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Analysis of variation of bovine viral diarrhoea virus E2 sequence following transplacental infection of cattle

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Abstract

The genetic and antigenic diversity observed in field isolates of bovine viral diarrhoea virus (BVDV) is thought to occur during acute infection because of the genetic stability observed in BVDV throughout the lifetime of persistently infected (PI) cattle. In this study, 15 cows in early pregnancy were inoculated with identical challenge doses obtained from a single infectious inoculum of the virologically cloned isolate Pe515nc. In order to examine the diversity that may develop in utero in the PI foetus, the variable E2 sequence of the virus isolated directly from the serum of each PI calf was compared. A high degree of sequence similarity was demonstrated, with 0–4 nucleotide differences out of 608 bases compared. Thus, the virus showed relatively few genomic changes in any of the PI calves, although we observed that the in utero environment did provide some opportunity for genetic variation to become established.
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Bovine viral diarrhoea virus (BVDV) causes great losses in the cattle industry world-wide due to a wide spectrum of clinical and pathological conditions, varying from reproductive failure, congenital abnormalities and general immunodepression to unthrifty calves and severe mucosal disease (Brownlie, 1990; Duffell and Harkness, 1985). In pregnant cows, acute BVDV infection will lead to transplacental infection of the foetus, which may develop immunotolerance against the virus and become persistently infected (PI) and a persistent excreter of the virus (McClurkin et al., 1984). PI animals are considered to be responsible for most of the spread of BVDV, whereas acutely infected animals usually are relatively inefficient in transmitting the virus (Houe, 1995; Corapi et al., 1988; Roeder and Harkness, 1986).

Field isolates of BVDV are characterized by their significant genetic, antigenic and pathogenic diversity (Hamers et al., 2001). The most variable sequences of the 12.5 kb RNA viral genome are those encoding the

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highly antigenic, major envelope glycoprotein E2 (Hertig et al., 1995). Within PI animals, only a relatively low level of viral diversity is detectable at different times (Collins et al., 1999; Mignon et al., 1990), due to the immunotolerance that keeps the infecting BVDV largely unaltered. Previous studies have looked at antigenic and sequence variation between a small number of PI animals, both cattle and sheep, within and between different farms. Similarities between the viral sequences in between three and six individual PI animals on single farms have led to the concept of herd-specific strains of BVDV (Desport et al., 1998; Hamers et al., 1998; Vilcek et al., 1999). The important role of PI animals in the spread of the virus has raised questions regarding the generation of these genetic differences. Acute infections with BVDV, as with other positive-strand RNA viral infections, favour survival of viral variants that are able to escape the immune response (Bolin and Ridpath, 1992), and therefore lead to a more diverse viral population. Thus, the diversity of BVDV is believed to be generated by passage of the virus between immunocompetent animals during acute rather than persistent infection (Hamers et al., 1998). However, it is not known at what rate the virus mutates in acutely infected cows or during the passage from infected cows to the early non-immunocompetent foetus. Are the viruses in PI offspring different from the original virus infecting the cows?

This study was designed to examine the genetic diversity of BVDV isolates generated in a larger number of PI calves following experimental inoculation of pregnant, immunocompetent but BVDV naïve cows with a defined viral inoculum.

Fifteen heifers of Norwegian Dairy Cattle, between 2 and 3 years of age, all proven negative for BVDV and antibodies against the virus, were inoculated with BVDV between days 74 and 81 of pregnancy. All the cows were infected during a 7-month period, and all delivered live PI calves that were proven antibody negative and BVDV positive in serum after birth. In general, the calves were small and unthrifty, but none had any severe signs of illness. They were given colostrum free from antibodies against BVDV, and housed in isolation from other animals. The animals included in this study and the infection experiment are more thoroughly described elsewhere (Stokstad and Loken, 2002).

All cows were infected with the same batch of BVDV, strain Pe515nc, with 5×10^6 TCID per cow (The Royal Veterinary College, UK). An aerosol spray with 5 ml of the inoculum was deposited approximately 15 cm into the nostril. Blood was collected from the calves at 3–5 months of age, and serum stored at $-70\,^{\circ}C$ until examination. RNA was extracted directly from the samples using RNA-stat (Ambion) according to the manufacturer's instructions. Reverse transcription was performed using Superscript II (Life Technologies) and random hexamer primers using standard procedures. A 744 base pair portion of the E2 coding sequence was amplified by Pfu polymerase (Stratagene) using primers SP6 (5′-CGAATTCATGAGGGGCCAGATGGTACAGGGC-3′) and SP3 (5′-GTCTACTAATCTGTAGCCAGTCTCATT-3′), corresponding to NADL position 2405–3149 (Collett et al., 1988). The sequence of the E2 amplimer was directly determined using the Thermosequenase kit with an ALF Express sequencer (Amersham Biosystems). Cy5-labelled internal primers (E2F2855: ATAGGATGGTCTGGGACCGTGAG, E2F2995: AAGTTCTAGGGGAGGATCTG, E2R3088: CCACACCACTTGGAAGATTC) were used to determine the consensus sequence in both directions. The data were analysed using Genetic Computer Group (GCG, Wisconsin) programs including gap, map, pileup and clustal available on the MRC HGMP-RC (Medical

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5'-
CCACTAGGAGCTGAAGGCCTTACCACTGTCTGGAAGGATTACTCACCTGAAATGACGCTGGAAGACACAATGGTCATAGC
CTCGTGCAGAGACGGTAAGTTTATGTACCTCTCAAGGTGCACGAGAGAAACTAGATATCTTGCAATTTTGCATTCAAGAG
CCTTACCGACCAGTGTGGTATTCGAAAAACTTTTTGAGGGGCAAAAGCAAGAGGACACAGTCGAAATGGATGACAACTTC
GAATTTGGACTCTGCCCATGCGATGCCAAGCCCATAGTAAGAGGGAAATACAATACAACACTGCTAAACGGACCGGCTTT
CCAGATGGTATGCCCCATAGGATGGACAGGGACCGTGAGCTGCATGTTAGCTAATAGGGATACCCTAGATACAGCAGTAG
TGCGGACATATAGGAGGTCTAGACCGTTCCCTTACAGGCAAGACTGTATCACTCAAAAAGTTCTGGGGGAGGATCTCTAT
GATTGTATTCTTGGAGGAAACTGGACTTGTATAACTGGGGACCAACTACAATACTCAGGAGGCTCTATTGAATCCTGCAA
GTGGTGTGGTTTTAAATTCCAAAGAAGTGAGGGATTACCACACTACCC-3'
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Fig. 1. Consensus sequence of the E2 region of bovine viral diarrhoea virus Pe515nc inoculum used to generate 15 persistently infected calves following inoculation of their mothers in early pregnancy. The sequence was generated by direct sequencing of the PCR product.

Research Council, Human Genome Mapping Project Resource Centre) server. The methods used meant that the consensus sequence of the quasi-species population was directly determined.

The direct sequencing of the E2 PCR products with labelled internal primers allowed comparison of the sequences corresponding to NADL bases 2519–3126 (Fig. 1). A very small number of ambiguities could not be resolved, but this probably represents the quasi-species nature of the sequences and the use of a direct sequencing strategy.

The number of sequence differences between the consensus in individual calves and the initial virus inoculum ranged from 0 to a maximum of 4 nucleotide differences in a window of 608 bases (Fig. 1 and Table 1). Some of these changes did translate to amino acid differences in 4 out of the 15 isolates, but a maximum of 3 amino acid differences were observed in 203 compared. The effects of these differences in amino acid sequence are difficult to predict given the paucity of information on the structure–function relationships of BVDV E2 protein, but it is conceivable that they would result in some antigenic changes.

These results reveal that the E2 sequences of BVDV in serum from 15 PI calves had a high degree of sequence identity to each other and to the virus with which the cows were inoculated in early gestation. Infection of these 15 BVDV naïve cows with the same viral inoculum did not lead to rapid and unrestricted generation of diversity. Sequences from 7 of 15 animals (2, 8, 9, 10, 11, 12 and 14) are identical and have the same sequence as the consensus inoculum sequence. Sequences from animals 5 and 7 are identical and differ from the above group by a single base change in 608 nucleotides. Sequences from animals 1, 3, 4, 6, 13 and 15 form no particular groupings and differ from the major group (2, 8, 9, 10, 11, 12 and 14) by 1 base change (animals 3, 4 and 13), 2 base changes (animal 6), 3 base changes (animal 1) and 4 base changes (animal 15). We observed 14 changes in a total of 15 × 608 bases sequenced which is 14/(15 × 608) = 0.15% variation overall. The maximum sequence divergence observed was between the inoculum and sequence from animal 15 where 4 base changes represents 0.65% divergence or 99.35% sequence identity. These differences occurred at 11 different positions within the E2 sequence which represents 11/608 = 1.8% of possible sites.

Quasi-species sequence variants of BVDV do exist both within virus in a PI animal and in culture (Desport et al., 1998; Collins et al., 1999). The range of variation generated during replication in a foetus prior to immunocompetence described here (0–4 bases different in 608 sequenced) is in the same range as that observed within a mature, immunocompetent PI animal (Collins et al., 1999). Diversity of individual virus

Table 1
The consensus sequence of the E2 region of bovine viral diarrhoea virus Pe515nc inoculum was compared to the same virus region from 15 persistently infected calves generated following inoculation of their mothers in early pregnancy

Animal number	Base differences	Nucleotide position and difference	Amino acid position and difference
1	3	110 T:G, 184 G:A, 212 A:G	22 L:R, 47 E:K, 56 E:G
2	0	–	–
3	1	372 T:A	–
4	1	392 C:T	116 T:I
5	1	2 C:T	–
6	2	2 C:T, 313 C:T	90 P:S
7	1	2 C:T	–
8	0	–	–
9	0	–	–
10	0	–	–
11	0	–	–
12	0	–	–
13	1	212 A:G	–
14	0	–	–
15	4	440 G:A, 584 G:A, 585 A:G, 588 T:K	132 Q:R, 180 R:K, 181 S:X

The data presented details the number and positions of differences between the Pe515nc consensus and the virus extracted directly from the serum of each animal. The sequence was generated by direct sequencing of the PCR product.

clones within a PI animal ranged from 1 to 12 base differences to the consensus sequence, but with a majority of clones showing 3–5 base differences in 680 bases sequenced (Collins et al., 1999). These two observations contrast with previously published work on the diversity observed between field isolates of BVDV (Hertig et al., 1995), in which variation in a 151 base sequence of E2 from 15 field strains was observed at 47% of possible sites and represented a diversity of 5–32% between different BVDV strains.

The current study was designed to investigate whether, during rapid virus growth in the BVDV naïve cow and foetus, different sequence variants would establish as the dominant population in different animals and then become fixed as the foetus developed immunocompetence. Viral sequence divergence was observed in 8 of 15 animals, but the maximum sequence diversity achieved was 0.65%.

The length of time between the inoculation of the cow and the development of foetal immunocompetence is uncertain, but in these cases, it was approximately 40–50 days during which the virus replicates in the cow, crosses the placenta and replicates within the foetus. In experimental infections, BVDV antigens have been found in the foetal liver and lung 14 days after inoculation of the cows and in the placenta 22 days post-infection (Fredriksen et al., 1999). In a PI animal, the virus will become fixed within a narrow range of sequence variation. The current results suggest that there was some generation of sequence divergence during replication within the 15 different cases as reported in several previous studies. Paton et al. (1995) report up to 3 nucleotides difference in 188 bases (1.59%) and Hamers et al. (1998) report up to 4 nucleotides difference in 389 bases analysed (1.02%). In the current study, the inoculum used represented a virologically cloned isolate. It is possible that this may have influenced our results where we observed a maximum divergence of 4 bases on 608 = 0.66%. This variation is in the same order of magnitude as described in other studies in animals from field outbreaks (Hamers et al., 1998; Paton et al., 1995), and supports the biological relevance of our model system. The significance of our study is that we investigated both a larger portion of the normally variable viral E2 sequence, a larger cohort of animals than previous studies and increased our opportunities for measuring diversity.

Similar studies with hepatitis C virus reveal that a maximal rate of sequence divergence of 0.68% (4 bases in 584) was observed in the conserved NS3 region of the virus (Martell et al., 1992). In comparison, previous studies of the NS3 region of BVDV indicate only 0.09% divergence but with rates of 0.49% for the most variable E2 region of the genome (Collins et al., 1999). This suggests that while BVDV sequences do change, the range of variation is lower than seen in some related RNA viruses.

The possibility of PCR-induced errors can never be eliminated in such studies, but Pfu polymerase is acknowledged to have a very low intrinsic error rate. This notwithstanding, few isolates did have single ambiguities that were not resolved by repeated sequencing. This may reflect errors, but given the quasi-species nature of the starting viral population, this probably reflects diversity within the original viral RNA sample. Previous studies have confirmed only 4 nucleotide changes/4230 bases sequenced = 0.09%, confirming a very low error rate in the RT and sequencing reactions which generated this data (Collins et al., 1999). The maximal rate of variation of E2 in the current study being 0.65% is slightly higher than described previously, but the two studies are different in examining either consensus or a number of individual clonal sequences.

The likelihood is that the viruses in each of the current 15 PI animals share great antigenic similarity, although this was not extensively tested. The possibility still remains that these changes could affect epitopes that would be recognised by an immunocompetent animal. However, the sequence similarity of these 15 virus isolates is much greater than would be observed for 15 field isolates of BVDV or in virus isolated from 15 different PI animals.

There is no reason to assume that the intrinsic error rate of the viral RNA polymerase has failed to generate variants. Therefore, the in vivo environment may have selected for the survival of viral genomes that have a well-adapted replicative fitness. The fact that the consensus sequence of the viral populations from the PI calves seems to be very similar suggests the absence of strong selective pressures to diverge. It can be assumed that diversity would be observed if the conditions of selection were to change, perhaps due to the presence of active antiviral immunity.

The current study does not support the hypothesis that the virus mutates rapidly during the acute infection of the mother of the PI animal, although it does confirm that some variation is generated and becomes fixed within the individual animals. This study was designed to investigate the hypothesis that the immune-privileged environment of the placenta and the early foetus is the major opportunity for BVDV variation to occur. The results show that a similar degree of variation is generated in these calves in utero as is observed within a mature PI animal (Collins et al., 1999). Many serial passages of the virus would be required to generate the sequence and antigenic diversity observed between field strains of BVDV. Further studies would be required to compare the rates of sequence variation during acute infections between cattle and that observed during vertical transfer from dam to foetus.

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