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# Epizootic haemorrhagic disease virus circulation in Tunisia

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**Keywords**
Epizootic haemorrhagic disease,
Epizootic haemorrhagic disease virus,
Tunisia.

**Summary**
Epizootic haemorrhagic disease virus (EHDV) was detected for the first time in Tunisia and in other Northern African countries in 2006. The objective of the present study was to investigate whether EHDV circulated in Tunisian livestock before and after the officially-reported outbreak of 2006. Thus, serum samples from cattle and dromedaries collected in different time periods (before and after 2006) and from different regions of Tunisia were screened for the presence of EHDV antibodies. Serological investigations conducted on cattle and dromedary sera collected in 2000 and 2001 demonstrated no virus circulation on these dates. However, viral circulation was evidenced in 2012 and 2013, although no EHDV cases were officially reported in these years. Serum-neutralization assessed on few ELISA positive samples, confirmed the presence of antibodies against EHDV serotype 6, which was the serotype involved in the EHDV outbreak in the Maghreb region in 2006.

## Indagine sulla presenza e circolazione del virus della Malattia emorragica epizootica in Tunisia

**Parole chiave**
Malattia emorragica epizootica,
Virus della Malattia emorragica epizootica,
Tunisia.

**Riassunto**
Il virus della Malattia emorragica epizootica (EHDV) è stato individuato per la prima volta in Tunisia e in altri Paesi del Nord Africa nel 2006. Scopo di questo studio è stato indagare l'eventuale circolazione dell'EHDV negli animali tunisini prima e dopo l'epidemia del 2006. I campioni di siero raccolti in diversi periodi (prima e dopo il 2006) e da diverse regioni della Tunisia da bovini e dromedari sono stati sottoposti a screening per la presenza di anticorpi nei confronti dell'EHDV. Le analisi sierologiche condotte sui campioni raccolti nel 2000 e nel 2001 non hanno evidenziato circolazione virale. È stata invece dimostrata la circolazione del virus nel 2012 e nel 2013, anche se in questi anni nessun caso di EHD è stato ufficialmente segnalato. Il saggio di sieroneutralizzazione, effettuato su alcuni campioni positivi al test ELISA, ha confermato la presenza di anticorpi nei confronti del sierotipo 6 del virus della malattia emorragica epizootica; lo stesso sierotipo che ha causato i focolai clinici verificatisi nella regione del Maghreb nel 2006.

Epizootic haemorrhagic disease (EHD) was first identified in 1955 in North America (Shope 1955). This is a non-contagious, infectious arthropod transmitted viral disease affecting wild and domestic ruminants, mainly deer. The acute form of the disease reported in deer is characterized by haemorrhages affecting several tissues and organs, including the skin and the heart. The peracute form is characterized by very severe oedema of the head and the neck accompanied by anorexia and respiratory distress (Savini *et al.* 2011). The EHD is caused by Epizootic haemorrhagic disease virus (EHDV), a species of the genus *Orbivirus* of the *Reoviridae* family that is closely related to Bluetongue virus (BTV) (Maclachan *et al.* 2004). Strains of EHDV from different parts of the world are classified into 7 different serotypes (Anthony *et al.* 2009 a, b) and their transmission occurs by biting midges belonging to the *Culicoides* genus (EFSA 2009, Savini *et al.* 2011). In 2006, a bluetongue-like disease was described in Tunisia, Algeria, and Morocco (OIE Disease Information). Laboratory analysis confirmed that this bluetongue-like disease was caused by EHDV serotype 6 (EFSA 2007, Ben Dhaou *et al.* 2016). Prior to 2006, even though several BTV outbreaks had occurred in the area and despite EHDV sharing a similar biological niche in terms of vectors and hosts as BTV, very few studies had been conducted in the region to investigate the epidemiology of EHDV.

As for its geographical location, Tunisia may represent a link between South and North Mediterranean shores. Considering that the vast majority of BTV strains responsible for clinical outbreaks in Europe have had a clear Northern African source and that they probably originated from sub-Saharan Africa (Lorusso *et al.* 2014), the establishment of an EHDV epidemiological picture from that area would be beneficial for global EHDV surveillance.

Therefore, in the present study first, we retrospectively analysed serum samples from cattle and free-ranging dromedaries from Tunisia that were collected prior to the EHDV outbreak of 2006. Second, we screened blood and serum samples that were collected immediately after the 2006 outbreak until recently in order to identify any sign of viral circulation.

Sera collected before 2006 consisted of 569 serum samples from cattle sampled in 2001 from the region of Beja (North part of Tunisia) and 84 serum samples from dromedaries collected in 2000 from the region of Medenine (South part of Tunisia). Sera that were collected after 2006, included 740 samples from cattle [13 collected in 2007 from Manouba (North part of Tunisia)], 577 collected in 2012 from Bizerte (North part of Tunisia), and 150 collected in 2012-2013 from scattered regions all over the whole Tunisian territory (Bizerte, Nabeul, Sousse, El kef, Beja, Sidi Bouzid, Kairouan, Kebili, and Tozeur), and 68 samples from dromedaries collected in 2012 from the region of Medenine (South part of Tunisia).

Serological and virological screening of sentinel animals was also performed: 25, 1-year old heifers from Bizerte (Northern part of Tunisia) were investigated. These animals were chosen because they were well identified since they were born in a farm owned by a state institution. All of these animals were tested, at the beginning of the study, by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) techniques in order to detect any presence of anti-EHDV or viral genome. All of them were negative. Then, these 25 heifers were sampled monthly from June to August 2013, twice a month from September to November 2013 (peak period of vector activity), and monthly from February to April 2014 (Table I). From each animal, 2 types of samples were collected: 1 consisting of serum and 1 consisting of whole blood with anticoagulant (EDTA).

Antibodies to EHDV VP7 protein were detected using a blocking ELISA (LSIVET EHDV Blocking, LSI, Lissieu, France). The assay was performed and analysed following the manufacturer's instruction (use of a positive cut-off value of the test of 60% blocking). All sera from sentinel animals and all positive EHDV ELISA samples were analysed in order to detect antibodies against BTV using the Bluetongue Competition IDVET kit (ID.vet Innovative Diagnostics, Grabels, France).

Serum-neutralization (SN) technique was used on some cattle sera that were detected positive by ELISA technique. The SN was conducted at the OIE reference Laboratory for BTV of Teramo, Italy, as described by Savini and colleagues (Savini *et al.* 2014). It permitted to determine whether the

**Table I.** *The figure shows the results of the Enzyme-linked immunosorbent assay run serum samples from cattle and dromedaries collected in Tunisia arranged according to animal species, date, and geographic region of sampling.*

| | Total sampled | Year of sampling | Geographic region | EHDV ELISA positive |
|---|---|---|---|---|
| Cattle | 569 | 2001 | North | 0 |
| | 13 | 2007 | North | 3 |
| | 577 | 2012 | North | 31 |
| | 150 | 2012 - 2013 | North, centre and south | 13 |
| | 25* | 2013 | North | 1 |
| | 25* | 2014 | North | 1 |
| Camels | 84 | 2000 | South | 0 |
| | | 2012 | South | 0 |

*Same animals sampled in 2013 and in 2014.

investigated Tunisian strains belonged to EHDV serotype 1, 2, 3, 4, 6 or 7.

Throughout the study, collected samples from sentinel animals were assessed by EHDV RT-PCR. Total RNA was extracted from 100 µl of blood using the Kingfisher 96 robot and the MagVet Universal isolation kit (LSI; reference: MV384) according to the manufacturer's instructions. Finally, the RNAs were eluted with 80 µl of ultrapure water and used in a commercial EHDV RT-PCR according to the manufacturer's instructions (LSI, Thermo Fisher, Lissieu, France). This kit allows the detection of all EHDV serotypes (by amplifying the EHDV S9 segment encoding VP6 protein) and does not cross-react with BTV. Five microliter of eluted RNA were denatured by heating to 95°C for 3 minutes in presence of 10% DMSO and added to 20 µl of EHDV mix. Sentinel blood samples were also assessed by RT-PCR that detects BTV according to a previously described protocol (Hoffman *et al.* 2010). This protocol amplifies the S10 viral segment and is able to detect all BTV-serotypes.

No antibodies against EHDV were detected in sera collected from cattle prior to 2002. All sera collected from dromedaries, before and after 2001, were also negative, EHDV ELISA results are shown in Table I. Thirty-one out of 577 sera collected from cattle in 2012 in Northern Tunisia were seropositive. The mean age of these seropositive animals was 1 and half year. Moreover, 13 out of 150 cattle sera collected from different regions of the country between 2012 and 2013, were also positive. All 25 sentinel animals were ELISA negative at the beginning of the survey (August 2013). In November 2013, seroconversion was detected in 1 of the 25 tested heifers. The same animal tested positive in the following examinations that were conducted in February, March, and April 2014.

All EHDV ELISA positive sera were negative by ELISA detecting anti-BTV.

Serum-neutralization test was performed in the OIE Reference Laboratory for Bluetongue (IZS Teramo, Italy) for 3 ELISA positive sera in order to identify the EHDV serotype. The first 2 of these 3 serum samples were collected from cattle tested in 2012 in Northern Tunisia; whereas the third was the serum of the seroconverted sentinel animal of 2013. Serum-neutralization revealed the presence of specific EHDV-6 antibodies with titres of 20 and 80 for the 2 animals that had been sampled in 2012, while the sentinel animal from 2013 was negative.

Real time PCR was assessed on EDTA blood samples collected from this sentinel animal, before and after seroconversion. However, viral RNA could not be detected.

A limitation of the study is the absence of sera collected in years immediately preceding the 2006 outbreak, so the presence of the virus between 2001 and 2006 cannot be excluded. Moreover, our study does not include samples between 2007 and 2012, so we cannot confirm or exclude any viral circulation in that period. All sera collected from dromedaries, before and after 2006, were negative for EHDV. The same result has been already described in studies in neighbouring countries; for example viral circulation has been detected in cattle and not in dromedaries in Algeria in 2008 (Madani *et al.* 2011). Also, viral circulation has not been detected in dromedaries in Morocco between 2003 and 2009 (Touil *et al.* 2012).

The presence of positive sera from 2012 in spite of the absence of officially reported clinical EHDV outbreak in the country was demonstrated. This phenomenon, which is likely due to silent viral circulation, demonstrates the utility of a larger number of sentinel animals placed all over the country for the monitoring of EHDV.

Such survey could be performed using serological methods, especially the ELISA technique that is less expensive. Molecular assays should be, then, performed to investigate samples from seroconverted animals. In the present study, molecular assay did not detect EHDV genome in blood samples collected from seroconverted animal. This could be explained by a low sensitivity of the used RT-PCR technique, by the degradation of viral RNA during transfer to the laboratory, or by a false positive ELISA result. However, this last hypothesis is to be considered with caution, because 3 samples, collected monthly (from February to April 2014) from the same animal, were ELISA positives. Serum-neutralization allowed the detection of antibodies against EHDV-6 in 2 of the 3 investigated samples. This EHDV serotype was the first involved in the 2006 EHDV outbreak (EFSA 2007, OIE 2006 a, b). Surprisingly, serum-neutralization was negative for the sample from the seroconverted animal. However, we can say with caution that it could be due to the infection with an EHDV strain belonging to serotype 5, which was not investigated in the SN test, or with another Orbivirus that cross-reacts with EHDV. The negativity of SN test can also be explained by a false positivity of the ELISA test.

Overall, our data suggest that a strict monitoring and early warning system has to be applied in Tunisia to control the circulation of some vector borne pathogens such as EHDV. For that purpose, strategies against vectors spread have to be developed and associated with the implementation of a surveillance program based on scattered sentinel animals all over the Tunisian territory. Such measures will certainly help to face the emergence of an EHDV outbreak in Tunisia and, by detecting the occurring serotype, it will address prophylaxis measures in the whole Mediterranean area.

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