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G Le Gall, E Boilletot, Jp Morisse. Viral haemorrhagic disease of rabbit: purification and characterization of a strain isolated in France. *Annales de Recherches Vétérinaires*, 1992, 23 (4), pp.381-387. hal-00902098

**HAL Id: hal-00902098**

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Submitted on 11 May 2020

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## Viral haemorrhagic disease of rabbit: purification and characterization of a strain isolated in France

G Le Gall \*, E Boilletot, JP Morisse

*Centre National d'Études Vétérinaires et Alimentaires,  
Laboratoire Central de Recherches Avicole et Porcine, BP 53, 22440 Ploufragan, France*

(Received 14 February 1992; accepted 5 June 1992)

**Summary** — The causative agent of rabbit viral haemorrhagic disease (VHD) was purified by CsCl density gradient centrifugation from liver homogenates of rabbits infected with a strain of VHD virus isolated in France. Electron microscopy observations revealed that the virions were icosahedral with a diameter of 33–35 nm, and with an average buoyant density of 1.33. Structural proteins were investigated by SDS–polyacrylamide gel electrophoresis and western blotting. The results indicated the presence of a major structural protein of 64 kDa and other minor proteins of molecular weight of 43, 36 and 32 kDa, the last presumably resulting from the proteolytic degradation of VP 64. These results correspond with the description made by the different authors who identify the VHD virus as a member of the Caliciviridae family.

**viral haemorrhagic disease of rabbit / viral protein / calicivirus**

**Résumé** — **Maladie hémorragique virale du lapin : purification et caractérisation d'une souche isolée en France.** *Dans le but de caractériser le virus responsable de la maladie hémorragique virale du lapin, des lapins ont été infectés expérimentalement avec une souche isolée en France. Le virus a été purifié sur gradient de chlorure de césium à partir de broyats de foies; des particules virales icosaédriques, d'un diamètre de 33-35 nm ont pu être observées en microscopie électronique dans la fraction correspondant à une densité de 1,33. L'analyse des protéines virales, après séparation électrophorétique sur gel de poly-acrylamide et immunotransfert, a révélé la présence d'une protéine majeure de 64 kDa ainsi que d'autres protéines mineures de poids moléculaire de 43, 36 et 32 kDa. La protéine de 32 kDa résulte vraisemblablement de la dégradation protéolytique de la VP 64. Les résultats obtenus montrent que la souche virale isolée en France présente les mêmes caractéristiques morphologiques, biochimiques et protéiques que celles des souches isolées dans d'autres pays européens dont l'appartenance à la famille des Caliciviridae a été clairement démontrée.*

**maladie hémorragique virale du lapin / protéines virales / calicivirus**

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\* Correspondence and reprints

## INTRODUCTION

Viral haemorrhagic disease (VHD) in rabbits has now been described by a number of different authors; it was first reported in China in 1984 (Liu *et al*, 1984) and subsequently in many other parts of the world. Contradictory results were first published on the classification of the aetiological agent, probably because it could not be cultivated in cell culture. The morphology of the viral particles and their presence at densities of 1.32 to 1.39 in CsCl gradient indicated that the causative agent of VHD might be a calicivirus (Ohlinger *et al*, 1990; Parra and Prieto, 1990; Rodak *et al*, 1990) or a parvovirus (Gregg and House, 1989; Xu *et al*, 1988). SDS-PAGE and western blot analysis of the structural protein indicated that VHD virus had either one major protein of 60 kDa (Ohlinger *et al*, 1990; Parra and Prieto, 1990) or 3 major proteins of 61, 38 and 52 kDa, the 61-kDa protein being dominant (Rodak *et al*, 1990). More recently, the genome of VHD virus has been molecularly cloned and sequenced by Meyers *et al* (1991), demonstrating that VHD is a calicivirus.

The aim of this present study was to purify a strain of VHD virus isolated in France from liver tissues of rabbits and to investigate its protein structure.

## MATERIALS AND METHODS

### *Viruses and antisera*

The VHD virus strain (No 88 265) was collected during an outbreak of the disease in France. Susceptible rabbits, 10 wk old and seronegative against VHD virus, were used to propagate the viral strain. Rabbits were inoculated by intramuscular route with infected liver homogenates prepared as follows: livers were mixed with 5

vol of stabilized phosphate buffer pH 7 (60 mM sucrose; 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 12 H<sub>2</sub>O; 3.8 mM KH<sub>2</sub>PO<sub>4</sub>), clarified by centrifugation (1 250 *g* for 15 min at 4 °C) and then filtered through 0.45 µm. Rabbits died within 2 days; livers were taken and stored at -20 °C.

Anti-VHD virus hyperimmune serum was obtained from rabbits immunized *via* the intranasal route with β-propiolactone-treated liver homogenates (0.3% for 2 h at 37 °C) and then boosted with non-inactivated liver homogenates from VHD-virus infected rabbits. A chicken hyperimmune anti-VHD serum was prepared using purified virus in CsCl gradient. Chickens were inoculated by intramuscular route 3 times, first with virus emulsified with an equal vol of Freund's adjuvant and twice with incomplete Freund's adjuvant. Feline calicivirus (FCV) and goat serum anti-FCV were kindly provided by Dr Chappuis (Institut Rhone-Mérieux, Lyon, France).

### *Concentration and purification of VHD virus*

For protein analysis, VHD virus was purified from the liver of infected rabbits. Livers from uninfected rabbits submitted to a similar procedure were used as controls. The livers (VHD virus-infected and VHD virus-negative) were diluted (1:5 w/v) in stabilized phosphate buffer and homogenized. Two vol chloroform were then added to 10 vol homogenate, and the mixture was shaken for 5 min at 20 °C. The suspensions were clarified by successive centrifugations at 3 000 *g* and 10 000 *g* for 30 min. The viral particles in the supernatant were collected by ultracentrifugation at 120 000 *g* for 180 min at 4 °C. The pellets were resuspended in Tris-CaCl<sub>2</sub> buffer pH 7.5 (0.005 M Tris, 0.1 M NaCl, 0.001 M CaCl<sub>2</sub>, 2 H<sub>2</sub>O) and layered on preformed CsCl gradients with a density of 1.17-1.42. The samples were centrifuged to isopycnic equilibrium at 75 000 *g* for 16 h at 4 °C. 0.5 ml-fractions were collected and the presence of VHD virus in each fraction was assayed using a haemagglutination test (HAT) and electron microscopy (EM) after elimination of CsCl by ultracentrifugation (12 000 *g* for 180 min); the pellets were resuspended in TNE buffer, pH 9.4 (0.01 M Tris, 0.1 M NaCl, 0.000 1 M EDTA).

### **Electron microscopy**

Samples in suspension were negatively stained with phosphotungstic acid (PTA) at 2% in distilled water, pH 6.45 and examined with a Jeol electron microscope (Model JEM 100 CX Jeol SA, Tokyo, Japan).

### **Haemagglutination test**

Haemagglutination test (HAT) using human type O red blood cells was performed as described by Le Gall *et al* (1990).

### **SDS-polyacrylamide gel electrophoresis**

SDS-PAGE analysis of virion proteins was performed in a Mini Protean II dual slab cell (Bio-rad, Ivry-sur-Seine, France) using 10 or 12% acrylamide separating gels overlaid with a 4% acrylamide stacking gel in a discontinuous buffer system described by Laemmli (1970). The samples were heated for 5 min at 95 °C in SDS-reducing buffer (0.5 mM Tris-HCl, pH 6.8; 2% SDS; 5% 2-β-mercaptoethanol; 10% glycerol; 0.001% bromophenol blue) and electrophoresed under a constant current of 100 volts through the stacking gel and then 200 volts in the separating gel. After electrophoretic separation, the gels were stained with Coomassie blue R250 (Bio-Rad, Ivry-sur-Seine, France) and the relative mobilities of the proteins determined by comparison with internal standard proteins of known molecular weight (Bio-Rad, Ivry-sur-Seine, France).

### **Western blot analysis**

Proteins separated by SDS-PAGE were transferred electrophoretically (1 h at 100 volts) into a nitrocellulose membrane using a modification of the method of Towbin *et al* (1979). Briefly, the blots were first treated with 3% bovin serum albumin (BSA) in PBS pH 7.2 for 2 h at room temperature to block non-specific staining. The blots were then washed 3 times in PBS, and incubat-

ed for 1 h with appropriate antiserum diluted at 1:100 in PBS-3% BSA. After washing, the blots were treated for 1 h with alkaline phosphatase conjugated anti-rabbit, anti-chicken or anti-goat IgG (Sigma, St Quentin Fallavier, France) used respectively at 1:2 500, 1:2 500 and 1:5 000 in PBS containing 3% BSA. The blots were again washed as described above and stained by the addition of bromochloroindolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT), as described by Harlow and Lane (1988).

## **RESULTS**

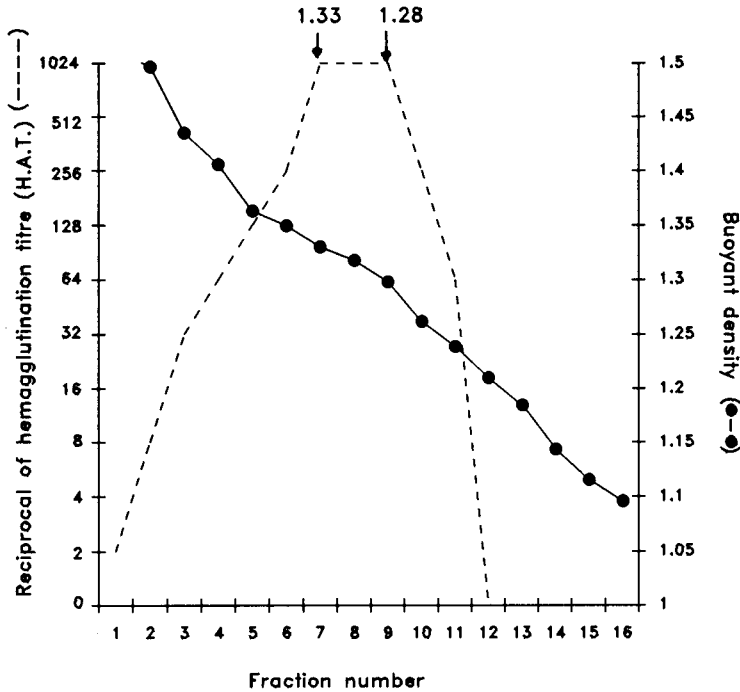
### **Virus purification**

The VHD virus was purified from liver samples of infected rabbits which exhibited the highest amount of VHD antigen by the HAT. In CsCl gradient, one HA activity peak was detected in fractions with a density between 1.25 and 1.33 (fig 1). Electron microscopic examination showed that viral particles were most numerous in fractions with an average buoyant density of 1.33. A mixture of empty and full particles was observed. The morphology of the purified VHD virus consisted of viral particles with cup-shaped depressions on their surface (fig 2). Their size was estimated at 33–35 nm in diameter.

The injection of purified material (1.33 density fraction) into susceptible rabbits reproduced the infection. The animals died within 72 h with characteristic lesions which included tracheal and lung congestion, degeneration of the liver and uncoagulated blood in the body cavities.

### **Structural proteins of VHDV**

After staining with Coomassie blue SDS-PAGE analysis of the structural proteins of VHD virus, showed different viral polypep-

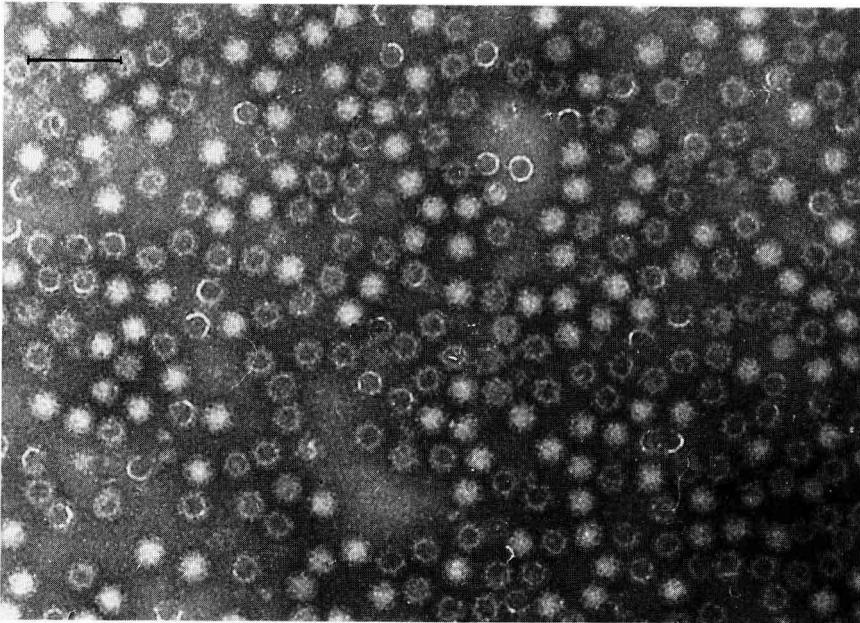


**Fig 1.** Detection of viral antigen in CsCl gradient: distribution of haemagglutination activity of VHDV extracted from liver of infected rabbit. (---) Haemagglutination activity; (●) buoyant density.

tides in purified virions: one major polypeptide with an estimated molecular weight (mw) of 64 kDa, and several minor distinct polypeptides with mw of 48 and 46 kDa respectively. These last 2 proteins were also present in liver homogenate from an uninfected rabbit, indicating that they were not specific to VHD virus (fig 3). In western blots, bands of 64, 43, 36 and 32 kDa reacted both with convalescent rabbit serum (fig 4) and with chicken hyperimmune anti-VHD virus serum (data not shown). The strongest reaction was against the 64-kDa structural polypeptide. Lower reactions were seen with the other proteins. The 64-kDa protein was not detected in any of the normal rabbit sera.

Proteins of VHD virus and feline calicivirus were co-electrophoresed on the same gel after fractionation by SDS-PAGE and were revealed by immunoblotting with their homologous serum. The major proteins of VHD virus and FCV were similar in size (VHD virus: 64 kDa; FCV 65–66 kDa) (fig 4).

Chicken hyperimmune anti-VHDV serum was used in western blots to detect the virus in a liver suspension of wild or domestic rabbits. The 64-kDa protein was constantly present in HAT and EM positive samples. Conversely, in HAT and EM negative samples, the protein 64-kDa protein was not detected. In HAT negative and EM positive samples, mw  $\approx$  32 kDa polypeptides were detected (data not shown).



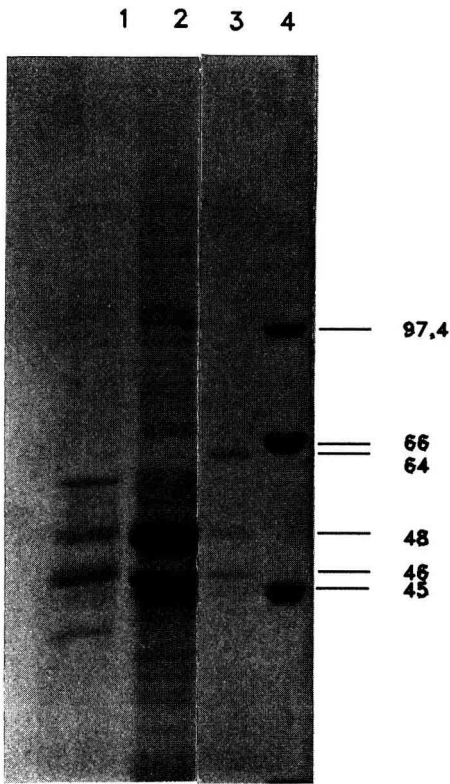
**Fig 2.** Morphology of VHD particles purified from liver of infected rabbits after negative staining with 2% phosphotungstic acid ( $\times 130\,000$ );  $be^{-} = 100\text{ nm}$ .

## DISCUSSION

The viral particles isolated from liver of VHD virus-infected rabbit presented the same morphological properties as those described by other authors (Ohlinger *et al*, 1990; Valicek *et al*, 1990; Rongling *et al*, 1991): the virions were icosahedral, non-enveloped and 33–35 nm in diameter (size varied between 29–40 nm according to the authors). Our studies indicate that the purified VHD has an average buoyant density of 1.33 in cesium chloride; prototype calicivirus are reported to have a buoyant density of 1.36–1.39. The average size (35–33 nm) and morphology are also comparable to that of other caliciviruses.

In western blot analysis, the presence of a major protein of 64 kDa suggests that

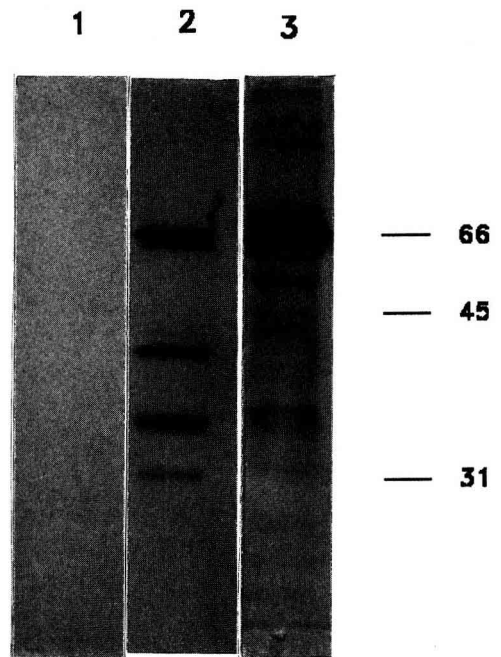
the VHD virus seems to be a calicivirus, one of the principal characteristics being that it possesses a single structural protein of 60–71 kDa (Schaffer, 1979). The 64-kDa protein was recognized by hyperimmune serum in our immunoblotting, suggesting that this major viral protein is highly immunogenic. Several proteins also reacted in western blot with hyperimmune serum (43, 36 and 32 kDa). The  $\approx 60$ –61 protein kDa has been described as the main polypeptide recognized in purified VHD virus preparation with convalescent serum (Ohlinger *et al*, 1990; Cappuci *et al*, 1991). Our results correspond with this observation, although the molecular weight of our major protein was slightly higher (64 rather than 60–61 kDa). Proteins with molecular weights of 43 and 36 kDa have



**Fig 3.** Polypeptide analysis of purified VHD virus by SDS-polyacrylamide gel (10%), after staining with Coomassie blue. lane 1: normal rabbit liver homogenate liver; lane 2: infected rabbit homogenate liver; lane 3: gradient purified virus; lane 4: molecular weight standards (kDa).

been observed by other authors in less purified VHD samples. Western blot of our rabbit sera and Spanish rabbit anti-VHD serum (Ovejero Laboratories, Leon, Spain) showed the same bands, indicating that the etiological agent was the same in these 2 countries (data not shown).

The degradation of the major protein has been described by Capucci *et al* (1991), who suggest that the proteolytic



**Fig 4.** VHD virus proteins were examined by western blotting after separation by SDS-PAGE (12%). Strips of nitrocellulose membrane were incubated with rabbit negative serum (lane 1) and with rabbit anti-VHDV serum (lane 2). FCV was also fractionated on the same gel and revealed by western blot with goat anti-FCV serum (lane 3).

degradation was probably due to the autolytic process or to physiological alterations in the host.

Chinese authors (Chuan *et al*, 1991) have now described the adaptation of VHD virus to a primary rabbit kidney cell line, designated DJRK, which would permit other useful biochemical and molecular analyses to be carried out. Moreover, it would be interesting to consider comparative

studies between VHD virus and other hepatitis viruses, such as EBHS (European brown hare syndrome) or human hepatitis E because of their pathological, clinical and epidemiological similarities.

## ACKNOWLEDGMENTS

The authors wish to thank Dr Chapuis and Dr Fargeaud of the Rhône-Merieux Institute (Lyon, France) for kindly supplying the feline calicivirus (FCV) and the goat anti-FCV serum.

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