Presence of bluetongue and epizootic hemorrhagic disease viruses in Egypt in 2016 and 2017

Sahar Ahmed, Mohamed Abd El-Fatah Mahmoud, Cyril Viarouge, Corinne Sailleau, Stéphan Zientara, Emmanuel Breard

PRESENCE OF BLUETONGUE AND EPIZOOTIC HEMORRHAGIC DISEASE VIRUSES IN EGYPT IN 2016 AND 2017

Sahar Ahmed[a], Mohamed Abd El-Fatah Mahmoud[b], Cyril Viarouge[c], Corinne Sailleau[c], Stephan Zientara[c], Emmanuel Breard[c]*

[a]Department of Cell Biology, Genetic Engineering and Biotechnology Division, National Research Centre, 12622 Dokki, Giza, Egypt

[b]Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Centre, 12622 Dokki, Giza, Egypt

[c] Université Paris Est, ANSES, ENVA, INRA, UMR 1161 VIROLOGIE, Laboratory for Animal Health, Maisons-Alfort, France.

***Correspondence:** Emmanuel.Breard@anses.fr

Short title: BT and EHD viruses in Egypt

Abstract

BTV and EHDV are closely-related orbiviruses that are transmitted between domestic and wild ruminants via the bites of hematophagous midges. Previous studies have reported seropositivity against BTV antibodies in sheep and goats in two Egyptian governorates (Beni Suef and Menoufia). However, no recent data are available on the BTV serotype(s) circulating in Egypt and the likely presence of EHDV has never been explored. This study investigated the presence of BTV and EHDV among cattle which had been found BTV-seropositive by ELISA method. These cattle living in proximity to sheep and goats previously found BTV-seropositive. These cattle displayed no clinical signs of BT but reproductive problems had been reported in herds. A total of 227 cattle blood samples were therefore collected in 2016 and 2017. Ninety-four of the 227 animals tested by a BTV ELISA were positive for BTV antibodies (41.4%). Of these 94 ELISA-positive cattle, only 83 EDTA-blood samples were available and therefore tested for BTV and EHDV genome detection by RT-PCR and sequencing.

Of the cattle sampled in 2016, results revealed that two were RT-PCR-positive for BTV and seven for EHDV. Sequencing showed the presence of EHDV-1 and BTV-3 genome sequences. EHDV-1 S2 shared 99.5% homology with an EHDV-1 S2 from a strain isolated in 2016 in Israel. BTV-3 S2 and S8 sequences shared more than 99.8% nucleotide similarity with the BTV-3 Zarzis S2 and S8 sequences (Tunisian BTV, also detected in 2016). Of the 66 blood samples tested following their collection in 2017, they were all EHDV-negative by RT-qPCR while five were BTV- positive by RT-qPCR. However, attempts to identify the BTV serotype of these five samples were unsuccessful. Only part of BTV S8 was sequenced and it showed 79% nucleotide similarity with S8 of atypical BTV serotypes (particularly with BTV-26 and another BTV serotype strain isolated from a sheep pox vaccine).

Overall, these findings demonstrate that both BTV and EHDV were circulating in Egypt in 2016 and 2017.

Abbreviations: BTV: Bluetongue virus; EHDV: Epizootic hemorrhagic disease virus; EHDV-1: EHDV serotype 1; BTV-3 or -26: BTV serotype 3 or 26; S: Segment; q: Real-time

Keywords: BTV, EHDV, Egypt, ELISA, genome detection

1. Introduction

Bluetongue (BT) and Epizootic hemorrhagic disease (EHD) are vector-borne viral diseases affecting domestic and wild ruminants, notifiable under OIE rules. BT and EHD viruses are species of the genus *Orbivirus*, with structural, antigenic and molecular similarities. Their genomes are composed of ten segments (S1 to S10) of double-stranded RNA which encode seven structural proteins (VP) and five nonstructural proteins (NS) (Ratinier et al., 2011). VP2 forms the outer capsid, and is therefore a target of neutralizing antibodies, as well as determining the virus serotype.

Both viruses induce variable clinical signs that mainly depend on the strain and the affected ruminant species (or breed). Both diseases have been reported to involve seasonal circulation and transmission by hematophagous midges (*Culicoides*) (Anthony et al., 2009; Maclachlan et al., 2009). Twenty-four traditional BTV serotypes in addition to novel and atypical serotypes have been identified to date (Bumbarov et al., 2016; Chaignat et al., 2009; Lorusso et al., 2018; Maan et al., 2011; Marcacci et al., 2018; Savini et al., 2017; Sun et al., 2016; Zientara et al., 2014). Since 2009, there has been a consensus recognizing seven EHDV serotypes (1, 2, and 4 to 8) (Anthony et al., 2009).

The global distribution of BT and EHD has recently expanded, possibly due to a variety of factors including increased global commerce and climate change (Guis et al., 2012; Maclachlan et al., 2009; Wilson and Mellor, 2009). In the last 20 years, various BTV serotypes have been reported in Europe and other countries bordering the Mediterranean basin, whereas EHDV has sporadically been detected in countries in the south or east of the Mediterranean basin (the Maghreb, Israel, Turkey, and Jordan) (Ben Dhaou et al., 2016; Temizel et al., 2009).

From 1998 to 2005, at least seven BTV incursions (involving five BTV serotypes (serotypes 1, 2, 4, 9 and 16)) spread across the Mediterranean basin (the Maghreb, Mediterranean islands and Southern Europe), inducing clinical signs in sheep and goats. These BTV strains entered the Mediterranean Basin and Europe through two main corridors: eastern via Turkey and Greece and

71 western via North Africa (Lorusso et al., 2014). In this last case, BTV first spread through North Africa,
72 then across multiple Mediterranean islands and finally, across southern European countries (Spain,
73 Italy and/or France).

74 In 2006, a BTV-8 strain emerged in Northern Europe and rapidly infected naive ruminants across
75 northern and eastern European countries. It was not only virulent in sheep but also, unexpectedly, in
76 cattle and its origin is still unknown. Experimental and field studies conducted on a large number of
77 abortions reported transplacental transmission of BTV-8 in cattle (Zanella et al., 2012). After
78 compulsory nationwide vaccination campaigns, Europe became free of BTV-8 in 2012. However, the
79 same BTV-8 strain re-emerged in 2015 in France (Breard et al., 2016), has since spread and is now (in
80 2019) present in mainland France, Switzerland and eastern Germany. A BTV-4 strain from Eastern
81 Europe (detected in Greece in 2014) moved westwards (through Balkan countries) and has been
82 found in Italy and mainland France in 2019 (Sailleau et al., 2018).

83 In 2018, an exotic serotype (BTV-3) was detected in sheep in Sardinia. This strain was shown
84 to be almost identical (>99% nucleotide identity) across all segments to the BTV-3 strain isolated in
85 2016 in Tunisia (Cappai et al., 2019). This new BTV-serotype incursion in the Mediterranean Basin,
86 spreading northwards, bears resemblance with the distinctive corridor where BTV-1, -2 and -4 strains
87 had previously been observed spreading from Africa into Europe (Lorusso et al., 2013). Indeed,
88 serotype 3 constitutes a possible future threat for Southern Europe.

89 Common to each *Orbivirus* is the variability of clinical outcomes after infection. In many cases,
90 BTV and EHDV induce mild or unapparent clinical infections, whereas in others they induce fever,
91 depression, respiratory distress and anorexia (MacLachlan et al., 2008; Maclachlan et al., 2009; Savini
92 et al., 2017). In the case of BTV, sheep are the most sensitive species. Although goats and cattle are
93 fully susceptible to BTV infection, they do not generally show signs of disease; it remains mild or
94 subclinical. Cattle show longer periods of BTV viremia than sheep and are considered reservoirs of
95 the virus. Experimental and field studies have reported clinical signs of BT in cattle and goats,

particularly when outbreaks have occurred in non-endemic areas (MacLachlan, 2004). Historically, EHDV has been associated with disease in wild cervids (Jessup, 1985; Odiawa et al., 1985). Severe clinical signs induced by EHDV have also been observed in infected cattle (Maclachlan et al., 2015; Omori et al., 1969). EHDV-6 or -7 has sporadically spread in countries surrounding the Mediterranean Basin and has induced clinical forms in cattle (Ben Dhaou et al., 2016; Temizel et al., 2009; Yadin et al., 2008). More precisely, in 2006, outbreaks of EHDV-6 were reported in cattle from Turkey, Morocco, Algeria, Tunisia and Jordan when an EHDV-7 strain was involved in outbreaks in Israel. The more recent outbreaks of EHD in the Mediterranean Basin were recognized clinically in 2015 In Israel involving an EHDV-6 strain (Golender et al., 2017). The introduction origins of these EHDV-6 and 7 strains detected in Mediterranean countries since 2006 are still unknown, however, analyses of their genome segments indicate a common "African/Arabian Peninsula and Indian Ocean Asia" origin (Ben Dhaou et al., 2016; Golender et al., 2017).

Studies concerning the presence of orbiviruses have been conducted in various countries neighboring Egypt and have confirmed the endemic nature of BT and EHD in these areas (Mejri et al., 2018; Sghaier et al., 2017). In Egypt, several serological studies reported BTV seroprevalence (Mahmoud and Khafagi, 2014; Mahmoud et al., 2017). In 1977, a BTV-4 strain from Egypt was isolated by the Pirbright Institute (ReoID). In 1987, BTV serotypes 1, 4, 10, 12 and 16 were identified through seroneutralization assays in retrospective studies from Egyptian sera (Ismail et al., 1987). Recent investigations carried out on sheep and goats have revealed the presence of antibodies against BTV in these domestic species in two Egyptian governorates (Beni Suef and Menoufia) (Mahmoud et al., 2017). Egyptian veterinary authorities wanted to determine whether BTV could have been involved in the reproductive problems reported in 2016 and 2017 in several herds located in these two Egyptian governorates. This study reports the results of the molecular detection of BTV and EHDV that was carried out on cattle samples collected in this context.

2. Material and methods

2.1. Animals and samples collected

A total of 227 cattle blood samples were collected from two herds in Beni Suef (72 animals sampled in 2016) and three herds in Menoufia (155 animals sampled in 2017). The animals were apparently healthy, but reproductive problems had been reported in these herds. Serum and EDTA-blood samples were stored in the National Research Centre (NRC) (Egypt) at -20°C until use.

2.2. ELISA

The ID Screen Bluetongue Competition ELISA kit (IDVet, Grabels, France) was used as per the manufacturer's instructions to initially detect BTV VP7 antibodies in all 227 serum samples. Eighty-three EDTA-blood samples from ELISA-positive cattle were then used for the molecular diagnosis of BTV and EHDV.

2.3. RNA extraction

A first batch of 17 RNA extracts came from 17 EDTA-blood samples collected in 2016 in Beni Suef. This RNA was purified in Egypt by the National Research Centre (NRC) using the TRIzol method. Two hundred and fifty µl of EDTA-blood was extracted using TRIzol LS Reagent as per the manufacturer's instructions. Total RNA was eluted into 30 µl of RNase-free water. The extraction products were then sent to ANSES's Laboratory for Animal Health (Maisons-Alfort, France) for RT-PCR assays and sequencing.

The second batch consisted of 66 EDTA-blood samples (from ELISA-tested cattle found positive for BTV) sent to ANSES's Laboratory for Animal Health in order for RNA extraction to be carried out using the MagVet Universal Isolation kit (Thermo Fisher Scientific, Lissieu, France). Total RNA was then extracted from 100 µl of EDTA-blood using an automated method (KingFisher Flex automated

145 extraction platform (ThermoFisher Scientific) and the MagVet Universal nucleic acid extraction kit
146 (Thermo Fisher)). Total RNA was eluted into 80 µl of RNase-free water.
147
148 **2.4. RT-PCRs**
149 Five µl of denatured RNA (heated at 95°C for 3 minutes) was then tested using each RT-PCR
150 method (real-time RT-PCR (RT-qPCR) or conventional RT-PCR). Commercial RT-qPCR kits were used
151 for pan-BTV detection (Adiavet BTV kit (Bio-X Diagnostics, Saint Brieuc, France)) and for detecting
152 BTV-1, -2, -4, -8, -9 or -16 (serotypes currently found in Europe) (VetMAX European BTV Typing Kit),
153 as per the manufacturer's instructions. An in-house BTV-3 RT-qPCR (Lorusso et al., 2018) was also
154 used.
155 In-house RT-qPCRs were used to specifically detect the EHDV genome; these RT-qPCRs were EHDV-
156 group or -serotype specific (Viarouge et al., 2015).
157 For each of the in-house RT-qPCRs targeting EHDV or BTV segments, different primers and
158 probe sets were designed in order to amplify part of the targeted gene using the AgPath One-Step
159 RT-qPCR Kit (Life Technologies). Amplifications were carried out using the same cycling parameters
160 for all RT-qPCR assays: 45°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s then
161 60°C for 1 min. These same cycling parameters were also applied when using commercial RT-qPCR
162 kits: in-house RT-qPCR methods have been developed to be used together, in a single amplification
163 run that could incorporate various commercial RT-qPCR mixes. Conventional RT-PCRs for BTV S2, S8,
164 S9 and S10 sequence amplifications (Breard et al., 2007; Sailleau et al., 2012; Viarouge et al., 2014)
165 were also performed using the Qiagen OneStep RT-PCR kit (Qiagen, France). The amplification
166 products were directly sequenced, in both directions, according to the Sanger method by Eurofins.
167
168 **2.5. Phylogenetic analyses**

Sequences were assembled and analyzed using EditSeq, SeqMan and Megalign Software (DNA Star Inc.) and aligned using ClustalW (DNAstar programs, Lasergene). The EditSeq program was used to split the sequence into individual files, SeqMan was used to assemble the reads of each sequence into contigs, and the Megalign program was then used to calculate sequence relationships, compare phylogenetic associations among BTV strains and construct phylogenetic trees based on the nucleotide sequences. The Megalign program uses the Neighbor-Joining method based on a matrix of "distances" between all sequences. The phylogenetic trees generated by Megalign are rooted trees, regardless of the method used for alignment.

2.6. Isolation assays

Isolation assays were carried out (at NRC and ANSES) on embryonated eggs (Clavijo et al., 2000) and KC cells (*Culicoides sonorensis* cell line)(Wechsler et al., 1989) using blood samples found positive for BTV or EHDV by RT-qPCR. The blood samples were inoculated onto KC cells. Briefly, a confluent monolayer of KC cells was inoculated with EDTA-blood samples diluted 10^{-1} in sterile PBS. The inoculum was removed 24 hours after inoculation and the cells were incubated with an appropriate cell culture medium (Schneider's Drosophila Media (ThermoFisher Scientific) and 10% of foetal calf serum). After 7 days of incubation at 28°C, KC cell culture supernatants were tested for the presence of BTV or EHDV RNA by RT-qPCR.

In parallel, groups of three embryonated chicken eggs were each inoculated intravenously with 0.1–0.2 ml of a 10^{-1} dilution of lysed blood samples. The eggs were incubated for 7 days at 35°C and examined daily using a cold candling lamp. The embryos that died within 24 h were discarded. After 1 week of incubation, the embryos were weighed and homogenized in Minimum Essential Medium (weight/volume: 1 / 10). Total RNA was then extracted from 100 µl of embryo lysate and tested by EHDV or BTV RT-qPCR.

3. Results and discussion

Serological analysis by ELISA revealed that 41.4% (94/227) of blood samples tested using the VP7 competitive ELISA were positive for BTV antibodies (Table 1). In each governorate, the percentage of cattle found positive for BTV by ELISA was similar (Table 1).

The BTV genome was looked for only in these ELISA-positive cattle. This limit was imposed due to findings from previous studies: when serum and EDTA-blood from BTV-infected animals (in the field) were tested, the animals that tested positive for BTV by RT-qPCR were mainly also found positive for BTV by the ELISA method (Breard et al., 2005; Verdezoto et al., 2018; Viarouge et al., 2014). An ELISA (commercial kit) is easy to perform in any laboratory and in our studies enables non-infected herds to be set aside in order not to undergo molecular analyses. Furthermore, when samples come from potentially endemic areas of BTV (Ecuador, Galapagos Islands and French Guiana in South America, Reunion Island in the Indian Ocean, and Tunisia in Africa), we systematically look for the EHDV genome or its antibodies (Ben Dhaou et al., 2016; Breard et al., 2004; Breard et al., 2005; Sailleau et al., 2012; Verdezoto et al., 2018; Vinueza et al., 2019). In all these studies, EHDV was always present in areas where BTV was also found. It is also common to find animals co-infected by both viruses.

Eighty-three EDTA-blood samples from these 94 ELISA-positive cattle were available and tested for BTV and EHDV genome detection by RT-qPCR. Of the first 17 RNA extracts sampled in 2016 and sent to ANSES, two head of cattle (C19 and C44) were positive by BTV group-specific RT-qPCR (Table 2). Both of these samples were also found to be positive by RT-qPCR specific to BTV-3 and negative by the RT-qPCR that amplified BTV-1, -2, -4, -8, -9, and -16. S2 (VP2) and S8 (NS2) sequences (754 bp (GenBank Accession no. MH706764) and 942 bp (GenBank Accession no. MH706766) respectively) were obtained by conventional RT-PCR from C44 RNA, showing 99.86% and 99.89% homology with the Tunisian BTV-3 strain (Zarzis) isolated in 2016 (Sghaier et al., 2017). A phylogenetic tree (Figure 1) illustrates the homology between Egyptian BTV-3 S2 and the S2 from BTV-3 strains isolated across

219 the world. These results demonstrate that the BTV-3 variant detected in Tunisia in 2016 was also
220 present in Egypt that same year and supports the hypothesis that BTV-3 Zarzis had moved westwards
221 after originating from North-East Africa.

222 A particular surprise was to detect a specific EHDV genome in seven cattle blood samples
223 collected in 2016. EHDV-1-specific RT-qPCR (Viarouge et al., 2015) allowed the EHDV serotype to be
224 determined for six of the seven pan-EHDV-positive cattle (Table 2). Only the sample with a Ct of 34.6
225 was not positive by EHDV-1 RT-qPCR, no doubt due to its low viral load. The tree is based on a single
226 sequence sample (longer sequences were obtained with RNA from C17). The serotype was confirmed
227 using conventional RT-PCR in three out of the seven EHDV-positive cattle and sequencing results
228 demonstrated that the amplification products (1839 bp, GenBank Accession no. MH706767) shared
229 99.5% homology with S2 of an EHDV-1 strain isolated in 2016 in Israel (GenBank Accession no.
230 MG808409), 95.86% homology with an EHDV-1 S2 isolated in Nigeria in 1967 and less than 87% with
231 other known EHDV-1 S2 sequences available in GenBank (Figure 2). One S2 sequence was obtained
232 from C20 and C21 (about 800 bp) with 100% homology with sequence S2 from C17. To our
233 knowledge, this study is the first report of EHDV-1 in North Africa. EHDV-6 and 7 have previously
234 been found in the Maghreb, Turkey and Israel (Ben Dhaou et al., 2016; Temizel et al., 2009; Yadin et
235 al., 2008) whereas EHDV has never yet been reported on any Mediterranean islands or in Europe.

236 Out of the 66 blood samples collected in 2017 in Menoufia and extracted in ANSES's
237 laboratory, five were found positive for BTV by RT-qPCR and all were negative for EHDV by RT-qPCR
238 (Tables 1 and 2). No amplification product was obtained when either classical or serotype-specific RT-
239 qPCR targeted BTV S2. In this study, the authors tried to determine the BTV serotype(s) by RT-qPCR
240 or conventional RT-PCR. Finding no success for some samples, we subsequently tried to amplify and
241 sequence BTV segments other than S2 (easier to amplify because they have more conserved
242 nucleotide sequences than S2) (Breard et al., 2005; Sailleau et al., 2012). The objective was then to
243 confirm, using a BTV genome sequence, the positive results obtained for the group by pan-BTV RT-

qPCR (targeting S10). Indeed, we tried to amplify S8, S9, and S10 , but only segment 8 amplification worked for one out of the five BTV-positive animals sampled in 2017 (914 bp, GenBank Accession no. MH706765). The S8 sequence showed 79.86% homology with S8 of a BTV strain isolated from a sheep pox vaccine (Bumbarov et al., 2016) and 78.99% with BTV-26 (Maan et al., 2011) (data not shown). These five RT-qPCR-positive blood samples obtained in 2017 were also negative with BTV-3 RT-qPCR. Considered as a whole, the BTV RT-PCR results suggest that other BTV serotypes were able to circulate at least in the Menoufia governorate in 2017, and that one of these BTV serotypes was atypical, like many other similar BTV serotypes recently discovered in the Mediterranean Basin and Europe (Chaignat et al., 2009; Lorusso et al., 2018; Schulz et al., 2016; Sghaier et al., 2017).

Unfortunately, no virus was isolated from EHDV- or BTV-positive blood samples, most likely due to their pre-analysis storage at -20°C or the fact that animals were sampled after the viremia period, when the virus had been neutralized but viral RNA was still present in the blood and detectable by RT-PCR. Isolating EHDV or BTV strains in most cases allowed us to obtain full EHDV or BTV genomes by the NGS method (Breard et al., 2016; Schulz et al., 2016; Verdezoto et al., 2018). Obtaining the full genome for every strain would have allowed us to verify that the Egyptian BTV-3, which has the same S2 and 8 as the Tunisian Zarzis strain, was not a reassortant virus for the other eight RNA segments.

4. Conclusions

This study showed that BTV-3 (Zarzis strain) and EHDV-1 were present in Egypt in 2016 in the same period during which BTV-3 was detected in Tunisia and EHDV-1 in Israel. These data confirm that Egypt constitutes an area of particular interest for investigating BTV and EHDV epidemiology in the Mediterranean Basin. Additional studies are necessary in order to estimate BTV and EHDV seroprevalence in this part of Africa, and to isolate BTV and EHDV strains so as to characterize the full genome of these orbiviruses present in the Mediterranean basin. Further collaboration must be

established between European and Mediterranean countries in order to control the spread of BTV more easily and effectively.

Funding: This work was supported by the EMHOTEP Program-Campus France (No. 35156PK) and by the H2020 PALE BLU project (Grant Agreement no. 727393).

Conflict of Interest Statement: Declarations of interest: none.

277 Table 1

278

year of sampling	governorate	BTV ELISA				PCR		
		number of cattle tested	positive	negative	% of positive cattle	number of ELISA-positive cattle tested	BTV-positive	EHDV-positive
2016	Beni Suef	72	28	44	38.9	17	2	4
2017	Menoufia	155	66	89	42.6	66	5	0
	Total	227	94	133	41.4	83	7	4

279

280

281

282 Table 2

283

Location	Date of sampling	Animal n°	Ct value with pan-BTV PCR	Ct value with pan-EHDV PCR	Serotype
Beni Suef	13/10/2016	C44	19.1	-	BTV-3 [a b]
	6/11/2016	C13	-	30	EHDV-1 [a]
		C17	-	26.7	EHDV-1 [a b]
		C19	31.6	-	BTV-3 [a]
		C20	-	26.8	EHDV-1 [a b]
		C21	-	26	EHDV-1 [a b]
		C7	-	34.6	undetermined
		C1	-	29.7	EHDV-1 [a]
		C9	-	27	EHDV-1 [a b]
Menoufia	27/07/2017	C60	31.7	-	undetermined
		C61	36.9	-	undetermined
		C69	29.4	-	undetermined [c]
		C86	31.2	-	undetermined
		C87	30.9	-	undetermined

284 [a]: determined with RT-qPCR.
285 [b]: determined with sequencing.
286 [c]: only NS2 sequence obtained.
287

Captions

Table 1: ELISA and PCR results from 277 Egyptian cattle sampled in 2016 and 2017 in two Egyptian governorates.

Table 2: RT-PCR and sequencing results of the 14 head of cattle found positive for BTV or EHDV by RT-qPCR sampled in 2016 and 2017 in Beni Suef and Menoufia governorates.

Figure 1: Phylogenetic tree of full-length segment 2, showing the relationships between EHDV-1 2016 Egypt (MH706767) segment 2 (bold letters) and homologous EHDV serotypes (Genbank). The phylogenetic tree of nucleotide sequences was constructed using MegAlign Clustal W method (DNASTAR software, Lasergene 8).

Figure 2: Phylogenetic tree of full-length segment 2, showing the relationships between BTV-3 Egypt (MH706764) segment 2 (bold letters) and homologous BTV-3 serotypes (Genbank). The phylogenetic tree of nucleotide sequences was constructed using MegAlign Clustal W method (DNASTAR software, Lasergene 8).

References

Anthony, S.J., Maan, S., Maan, N., Kgosana, L., Bachanek-Bankowska, K., Batten, C., Darpel, K.E., Sutton, G., Attoui, H., Mertens, P.P., 2009. Genetic and phylogenetic analysis of the outer-coat proteins VP2 and VP5 of epizootic haemorrhagic disease virus (EHDV): comparison of genetic and serological data to characterise the EHDV serogroup. Virus Res 145, 200-210.

Ben Dhaou, S., Sailleau, C., Babay, B., Viarouge, C., Sghaier, S., Zientara, S., Hammami, S., Breard, E., 2016. Molecular characterisation of epizootic haemorrhagic disease virus associated with a Tunisian outbreak among cattle in 2006. Acta Vet Hung 64, 250-262.

Breard, E., Sailleau, C., Hamblin, C., Graham, S.D., Gourreau, J.M., Zientara, S., 2004. Outbreak of epizootic haemorrhagic disease on the island of Reunion. Vet Rec 155, 422-423.

Breard, E., Sailleau, C., Hamblin, C., Zientara, S., 2005. Bluetongue virus in the French Island of Reunion. Vet Microbiol 106, 157-165.

Breard, E., Sailleau, C., Nomikou, K., Hamblin, C., Mertens, P.P., Mellor, P.S., El Harrak, M., Zientara, S., 2007. Molecular epidemiology of bluetongue virus serotype 4 isolated in the Mediterranean Basin between 1979 and 2004. Virus Res 125, 191-197.

Breard, E., Sailleau, C., Quenault, H., Lucas, P., Viarouge, C., Touzain, F., Fablet, A., Vitour, D., Attoui, H., Zientara, S., Blanchard, Y., 2016. Complete Genome Sequence of Bluetongue Virus Serotype 8, Which Reemerged in France in August 2015. Genome Announc 4.

Bumbarov, V., Golender, N., Erster, O., Khinich, Y., 2016. Detection and isolation of Bluetongue virus from commercial vaccine batches. Vaccine 34, 3317-3323.

Cappai, S., Rolesu, S., Loi, F., Liciardi, M., Leone, A., Marcacci, M., Teodori, L., Mangone, I., Sghaier, S., Portanti, O., Savini, G., Lorusso, A., 2019. Western Bluetongue serotype 3 in Sardinia, diagnosis and characterization. Transbound Emerg Dis.

Chaignat, V., Worwa, G., Scherrer, N., Hilbe, M., Ehrensperger, F., Batten, C., Cortyen, M., Hofmann, M., Thuer, B., 2009. Toggenburg Orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. Vet Microbiol 138, 11-19.

Golender, N., Khinich, Y., Gorohov, A., Abramovitz, I., Bumbarov, V., 2017. Epizootic hemorrhagic disease virus serotype 6 outbreak in Israeli cattle in 2015. J Vet Diagn Invest 29, 885-888.

Guis, H., Caminade, C., Calvete, C., Morse, A.P., Tran, A., Baylis, M., 2012. Modelling the effects of past and future climate on the risk of bluetongue emergence in Europe. J R Soc Interface 9, 339-350.

Ismail, J.M., Martin, J., Nazmi, A., 1987. Bluetongue neutralization test with different virus under variable conditions. Agric Res Rev Egypt 65, 867-872.

Jessup, D.A., 1985. Epidemiology of two orbiviruses in California's native wild ruminants: preliminary report. Prog Clin Biol Res 178, 53-65.

Lorusso, A., Sghaier, S., Ancora, M., Marcacci, M., Di Gennaro, A., Portanti, O., Mangone, I., Teodori, L., Leone, A., Camma, C., Petrini, A., Hammami, S., Savini, G., 2014. Molecular epidemiology of bluetongue virus serotype 1 circulating in Italy and its connection with northern Africa. Infect Genet Evol 28, 144-149.

Lorusso, A., Sghaier, S., Carvelli, A., Di Gennaro, A., Leone, A., Marini, V., Pelini, S., Marcacci, M., Rocchigiani, A.M., Puggioni, G., Savini, G., 2013. Bluetongue virus serotypes 1 and 4 in Sardinia during autumn 2012: new incursions or re-infection with old strains? Infect Genet Evol 19, 81-87.

Lorusso, A., Sghaier, S., Di Domenico, M., Barbria, M.E., Zaccaria, G., Megdich, A., Portanti, O., Seliman, I.B., Spedicato, M., Pizzurro, F., Carmine, I., Teodori, L., Mahjoub, M., Mangone, I., Leone, A., Hammami, S., Marcacci, M., Savini, G., 2018. Analysis of bluetongue serotype 3 spread in Tunisia and discovery of a novel strain related to the bluetongue virus isolated from a commercial sheep pox vaccine. Infect Genet Evol 59, 63-71.

Maan, S., Maan, N.S., Nomikou, K., Batten, C., Antony, F., Belaganahalli, M.N., Samy, A.M., Reda, A.A., Al-Rashid, S.A., El Batel, M., Oura, C.A., Mertens, P.P., 2011. Novel bluetongue virus serotype from Kuwait. Emerg Infect Dis 17, 886-889.

MacLachlan, N.J., 2004. Bluetongue: pathogenesis and duration of viraemia. Vet Ital 40, 462-467.

MacLachlan, N.J., Crafford, J.E., Vernau, W., Gardner, I.A., Goddard, A., Guthrie, A.J., Venter, E.H., 2008. Experimental reproduction of severe bluetongue in sheep. Vet Pathol 45, 310-315.
Maclachlan, N.J., Drew, C.P., Darpel, K.E., Worwa, G., 2009. The pathology and pathogenesis of bluetongue. J Comp Pathol 141, 1-16.
Maclachlan, N.J., Zientara, S., Savini, G., Daniels, P.W., 2015. Epizootic haemorrhagic disease. Rev Sci Tech 34, 341-351.
Mahmoud, M.A., Khafagi, M.H., 2014. seroprevalence of bluetongue in sheep and goats in Egypt. veterinary world 7, 205-208.
Mahmoud, M.A.E., Elbayoumy, M.K., Sedky, D., Ahmed, S., 2017. Serological investigation of some important RNA viruses affecting sheep and goats in Giza and Beni-Suef governorates in Egypt. Vet World 10, 1161-1166.
Marcacci, M., Sant, S., Mangone, I., Goria, M., Dondo, A., Zoppi, S., van Gennip, R.G.P., Radaelli, M.C., Camma, C., van Rijn, P.A., Savini, G., Lorusso, A., 2018. One after the other: A novel Bluetongue virus strain related to Toggenburg virus detected in the Piedmont region (North-western Italy), extends the panel of novel atypical BTV strains. Transbound Emerg Dis 65, 370-374.
Mejri, S., Dhaou, S.B., Jemli, M., Breard, E., Sailleau, C., Sghaier, S., Zouari, M., Lorusso, A., Savini, G., Zientara, S., Hammami, S., 2018. Epizootic haemorrhagic disease virus circulation in Tunisia. Vet Ital 54, 87-90.
Odiawa, G., Blue, J.L., Tyler, D.E., Shotts, E.B., 1985. Bluetongue and epizootic hemorrhagic disease in ruminants in Georgia: survey by serotest and virologic isolation. Am J Vet Res 46, 2193-2196.
Omori, T., Inaba, Y., Morimoto, T., Tanaka, Y., Ishitani, R., 1969. Ibaraki virus, an agent of epizootic disease of cattle resembling bluetongue. I. Epidemiologic, clinical and pathologic observations and experimental transmission to calves. Jpn J Microbiol 13, 139-157.
Ratinier, M., Caporale, M., Golder, M., Franzoni, G., Allan, K., Nunes, S.F., Armezzani, A., Bayoumy, A., Rixon, F., Shaw, A., Palmarini, M., 2011. Identification and characterization of a novel non-structural protein of bluetongue virus. PLoS Pathog 7, e1002477.
Sailleau, C., Breard, E., Viarouge, C., Gorlier, A., Leroux, A., Hirchaud, E., Lucas, P., Blanchard, Y., Vitour, D., Grandcollot-Chabot, M., Zientara, S., 2018. Emergence of bluetongue virus serotype 4 in mainland France in November 2017. Transbound Emerg Dis 65, 1158-1162.
Sailleau, C., Zanella, G., Breard, E., Viarouge, C., Desprat, A., Vitour, D., Adam, M., Lasne, L., Martrenchar, A., Bakkali-Kassimi, L., Costes, L., Zientara, S., 2012. Co-circulation of bluetongue and epizootic haemorrhagic disease viruses in cattle in Reunion Island. Vet Microbiol 155, 191-197.
Savini, G., Puggioni, G., Meloni, G., Marcacci, M., Di Domenico, M., Rocchigiani, A.M., Spedicato, M., Oggiano, A., Manunta, D., Teodori, L., Leone, A., Portanti, O., Cito, F., Conte, A., Orsini, M., Camma, C., Calistri, P., Giovannini, A., Lorusso, A., 2017. Novel putative Bluetongue virus in healthy goats from Sardinia, Italy. Infect Genet Evol 51, 108-117.
Schulz, C., Breard, E., Sailleau, C., Jenckel, M., Viarouge, C., Vitour, D., Palmarini, M., Gallois, M., Hoper, D., Hoffmann, B., Beer, M., Zientara, S., 2016. Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica, France. J Gen Virol 97, 2073-2083.
Sghaier, S., Lorusso, A., Portanti, O., Marcacci, M., Orsini, M., Barbria, M.E., Mahmoud, A.S., Hammami, S., Petrini, A., Savini, G., 2017. A novel Bluetongue virus serotype 3 strain in Tunisia, November 2016. Transbound Emerg Dis 64, 709-715.
Sun, E.C., Huang, L.P., Xu, Q.Y., Wang, H.X., Xue, X.M., Lu, P., Li, W.J., Liu, W., Bu, Z.G., Wu, D.L., 2016. Emergence of a Novel Bluetongue Virus Serotype, China 2014. Transbound Emerg Dis 63, 585-589.
Temizel, E.M., Yesilbag, K., Batten, C., Senturk, S., Maan, N.S., Mertens, P.P.C., Batmaz, H., 2009. Epizootic hemorrhagic disease in cattle, Western Turkey. Emerg Infect Dis 15, 317-319.
Verdezoto, J., Breard, E., Viarouge, C., Quenault, H., Lucas, P., Sailleau, C., Zientara, S., Augot, D., Zapata, S., 2018. Novel serotype of bluetongue virus in South America and first report of epizootic haemorrhagic disease virus in Ecuador. Transbound Emerg Dis 65, 244-247.

Viarouge, C., Breard, E., Zientara, S., Vitour, D., Sailleau, C., 2015. Duplex Real-Time RT-PCR Assays for the Detection and Typing of Epizootic Haemorrhagic Disease Virus. PLoS One 10, e0132540.
Viarouge, C., Lancelot, R., Rives, G., Breard, E., Miller, M., Baudrimont, X., Doceul, V., Vitour, D., Zientara, S., Sailleau, C., 2014. Identification of bluetongue virus and epizootic hemorrhagic disease virus serotypes in French Guiana in 2011 and 2012. Vet Microbiol 174, 78-85.
Vinueza, R.L., Cruz, M., Breard, E., Viarouge, C., Zanella, G., 2019. Bluetongue virus and epizootic hemorrhagic disease virus survey in cattle of the Galapagos Islands. J Vet Diagn Invest, 1040638718824630.
Wechsler, S.J., McHolland, L.E., Tabachnick, W.J., 1989. Cell lines from Culicoides variipennis (Diptera: Ceratopogonidae) support replication of bluetongue virus. J Invertebr Pathol 54, 385-393.
Wilson, A.J., Mellor, P.S., 2009. Bluetongue in Europe: past, present and future. Philos Trans R Soc Lond B Biol Sci 364, 2669-2681.
Yadin, H., Brenner, J., Bumbrov, V., Oved, Z., Stram, Y., Klement, E., Perl, S., Anthony, S., Maan, S., Batten, C., Mertens, P.P., 2008. Epizootic haemorrhagic disease virus type 7 infection in cattle in Israel. Vet Rec 162, 53-56.
Zanella, G., Durand, B., Sellal, E., Breard, E., Sailleau, C., Zientara, S., Batten, C.A., Mathevet, P., Audeval, C., 2012. Bluetongue virus serotype 8: abortion and transplacental transmission in cattle in the Burgundy region, France, 2008-2009. Theriogenology 77, 65-72.
Zientara, S., Sailleau, C., Viarouge, C., Hoper, D., Beer, M., Jenckel, M., Hoffmann, B., Romey, A., Bakkali-Kassimi, L., Fablet, A., Vitour, D., Breard, E., 2014. Novel bluetongue virus in goats, Corsica, France, 2014. Emerg Infect Dis 20, 2123-2125.

Reference Website

ReoID. Phylogenetic sequence analysis and improved diagnostic assay systems for viruses of the family *Reoviridae.* Peter. P. C. Mertens and Houssam Attoui (accessed 04 March 2019). (http://www.reoviridae.org/dsrna_virus_proteins/reoid/btv-4.htm#EGY1977/01).

Figure 1

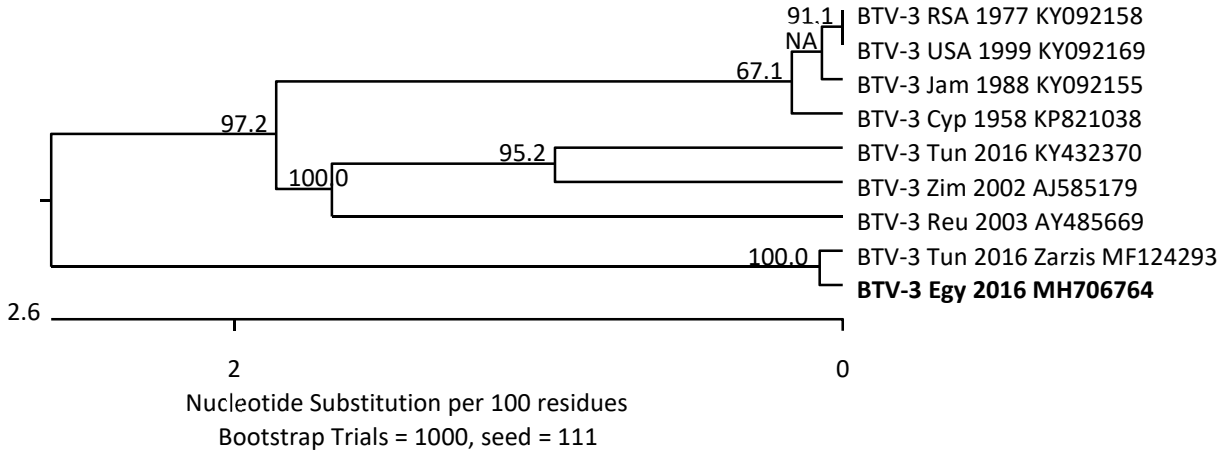
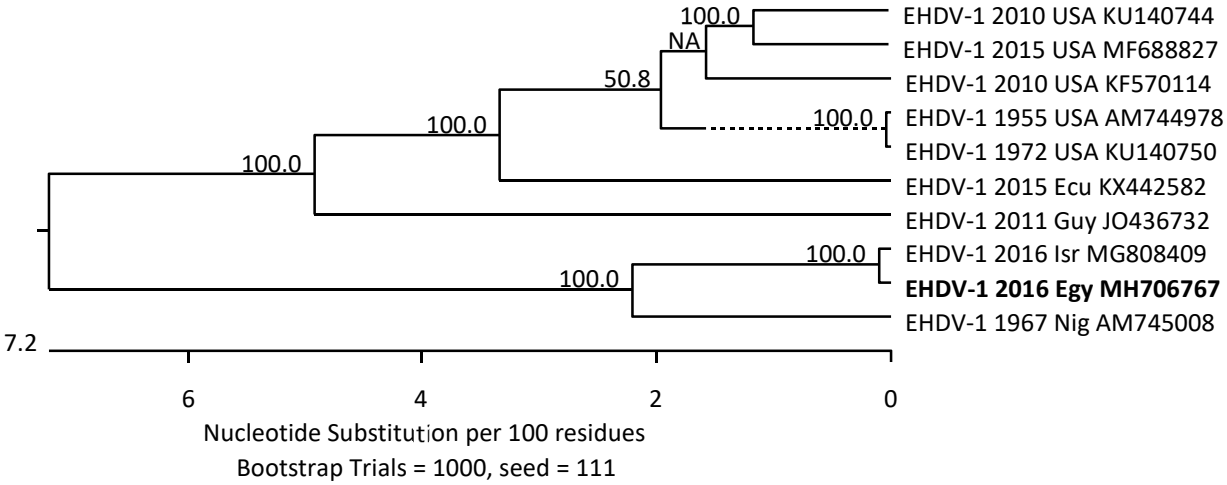

91.1
NA
67.1
97.2
95.2
100.0
100.0
BTV-3 RSA 1977 KY092158
BTV-3 USA 1999 KY092169
BTV-3 Jam 1988 KY092155
BTV-3 Cyp 1958 KP821038
BTV-3 Tun 2016 KY432370
BTV-3 Zim 2002 AJ585179
BTV-3 Reu 2003 AY485669
BTV-3 Tun 2016 Zarzis MF124293
BTV-3 Egy 2016 MH706764
2.6
2
0
Nucleotide Substitution per 100 residues
Bootstrap Trials = 1000, seed = 111

Figure 2


100.0
NA
50.8
100.0
100.0
100.0
100.0
EHDV-1 2010 USA KU140744
EHDV-1 2015 USA MF688827
EHDV-1 2010 USA KF570114
EHDV-1 1955 USA AM744978
EHDV-1 1972 USA KU140750
EHDV-1 2015 Ecu KX442582
EHDV-1 2011 Guy JO436732
EHDV-1 2016 Isr MG808409
EHDV-1 2016 Egy MH706767
EHDV-1 1967 Nig AM745008
7.2
6
4
2
0
Nucleotide Substitution per 100 residues
Bootstrap Trials = 1000, seed = 111