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Research paper

Bovine viral diarrhea virus genomic variation within persistently infected cattle

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is a single stranded RNA virus in the family *Flaviviridae* that causes a form of persistent infection. If a fetus is infected *in utero* during the first 120 days of gestation the resulting calf will be immunotolerant to the infecting strain and maintain the virus for life. These animals are epidemiologically important in maintaining BVDV on farms, but also present a unique opportunity to study quasispecies *in vivo* in the absence of significant selection by the host adaptive immune response. We used deep sequencing and novel analytical methods to characterize the viral populations within the mesenteric lymph nodes of 10 persistently infected animals. Our results indicate that the pattern of variability across the viral genome from animal to animal is very consistent within BVDV subgenotypes. However, the individual mutations that constitute this variation are not necessarily the same in each animal. Even in the absence of significant immune selection the structural genes of BVDV vary more extensively than the non-structural genes. These findings could be useful for future vaccine design against BVDV as well as for measuring and understanding patterns of variation in other ssRNA viruses, especially those that belong to the family *Flaviviridae*.

1. Introduction

Single stranded RNA (ssRNA) viruses, including the genus *Pestivirus* within the family *Flaviviridae*, of interest in this study, are an important group of infectious agents that have in general rapidly evolving genomes. Their use of error prone RNA-dependent RNA polymerases results in the introduction of many more mutations during each replication cycle than other groups of organisms (Sanjuan et al., 2010). This, in combination with their rapid generation times and large population sizes within an infected host, allows these viruses to generate diverse collections of genomes that are only restricted in variability by their own tolerance of mutations and the selective pressures exerted by the host (Biebricher and Eigen, 2006). Almost all possible genomic variations exist at any given time in these populations, resulting in a group of viruses that are well suited to changing environments. The resulting swarm of viral genomes is often referred to as a quasispecies. Viral quasispecies have been extensively reviewed (Domingo et al., 2012). When selective pressures change, such as during the development of an adaptive immune response, the frequencies of all mutants will change to optimize the viral population structure, or mutant spectra, to adapt to its changing environment. The quasispecies theory indicates this as a key aspect of these populations; the swarm of mutants is subject to evolutionary events, not each individual viral particle (Domingo et al., 2012). The implications of quasispecies theory helps to explain how and why ssRNA viruses are highly capable of altering their cell tropism or host preference, as well as the difficulty for the host to induce and maintain long term immunity against these pathogens because some mutations allow for viral immune escape.

The study of viral quasispecies in natural environments is often difficult due to a variety of factors. Rapid host immune responses that quickly reduce viral loads and limit the duration of infection make it challenging to assess how viral quasispecies evolve. Although *in vitro* experiments have explained many aspects of viral quasispecies behaviour (Domingo et al., 2012), these studies are limited by their experimental methodology and do not necessarily represent the true behavior of these populations *in vivo*. In other experiments based on *in vivo* sampling the host immune response plays a vital role in shaping the mutant spectra, altering the inherent structure of the quasispecies and

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thereby skewing the analysis.

Bovine viral diarrhea virus (BVDV) persistent infection (PI) is in that sense unique. The virus can infect its natural host indefinitely with limited or no adaptive and innate immune response due to induced immunotolerance and active immunosuppression by the virus, respectively. BVDV is a ssRNA virus that infects cattle worldwide (Ridpath, 2010). As a member of the *Flaviviridae* family it is related to human pathogens such as Hepatitis C virus, Dengue virus and Yellow Fever virus as well as animal pathogens such as Classical Swine Fever Virus (CSFV). It causes economically significant losses but often remains subclinical on farms (Lindberg and Alenius, 1999). In conjunction with its unique infection cycle that relies on persistence in some hosts it likely cannot be eradicated using vaccination alone (Brownlie, 2014). Transient BVDV infections account for the majority of cases (Houe, 1995) and occur due to horizontal transmission of the virus from an infected to a susceptible animal (Thurmond, 2005). However, PI animals are required to maintain BVDV within a herd (Lindberg and Alenius, 1999). PI calves are the result of an *in utero* infection during the first 120 days of gestation with a noncytopathic biotype of BVDV (Liebler-Tenorio, 2005). Such calves are born immunotolerant to the infecting viral strain and will sustain viral replication and excretion for the rest of their lives. They shed significantly more virus than their transiently infected peers (Thurmond, 2005), continuously re-infecting other susceptible members of the herd. They also appear to act as an important source of novel viral variability (Dow et al., 2015). This variability appears to fluctuate over time for reasons that are not known, as demonstrated with PI of the related 'HoBi'-like *Pestivirus* (Weber et al., 2017). Their lack of robust innate and adaptive immune responses leads to a reduction in selective pressure that likely permits a larger collection of viral genomic variants to exist than in transiently infected animals. BVDV PI animals provide an opportunity to describe a quasispecies population structure *in vivo* in the absence of significant immune selection. As such, BVDV PI can be used as a tool to study ssRNA viral quasispecies in its natural host and better describe the behavior of a member of this important group of viral pathogens.

This study used a group of PI animals to assess the intra-host variability of BVDV. Through next generation sequencing we could define at a very high resolution the viral population that exists within these animals and compare the resulting variant profiles to each other to assess the common features of the BVDV quasispecies among different PI animals. This study provides a unique insight into the evolutionary capacity of this ssRNA virus using deep sequencing and discusses some of the implications of this variability in the context of BVDV and quasispecies in general.

2. Methods

2.1. Sample collection

The animals included in this study have been described in a previous publication with an identical naming scheme (Dow et al., 2015). See Table 1 for a summary of all PI animals and their relationships. As part of a larger sample collection, mesenteric lymph nodes were collected from each animal and frozen at − 80 °C until used. All animal protocols were reviewed and approved by the University of Calgary Animal Care Committee under protocol AC14-0144. To reduce cross contamination incurred during sample collection, the exterior of the tissue sample was removed before sample processing. About 1cm^3 of tissue was then crushed in 1 mL of sterile PBS and centrifuged at > 13,000 × *g* for 5 min. RNA was extracted from the supernatant using the E.Z.N.A. Viral RNA kit (Omega Biotek, Norcross, GA USA) and stored at − 80 °C until used.

2.2. DNA sequencing

The entire viral genome was amplified using a series of four overlapping RT-PCRs. The primer sequences and sources can be found in Table S1. These reactions were performed using PrimeScript One-Step RT-PCR kit (Takara Bio USA, Inc., Mountain View, CA, USA). The E.Z.N.A. Gel Extraction kit (Omega Biotek, Norcross, GA, USA) was used to purify the amplicons. These were quantified using the Qubit 2.0 and Qubit HS dsDNA kit (Thermo Fisher Scientific, Waltham, MA, USA), pooled at equimolar concentrations adjusting for amplicon size to ensure the same number of DNA molecules for each fragment were present in the pool. Pooled amplicons were prepared for NGS using the Nextera XT DNA Library Preparation kit (Illumina, San Diego, CA, USA). The dual-indexed library was sequenced on a MiSeq (Illumina, San Diego, CA, USA) using a v2 500-cycle cartridge and paired-end, 250 bp reads.

Table 1
Summary of all cattle included in this study.

PI ID	Farm ID	Family ID	BVDV subgenotype
1	1	NA	1a
2	1	F1-G1	1a
3	1	F1-G2	1a
4[a]	1	F1-G3	1a
5	2	NA	1b
6	3	NA	1b
7	3	NA	1b
9	5	F2-G1	1b
10[b]	5	F2-G2	1b
11	6	NA	1b

NA: not applicable, not part of a family. Unique PI ID numbers were assigned to animals as well as an additional designation (Family ID) for those animals that were part of a family within this sample. F denotes the family and G denotes the generation. For example, F1-G2 is the second generation of family 1. Furthermore, PI4 is the fetus of PI3 and PI10 is the fetus of PI9.

[a] Fetus in approximately the fourth month of gestation.

[b] Fetus in approximately the eighth month of gestation.

2.3. Data analysis

Short reads were mapped to the BVDV SD-1 reference genome (GenBank accession: M96751) using the Geneious assembler (Kearse et al., 2012). VirVarSeq was used to call both codon- and amino acid-level variant frequencies while accounting for nucleotide Q-scores using default settings (Verbist et al., 2015). The developers of this tool have demonstrated it to have good specificity and sensitivity down to single-nucleotide polymorphism (SNP) frequencies of 0.5% compared to other commonly used SNP detection tools. The published script was modified to use the Geneious assembler rather than BWA (Li and Durbin, 2009) as it was found to map a higher proportion of reads to the reference genome. Using default parameters, the Geneious assembler mapped between 55% and 92% of reads for each animal with a mean rate of 74% and median rate of 74% across all animals. The resulting variant frequencies were used to construct a group variant profile which consisted of the mean frequency of each detected variant at each position of the viral genome across all animals in the group. All comparisons were made to this group variant profile. Groups of animals were varied and consisted of the entire collection of animals and the animals divided by BVDV sub-genotype (1a or 1b).

Comparisons at the nucleotide level were done animal by animal using a script developed for this purpose. A variant profile was constructed for each animal which consisted of the frequency of each detected nucleotide variant at each position in the viral genome. A group profile was then built which consisted of the mean frequency of each detected nucleotide variant at each position of the viral genome across all animals in the group. The absolute difference between each animal-specific frequency and the same frequency in the group profile was then calculated. These differences were averaged at each position in the viral genome and used to generate the figures shown below.