# Identification and Characterization of the Virus Causing Rabbit Hemorrhagic Disease

VOLKER F. OHLINGER,* BERND HAAS, GREGOR MEYERS, FRANK WEILAND, AND HEINZ-JÜRGEN THIEL

*Federal Research Centre for Virus Diseases of Animals, D-7400 Tübingen, Federal Republic of Germany*

Liver tissue from animals that died of rabbit hemorrhagic disease (RHD) was used to identify the causative agent. After extraction of liver homogenates and sucrose density gradient ultracentrifugation, distinct bands were obtained. The respective gradient fractions reacted positively in an enzyme-linked immunosorbent assay as well as in hemagglutination assays and were infective for rabbits. These fractions contained virions which had a diameter of 40 nm and resembled morphologically those of the family *Caliciviridae*. By immunoblotting, a major structural protein with a molecular weight of 60,000 was identified. Highly pure RNA of about 8 kilobases was isolated from virions. Labeled cDNA synthesized from virion RNA detected two RNAs of 8 and 2 kilobases in Northern (RNA) blots of liver RNA from animals infected with RHD virus. Finally, isolated virion RNA injected into the liver of rabbits produced a disease with clinical symptoms and pathological findings typical of RHD. We conclude that a calicivirus represents the causative agent of RHD.

A recently emerging contagious disease of rabbits that was first described in China in 1984 as rabbit hemorrhagic disease (RHD) (7), hemorrhagic septicemia, or infectious necrotizing hepatitis currently causes severe losses in the whole of Eastern Europe and many countries of Western Europe. The disease is characterized by high morbidity and mortality in adult animals, whereas infected rabbits below the age of about 2 months usually survive (23). After experimental infection adult rabbits usually die within 48 to 72 h and show characteristic pathological lesions. A specific diagnosis can be obtained, since homogenates of some organs contain high titers of hemagglutinating activity with human erythrocytes (23) and also react in an enzyme-linked immunosorbent assay (ELISA). Surviving animals exhibit high titers of anti-RHD antibodies, as measured by hemagglutination inhibition tests (9) or the ELISA. Protection can be induced by vaccination with Formalin-inactivated liver homogenates of infected animals (15). Until now the exact nature of the causative agent remained unclear, although a parvoviruslike agent (5, 22), a picornavirus (23), and a calicivirus (20) had been connected with the disease. In the present study the causative agent of RHD is characterized.

## MATERIALS AND METHODS

**Virus, animals, and antisera.** Virus isolated from an RHD virus (RHDV)-infected rabbit in the Federal Republic of Germany (FRG) was passaged in rabbits twice by intramuscular injection of filtered liver homogenates from infected animals.

The animals showed characteristic pathologic lesions, such as a pale, fragile liver, often with accentuation of the lobular markings and sometimes interspersed with hemorrhages, an enlarged, congested spleen, reddish speckled kidneys, and lungs with hemorrhagic lesions of different degrees.

Convalescent-phase sera were obtained from rabbits infected at 4 to 6 weeks of age. To obtain additional anti-RHD sera, we immunized rabbits subcutaneously with Formalin-treated (0.4%, 24 h, 37°C) liver homogenates and boosted

them with noninactivated liver homogenates from RHDV-infected rabbits.

**Density gradient centrifugation.** Supernatants from clarified (2,000 × $g$) homogenized liver tissue were layered onto a cushion of 17% (wt/vol) sucrose in phosphate-buffered saline (PBS) (pH 7.2) and centrifuged for 2 h at 25,000 rpm in a TST 28.38 rotor (Kontron, Zurich, Switzerland). The pellet was suspended in PBS and extracted twice with Freon 113 (Serva, Heidelberg, FRG). The aqueous phases were saved for further purification.

Cesium chloride density gradient centrifugation was performed after the addition of 1 ml of the aqueous phase to 10 ml of a 43% (wt/vol) cesium chloride solution in 0.2 M Tris hydrochloride buffer (pH 7.2). The mixture was adjusted to a density of 1.33 g/cm$^3$ and centrifuged at 48,000 rpm for 60 h in a TST 55.5 rotor (Kontron).

To determine the sedimentation coefficient of the virus particles, we layered 1 ml of the Freon 113-extracted material on top of a 15 to 30% (wt/vol) sucrose density gradient. After centrifugation for 4 h at 25,000 rpm in a TST 28.38 rotor, bands were visualized in scattered light and the sedimentation coefficient was estimated as described previously (12).

**Electron microscopy.** Samples in suspension were negatively stained with 1% uranyl acetate. Specimens were examined with a Zeiss electron microscope (model 109), and magnification was determined with a cross grating (2,160 lines per mm; Agar Scientific, Ltd., Stansted, Essex, United Kingdom).

**Hemagglutination tests and ELISA.** Hemagglutination was performed in U-bottomed microdilution plates (Greiner, Nürtingen, FRG) with equal amounts of antigen dilutions and 0.5% suspensions of group O human erythrocytes in 0.05 M NaH$_2$PO$_4$ · Na$_2$HPO$_4$–0.15 M NaCl (pH 6.4) at room temperature.

For the ELISA, 96-well flat-bottomed plates (Nunc I; Nunc, Roskilde, Denmark) were coated with rabbit hyperimmune serum in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 4°C for 16 h. Antigen samples were serially diluted 1:2 in PBS–0.1% Tween 20 and incubated at 37°C for 1 h. After being washed, the plates were incubated first with biotin-conjugated rabbit immunoglobulin G (in PBS–0.5% casein,

---

* Corresponding author.

37°C, 1 h) purified from anti-RHD convalescent-phase sera and then with peroxidase-conjugated streptavidin (Dianova, Hamburg, FRG) (in PBS–0.1% Tween 20, 30 min). *O*-Phenylenediamine (Sigma, Deisenhofen, FRG) was used as the substrate.

**Western blot (immunoblot).** Supernatants from homogenized tissues, clarified at 10,000 × *g*, or gradient-purified material was separated by electrophoresis under reducing conditions in a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (6). The gels were equilibrated in renaturation buffer (4 M urea, 50 mM NaCl, 10 mM Tris hydrochloride, 0.1 mM dithiothreitol [pH 7]) for 30 min and then incubated in electrophoresis buffer without SDS for an additional 30 min. Electrophoretic transfer to nitrocellulose membranes (Schleicher & Schuell, Dassel, FRG) was performed in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Munich, FRG) at 2 A for 1 h. Nonspecific binding of proteins to the membrane was blocked by incubation with 3% bovine serum albumin (Sigma) in PBS for 2 h. Biotinylated rabbit anti-RHD antisera diluted in 1% bovine serum albumin in PBS were allowed to bind overnight, and specific binding was detected with peroxidase-conjugated streptavidin (Dianova) and 2,2-chloronaphthol (Sigma) as the substrate.

**Preparation of RNA, agarose gel electrophoresis, and Northern (RNA) blotting.** Total cellular RNA from liver as well as RNA from virions was isolated by the guanidine thiocyanate method (4). Liver material, homogenized in the presence of liquid nitrogen, and pelleted virions were suspended in 4 M guanidine thiocyanate–0.5% sarcosyl–25 mM lithium citrate (pH 7.0)–0.1 M mercaptoethanol. The lysate was layered onto a cushion of 5.7 M CsCl–5 mM EDTA (pH 7.5) and centrifuged at 32,000 rpm in an SW41 Ti rotor (Beckman, Munich, FRG) for 24 h at 20°C. The RNA pellet was suspended in water and precipitated with 0.2 M potassium acetate (pH 5.6)–2.5 volumes of ethanol for 1 h at −70°C. For RNA preparation from density gradient-purified virions in some instances, 10 μg of yeast tRNA (Boehringer GmbH, Mannheim, FRG) was added as the carrier for ethanol precipitation. RNA (1 to 10 μg) was denatured for 40 min at 56°C in a total volume of 12 μl of glyoxylation mixture (10 mM sodium phosphate [pH 6.8], 1.2 M glyoxal). Glyoxylated RNA was separated in 1% phosphate-buffered agarose gels (10 mM sodium phosphate [pH 6.8]) containing 5.5% formaldehyde (3). An RNA ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used as a size marker. RNA gels were stained with acridine orange (3). Northern transfer to nylon membranes (Duralon; Stratagene, La Jolla, Calif.) was performed essentially as described previously (10).

For cDNA synthesis, RNA from pelleted virions (approximately 0.5 μg of RHDV RNA), 0.05 μg of random hexanucleotide primer (Pharmacia, Uppsala, Sweden), and 0.1 μg of oligo(dT) primer (Pharmacia) in 20 μl of water were heated to 65°C for 10 min and then chilled on ice. First-strand buffer was added, resulting in final concentrations of 50 mM Tris hydrochloride [pH 8.3]; 30 mM KCl; 8 mM MgCl$_2$; 1 mM dithiothreitol; 1 mM each dATP, dGTP, and dTTP; 30 μCi of [α-$^{32}$P]dCTP [Amersham Ind. plc., Buckinghamshire, United Kingdom]; 500 U of RNA-guard [Pharmacia] per ml and a total volume of 32 μl. Avian myeloblastosis virus reverse transcriptase (35 U) (Life Sciences, Inc., St. Petersburg, Fla.) was added. After 1 h at 43°C the reaction was stopped, free label was removed by spun-column chromatography (10), and the probe was heat denatured (95°C for 5 min).

The labeled cDNA probe was hybridized to Northern filters in hybridization mixture (0.5 M sodium phosphate, 1 mM EDTA, 7% SDS [pH 7.2]) at 68°C for 12 to 14 h. Membranes were washed at 68°C once in 40 mM sodium phosphate–1 mM EDTA–5% SDS (pH 7.2) for 30 min and twice in 40 mM sodium phosphate–1 mM EDTA–1% SDS (pH 7.2) for 30 min each time and subsequently exposed to Kodak X-Omat AR films.

**Injection of viral RNA into liver.** After laparotomy of narcotized adult rabbits, 7.5 μg of RNA (isolated from highly purified virions) in a buffer used for transfection experiments (21) was injected into the left lobe of the liver.

## RESULTS AND DISCUSSION

**Detection of viral antigen and purification of the virus.** Epidemiological studies and animal tests conducted after the first outbreaks of RHD in Germany showed that incubation time, clinical symptoms, and pathological lesions were very similar to those described in China in 1985 (23) for RHD. The identity of the diseases was confirmed by serological studies.

Hemagglutination activity with human erythrocytes was consistently found in liver, spleen, lungs, and kidneys of rabbits with peracute or acute RHD. Thus, hemagglutination was used as a main diagnostic procedure for RHD (23). In addition, RHD-specific antibodies obtained after immunization or infection reacted with liver homogenates from RHDV-infected animals in an ELISA (data not shown). Therefore, hemagglutination tests and an ELISA were used to monitor purification of the virus.

Liver material, which in both assays consistently exhibited the highest amounts of RHDV antigen, was used for purification of the virus. Density gradient centrifugation in cesium chloride solution resulted in a single visible band ranging from 1.32 to 1.34 g/cm$^3$, confirming previous reports (23). Sucrose density gradient centrifugation yielded three bands with estimated sedimentation coefficients of 175S, 136S, and 100S. These values are in good agreement with those previously published for RHDV (23) and also correspond to those described for caliciviruses (18). Heterogeneous densities resulting in distinct bands have also been published for other caliciviruses (14) because of loss of RNA (19), different pH values (17), and storage conditions (8).

Sucrose density gradients were fractionated, and aliquots were tested by hemagglutination and an ELISA for the presence of RHDV (Fig. 1). Hemagglutination titers of >1:128 were found in fractions 39 to 82 and peaked at fractions 47 to 50 (peak I) (1:2,048) and 71 to 76 (peak II) (1:8,192). In the ELISA, fractions 41 to 82 were also positive, showing absorption peaks at fractions 41 to 52 (peak I) and 57 to 82 (peak II). Peak I fractions from hemagglutination tests and the ELISA correlated with visible band I of the sucrose density gradient, and peak II fractions correlated with bands II and III of the sucrose density gradient.

To study whether the material from the distinct bands (peaks I and II) can induce the disease, we injected aliquots intramuscularly into rabbits. All the animals died within 3 to 6 days postinjection, with typical symptoms of peracute or acute RHD. Animals injected with pooled nonpeak fractions did not show any signs of disease.

**Detection of viral particles by electron microscopy.** The results of hemagglutination assays and the ELISA indicated the presence of RHDV antigen in distinct bands and therefore suggested the presence of viral particles. Aliquots from gradient fractions were analyzed by electron microscopy. Virus particles of uniform size were detected in fractions
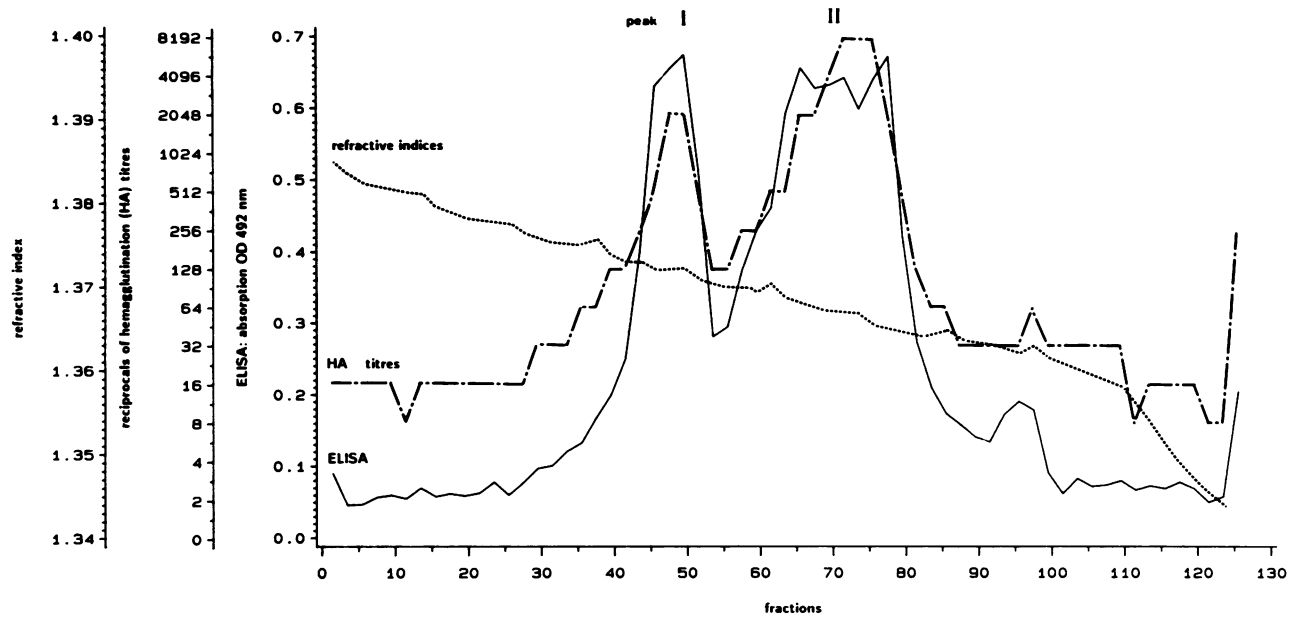
FIG. 1. Detection of viral antigen. Extracted liver material from a rabbit infected with RHDV was separated by sucrose density gradient ultracentrifugation. After fractionation, each 200-μl fraction was tested for the presence of RHDV antigen by hemagglutination tests and an ELISA. OD, Optical density.

corresponding to all three visible bands in sucrose gradients, indicating the existence of 175S, 136S, and 100S particles. Preparations of 175S particles (peak I in Fig. 1) showed the highest degree of homogeneity and purity (Fig. 2). The virions were 40 nm in diameter and displayed a clearly structured surface consisting of regularly arranged cup-shaped depressions, consistent with previous reports (20, 23). Morphology and size were in agreement with those recorded for caliciviruses (18).

**Detection of viral proteins.** To detect virus-specific proteins in livers from animals with RHD, we examined virus-containing fractions by SDS-polyacrylamide gel electrophoresis and Western blotting with anti-RHD sera. A prominent protein with an apparent molecular weight of 60,000 (60K protein) (Fig. 3, lane 1) was seen after Coomassie blue staining following SDS-polyacrylamide gel electrophoresis of sucrose density gradient-purified particles from liver homogenates of animals with RHD. Western blotting with
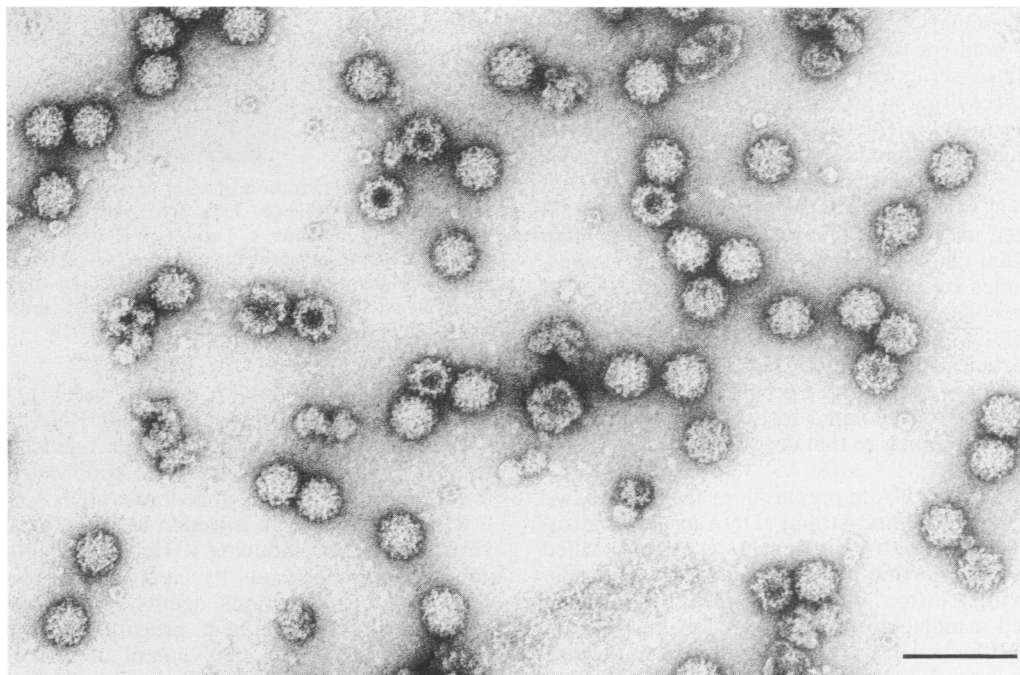


FIG. 2. Electron microscopy of purified virions after negative staining with 1% uranyl acetate. Bar, 100 nm.
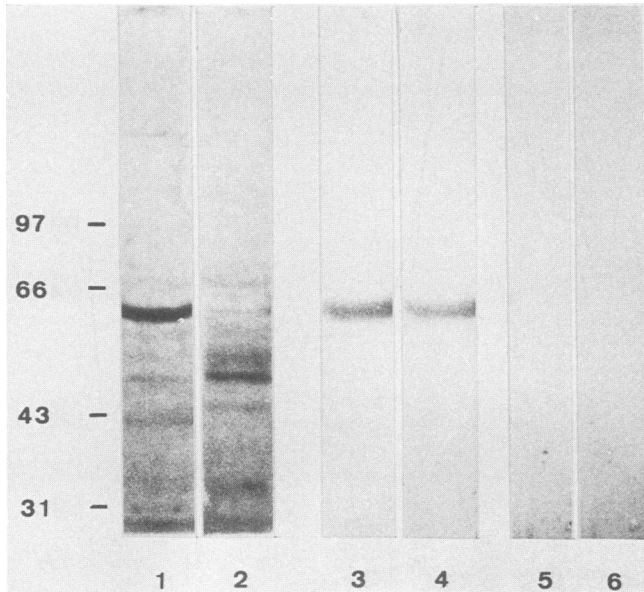
FIG. 3. Detection of viral proteins. Highly purified virions (lanes 1, 3, and 4) and respective preparations from healthy rabbits (lanes 2, 5, and 6) were subjected to SDS-polyacrylamide gel electrophoresis (lanes 1 and 2) and Western blotting (lanes 3, 4, 5, and 6). Convalescent-phase serum from FRG (lanes 3 and 5) and anti-RHD serum from the People's Republic of China (lanes 4 and 6) were used for the detection of viral proteins. Numbers indicate the molecular weights in thousands of the SDS-polyacrylamide gel electrophoresis standards (Bio-Rad).

anti-RHD serum demonstrated the presence of an antigen which had a similar apparent molecular weight and was the only protein detected in purified virions (Fig. 3, lanes 3 and 4). This 60K band was not observed in control preparations derived from noninfected livers (Fig. 3, lanes 2, 5, and 6).

In search of additional infectious agents in the livers of RHDV-infected animals, we examined all fractions of the sucrose density gradient (Fig. 1) in Western blots by using convalescent-phase sera. Two consecutive fractions each were combined, resulting in a total of 65 samples. The 60K protein was only found in fractions shown previously to be positive in hemagglutination tests and the ELISA. There was no indication of other virus-specific proteins (data not shown). Thus, a single apparently virus-specific protein could be detected in fractions previously shown to contain virus particles that cause RHD and resemble caliciviruses. Calicivirus particles are composed of only a single structural protein of about 60K to 71K (2, 19).

**Demonstration of viral RNA.** RNA was isolated from purified 175S particles (peak I fractions), and the size of the viral RNA was analyzed by agarose gel electrophoresis (Fig. 4). The viral RNA was calculated to be about 8 kilobases (Fig. 4, lane 3), a size similar to that described for RNA from a feline calicivirus (13).

To demonstrate viral RNA in preparations from livers, we used radioactively labeled first-strand cDNA as a hybridization 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 recognized a molecule of about 2 kilobases (Fig. 5, lane 3). After a prolonged exposure time, a smear in the size range of about 8 to 0.2 kilobases (Fig. 5, lane 3*) and resembling the signal obtained with genomic viral RNA
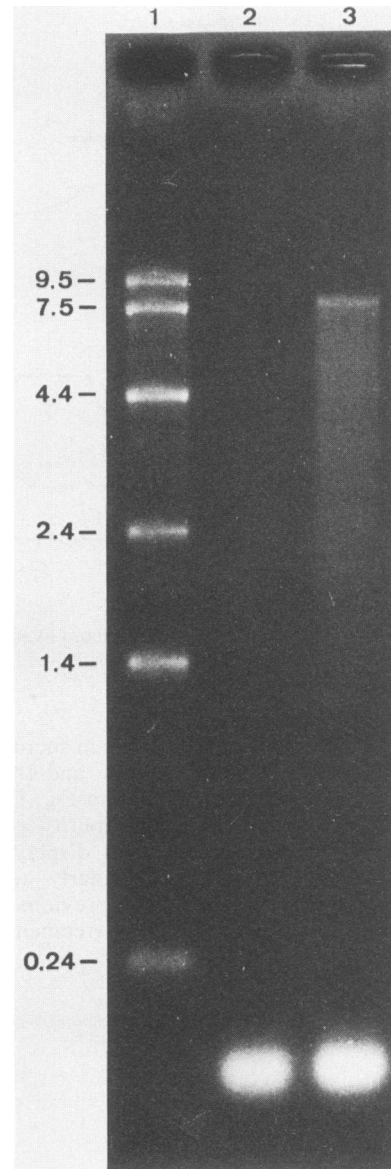


FIG. 4. Demonstration of viral genomic RNA. RNA was separated electrophoretically in a 1% agarose gel and stained with acridine orange. Lanes: 1, 1 μg of an RNA ladder; 2, 5 μg of yeast tRNA; 3, approximately 0.5 μg of RNA extracted from sucrose density gradient-purified virions (peak I) and supplemented with 5 μg of yeast tRNA as the carrier. Numbers indicate the sizes in kilobases of the RNA ladder.

could be detected (Fig. 5, lanes 1 and 1*). In contrast, no specific signal could be detected with RNA from the liver of a noninfected animal (Fig. 5, lanes 2 and 2*). In the feline calicivirus system, an RNA of 2.4 kilobases has been shown to represent the major subgenomic mRNA (13).

**RHD resulting from injection of viral RNA.** Although the virus preparation inducing RHD after injection into rabbits appeared homogeneous by electron microscopy, the presence of other infectious agents had to be excluded. In addition, the nucleic acid preparation from virions was highly pure, but the involvement of a DNA virus in the development of RHD could not be definitively ruled out.

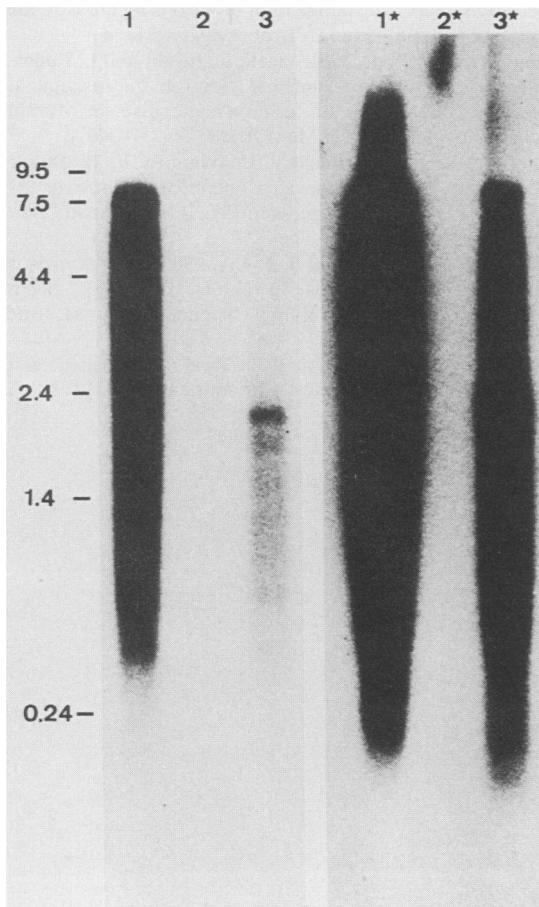As a direct approach, three rabbits negative for anti-RHD

FIG. 5. Demonstration of viral RNA by hybridization. Shown are the same Northern blots after two different exposure times (1 h for lanes 1, 2, and 3 and 12 h for lanes 1*, 2*, and 3*). Radioactively labeled first-strand cDNA synthesized from RNA extracted from sucrose density gradient-purified virions was hybridized to 0.5 µg of RNA extracted from purified virions (lanes 1 and 1*) and 10 µg of RNA isolated form the liver tissue of noninfected (lanes 2 and 2*) or RHDV-infected (lanes 3 and 3*) animals. Numbers indicate the sizes in kilobases of an RNA ladder.

antibodies were injected intrahepatically with RNA isolated from purified calicivirus particles. Three days after injection, two of three animals died, with pathological findings typical of RHD.

Organs obtained from these animals were investigated further. In homogenates of liver and spleen, hemagglutination titers of >1:4,096 were recorded. Supernatants of clarified homogenized liver, spleen, lung, and kidney tissues were positive in the ELISA. In Western blot analyses, the single 60K protein was again demonstrated. Furthermore, virions which were isolated after density gradient centrifugation from liver tissue and analyzed by electron microscopy showed the typical morphology of a calicivirus. The virus particles thus showed characteristics identical to those from which the RNA was prepared.

The results obtained so far show clearly that a member of the family Caliciviridae is the causative agent of RHD. Parvoviruses can be excluded because of the production of RHD after injection of the calicivirus RNA into rabbits, and picornaviruses can be excluded by the detection of subgenomic RNA and the presence of a single capsid protein.

Further efforts will be directed toward molecular cloning and sequencing of the viral genome. The development of molecular probes will be important not only for diagnostic purposes but also for the investigation of an assumed connection between RHD and European brown hare syndrome, a disease that resembles RHD with respect to pathological lesions, hemagglutination activity, and the morphology of particles found in livers of diseased hares.

The poorly studied family Caliciviridae will probably be enlarged by a human pathogen, the so-called hepatitis E virus. The respective disease has been described as one form of non-A, non-B hepatitis (16). After intravenous application, caliciviruslike particles produced non-A, non-B hepatitis in nonhuman primates (1). The histopathological lesions were quite similar to those seen in rabbits after RHDV infection (11). The characterization of this virus is in progress, and it will be exciting to compare the individual members of the family at the molecular level.

## LITERATURE CITED

1. Bradley, D. W., K. Krawczynski, E. H. Cook, Jr., K. A. McCaustland, B. H. Robertson, C. D. Humphrey, M. A. Kane, J. Spelbring, I. Weisfuse, A. Andjaparidze, M. Balayan, H. Stetler, and O. Velazquez. 1988. Enterically transmitted non-A, non-B hepatitis: etiology of disease and laboratory studies in nonhuman primates, p. 138–147. In A. J. Zuckerman (ed.), Viral hepatitis and liver disease. Alan R. Liss, Inc., New York.
2. Burroughs, J. N., T. R. Doel, C. J. Smale, and F. Brown. 1978. A model for vesicular exanthema virus, the prototype of the calicivirus group. J. Gen. Virol. 40:161–174.
3. Carmichael, G. C., and G. K. McMaster. 1980. The analysis of nucleic acids in gels using glyoxal and acridine orange. Methods Enzymol. 65:380–391.
4. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299.
5. Gregg, D. A., and C. House. 1989. Necrotic hepatitis of rabbits in Mexico: a parvovirus. Vet. Rec. 125:603–604.
6. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
7. Liu, S. J., H. P. Xue, B. Q. Pu, and N. H. Qian. 1984. A new viral disease in rabbits. Anim. Husb. Vet. Med. 16:253–255.
8. Love, D. N., and R. F. Jones. 1974. Studies on the buoyant density of a feline calicivirus. Arch. Gesamte Virusforsch. 44:142–143.
9. Maess, J., S. Matthes, and G. Flauss. 1989. Serologische Untersuchungen über das Vorkommen der infektiösen hämorrhagischen Erkrankung der Hauskaninchen (rabbit viral haemorrhagic disease) in Norddeutschland. Tieraerztl. Umsch. 44:423–425.
10. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. Marcato, P. S., C. Benazzi, G. Vecchi, L. della Salda, P. Simoni, P. Aiello, and G. Tumino. 1988. L'epatite necrotica infettiva del coniglio. Riv. Coniglicoltura 9:59–64.
12. McEwen, C. R. 1967. Tables for estimating sedimentation through linear concentration gradients of sucrose solution. Anal. Biochem. 20:114–149.
13. Neill, J. D., and W. L. Mengeling. 1988. Further characterization of virus-specific RNAs in feline calicivirus infected cells. Virus Res. 11:59–72.
14. Oglesby, A. S., F. L. Schaffer, and S. H. Madin. 1971. Biochemical and biophysical properties of vesicular exanthema of swine

virus. Virology **44:**329–341.

15. **Pages Mante, A.** 1989. Consideraciones técnicas de la sueroterapia y de la profilaxis vacunal en la enfermedad hemorrágica virica del conejo (R.H.D.V.). Med. Vet. **6:**285–291.

16. **Purcell, R. H., and J. R. Ticehurst.** 1988. Enterically transmitted non-A, non-B hepatitis: epidemiology and clinical characteristics, p. 131–137. *In* A. J. Zuckerman (ed.), Viral hepatitis and liver disease. Alan R. Liss, Inc., New York.

17. **Rowlands, D. J., D. V. Sangar, and F. Brown.** 1971. Buoyant density of picornaviruses in caesium salts. J. Gen. Virol. **13:**141–152.

18. **Schaffer, F. L.** 1979. Caliciviruses, p. 249–284. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 14. Newly characterized vertebrate viruses. Plenum Publishing Corp., New York.

19. **Schaffer, F. L., and M. E. Soergel.** 1976. Single major polypeptide of a calicivirus: characterization by polyacrylamide gel

20. **Smid, B., L. Valicek, J. Stepanek, E. Jurak, and L. Rodak.** 1989. Experimental transmission and electron microscopic demonstration of the virus of haemorrhagic disease of rabbits in Czechoslovakia. J. Vet. Med. Ser. B **36:**237–240.

21. **Van Der Werf, S., J. Bradley, E. Wimmer, F. W. Studier, and J. J. Dunn.** 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**2330–2334.

22. **Xu, W. Y., N. X. Du, and S. J. Liu.** 1988. A new virus isolated from haemorrhagic disease in rabbits, p. 456–461. *In* Proceedings of the 4th World Rabbit Science Congress, Budapest, Hungary, 1988.

23. **Xu, Z. J., and W. X. Chen.** 1989. Viral haemorrhagic disease in rabbits: a review. Vet. Res. Commun. **13:**205–212.