sucrose density gradient purified virions was hybridised to 0.5 μg of RNA extracted from purified virions (lanes 1 and 1a) and 10 μg of RNA isolated from liver tissue of uninfected (lanes 2 and 2a) or VHDV-infected (lanes 3 and 3a) animals. Numbers indicate the size in kb of an RNA ladder (BRL, GB).
Furthermore, these viral particles are composed of a single structural protein with an MW of about 60 kDa (8, 10, 29, 30, 34), which is characteristic of caliciviruses. MAbs raised against VHDV reacted in Western blotting with the 60 kDa protein (34; Capucci, personal communication). In addition, one of the MAbs (8) apparently neutralises the virus, as passive immunisation with this MAb led to protection against lethal infection with VHDV (Capucci and Ronsholt, personal communications).

The VHDV genome was demonstrated by using either purified virions or liver tissue. The genomic RNA found in virions was approximately 8 kb, while RNA preparations from liver also contained a putative subgenomic RNA of 2 kb. RNAs of similar sizes have been described in the FCV system (25). Finally, the RNA from VHD virions was shown to be infectious for rabbits after intrahepatic injection. Considered together, these results clearly show that a member of the Caliciviridae family is the causative agent of VHD. Other lines of evidence strongly suggest that a calicivirus different from VHDV causes EBHS.

The first nucleotide sequences of parts of the FCV genome have recently been published (9, 24). It is clear now that FCV nonstructural proteins are encoded in the 5' region of the genome (24). According to an analysis of the deduced amino acid sequences, FCV nonstructural proteins contain motifs which are also found in picornaviruses (24). The only structural protein from caliciviruses is apparently translated from the major subgenomic RNA of approximately 2.4 kb (2 kb for VHDV), which is colinear to the 3' region of the genomic RNA (24, 25). This is similar to alphaviruses, the subgenomic RNA of which is also derived from the 3' end of the viral genome. FCV, and probably caliciviruses in general, are therefore quite different from other positive-stranded RNA viruses. It has been suggested that caliciviruses represent an intermediate family between the alphaviruses and the picornaviruses (24). It will be important to include other caliciviruses in such studies, in particular to compare nucleotide and deduced amino acid sequences with those of FCV. No sequence information about the structural protein of any calicivirus is available at this point. In addition, the genome organisation of caliciviruses remains to be established; for example, very little information is available about the VPg of caliciviruses, the protein which is covalently linked to the 5' end of the viral genome (6). The molecular biology of caliciviruses, including VHDV and EBHSV, is undoubtedly at a very early stage.

The Caliciviridae family, to date insufficiently studied, may be enlarged by two human pathogens, the Norwalk agent (43) and the so-called hepatitis E virus (33). The latter disease has been described as one form of non-A non-B hepatitis (32). After intravenous application, calicivirus-like particles produced non-A non-B hepatitis in nonhuman primates (5). The histopathological lesions were quite similar to those seen in rabbits after VHDV infection (22). The characterisation of both human viruses is in progress and should lead to exciting comparisons among the individual members of the family at the molecular level.

Résumé: Les auteurs ont identifié le virus de la maladie hémorragique virale du lapin (VHDV) dans le foie de lapins ayant succombé à la maladie. A partir d'extraits homogènes de cet organe, qu'ils ont soumis à une ultracentrifugation en gradient de densité, les auteurs ont obtenu des particules virales de 1,32 à 1,38 g/ml, dont le coefficient de sédimentation varie de 100 S à 175 S. Les virions isolés ont 40 nm de diamètre. Les particules obtenues ressemblent, d'un point de vue morphologique, à celles de la famille des Caliciviridae ; elles ont réagi positivement tant à l'épreuve ELISA qu'au test d'hémagglutination, et elles sont infectieuses pour le lapin. Par immunotransfert, une protéine structurale majeure, d'un poids moléculaire de 60 kDa, a été identifiée. Un ARN très pur de 8 kb environ a été isolé à partir des virions. A partir de Northern blots d'ARN provenant de foie de lapins infectés, il a été possible de différencier deux ARN de 8 et 2 kb à l'aide d'ADNc marqués. Finalement, le virus à ARN, injecté directement dans le foie du lapin, a provoqué une maladie dont les symptômes cliniques et les lésions histologiques sont typiques de la VHD (VHDV). Un calicivirus, auquel on a d'abord donné l'appellation de «virus de la maladie hémorragique du lapin» (RHDV), a donc pu être identifié comme responsable de la VHD. Le virus du syndrome du lièvre brun européen (EBHS) est également un calicivirus (EBHSV). Les virus VHDV et EBHSV sont deux membres de la famille des Caliciviridae ; différentes voies de recherche démontrent clairement leur parenté.

MOTS-CLÉS : Calicivirus - Maladie hémorragique virale du lapin - Syndrome du lièvre brun européen.


Resumen: Los autores identificaron el virus de la enfermedad hemorrágica viral del conejo (VHD) en el hígado de conejos muertos de esta enfermedad. Al cabo de la ultracentrifugación con gradient de densidad a la cual fueron sometidos los extractos homogéneos de hígado, se obtuvo unas partículas virales de 1,32 a 1,38 g/ml de densidad, cuyos coeficientes de sedimentación pueden variar de 100 S a 175 S. Los viriones aislados tienen un diámetro de 40 nm. Estas partículas se parecen, morfológicamente, a las pertenecientes a la familia de los Caliciviridae. Han reaccionado positivamente tanto a la prueba ELISA como a la prueba de hemaglutinación, y son infecciosas para los conejos. La técnica de «immunoblotting» permitió identificar una proteína estructural mayor, cuyo peso molecular es de 6 kDa. Un ARN muy puro de 8 kb aproximadamente fue aislado a partir de los viriones. Un ADNc reconocido del virión ARN, detectó dos ARN de 8 y 2 kb respectivamente, mediante «Northern blot» aplicado al ARN de hígado de animales infectados por el virus de la VHD (VHDV). Finalmente, los viriones ARN anteriormente aislados fueron inyectados directamente en el hígado de conejos, y produjeron una enfermedad cuyos síntomas clínicos y lesiones histológicas son típicos de la VHD. Se pudo entonces identificar el virus responsable de la VHD como un calicivirus, al cual se dio originalmente la apelación de «virus de la enfermedad hemorrágica del conejo» (RHDV). El virus del síndrome de la liebre parda europea (EBHS) también es
un calicivirus (EBHSV). Ambos virus VHDV y EBHSV pertenecen a la familia de los Caliciviridae; distintas vías de investigación demostraron claramente su parentezco.

PALABRAS CLAVE: Calicivirus - Enfermedad hemorrágica viral del conejo - Síndrome de la liebre parda europea.

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REFERENCES


