Molecular evolution of American field strains of Bluetongue and Epizootic haemorrhagic disease viruses

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Bluetongue,
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Summary
Recent *Orbivirus* occurrences in the Americas have been investigated using whole genome amplification and sequencing followed by phylogenetic analysis. The Bluetongue virus (BTV) and Epizootic haemorrhagic disease virus (EHDV) whole genomes were amplified without prior sequence knowledge and deep sequenced. This technology was applied to evaluate BTV-3 isolates spanning 4 decades from Florida, Arkansas, Mississippi, South Dakota, Central America, and the Caribbean Basin. The results of the dataset analysis are consistent with the hypothesis that these viruses were introduced into the United States from Central America and the Caribbean Basin. A similar analysis has been performed on a recent BTV-2 isolate from California. It indicates that the BTV-2 strain was likely introduced into Florida and then moved South to the Caribbean and West to California. A historical (1955-2012) molecular characterisation of EHDV strains was also completed, and subsequently used as reference sequence for comparison of genomes from recent 2012 cattle isolates associated with clinical disease. Finally, this analysis was performed on BTV-11 isolated from 2 canine cases and demonstrated that the genome sequences of the virus isolates from these cases were almost identical. These studies indicate the value of this technology in understanding virus epidemiology and ecology.

Evoluzione molecolare di ceppi americani dei virus della Bluetongue (BTV) e della Malattia emorragica epizootica (EHDV)

Parole chiave
Evoluzione molecolare,
Virus della Bluetongue,
Virus della Malattia emorragica epizootica.

Riassunto
Lo studio ha preso in esame le recenti incursioni di *Orbivirus* registrate nel continente americano. Ceppi del virus della Bluetongue (BTV) e del virus della Malattia emorragica epizootica (EHDV) responsabili delle incursioni sono stati amplificati e completamente sequenziati con nuove tecniche che non necessitano di conoscere a priori le sequenze. È stata effettuata l'analisi filogenetica dei ceppi di BTV-3 che hanno circolato per decenni in Florida, Arkansas, Mississippi, Dakota del Sud, America Centrale e nel bacino dei Caraibi. I risultati confermano l'ipotesi che questi ceppi siano stati introdotti negli Stati Uniti dall'America Centrale e dal bacino caraibico. Un'analisi simile condotta sul ceppo BTV-2, isolato di recente in California, ha evidenziato come, probabilmente, sia stato introdotto in Florida per poi raggiungere, a sud, i Caraibi e, a ovest, la California. Questo studio ha inoltre completato la caratterizzazione molecolare dei ceppi storici (1955-2012) di EHDV che è stata successivamente utilizzata come sequenza di riferimento per confrontare il genoma dei ceppi responsabili dei casi clinici osservati nei bovini nel 2012. L'analisi è stata condotta anche sui ceppi di BTV-11 isolati da 2 casi di infezione nel cane dimostrando una completa identità nell'allineamento delle sequenze. Lo studio mostra il valore della tecnologia adottata nella comprensione dell'epidemiologia e dell'ecologia dei virus presi in esame.

Introduction

The molecular epidemiology of Bluetongue (BT) and Epizootic haemorrhagic disease (EHD) viruses (EHDV) has been used to evaluate the movement of these viruses and these techniques provide insights into potential biological mechanisms. So far this has been done primarily on the single gene level. Phylogenetic studies targeting the L3 gene from various BT viruses (BTV) were the first studies to demonstrate geographic genotypes (Gould 1987). This gene was also used to provide insight into the probable origin of BT virus serotype 2 (BTV-2) introduced in Florida in 1983 (Pritchard *et al.* 1995). A study, conducted using the S7 gene, demonstrated a tendency for geographic genotypes, but not an absolute separation of virus genotypes (Wilson *et al.* 2000). Similarly, a phylogenetic analysis of S7 from Chinese BTV isolates demonstrated the gene to be relatively conserved, yet not consistent for geographic genetic typing (Bonneau *et al.* 2000). The S10 gene, however, did demonstrate geographic genetic types (Balasuriya *et al.* 2008). These particular genes are interesting to study because they encode viral proteins related to the insect vector infectivity. The L3 and S7 genes encode the inner core proteins, which is all that is required for virus infectivity (Mertens *et al.* 1987). The S7 encodes the virus attachment protein for the *Culicoides* vector insects (Xu *et al.* 1997). Similarly, the S10 encodes the NS3/3a that is associated with virus budding (Hyatt *et al.* 1993, Jensen *et al.* 1994). Studies of the L2 gene that encodes VP2 from California BTV strain field isolates revealed closely related yet distinct L2 genes and predicted VP2 sequences (de Mattos *et al.* 1994 a, b). The double strand nature of the RNA genome and the requirement of the virus to infect both vertebrate and invertebrate cells likely affect the genetic variability of these viruses.

The development of the single primer ligation – whole genome amplification protocol (Potgieter *et al.* 2009) – allowed for the complete sequences of BT and EHD viruses to be determined without prior sequence knowledge. This has been further enhanced by the availability of next generation sequencing technologies. This technology has been applied to several independent BTV and EHDV studies to investigate the molecular evolution and putative origins of a number of isolated strains. These studies demonstrate the value of this technology in understanding virus epidemiology.

Materials and methods

Virus and RNA purification

The viruses were obtained from the Arthropod-Borne Animal Diseases Research Unit (ABADRU) collections or through various collaborations. The double stranded RNA was further purified as described previously (Wilson 1990) and purity assessed with agarose gel electrophoresis and UV absorbance.

Whole genome amplification and sequencing

The whole genome amplification was done essentially as described previously (Potgieter *et al.* 2009) with modifications described by Gaudreault and colleagues (Gaudreault *et al.* 2014, Gaudreault *et al.* 2015, Wilson *et al.* 2015). The sequences were assembled and analysed using Geneious 7.0 (Biomatters Ltd, Aukland, New Zealand). Sequence alignment was performed with Muscle or ClustalW and consensus phylogenetic trees constructed using the Neighbor-joining analysis. Bootstrap confidence values were generated using 1,000 replicates (Biomatters Ltd, Aukland, New Zealand).

Results

BTV-3 in the Americas

An intra-American Bluetongue project was conducted in the late 1980's to investigate the epidemiology of BTV in Central America and the Caribbean Basin (Homan *et al.* 1990, Gibbs *et al.* 1992). During this study, BTV-3 was first reported in this region, it subsequently slowly spread to different islands over time. It was also noted that *Culicoides insignis* was the predominant vector species in the region (Greiner *et al.* 1993). This species is also present in the South-Eastern United States (US), although the primary vector species throughout the US is *Culicoides sonorensis*. For clarification, this species was originally classified as part of the *Culicoides varipennis* complex that was comprised of 3 subspecies, *sonorensis*, *varipennis*, and *occidentalis*. These subspecies were elevated to the species level in 2000 (Holbrook *et al.* 2000), creating some confusion in the literature. In 1999, BTV-3 was isolated from samples from clinically ill sheep. The sheep had been recently moved to Florida from New Hampshire and were submitted to the USDA National Veterinary Services Laboratory (NVSL). Although still considered exotic to the US, multiple identifications of BTV-3 in ruminants from Southern and Mid-Western states have been made since 1999 (Johnson *et al.* 2007). Nine other exotic BTV serotypes have been isolated from samples submitted for diagnostic and export testing from the Southern US. Bluetongue virus serotype 3 has been isolated multiple times and was isolated the farthest North in the US of the exotic serotypes (Ostlund 2013) To investigate possible genetic

changes that may have allowed BTV-3 to move across the country, whole genome sequencing was performed on 17 isolates from the Caribbean Islands up to South Dakota, a Northern US state. A preliminary bioinformatic analysis did not reveal any remarkable or consistent genetic changes. Since there is not an active surveillance program in the US, it is unclear whether these isolations are the result of new introductions or whether BTV-3 has become established in the US.

BTV-2 in the Americas

BTV-2 has been recognised in the US since 1982 (Gibbs *et al.* 1983, Collisson *et al.* 1985, Greiner *et al.* 1985). After 1985, BTV-2 was not detected again until 1999, circulating in Florida, where it was originally identified (Mecham and Johnson 2005). In 2010, BTV-2 was detected in California for the first time from a dairy heifer during a surveillance study of 4 farms in the Northern Sacramento Valley (Mayo *et al.* 2012, MacLachlan *et al.* 2013). Whole genome sequencing and phylogenetic analysis indicated that the BTV-2 strain isolated in California was most closely related to BTV strains detected in the South-Eastern US during 1999 (MacLachlan *et al.* 2013, Gaudreault *et al.* 2014). These included BTV-13 and BTV-17 strains in addition to BTV-2, and a BTV-6 strain isolated in 2006 (MacLachlan *et al.* 2013, Gaudreault *et al.* 2014), but not BTV strains from the western states. An isolate of BTV-2 from Panama in 1990 was closely related to other BTV-2 strains, yet branched distinctly from the Republic of South Africa prototype and US BTV strains (Gaudreault *et al.* 2014). This analysis further supports the hypothesis that reassortment among strains regardless of serotype is a common feature of BTV evolution, and demonstrates the value of complete genetic analysis as an effective method for better understanding the relationships, evolution, and origins of novel virus isolates.

BTV-11 canine isolates in the US

Bluetongue virus serotypes 10, 11, 13, and 17 occur throughout the US, with their prevalence varying markedly between transmission seasons (MacLachlan *et al.* 2009). Cases of BTV-11 infection in domestic dogs have been reported and were previously linked to a BTV-11 contaminated multi-component modified live vaccine (Akita *et al.* 1994, Evermann *et al.* 1994, Wilbur *et al.* 1994) or consumption of contaminated raw meat (Alexander *et al.* 1994, MacLachlan *et al.* 2009). More recently, two separate cases of BTV-11 were isolated from aborted domestic canine foetuses in the Midwestern US from 2011 and 2012, with no prior history of BTV-contaminated vaccination or raw meat diets (Dubovi *et al.* 2013). The two recent canine isolates from Kansas and Texas, and six additional BTV-11 field isolates from wild and domestic ruminant species isolated from Texas, Florida and Washington State during 2011 to 2013 were subjected to whole-genome sequencing and phylogenetic analysis. The results indicate that each of the BTV-11 isolates from the aborted canine foetuses are nearly genetically identical to each other and to other BTV-11 field isolates sequenced from the same area, but not to field isolates from the more distant states. Thus, results from this analysis support the possibility of vector-transmission of a field strain of BTV to domestic dogs.

EHDV-1 and EHDV-2 in the US

Epizootic haemorrhagic disease virus in the US has been associated with haemorrhagic disease of white-tailed deer and serotype 1 and 2 (Savini *et al.* 2011). In 2006, serotype 6 was first isolated in the US (Allison *et al.* 2010). To understand the molecular evolution of EHDV's in the US, the whole genomes of 22 strains of EHDV-1 and EHDV-2 were determined. While these data were being analysed, an outbreak of BT-like disease occurred in cattle, which was found to be associated with EHDV-2. Therefore, an additional 15 strains isolated from cattle in 2012 were sequenced. The data did not reveal geographic genetic types as noted previously (Cheney *et al.* 1995, Cheney *et al.* 1996). However, data did reveal accumulation of chronologic genetic changes. A previous study suggested that EHDV-6 reassortment required the presence of S7 from EHDV-2 (Anbalagan *et al.* 2014). In this study, little evidence of reassortment between EHDV-1 and EHDV-2 was observed. This is likely because EHDV-2 is the more commonly isolated serotype and concurrent outbreaks of both serotypes are rare. The 2012 cattle isolates were all associated with recent EHDV genetic clades. During that time, conditions were ideal for vector-borne disease transmission and animals were under stress due to drought. Therefore, it is likely that environment rather than viral genetics contributed more to the clinical outcome, but viral genetics could have also contributed. Unfortunately, it is very difficult to reproduce disease in cattle experimentally to investigate this issue.

Discussion

Whole genome amplification and next generation sequencing provide new insights into the epidemiology of orbiviral diseases. This article summarises recent studies conducted using this technology. Data clearly indicate the potential

to predict the origin of new strains isolated from previously naïve regions. In other cases, data are less enlightening, at least with standard bioinformatics applied to the data available so far. It is clear that the analysis is limited by the available information. As orbiviral sequence data become more available and shared through international databases, these types of analyses will be more informative.

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