Microbial Metagenomic Approach Uncovers the First Rabbit Haemorrhagic Disease Virus genome in Sub-Saharan Africa

Anise N. Happi[1*], Olusola A. Ogunsanya[1], Judith U. Oguzie[2,3], Paul E. Oluniyi [2,3], Alhaji S. Olono[1], Jonathan L. Heeney[4], Christian T. Happi[2,3*]

1. Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria. Email: anisehappi@yahoo.com, shorlar7@gmail.com, halhaagy1@gmail.com

2. Department of Biological Sciences, Faculty of Natural Sciences, Redeemer's University, Ede, Nigeria.

3. African Centre of Excellence for Genomics of Infectious Diseases (ACEGID), Redeemer's University, Ede, Osun State, Nigeria. Email: oguziej@run.edu.ng, oluniyip@run.edu.ng, happic@run.edu.ng

4. Lab of Viral Zoonotics, Department of Veterinary medicine, University of Cambridge, Cambridge, UK. Email: jlh66@cam.ac.uk

* Corresponding authors: **Anise Happi**, Email: anisehappi@yahoo.com and **Christian Happi**, Email: happic@run.edu.ng

ABSTRACT

Rabbit Haemorrhagic Disease (RHD) causes high morbidity and mortality in rabbits and hares. Here, we report the first genomic characterization of Rabbit Hemorrhagic Disease Virus (RHDV) from sub-Saharan Africa. While suspected, only a single PCR finding without sequence confirmation or characterization has been reported. Here, we used a microbial metagenomic approach to confirm and characterize pathogens causing the suspected outbreak of RHD in Ibadan, Nigeria. The liver, spleen, and lung samples of five rabbits from an outbreak in 2 farms in Ibadan, Nigeria were analyzed for the vp60 gene of RHDV by RT-PCR. Subsequently, Next Generation Sequencing revealed one full and two partial RHDV2 genomes on both farms. Phylogenetic analysis showed close clustering with RHDV2 lineages from Europe, in particular, 98.6% similarity with RHDV2 in the Netherlands, and 99.1 to 100% identity with RHDV2 in Germany, suggesting potential importation from Europe. The detection of twelve unique mutations in RHDV2 sequences from the Ibadan outbreak compared to other RHDV2 sequences in the same clade suggests significant genetic diversity of the GI.2 strains in Nigeria. This highlights

the need to further understand the genetic diversity of Lagoviruses to, inform vaccine development, and for accurate tracking, monitoring, and control of outbreaks in Africa.

Key words: Rabbit Haemorrhagic disease, Nigeria, RHDV2

Introduction

Rabbit haemorrhagic disease (RHD) is a highly infectious and deadly viral haemorrhagic disease of rabbits. The disease is caused by Rabbit haemorrhagic disease virus (RHDV) a *Lagovirus* of the *Caliciviridae* family[1]. The virus causes a high morbidity and mortality rates, killing more than 90% of infected adult animals in 2-3 days following infection[2,3]. This disease causes economic losses to the rabbit meat and fur industry and great negative ecological impact in wild rabbit populations[3, 4]. RHD is among the diseases notifiable to the World Organization for Animal Health (OIE).

Transmission is via nasal, oral, conjunctival routes with possible vector-borne transmission reported[5]. The virus is also shed through excretions from infected animals[6]. The lesions of RHD include petechial hemorrhages in multiple organs as a result of virus-induced hyper-coagulopathy. The most severe form of these lesions appears in the liver, trachea, and lungs[7]. RHDV also promotes fatal hepatitis in adult rabbits[8].

The RHDV genome is positive sense and single stranded RNA of approximately 7437 nucleotides in length[1]. There are two open reading frames (ORFs); ORF1 encoding the nonstructural proteins (RNA-dependent RNA polymerase and the major capsid protein VP60) and ORF2 encoding the VP10 protein a minor structural protein[9,10].

RHD was first reported in China in 1984[11]: where over 140 million rabbits were killed in the course of an outbreak[12]. Subsequent spread of the virus was reported in Europe and other continents[13-19]. In Africa, RHDV has been reported since the late 1980s[20], but molecular characterization and diversity of the circulating variants and their impact are yet to be understood.

There are six serogroups (G1-G6) RHDV[21] and one serotype[22] before 2010. These groups are known as "classical RHDV". A new strain RHDV2 (GI.2 or RHDVb) was discovered in France in 2010[23] and is now reported in many parts of Europe[22, 24-26] and other parts of the world[27, 28]. RHDV2 was responsible for major outbreaks causing deaths in previously vaccinated adult rabbits as well as younger rabbits known to be immune to classical RHD[22, 23, 29]. This strain is now reported in Europe, Australia, Canada and more recently the USA[27, 30, 31]. RHD has been reported in some African countries like Tunisia, Morocco, Cape Verde and Egypt[7, 32-34]. In sub-Saharan Africa, outbreaks of GI.2 were reported and confirmed by enzyme immunoassays (EIA) in domestic rabbits from Benin Republic[34], and PCR from Cote d'Ivoire[30]. However, to date there are no available virus genome data from any Sub-Saharan African country, rendering it difficult to trace their origin, evolution and genetic diversity.

Following devastating outbreaks of suspected cases of RHD affecting several farmers from the southwestern region of Nigeria, there was considerable loss of investment with no surviving rabbits in some of the establishments. During the decline in the number of cases in August 2020, two farms (Farm A and Farm B) reported high mortalities and

were visited to carry out post-mortem examinations and sampling for molecular diagnosis. During the outbreak, farmers had observed symptoms similar to those caused by RHDV. The fatalities affected exotic breeds of rabbits of all ages and both sexes. The symptoms reported by farmers were anorexia (a day prior to death), lacrimal discharge, lethargy, bleeding from the oral and nasal orifices and sudden death.

Here we report the use of microbial metagenomics sequencing to uncover the first genomic characterization and whole genome sequencing of the RHDV2 responsible for an outbreak of RHD in Sub-Saharan Africa.

Results

Gross Post mortem findings

The carcasses were found to be in good body condition and slightly autolyzed (5/5). The oral and ocular mucous membranes were mildly pale (5/5). There were a few multifocal widespread petechial haemorrhages on the ventral and dorsal abdominal subcutaneous muscle (2/5), as well a few multifocal petechial haemorrhages on the pleura surface of the lungs (2/5).

RT-PCR

Two samples (RT2 and RT4) out of five were positive by RT-PCR for RHDV targeting the VP60 gene (Table 1). Bands were confirmed on electrophoresis at regions between 1500 and 2000bp (Results not shown).

Sequencing

We obtained one full genome (sample RT5) and two partial sequences (samples RT4 and RT2) (Table 1). Metagenomics analysis identified the presence of Rabbit haemorrhagic disease virus (RHDV) reads in three samples (RT2, RT4 and RT5) out of five samples sequenced. Analysis of the sequence data revealed that all three of our sequences belong to genotype RHDV2 also known as GI.2 or RHDVb. BLAST analysis revealed a sample RT5 shared a 98.58% identity with a RHDV2 strain from the Netherlands, RT4 shared a 99.05% identity with a RHDV2 strain from while RT2 shared a 100% with the same RHDV2 strain from Germany as RT4 (Figure 1). Phylogenetic analysis confirmed the results of our BLAST analysis demonstrated that the sequences from these animals belong to the RHDV2 genotype as they clustered together in the same clade with previous RHDV2 sequences from Europe and other parts of Africa (Figure 1). Analysis of Single nucleotide polymorphisms (SNPs) in the RHDV2 genome of sample RT5 obtained from our study, revealed 11 unique mutations resulting in amino acid changes. These are: Thr179Ile, Leu216Ser, Leu342Ser, Arg827Lys, Gln928Arg, Thr1337Ile, Thr1742Ile, Leu1929Pro, Ser1978Phe, Val2104Ala, Val2127Ala. Four of these mutations (Leu1929Pro, Ser1978Phe, Val2104Ala, Val2127Ala) occur in the region of target for the RT-PCR primer. Sample RT4 had a unique mutation Pro440Leu compared to other RHDV2 sequences they clustered with on the tree.

Table 1: Sample Demographics

Sample ID	Sex	Age	RT-PCR	Genome Sequence
RT1	Female	8 weeks	Negative	No
RT2	Male	8 weeks	Positive	Yes (partial)
RT3	Male	5 weeks	Negative	No
RT4	Male	3 weeks	Positive	Yes (partial)
RT5	Male	Adult	Negative	Yes (full)

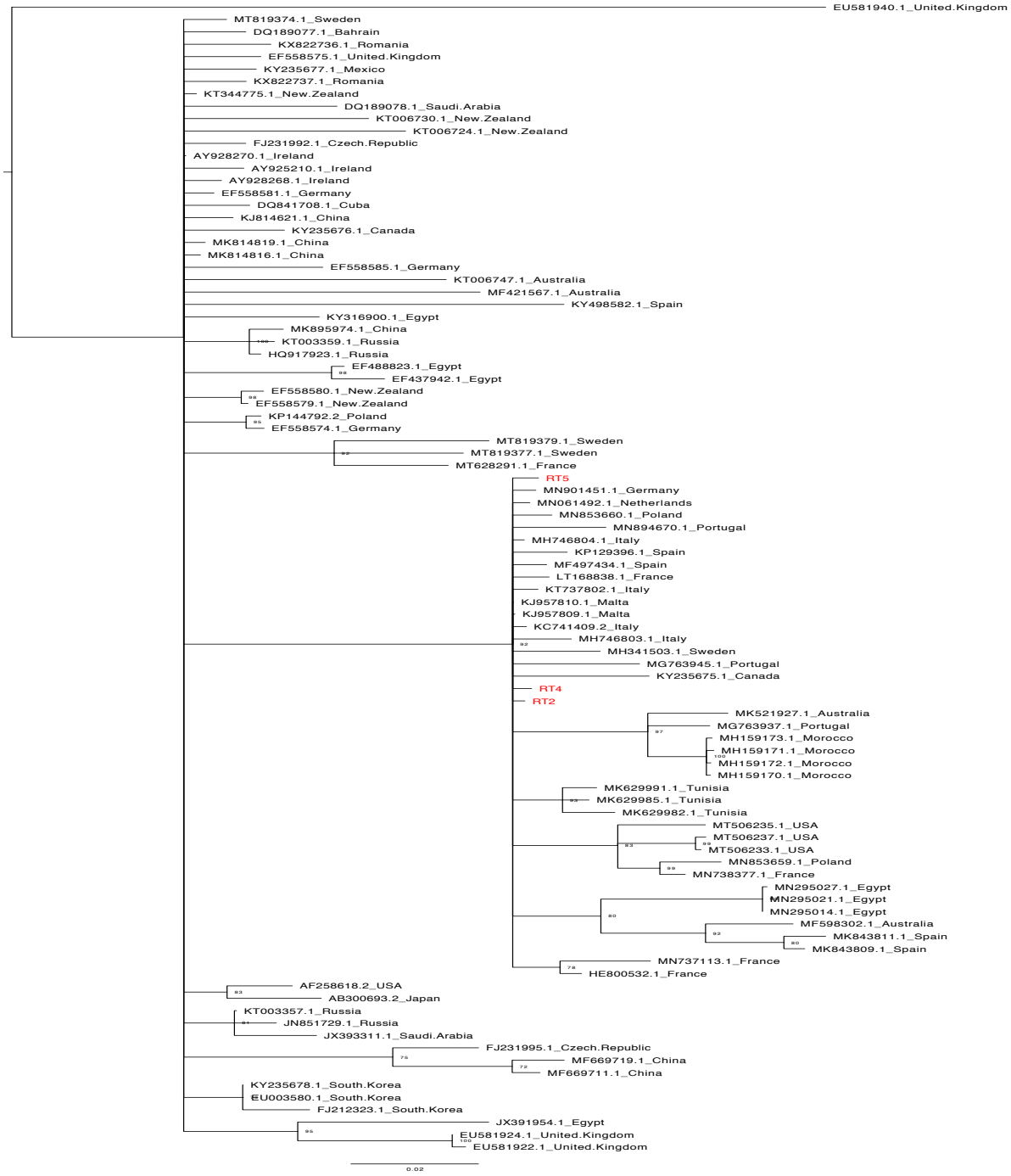

Figure 1: Phylogenetic relationship between the sequences from this study (coloured red) and representative RHDV sequences obtained from the NCBI database

173
174 **Discussion**
175 Our initial suspicion of RHD based on case history, the extent of morbidity and mortality
176 and post mortem findings was confirmed by RT-PCR in two out of five tissue samples.
177 Further metagenomic sequence analysis resulted in three genome (one full and two
178 partial) assemblies.
179 To the best of our knowledge, this study represents the first full genome sequence of
180 Rabbit Hemorrhagic Disease Virus (RHDV) in sub-Saharan Africa. Bioinformatics
181 analysis of the virus genomes revealed that the three sequences from this study belong to
182 genotype *Lagovirus* europaeus/GI.2 also known as RDHV2 or RHDVb. This genotype
183 was first identified in 2010 as a novel pathogenic form of *Lagovirus* in France[8] after
184 which it spread rapidly through Europe and other parts of the world (Oceania, North
185 America and Africa)[27, 28, 30-33]. It has also been reported to replace the former circulating
186 GI.1 strain in Australia and Portugal[35, 36]. Phylogenetic analysis showed that our
187 sequences clustered closely with previous sequences from Europe. BLAST analysis
188 revealed that sample RT5 shared a 98.58% identity with a RHDV2 strain from the
189 Netherlands, RT4 shared a 99.05% identity with a RHDV2 strain from Germany while
190 RT2 shared a 100% with the same RHDV2 strain from Germany as RT4 suggesting that
191 the virus was most likely imported into the country from Europe.
192 The *Lagovirus* europaeus/GI.2 strain is known to dominate in most regions it has been
193 found and most recently in Morocco[37]. RHDV has been circulating in some African
194 countries and more recently, there have been reports of GI.2 outbreaks in a few countries
195 from North Africa (Tunisia, Egypt and Morocco)[37].
196 RHDV strains have yet to be characterized in Sub-Saharan African despite its devastating
197 effect on rabbit farms and the industry. Given the growing distribution and the
198 devastating effect, there is a need for heightened surveillance. In Australia, it spread to all
199 states and territories and rapidly became the dominant circulating strain within 18 months
200 of initial detection[36]. Ongoing surveillance and genomic sequencing are therefore needed
201 to understand the interaction of the various *Lagoviruses* in Nigeria and their impact on
202 host populations. Next generation sequencing of more samples is also needed in order to
203 determine the time of introduction of the virus into the country and how it is spreading
204 through the country.
205 Our phylogenetic analysis also revealed high genetic diversity of the GI.2 strain as shown
206 by the number of branching events within the clade. In addition, the high number of
207 unique mutations observed in the sequences from this study further emphasizes this
208 diversity, which is likely due in part to the rapid spread and evolution of this virus. Also,
209 it may explain why it affects rabbits of all ages more or less equally in our communities.
210 The fact that some of the mutations occurred in the region of target of the RT-PCR
211 primers may be the reason why some of the RT-PCR were negative (RT5), and yet
212 yielded a full genome after NGS and metagenomic analysis. This finding emphasizes the
213 need to develop primers for diagnosis based on RHDV sequences from the country of
214 outbreak in order to avoid false negative RT-PCR results. Compiled genomic data should
215 be carefully considered when developing diagnostics and vaccines.
216 The findings from this study is a significant landmark in the field, as it has revealed the
217 circulation of the RHDV2 in Nigeria and reports the first genomics characterization of
218 RHDV in sub-Saharan Africa. The close sequence homology suggests that the virus was

most likely imported from Europe. In addition, the high genetic diversity of the GI.2 strains found highlights the need for characterization of many more samples across sub-Saharan Africa and in high incidence zones to guide the development of improved diagnostics and vaccines.

The detection of RHDV2 on unbiased metagenomic sequencing, as shown in this study illustrates the power of genomics in explaining a suspected outbreak. This ability to rapidly identify and characterize an emerging virus (RHDV 2) – in an unusual cluster– highlights the value of in-country genomics capacity. Serology using ELISA and RT-PCR are the current methods of choice for RHDV diagnosis in Sub-Saharan Africa. These diagnostic methods despite their limitations are done in very few selected laboratories. The integration of genomics capacity into the established, but siloed, pathogen-specific diagnostic platforms provides exciting opportunities for Veterinary public health surveillance.

METHODS

Post Mortem and Sample collection

In August 2020, following reports of devastating outbreaks of suspected Rabbit Haemorrhagic Disease (RHD) in Rabbit farms around, Ibadan, South-western region of Nigeria, post mortem was carried out on four carcasses from farm A and one carcass from farm B. Signalment from farm A consists of 1 female (8 weeks old) and 3 males (8 weeks, 5 weeks and 3weeks old). The breeds on the farm were Hyla. Farm B consists of one adult male Hyla. Tissue sections were collected into RNAlater from the five (5) rabbit carcasses suspected to have died from RHD. The samples were tagged RT1, RT2, RT3, RT4 (farm A) and RT5 (farm B). Tissues (liver, spleen, lungs) of each animal were pooled for RT1 - RT4 while only liver was collected for RT5. The samples were then maintained in a cold chain during transportation back to the laboratory for molecular analysis.

RNA Extraction and RT-PCR

Samples were first washed in PBS and thereafter homogenized and macerated. Total RNA was extracted from tissues macerated in TRIzol using QIAamp Viral RNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Extracted RNA was stored in -20°C until RT-PCR and sequencing.

RT-PCR was performed on extracted RNA based on modified established protocol[14] with primers targeting the VP60 gene. One-step SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, USA) was used for PCR amplification targeting the VP60 capsid protein giving a 1740 base pair product[11]. The RT-PCR final reaction volume of 25ul was made up of 12.5 µl of 2X reaction mix, 1.25µl of 20µM each of forward and reverse primers, 1µl SuperScript™ III RT/Platinum™ Taq Mix, 1µl RNA template and MgSo4 optimization to a final concentration of 2.5µM and nuclease free water to make up the reaction volume. Our thermocycling condition includes; 55 °C for 30 min, 95 °C for 15 min, then 40 cycles at 95 °C for 1 min, 58 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products were viewed in 1% gel electrophoresis (Results not shown).

264 ***Next Generation Sequencing and Bioinformatics Analysis***
265 Nextera XT sequencing Libraries were made based on established unbiased protocol[38].
266 Briefly, RNA was turbo DNase treated to remove contaminating DNA, and converted to
267 cDNA. Nextera XT sequencing libraries were made from cDNA. Libraries were
268 sequenced using the Miseq illumina platform in our sequencing platform at the African
269 Centre of Excellence for Genomics of Infectious Disease (ACEGID), in Ede, Osun State,
270 Nigeria.
271 Following sequencing, raw reads from the next-generation sequencing machine were
272 uploaded to our cloud-based platform (DNAnexus, www.dnanexus.com). Quality control
273 was carried out on the raw reads using fastqc
274 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). Metagenomics analysis
275 was carried out using Kraken2[39].
276 RHDV genomes were assembled using our publicly-available software viral-ngs v2.1.8
277 (https://github.com/broadinstitute/viral-ngs) implemented on DNAnexus.
278 Following BLASTn analysis, ninety representative RHDV sequences were obtained from
279 the NCBI database and aligned with our three sequences using MAFFT v7.388[40] with
280 further adjustment made manually as necessary in Geneious[41]. A neighbour-joining tree
281 was generated also in Geneious from a distance matrix corrected for nucleotide
282 substitutions by the Tamura-Nei model. The tree was viewed and manually edited using
283 FigTree (http://tree.bio.ed.ac.uk/software/figtree/). We aligned the three sequences from
284 this study with all sequences in the same RHDV2 clade on the tree to check for amino
285 acid mutations specific to our new sequences from Nigeria. We also mapped the RT-PCR
286 primers to our full genome obtained from this study to check if any mutation observed
287 occurred in the region the primers bind to.
288
289
290 Data Availability
291 All sequences from this study have been submitted to the National Center for
292 Biotechnology Information (NCBI) database with accession numbers MW123059 -
293 MW123061.
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Authors Contribution

A.N.H.- Initiated the work, performed the necropsy examination, advised on NGS analysis and co wrote the first draft. O.A.O.-performed the necropsy examination and sample collection, performed RT-PCR and analysis, and co-wrote the first draft. J.U.O. - performed the RT-PCR and NGS experiments and co-wrote the first draft. P.E.O. – performed Bioinformatics analysis of NGS data and discussed them. A.S.O.-participated in the necropsy examination and sample collection, revised the first draft. J. L. H.- Reviewed the manuscript. C.T.H.-Guided the NGS experiments, genomic data analysis and discussion, review the manuscript. All authors contributed to the final version of the manuscript.

Competing Interest

The authors declare no competing interest.

Additional Information: No additional information available