Quasispecies in the 5′ untranslated genomic region of bovine viral diarrhoea virus from a single individual

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The variability of the 5′ untranslated genomic region (5′UTR) of bovine viral diarrhoea virus (BVDV) RNA obtained from a single individual was analysed. Lung, kidney and spleen tissues from a naturally infected foetus were used as the source of viral RNA. A fragment of 288 bases of the internal ribosome entry site from the BVDV 5′UTR was amplified by RT–PCR using a proofreading DNA polymerase. PCR products were cloned into pGem and, subsequently, transformed into Escherichia coli. The single-strand conformational polymorphisms of 158 lung-derived clones were analysed; a total of 11 banding patterns was observed. DNAs corresponding to all patterns were sequenced. Of the randomly selected clones, 11 and 10 clones derived from the kidney and spleen, respectively, were also sequenced. All sequences presented differences ranging from 1 to 6 nt substitutions. Analysis of the secondary structure of the variant sequences and comparisons to variant nucleotide sites from the 5′UTR of several BVDV isolates showed that the observed changes were almost free of randomness. Clustering and phylogenetic analyses suggested the existence of low-kinetic variants. BVDV quasispecies may be involved in establishing persistent infections by means of eluding maternal antibodies. The methods described here may be adapted easily both to analyse large numbers of samples from other genomic regions and for the study of BVDV quasispecies evolution in other systems.

Introduction

Bovine viral diarrhoea virus (BVDV) is a positive-stranded RNA virus, which, together with Classical swine fever virus and Border disease virus, constitute the genus Pestivirus. The pestiviruses cluster with the genera Hepacivirus (Hepatitis C virus, HCV) and Flavivirus in the family Flaviviridae (Pringle, 1999).

Most of the infections caused by BVDV are of the acute type but around 0·1–1·8% of animals are persistently infected (Houe, 1999), representing the way in which the virus spreads and is maintained in natural populations. This persistent infection is the consequence of an in utero infection (around days 30–150 of gestation), when the foetus is unable to mount an immune response. Unlike other RNA viruses in which antigenic evolution appears to act in the maintenance of persistence, persistence of BVDV is thought to be favoured by the existence of an antibody-resistant virus population (Hertig et al., 1995; Paton et al., 1994). Nevertheless, viral genetic change has been reported in viruses from persistently infected animals, although the biological significance of these changes still requires elucidation (Collins et al., 1999).

During viral genome replication, variant mutants are spontaneously generated. Some of these variants may differ in replication efficiency or fitness (Holland et al., 1991; Martínez et al., 1991; Domingo & Holland, 1997). Knowledge of virus genetic heterogeneity in a single carrier individual may be fundamental in studying how genomic changes influence the ability of the virus to cause disease, which might be especially important to elucidate a pathogenic process caused by viral genomic variation. It may be also of value in understanding the evolution of the virus, designing vaccines and/or improving diagnostic assays. Intra-host genetic heterogeneity has been
investigated widely in HCV, for which BVDV is a model (Oshima et al., 1991; Martell et al., 1992; Kato et al., 1992a, b; Enomoto et al., 1994; Okamoto et al., 1992; Okada et al., 1992; Taniguchi et al., 1993; Katayama et al., 1998; Toyoda et al., 1998; Jang et al., 1999; Farci et al., 2000).

The genome of BVDV is about 12 kb long; it has a single open reading frame and two untranslated regions (UTRs) at the 5′ and 3′ ends (reviewed by Meyers & Thiel, 1996). The 5′UTR is the most conserved region of the genome (De Moerlooze et al., 1993). It has a highly structured internal ribosome entry site (IRES) (Poole et al., 1995; Chon et al., 1998; Le et al., 1998), is involved in the regulation of genome replication and gene expression (Becher et al., 2000; Yu et al., 2000) and has some possible markers of virulence (Topliff & Kelling, 1998). Furthermore, the 5′UTR has been used widely in studies of evolution, epidemiology and taxonomy (Harasawa & Giangaspero, 1998; Hofmann et al., 1994; Sakoda et al., 1999; Baule et al., 1997; Ridpath et al., 1994; Pellerin et al., 1994; Harpin et al., 1995; Jones et al., 2001). In both picornaviruses and hepacviruses, the 5′UTR is related to tropism and pathogenesis (Lerat et al., 2000; Nakajima et al., 1996; Funkhouser et al., 1999).

Previous reports of BVDV quasispecies variability consist of studies on genomic regions that encode proteins E2 and NS3 of a virus from a persistently infected cow (Collins et al., 1999) and observations of the occurrence of stabilizing mutations over several cell passages of a recombinant BVDV (Becher et al., 2000). We described the genetic heterogeneity in the 5′UTR from a cell-passaged isolate (Jones & Weber, 2001). To our knowledge, the variability of the BVDV 5′UTR within a single individual has not been analysed. The aim of this work was to describe 5′UTR quasispecies in BVDV. Tissues from a field-infected foetus were used as a source of viral RNA to investigate the variability of the BVDV 5′UTR.

Methods

**Diagnosis of natural infection with BVDV in foetuses.** Foetal organs were obtained from the cell culture section of our institute. Primary cell cultures are tested routinely for the presence of BVDV by direct immunofluorescence and viral genome detection by RT–PCR. For this study, the presence of viral nucleic acids directly in foetal tissues corresponding to BVDV-immunofluorescence and PCR-positive primary cultures was analysed as follows: tissues from organs were homogenized in 5 vols of a guanidinium thiocyanate-based buffer (ITG buffer) (4 M guanidine thiocyanate and 0·1 M Tris–HCl, pH 7·8) and clarified for 10 min at 10000 g at 4 °C using a Sorvall SM20 rotor. RNA was obtained by adding 400 µl di-distilled water (ddH₂O) to 100 µl of the preparation and then extracting nucleic acids by acid phenol–chloroform extraction and ethanol precipitation (Sambrook et al., 1989). Pellets were resuspended in 25 µl ddH₂O and suspensions were used for cDNA synthesis and PCR amplification with primers 324 and 326 directed to the 5′UTR (Vilcek et al., 1994), as described elsewhere (Jones & Weber, 2001; Jones et al., 2001).

**Cloning.** Viral RNA was partially purified from 10% homogenates of BVDV-positive foetal tissues in Eagle’s minimal essential medium. Virus suspensions were concentrated by adding 125 µl cold NaCl (4 M) and 0·1 g PEG₆₀₀₀ to 875 µl homogenate. Following thorough mixing, the preparation was incubated for 12 h at 4 °C and then pelleted by centrifugation at 4 °C in a microcentrifuge for 15 min. Pellets were washed with TE (0·01 M Tris, pH 7·8, and 0·005 M EDTA) and resuspended in 400 µl of TE by gently pipetting up and down. The suspension was used immediately for RNA extraction by digestion with proteinase K followed by standard acid phenol–chloroform extraction and ethanol precipitation (Sambrook et al., 1989). Pellets were resuspended in 5 µl ddH₂O and used immediately for cDNA synthesis. cDNA synthesis and PCR were carried out as described above but PCR was conducted using Pfu DNA polymerase (Promega) and the number of cycles was 20 instead of 35 in order to minimize the frequency of in vitro-generated variability. Reactions were performed following the manufacturer’s instructions, with the above-mentioned cycling conditions. It must be stated that RNA dilutions of up to 1:100 still give positive amplification bands in RT–PCR. This ensures that an amplification bottleneck is not biasing the complexity of the mutant spectrum. PCR products were purified from agarose gels using a commercial kit. Purified DNA was adenylated by treating with Taq DNA polymerase and cloned into a pGem-T Easy System II kit (Promega). Cloning in Escherichia coli, strain J109, was carried out as suggested by the manufacturer except that the 37 °C incubation step prior to plating in ampicillin-containing medium was only for 5 min to avoid duplicated clones. Bacterial colonies were double screened by blue/white standard selection followed by insert-specific PCR testing. Recombinant plasmids were extracted from white colonies by the Turboprep method (Woodford & Usdin, 1991) and used as templates for PCR amplification with primers 324 and 326. PCR conditions were as described above.

**Single-strand conformational polymorphism (SSCP) analysis.** PCR products from the plasmid preparation described above were analysed by SSCP, as described previously (Jones & Weber, 2001). Briefly, DNA was denatured using formamide and electrophoresed immediately in both 15% acrylamide and acrylamide–glycerol (15 and 5%, respectively) minigels at 200 V for 3 h. Gels were silver stained.

**Sequence analysis.** Direct sequencing of both strands of purified PCR products from variant clones was performed by the dideoxy-terminator method. Sequencing was performed using an ABI373 sequencer. Sequences were aligned using clustalw software (Thompson et al., 1994). Clustering of sequences was analysed using the partition analysis of quasispecies (PAQ) program (Bacca et al., 2001). Phylogenetic analysis was performed using the dnaps and dnaplus programs of the PHYLIP package (Felsenstein, 1993). Resampling of the original alignment for bootstrap analysis was performed with the SEQBOOT routine from PHYLIP.

Results

In this work we analysed the quasispecies structure of a BVDV obtained from lung, kidney and spleen tissues of a field-infected foetus that was approximately 4 months old. Virus from the lung was analysed thoroughly by RT–PCR–SSCP as follows: four different RT–PCRs (A–D) were achieved using viral RNA extracted from a 10% lung homogenate. PCR products from these reactions were cloned and inserts from 158 clones were submitted to SSCP analysis. A total of 11
different banding patterns was detected (Fig. 1a, b). There was one major pattern, Lung_M (Fig. 1a, b), represented by 125 clones (Fig. 1c). Patterns Lung_Q1, _Q4 and _Q5 had 11, 5 and 9 representative clones, respectively (Fig. 1c). These SSCP patterns were independent of the RT–PCR from which each clone was derived (Table 1). SSCP profiles Lung_D39, _D44, _D13, _A30, _C15, _C31, _C23 and _C17 were represented by just one clone each.

DNAs corresponding to all SSCP patterns from the lung-derived clones were sequenced. Quasispecies from the spleen and kidney were analysed by sequencing 11 and 10 randomly selected clones, respectively. Fig. 2 compares all of the sequences. Sequences from the kidney appear to be slightly more variable than sequences from the other organs, as there were five identical sequences (represented by Kidney_M) from a total of 10 samples (50%) in the kidney-derived clones, while among the spleen-derived clones, identical sequences (represented by Spleen_M) were 8 from a total of 11 samples (72%). Furthermore, variant sequences from the kidney appear to be more related to minor groups from the lung (Lung_Q1, _Q4 and _Q5) than to the major sequence (Fig. 3). Sequences Lung_M, Kidney_M and Spleen_M were identical (Fig. 2).

The ‘non-randomness’ of the observed substitutions was assessed by two different approaches: we tested whether nucleotide changes in variant clones affected the RNA base-pairing in the IRES structure and we compared the observed nucleotide substitutions with those of sequences described previously from different BVDV isolates. All changes, except those from positions 76, 163 and 228 of our sequences, were Watson–Crick base-pairing compliant [considering A–U, G–U and G–C as allowed base pairs and following the IRES structure proposed by Brown et al. (1992), Deng & Brock (1993) and Chon et al. (1998)] or were located in non-base-paired sectors of the molecule (Fig. 2). It must be noted that changes in positions 76, 163 and 228 were all of the type U to C (see also Fig. 3) and that the net result of all these changes is the formation of Watson–Crick base pairs, which are the most stable and thermodynamically favored type of base pairs in RNA interference (RNAi) (Brown et al., 1992). A change from U to C is thus expected to enhance the stability of the RNA structure, facilitating the binding of the viral IRES with the host cell machinery, leading to increased viral replication.

### Table 1. Composition of patterns Lung_Q1, _Q4 and _Q5 as ascertained by RT–PCR

<table>
<thead>
<tr>
<th>SSCP pattern</th>
<th>Clone</th>
<th>RT–PCR</th>
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<tbody>
<tr>
<td>Lung_Q1</td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Lung_B6</td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Lung_C4</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Lung_C7</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Lung_C38</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Lung_D3</td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Lung_D9</td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Lung_D19</td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Lung_D25</td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Lung_D32</td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Lung_D39</td>
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<td>D</td>
</tr>
<tr>
<td>Lung_A40</td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Lung_C6</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Lung_C27</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Lung_D33</td>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Lung_D40</td>
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</tr>
<tr>
<td>Lung_A5</td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Lung_A27</td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Lung_A43</td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Lung_B35</td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Lung_C25</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Lung_C43</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Lung_D14</td>
<td></td>
<td>D</td>
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<tr>
<td>Lung_D22</td>
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<tr>
<td>Lung_D29</td>
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Fig. 2. Differences between 5'UTR sequences from lung, kidney and spleen. The multiple nucleotide sequence alignment was obtained using CLUSTALW. Dots indicate identity with upper sequence; dashes indicate gaps introduced to maximize alignment. Names are as described in Fig. 1 and are discussed in the text. Kidney_M and Spleen_M: major sequence from kidney and spleen, respectively. Variant positions and positions with which those form base-pairings are connected by lines. Positions 75, 103, 104 and 240 are located in non-base-paired sectors.
BVDV quasispecies

Fig. 3. Phylogenetic hypothesis for all identified quasispecies. Substitutions are mapped on the cladogram. Nucleotide substitutions (\(r\)), reversions (\(X\)) and parallelisms (\(s\)) are indicated; numbers indicate the alignment position in which the substitution occurs. Polarity was determined using the major sequence as outgroup. Note that Lung..A30 may also be assigned to the group defined by character 76. Bootstrap (1000 resamplings) value for M and Q groups is indicated in parentheses.

independent changes is the substitution of an U–A pairing group by a C–A pair. We compared our alignment (Fig. 2) to an alignment of 5'UTR sequences from 91 BVDV isolates, selected randomly from GenBank. Variable positions 9, 58, 62, 81, 103, 163, 225, 228, 230 and 240 from our alignment correspond with variable positions in the alignment of the randomly selected sequences. Furthermore, the changes observed in these positions are similar to those observed for the different strains (Table 2). Aside from these criteria, we reinforced the non-randomness of changes by the observation of a high covariance at positions 9 and 58, the appearance of similar quasispecies from different RT–PCRs and the existence of equal variant sequences in different organs. From the analyses described above, we conclude that positions 9, 58, 62, 81, 103, 225, 230 and 240 are almost free of randomness; positions 75, 77, 104, 117, 163 and 228 satisfied one of our criteria, while changes at position 76 may be due to experimental artefacts. It must be emphasized that changes from T to C at position 76 were detected in five independent PCR events: A–D, represented by Lung..Q1, ..A30 and ..C15, plus clone Spleen..77. We avoided making speculations based on evidence provided just by this position.

We analysed the grouping of the quasispecies sequences using PAQ, which brings up a clustering structure based on global similarity between the input sequences. The main differences between PAQ and other agglomerative methods are its non-hierarchical clustering algorithm and the fact that PAQ allows for overlapping clusters. Phylogenetic analysis was performed using the DNA pars and DNA ml programs of the PHYLIP package. Fig. 3 depicts a proposed phylogeny for the quasispecies under study. The phylogenetic analysis approaches used (parsimony and maximum-likelihood methods) both identified groups M and Q (Fig. 3) but failed to resolve them further. PAQ also identified groups M and Q (data not shown). Groups obtained by PAQ did not differ from those identified by phylogenetic analysis (data not shown).

Discussion

In this work, we showed that natural BVDV infections exist as a mutant spectrum. In a previous work from Becher et al. (2000), a mutant spectrum was generated from an infectious clone. These two observations justify the use of quasispecies terminology for BVDV.

The 5'UTR was chosen for this analysis of quasispecies for several reasons: (i) it is the most conserved region of the pestivirus genome (therefore, RT–PCR amplification is efficient and almost free of subpopulation selection due to the

### Table 2. Comparison of quasispecies substitutions with changes in homologous positions from different isolates

<table>
<thead>
<tr>
<th>Position</th>
<th>9</th>
<th>58</th>
<th>62</th>
<th>75</th>
<th>76</th>
<th>81</th>
<th>103</th>
<th>104</th>
<th>117</th>
<th>163</th>
<th>225</th>
<th>228</th>
<th>230</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>GenBank</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Variant</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>T</td>
<td>A</td>
<td>G</td>
<td>A</td>
<td>C</td>
</tr>
</tbody>
</table>

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differential annealing of primers); (ii) the use of more variable regions for this type of analysis is not straightforward, as the presence of a large number of variants may induce artefactual polymorphisms related to sampling error; and (iii) the highly structured nature of the 5’UTR allows the randomness of the substitutions among variant sequences to be tested.

It is well known that experimentally generated variability is a major problem in studying virus quasispecies (reviewed by Smith et al., 1997). The low proportion of randomness in the data presented here may be attributable to two principal facts: (i) Pfu polymerase has a 90% reduction in the error rate when compared with Taq polymerase (Lundberg et al., 1991); and (ii) the step of virus concentration, since using high concentrations of virus appears to reduce the likelihood of introducing experimental artefacts (Laskus et al., 1998). A work presented by Arias et al. (2001), which compared the performance of molecular and biological cloning for studying foot-and-mouth disease virus quasispecies, showed similar results. These authors observed that biological and molecular clones provide indistinguishable definitions of the mutant spectrum. We chose to clone RT–PCR products obtained directly from animal organs in order to avoid the selection of virus subpopulations by replication in cell culture.

The genomic sector analysed here spans residues 128–372 of the NADL reference strain sequence (Coletti et al., 1988). As expected, the observed nucleotide changes do not appear to affect structures essential for IRES function (Chon et al., 1998). The BVDV IRES has been shown to act in RNA stability, RNA replication, translation of the viral polyprotein and encapsidation of the viral genome (Becher et al., 2000; Yu et al., 2000). 5’UTR variants could be related with the existence of different environmental constraints in different tissues, i.e. conditions in different tissues might favour the presence of different virus quasispecies acting as selective pressures over some 5’UTR sectors. However, we may not conclude that the changes observed in the 5’UTR have an adaptive function, as quasispecies evolution in the E2-encoding region may occur in the absence of an apparent selective force (Collins et al., 1999). In addition, changes in any region of the genome may be linked with changes in another region (i.e. if changes occurred at the same time).

In HCV, phylogenetic analysis has been used to demonstrate the occurrence of compartmentalized virus replication through the identification of monophyletic groups exclusive to different cells (Rogue Afonso et al., 1999). Although we were able to detect sequences exclusive to different organs, quasispecies did not form any kind of groups according to the organ of origin, neither by phylogenetic- (Fig. 3) nor by similarity (pAQ)-clustering analysis (data not shown). These may indicate either the non-existence of clonal lineages replicating in different organs or its existence at proportions not detectable by the strategy implemented in this work. In any case, the detection of unique sequences in different organs is a proof of the existence of variants occurring in low frequencies. That is, representatives of some lineages are in minor numbers and thus have less chance of being detected. Thus, our data suggest that BVDV could replicate at high rates in some tissues (in blood cells, for example, as there are evidences of the presence and ability of replication of BVDV in white blood cells) (Truitt & Schechmeister, 1973; Ohmann, 1983; Brodersen & Kelling, 1998), while replication rates in some cells may be very low. Low-kinetic 5’UTR subpopulations have been detected in HCV and have been suggested to act in avoiding immune surveillance (Jang et al., 1999).

Immunity does not always protect the foetus from congenital infection (van Oirschot et al., 1999). Ability to elude antibodies may be an explanation for transplacental infections in seropositive dams. Previous works suggest that the genetic variability of the 5’UTR correlates with antigenic characteristics (van Rijn et al., 1997; Flores et al., 2000; Nagai et al., 2001). The diversity observed by us in the 5’UTR might be an indirect evidence of a high variability in other genomic regions, which may be a strategy for excluding some virus variants from immune system recognition. Previous works have shown the existence of herd-specific BVDV strains (Paton et al., 1994), apparently maintained by persistently infected animals (Hamers et al., 1998). If these persistently infected animals are born from vaccinated cows, then the herd may be a focus for spreading vaccine-resistant BVDV variants.

BVDV has been used as a model for HCV (Weiner et al., 1991; Ruibal Brunet et al., 1999; Prince et al., 2000; Baginski et al., 2000; Zitzmann et al., 1999). Since BVDV is much easier to be passaged in cell culture than HCV, it may be a good candidate for studies of the relationship between virus quasispecies and virus behaviour under particular conditions; for instance, the presence of an antiviral compound. Results originating from this type of study may serve as a preliminary test prior to the analysis of HCV.

The strategies used in this work may be adapted easily both to analyse large numbers of samples of other genomic regions and for the study of BVDV quasispecies evolution in systems different from infected animals. Further work is necessary to elucidate the biological role of quasispecies in BVDV. A deeper knowledge of the genetic variability of BVDV will be of great importance for classification, diagnosis, vaccination and understanding some as yet unknown aspects of BVDV biology.

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