Enzyme-linked immunosorbent assay of antibodies to rabbit haemorrhagic disease virus and determination of its major structural proteins

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A ELISA was developed for the determination of antibodies to rabbit haemorrhagic disease virus (RHDV) in whole blood and blood serum of rabbits. Naturally acquired antibodies were detected in 19.4% of blood samples collected from 1461 rabbits in 43 farms apparently free of the disease, 19.7% samples were doubtful and 60.9% of the rabbits were free of antibodies to RHDV. Their presence has a considerable effect on the resistance of rabbits to infection with RHDV. Antibodies were also found in rabbit blood serum samples collected up to 12 years before the first outbreaks of RHD were reported. Up to 14 viral protein antigens were determined by PAGE and Western blot analysis, of which three with M, values of 61K, 38K and 52K were major proteins, the 61K being dominant. Our hyperimmune sera, a Chinese reference serum and sera with positive antibody titres, including those collected several years before the first outbreaks of RHD, reacted identically with these antigens in the Western blot analysis. The data obtained suggest that naturally acquired antibodies are a product of a specific response to prior infection with an avirulent strain of the virus.

Introduction

The first outbreaks of rabbit haemorrhagic disease (RHD) were recorded in China in 1984, reportedly in connection with the import of Angora rabbits from Germany (Liu et al., 1984; Xu et al., 1988). The causal agent was demonstrated to be a virus with a diameter of 28 to 33 nm. The Chinese authors also published the first data on the production and properties of an inactivated vaccine from organs of infected rabbits (Liu et al., 1984; Gu et al., 1986; Du et al., 1986; Cao et al., 1986; Wei et al., 1987), as well as demonstrating the virus antigen and specific antibodies by haemagglutination and haemagglutination-inhibition tests (HIT) (Liu et al., 1984; Pu et al., 1985; Du et al., 1986; Xu et al., 1988). The classification of the virus by these authors is contradictory, some designating it as an RNA and others as a DNA virus (Liu et al., 1984; Du et al., 1986; Shen et al., 1986; Xu et al., 1988).

An acute, infectious disease of rabbits, with a high mortality and clinical and necropsy signs identical to those described by the Chinese authors, emerged in Europe in 1987 to 1988. The first information published by European authors (Górski et al., 1988; Ševevičková et al., 1988; Löüger et al., 1989) was based mostly on the data published in China, including the contradictory classification of the causal agent. Recently, Šmíd et al. (1989) investigated the virion structure by electron microscopy and designated the causal agent as a possible member of the calicivirus group. A similar conclusion was reached by Ohlinger et al. (1989). Such classification is supported by the identical density in CsCl gradients (1.36 to 1.39 g/ml) of caliciviruses and RHDV (Broughs et al., 1978; Cubitt, 1987; Deng et al., 1987; Xu et al., 1988) and the identical M, values of major structural proteins of RHDV and the feline calicivirus (Ohlinger et al., 1989). However, the dominant protein typical for caliciviruses was not among the seven RHDV polypeptides detected by Xu et al. (1988).

The aims of the present study were: (i) to develop an ELISA for the detection of antibodies to the RHD agent which would replace the less sensitive haemagglutination-inhibition test and (ii) to identify the major structural proteins of RHDV and to determine the specificities of positive rabbit sera of various origins.

Methods

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Methods

Animals and blood sample collecting. Blood samples taken from 1461 rabbits were examined for the presence of antibodies by ELISA. The samples, collected from 43 rabbit farms free of clinical RHD, were from animals of both sexes, older than 2 months and with a live weight over 2 kg. Approximately 10 μl of blood was withdrawn from the ear vein into a heparinized capillary tube and suspended in a special collecting-and-dosing test tube in 1 ml of a diluent (Rodák et al., 1985). After a quick freezing and thawing, the sample, diluted 1:100, was used in ELISA immediately. Convalescent and hyperimmune sera and a
reference serum obtained from China were used as positive controls. We wish to thank Dr X. X. Wang from the National Control Institute of Veterinary Bioproducts and Pharmaceuticals, Beijing, China, for the reference serum.

A selected set of 42 stored rabbit sera was also examined by ELISA. The set included 33 samples collected between 1975 and 1983 and nine samples collected between 1984 and 1987. The rabbits, purchased from small farms, were kept in the premises of the Veterinary Research Institute and used to produce hyperimmune sera to various virus antigens or immunoglobulin preparations. The sera, stored freeze-dried at 4 °C or, after reconstitution, at -20 °C, remained clear and retained their specificity over the long storage period.

**Virus preparation and purification.** The RHDV strain CAPM V-351 (rabbit calicivirus) was prepared from the supernatant of an organ suspension of a rabbit which died following experimental infection (Smid et al., 1989). The suspension contained 10³ LD₅₀/ml. Liver, kidneys and spleen of rabbits dying as a result of experimental infection with this strain, were homogenized in a five-fold volume of phosphate-buffered saline (PBS) and used for obtaining a purified virus suspension. One to 2 volumes of chloroform were added to 10 volumes of the homogenate and the mixture was shaken for 5 min at 20 °C. Supernatants were withdrawn after 15 min centrifugation at 3000 g and further purified by ultracentrifugation (2 h at 28000 r.p.m.; SW-28, Beckman) in a 3 ml cushion of CsCl with a density of 1.3 g/ml. The purified RHDV was obtained after resuspension of the pellet in 2 to 3 ml PBS and removal of traces of rabbit IgG by affinity chromatography.

**Hyperimmune sera and conjugates.** Specific antibodies were isolated from swine serum to rabbit IgG (SwARIgG) by affinity chromatography (CNBr Sepharose 4B, Pharmacia) and conjugated with horseradish peroxidase (HRP, RZ = 3:0; Boehringer) by the periodate method (Boorsma & Streefkerk, 1979). Stock conjugate solution (HRP-SwARIgG), containing 2 mg IgG/ml, was diluted 1:1000 to 1:2000 in PBS containing 0.1% Tween 80 and 1% lactalbumin hydrolysate (PBST-LAH). Radiolabelled RHDV was obtained after resuspension of the pellet in 2 to 3 ml PBS and removal of traces of rabbit IgG by affinity chromatography.

**ELISA and interpretation of results.** Fifty μl of purified RHDV suspension (diluted 1:500 with 0.05 m-carbonate-bicarbonate buffer pH 9.6, was pipetted into each well of microtitration plates (Koh-I-Noor) and left to adsorb at 4 °C overnight. Two drops (approximately 100 μl) of the basic dilution (1:100) of the haemolysed blood sample were added to each well in the pair of microtitration plates in the pilot examination of field samples, and serial twofold dilutions were prepared with PBST-LAH for antibody titration. 100 μl of HRP-SwARIgG was added to each well after 1 h incubation at 37 °C in a wet chamber and the plates were incubated for another hour under the same conditions. Each incubation was followed by triple washing with PBST. The absorbance of each sample was measured at 492 nm using a spectrophotometer (Titertek MCC) 1 h after the addition of 100 μl of substrate solution (5-aminosalicylic acid). Positive and negative reference samples were included in each set of samples. PBST-LAH was used instead of blood or serum sample in wells serving as blanks.

The rabbit blood samples, diluted 1:100, were classified by their antibody titres. A492 values up to 1:1000 were recorded in most of the samples in the basic dilution of 1:100 and antibody titres exceeded 1:3200. The absorbance of each sample was measured at 492 nm using a spectrophotometer (Titertek MCC) 1 h after the addition of 100 μl of substrate solution (5-aminosalicylic acid). Positive and negative reference samples were included in each set of samples. PBST-LAH was used instead of blood or serum sample in wells serving as blanks.

**HIT.** The presence of RHDV antibodies in rabbit blood sera was demonstrated by HIT, using a 0.5% suspension of human group O erythrocytes and 4 haemagglutinating units of RHDV. Six wells (4 mm diameter, 6 mm apart) were punched in 0.75% agarose gel to form a rosette arrangement. Each outside well was filled with twofold serially diluted test serum; purified RHDV suspension was pipetted into the central well. The agarose gel was examined after 48 h incubation in a wet chamber at 20 °C.

**PAGE.** Discontinuous SDS–PAGE, as described by Laemmli (1970), was used for the determination of major structural proteins of RHDV. Samples (organ homogenates, purified RHDV, rabbit IgG, low Mr standards from Pharmacia) were boiled at 100 °C for 3 to 5 min and fractionated in 10% gel at 120 V, 25 mA. The gel was cut into two parts when the electrophoresis was finished and the first was stained with Coomassie blue R 250 or with silver (Heukeshoven & Dernick, 1985). Fractionated proteins from the second part were transferred electro-photorethetically (2 to 16 h at 20 V, 50 to 200 mA) onto a nitrocellulose (NC) membrane (Sartorius SM 113). After blocking in PBST-LAH, the membranes were incubated with hyperimmune or the examined rabbit sera diluted 1:500 with PBST-LAH at 20 °C for 1 h. The NC membranes were then incubated for 1 h with HRP-SwARIgG, and stained with a substrate solution (3,3′-diaminobenzidine, Merck). The reaction was stopped by the addition of 0.5% sodium azide and the NC membranes were dried.

**Results**

**Antibody levels in field samples**

Blood samples, collected from 1461 rabbits in 43 farms apparently free of RHD, were examined for the presence of antibodies to RHDV by ELISA (Table 1). Naturally acquired antibodies were detected in samples from 283 animals (19.4%). Low antibody titres and doubtful results were found in 288 samples (19.7%) and 890 samples (60.9%) were free of antibodies. Of the 43 farms only 10 had rabbits with negative RHDV antibody titres (Table 1).

**Antibody levels in laboratory rabbits**

The set of sera examined included the positive reference serum obtained from China, sera of vaccinated rabbits and survivors after experimental infection and hyperimmune sera prepared in our laboratory. A₄₉₂ values higher than 1.0 were recorded in most of the samples in the basic dilution of 1:100 and antibody titres exceeded 1:3200. The antibody titre of the Chinese reference serum was 1:25600 and those of our hyperimmune sera reached values of up to 1:102400 and exceptionally even 1:409600. Another set included sera collected during the periods 1975 to 1983 and 1984 to 1987. No difference in the proportion of positive and negative samples was found between these two subsets. The presence of naturally acquired antibodies was detected in 32 of the 42 samples (76.2%). Of these 16 had high and 16 had low antibody levels. Doubtful results were obtained from six samples (14.3%) and the remaining four (9.5%) were free of antibodies to RHDV.
RHDV antibodies and structural proteins

Table 1. Determination of natural RHDV antibodies in the blood of non-infected rabbits by ELISA

<table>
<thead>
<tr>
<th>Percentage of positive rabbits in farms</th>
<th>No. of farms</th>
<th>No. of rabbits</th>
<th>No. of samples with A492* of</th>
<th>No. of</th>
<th>No. of</th>
<th>No. of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.1 (negative)</td>
<td>0.1 to 0.3 (doubtful)</td>
<td>&gt;0.3 (positive)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>283</td>
<td>257</td>
<td>26</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>14</td>
<td>545</td>
<td>415</td>
<td>84</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>19</td>
<td>633</td>
<td>218</td>
<td>178</td>
<td>237</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>1461</td>
<td>890</td>
<td>288</td>
<td>283</td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td></td>
<td></td>
<td>60.9</td>
<td>19.7</td>
<td>19.4</td>
<td></td>
</tr>
</tbody>
</table>

* ELISA A492 at a sample dilution of 1:100.

Immunodiffusion and haemagglutination-inhibition tests

Both of these tests were used for the examination of rabbit sera for the presence of antibodies to RHDV before the ELISA technique was developed. Precipitation lines were formed by highly positive sera diluted 1:4 to 1:16 and HIT titres ranged between 1:2048 and 1:4096. Results of both tests correlated well with those of ELISA.

Analysis of structural proteins of RHDV

A pronounced band representing the major virus protein with an Mr of 61K and a number of further protein bands (Fig. 1 and 2) were detected after fractionation of the purified RHDV by discontinuous SDS–PAGE and staining with Coomassie blue or silver. These structural proteins could also be identified by immunoblotting (Western blot) using sera of convalescent or hyperimmunized rabbits. Three to 14 proteins could be detected depending on the concentrations of reaction components used. Rabbit sera reacted most intensely with the dominant 61K protein and in descending order of intensity with the 38K and the 52K proteins. Weak reactions were seen with a number of other proteins ranging from 28K to 61K.

The specificity of the three major protein antigens of RHDV was confirmed by Western blot analysis. No bands could be detected (Fig. 1b, lanes 8 to 12) by HRP–SwARlgG on NC membranes incubated with negative or doubtful sera or with PBST–LAH alone. This control was necessary to exclude confusion of the 52K antigen with rabbit heavy-chain I gG (Fig. 2, lane 1). The high concentration of RHDV in the liver tissue of rabbits dying after experimental infection, and the sensitivity of Western blot analysis were documented by the demonstration of virus antigens in high dilutions (150 × ) of liver tissue (Fig. 2a, b).

The reactions of hyperimmune or convalescent rabbit sera with the virus antigens were similar to that of

Table 2. Determination by ELISA of RHDV antibody titres in rabbit sera collected in various periods and examined by Western blot analysis (Fig. 1)

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Year of serum collection</th>
<th>A492 at dilution 1:100</th>
<th>Antibody titre</th>
<th>Serum specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1988</td>
<td>1.405</td>
<td>204800</td>
<td>RARHDV – rabbit anti-rabbit haemorrhagic disease virus, own hyperimmune serum</td>
</tr>
<tr>
<td>2</td>
<td>1989</td>
<td>1.360</td>
<td>25600</td>
<td>RARHDV – Chinese reference serum</td>
</tr>
<tr>
<td>3</td>
<td>1978</td>
<td>1.093</td>
<td>1600</td>
<td>Rabbit anti-bovine IgA immunoglobulin</td>
</tr>
<tr>
<td>4</td>
<td>1980</td>
<td>1.275</td>
<td>6400</td>
<td>Rabbit anti-bovine IgG1 immunoglobulin</td>
</tr>
<tr>
<td>5</td>
<td>1986</td>
<td>1.281</td>
<td>600</td>
<td>Normal rabbit serum</td>
</tr>
<tr>
<td>6</td>
<td>1986</td>
<td>1.356</td>
<td>6400</td>
<td>Rabbit anti-rhabdovirus carpio</td>
</tr>
<tr>
<td>7</td>
<td>1989</td>
<td>1.254</td>
<td>3200</td>
<td>Normal rabbit serum</td>
</tr>
<tr>
<td>8</td>
<td>1988</td>
<td>0.219</td>
<td>Dubious</td>
<td>Normal rabbit serum</td>
</tr>
<tr>
<td>9</td>
<td>1989</td>
<td>0.012</td>
<td>Negative</td>
<td>Normal rabbit serum</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.000</td>
<td>Blank</td>
<td>Rabbit serum replaced by PBST–LAH</td>
</tr>
<tr>
<td>11</td>
<td>1989</td>
<td>0.165</td>
<td>Dubious</td>
<td>Normal rabbit serum</td>
</tr>
<tr>
<td>12</td>
<td>1975</td>
<td>0.094</td>
<td>Negative</td>
<td>Rabbit anti-swine IgM immunoglobulin</td>
</tr>
</tbody>
</table>
Results of the examination of selected sera by ELISA and Western blot analysis are given in Table 2 and Fig. 1.

Discussion

The ELISA technique described here proved useful for vaccine potency tests, the selection of rabbits suitable for experimental infection, the determination of antibodies after infection and for health checks in rabbit farms not affected by RHD so far. Naturally acquired antibodies were detected in 19.4% of 1461 rabbits from 43 farms free of RHD. The results were doubtful in 19.7% and the remaining 60.9% of rabbits were negative. These results agree with the data of Chinese authors, who detected antibodies to RHDV in 17.4% of rabbits by HIT (Pu et al., 1985).

The considerable protective effect of these antibodies against RHDV infection was confirmed in another study. Death rates after experimental infection were 4-3%, 22.2% and 97.2% in the groups of rabbits with high, low and zero antibody levels, respectively (B. Šmid and others, unpublished results).

Three proteins of M₁, 61K, 52K and 38K were clearly detected by SDS–PAGE and Western blot analysis and of these the 61K protein was predominant. However, up to 14 proteins with M₁ values ranging between 28K and 61K, reacting with various intensities with convalescent and hyperimmune rabbit sera, could be detected when the sensitivity of Western blot analysis was increased.

Chinese reference serum in Western blot analysis. Similar reactions were also seen with ELISA-positive rabbit sera, collected between 1975 and 1987.
The $M_r$ values of the two major structural proteins, 61K and 38K, were confirmed in repeated experiments and identical results were obtained with both the purified RHDV and a liver homogenate from an infected rabbit (Fig. 1 and 2). In liver homogenates, the third protein (52K) showed slight differences in migration, which were probably caused by the higher protein concentration in these samples. Our results differ considerably from those published by Xu et al. (1988), who detected seven major structural proteins with $M_r$ values of 17K to 75K, of which only two (51-9K and 51-3K) resembled our 52K protein. Ohlinger et al. (1989) demonstrated two major proteins (60K and 44K) in purified RHDV and a further six proteins (24K to 120K) in liver tissue from an infected rabbit. These data agree better with our results, although the $M_r$ of the second protein is different (38K rather than 44K).

These findings justify the classification of RHDV as a calicivirus, as the $M_r$ of the dominant calicivirus protein determined by SDS–PAGE is 60K to 65K and approaches the lower limit of this range in a discontinuous system (Bachrach & Hess, 1973; Burroughs & Brown, 1974; Burroughs et al., 1978).

Results of comparative examinations of our sera and the Chinese reference serum by Western blot analysis and ELISA have confirmed that the disease reported from China and that occurring in Europe are caused by a similar agent. However, we consider it more significant that sera collected from laboratory rabbits between 1975 and 1987, in which the presence of naturally acquired antibodies was later demonstrated by ELISA, reacted with the same virus proteins. The prevalence of positive samples (76-2%) is probably due to factors that enhance the risk of infection in laboratory rabbits (lifespan mostly 1 year or more, frequent translocations, continuous supply of animals from various sources, etc.). The agreement between ELISA and Western blot analysis not only confirms the specificity of ELISA, but also suggests that rabbit colonies had been infected with a viral agent containing the same structural proteins as RHDV, but having a lower virulence and causing no serious problems, many years before RHD was identified in China.

The connection between the import of Angora rabbits to China and the emergence of RHD (Liu et al., 1984; Xu et al., 1988) is interesting, but may be coincidental.

Reliable data supporting the theory of mutation in an originally low virulent rabbit calicivirus are not available so far.

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References


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