A new variant of the viral haemorrhagic disease of rabbits virus

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Summary: A new variant of viral haemorrhagic disease of rabbits (VHD) virus, recently detected in Poland and called Blaszki (BLA), gives positive results in enzyme-linked immunosorbent assay (ELISA) and exhibits viral protein of 60 kilodaltons (VP 60), as detected by Western blot analysis. This BLA variant of VHD virus has caused high morbidity and mortality in rabbits, as have other reported variants with similar clinical signs and pathological lesions, but – in contrast to other variants – the BLA variant gave negative results in the haemagglutination test. This development indicates the limitations of haemagglutination testing in the diagnosis of VHD.

KEYWORDS: Enzyme-linked immunosorbent assay – Haemagglutination test – Liquid immunochromatography – Viral haemorrhagic disease of rabbits virus – Western blot analysis.

INTRODUCTION

Viral haemorrhagic disease of rabbits (VHD), which was first detected in China in 1984 (13), causes serious losses in rabbit populations in many European countries. The disease is characterised by high morbidity and mortality in adult animals, whereas young rabbits aged less than two months usually survive (20). Both experimental and natural infections cause death within 48 to 72 hours, with characteristic clinical signs and pathological lesions. Surviving animals show high titres of antibodies to VHD virus (VHDV) in sera. Protection can be successfully induced by vaccination with formalin-inactivated liver homogenate of infected and dead animals. VHD is usually diagnosed by using the haemagglutination test (HA), due to the ability of VHDV to agglutinate human erythrocytes, or by enzyme-linked immunosorbent assay (ELISA). The principal VHDV protein is viral protein (VP) 60, with a molecular weight (mw) of approximately 60 kilodaltons (kDa). In the present study, a new variant of VHDV is reported. This variant, recorded in Poland and called Blaszki (BLA), can be detected by ELISA but gave negative results in the HA test.

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MATERIALS AND METHODS

Animals

The BLA-VHDV was found on a small farm (of about a hundred rabbits) in the centre of Poland. Within five days of the outbreak, 100% of the animals died, exhibiting characteristic clinical signs of VHD and pathological lesions. Experimental inoculation of six control rabbits was performed by intradermal injection of liver homogenate from dead animals.

Diagnostic tests

Haemagglutination tests were performed using type O human erythrocytes, suspended in phosphate-buffered saline (PBS), pH 7.4 (7). The tests were conducted in microtitre plates at room temperature. The results of the HA tests were highly reproducible. Before the discovery of BLA-VHDV, the results using HA and ELISA had been observed to concur perfectly. ELISA tests were performed in 96-well flat bottom plates using purified antigen and reagents (3). The microplate reader was employed for ELISA evaluation.

Sera

The hyperimmune sera used in this study were developed for HA-positive VHDV by immunising the rabbits or guinea-pigs with antigen purified by sucrose density gradient centrifugation (8).

Antigen isolation

The antigen was isolated from liver homogenates by both density gradient centrifugation and affinity chromatography (8, 19). The immunoaffinity chromatography method employed Sepharose 4B gel with covalently linked antibodies against VHDV. These antibodies were isolated from hyperimmune sera of rabbits or guinea-pigs with protein A cartridges and bound to cyanogen bromide-activated Sepharose 4B. The gel was packed into a C10/10 column and connected to the chromatography system. Supernatant from clarified (2,000 x g for 2 h) homogenised liver tissue was filtered through a 0.22 μm filter and passed through the column equilibrated with PBS. The column was extensively washed with PBS, to remove non-specifically bound proteins, and specifically bound antigen was eluted with citrate buffer, pH 4.0. The eluted antigen was dialysed overnight against PBS, tested by both HA and ELISA tests and stored in small aliquots at -70°C. To avoid cross-contamination, different prepared columns were used for different variants of VHDV. No difference in antigen purity, activity or yield was observed when immunoglobulin from rabbits or guinea-pigs was used.

Analytical methods

The analytical procedures used in this study have been described in detail (8). The concentration of purified antigen was determined with a calculator which is designed for deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) and protein determination. Purified antigens were analysed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide gel), using the Laemmli system (12).
Separated proteins were stained with Coomassie blue or Ponceau S dyes, analysed and imaged, using an electrophoresis analyser. The proteins separated by SDS-PAGE were electrophoretically transferred from gel onto polyvinylidine difluoride membrane (PVDF) for immunoblotting (18). Immediately after the transfer was completed, the membrane was incubated with 1% bovine serum albumin to block free binding sites and incubated with hyperimmune rabbit serum. After extensive washing, membranes were incubated with the second goat anti-rabbit immunoglobulin G (IgG), conjugated with alkaline phosphatase, and washed and stained with 5-bromo-4-chloro-3-indoly1 phosphate/nitro-blue tetrazolium (BCIP/NBT) substrate for alkaline phosphatase.

RESULTS AND DISCUSSION

In Poland, VHDV was first isolated and characterised in 1988. The VHDV strains found in two distinct outbreaks were immunologically identical but purified antigens were called SGM and KGM to identify the origins of the strains (9). In following years, other VHDV outbreaks occurred in Poland and these antigens (PD, ZD and MAL) were also purified and characterised (6, 10, 11).

At the same time, VHD was diagnosed in many other European countries, where the disease was endemic (1, 5, 14, 15, 16, 17).

All reported VHDV strains were immunologically indistinguishable when hyperimmune sera were used, and all were able to agglutinate human erythrocytes. This feature is commonly used in a simple diagnostic test (the HA test). Capucci et al., however, found that, of the samples which gave positive results in ELISA, approximately 5% gave negative results when HA was used (2). VP 60 could not be detected by Western blot in any of these HA-negative samples, but products of degradation of 26-28 kDa protein were detected. Similar results, indicating the existence of non-haemagglutinating strains of VHDV, have also been reported in the United Kingdom by Chasey et al. (4).

The new variant of VHDV, BLA, recently discovered in Poland, can be detected by both ELISA and Western blot analysis with VP 60 as the major antigen, but this new variant can give negative results to HA. This strain incurred 100% mortality on the farm during the five days of the outbreak. The rabbits died with characteristic clinical signs of VHD and specific pathological lesions in the lungs and liver.

Five of six experimentally infected rabbits died within 48 hours, and demonstrated the same clinical signs and pathological lesions as the field cases. The V HDV isolated from experimentally infected animals still gave negative results to HA, but gave positive results in both ELISA and Western blot analyses with major structural protein 60 kDa (VP 60).

To ensure that the new feature of the BLA variant was not caused by contamination, which can be transferred from tissue during the virus purification by gradient centrifugation, the authors decided to employ immunoaffinity chromatography. It was found that the immunoaffinity-purified KGM and SGM variants of VHDV were still HA-positive, whereas BLA remained HA-negative.

The Western blot analyses demonstrated VP 60 to be a principal antigen in the virus variants studied, including the BLA variant (Fig. 1).
FIG. 1

Comparison of Western blot results of the immunoaffinity-purified KGM, SGM and BLA variants of viral haemorrhagic disease of rabbits virus (VHDV)

Samples were electrophoretically separated in 12% gel and transferred onto polyvinylidene difluoride membrane. Antigens were reacted with hyperimmune rabbit serum, followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase

Lanes 1-3: KGM, SGM and Blaszki (BLA) variants of VHDV, respectively
Lane 4: molecular weight standards

In summary, the new variant of VHDV which has recently been isolated gives negative results using the HA test; this negative response is not a result of the VP 60 degradation process. It is possible that the BLA antigen could be distinguished from HA-positive antigens by identifying specific monoclonal antibodies. The most important information for veterinary practice is that the HA test is of limited value in the diagnosis of VHD.

ACKNOWLEDGEMENT

This study was partially supported by Project No. 055/1 of the National Veterinary Research Institute in Pulawy, Poland.

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Résumé : Un nouveau variant du virus de la maladie hémorragique virale du lapin, récemment décelé en Pologne et nommé Blaszki (BLA), est mis en évidence par l’épreuve immuno-enzymatique (enzyme-linked immunosorbent assay : ELISA) ; quant à la protéine virale de 60 kilodaltons qu’il contient (VP 60), elle est mise en évidence par la technique du « western blot ». Ce variant BLA du virus de la maladie hémorragique virale du lapin a entraîné une morbidité et une mortalité élevées, à l’instar d’autres variants s’accompagnant de signes cliniques et de lésions histologiques similaires mais, contrairement à ces derniers, le variant BLA n’est pas mis en évidence par le test d’hémagglutination, montrant ainsi les limites de cette méthode pour le diagnostic de la maladie.


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Resumen: Una nueva variante del virus de la enfermedad hemorrágica viral del conejo (EHV), descubierta recientemente en Polonia y denominada Blaszki (BLA), es positiva al ensayo inmunosorbente asociado con enzimas (enzyme-linked immunosorbent assay, ELISA) y exhibe la proteína vírica de 60 kilodaltones (VP 60), como se ha puesto de manifiesto mediante la prueba del western blot. La variante BLA del virus de la EHV ha causado una elevada morbilidad y mortalidad entre los conejos, al igual que otras variantes ya descritas que provocan síntomas clínicos y lesiones patológicas similares. Sin embargo, y en contraste con otras variantes, la BLA arrojó un resultado negativo a la prueba de hemaglutinación. Ello pone de relieve las limitaciones de esta técnica en lo que concierne al diagnóstico de la EHV.


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REFERENCES


