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## Co-circulation of bluetongue and epizootic haemorrhagic disease viruses in cattle in Reunion Island

Corinne Sailleau<sup>a,\*</sup>, Gina Zanella<sup>a</sup>, Emmanuel Breard<sup>a</sup>, Cyril Viarouge<sup>a</sup>, Alexandra Desprat<sup>a</sup>, Damien Vitour<sup>a</sup>, Micheline Adam<sup>a</sup>, Laurent Lasne<sup>b</sup>, Arnaud Martrenchar<sup>c</sup>, Labib Bakkali-Kassimi<sup>a</sup>, Laura Costes<sup>b</sup>, Stéphan Zientara<sup>a</sup>

<sup>a</sup> French Agency for Food, Environmental and Occupational Health and Safety (ANSES), 23 Avenue du Général de Gaulle 94706 Maisons-Alfort Cedex, France

<sup>b</sup> Directorate for Food, Agriculture and Forestry – Parc de la Providence 97488 Saint Denis Cedex, France

<sup>c</sup> General Delegation of French Overseas Regions, 27, rue Oudinot, 75007 Paris, France

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### ABSTRACT

Bluetongue virus (BTV) and epizootic haemorrhagic disease virus (EHDV) in deer have already been isolated in Reunion Island and have caused more or less severe clinical signs in cattle (EHDV) or in sheep (BTV), as observed in 2003. In January 2009, cattle in Reunion Island showed clinical signs suggesting infection by one or the other of these arboviral diseases. A study was set up to determine the etiology of the disease. Analysis by reverse transcriptase–polymerase chain reaction (RT-PCR) performed on blood samples from 116 cattle from different districts of the island detected the presence of the EHDV genome in 106 samples and, in 5 of them, the simultaneous occurrence of BTV and EHDV. One strain of EHDV (7 isolates) and one of BTV were isolated in embryonated eggs and a BHK-21 cell culture. Group and subgroup primer-pairs were designed on the segment 2 sequences available in GenBank to identify and type the EHDV strains.

Phylogenetic analysis of the genomic segment 2 (encoding the VP2 serotype-specific protein) of the isolates confirmed the serotypes of these two orbiviruses as BTV-2 and EHDV-6 and allowed them to be compared with previously isolated strains.

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### 1. Introduction

Bluetongue virus (BTV) and epizootic haemorrhagic disease virus (EHDV) belong to the family *Reoviridae* and the genus *Orbivirus* (Verwoerd and Erasmus, 2004; Maclachlan and Osburn, 2004). These two viruses have structural, antigenic and molecular similarities. Both viruses are transmitted to their host range (ruminants) by the bites of *Culicoides* midges. Serological and molecular techniques for the laboratory diagnosis of these two diseases are very similar.

Both viruses have seven different structural proteins (VP1 to VP7) divided into two capsids (Roy, 2005). The

outer capsid consists of VP2 and VP5. The VP2 protein, the major constituent of the outer capsid, is exposed on the surface of the virus particle and determines the serotype-specific antigen. Twenty-four BTV serotypes and 7 EHDV serotypes have been identified (Anthony et al., 2009). The specific antigens of each serotype induce the production of serotype-specific neutralising antibodies. Segment 2, which encodes VP2, is favoured for conducting studies of genetic variability between different serotypes.

EHDV primarily infects wild ruminants such as deer, which develop the most severe clinical signs. Several strains – including strains of EHDV serotype 2 (Ibaraki strain), 6 and 7 – can also cause a severe disease in cattle; clinical signs observed are similar to those caused by the European strain of BTV-8. EHD is endemic in the United States and Canada, where it causes clinical signs exclusively in certain species of deer. In Australia, Southeast

\* Corresponding author. Tel.: +33 149772707; fax: +33 143689762.

E-mail address: [corinne.sailleau@anses.fr](mailto:corinne.sailleau@anses.fr) (C. Sailleau).

Asia, sub-Saharan Africa and, since 2006, in the Maghreb countries (serotype 6) and the Arabian Peninsula (serotype 7), it occurs in outbreaks and causes seasonal clinical signs in cattle.

In January 2009, cattle in Reunion Island showed clinical signs of hyperthermia, anorexia, congestion and petechia of the oral mucosa, prostration and nasal discharge. Some animals had a severe dyspnea, facial oedema and lameness. The morbidity rate was 0.8% and the case fatality rate was estimated at 11%. This clinical pattern could be attributed to BTV or EHDV.

In this article, we present a study, which was set up in February 2009 to determine the etiology of this disease and to quantify the clinical signs displayed by cattle in 2009 in Reunion Island. The laboratory diagnostic techniques developed and used to identify viruses responsible for the observed pathology are described.

## 2. Materials and methods

### 2.1. Clinical studies

Private veterinarians participating in the study were asked to take blood samples from at least 100 cattle showing EHD clinical signs. They also had to fill out a detailed questionnaire on clinical signs and lesions observed.

### 2.2. Blood samples

EDTA blood samples were collected from cattle displaying clinical signs, for viral isolation and RT-PCR assays.

### 2.3. Nucleic acid sample preparation, RT-PCR reactions and sequencing

Total RNA was extracted from 100  $\mu$ l of blood samples using the QIAcube robot (QIAGEN) and the QIAamp Viral kit (QIAGEN; reference: 52906) according to the manufacturer's instructions. Finally, the RNAs were eluted with 50  $\mu$ l of ultrapure water and used in specific EHDV and BTV RT-PCR.

#### 2.3.1. BTV group-specific RT-PCR

Reverse transcription (RT) and amplification were performed using a commercial real-time RT-PCR kit (ADI-352, AES) according to the manufacturer's instructions. This kit allows all 24 serotypes to be detected (by amplifying BTV segment 10 which encodes NS3).

#### 2.3.2. EHDV group-specific RT-PCR

Group-specific primer-pairs (EHDVF617 cgc aca Rga Kac Rccaca; EHDVR1070 ccc tca cat/c ata ggc gtt tt) derived from the nucleotide sequence data of genome segment 7 of EHDV available in GenBank were used in conventional RT-PCR using the one-step RT-PCR Kit (Qiagen, Courtaboeuf, France). Briefly, 2.5  $\mu$ l of denatured RNA were added to a mixture containing 15.2  $\mu$ l of RNase-free water, 5  $\mu$ l of 5X QIAGEN one-step RT-PCR buffer, 1  $\mu$ l of dNTP mix (400  $\mu$ M of each dNTP), 0.6  $\mu$ M of each primer, 1  $\mu$ l of QIAGEN one-step RT-PCR Enzyme. The amplification was carried out according to the following cycling parameters: 50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C. A quota of ten microlitres of each RT-PCR product was analysed by electrophoresis on a 2% agarose gel.

#### 2.3.3. EHDV subgroup-specific primers

The alignment (Megalign) of the segment 2 nucleotide sequences available in GenBank shows 4 clusters (Fig. 1). Sequence data were used to design one primer-pair for each cluster (Table 1) allowing the amplification of each serotype for each group. These primers were selected in a conserved region of each group. These four primer-pairs were used in RT-PCR assays using the same protocol as described above. In order to determine the serotype, each amplification product was sequenced.

#### 2.3.4. Sequencing of amplified products

2.3.4.1. *EHDV Sequencing.* Amplified RT-PCR products were sequenced directly, in both directions, using the primer-pairs (Table 1) (Cogenics). Sequences were assembled by SeqMan (DNASTar programs, Lasergene) and compared (Blastn 2.2.23) to the homologous sequences available in

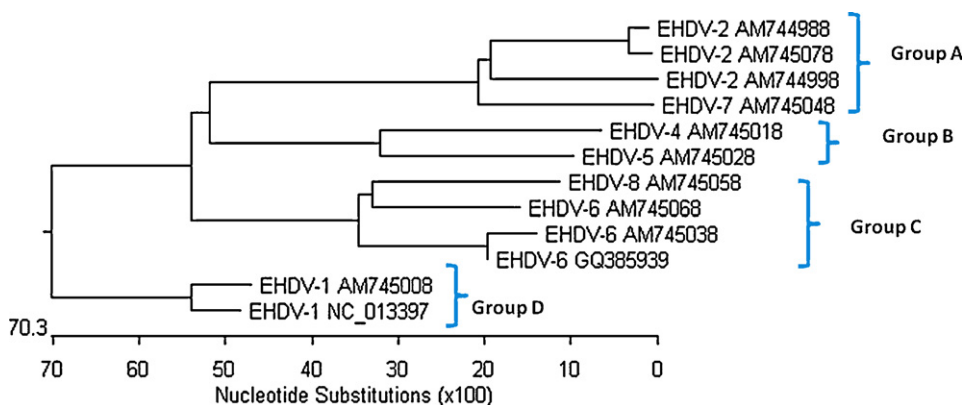


Fig. 1. Phylogenetic tree of full-length segment 2 sequences showing relationships between EHDV serotypes (GenBank). The serotypes of EHDV are associated in 4 Clusters (A, B, C and D).

**Table 1**  
EHDV subgroup-specific primers.

	Forward primer	Sequence (5–3')	Localisation	Reverse primer	Sequence (5–3')	Localisation	Amplified product size	Serotype amplified
Group A	EHDAF	ATACGCCGATATGACTGG	255–272	EHDAR	GATGATAATTCCTTATGTTTCG	1041–1020	786	EHDV2 EHDV7
Group B	EHDBF	ATTATGAGATTTTCATTATGGG	2479–2500	EHD BR	AGCTTCAGTCACGAAGC	3001–2985	522	EHDV4 EHDV5
Group C	EHDCF	GTAAATTATTCAGGATGG	1–20	EHD CR	TTCCATATTCGGTTTAATCC	391–372	391	EHDV6 EHDV8
Group D	EHDDF	GTAAATTGTGTCAGGATGG	1–20	EHD DR	CCGTCTACCCATTTTCATCC	820–801	820	EHDV1

GenBank. Based on the segment 2 nucleotide sequences of identified serotypes available in GenBank, primers were designed to sequence the full length segment 2 nucleotide sequences. Sequence alignments were performed using MegAlign-Clustal V method (DNAsar software).

**2.3.4.2. BTV sequencing.** Based on the segment 2 nucleotide sequences of BTV-2 available in GenBank, primers were designed to sequence the segment 2 of the isolated strain.

#### 2.4. Virus isolation

Individual groups of three embryonated chicken eggs (ECE) were each inoculated intravenously with 0.1–0.2 ml of a  $10^{-1}$  dilution of washed and lysed blood (Clavijo et al., 2000). The eggs were incubated for 5 days at 35 °C and examined daily using a cold candling lamp. The embryos that died within 24 h were discarded. The embryos that died between days 2 and 5 were removed and homogenised in a pestle with sterile sand and Eagle's MEM, supplemented with 100 µg/ml streptomycin and 100 IU/ml penicillin, using a mortar to give a final 10% suspension. The tissue homogenates were clarified by centrifugation at  $2000 \times g$  for 10 min at 4 °C and inoculated into BHK-21 cells. Inoculated flasks were incubated at 37 °C and examined under a microscope every day for 7 days to check for the appearance of cytopathic effects (CPE).

#### 2.4.1. Virus neutralisation test

Virus neutralisation tests (VNT) were carried out using methods similar to those described in the OIE Manual of Standards for diagnostic tests and vaccines (OIE, 2009). All assays were carried out in microtitre plates. Constant amounts of each BTV type-specific antiserum (50 µl/well) were added to a tenfold dilution series of each virus sample (50 µl/well) and the serum–virus mixtures incubated for 1 h at 37 °C. BHK-21 cells (100 µl/well), diluted in Eagle's MEM, supplemented with 5% of foetal calf serum, 100 µg/ml streptomycin and 100 IU/ml penicillin, were then added at a concentration of  $10^5$  cells/ml. Microtitre plates were sealed, incubated at 37 °C for 7 days and examined under a microscope each day to check for CPE. The BTV type-specific antiserum that reduced the virus titre by at least two  $\log_{10}$  compared with the virus control in the absence of any antiserum designated the virus serotype.

### 3. Results

#### 3.1. Clinical signs and epidemiological data

The age of affected animals ranged from 6 months to 10 years. All were of European breeds (Holstein, Limousine, Blonde d'Aquitaine and Aubrac) and cross-bred cattle. Questionnaires on clinical signs were filled out for 67 cattle, all of which were EHDV positive. Fig. 2 summarises the percentage of clinical signs associated with EHDV

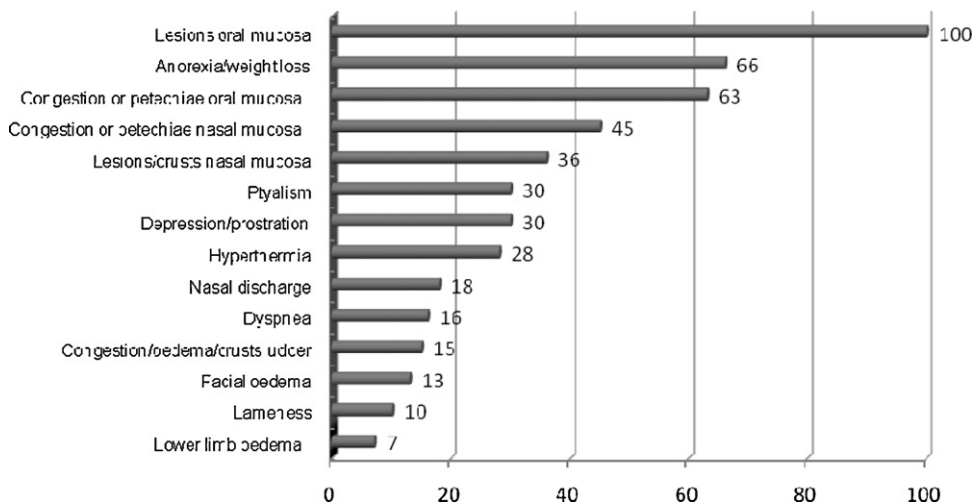


Fig. 2. Percentage of EHDV clinical signs found in 67 cattle in Reunion Island during the 2009 epizootic.

**Table 2**  
Distribution of EHDV and BTV RT-PCR-positive cattle.

District	Number of sampled cattle	Number of EHDV RT-PCR+ cattle	Number of EHDV RT-PCR+ and BTV RT-PCR+ cattle
Le Tampon	73	68	0
L'Entre-Deux	3	3	0
Les Trois-Bassins	3	3	0
Saint-Joseph	2	2	0
Saint-Leu	30	26	5
Saint-Louis	1	0	0
Saint-Pierre	4	4	0
Total	116	106	5

observed in these animals. The most common clinical signs were: anorexia, weight loss, pyalism, depression and hyperthermia. Congestion, petechiae and lesions (erosions or ulcers) in oral and nasal mucosae were the most frequent lesions observed. A reduction in the milk yield was reported in 7 of the 20 dairy cows (Holstein) included in the sample. The five beef cows that were co-infected with EHDV and BTV showed congestion, petechiae or lesions in oral and nasal mucosae.

### 3.2. RT-PCR analysis

A total of 116 cattle, belonging to 57 farms spread across 7 districts of the island, were blood sampled. Ninety one percent ( $n = 106$ ) were EHDV positive and 4% ( $n = 5$ )

were both BTV and EHDV positive (Table 2). The latter five animals were from the same breed (Limousine).

### 3.3. Viral isolation

Eleven blood samples caused haemorrhagic lesions on embryos and CPE on BHK-21 after the first passage. On the basis of the PCR results using EHDV- and BTV-specific RT-PCR, 7 strains were identified as EHDV, one strain was identified as BTV and co-infection with EHDV and BTV strains was found in three samples.

### 3.4. Virus serotyping and sequence analysis

#### 3.4.1. BTV strain

On the basis of the VNT results, the BTV strain was identified as BTV-2. This result was confirmed by sequence analysis of segment 2 (GenBank accession number HQ222824). The comparison with corresponding sequences available in GenBank showed identity from 79.5% to 87.9% (nucleotides) and from 91% to 96.3% (amino acids) (Table 3).

#### 3.4.2. EHDV strains

RT-PCR using four primer-pairs (Table 1) performed on total RNA extracted from 100  $\mu$ l of EHDV infected cell culture or directly from EHDV infected blood allowed the amplification of 7 products only with the primer-pair (group C). The sequence determination and alignment

**Table 3**  
Nucleotide and amino acid identity (%) of genome segment 2 and VP2 of BTV-2 Reunion compared with the cognate region in other BTV-2 strains.

	Nucleotides					
	BTV-2 Reunion	BTV-2 Martinique	BTV-2 France	BTV-2 Italy	BTV-2 USA	BTV-2 SA
BTV-2 Reunion (HQ222824)		79.5	87.4	87.2	87.9	87.8
BTV-2 Martinique (HQ222820)	91		79.6	79.4	80.4	80.5
BTV-2 France (AF356601)	96.3	90.9		99.7	96.3	96.5
BTV-2 Italy (AM773698)	96	90.6	99.6		96	96.2
BTV-2 USA (AY855265)	96.3	96.3	98.2	97.2		99.5
BTV-2 SA (AY855265)	96.2	90.7	98.3	98	99.8	
	Amino acids					

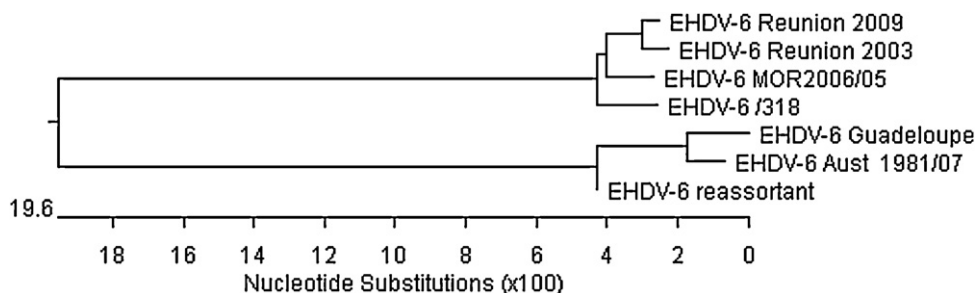


Fig. 3. Phylogenetic tree of full-length segment 2 sequences showing relationships between EHDV-6 strains (clustal analysis).

**Table 4**

Nucleotide and amino acid identity (%) of genome segment 2 and VP2 of EHDV Reunion compared with the cognate region in other EHDV strains (GenBank accession number in brackets).

	Nucleotides						
	EHDV-6 Reunion 2009	EHDV-6 Reunion 2003	EHDV-6 MOR 2006/05	EHDV-6 318	EHDV-6 AUS1981/07	EHDV-6 Guadeloupe	EHDV-6 <sup>a</sup> reassortant
EHDV-6 Reunion 2009 (HQ848379)		98.8	97.1	96.5	68.4	68.2	71.2
EHDV-6 Reunion 2003 (HQ222817)	98.9		97.1	96.5	67.8	67.6	70.6
EHDV-6 MOR 2006/05 (HM156730)	98.9	98.1		96.5	67.8	67.6	70.6
EHDV-6 /318 (AM745068)	98.9	98	98.8		68.1	68.1	71
EHDV-6 AUS1981/07 (AM745038)	77	76	77	76.7		97.2	98.1
EHDV-6 Guadeloupe (HQ 848380)	76	76.1	76.4	76.2	96.9		96.8
EHDV-6 reassortant <sup>a</sup> (GQ285939)	80.8	79.9	80.3	80.8	97.9	95.6	
	Amino acid						

<sup>a</sup> Partial sequence (1014 nucleotides and 338 aa).

(Blastn) of 391 pb amplified products indicated identity of 97% to EHDV-6/strain 318 (AM775068). Sequences of each isolate were identical to each other.

The full length seg-2 sequence of one isolate was determined (HQ848379). Clustal alignment with the cognate gene (EHDV-6) present in GenBank indicated between 68.2% and 98.8% identity (76% and 98.9% amino acid identity) (Table 4 and Fig. 3).

#### 4. Discussion

The number of clinical cases during the 2009 EHDV epizootic was estimated to be at least 270 in Reunion Island. Only 116 were analysed in our study. Of the 116 animals tested, 91% were RT-PCR EHDV positive and 4% were both RT-PCR EHDV and BTV positive. These results demonstrate the involvement of EHDV in observed “BTV-like” clinical signs. It is very likely that other animals on the island were co-infected with EHDV and BTV. The presence of these two orbiviruses has been reported several times in Reunion Island. From 1974 to 1979, serological data indicated the presence of BTV on the island (personal data). In January 2003, EHDV caused a severe epizootic in cattle (Breard et al., 2004) and in the same year an outbreak of bluetongue was described in a flock of Merino sheep (Breard et al., 2005). In 2008, a serological survey of over a hundred cattle from Reunion Island conducted by the Onderstepoort Veterinary Institut (South Africa) showed that 88% of the animals were seropositive, as determined by BTV-ELISA. In SNT assays, high titres ( $\geq 1/80$ ) were obtained against BTV 2, 3, 9, 10, 12, 13, 14, 15 and 16, and lower but significant titres ( $1/20 \leq \text{titre} \leq 1/40$ ) against BTV 1, 5, 8, 20, 21, 23 and 22 (personal data). These results suggest that more than a dozen serotypes have circulated or circulate in recent years on the island.

EHDV clinical signs in cattle, involving EHDV-2, 6 and 7, had previously been reported in Japan (1959/60 and 1997), Reunion Island (2003), Morocco (2006), Israel (2006) and Turkey (2007) (Inaba, 1975; Breard et al., 2004; OIE, 2006;

Yadin et al., 2008; Temizel et al., 2009) and were very similar to those observed and quantified in our study.

Since, to our knowledge, the BTV-2 serotype has never caused clinical signs in cattle, it is likely that the lesions displayed by the EHDV and BTV2 co-infected cows found in our study were due to EHDV. Moreover, the serological findings indicate that BTV2 is circulating silently on the island. Unfortunately, questionnaires were not filled out for ten EHDV-negative animals showing clinical signs that may have allowed us to propose a differential diagnosis.

The analysis of genome segment 2 sequence of the EHDV strain performed in this study showed a high homology in nucleotides and amino acids (98.8% and 98.9%, respectively) with the homologous genes and proteins from the strain that caused an epizootic on the Island in 2003 (Breard et al., 2004). These data suggest that the same strain has been circulating on the Island for 7 years. In the period of time between the two epizootics, no clinical signs were reported. We can assume that sporadic cases have occurred but have gone unnoticed. Because of the lack of serological data from 2003 to 2009, it is impossible to determine whether any change has occurred in the EHDV seroprevalence rate.

Sequence comparison with other EHDV-6 strains showed a high identity to the “western virus” (Anthony et al., 2009) EHDV-6 strain 318 (nucleotides: 96.5%, amino acids: 98.9%) and a lower identity (68.2% to 71.2% in nucleotides and 76% to 80.8% in amino acids) to “eastern” viruses (Maan et al., 2010) EHDV-6 AUS1981/07, EHDV-6 (reassortant) isolated in USA in 2006, and EHDV-6 detected in Guadeloupe in 2010.

These sequence data indicate that the EHDV strain circulating in Reunion Island is closely related to the EHDV-6 that circulated in the Mediterranean Basin in 2006–2007 (nucleotide and amino acid identity of 97.1% and 98.9%, respectively, to isolate MOR2006/05 Table 4). Further sequence analyses of the whole genome are needed to determine if the two strains have the same origin.

In this study we also described the presence of one BTV-2 isolate. Despite serological data in 2009 showing the recent circulation of BTV-2 on the island (personal data), this serotype has never been isolated. In 2003, BTV-3 was responsible for an outbreak in sheep (Breard et al., 2005). The comparison of nucleotide sequences of segment 2 of the BTV-2 strain with the homologous sequences present in GenBank shows rates of divergence at least equal to 12% or greater than those usually observed. This result does not allow us to put forward a hypothesis concerning the origin of the BTV-2. The isolation of this virus, even if it is not responsible for the clinical signs observed, confirms the enzootic nature of the BTV in Reunion Island.

When this study was conducted, no serological or molecular tools were available for EHDV typing. Only a few group-specific gel-based RT-PCR assays (Aradaib et al., 1995, 2003) had been developed and no reference serum was available for the VNT. In this study, we describe a new group-specific gel-based RT-PCR for detecting all EHDV serotypes and a method to easily identify the EHDV serotypes. For this purpose, we selected sub-group primers, which allowed the amplification of serotypes in each cluster (Table 1). These RT-PCRs, supplemented by nucleotide sequencing, were successfully used to identify the serotypes of EHDV directly isolated from blood or infected culture cells. Recently, new group-(real time) or type-(conventional) specific RT-PCR assays have been developed (commercial assay – LSI) (Wilson et al., 2009; Clavijo et al., 2010; Maan et al., 2010) which allow rapid and sensitive detection and identification of recent isolates from the Mediterranean region and North America. Our method for identifying EHDV serotypes (PCR + sequencing) uses primers selected in more highly conserved genome regions of segment 2 than those described in Maan et al. (2010). The use of a RT-PCR that is less selective (amplification of more than one serotype) can be an advantage in the case of mutations on the segment 2 which could cause the type-specific RT-PCR to fail. Moreover, the sequence data allow both formal identification of the serotype and provide useful information about the strain. Phylogenetic comparison of segment 2 sequences helps to understand the epidemiology of the virus.

As with the EHDV-6 reported in the Mediterranean Basin, the EHDV-6 strain isolated in Reunion Island was shown to be linked to severe clinical signs in cattle. This had not been described with the EHDV-6 reported in Australia during 1977–1982 (Parsonson and Snowdon, 1985) or with the new EHDV-6/EHDV-2 reassortant isolated from white-tailed deer (Allison et al., 2010).

How and when EHDV was introduced onto the island remains unknown. As with BTV, it is likely that it was introduced by infected animals from Africa, Madagascar or Australia over the course of many years. Since the beginning of the 1990s, introduction of animals from these countries has stopped and imports were all from France (free of EHDV). A wind-borne introduction of infected midges is unlikely, given the considerable distance from Africa – Reunion island lying 800 km to the east of Madagascar – and the prevailing southeasterly trade winds. We can therefore assume that the virus remained present on the island. The possible role of Sika

deer, reared on the island, in the EHDV epidemiology remains to be determined.

The general consensus is that, for both BTV and EHDV, the serotype alone does not determine the virulence of a strain (Maclachlan et al., 2009), but rather that other factors, such as breed, play an important role. In our study, however, we observe no link between the factors of either age or breed and the presence or absence of clinical signs.

Finally, the simultaneous presence of several strains of orbivirus (EHDV and/or more than one serotype of BTV) reveals that co-infection of animals (BTV/BTV or BTV/EHDV), as observed during this clinical episode (5 cattle infected with both viruses), is possible. Furthermore, this could favour reassortment mechanisms that may lead to the emergence of new virulent strains.

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