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Antigenic and genetic characterisation of border disease viruses isolated from UK cattle

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Abstract

Available empirical data on the natural occurrence of ruminant pestiviruses has shown that bovine viral diarrhoea virus (BVDV) is nearly exclusively found in cattle, whereas both border disease virus (BDV) and BVDV can be isolated from sheep. During routine genetic typing of pestivirus RNA from UK cattle diagnosed as BVDV positive between 2006 and 2008, five samples that were classified as BDV positive yielded positive virus isolates in cell cultures. The samples originated from animals that had shown signs typical for BVD. Phylogenetic analysis of the bovine BDVs showed that two belonged to the BDV-1a group and three to the BDV-1b group, thereby matching the genetic diversity seen for previously described UK ovine BDVs. Antigenic typing with a set of monoclonal antibodies (MABs) showed that all bovine BDVs lacked one or more epitopes conserved among ovine BDV-1 isolates, and that they had gained reactivity with at least one BVDV-1 specific MAB. Serial passaging of two of the virus isolates in ovine cell cultures did not change the epitope expression pattern. These findings suggest that the presumed natural resistance of cattle against infection with BDV no longer holds. A consequence of this is that BVD diagnostic assays should be checked for their ability to also detect BDV, and also highlights the need for monitoring of the BDV status in sheep that may be in contact with cattle in areas with organised BVD control programmes.

Keywords: Pestivirus; Border disease virus; Cattle; Genetic typing; Monoclonal antibody
1. Introduction

Pestiviruses cause economically important diseases in domestic ruminants and pigs worldwide. There are currently four recognized members of the genus Pestivirus, family Flaviviridae, three of which infect ruminants naturally; bovine viral diarrhoea virus (BVDV) 1, BVDV-2 and border disease virus (BDV) (Thiel et al., 2005). Acute BVDV infection in cattle can vary from sub-clinical infection to mild clinical disease with inappetence and transient fever, or with more specific signs such as diarrhoea, respiratory or reproductive dysfunction. Vertical transmission of virus in pregnant animals to their unborn young that can result in abortion, mummification of the foetus, congenital defects, stillbirth or the birth of persistently infected calves immunotolerant to BVDV (PI). PI animals have been shown to excrete high levels of infectious virus in the absence of clinical disease. Identification of persistently infected animals within the herd is obviously of great economic importance. The presence of BVDV-1 infection in cattle has been detected worldwide. In contrast BVDV-2 infection has only been seen since the 1990s when it was detected in cattle in North America (Pellerin et al., 2000). More recently, BVDV-2 isolates have been detected in Europe, Japan, Korea and South America (Vilcek et al., 2001; Nagai et al., 2001; Flores et al., 2000).

Border disease is principally a disease of sheep that shares some of the characteristics of BVD in cattle, but with more pronounced emphasis on reproductive disease such as abortion, and birth of stillborn or mummified fetuses. It can also cause the birth of small weak lambs that suffer with ‘hairy-shaker’ syndrome named due to the hairy fleeces and tremors suffered by the lambs (Nettleton et al., 1992). Birth of PI animals is common in sheep but more rare in goats.

The Pestivirus genome consists of a single stranded positive-sense RNA of approx 12.3 kb in length, encoding for a single open reading frame that is flanked by a 5’ and 3’ untranslated regions (UTR). The genome codes for 4 structural proteins, the capsid (C) and three envelope
proteins (\(E_{\text{ms}}^+, E1\) and \(E2\)), plus several non-structural proteins (Thiel et al., 2005).

Phylogenetic studies generally classify virus isolates based on sequences generated from the 5'-UTR or N\(^{\text{pro}}\). BDV isolates have been divided into six phylogenetic groups; BDV-1 has been detected in sheep from the USA (Sullivan et al., 1997), the UK (Vilcek et al., 1997), Australia (Becher et al., 1994) and New Zealand (Vilcek et al., 1998); BDV-2 in ruminants in Germany (Becher et al., 2003); BDV-3 in Switzerland (Stalder et al., 2005) and Austria (Krametter-Froetscher et al., 2007), BDV-4 in Spain (Valdazo-Gonzalez et al., 2007) and BDV-5 and -6 in France (Dubois et al., 2008). Isolates from Turkey form a possible seventh group, BDV-7, that remains to be fully characterised (Oguzoglu et al., 2008).

Historically pestiviruses were named after the animal host species they were isolated from. However, the ability of interspecies transmissibility has been shown both naturally and experimentally (Paton et al. 1995; 1997). For instance, BVDV also infects sheep, goats, wild ruminants and pigs (Becher et al., 1997). In sheep, BVDV-1 has been detected in Germany, Sweden, UK and the United States (Becher et al., 1994; Vilcek et al., 1997; Willoughby et al., 2006) and BVDV-2 in sheep in Germany, UK and the US (Becher et al., 1995, Sullivan et al., 1997, Vilcek et al., 1997). Conversely, classical swine fever virus (CSFV) has never been isolated from naturally infected cattle, although an experimental study has shown cattle to be susceptible to CSFV (Dahle et al., 1987).

Although BVDV is commonly seen in sheep there is very little evidence of natural infection with BDV in cattle. One exception is the Australian V/TOB isolate, which W.A. Snowdon (1973) described as antigenically different from other bovine isolates, and that later was genotyped as BDV by Becher et al. (1997). More recently, one case of BDV in cattle was identified during routine testing in Austria as part of their BVDV control program (Hornberg et al., 2009).
During recent screening for exotic pestiviruses in UK cattle samples positive for BVDV antigen, six cases were found to be BDV positive by TaqMan RT-PCR. Here we describe the genetic and antigenic typing of five bovine BDV isolates from the UK.

2. Material and methods

2.1 Origin of BDV isolates

Three suspect BVD cases were identified as being caused by BDV by a routine TaqMan RT-PCR genotyping as reported by Cranwell et al. (2007). Three further cases have tested positive for BDV; one case in 2006 by RT-PCR of spleen tissue from an aborted foetus, and in 2008 two more cases initially diagnosed as BVDV positive by antigen ELISA (Serelisa BVD/MD Ag, Synbiotics, France) were typed as BDV positive by the TaqMan RT-PCR (Table 1).

Information on the case histories and potential epidemiological links between cattle and sheep on the farms where the isolates came from was obtained either by farm visits or sought from the private veterinary surgeons attending the cases.

2.2 Virus isolation

Either plasma or clarified supernatant from homogenized tissue samples were set up for virus isolation by inoculation onto pestivirus-free foetal bovine turbinate (fBT) cells, maintained in minimum essential medium containing 10% foetal bovine serum proven free from pestiviruses and pestivirus neutralizing antibodies. After a further virus passage on fBT cells, samples were fixed and immunostained using an unconjugated pool of pan-pestivirus reacting monoclonal antibodies (MABs) (WB103/WB105/WB112) and a peroxidase-linked secondary mouse antibody as described by Paton et al. (1994).
2.3 Genetic typing

RNA was extracted from infected fBT cells using Trizol® (Invitrogen, UK) and RNA was eluted in 100μl nuclease-free water. Reverse transcription (RT) was carried out using the Superscript™ II Reverse Transcriptase kit (Invitrogen, UK) as per the manufacturer’s protocol. A 242-246 nucleotide (nt) fragment of the 5’-UTR was amplified using primers 324/326 (Vilcek et al., 1994) and Taq DNA polymerase (Promega, UK). The N\textsuperscript{pro} gene was amplified with primers 320F (5’-GCCTGATAGGGTGYWGCAGAG-3’, forward) and 1040R (5’-TTYCCTTTCTTCTTYACCTGGTA-3’, reverse). Sequencing reactions based on purified amplicons were set up in both directions using both the PCR primers and the 356F / 1027R pair (Valdazo-Gonzalez et al., 2007) using a chain termination protocol based on fluorochrome labeled dideoxynucleotides (Cogenics, UK). Assembled sequences were aligned with matching sequences of BDV and BVDV reference strains using Clustal X (Thompson et al., 1997). Transition/transversion ratios were calculated with Puzzle4 (Strimmer and von Haeseler, 1997) and phylograms were calculated using components of the PHYLIP package (Felsenstein, 1989). Phylogenetic analyses were carried out using the maximum likelihood distance matrix method using 1000 replicates for calculation of bootstrap values. Trees were drawn with TREEVIEW (Page, 1996) and edited with Freelance Graphics. Nucleotide sequences of the new BDV strains have been submitted to GenBank; accession numbers are given in Table 1.

2.4 Antigenic typing

fBT cells infected with each virus isolate were acetone fixed and immunostained using a selection of monoclonal antibodies (MABs) raised against different pestiviruses (BDV, BVDV-1, BVDV-2 and CSFV) (Edwards et al., 1988; Edwards and Sands 1990; Paton et al., 1994). The staining intensity was graded as negative, weak positive and strong positive with staining of less than 50% of the monolayer also being recorded. Ten serial passages were
carried out on two of the isolates using pestivirus-free sheep choroid plexus (SCP) cells maintained in minimum essential medium containing 10% foetal bovine serum proven free from pestiviruses and neutralizing antibodies to pestiviruses. Each passage was fixed, immunostained and results recorded as described for the isolates grown in fBT cells.

3. Results

3.1 Case histories

The available case history information was variable. For the adult animals the presentation was undistinguishable from typical signs seen in animals PI with low-virulent BVDVs (Table 1). Four of the cases came from farms that also kept sheep, but the degree of contact appeared to have been variable. There was no record of recent border disease among the sheep on any of the farms involved, nor any obvious signs of BVD in other cattle.

3.2 BDV isolates

A pestivirus was isolated from five of the six cases that had been recorded positive by RT-PCR. The sixth sample remained negative after two virus isolation attempts with spleen samples from the aborted foetus, probably due to the cytotoxicity of the available sample. When cultured on fBT cells, all five BDV isolates viruses were of the non-cytopathogenic biotype.

3.3 Genetic typing

The genetic analysis of partial 5’-UTR sequences showed that all five bovine BDV isolates grouped with other UK ovine BDV-1 sequences. Three isolates clustered within the BDV-1b viruses, together with isolate K1729/3 from 1987, whilst the other two isolates were allocated to different clusters within the BDV-1a viruses (Fig. 1). Pairwise sequence comparison with the closest matching ovine BDV 5’-UTR sequence showed between two to five nucleotide substitutions, corresponding to sequence identities between 97.9 and 99.2 %. Within the
group of bovine BDVs sequence identities ranged from 97.1 to 88.5%. A partial 5'-UTR nucleotide sequence (140 nucleotides) derived from a PCR amplicon of the virus isolation negative case showed greatest similarity to that of isolate 1505744, but with a significantly lower sequence identity (81%).

Phylogenetic analysis based on full length \(N^{pro}\) sequences resulted in the same classification of the five bovine BDVs as obtained with the partial 5'-UTR sequences, but with much higher bootstrap values (Fig. 2). Pairwise sequence identities for the group of bovine BDVs ranged from 98.4 to 75.4%, with the same order of sequence identity as seen for the partial 5'-UTR sequences.

### 3.4 Antigenic typing

The five bovine BDV isolates were most similar to ovine BDVs by immunostaining, although an unusual reactivity pattern was observed compared to the BDV reference strains (Fig. 3). The otherwise broadly reactive BDV MAB WS381 failed to detect four of the five bovine BDV isolates. Similarly, MAB WB160 did not recognise three of the bovine BDVs. Most of the bovine BDVs were immunostained by MABs WB214, CA1, and CA39, which have been known as BVDV-1 specific. In general, the staining intensity with the anti-BDV MABs was more variable for the bovine BDVs than for the ovine BDVs. The immunostaining pattern of the bovine isolate 1505744 was more similar to BDV reference strains than the other four bovine BDVs.

Two of the bovine BDV isolates, 1062689 and 1118212, were maintained for up to 10 passages on SCP cells to assess any changes in the MAB reactivity pattern. No change in the MAB reactivity pattern was seen for either of these isolates (data not shown).
4. Discussion

It has been well documented that BVDV not only infects cattle but also other members of the Order Artiodactyla, whilst BDV has mainly only been found in small ruminants. This study describes antigenic and genetic properties of the first BDVs isolated from UK cattle. Previous in vivo transmission studies of BVDV between cattle and sheep showed host animal dependent reversible changes in the MAB reactivity pattern (Paton et al., 1997). To avoid artificial induction of an ovine antigenic profile, the cattle BDV isolates were maintained in bovine cell cultures for the antigenic characterization. Although variable, the MAB reactivity pattern of the cattle isolates revealed an antigenic profile most similar to ovine BDV reference strains. The most striking exception was that nearly all the bovine BDVs expressed the BVDV-1 specific WB214, CA1 and CA39 epitopes, which reside within the major envelope protein E2. Interestingly, these epitopes were the only ones that were expressed exclusively in a bovine environment in the study by Paton et al. (1997). This may indicate that the WB214, CA1 and CA39 epitopes are directed against cell surface receptors important for replication in bovine, but not ovine cells. These changes, along with the general lower reactivity against BDV specific MABs indicate that BDVs benefit from adapting antigenically when shifting to a new host environment. However, this would not explain why antigenic differences in non-structural proteins were seen – whilst the NS3 specific MAB WB160 has been known to recognise most BVDV-1 and BDV isolates, three of the five bovine BDVs were not immunostained with this MAB. The observation that the “bovine MAB reactivity pattern” did not alter after 10 passages in ovine cell cultures is consistent with the previously observed delayed reversion to an ovine antigenic pattern of a bovine BVDV after transfer from cattle to sheep (Paton et al., 1997). This indicates that the antigenic adaptation after host animal changes may be a clonal selection process that takes some time, in the absence of immune selective pressure.
Phylogenetic analysis showed that the bovine BDV isolates grouped together with previously described UK ovine BDV-1 isolates (Vilcek et al. 1997). This genetic classification is not unlikely considering the expected continued field circulation of UK BDVs since they were isolated 15-30 years ago. Based on the partial 5'-UTR sequences, the best sequence match for the bovine BDVs was for the group of three BDV-1b’s with a Scottish isolate obtained 20 years ago, whereas the remaining two bovine BDVs were allocated to two different groups of BDV-1a’s. The same genotyping pattern was seen in the Npro based phylogram, with sufficient statistical support to verify the 5'-UTR based classification. Direct comparison of Npro sequences of the bovine BDVs with their closest matches in the 5'-UTR phylogram was not possible since the latter Npro sequences were not available from GenBank. Interestingly, the Australian V-TOB BDV isolate, reported to have originated from a case of acute mucosal disease, and being antigenically distinct from contemporary BVDV isolates (Snowdon, 1973), is also a BDV-1a, but genetically different from the bovine BDVs characterised in this paper.

Based on the relatively high genetic similarity of the isolates 1062689, 1502304 and 1505744, it is possible that they might have emerged from the same parental virus strain, although the differences in the MAB reactivity patterns did not support this. On the other hand, from the more similar MAB profile of isolates 1118212 and 1376527 one might assume they are more closely related than the bovine BDV-1b isolates, but the genotyping does not support this either. However, it should be noted that the genetic typing is based on non-coding nucleotide sequences, as opposed to antigenic properties that to a large extent reside in structural glycoproteins involved with host cell receptor binding.

Recently a wide genetic diversity of BDV isolates has been described. Although all current BDV reference strains belong to the BDV-1 cluster (Thiel et al., 2005), several new genetic groups have now been described, most recently in southern Europe and around the Mediterranean (De Mia et al., 2005; Thabti et al., 2005; Valdazo-Gonzalez et al., 2007;
Dubois et al., 2008; Oguzoglu et al., 2009). To some extent there appears to be a geographical bias in the distribution of BDVs in sheep. In the UK, BDV-1 is the only genetic group that has been found (Vilcek et al., 1997), but this group has also been identified in Australasia and the Americas. The genetic groups BDV-2 to -6 have only been described regionally in Germany, Switzerland and Austria, Spain and France, respectively (Becher et al., 2003; Stalder et al., 2005; Krametter-Froetscher et al., 2007; Valdazo-Gonzalez et al., 2007; Dubois et al., 2008). Of these, only BDV-1 and BDV-3 have been isolated from cattle in Australia, the UK, Italy and Austria (Snowdon, 1973; Cranwell et al., 2007; Schirrmeier et al., 2008; Hornberg et al., 2009), and on each occasion the bovine BDV isolates were of the common local genotype. Thus it seems like the BDV strain is less important than how sheep and cattle are managed. A sufficiently close epidemiological contact between the two animal species appears to be the most important risk factor for interspecies transfer. In the UK, sheep and cattle are kept in relatively close contact on many farms, including the majority of the cases investigated here. It is not unlikely that this also is a contributing factor to the high prevalence of BVDV in sheep in the UK (Vilcek et al., 1997); if so, detection of BVDV in sheep in other countries could be an indicator of likely presence of local BDVs in cattle as well.

When a pestivirus is isolated from particularly a novel host animal, it is important to ensure that the virus actually originated from the animal and is not a laboratory contamination. Cell cultures and foetal calf serum used to supplement cell culture medium have frequently been shown to be contaminated with BVDV (Bolin et al., 1994; Sandvik et al., 1997; Schirrmeier et al., 2004). This may be one explanation of the general high prevalence of pestivirus infections worldwide, and also of some instances where the origin of sets of isolates has been questioned (Vilcek et al., 1997; Vilcek and Paton, 2000). This potential problem can be overcome by detection of pestiviruses by RT-PCR directly in original sample material, and with identity confirmation by partial sequencing of PCR products. Following this approach,
and demonstrating that the five bovine BDVs characterised in this study although genetically
distinguishable were allocated to three different phylogenetic clusters, it is highly likely that
BDV has jumped host species from sheep to cattle on at least three different occasions in the
UK. This route of infection is supported by the findings of Schirrmeier et al. (2008) and
Hornberg et al. (2009), who both also detected BDV-3 directly in blood samples by RT-PCR
and sequencing.

Detection of natural infection of cattle with BDV highlights the needs for BVDV diagnostic
tests that are able to detect all three ruminant pestiviruses; BVDV-1 and -2 as well as BDV.
The majority of the cases described here were originally diagnosed as BVDV antigen ELISA
positive, using an assay specific for the NS2-3 antigen. Being the most conserved of all
pestivirus proteins, NS2-3 is the most likely viral antigen to be detected by broadly reactive
MABs. Recently BVDV antigen ELISAs targeting the E^{ms} glycoprotein have become more
popular. It may be difficult to know to what extent E^{ms} ELISAs will detect bovine BDVs,
since the antigenic profile of the latter differed from BDV reference strains. Diagnostic RT-
PCR assays generally have a high sensitivity, and most assays targeting the 5' UTR by use of
Pestivirus generic primers should be able to amplify BDV cDNA, but it is important to ensure
that the fluorogenic detection probes in use also recognise BDV.

Lastly, from a disease control point of view, it would be of interest to assess to what extent
inactivated BVDV vaccines are able to provide immunoprotection against BDVs, of both
ovine and bovine origin. Since sheep previously have been known to be infected with BVDV,
sheep should not be considered a greater risk for BVD control programmes after natural
infection of cattle with BDV has been described, since all domestic inter-ruminant contact
should be considered a risk for successful eradication of BVD.

5. Conclusion
Molecular characterisation of five BDV-1s from naturally infected cattle has indicated that they are likely to have jumped host species from sheep to cattle within the UK. These bovine BDVs showed an antigenic profile differing from that of ovine BDVs, with lower reactivity to BDV specific MABs, whilst they were expressing epitopes normally only seen in BVDV-1. The previously assumed natural resistance of cattle against infection with BDV therefore no longer holds.

Acknowledgements

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References


Table 1. Case history details of bovine border disease virus isolates characterised in this study.

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<th>Isolate</th>
<th>Year</th>
<th>County</th>
<th>Clinical signs, age</th>
<th>Contact with sheep</th>
<th>Sample</th>
<th>GenBank Accession no.</th>
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<td>2006</td>
<td>West Yorkshire</td>
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<td>Blood</td>
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<td>Powys</td>
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Fig. 1.
Phylogenetic tree constructed from partial 5’UTR sequences (241-247 nucleotides) of five UK bovine Border disease viruses (BDV) (bold & underlined typeface), BDV and Bovine viral diarrhoea virus (BVDV) reference strains (bold typeface), previously described UK ovine BDVs (Vilcek et al., 1997), and representatives for other BDV genetic groups (Valdazo-Gonzalez et al., 2007; Dubois et al., 2008). BVDV strain NADL was used as outgroup species.

Fig. 2.
Unrooted phylogenetic tree based on full Npro sequences (504 nucleotides) of five UK bovine Border disease viruses (BDV) (bold & underlined typeface) plus representatives of other BDV genetic groups as well as Classical swine fever virus, Bovine viral diarrhoea virus and various other pestivirus. Literature references to sequences not included in Fig. 1 have been described in Valdazo-Gonzalez et al., (2007), plus in Dubois et al., (2008). The BDV-1 branch is reproduced at 200% scale for clarity.

Fig. 3.
Reactivity of MABs to bovine BDV isolates, ovine BVD isolates, BVDV-1 and BVDV-2. MABs used were raised against various epitopes; anti-E2 (WS381, WS384, WB166, WB214, WH303); anti-Ems (WS363, WS368); anti-NS2/3 (WB160, WA437, WA443). Black boxes indicate positive reaction between the MAB and virus and a grey box indicates a weak or partial positive reaction.
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<th>NS2-3</th>
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