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Article

Epizootic Haemorrhagic Disease virus serotype 8 in Tunisia, 2021

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Abstract: Epizootic haemorrhagic disease (EHD) is a Culicoides-borne viral disease caused by epizootic haemorrhagic disease virus (EHDV) and associated with clinical manifestations in cervids and bovids. In late September 2021, EHDV was reported in cattle farms in central/western Tunisia. It rapidly spread throughout the country with more than 200 confirmed outbreaks. A combination of classical and molecular techniques was applied to characterize the causative virus as a member of EHDV-8 serotype. This is the first evidence of EHDV- 8 circulation since 1982 when the prototype EHDV-8 strain was isolated in Australia. This work highlights the urgent need for vaccines for a range of EHDV serotypes.

Keywords: EHDV; Tunisia; virus characterization; EHDV serotype 8; circulation

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1. Introduction

The Epizootic haemorrhagic disease (EHD) is a World Organization for Animal Health (WOAH, founded as OIE)-listed vector-borne disease of wild and domestic ruminants caused by EHD virus (EHDV). This virus belongs to the genus Orbivirus of the family Sedoreoviridae (ICTV, https://ictv.global/report/chapter/sedoreoviridae/sedoreoviridae/orbivirus) and is closely related to Bluetongue virus (BTV) [1]. Both viruses are transmitted by Culicoides biting midges (Diptera, Ceratopogonidae). EHD is a common disease in wild ruminants, particularly among white-tailed deer (Odocoileus virginianus) in North America, while mule deer (Odocoileus hemionus) and pronghorn (Antilocapra americana) are affected to a lesser extent. EHDV infection in deer often results in high levels of mortality associated with high fever, lethargy, oedema, ulcerations of the dental pad and oral mucosa, haemorrhaging of the heart, lungs, major blood vessels and other tissues. Historically, less severe or asymptomatic infections are observed in cattle (Bos taurus), which are considered to be the reservoir host for the virus [2-5]. EHDV has been detected globally

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across tropical and temperate regions of the Americas, Asia, Africa, Australia, and Middle East. Unlike BTV, EHDV has never been described in the European Union.

EHDV comprises 10 linear segments of double-strand RNA, coding seven structural (VP1–VP7) and four non-structural (NS1–NS4) proteins [6]. The outer capsid protein - VP2 - is the primary determinant of serotype specificity. To date, seven distinct serotypes, numbered 1, 2 and 4 to 8 have been described. However, the serological relationship between individual viruses has not been fully determined and serotype reclassification has taken place, with the previous serotype 3 now recognized to be part of EHDV serotype 1, and EHDV-318 (also referred to as EHDV-9) now referred to be EHDV-6 [7]. In addition, the highly virulent Ibaraki virus, first identified in cattle in Japan in 1959, is now considered a member of EHDV-2 [8]. A number of putative novel serotypes have also been described [9, 10]. The only commercially available vaccines are a monovalent live-attenuated vaccine and a bivalent (EHDV-2/bovine ephemeral fever virus) inactivated vaccine produced in Japan for the control the Ibaraki strain of EHDV-2. In addition, EHDV is controlled in North America by using autogenous vaccines [11].

In the past two decades there has been a gradual increase in outbreaks of EHD in cattle. The circulation of different EHDV serotypes have been documented in Ecuador (EHDV-1) [12] the island of Mayotte (EHDV-6, and 7) [13], Trinidad (EHDV-6), French Guiana (EHDV-1 and 6) [14], Egypt (EHDV-1) [15], Israel (EHDV-1, -6 and -7) [16-19], Maghreb (EHDV-6) [20, 21], Arabian Peninsula and African countries [4], and China (EHDV-7) [22]. In these outbreaks, the cattle showed BTV-like clinical signs including fever, anorexia, facial oedema, dysphagia, ulcerative and necrotic lesions of the mouth, reduction in rumination, respiratory distress, hyperaemia of teats and udder, difficulty swallowing, lameness and drop in milk production. Death, abortions, and stillbirths were also reported in some cases.

In Tunisia, the first EHDV outbreak was described in 2006. It caused high mortality, morbidity rates and economic losses for different cattle farms. Based on sequencing analyses of segment 2 (Seg-2), EHDV Tunisian strains were classified as serotype 6 (EHDV-6) and were closely related to other EHDV-6 strains circulating in the Mediterranean basin [20]. In late September 2021, EHDV was reported in cattle farms in central/western Tunisia and rapidly spread across the Northern and Eastern regions between October and November, with more than 200 confirmed outbreaks. Herein, we used a combination of classical and molecular techniques to characterize the causative virus of the 2021 outbreak.

2. Materials and Methods

2.1. The Ethical statement

The study did not involve any animal experimentation. Blood and serum samples were collected by the Tunisian Veterinary Services within the context of outbreaks investigation, following standard procedures, such that no ethical approval was required.

2.2. Samples

From late September to November 2021, a total of 174 whole blood and 241 serum samples were collected from cattle and delivered to the “Institut de la Recherche Vétérinaire de Tunisie” (IRVT), Tunisia. Cattle showed clinical signs including fever, conjunctivitis, lacrimation, drooling, erythema of nasal and oral mucosa and teat erosions (Figure 1). Most of the clinical cases were observed in late September 2021 in different delegations (the second level administrative divisions of Tunisia after the 24 governorates). The first outbreaks were reported in Central/Western Tunisia, and rapidly spread over a large part of the country with more than 200 outbreaks notified.

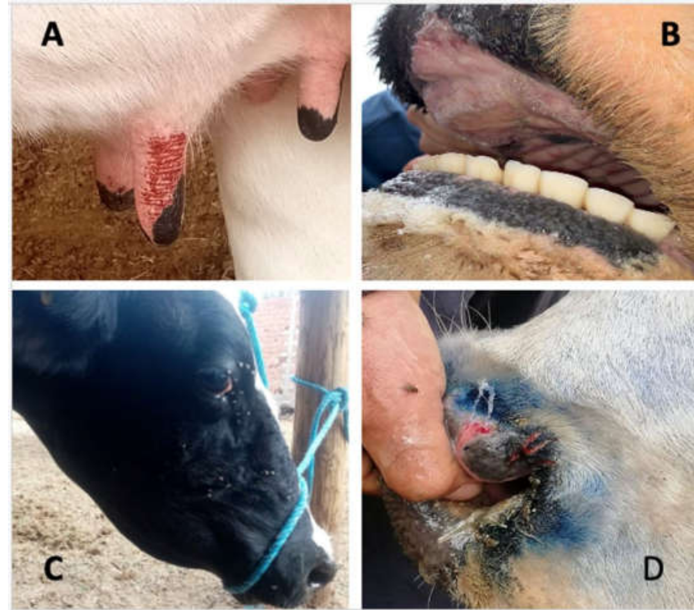

Figure 1. Clinical signs in cattle. (A) Teat erosions, (B) Oral congestion and erosions, (C) Submandibular oedema, conjunctivitis, and lacrimation, (D) Nasal discharge and mucosal erosion.

2.3. Serological tests for EHDV

A total of 241 serum samples were screened for the presence of EHDV specific antibodies using a competitive ELISA (c-ELISA) test (Innovatives Diagnostics, Grabels, France). Of these, positive serum samples were tested using serum-neutralization (SN) tests against all reference EHDV serotypes, as described in the WOAH Manual.

2.4. EHDV detection by real time RT-PCR and genotyping

Total RNA was extracted from the blood samples using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) and then tested for EHDV genome presence by real time RT-PCR using the VetMAX™ EHDV Kit (rRT-PCR; Thermo Scientific™ Waltham, MA, USA). This assay can detect a portion of Seg-9 of the 8 EHDV serotypes. EHDV-positive samples were further tested by means of genotype-specific assays targeting Seg-2 (encoding VP2) of the 8 EHDV serotypes (rRT-PCRVP2) [23,24].

2.5. Virus Isolation and virus neutralization test (VNT)

Sixteen whole blood samples with the lowest EHDV rRT-PCR CT values were selected for virus isolation on BSR cells monolayers [25]. Isolates positive for EHDV by rRT-PCR were tested [26] by VNT, in presence of reference sera, for serological serotype characterisation. Four neutralizing units of reference sera (50 µL) were added to a 10 fold dilution series of EHDV (50 µL). The serum virus mixtures were incubated for 1 h at 37 °C. BSR cells (20,000 cells in 100 µL/well) were added to each well and plates were incubated for 6 days at 37 °C with 5% CO2. A two way reduction of at least 2 logs is considered to classify a virus as belonging to the serotype of the antibody which neutralized it.

2.6. Shotgun (SG) metagenomic analysis by Oxford Nanopore MinION

Three samples with different rRT-PCR CT values (20-24-28) were selected for metagenomic shotgun (SG) analysis using the portable Oxford Nanopore MinION device, to simulate a field-deployable whole genome sequencing scenario. Total RNA was treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA) at 37°C for 20 min and then purified by RNA Clean and Concentrator-5 Kit (Zymo Research). RNA was then used for the assessment of sequence-independent single-primer amplification protocol (SISPA) with some modification [27]. The PCR products were purified by ExpinTM PCR SV

(GeneAll Biotechnology CO., Seoul, Korea) and then quantified using the Qubit® DNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation was performed in approximately 20 minutes using the SISPA products and Rapid Barcoding Kit 96 (Oxford Nanopore, UK) according to manufacturers' protocol. After flowcell priming, the library pool was loaded onto flowcell R9.4.1 (FLO-MIN106). The run parameters including the duration time (24 hr) and basecaller model (Fast basecalling) were set-up operating onto MinKNOW software. Fastq WIMP analysis was launched on EPI2ME platform to perform taxonomically classification of the fastq files in real time.

2.7. Illumina sequencing

To characterize the genome constellation of the novel EHDV, six whole blood samples with the lowest rRT-PCR CT values were selected for Illumina sequencing. Total RNA was treated and purified as previously described and then used for the assessment of SISPA [27]. The PCR products were purified by ExpinTM PCR SV (GeneAll Biotechnology CO., Seoul, Korea) and then quantified using the Qubit DNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation was performed using Illumina DNA Prep, (M) Tagmentation (96 Samples) (Illumina Inc., San Diego, CA) according to manufacturers' protocol. Sequencing was performed on the NextSeq500 (Illumina Inc., San Diego, CA, USA) by the NextSeq 500/550 Mid Output Reagent Cartridge v2 (300 cycle) (Illumina Inc., San Diego, CA, USA) and standard 150 bp paired-end reads. After quality check and trimming of raw reads data using FastQC v0.11.5 and Trimmomatic v0.36, respectively, host depletion was performed by Bowtie2 [28]. The remaining reads were used for de novo assembly using SPAdes 3.5 [29] and the consensus sequences of each genome segment were blasted against the NCBI database to identify related EHDV sequences.

2.8. Phylogenetic analysis

EHDV nucleotide sequence data was obtained from NCBI (n=1,361 download 22 March 2022). The sequence data was separated according to genome segment. Sequences that were too short or had no sample date were excluded. The final data set comprised: segment 1 n=84, segment 2 n=111, segment 3 n=96, segment 4 n=79, segment 5 n=81, segment 6 n=88, segment 7 n=96, segment 8 n=73, segment 9 n=84, and segment 10 n=73 sequences (total 866 sequences). A multiple sequence alignment was performed using MAFFT [30,31] with the FFT-NS-2 algorithm and manually inspected in Geneious Prime 2005-2022. Phylogenetic trees were estimated using the maximum likelihood method implemented in IQ-TREE utilizing the best-fit model of nucleotide substitution and ultrafast bootstrapping [32-34]. Trees were visualized in FigTree v1.4.4 (https://github.com/rambaut/figtree/releases).

3. Results

3.1. Serological tests results

Serum samples were only used for serological tests. Of 241 serum samples tested by c-ELISA, 160 were positive for EHDV antibodies. Of these, 30 samples had sufficient remaining material to also be tested by serum-neutralization for serotype-specific EHDV antibodies. The 30 EHDV positive sera showed neutralization only for EHDV-6 and EHDV-8 reference isolates but not for the other EHDV serotypes tested. Five of 30 samples were positive for EHDV-6-specific Abs only, and an additional 18/30 were positive for both, EHDV-6 and EHDV-8 specific Abs (Figure 2A). The remaining 7 sera were negative for either serotype likely due to lower sensitivity of the neutralizing antibody assay at early stages of infection. No significant difference was found in the titres of the sera from cattle obtained with the two reference strains EHDV-6 and 8, suggesting serological homogeneity.

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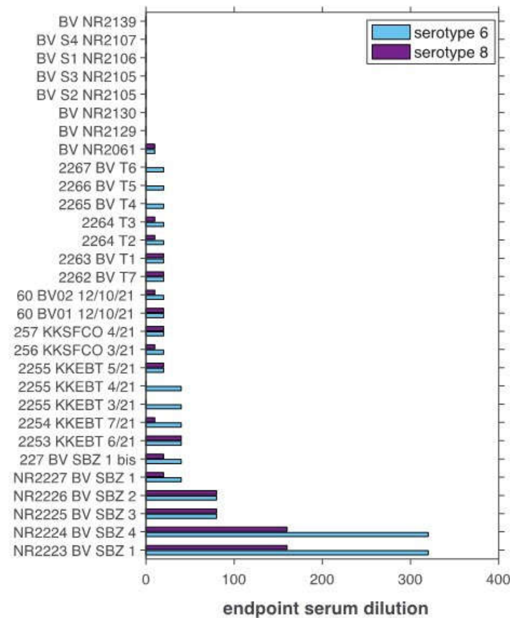

Figure 2A. Virus neutralisation assays. (A) EHDV serum neutralization test results using serotype 6 (cyan) and serotype 8 (purple) reference sera. (A) Endpoint serum dilutions for 30 serum samples. The titres represent the reciprocal value of the endpoint serum dilution.

3.2. Virus isolation and VNT

Of the 55 EHDV rRT-PCR positive samples, 16 with C_T values of 20-23 were used for virus isolation on BSR cells. Virus isolation was successful only for 5 samples and the presence of EHDV in both was confirmed by rRT-PCR. Two isolates (#13443 and #13446) were used in VNT against EHDV serotype 6 and 8 reference antiserum. Both samples showed a 2-log or higher reduction in neutralisation against serotype 8 reference antiserum, but only a 0.37 and 0.85-log reduction in neutralisation against serotype 6 reference antiserum (Fig. 2B).

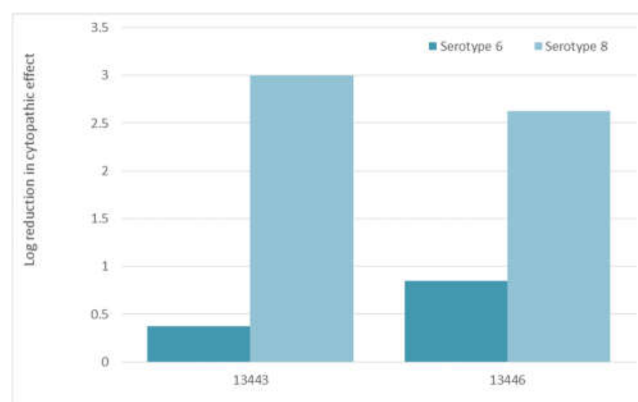

Figure 2B. Values represent the log reduction in cytopathic effects (neutralisation). A two-way reduction of at least two logs is considered to classify a virus as belonging to the serotype of the antibody which neutralized it.

3.4. EHDV genome identified by shot gun metagenomics

Three EHDV samples with C_T values of 20, 24, and 28 were processed for shot gun metagenomics sequencing. Detectable sequence reads classified as EHDV were produced within twenty minutes from the beginning of the run. At the end of the run (after 24h), the number of reads classified as EHDV per sample was 47,060 (C_T value 20), 23,555 (C_T value 24), and 1,980 (C_T value 28) with an average quality score of 10.64. Unfortunately, it was not possible to genetically characterise the EHDV serotype as the database related to

the What's in my Pot? (WIMP) application does not include EHDV reference sequences for all EHDV serotypes.

3.5. The whole genome sequence of EHDV-8/17 TUN2021 strain

Six samples with the lowest C_T values were selected for whole genome sequencing using the Illumina MiSeq technology. The total number of raw reads produced from each sample varied from 1,717,478 to 3,078,122 with average length of 143 bp. The de novo assembly produced nearly complete consensus sequences for each genome segment for all six samples. Notably, all sequences obtained showed >99.9% of nt identity with each other (excluding missing regions due to sequencing failure). Isolate EHDV-8/17 TUN2021 had the best overall horizontal and vertical coverage and was therefore considered representative and hence deposited in GenBank (acc. nos OP381190-OP381199).

A *Blastn* and *blastp* search was performed for all ten segments of EHDV-8/17 TUN2021. For segment 2 only one match was found with *blastn* with only 77% identity to a serotype 8 isolate from Australia 1982 (Table). This is the only serotype 8 isolate ever reported. Three segments (5, 7, and 8) where closest to a South Africa EHDV-6 isolate from 1996, with ~98% identity. Segments 1 and 9 were genetically close to a serotype 7 isolate from Israel 2006 (also ~98% identity), with segments 4 and 10 closest to a serotype 1 isolate from Nigeria 1967 IbAr22619 (93% and 95% identity, respectively). Finally, segment 3 was closest to a Tunisian serotype 6 isolate, while segment 6 was closest to the reference serotype 8 from Australia, but with only 71.53% identity indicating the Australian virus is not directly ancestral to that directly responsible for the Tunisian outbreak.

The sequences obtained from the *Blast* output were combined with other publicly available sequence data for EHDV as well as with the sequences obtained in the current study. Segment 2 (n=111), that encodes VP2, was used to assign the virus sequences into different serotypes (Figure 3A). Accordingly, the segment 2 phylogeny placed the sequences obtained in the study here as closest to the serotype 8 sequence from Australia isolated in 1982, consistent with the *blastn* output (Figure 3A). The phylogenetic analysis of segment 1 (VP1) and 9 (VP6) placed the Tunisian isolates into a monophyletic group with serotype 7 isolates from Israel 2006. The phylogenetic tree for segment 3 placed the Tunisian viruses with a serotype 6 isolate from Tunisia from 2006. For segment 5 (NS1), 7 (VP7), and 8 (NS2) the phylogeny placed the Tunisian isolate closest to a serotype 6 isolate from South Africa. The segment 10 (NS3) sequence fell closest to serotype 1 viruses from Nigeria 1967. Interestingly, the segment 4 (VP4) phylogeny showed that the Tunisian viruses fell into a distinct monophyletic group. Similarly, in the segment 6 (VP5) phylogeny, the Tunisian viruses formed a sister clade to other viruses including the reference EHDV-8 from Australia (Figure 3B).

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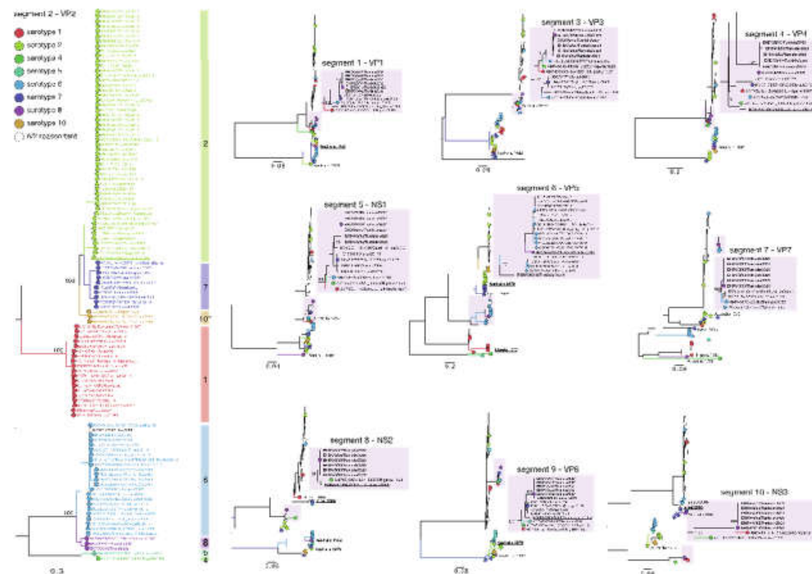

Figure 3. Phylogenetic trees for EHDV nucleotide sequences. (A) A maximum likelihood tree for segment 2 nucleotide sequences (n=111) was estimated using IQ-TREE, employing the best-fit model of nucleotide substitution. The different serotypes are indicated. Note, serotype 10 has only recently been reported from isolates in China (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7706924/), (B) Phylogenies for all other segments with clades containing Tunisian sequences from 2021 shown enlarged; segment 1 n=84, segment 3 n=96, segment 4 n=79, segment 5 n=81, segment 6 n=88, segment 7 n=96, segment 8 n=73, segment 9 n=84, and segment 10 n=73 sequences. Trees are annotated according to serotype (segment 2) and sequences from the study here are shown in bold (Tunisia 2021) and *blastn* matches are underlined (Table). Branch length indicates the number of nucleotide substitutions per site.

Table 1. Blast results for nucleotide and amino acid sequences for the Tunisia 2021 strain.

ID	Segment	blastn output strain (accession number)	% Identity
17	1	Israel 2006 ISR2006/04 serotype 7 (KM391733)	98.58
17	2	Australia 1982 CPR_3961A serotype 8 (AM745058)	77.0
17	3	Tunisia 2006 2577 serotype 6 (KC986825)	96.59
17	4	Nigeria 1967 IbAr22619 serotype 1 (AM745010)	93.44
17	5	South Africa 1996 M44/96 serotype 6 (HM636911)	97.55
17	6	Australia 1982 CPR_3961A serotype 8 (AB078633)	71.53
17	7	South Africa 1996 M44/96 serotype 6 (HM636913)	98.01
17	8	South Africa 1996 M44/96 serotype 6 (HM636914)	98.31
17	9	Israel 2006 ISR2006/04 serotype 7 (KM391738)	97.63
17	10	Nigeria 1967 IbAr22619 serotype 1 (AM745016)	95.95

4. Discussion

We describe the identification of EHDV serotype 8 in Tunisia causing outbreaks in cattle. In addition to the threat posed to Tunisian livestock, the geographic proximity to Europe represents a clear risk for its even wider spread. Notably, EHDV and BTV occupy a similar biological niche and the latter spreads repetitively to Europe via *Culicoides* in sandstorms. Surveillance is therefore crucial to mitigate disease spread across the continents.

Our initial serological investigations (ELISA and serum neutralization) performed on serum samples allowed accurate identification of the serotype. Serologically, the difference between the titres obtained against the two reference EHDV-6 and 8 strains was not significant, and therefore initial diagnosis was made based upon the most realistic scenario, with EHDV-6 as the serotype in question as this has been circulating in Tunisia previously (2006). In addition, serotype 8 virus had only been documented once before in

Australia in 1982. However, our genome sequencing analysis revealed that the Tunisian strain identified here was closest to this Australian isolate and the virus neutralization results also demonstrated that the EHDV Tunisian strain belongs clearly to EHDV serotype 8.

In combination, our phylogenetic results suggest that EHDV-8 TUN2021 has a genome constellation composed by of gene segments that are genetically close to homologous segments of EHDV strains identified in the African continent (segments 1, 3, 4, 5, 7, 8, 9, and 10), with the exception of segments 2 and 6 that were more closely related to the only EHDV-8 strain ever isolated from Australia in 1982. Importantly, however, this does not mean that segments 2 and 6 of the Tunisian strain have an Australian origin as geographic sampling of these viruses is so limited, with only a few available sequences available for most years. Indeed, it is striking that the most recent sequences deposited on NCBI prior to 2021 are isolates from Florida from 2019, sampled during an outbreak in white-tail deer [35]. In addition, only one other sequence was available from Tunisia, serotype 6 from 2006, and only four sequences were available from other African countries.

Another notable result was that the currently available molecular PCR-based typing methods failed at accurate EHDV serotyping, most likely due to the genetic variation in the VP2 nucleotide sequence between EHDV-8 TUN2021 and the EHDV-8 Australia 1982 isolate used as reference for serotype 8 specific assays. In addition, using a portable MinION device for ‘on-site’ shot gun sequencing and characterisation also failed to classify the serotype accurately due to a lack of appropriate reference sequences. We hope that our study will aid in updating these methods, as the MinION would be an extraordinary tool to boost early detection, notification, and assessment of containment measures for future outbreaks.

Currently, the only valid strategy for EHD prevention is vaccination. Unfortunately, vaccine development for EHDV remains poor. EHD vaccination is currently achieved in USA by means of autogenous vaccines which are mostly used by deer farmers, and in Japan, where a monovalent live-attenuated and a bivalent (EHDV-2/bovine ephemeral fever virus) inactivated vaccines have been produced for the control of Ibaraki strain of EHDV-2 [4]. However, these vaccines do not permit the differentiation between infected and vaccinated animals (DIVA), which is necessary for control strategies. Interestingly, an engineered vaccine using baculovirus-expressed EHDV-2 and EHDV-6 rVP2 was recently produced [36] and shown to induce homologous virus-neutralizing antibodies in cattle and mice. Hence, vaccines can reduce disease burden and infection rate, although they are serotype specific. The reported outbreaks of EHDV in Tunisia, and the potential co-circulation of multiple serotypes, highlight the need for prompt production and release of new vaccines. As this EHDV-8 strain has been shown to induce significant clinical signs, including mortality, in infected cattle, we strongly believe that countermeasures must be taken including the establishment of EHDV surveillance in the European regions previously shown to be at high risk of *Orbivirus* incursions from Northern Africa [37-40], entomological and surveillance studies to assess the biological vector of EHDV-8 in Tunisia and the circulation of this virus in other animal species including camelids [41], respectively, and the production of specific EHDV-8 vaccines able to protect animals from clinical disease. In this regard, it should be noted that a vaccine designed on EHDV-6 could be also effective at preventing EHDV-8 clinical disease, but this must be proven in dedicated experimental in vivo trials.

In summary, we present here a new EHDV-8 isolated from an outbreak in Tunisia, which is only the second strain characterized belonging to this serotype. The neutralization results confirm that this EHDV-8 strain is close to serotype 6 and belongs to the nucleotype B as previously described [7].

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Data Availability Statement: The data presented in this study are available upon request to the corresponding author. Nucleotide sequences of viral strains are available on GISAID.

Conflicts of Interest: The authors declare no potential conflict of interest with respect to the research, authorship and/or publication of this article. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply a recommendation or endorsement.

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