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Research Article

Antigenic Diversity of Bovine Viral Diarrhoea Viruses (BVDV) Isolated in Turkey

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Abstract: Sixty BVDV field strains isolated from different regions of Turkey were antigenically characterised with a panel of 16 monoclonal antibodies (mAbs) specific to viral proteins E2 and NS2-3. MAb recognition patterns were detected by an indirect peroxidase-linked antibody assay and data were subjected to computer analysis for discussing herd specificity of viral strains isolated from immunotolerant, persistently infected (IPI) cattle. For that purpose, phylogenic and statistical analyses were performed. A high level of antigenic diversity was detected among the isolates. Diversity was also exhibited in IPI strains originating from the same herd. In some herds, viruses were divided into 2 viral subpopulations that were antigenically distinct from each other. The viruses in the subpopulations were significantly different from the viruses in the twin-subpopulation, as well as from viruses in other herds. This study demonstrated epitopic properties of Turkish BVDV field strains; yet, the results of this study do not support the concept of herd specificity of the virus.

Key Words: Bovine viral diarrhoea, BVDV, monoclonal antibodies, antigenic diversity, phylogenetic analysis, herd specificity

Türkiye'de İzole Edilen Bovine Viral Diarrhoea Virus (BVDV) İzolatlarında Antijenik Farklılıklar

Özet: Bu araştırmada Türkiye'nin değişik bölgelerinden elde edilmiş olan 60 BVDV izolatının E2 ve NS2-3 proteinlerine spesifik 16 monoklonal antikorla (mAb) antijenik karakterizasyonu yapılmıştır. MAb reaksiyonları indirekt immunoperoksidaz tekniği ile belirlendi. Veriler immunotolere persiste enfekte (IPI) bireylerden elde edilmiş izolatların sürü spesifitesini belirlemek amacıyla kullanıldı. Bu amaca yönelik olarak filogeni analizi ve istatistik analizler gerceklestirildi. Izolatlar arasında önemli düzeyde antijenik farklılıklar olduğu ve bu farklılıkların aynı sürüdeki IPI sığırlardan izole edilmiş viruslarda da bulunabileceği saptandi. Bazı sürülerde virus izolatlarının antijenik olarak farklı 2 alt populasyona ayrıldığı tespit edildi. Alt popülasyonda yer alan virusların sürüdeki diğer alt popülasyondan ve diğer sürülerdeki viruslardan önemli düzeyde farklı olduğu belirlendi. Bu araştırmada elde edilen veriler Türkiye'de izole edilmiş BVDV saha izolatlarının epitopik özelliklerini ortaya koymaktadır. Araştırma bulguları virusun sürü spesifitesi kavramını desteklememektedir.

Anahtar Sözcükler: Bovine viral diarrhoea, BVDV, monoklonal antikorlar, antijenik farklılık, filogenetik analiz, sürü spesifitesi

Introduction

Bovine viral diarrhoea virus (BVDV) causes different clinical manifestations in cattle, ranging from mild respiratory disease to foetal death and mucosal disease. BVDV is a member of the genus *Pestivirus*, of the family *Flaviviridae.* The virus contains a single stranded RNA genome 12.5 kb in length, with positive polarity, flanked by non-translational regions at 5' and 3' ends (5'UTR and 3'UTR). Two different genotypes of the virus, BVDV-1 and BVDV-2, have been distinguished by sequence analyses of 5'UTR (1), and 2 biotypes, cytopathogenic (cp) and non-cytopathogenic (ncp), have been recognised according to their effects on cultured cells. BVDV virions consist of 4 structural proteins, namely nucleocapsid C protein and the envelope glycoproteins E^{rns}, E1, and E2. The majority of viral epitopes related to virus neutralisation are located on glycoprotein E2 (gp53). BVDV isolates and laboratory strains may have important

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differences in their genomic composition (2) and most mutation-originated changes are located on glycoprotein E2. Therefore, the reactivity of monoclonal antibodies (mAbs) directed to glycoprotein E2 is a very important tool for the characterisation of BVDV strains.

Infection of pregnant cows by ncp strains during the first trimester of gestation may result in the birth of immunotolerant persistently infected (IPI) calves (3). IPI animals, shedding live virus in high titres throughout their lives, are the main source for introduction and survival of the virus in cattle herds (3). Superinfection of IPI calves by an antigenically related cytopathogenic BVDV strain results in mucosal disease. It was hypothesised that the presence of IPI animals could lead to the generation of herd-specific strains in cattle herds (4).

The objectives of this study were to characterise 60 BVDV isolates taken from 12 different herds in Turkey during field screening activities using mAbs, and to analyse antigenic relationships among those isolates as an epidemiological tool.

Materials and Methods

Viral Isolates, Cell Line, and Monoclonal Antibodies

A total of 60 BVDV field isolates were received from the virus collection of the Virology Department of Ankara University, Faculty of Veterinary Medicine. These viruses were isolated between 1997 and 2000 from 12 Turkish dairy herds, of which 11 were closely managed state farms and one was privately owned (herd D) (5,6, unpublished data). These herds were coded with the letters (A to M) and viral isolates from each herd were identified with a number, e.g., A1, A2. Of all the viral isolates, 18 were from IPI cattle (Figure 1), while 1 isolate was from a clinical mucosal disease case from herd A. There were 2 more isolates taken from IPI animals in herd A. The herds in which BVDVs were isolated were located in eastern (herds C and G), central (herds D, F, K, L, and M), western (herd B), southern (herds A and H), and northern (herds E and J) regions of Turkey. The NADL strain of BVDV was also used in the study. Virus replication was confirmed by direct peroxidase linked antibody assay (dPLA) (7).

The Madin-Darby Bovine Kidney (MDBK) cell line, previously proved to be free from endogenous BVDV infection, was used in the study in Dulbecco's MEM by the addition of 10% foetal calf sera that was free of BVDV antigens and antibodies. A panel of 16 mAbs, which was kindly provided by Prof. V. Moennig (Virology Institute of Hannover Veterinary School, Germany), was used in the antigenic characterisation step (Table).

Biotype Characterisation and Purification of Mixed Biotypes

Biotype characterisation of the viral isolates was performed by immunoplaque assay (8). From 1 isolate which, was a mixture of both biotypes, the cp biotype was purified using a plaque assay, while the ncp biotype was purified by the limiting dilution technique (9). Purified biotype clones are shown as A3 and A4 (Figure 1).

Antigenic Characterisation

For this purpose, 100$TCID_{50}$ dilutions of viruses were placed into microtitre plate wells (100 µl volume) in duplicates and mixed with MDBK cells at 15.00 cells/well. Plates were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h and heat fixed before 1 h incubation with mAbs. Binding of mAbs was determined by a secondary antibody (biotinylated anti-mouse IG) and streptavidin-biotinylated-peroxidase complex (Amersham, Germany). The substrate solution included 2 mg of 3-amino-9-ethyl-carbazole (Sigma, Germany) dissolved in 0.3 ml of dimethyl-formamide (Merck, Germany), 4.7 ml of 0.05 M acetate buffer (pH 5), and 0.03% H_2O_2 (v/v). Results were evaluated with an inverted microscope.

Table. Parental virus strain and protein specificity of monoclonal antibodies used in the study.

Monoclonal Antibody	Homologue Strain	Protein Specificity
C16	NADL	NS2-3
CT2, CT3, CT6, CT9	A1138/69	E2
CA1, CA3	NADL	E2
CA25, CA34, CA36, CA39	7443	E2
CA73, CA78, CA80, CA82	Singer	E2
PX1	0712/80	*

* Protein specificity of this mAb has not been determined.

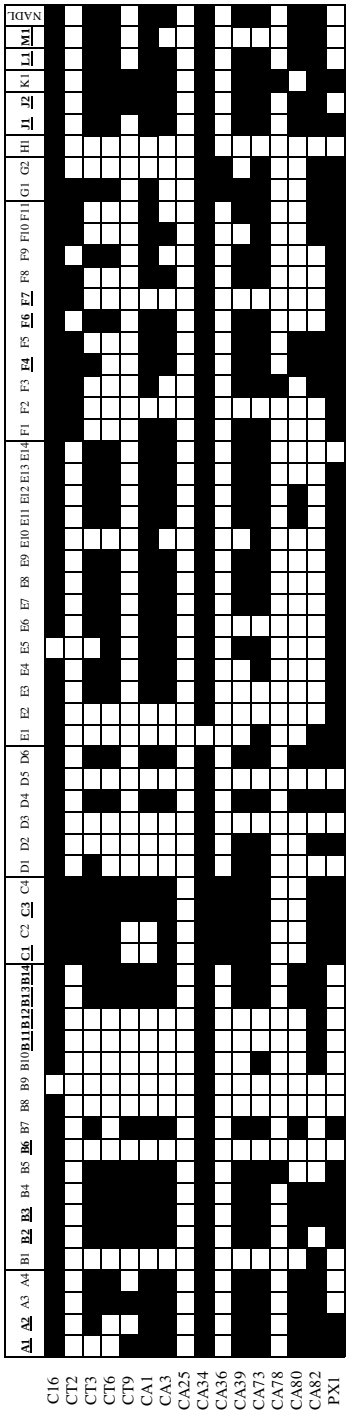

Figure 1. Monoclonal antibody binding patterns of Turkish BVD viruses.
The top line includes virus codes given. mAbs are on the left. Dark boxes indicate positive reactions; clear boxes are negative and hatched boxes indicate doubtful results. Viruses with the same letter code originated from the same herd. Underlined virus codes (e.g. A1) determine IPI animals.

Phylogeny and Statistical Analysis

To determine the antigenic relationships among viral isolates, the Pars (1-0) program of the PHYLIP (V 3.64) phylogeny inference package was employed. For entering the data, results of virus/mAb reaction received from antigenic characterisation attempts were scored as positive = 1, negative = 0, or doubtful = P. A tree diagram was drawn with Treeview software 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rad/treeview.html). In order to analyse herd specificity of BVDV, mAb recognition patterns of 18 BVDV isolates belonging to IPI animals were compared (Figure 1). Chi-square and Fisher's exact tests (in GraphPad InStat software V 2.02) were employed to analyse intra-herd antigenic variations, as well as inter-herd antigenic variations.

Results

Virus Growth

During 3 consecutive passages no cytopathogenic changes were observed in MDBK cell cultures infected with 59 viral isolates. One isolate from herd A and the NADL strain of BVDV produced a cytopathogenic effect (cpe) in cultured cells.

Biotypic and Antigenic Characterisation

One isolate from herd A (mucosal disease case), which was previously determined to produce cpe, contained both biotypes (cp + ncp) of BVDV, though the remaining 59 isolates were in the ncp biotype. Highly divergent reaction patterns were revealed from the antigenic characterisation studies summarised in Figure 1. Only mAb C16 reacted with all of the viruses evaluated, while mAb CA25 bound to none of the viruses tested. MAb CA34 reacted with all except one virus and mAb PX1 recognised more than 80% of the isolates. No single viral isolate reacted with all of the mAbs used. Important differences in reaction patterns indicating antigenic variations were also detected among viruses that originated from the same herd (e.g., viruses B5-B6). No herd-specific pattern was recognised, but interestingly a unique specificity was attributed to mAb CA36. This mAb was bound to either all or none of the viruses from an individual herd.

Phylogeny Analysis and Herd Specificity of Persistent Viruses

A phylogeny tree was generated using data computed with the Pars program of PHYLIP (Figure 2). Three major groups of viruses were generated in this consensus tree, and some subgroups were also stated in sub-trees.

By analysing data from IPI animals, there were no significant intra-herd differences among the viral populations of herds A, C, J, L, and M, but 2 distinct antigenic patterns were observed within herds B and F. Isolates B2, B3, B13, and B14 had a closely related pattern (sub-population B_1), while isolates B6, B11, and B12 created a different mAb recognition pattern (sub-population B_2). This was also the case for herd F (sub-population F_1: F4 and F6; sub-population F_2: *F7*). Statistical analyses of sub-populations showed a significant difference ($P < 0.001$ for B_1-B_2; $P < 0.05$ for F_1-F_2). Thus, these viral sub-populations were regarded as individual herd patterns for comparison with other herds. Statistical analyses showed that sub-populations B_2 and F_2 were significantly different from the viruses taken from other herds, as well as from sub-populations B_1 and F_1 (Figure 3). There was no statistical difference among viral populations if sub-populations B_2 and F_2 were ignored ($P > 0.05$).

Discussion

Diversity in antigenic composition of BVDV field strains is an important issue, especially for disease control and vaccination schedules. Antigenic characteristics of BVDVs from different countries have been described elsewhere (10-13). A preliminary description of antigenic properties of some Turkish BVDV isolates has been previously published (9). The present study examined a large number of viral isolates from dairy herds located in various regions of Turkey and compared their antigenic properties.

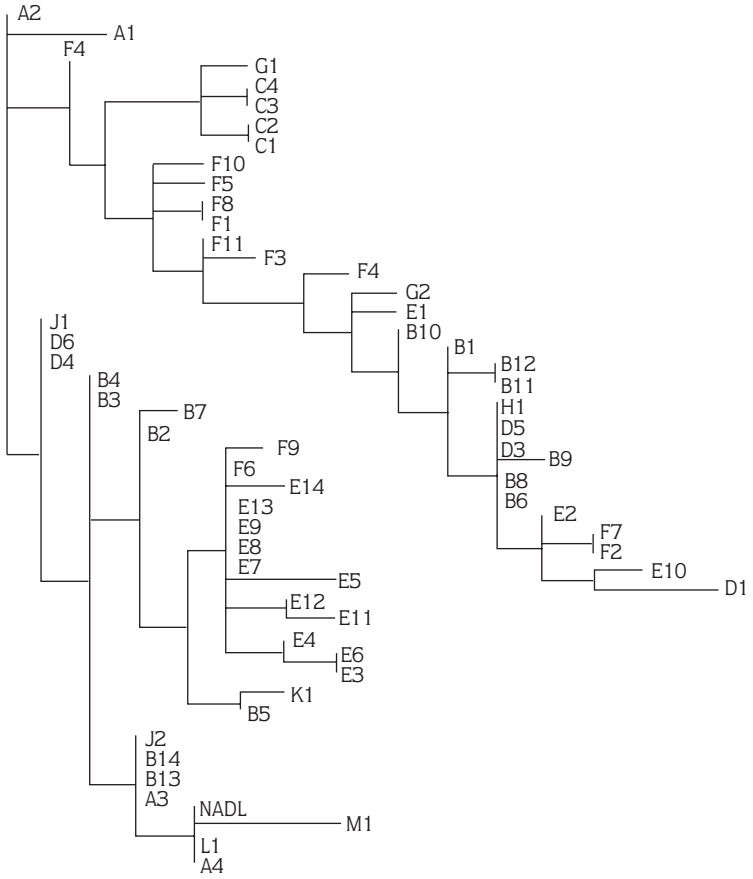

Figure 2. Parsinomy tree of viruses on the basis of mAb-epitope recognition patterns.

	M	L	J	F_2	F_1	C	B_2	B_1
A	ns	ns	ns	*	ns	ns	***	ns
B_1	ns	ns	ns	***	ns	ns	***	
B_2	*	***	***	ns	***	***		
C	ns	ns	ns	***	ns			
F_1	ns	ns	ns	*				
F_2	ns	*	**					
J	ns	ns						
L	ns							

Figure 3. Cross sectional description of statistical relationships among persistent BVDV viral populations on the basis of antigenic similarity. Letter codes given at the top and left represent herds in which IPI animals had been detected. Codes in italic identify viral subpopulations in the herd. Asterisks refer antigenic differences significant at * $P < 0.05$, ** $P = 0.01$ and *** $P = 0.001$; ns: not significant ($P > 0.05$).

All of the viral isolates and NADL strain reacted with mAb C16, which is known as a pestivirus-specific mAb (14). MAb CA34 was found to bind to all viruses except one isolate. In a previous study, a pool of these mAbs (C16 and CA34) successfully recognised a high percentage of field isolates (7). In our preliminary report (9), mAb CA82 bound to all viruses tested. The reactivity percentage of this mAb decreased to 52.4% by increasing the number of viral isolates tested. In contrast to a previous study (15), presently there was no positive reaction evaluated with mAb CA25. Among the other mAbs, the lowest binding ability to the viruses was exhibited by mAbs CA78 (4.9%) and CA36 (10%). These findings denote the presence of a high-level of antigenic diversity among Turkish BVDV isolates. The most conserved epitope on glycoprotein E2 that was reactant to mAb CA34 was shared by 98% of the isolates.

Pituco (16) reported that mAb PX1 binds to all BVDV strains isolated in Germany before 1988, but does not react with some viruses isolated after that time. In the present study, mAb PX1 did not react with approximately 20% of the Turkish isolates. In terms of glycoprotein E2, there is a great heterogeneity in the antigenic composition of the isolates (Figure 1). Some viruses were recognised by only one mAb, while others reacted with a number of mAbs. This distinction was also shown on a herd basis. Viruses from herds B and F were divided into 2 separate viral sub-populations with high levels of antigenic variation. Thus, it can be postulated that 2 different persistent virus sub-populations can circulate in the same herd. Meanwhile, dates of birth (ranging from 1995 to 1999, data not shown) of IPI animals in herd B indicated long-term circulation of both viral sub-populations in this herd. Therefore, our findings do not support the hypothesis of "immunological elimination of divergent IPI strains", which was previously suggested (4).

Antigenic stability of BVDV strains in individual IPIs was previously demonstrated (9). In the present study, statistical analysis did not show a close relationship between herd of origin and mAb recognition patterns. Moreover, it is clear that various BVDV strains, antigenically distinct from each other, could persistently circulate in the same herd. Thus, the epitopic map of BVDV isolates does not favour the concept of herd specificity. The term herd specificity may be addressed after sequence analysis of the 5'UTR genomic region, which is more stable than the genomic regions coding for structural proteins.

The 11 state farms (the subject of this study) had some administrative, biological, and technical connections. In farm J, there was a frozen sperm production centre, which functioned as a sperm bank and provided service to other state farms all around the country. In some periods, young bulls were transferred to this centre from other farms. It can be speculated that BVDV strains circulating in one of these farms could have been transferred to another by transferring IPIs or transiently infected bulls, or by the frozen sperm of those animals. Thus, viruses within various antigenic compositions could have been circulated among related farms, causing generation of viral sub-populations.

As pointed out from herd records, there were many imported heifers in farm D. In fact, a large number of live animals have been imported to Turkey from European countries during the last few decades; therefore, detection of many divergent antigenic patterns among local BVDV isolates may indicate the introduction of some BVDVs by animal importation. This could also be the case for biological products, such as vaccines and foetal calf sera contaminated with BVDV.

It is well known that variant BVDV strains may escape the immunological response of cattle (17). As a consequence of this study, it was shown that a high level of antigenic diversity may allow maintenance of infection

in cattle herds around the country. This situation could possibly reduce the efficiency of vaccination schemes and may be important for control of BVDV infection.

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