Acta Veterinaria-Beograd 2022, 72 (4), 433-441
UDK: 636.92.09:616.98: 578.835.3(6-17)
578.835.3:575.2(6-17)
DOI:10.2478/acve-2022-0035

*Research article*

# CONFIRMATION OF THE RABBIT HEMORRHAGIC DISEASE VIRUS TYPE 2 (GI.2) CIRCULATION IN NORTH AFRICA

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(Received 24 July, Accepted 02 November 2022)

Rabbit hemorrhagic disease (RHD) is a highly contagious viral disease that causes fatal acute hepatitis in domestic and wild lagomorphs. It has taken on major economic importance in countries like Morocco. In addition to the classical virus (RHDV), a novel emerged genotype (RHDV2) is circulating, especially in the north shore of the Mediterranean basin since 2010. Many small animal farmers reported clinical cases from several rabbitries in Agadir (Morocco) despite systematic vaccination against the RHDV. The main objective was to characterize the current RHDV strains circulating in the studied area to help to choose an adequate vaccine. For that, we extracted viral RNA from rabbit livers, carried out the PCR analyses, and we sequenced the viral structural capsid protein (VP60) of the RHDV. The phylogenetic analysis results allowed us to state that the novel genotype (RHDV2) is circulating in the studied geographical area, and to characterize the isolated sequences. As a conclusion, we recommend updating RHD epidemiological relating data and reviewing the vaccine protocols by both targeting RHDV (GI.1) and RHDV2 (GI.2) in any future preventive program.

**Keywords:** Rabbit Hemorrhagic Disease; Morocco; RHDV2 (GI.2); VP60; Phylogeny

## INTRODUCTION

Cuniculture is an agricultural activity that generates income and contributes to food security especially in rural areas around the world. According to the Food and Agriculture Organization (FAO), Leporids production amounts to 299,945,000 heads globally and 15,964,000 heads in Africa [1]. This activity remains very dependent on the health management of the rabbitries, sometimes shaken by epizootic crises caused by certain infectious diseases such as myxomatosis and rabbit hemorrhagic disease (RHD) [2].

Since its appearance in 1984 in China, RHD has continued to cause damage in lagomorphs around the world [3]. It is, etiologically, caused by an RNA virus belonging to the Caliciviridae family, genus *Lagovirus* and named Rabbit Hemorrhagic Disease

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Virus (RHDV). In infected animals, this highly contagious virus (RHDV) causes a specific hemorrhagic syndrome with a fatal outcome that is characterized by hepatic necrotic lesions [4,5]. The disease is considered to be a major threat for domestic rabbitries, as well as for the population dynamics of lagomorphs in the wild [6,7]. If this hazard is classically thwarted by the use of classical vaccines, these must change in relation to the evolution of the viruses [8].

In fact, a novel emerging virus named RHDV2 occurred in France since 2010 causing significant economic losses in industrial rabbitries, with remarkable damage to young animals even though conventional vaccines are systematically used [9]. The RHDV2 has since spread to other parts of Europe and the rest of the world, it has been reported later in Spain [10], Portugal [11], Italy [12], UK [13], Sweden [14], Australia [15], USA [16], Singapore [17], etc. Since 2017, an updated taxonomy classification based on phylogeny and genetic distances, states that the two genotypes "RHDV (GI.1) and RHDV2 (GI.2)" are a part of the RHDVs (GI) genogroup beside the EBHSV (GII) (European brown hare syndrome virus) genogroup both belonging to the same virus species "*Lagovirus europaeus*" among the Caliciviridae family [18]. In Morocco, the first reported occurrence of atypical forms of RHD already dates back to 2017 [19]. Since then, several breeders across the country have reported mortalities suggestive of RHD, especially in young animals, even though they are vaccinated against the classical strains (RHDV), which push us to speculate on an eventual mismatching between field and vaccine strains. In April 2021, investigations related to this present study, were carried out on a rabbitry in Ouled Teima (Agadir region) after having observed an epidemio-clinical profile of RHD.

Contributing to the resolution of this health problem, this current work aims to characterize the field-isolated strains using the molecular tool by sequencing the gene coding for the viral capsid structural protein (VP60) and conduct a molecular epidemiological study.

## MATERIAL AND METHODS

### Viral RNA extraction and PCR amplification

Livers were collected in rabbitries, located in the Agadir area, from autopsied animals that have probably succumbed from RHD in April 2021. In the laboratory, the RNA was extracted from liver triturates using a silica-based column kit (NucleoSpin® RNA virus, MACHEREY-NAGEL, Germany).

The molecular diagnosis of RHD was conducted by RT-PCR using the OIE reference laboratory protocol [20]. Briefly, a one-step RT-PCR was performed employing the cited protocol primers capable of detecting both RHDV and RHDV2 (forward: 5'-CCT-GTT-ACC-ATC-ACC-ATG-CC-3'; reverse: 5'-CAA-GTT-CCA-RTG-SCT-GTT-GCA-3'), used at final concentration of 0.6 μM each in AgPath-ID One-Step RT-PCR (Ambion® ABI, USA) mastermix. The thermo-profile starts with 50°C for

30 min (retrotranscription), followed by initial denaturation at 95°C for 15 min, then 40 polymerization cycles, 30 s each, of denaturation at 95°C, annealing at 62°C and extension at 72°C, with a final extension at 72°C for 10 min. PCR amplicons were visualized by electrophoresis in a 2% agarose gel; positive samples were identified by the presence of a band of ~348 bp. To verify the PCR products, they were subject to sequencing using the same primers. Eleven sequences were obtained and deposited in GenBank database with assigned accession numbers [MZ451407 - MZ451417].

### DNA sequencing and phylogeny

In order to proceed to the molecular epidemiology analyses, the viral structural capsid was targeted, adopting the PCR protocol of Le Gall-Reculé et al. [12] using the described primers: "14U1" (5'-GAA-TGT-GCT-TGA-GTT-YTG-GTA-3') and "RVP60-L1" (5'-CAA-GTC-CCA-GTC-CRA-TRA-A-3'), which amplify a 794 bp sequence located in the C-terminal of the gene encoding VP60 of RHDV2, that then were sequenced by the Sanger method. Six sequences were deposited in GenBank and assigned the following accession numbers [MZ451401- MZ451406].

The BLAST algorithm of the NCBI network server [21] was solicited for the research of similarities and to retrieve reference sequences belonging to the two pre-identified types RHDV and RHDV2 (15 annotated sequences for RHDV (GI.1) genotype and 20 sequences for RHDV2 (GI.2) genotype), plus a reference sequence of the European Brown Hare Syndrome Virus (EBHSV) as out of the group. The preliminary alignments were done by BioEdit v7.2.6 software, and the advanced ones were done on MAFT server [22]. The pairwise distances calculating and the phylogenetic tree drawing were computed using MEGA v7.0 software.

### Ethical approval

Having in mind that we used liver from autopsied animals, we did not need an ethical permission of any authority in our country.

## RESULTS

Initially, we were able to confirm the diagnosis of the RHD when the six sequences [MZ451401- MZ451406] were blasted in GenBank and showed similarities with the annotated RHD sequences. Secondly, to investigate the circulation of RHDV2 in Morocco, a maximum-likelihood phylogenetic tree was raised using the RHDV VP60 sequences giving up two distinguished groups (RHDV and RHDV2) as expected (Figure 1).

At first sight, we noticed that our six sequences clustered in the RHDV2 (GI.2) genotype clade alongside the other Moroccan and Iberian sequences. Furthermore, the overall mean distance computed on MEGA7 using the Kimura 2-parameters

model [23], across the 20 RHDV2 sequences was p = 0.038 equal to 96.2% of similarity. The estimates of evolutionary divergence between sequences give us more information about distances within the sequences belonging to RHDV2. In fact, using the same model as above, the calculating of pairwise distances showed that: the closest sequences to ours were successively [MH159170-MH159171] isolated from Morocco in 2017 with p = 0.029 [19], [KT000342] with p = 0.034 from continental Portugal [24], [KP862921-KP862933] with p = 0.037 from Azores archipelago in Portugal [25], and [KY783700] with p = 0.039 from Madeira Island in Portugal [26].

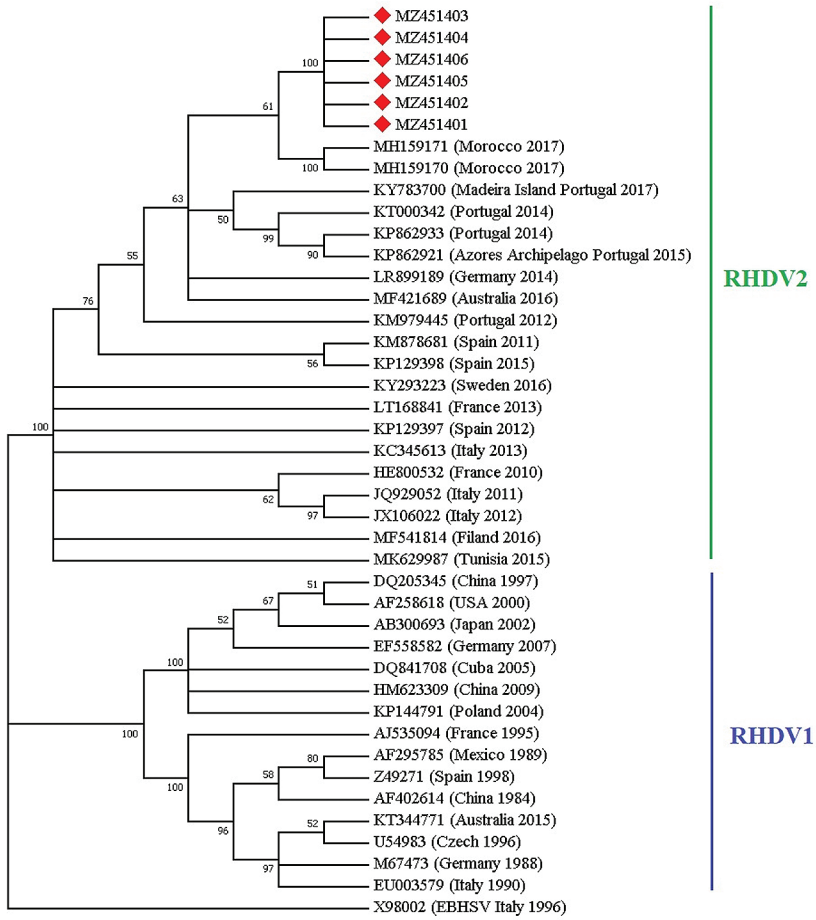


**Figure 1.** The phylogenetic condensed tree, dressed by the Maximum Likelihood method (ML) based on the Kimura 2-parameter model. The percentages of replicate trees in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 42 nucleotide sequences (6 from the current study, and 36 from Genbank). Evolutionary analyses were conducted in MEGA7.

## DISCUSSION

After its first emergence from France in 2010 [9], the RHDV2 (GI.2) spread around in Western Europe [10,12,13,27], and the other parts of the globe: America [28], Australia [29], Asia [30], and Africa. Many African countries had notified the RHDV2 occurrence such as Tunisia [31], Egypt [32], and Nigeria [33]. Given its special geographic position and proximity to Europe, Morocco as a gate of Africa was expecting the arrival of the new genotype due to the important trade flow in the Strait of Gibraltar (movement of humans and animals). In fact, the earliest alert came from the Iberian Peninsula (as seen above), from the Canary Islands [34], and from the first report of Lopes et al. [19]. Now as confirmed by the current study, the novel genotype (RHDV2: GI2) is effectively circulating in the Moroccan geographical area.

This new fact should be taken into consideration when dealing with this disease. Once in the environment, the virus could be hosted by domestic rabbits as well as wild lagomorphs that play an important role in the disease epidemiology [7,35]. In addition, the RHDV is an RNA virus that undergoes a continuous evolution process impacting its antigenicity and the choice of vaccine strains as the ultimate preventive goal [36,37].

Even though the cuniculture activity is mainly artisanal across all of Africa, the northern countries are well ahead in it [38]. In Morocco, with an average consumption of 0.7 kg/year/person, this agricultural activity has experienced moderate changes in recent years in terms of the modernization of small livestock farming units [39]. Developing this economic activity passes through the fight against the diseases that threaten it, which involves the implementation of efficient tools for diagnosis, control, and prevention of certain infectious diseases such as RHD, by the implementation of biosecurity measures as a first step, and by vaccination of animals against this highly contagious disease.

## CONCLUSION

The choice of the type of vaccine must be done carefully in order to immunize the animals against the strains present in their environment. For this, and in the light of the results of our study, we recommend updating the vaccine protocols, not only by vaccinating against the classical RHDV (GI.1) but also against the newly detected RHDV2 (GI.2) and why not a bivalent vaccine. Hence, we hope that the results of this survey will help update the epidemiological data relating to this virus and enlighten operators in the field to make the right decisions in terms of disease control measures, in particular the choice of adequate vaccines.

**Acknowledgements**

We gratefully thank the joint FAO-IAEA Division of Nuclear Techniques in Food and Agriculture for assuring the genetic sequencing service.

**Authors' contributions**

AA collected samples. AS carried out the laboratory analyses (molecular biology & phylogeny). AS and AA participated in the design of the study, then prepared and critically revised the manuscript. All authors read and approved the final manuscript.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## POTVRDA KRETANJA VIRUSA HEMORAGIJSKE BOLESTI ZEČEVA TIP 2 (GI.2) U SEVERNOJ AFRICI

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Hemoragijska bolest zečeva (RHD) je veoma kontagiozna virusna bolest koja uzrokuje fatalni akutni hepatitis kod domaćih i divljih lagomorfa. Bolest ima veliki ekonomski značaj u zemljama poput Maroka. Pored klasičnog virusa (RHDV), novi genotip

(RHDV2) cirkuliše, posebno na severnoj obali Mediteranskog basena od 2010. godine. Mnogi farmeri su prijavili kliničke slučajeve na nekoliko farmi zečeva u Agadiru (Maroko) uprkos sistematskoj vakcinaciji protiv RHDV. Glavni cilj ovog rada je bio da se okarakterišu trenutni RHDV sojevi koji kruže u proučavanom području kako bi se pomoglo u odabiru adekvatne vakcine. Izolovana je virusna RNK iz jetre kunića, izvršene PCR analize i sekvencioniran virusni strukturni kapsid protein (VP60) RHDV. Rezultati filogenetske analize su nam omogućili da konstatujemo da novi genotip (RHDV2) cirkuliše u proučavanom geografskom području i da karakterišemo izolovane sekvence. Kao zaključak, preporučujemo ažuriranje epidemioloških podataka o RHD i reviziju protokola vakcine ciljajući RHDV (GI.1) i RHDV2 (GI.2) u budućim preventivnim programima.