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To cite this version:
Vf Ohlinger, B Haas, Hj Thiel. Rabbit hemorrhagic disease (RHD): characterization of the causative calicivirus. Veterinary Research, BioMed Central, 1993, 24 (2), pp.103-116. <hal-00902110>

HAL Id: hal-00902110
https://hal.archives-ouvertes.fr/hal-00902110
Submitted on 1 Jan 1993

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Rabbit hemorrhagic disease (RHD): characterization of the causative calicivirus

VF Ohlinger *, B Haas, HJ Thiel

Federal Research Centre for Virus Diseases of Animals Tübingen, PO Box 1149, D-7400 Tübingen, Germany

(Received 6 July 1992; accepted 23 July 1992)

Summary — Rabbit hemorrhagic disease (RHD) which was first recognized in China in 1984 spread via Eastern Europe to many countries of Western Europe and other parts of the world. The analysis of the virus outlined in this review comprises: 1) physico-chemical properties, 2) electron microscopy including immunoelectron microscopy, 3) demonstration of capsid protein, 4) in vivo neutralization with monoclonal antibodies (mabs), 5) infectivity of purified RNA, and 6) characterization of the viral genome. Also included are clinical, pathological and epidemiological findings, different diagnostic methods as well as disease control measures. Finally, similarities between RHD and the European brown hare syndrome (EBHS) are pointed out. The latter disease is caused by a calicivirus different from RHDV.

rabbit hemorrhagic disease / etiology / epidemiology / diagnosis / calicivirus

Résumé — Maladie hémorragique du lapin : caractérisation du calicivirus responsable. La maladie hémorragique du lapin, qui a été signalée pour la première fois en Chine en 1984, s'est étendue par l'Europe de l'Est à de nombreuses régions d'Europe Occidentale et à d'autres parties du monde. L'analyse du virus exposée dans cette revue comprend (1) les propriétés physicochimiques, (2) l'étude en microscopie électronique incluant l'immunoélectronmicroscopie, (3) la mise en évidence de la protéine de capsid, (4) la neutralisation par des anticorps monoclonaux, (5) l'infectivité de l'ARN purifié, et (6) la caractérisation du génome viral. Les résultats concernant les études cliniques, la pathologie et l'épidémiologie ainsi que les différentes méthodes de diagnostic et les mesures de contrôle de la maladie sont également présentées. Enfin sont signalées les similitudes entre la maladie hémorragique et le syndrome du lièvre brun européen. Celui-ci est causé par un calicivirus différent du virus de la maladie hémorragique du lapin.

maladie hémorragique du lapin / étiologie / épidémiologie / diagnostic / calicivirus

* Correspondence and reprints
INTRODUCTION

Within the last decade 2 new viral diseases causing acute liver necrosis with high mortality in leporides have been reported from many countries around the world. In 1984 rabbit hemorrhagic disease (RHD), which by OIE is named "viral hemorrhagic disease" (VHD), was first recognized in the People’s Republic of China (Liu et al, 1984), whereas another viral epizootic disease, called European brown hare syndrome (EBHS) has caused severe losses in European brown hares since the beginning of 1980 (Gavier-Widen and Mörner, 1991; Lölliger and Eskens, 1991). In this review findings concerning the etiological agent, clinical symptoms, diagnosis, pathology and epidemiology of RHD are outlined and some interesting similarities with EBHS are discussed.

CLINICAL SYMPTOMS

Infection usually leads to peracute or acute RHD. Whereas no or indistinct clinical signs are found before death for peracute RHD, anorexy, apathy and tachypnoea can be detected in acute cases (Boujon et al, 1989; Ohlinger et al, 1989; Xu and Chen, 1989; Du, 1990; Schirrmeier et al, 1990; Marcato et al, 1991). Furthermore, the rabbits show clonal convulsions, lateral torsion of the head, paresis and sometimes bloody or bloody-foamy nasal excretions. The animals show fever but shortly before death subnormal body temperature (Xu and Chen, 1989). Experimental intranasal and intramuscular infections usually result in death after 48–72 h, but sometimes animals die after 8 d. During an epidemic subacute cases with limited anorexy, apathy and convalescence have been reported (Marcato et al, 1991). Some of these rabbits show icteric mucosa and die several weeks after infection. Interestingly, rabbits up to 4 wk of age do not exhibit clinical signs and usually survive infection.

PATHOLOGY

Pathological alterations varied from inapparent to highly significant (Ohlinger et al, 1989). Enlarged vessels were observed especially in the abdominal cavity. As a rule the coagulation of blood was inhibited significantly (Xu and Chen, 1989). Sometimes a hydrothorax and in few cases an icterus were found. The lungs, liver, kidneys and spleen were usually affected (Marcato et al, 1988, 1989, 1991; Boujon et al, 1989; Ohlinger et al, 1989; Xu and Chen, 1989; Albert and Wenzel, 1990; Schirrmeier et al, 1990; Nowotny et al, 1990). The lungs showed hemorrhagic lesions to varying degrees. Bloody foam was generally detected in the trachea and in the bronchi. Hemorrhages were also found in the tracheal mucosa. The liver was pale and fragile with accentuation of the lobular markings, or the organ was enlarged and dark reddish discolored. The kidneys were enlarged and speckled reddish, but sometimes only few petechiae were observed. Usually the spleen was enlarged and also dark reddish discoloured. Histopathologically, in the lungs alveolar oedema, hemorrhages and infiltrates of granulocytes were detected. The submucosal capillaries of the trachea were congested, and sometimes infiltrates of rounded cells were found. Diffuse or focal infiltrates of granulocytes and single hemorrhages were also detected in the liver early after infection. Furthermore, degenerative perilobular alterations in hepatocytes with strongly homogenous or pale vacuolized cytoplasm, nuclear pyknosis, karyorrhexis or karyolysis were observed. The kidneys showed focal hemorrhages, hyperaemia, sometimes glo-
merular hyalinic thrombosis, dilated tu-
bules and lymphocytic infiltrates. The
spleen was highly hyperaemic with singu-
lar follicular karyorrhexis. By electron mi-
croscopy strongly degenerative and ne-
crotic alterations were detected in
hepatocytes (Marcato et al, 1991). Virus-
like electron dense particles were detected
intracellularly at the beginning of infection,
later on within the cytoplasm. Similar parti-
cles were also found in endothelial cells.

RHD was characterized by the rapid
course of the disease. Fibrinous thrombi
within the capillaries of most organs, a
thrombocytopenia, as well as prolonged
prothrombin- and thrombin-times indicated
a consumption coagulopathy (Xu and
Chen, 1989). This disseminated intravas-
cular coagulation (DIC) could be caused
either by endothelial destruction or by ne-
crosis of the liver. Thus, the hemorrhagic
syndrome would be due to a primary or
secondary disorder of coagulation factors

ETIOLOGY

Early after outbreak of the RHD, epidemic
experimental infections were performed
with filtrated and chloroform treated organ
homogenates; it was concluded that a non-
enveloped virus was the causative agent
of RHD (Ohlinger et al, 1989; Xu and
Chen, 1989). Homogenates from various
organs, mainly from liver of infected rab-
bits, agglutinate human erythrocytes up to
high titres, indicating the presence of high
amounts of viral antigens. Accordingly the
liver of dead rabbits was used to isolate
the causative virus usually by a method
similar to that described by Ohlinger et al
(1990a).

Briefly, liver homogenate from infected
animals was centrifuged at low speed and
the supernatant layered on top of a su-
crose cushion. After ultracentrifugation, the
pellet was resuspended and extracted with
Freon –113. Density gradient centrifuga-
tion using cesium chloride resulted in a vis-
ible band at a density of 1.31 to 1.36 g/ml
(Deng et al, 1987; Granzow et al, 1989;
Smid et al, 1989; Xu and Chen, 1989; Oh-
bands were found after sucrose density
gradient centrifugation representing parti-
cles with sedimentation coefficients of 175
S, 136 S and 100 S. Similar values be-
tween 150–200 S were reported by others
(Deng et al, 1987; Granzow et al, 1989;
Capucci et al, 1990; Liebermann et al,
1991). The density gradient fractions corre-
lying with visible bands showed the high-
est activity with convalescent sera in ELI-
SA as well as in hemagglutination tests
(Ohlinger et al, 1990a). Bands of similar
density and sedimentation coefficients
were also found for caliciviruses (Schaffer,
1979). Different bands of calicivirus parti-
cles have been shown to be due to loss of
RNA (Schaffer and Soergel, 1976), or dif-
ferent pH values (Rowlands et al, 1971).

Material obtained from the bands after
density gradient centrifugation was nega-
tively stained in suspension and analyzed
by electron microscopy (EM). Virions of
uniform size were detected in all samples,
but preparations of 175 S particles showed
the highest degree of homogeneity and
purity. The virions were 40 nm in diameter
(Ohlinger et al, 1990a) and displayed a
clearly structured surface consisting of reg-
ularly arranged cup-shaped depressions
(Granzow et al, 1989; Ohlinger et al, 1989,
1990a; Smid et al, 1989; Soike et al, 1989;
Capucci et al, 1990; Du, 1990; Tesouro-
Vallejo et al, 1990; Valicek et al, 1990;
Marcato et al, 1991; Park et al, 1991; Ro-
dak et al, 1991). Following a 5-fold axis of
symmetry, 10 short projections most parti-
cles exhibit. Few particles showed 3- or 2-
fold axes of symmetry (Capucci et al,
1991; Rodak et al, 1991) and smaller parti-
icles with a smooth surface were found in proteolytically degraded preparations (Capucci et al., 1991). Whereas the electron micrographs from several authors showed caliciviral particles, the reported size of the virions varied from 29–40 nm in diameter. These variations can either be explained to be dependent on different staining methods, or on non-uniform presentations of the projections. Rodak et al. (1991), Marcato et al. (1991) and Park et al. (1991) demonstrated specific immune complexes by EM, which had been prepared after incubation of such virions with convalescent sera.

To detect virus specific proteins in liver tissue from infected rabbits, materials from a sucrose gradient were first separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Fractions with highest hemagglutinating activity resulted in a single visible band after Coomassie blue staining (Ohlinger et al., 1990a). Western blot analyses of such fractions with convalescent sera again resulted in a single protein band with an identical apparent molecular weight of 60–61 kDa; such a protein could not be observed with RHD-antibody negative sera as well as with material from non-infected rabbits (Ohlinger et al., 1990a). Others performed equivalent experiments and reported similar molecular weights (Capucci et al., 1990; Rodak et al., 1990a).

Virions of the Caliciviridae family consist primarily of RNA and 180 copies of a single capsid protein with a molecular weight of 60–71 kDa (Schaffer and Soergel, 1976; Francki et al., 1991). The results outlined so far for RHD virus (RHDV) are consistent with its preliminary status as a calicivirus.

In less pure preparations of virions additional proteins with apparent molecular weights of 24–25 kDa and 35–44 kDa were found (Ohlinger et al., 1989; Capucci et al., 1990; Rodak et al., 1990a, 1990b). Proteins with apparent molecular weights of 120, 85–87, 52–56, 29, and < 20 kDa were detected only in homogenates from livers of infected rabbits and may represent nonstructural proteins (Ohlinger et al., 1989; Parra and Prieto, 1990). Proteins of similar molecular weights were previously described for other caliciviruses (Black and Brown, 1975/1976; Fretz and Schaffer, 1978). So far, nonstructural proteins encoded by caliciviruses have not been identified.

Capucci et al. (1991) produced about 50 monoclonal antibodies (mabs) directed against RHDV. Eight of these mabs were further characterized and reacted with organ homogenates from infected animals in a double sandwich-ELISA, using polyclonal trapping sera. Two of these mabs reacted exclusively with RHDV, whereas the other 6 mabs showed crossreactivity with EBHS virus (EBHSV). When purified intact virions were employed, only the 2 non-crossreactive mabs exhibited strong binding (Capucci et al., 1991).

After simultaneous injection of RHDV and single mabs into rabbits, only one of the RHDV specific mabs (1H8) mediated strong in vivo-neutralizing activity. In vivo-neutralizing activity was not detected with the crossreactive mabs (Capucci et al., 1991).

These results led to the hypotheses that RHDV specific mabs are directed against external epitopes and that the crossreactive mabs recognize internal epitopes (Capucci et al., 1991). Whereas Capucci et al. (1991) found only 3 crossreactive mabs to be positive by Western blot, Ohlinger et al. (1990b) could show by Western blotting the binding of the in vivo-neutralizing mab 1H8 to the 60 kDa protein. The mabs, which did not react by Western blot, also recognize the capsid protein as shown by immunoprecipitation and subsequent demonstration by Western blot (Ohlinger and Thiel, 1991). Rodak et al. (1990a, 1990b)
also produced mabs against RHDV, which reacted in ELISA and recognized a 60 kDa and 38 kDa protein by Western blotting.

**MOLECULAR BIOLOGY**

The findings outlined so far are consistent with the idea that a member of the family Caliciviridae represents the causative agent of RHD. However, up to this point data about the genetic material of the virus are missing. Caliciviruses represent plus strand RNA viruses; the genomic RNA has a size of \( \approx 8 \) kb. Virus infected cells contain 1 additional RNA with a size of \( \approx 2.4 \) kb (Ehresmann and Schaffer, 1977; Black et al., 1978; Neill and Mengeling, 1988; Carter, 1990).

It was first shown that the RNA from purified RHD virions has a size of \( \approx 8 \) kb. Liver tissue from infected animals contained in addition to the genomic RNA a subgenomic RNA with a size of 2.2 kb. The demonstration of genomic and subgenomic RNA with expected sizes suggested again a calicivirus as the causative agent of RHD (Ohlinger et al., 1990a).

After reverse transcription of the RHDV genome, the cDNA was cloned in a bacteriophage vector. To detect virus specific clones a radioactive cDNA probe was employed, which was obtained after incubation of the reverse transcriptase with RNA from purified virions. Following cloning of the whole RHDV genome as cDNA the complete nucleotide sequence was determined; this represents the first complete sequence of a calicivirus (Meyers et al., 1991a). The RHDV genome comprises 7 437 nucleotides (without polyA tail) and contains one long open reading frame. The nonstructural proteins are encoded in the 5' region of the genome, whereas the 60 kDa capsid protein is encoded in the 3' region. The 2.2 kb subgenomic RNA mentioned above is colinear with the 3' region of the genome and encodes the capsid protein. Sequence comparisons with feline calicivirus (FCV) and RHDV indicated regions of significant homology between nonstructural proteins (Meyers et al., 1991a). In the meantime additional sequences of the capsid protein genes from FCV and RHDV have become available, indicating a higher degree of homogeneity among RHDV isolates than among FCV isolates (Neill et al., 1991; Tohya et al., 1991; Milton et al., 1992).

Further studies showed that genomic as well as subgenomic RNA from RHDV are protein-linked (Meyers et al., 1991b). Equivalent findings have been reported for the genomic RNAs of other caliciviruses. In analogy to picornaviruses, which also represent a virus family with plus-strand RNA genome and a protein covalently linked to the 5' end of the genome, the respective polypeptide was termed VPg. In contrast, almost all eukaryotic mRNAs possess a cap at the 5' end.

**CONCLUSIONS CONCERNING THE CAUSATIVE AGENT OF RHD**

The above outlined analysis of the virus comprises so far: 1) physico-chemical properties, 2) electron microscopy including immunoelectron microscopy, 3) demonstration of one capsid protein, 4) *in vivo* neutralization with a mab which recognizes the capsid protein, 5) infectivity of purified viral RNA after intrahepatic injection, and 6) characterization of viral nucleic acids. All of the results show that RHD is caused by a calicivirus. One of our RHDV cDNA clones has been made available to a research group in the People’s Republic of China to test the conflicting findings of scientists from China (Xu et al., 1988) and also from the USA (Gregg and House, 1989).
DIAGNOSIS

Together with the description of the first RHD outbreaks, Liu et al (1984) reported hemagglutination of human erythrocytes after incubation with diluted organ homogenates from infected rabbits. Later the hemagglutination test was frequently recommended by several authors as a diagnostic test for the detection of RHDV antigen (Pu et al, 1985; Ohlinger et al, 1989; Schirrmeier et al, 1990; Schlüter et al, 1990) and it is still being used as a simple test. Whereas the test appears to be independent of the blood group of human erythrocytes, no or only weak reactions are found with erythrocytes from other species (Xu, 1991). The test is performed as a microtest, at pH values of 6–7.2 and with 0.5 to 1% erythrocytes in phosphate-buffered saline. Between incubation temperatures of 4–37 °C no significant variations in the titres were found (Capucci et al, 1991). The hemagglutinating activity is stable against ether, chloroform, and for 60 min against temperatures up to 50 °C. No significant loss of activity was found with organ material, which was stored at 4 °C for several months (Capucci et al, 1991). Xu (1991) reported hemagglutination titres of = 10 x 218 in liver homogenates, spleen and peripheral blood. Significantly lower titres were found in other organ homogenates.

At the Federal Research Centre for Virus Diseases of Animals in Tübingen, the hemagglutination test (HAT) was performed with human erythrocytes at 1% in phosphate buffered saline, pH 6.4; about 2% of organ homogenates from experimentally infected rabbits reacted negatively in HAT.

For the detection of RHDV antigens, sandwich-ELISAs have also been reported (Ohlinger et al, 1990a; Capucci et al, 1991). In all these assay systems, rabbit antisera to RHDV were bound to ELISA-plates to trap RHDV. Thereafter, the bound antigen was detected using either guinea-pig antisera to RHDV (Ronsholt, personal communication) or biotinylated anti-RHDV IgG purified from RHDV-convalescent rabbits (Haas et al, unpublished observations) followed by peroxidase-conjugate and substrate (Ohlinger et al, 1990a). The peroxidase was also directly conjugated to RHDV specific IgG (Capucci et al, 1991). Normal rabbit sera were used to exclude nonspecific reactions. An increase in specificity and sensitivity was reported by using mabs against RHDV instead of polyclonal antisera (Capucci et al, 1991). These mabs were either directly conjugated to alkaline phosphatase (Capucci et al, 1991), or detected by peroxidase conjugated anti species sera (Haas and Ohlinger, 1990).

After ELISAs became available for the detection of RHDV antigen, the sensitivity of the HAT is being discussed much more critically. Capucci et al (1991) performed a comparative survey of 1 000 samples, which were tested by immunoelectron microscopy (IEM), antigen-ELISA and HAT; after direct comparison of the 3 techniques, they reported that HAT resulted in 8% of the samples being false positive and 9% being false negative. Similar results were obtained at our institute. HAT-titres up to 1 in 500 should be considered questionable; for example nonspecific HAT positive results can be due to Pasteurella spp. Western blotting experiments demonstrated that HAT-negative samples, which reacted positive in ELISA, contained a degraded 60 kDa RHDV-specific protein (Capucci et al, 1991). The presence of hemagglutination inhibiting antibodies in virus positive samples has also been discussed to interfere with the detection of hemagglutinating activity (Biermann et al, 1992). Especially with regard to samples which have undergone degradation the ELISA is thus certainly preferable to HAT.
Direct immunofluorescence (Nowotny et al., 1990; Rodak et al., 1991) or peroxidase procedures are also used to detect viral antigen in organ material. However, strong nonspecific fluorescence is commonly observed when cryosections of infected, highly necrotic livers and various fluorescence-conjugates are used. Therefore, the fluorescence has to be evaluated very carefully and it is not used for routine diagnosis in our institute. Peroxidase staining has also been reported for the detection of viral antigens (Marcato et al., 1988; Mandelli et al., 1990; Stoerckle-Berger et al., 1990; Park and Itakura, 1992).

Several groups failed to propagate RHDV in tissue culture cells. Interestingly, Ji et al. (1991) reported the establishment of a permissive rabbit kidney cell line (DJRK). Data obtained by electron microscopy and immunofluorescence, successful protective immunization, as well as infection with material obtained from the 10th to 16th passage of infected DJRK cells, suggested replication of RHDV in cell culture. However, the titres of infectious virus are apparently extremely low. Additional experiments have to be performed, especially direct demonstration of viral nucleic acid and virus encoded protein(s).

HAT and ELISAs established for diagnosis of RHD were also employed for detection of EBHSV antigen(s) in diagnostic samples. Using mabs against RHDV, which are crossreactive with EBHSV, the ELISA can be used as a sensitive assay for the detection of EBHSV antigens. RHDV specific mabs and mabs crossreactive to EBHSV allow discrimination between RHDV and EBHSV antigens in diagnostic samples (Ohlinger et al., 1990b; Capucci et al., 1991). Comparative studies by Capucci et al. (1991) resulted in much higher sensitivity and specificity of the ELISA when compared to the HAT. Hemagglutinating activities were found with semi-purified preparations of EBHSV, when the test was performed on ice. Only 50% of the samples, which reacted positively in ELISA and by IEM, showed in HAT low titres of hemagglutinating activity.

In order to detect antibodies to RHDV, the hemagglutination inhibition test (HIT), indirect ELISAs, as well as the competition ELISAs are used. The HIT is performed with 0.5–1% of human erythrocytes in phosphate buffered saline and 4 to 16 hemagglutinating units of RHDV antigen (Maess et al., 1989; Ohlinger et al., 1989; Sodan et al., 1990; Capucci et al., 1991; Xu, 1991). To eliminate nonspecific hemagglutination inhibition activities, the sera have to be pretreated by incubation at 56°C for 30 min, treatment with potassium tetraiodoaldo, kaolin or preadsorption to erythrocytes. The pretreatment of test samples interferes with the examination of large numbers of diagnostic sera and the HIT is difficult to standardize. Therefore various ELISAs for the detection of antibodies to RHDV were established. Rodak et al. (1990a) and Schirrmeier et al. (1990) employed an indirect ELISA. In this test purified RHDV antigens are coated to ELISA plates and antibodies to RHDV are detected by anti-species enzyme conjugates and substrate; such an ELISA is commercially available. To avoid laborious purification steps of the antigen and to increase the specificity, ELISAs were established to detect antibodies by competition. Competitive antisera to RHDV were raised in guinea pigs and detected with anti-species enzyme conjugates (Ronsholt, personal communication). Alternatively sera derived from convalescent rabbits were either directly conjugated to enzymes (Capucci et al., 1991) or biotinylated and subsequently detected by streptavidin-enzyme conjugates (Ohlinger et al., 1990a). In our ELISA, RHDV antibody-negative sera resulted in < 30% inhibition compared to values derived from phosphate buffer controls. Thus, this 30% inhibition was taken as cut-off value to differentiate between RHDV antibody-positive and -negative samples.
sera were tested in a comparative survey using our competitive ELISA and the HIT. In both systems 77 sera reacted positive and 219 sera gave negative results. Seven sera, which originated from infected holdings, but were negative in the HIT, reacted positively in the ELISA. After comparative titration of 52 sera in the HIT and the ELISA, we found a coefficient of correlation of 0.62.

Animals which survived an infection with RHDV or infected juvenile rabbits developed antibodies to RHDV, which were detectable at d 5 post infection. These antibody titres reached a plateau one week after infection and persisted for at least 8 months.

Surprisingly, antibodies to RHDV were found in sera, which were taken prior to the first reports of viral hemorrhagic diseases of lagomorphs in Europe between 1960–1980, and also in rabbit sera, which came from holdings with no history of RHDV (Ohlinger et al, 1989; Rodak et al, 1990a; Nowotny et al, 1992). Three sero-positive rabbits from such a holding survived experimental challenge infections with RHDV, whereas the control rabbits died. Rodak et al (1991) challenged 121 non-RHD diseased antibody positive rabbits and correlated the anti-RHDV antibody titres with the rates of survival. Eight sero-negative rabbits were introduced into a clinically unsuspicious holding, which comprised rabbits with antibodies to RHDV, seroconverted without any clinical symptoms of RHD (Capucci et al, 1991). Taken together these findings indicate the existence of an apathogenic virus which is related to RHDV.

**EPIDEMIOLOGY**

Rabbit hemorrhagic disease was first reported in the People's Republic of China in 1984 (Liu et al, 1984) and connected to an import of angora rabbits from Germany (Xu et al, 1988). Further outbreaks occurred in Korea (An et al, 1988), in Italy (Cancelotti et al, 1988, 1991) and in nearly all European countries (Morisse, 1988; Argüello Villares et al, 1989; Löigler et al, 1989; Peschev et al, 1989; Smid et al, 1989; Soike et al, 1989; Boujon et al, 1989; Kölbl et al, 1990; Mocsari, 1990; Schlüter and Schirmeier, 1991; OIE, 1992). RHD epidemics were also described in Mexico (Gregg and House, 1989) and in Israel. Most investigators suppose that the spread of the disease started with exported rabbit meat from China. In contrast, Löigler and Eskens (1991) hold unrecognized caliciviral infections of wild-life lagomorphs in Europe responsible for RHD. However RHD appeared in wildlife rabbits at the same time as in domestic rabbits. In contrast EBHS was recognized in European brown hares prior to the first reports of RHD. Following the hypothesis of Löigler and Eskens, EBHS and RHD should show epidemiological correlations and one would expect that the viruses can overcome species barriers. After infection with material from EBHS diseased hares, Morisse et al (1991) as well as Di Modugno and Nasti (1990) reported clinical symptoms in rabbits identical to RHDH, and Du (1991) as well as Nowotny et al (1992) found clinical signs identical to EBHS in hares infected with material from rabbits with RHD. However, most of the infection experiments across species failed (Capucci et al, 1991; Ohlinger, unpublished results). Furthermore, EBHS was observed in Scandinavian countries several years before the first outbreaks of RHD occurred and from most of these countries also no RHD was reported in wild rabbits.

Lagomorphs are the only animals which are susceptible to experimental infections with RHDV and EBHSV (Xu and Chen, 1989; Smid et al, 1991; Xu, 1991). Other species like rats, mice, hamster, guinea
pigs, horses, donkeys, cattle, buffaloes, sheep, goats, dogs, cats and chicken were tested with negative results. Furthermore there are no indications for RHD and EBHS as zoonoses. Significant differences between various races of rabbits (Xu, 1991) or between males and females (Pages Mante, 1989) have not been found. Using an RHDV isolate from Spain, Pages Mante (1989) reported a higher susceptibility of wild rabbits to experimental infections when compared with rabbits from holdings.

The disease is spread by oral, nasal or parenteral transmission. The virus is excreted with all secretions and excretions of diseased rabbits. In the field, the fecal–oral way of transmission is probably the most important one (Morisse et al, 1991). Gregg et al (1991) found that faeces from surviving rabbits were infectious for susceptible animals up to 4 wk after infection. In experiments with 3 susceptible rabbits, which were held in contact with young RHD convalescent animals (4 wpi) no clinical symptoms and no seroconversion could be detected even after immunosuppression of the convalescent rabbits (Haas, unpublished observations). Other ways of transmission could be contamination of feed, materials and persons (Erber et al, 1991), which was shown to be important especially in small extensive, non-commercial holdings. The significance of wild rabbits with regard to contamination of green feed as well as epidemiology of RHD is described by Maess et al (1990). The importance of insects and rodents as passive vectors is unclear (Cancelotti and Renzi, 1991; Gehrmann and Kretzschmar, 1991). RHDV remained infectious for 225 d at 4 °C and for 2 d at 60 °C. Infective particles could be demonstrated in dried material for 105 d at room temperature (Smid et al, 1991). Furthermore, RHDV is resistant to pH 3.0 (Xu and Chen, 1989).

After introduction of RHD into free regions, the virus shows rapid spreading and leads to epidemics with high morbidity and mortality with rates up to 100% for adult rabbits (Xu and Chen, 1989). In endemic regions, the morbidity and mortality are significantly lower due to higher frequencies of anti-RHDV-antibody positive animals.

**DISEASE CONTROL**

Effective disease control measures can so far only be taken for non-wildlife rabbits and hares. Vaccination with an inactivated vaccine is currently the most effective way to control RHD. All the available vaccines are derived from organ homogenates, which are prepared from infected rabbits. The virus is inactivated by different methods, like β-propiolactone-, formaldehyde-, ethylenediamine- (Pages Mante, 1989; Argüello Villares, 1991; Smid et al, 1991) or heat (68 °C) treatment (Haralambiev et al, 1990, 1991a, 1991b). Whereas Argüello Villars (1991) added aluminium hydroxide, Pages Mante (1989) used an oil adjuvant. Even after multiple vaccinations, no side effects were reported except for rare local reactions. Rabbits can be vaccinated after weaning, and protective immunity lasts from 6 months up to over 1 year (Argüello Villars, 1991). Huang (1991) and Peschlefski et al (1991) reported protective immunity induced by hyperimmune sera. Haralambiev et al (1991a) detected protective immunity in rabbits only 2 d after vaccination. Since sera from these animals contained no detectable antibodies to RHDV, protection may also be explained by other means.

Antibodies to RHDV induced after either vaccination or infection cannot be differentiated by the various serological assays. Vaccines can thus not be used in commercial holdings, which want to export seronegative rabbits. To avoid the introduction of RHDV into such holdings the following pro-
Phylactic control measures are recommended:

- rabbits, which have to be introduced into the holding should be seronegative to RHDV and taken into quarantine for a period of = 2 wk. Sentinel rabbits should be added;
- avoid contact to wild rabbits and hares as well as to rabbits from non commercial holdings;
- avoid green feed, use prepared feed;
- take care of high standards of hygiene, like change of cloths, use of shower, etc.

In Germany and other countries RHD is a notifiable disease. During an outbreak, RHD can be eradicated by stamping out affected holdings as was exercised in Mexico. If an eradication is not possible due to interactions with wild rabbits, the following control measures are recommended:

- killing of diseased and RHD suspicious rabbits and innocuous disposal of the carcasses;
- cleaning and disinfection procedures using 10% formaldehyde, 2% NaOH (Xu and Chen, 1989) or other disinfectants, which are recommended for use and effective against nonenveloped viruses;
- vaccination of the rabbits within the holding, in contact holdings or in affected regions, which are suspicious of becoming infected;
- no introduction of rabbits of unknown origin.

OUTLOOK

The virus family Caliciviridae presently consists of several members, like vesicular exanthema virus of swine, feline calicivirus, San Miguel sealion virus and calicivirus of dogs. The Calicivirus study group recently included RHDV into the family.

The studies on EBHSV demonstrate that this virus also represents a calicivirus (manuscript in preparation). An infectious agent isolated from man, the Norwalk agent, is now also a member of the virus family.

Surprisingly little is known about caliciviruses with regard to genome organization, strategy of gene expression, replication as well as synthesis of virions. Such studies are not only of basic interest but will also be important for applied approaches like development of recombinant vaccines. An animal virus could serve as a model for agents which are relevant for man. Finally the techniques of molecular biology may allow to isolate the so far hypothetical rabbit calicivirus. Such an approach may not only answer the question whether RHDV initially originated from rabbits imported to China from Western Europe, but also provide a tool to study the molecular basis of RHDV pathogenicity.

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