

# The first study of bovine immunodeficiency virus (BIV) and bovine viral diarrhea virus (BVDV) co- infection in industrial herds of cattle in two provinces of Iran

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## Abstract:

**BACKGROUND:** BIV is a well-known bovine immunosuppressive cause, but its pathogenesis has not been well characterized. It seems that it is possible that cofactors such as co- infection with other bovine viral pathogens may play a role in enhancing the pathogenesis of BIV infection; BVDV also has immunosuppressive effects. **OBJECTIVE:** The aim of this study was determination of possible correlation between BIV and BVDV infections. **METHODS:** Blood samples were randomly collected from a total of 1800 cattle in dairy industrial farms in Isfahan and Chaharmahal va Bakhtiari provinces of Iran. First BIV or BVDV positive sera were screened by ELISA, and then samples were analyzed to detect BIV proviral DNA or BVDV RNA, using PCR. **RESULTS:** Out of 1800 blood samples, 19 (1.06%) samples were BVDV positive, while BIV positive samples were 10 (0.55%). Nine (0.5%) samples contained both BIV and BVDV genomes and were positive in ELISA, while one of the samples (0.05%) was only BIV positive. **CONCLUSIONS:** In this study, there was a statistically significant relationship between BIV status and BVDV infection using Chi square and Pearson's correlation coefficient test ( $p=0$ ,  $r=0.65$ ).

## Introduction

Bovine immunodeficiency virus (BIV) is an infectious pathogenic lentivirus in the family Retroviridae. BIV infections are lifelong and generally subclinical (Amborski et al., 1989; Belloc et al., 1996). There are some evidences that BIV can cause immunosuppression with increased incidences of secondary bacterial infections in herds with high seroprevalences (Burkala et al., 1999; Carpenter et al., 1992; Cyrcoats et al., 1994; Evermann et al., 1997; Gonda et al., 1987; Gonda et al., 1994; McNab et al., 1994; Fakur et al., 2008) or following experimental infections (Yilmaz et al., 2008). It has also been suspected that the stress of parturition in BIV infected cows is associated with the progression of other bovine viral and bacterial infections (Cyrcoats et al., 1994; Gonzalez et al., 2001a,b).

Bovine viral diarrhea virus (BVDV) is one of the most important viral pathogens of cattle, affecting herds worldwide and causing significant economic impacts. Many production losses from BVDV occur (e.g. reduced milk production and conception rate, respiratory disorders, and increased susceptibility to other disease) (Lambeth et al., 2007).

While pathogenic and economic effects of BVDV are known clearly, the role of BIV in animal disease remains controversial. Since both these viruses have suppressor effects in the bovine immune system, existence of a synergism between BVDV and BIV is possible in the co- infected cases. Therefore, we proposed that each virus may predispose cattle to other infection. In this study, we tried to determine a correlation between these active infections without focusing on how this synergism occurs.

## Materials and Methods

**Herd management and size:** The samples were obtained from dairy industrial provinces in Isfahan and Chaharmahal va Bakhtiari provinces of Iran from 2008 to 2009 from 1800 cattle. These industrial herds use more advanced technology with average milk production from about 4300 to 7900 Kg/cow/year. The populations of these industrial farms were between 100 and 7500. The total 20 herds for sampling were categorized by density such as: 8 small (100-500), 9 medium (500-2000), and 3 large herds ( $\geq 2000$ ). We eliminated the effect of cows' age on BVDV or BIV prevalence by selecting the cattle in the same age ( $2 < \text{age} < 3$  years). The cow population of the tested herds included was 1800. We collected samples from all of these farms so that one-tenth of the population between 2 and 3 years old from each farm to be sampled. All of the cows were Holstein breed. They were housed in an intensive system. About 95% of the herds had free-stall system. The calves were kept in individual boxes. All of the female cows were vaccinated against brucellosis. The animals were immunized against foot and mouth disease and clostridial diseases according to routine schedule in Iran. All of the herds used artificial insemination.

**Blood sampling and DNA/RNA extraction:** The samples were obtained from dairy industrial provinces in Isfahan and Chaharmahal va Bakhtiari provinces of Iran during the period of 2008-2009 from 1800 cattle and then were centrifuged (2000 rpm/50 min) to obtain serum samples. Blood samples were randomly collected from cows that were between 2 and 3 years old (We gave a number to each cow, then using SPSS v. 16, from the transform menu-->random number generator-->under the Active Generator Initialization, clicking on random, we selected a random number). The sera were stored at  $-20^{\circ}\text{C}$  until further use. All serum samples were analyzed to detect anti BIV and BVDV antibodies applying Lab-ELISA and I-ELISA respectively. For PCR assay confirming the BIV serology results, blood samples with EDTA were obtained from seropositives and seronegatives dairy cows, and Genomic DNA was extracted from peripheral PBMC using the DNA isolation kit for mammalian whole blood (Roche Applied Science) according to the

manufacturer's directions within 48h. For RT-PCR assay confirming the BVDV results in ELISA, blood samples with Heparin Sodium were obtained from seropositives. Seronegatives dairy cows and total RNA was extracted from sera using the Qiagen RNA extraction kit (Qiagen RNeasy Mini kit, catalogue number: 52906) according to the manufacturer's directions within 24 h. Then cDNAs were made using Fermentas cDNA synthesis kit (catalogue number: K1622) according to the manufacturer's instructions.

**Detection of anti- BIV antibodies by labeled avidin- biotin enzyme-linked immunosorbent assay (Lab-ELISA):** Serological analysis was performed on 1800 serum samples using a synthetic peptide derived from the available sequence of the transmembrane (TM) glycoprotein of BIV-FL112, produced at the Veterinary Laboratories Agency, Weybridge, Surrey, UK (Scobie et al., 1999). The results were expressed as the absorbance at 405 nm. A sample to positive ratio was calculated based on the positive and negative control sera included (Generous donations from Jean Pierre Frossard -Veterinary Laboratories Agency, UK) in each plate (Scobie et al., 1999).

**Detection of anti- BVDV antibodies by I-ELISA:** Samples were tested with an indirect ELISA (SVANOVIR™ BVDV-Ab ELISA, Svanova Biotech AB, Sweden, cat. no: P06029) according to the manufacturer's instructions.

Sample and reference optical density (OD) values were corrected before interpretation by subtracting the OD values of the corresponding wells containing the control antigen. The antibody titer was interpreted on the basis of the percentage positivity (PP) by dividing the sample OD values by positive reference sample OD values. According to the kit's instructional manual, the criteria for a sample to be assessed as positive was  $\text{PP} \geq 14$ .

**Detection of BIV by PCR assay:** The presence of BIV was detected using the Gene Pak DNA PCR test kit specific for the gag gene of BIV (catalogue number 12134 and from Isogene Lab Ltd, Moscow). The assay was performed according to the manufacturer's instructions. The PCR products were visualized after electrophoresis in 1.3% agarose by staining with ethidium bromide and compared to DNA markers (50 base pair ladder, Fermentas).

Briefly, in PCR test for detection of BIV proviral DNA, each PCR microtube contained 10  $\mu\text{L}$  PCR

diluents, 5  $\mu$ L Master mix and 5  $\mu$ L DNA sample. The thermal cycling conditions for the amplification were 1 cycle for 2 min at 95°C, 30 cycles of 45s at 95°C, 45s at 58°C and 60s at 74°C, with a final extension step of 2 min at 74°C. Positive and negative controls (Generous donations from Jean Pierre Frossard - Veterinary Laboratories Agency, UK) were included in each analysis. Six microliters of the amplified products were loaded on a 1.3% agarose gel and were visualized by staining with ethidium bromide and compared to DNA markers (50 base pair ladder, Fermentas).

**Detection of BVDV by RT-PCR assay:** The optimized RT-PCR assay was used to screen pooled sera under diagnostic laboratory conditions. A volume of 100  $\mu$ L of serum from each cow was pooled in groups of 10 samples, and RNA was extracted according to the manufacturer's instructions (refer to the Qiagen RNeasy Mini Kit protocol available online). For RT-PCR, a BVDV specific PCR was used as Pfeijer et al. described (Pfeijer et al., 2000). The BVDV reactive 324 (5'-ATG CCC TTA GTA GGA CTA GCA -3') and 326 (5'-TCA ACT CCA TGT GCC ATG TAC -3') primers (17) flank a 288bp DNA fragment were selected. The amplification mixtures (50  $\mu$ L) consisted of 5  $\mu$ L 10 x reaction PCR buffer (Promega), 5  $\mu$ L of 25 mM MgCl<sub>2</sub>, 1  $\mu$ L of 2mM each dNTP (Pharmacia), 15pmol of each primer, 1U Taq DNA polymerase (Promega), and 3  $\mu$ L cDNA. Positive and negative controls were provided by the manufacturer were included in each test. In vitro, amplifications were performed in a Thermal Cycler (Corbett Research, Australia) using the following thermal profile: denaturation at 94°C for 1 min, annealing for 1 min at 56°C, extension at 72°C for 1 min. After 36 cycles, the last extension step was prolonged for 7min.

After revealing the BVDV positive pool samples in the RT-PCR test, the same test was performed on each of the 10 samples in a positive pool sample separately.

**Statistical analysis:** The results were analyzed using Chi square and Pearson's correlation coefficient tests by using SPSS software v.16.

## Results

**Co-infection and statistics:** The rate of active

infections of BIV and BVDV in dairy farms in Isfahan and Chaharmahal va Bakhtiari provinces were 0.55% (No. 10) and 1.06% (No. 19), respectively (Table 1 and 2). Nine samples out of 1800 bovine sera (0.5%) were positive for both BIV and BVDV at the same time. They contained both BIV proviral DNA and BVDV RNA and were also positive in ELISA test. One sample (0.05%) was only BIV positive (Table 4). Out of BIV positive samples (n=10) 9 samples (90%) were BVDV positive in ELISA and PCR tests, while among BIV negative samples (n=1790) 10 (0.55%) samples were BVDV positive. In this study, there was a statistically significant relationship between BIV status and BVDV status using Chi square and Pearson's correlation coefficient test ( $p=0$ ,  $r=0.65$ ) (table 4).

**I-ELISA:** Of the 1800 samples, 19 (1.06%) were BVDV seropositive using I-ELISA test, while 10 (0.55%) samples were positive in BIV Lab-ELISA test. In ELISA tests performed in this study, 9 (0.5%) sera had antibodies against both BIV and BVDV. S/P ratios of the BIV positive samples were from 0.27 to 1.86, while BVDV positive PP values were from 20 to 124. Tables 2 and 3 show the results.

**PCR:** The presence of BIV provirus was detected using PCR test specific for the gag gene of BIV in peripheral blood mononuclear cells (PBMCs) from the bovine samples using the Gene Pak DNAPCR test kit. The BIV-specific band with the size of 298bp was detected in DNA positive control sample. The positive PCR products were in the same size as those from the positive control sample, while as expected, a 288bp DNA fragment was amplified in BIV-positive samples using the general BVDV primers 324 and 326. This band also was detected in positive control sample for BVDV.

## Discussion

In this study, we found a consistency between serological and genomic detection of BVDV and BIV results.

Seroepidemiological studies of BIV infections in cattle have been reported in many countries (Amborski et al., 1989; Baron et al., 1998; Belloc et al., 1996; Burkala et al., 1999; Carpenter et al., 1992; Cyrcoats et al., 1994; Evermann et al., 1997; Gonda et al., 1987; Gonda et al., 1994; McNab et al., 1994;

Table 1. Co-infection of BVDV and BIV with PCR and ELISA tests: Using ELISA and PCR tests. Out of 1800 cattle 9 (0.5%) samples were positive for both BIV and BVDV.

BIV seronegative		BIV positive		Total	
Number of samples	BVDV positive samples	Number of samples	BVDV positive	Number of samples	BVDV positive
1790	10 (0.55%)	10	9 (0.5%)	1800	19 (1.06%)

Table 2. Seroprevalence of BVDV and BIV in Isfahan and Chaharmahal va Bakhtiari areas.

BIV Prevalence		BVDV Prevalence	
Number of samples	BIV seropositive samples	Number of samples	BVDV seropositive samples
1800	10 (0.55%)	1800	19 (1.06%)

Table 3. CODs and PP values for BVDV seropositive samples: Sample and reference optical density (OD) values were corrected before interpretation by subtracting the OD values of the corresponding wells containing the control antigen. The antibody titer was interpreted on the basis of the percentage positivity (PP) by dividing the sample OD values by positive reference sample OD values. According to the kit's instructional manual, the criteria for a sample to be assessed as positive was  $PP \geq 14$ .

No	Location of the herd	Sample number	PP
1	Isfahan	135	124
2	Isfahan	138	24
3	Isfahan	140	20
4	Isfahan	201	25
5	Isfahan	941	20
6	Isfahan	945	30
7	Isfahan	949	25
8	Isfahan	1292	100
9	Isfahan	1293	110
10	Isfahan	1294	26
11	Isfahan	1	35
12	Isfahan	2	43
13	Isfahan	3	27
14	Isfahan	4	65
15	Isfahan	5	54
16	Chaharmahal Bakhtiari	6	76
17	Chaharmahal Bakhtiari	7	62
18	Chaharmahal Bakhtiari	8	35
19	Chaharmahal Bakhtiari	9	47

Table 4. Statistical results for co- infection of BVDV and BIV. Out of 1800 cattle 9 (0.5%) samples contained both BIV and BVDV genomes and were positive in ELISA while one of samples (0.05%) was only BIV positive.

	BVDV positive	BVDV negative	p-value
BIV negative	10	1780	0
BIV positive	9	1	0

Fakur et al., 2008; Yilmaz et al., 2008). Despite the worldwide distribution of BIV infection, whether the presence of BIV in a host leads to primarily pathologic changes or can cause secondary bacterial and/or viral infections as a predisposition factor has not been fully elucidated. Under practical conditions, infection with BIV has a different effect on the host than has been observed under experimental conditions. The presence of BIV combined with the stresses associated by parturition and a modern dairy production system was considered causal for the development of secondary diseases in immunocompromised cattle. The frequent development of concurrent infections in BIV-infected animals suggested that persistent BIV infection had a role in reducing functional immune competence, in accordance with other studies.

It has been hypothesized that infection with BIV, and potential consequent immunosuppression, might predispose cattle to infection by other agents (Nikbakht Borujeni et al., 2010). The co-infection of BIV and BVDV in dairy cattle in Iran is not reported.

In our study, the overall BIV-seroprevalence in industrial dairy farms was 0.55%. The prevalence of BVDV active infection in industrial farms was 1.06%. Therefore, BVDV active infection is more common than BIV infection in the Iranian cattle in the studied industrial farms. Previously, the presence of antibodies against BIV in dairy cattle of non industrial farms in Iran was reported by Nikbakht Borujeni et al., (2010) and Tajbakhsh et al., (2010). The BIV seroprevalence in these studies were 20.3% and 60%, respectively, which are much more than expected in the world average (4 - 5%).

Also, in the previous studies performed by Iranian researchers (Fakur et al., 2008; Badiei et al., 2010; Morshedi et al., 2004) on BVDV seroprevalence, the rate of infection in non industrial farms in Shiraz, Urmia, and Sanandaj provinces of Iran were 37-86%, 31.38% and 27.7%, respectively. Previous studies of these viruses have done in non- industrial farms in Iran, so these findings may vary with our results. The main cause of difference was interpretation of results. We only consider active infection which showed both anti - BVDV antibodies and viral RNA, but in previous published data, the researchers recorded only seropositive which had only antibodies and it showed previous or transient infection. In the study



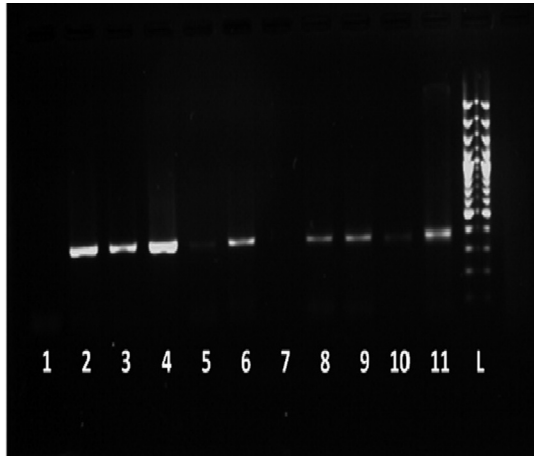


Figure 1. BIV-gag PCR amplification products following electrophoresis. L= 50bp DNA ladder, 11= positive control (BIV infected animal), 1= negative control (uninfected animal), 2 to 10 samples (Iranian animals), 7= a negative sample.

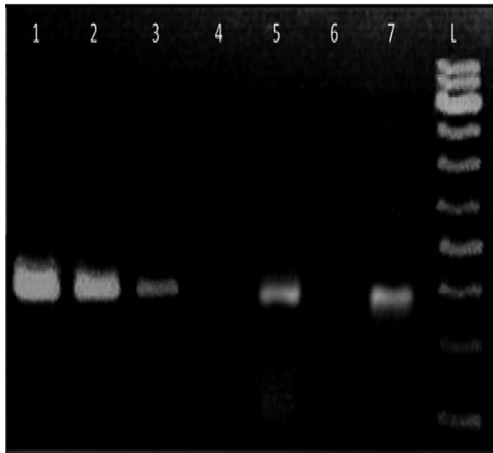


Figure 2. BVDV RT-PCR amplification products following electrophoresis. (BVDV RT-PCR test was performed on BIV positive samples). L= 100bp DNA ladder, 7= positive control (BVDV infected animal), 6= negative control (uninfected animal), 1,2,3,5 are BVDV positive samples.

performed by Badiei et al. in Shiraz, cows in semi-industrial herds were tested (the populations of the herds were between 50 and 1700). Talebkhan Garoussi et al. (Talebkhan Garoussi et al., 2009) have found higher BVDV seroprevalence among the industrial dairy cattle herds in suburb of Mashhad in Iran (72.25%). As mentioned above, the main cause of difference between our study and Talebkhan Garoussi et al.'s survey was using the method for finding positive cows. They had just used serology to find seropositive cows, while in this study we try to find active infection with BVDV. However, we have eliminated the age effect on prevalence of these two

infections by selecting an age group.

The aim of this study was not to investigate the prevalence of BVDV or BIV in herds of Isfahan and Chaharmahal va Bakhtiari provinces of Iran; we were looking for a meaningful relationship between co-infection with both viruses. So far, co-infection of BVD and BIV has not been studied. Carpenter et al. for characterization of early pathogenic effects after experimental infection of calves with BIV attempted to separate the effects of BVDV and BIV in their study. The authors believed that fever and leucopenia are characteristic of acute infection with BVDV that is a common contaminant of cell culture and previous infection with this virus is causing confusion in identifying the exact symptoms of the BIV.

In fact, they were convinced that no synergism existed between these two viruses, but they saw that in the cattle which was co-infected with BIV and BVDV or cell cultures that were contaminated with BVDV, BIV has caused more changes. The pathogenesis of the BIV infection has not been well characterized. Experimentally-infected animals did not develop immunodeficiency. It is possible that co-factors may play a role in enhancing the pathogenesis of BIV infection, and one of these co-factors could be bovine viral diarrhea virus (BVDV) because of its immunosuppressive effects. (Carpenter et al., 1992)

In the present study, a seroepidemiological survey of BIV and BVDV was performed to determine a correlation between BIV and BVDV infections. We found a statistically significant relationship between these viruses infections.

Overall, among 1800 cattle tested, 19 (1.06%) were BVDV positive and 9 (0.5%) animals were positive to both BIV and BVDV. The statistical analysis shows a p value less than 0.05 ( $p=0.0$ ) for the chi square test, and r equal to zero. These indexes demonstrated there is an association between BIV and BVDV infections.

This study had some limitations. First, it was difficult to know whether the disorders observed were due to BIV or BVDV infections alone; because of the fact that BIV-positive or BVDV positive cattle were not further analyzed for other infectious agents like viruses or bacteria that may play a role in that kind of clinical disorder. Second, it is difficult to select uniform patient and control populations in animal studies. Therefore, control animals were selected

from among BIV-seronegative cattle from the same herds including BIV-positive animals, because of the fact that some factors, i.e. climate, magnitude of farm, and management, are well known to affect the health status of dairy cattle. Third, the number of lactations could have been recorded in the present study was limit.

Co-factors such as BVDV infection may enhance the pathogenesis of BIV infection, and BIV can be a risk factor for other infections such as BVD. It is difficult to distinguish in cattle co-infected with these viruses, which of them predispose the infection with the other. On the other hand, we did not determined viral cytopathogenicity. While pathogenic and economic effects of BVDV are known clearly, the role of BIV in animal disease remains controversial. Since these two viruses have suppressor effects in the bovine immune system, the existence of a synergism between BVDV and BIV is hypothetically possible in the co-infected. In this study, we tried to determine a correlation between these infections without focusing on how this synergism occurs. We found a statistically significant relationship between BIV status and BVDV status using Chi square and Pearson's correlation coefficient test ( $p=0$ ,  $r=0.65$ ) and high co-infection rate of these 2 viruses can support our hypothesis, but it needs further studies.

## Conclusions

In this study, the statistical analysis shows  $P=0.0$  for the chi square test, and  $r=0.65$  demonstrated there is a statistical association between BIV and BVDV. So, the existence of a synergism between BVDV and BIV is possible in the co-infected cases.

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## اولین مطالعه بررسی عفونت توأم فارم‌های صنعتی پرورش گاو با ویروس‌های عامل نقصان ایمنی گاو و اسهال ویروسی گاوها در دو استان ایران

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### چکیده

**زمینه مطالعه:** ویروس عامل نقصان ایمنی گاو (BIV)، یکی از بارزترین عوامل سرکوب‌کننده سیستم ایمنی گاو محسوب می‌شود، اما روند بیماری‌زایی این ویروس تاکنون به درستی شناخته نشده است. به نظر می‌رسد که کوفاکتورهایی نظیر آلودگی توأم با سایر عوامل ویروسی بیماری‌زای گاو در تقویت روند بیماری‌زایی پس از عفونت با BIV نقش داشته باشد. ویروس عامل اسهال ویروسی گاوها (BVDV) نیز آثار سرکوب‌کنندگی ایمنی دارد. **هدف:** هدف این مطالعه تعیین ارتباط احتمالی بین عفونت‌های BIV و BVDV بود. **روش کار:** نمونه‌های خون به طور تصادفی از مجموع ۱۸۰۰ گاو در فارم‌های صنعتی استان‌های اصفهان و چهارمحال و بختیاری جمع‌آوری شدند. در ابتدا، نمونه‌های سرمی از نظر آلودگی به ویروس‌های BIV و BVDV با آزمون الایزا غربالگری شد. سپس، به منظور شناسایی DNA پرو ویروسی BIV و RNA ویروس BVDV با آزمایش PCR بررسی شدند. **نتایج:** از میان ۱۸۰۰ نمونه، ۱۹ مورد (۱/۰۶٪) BVDV مثبت بودند، در حالی که تعداد موارد آلوده به BIV ۱۰ مورد (۰/۵۵٪) بود. نه نمونه (۰/۵٪) آلودگی ژنومی به هر دو ویروس را نشان دادند در حالی که تنها یک نمونه (۰/۰۵٪) فقط آلودگی به BIV را نشان داد. **نتیجه‌گیری نهایی:** در این مطالعه با استفاده از آزمون‌های آماری کای اسکوار و ضریب همبستگی پیرسون ارتباط معنی‌دار آماری بین وضعیت آلودگی به BIV و BVDV مشاهده شد ( $p=0$ ،  $r=0/65$ ).

واژه‌های کلیدی: BIV، BVDV، عفونت توأم

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