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Bovine viral diarrhea virus antigen status in milk and blood serum: Implications for effective screening and risk factors analysis in dairy cows

Md Ashek Ullah[1, †], SM Nazmul Hasan[1, †], Md. Rafiqul Islam[2], Nurejunnati Jeba[1], Sharmin Akter[3], Sukumar Saha[1], Md. Golzar Hossain[1, *]

[1]Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[2]Department of Anatomy and Histology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[3]Department of Physiology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

***Corresponding author**
Md. Golzar Hossain, PhD
Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
Email: mghossain@bau.edu.bd

[†]These authors contributed equally.

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is a significant viral pathogen affecting cow populations worldwide. This study was conducted in the Bagha Bari area of Sirajganj District, Bangladesh, and investigated the prevalence of BVDV antigen in 54 dairy cattle exhibiting reproductive, milk production, and congenital abnormalities. Milk and blood samples were collected from dairy cows without a history of BVDV vaccination. The research work employs indirect enzyme-linked immunosorbent assay (ELISA) to detect viral antigen, and the results reveal a significant discrepancy in BVDV antigen positivity between milk (55.56%) and serum (92.60%) samples from the same animals. Interestingly, animals negative in one sample type tested positive in the other, indicating potential localized viral presence or shedding variations. ELISA titers differed between serum and milk samples, highlighting variations in viral dynamics within bodily fluids. Risk factor analysis shows an association between BVDV positivity and pregnancy. In conclusion, this study advocates a comprehensive testing approach involving both serum and milk samples for effective BVDV screening. Future studies should involve larger, geographically varied populations and incorporate molecular diagnostic tools, such as PCR, to enhance accuracy.

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INTRODUCTION

Bovine viral diarrhea (BVD) or mucosal disease is an illness in cattle stemming from the BVDV. The virus is prevalent and poses a risk of infection to the majority of cattle herds, resulting in significant financial losses due to its severity [1]. BVDV belongs to the family *Flaviviridae* and the genus *Pestivirus*, consisting of four species: BVD virus 1 (BVDV1), BVD virus 2 (BVDV2), classical swine fever virus, and border disease virus [2]. A novel virus named HoBi-like BVDV3 has recently been identified in Thailand, Europe, and Brazil [3, 4]. It is a small, enveloped virus with a single-stranded positive s-nse RNA, and its RNA genome size is approximately 12.5 kb [5]. Due to the wide-ranging nature of the virus, its ease of transmission, and the absence of effective treatments, BVDV has become a pervasive and highly consequential disease in the global cattle population [6-8].

BVDV infection can lead to significant financial losses, negatively impacting various aspects of cattle health and productivity. This includes reduced fertility and milk production, delayed fetal growth, symptoms like diarrhea and respiratory issues, and reproductive problems such as abortion, birth defects, embryo loss, mummification, and stillbirth [9]. BVDV also disrupts the immune system, increases susceptibility to

other infections, impairs overall herd performance, and is particularly concerning due to the development of persistent infection (PI) in calves [10, 11]. In some instances, animals may not show any clinical signs but undergo immunosuppression [12]. When animals contract noncytopathic BVDV during the early stages of pregnancy, it can result in the birth of persistently infected animals, contributing to viral transmission within the herd [13]. Therefore, identifying and removing these animals is crucial for successfully implementing eradication programs.

Although healthy and immunocompetent cattle, including late-term fetuses, can experience acute BVDV infection and develop antibodies in response (seroconversion), the primary transmission and perpetuation of the disease within cattle populations occur through persistently infected (PI) individuals. The persistence of infection is established through fetal infection during the early stages of gestation [14]. Cattle with immunotolerance can release the virus through secretions and waste over an extended period, significantly contributing to the spread of BVDV infections among herds. Due to the economic losses associated with BVD, it is crucial to have precise and sensitive diagnostic techniques that can quickly detect and eliminate persistently infected carriers within herds [15].

The initial documentation of BVDV infection in the Indian subcontinent was recorded in 1982 in the state of Orissa [16]. A study from 2000 to 2002 demonstrated that the BVDV-1 strain was prevalent in India. However, in 2011, Indian cattle were found to be infected with a different strain called BVDV-2 [17]. The recent discovery of the uncommon HoBi-like pestivirus or BVDV-3 variant in Bangladesh necessitates additional monitoring to assess its influence on the livestock industry [18]. On the other hand, the antigen-capture enzyme-linked immunosorbent assay exhibits high sensitivity, specificity, and repeatability in detecting BVDV antigens. It is a reliable and cost-effective technique for identifying persistently infected cattle, with ease of transfer and implementation [19, 20]. Therefore, the current study was designed to detect BVDV Ag using antibody-coated ELISA from dairy cattle with histories associated with BVDV infection in the Sirajganj District of Bangladesh.

MATERIALS AND METHODS

Ethical approval

Ethical clearance for handling animals and experimental procedures was obtained from the institutional ethical committee (AWEEC/2023(64)).

Data collection

The study was conducted on a total of 54 dairy cattle in the Bagha Bari area of the Sirajganj District of Bangladesh. Milk and blood samples were obtained from 54 dairy cows of various ages with a history of either repeat breeding, abortion, or reduced milk production. None of the dairy cows had a history of BVDV vaccination. The breeding type and pregnancy condition were also recorded, and these data were collected using a predefined questionnaire.

Sample collection and preparation

The milk samples were centrifuged at 600 g for 5 minutes at a temperature of 4 °C to separate the somatic cell fat and fatless milk. The fatless milk was collected and used

directly for ELISA or stored at -80 °C. The blood was collected from the jugular vein, and the animal was kept at room temperature for 1 hour to allow clotting. After clotting, the serum was separated. The clear straw-colored serum was transferred into a 1.5 ml Eppendorf tube and centrifuged at 600 g for 5 minutes at a temperature of 4 °C to remove the remaining red blood cells (RBC). The supernatant (serum) was then transferred into a new tube and stored at -80 °C or used directly for ELISA.

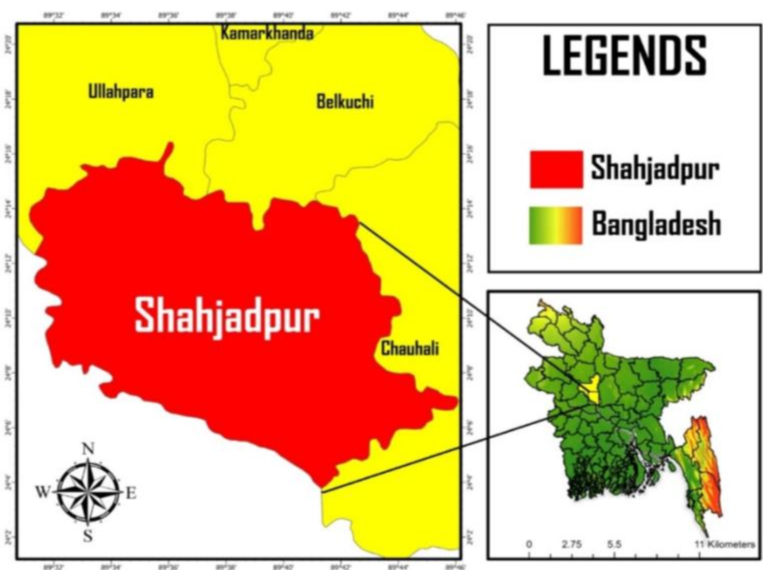

Figure 1. The study area map was created by ArcGIS Pro (ESRI, USA) based on the Geographical Information System (GIS).

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) (Tianjin Geneally Biotechnology Co., Ltd, Tianjin, China) was conducted using the manufacturer's recommended protocol with slight modifications. Briefly, all the steps of the ELISA were conducted at room temperature unless otherwise specified. First, 50 µl of each sample, positive control, and negative control were poured into the respective wells of the ELISA plate, with one well kept as a blank control. Then, 100µl of enzyme-conjugate was added to each well containing the sample, positive control, and negative control, excluding the blank one. The plate was covered with an adhesive strip, gently mixed for a few seconds using a shaker, and incubated for 60 minutes at 37 °C in a humidified incubator. The sample-enzyme-conjugate mixture was then carefully aspirated from every well, and the wells were washed with 1X wash buffer four times. Each well was filled with 350 µl wash buffer during each wash. The plate was flipped upside down to eliminate any remaining moisture by gently tapping it onto absorbent paper or paper towels until no visible dampness remained. Afterward, 50µl of substrate A and 50µl of substrate B were added to each well, gently mixed, and the plate was incubated for 15 minutes at 37°C. Subsequently, 50µl of stop solution was added to each well, and the plate was gently tapped to ensure proper mixing and uniformity. Finally, the optical density (OD) was measured at a wavelength of 450 nm using a microtiter plate reader (Stat Fax 4200, USA). The positivity of the samples was calculated based on the cut-off value according to the manufacturer's guidelines, and samples exhibiting S-N values of 0.277 or lower were categorized as negative for BVDV antigen, while samples with S-N values >0.277 were classified as positive.

Statistical analysis

Data was analyzed using MS Office Excel. The Chi-Square test was performed using an online tool (https://www.socscistatistics.com/). A statistical significance level of 0.05 was set for risk factor determination.

RESULTS

Status of BVDV antigen in milk and serum samples

A total of 108 samples (54 milk and 54 serum) from 54 cows with a history of repeat breeding, abortion, decreased milk production, and congenital defects were collected. These samples were subjected to an indirect ELISA to detect viral antigens (Figure 2A). The BVDV antigen level, as detected by ELISA, demonstrated that 55.56% (30/54) of milk samples were positive, whereas 92.60% (50/54) were positive in the case of serum samples, even though both types of samples were collected from the same animals (Figure 2B). Overall, 100% of the suspected animals were BVDV Ag positive based on both serum and milk sample analysis. Interestingly, animals that tested negative for BVDV Ag in serum samples were found to be positive by ELISA in milk samples. Similarly, animals that tested negative in milk samples showed BVDV Ag positivity in serum samples. However, the original titer of the ELISA in serum samples ranged from 0.458 to 2.332, whereas in milk samples, it ranged from 0.295 to 2.715 (Figure 2A and Supplementary Table 1). Therefore, it may be concluded that either serum alone or both serum and milk samples should be tested for screening BVDV-suspected animals.

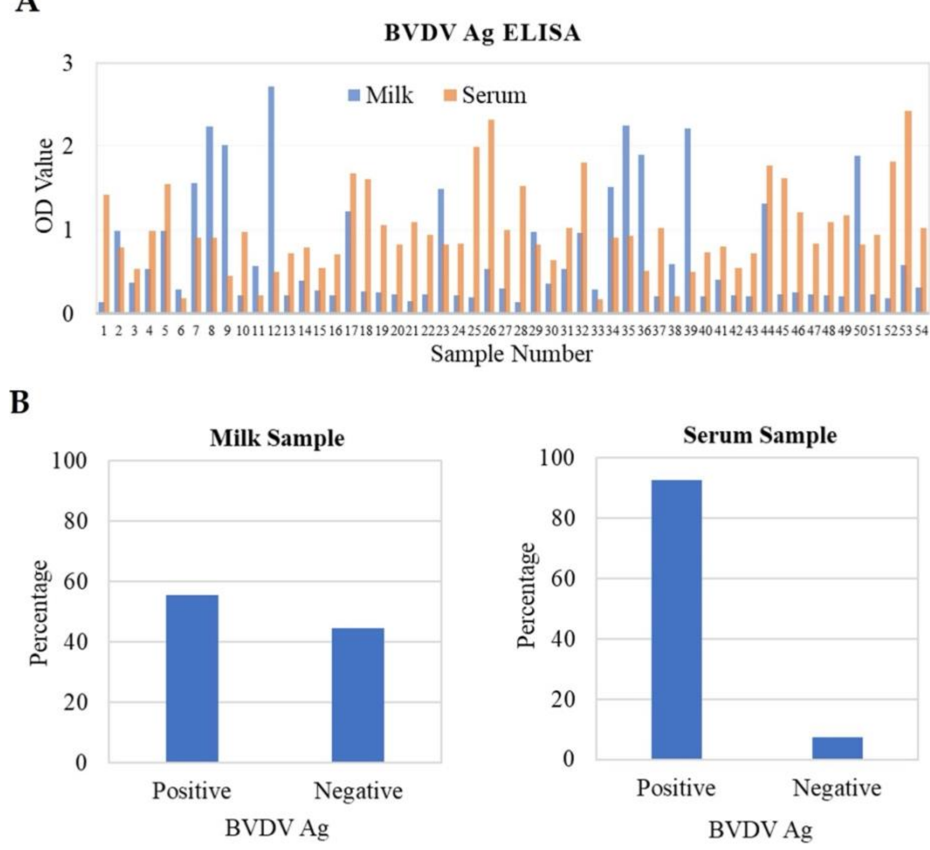

Figure 2. BVDV antigen status in milk and serum samples of the BVDV suspected cows and their prevalence. A) BVDV Ag ELISA was conducted according to the manufacturer's guidelines, and OD values were recorded. B) The percentages of positive and negative cows based on milk and serum samples.

Risk factors and clinical symptoms associated with BVDV infected cows

The various symptoms and factors associated with BVDV persistent infection, such as age, pregnancy, decreased milk production, repeat breeding abortion, and congenital defects, were analyzed [21]. The age of the animals ranged from 3 to 13 years, and the data, along with samples, were collected randomly without any bias. Cows were categorized into two groups based on age: ≤7 years (38) and >7 years (16). Overall, 70.37% (38/54) of the cows were 7 years old or younger, while 29.65% (16/54) were older than 7 years. However, 52.63% of cows aged ≤7 years showed BVDV Ag positive, whereas 62.5% of cows older than 7 years tested positive for BVDV Ag (Figure 3A). Among the 26 pregnant cows, 22 tested positive for BVDV antigen, while only 8 out of 28 nonpregnant animals were BVDV Ag positive (Figure 3B). Therefore, it can be concluded that BVDV Ag positivity is significantly higher ($p < 0.05$) in pregnant animals.

On the other hand, decreased milk production was observed in 30 cows, of which only 40% (12/30) tested positive for BVDV antigen. In contrast, 75% (18/24) of cows with no history of decreased milk production were found to be viral antigen negative (Figure 3C). Lastly, we observed congenital defects in the cows, and only 10 out of 54 showed various congenital defects. Among cows with congenital defects, only 20% (2/10) tested positive for BVDV antigen, while 63.64% (28/44) of those without congenital defects showed BVDV antigen positivity (Supplementary table 1 and Figure 3D).

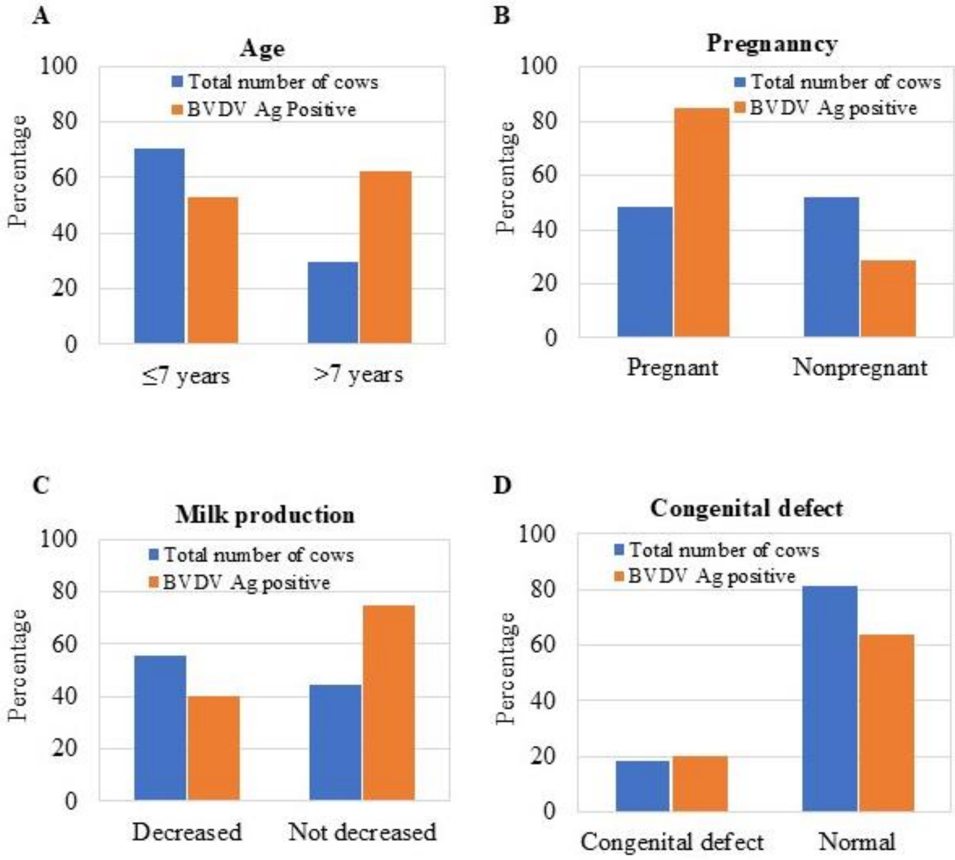

Figure 3. Risk factors and clinical signs associated with BVDV infected cows include A) age, B) Pregnancy, C) milk production, and D) Congenital effects. Data were collected as mentioned in the questionnaire, and ELISA was conducted according to manufacturer's guidelines, and samples exhibiting S-N values of 0.277 or lower were categorized as negative for BVDV antigen, while samples with S-N values >0.277 were classified as positive. Data were prepared in MS Office Excel, and a Chi-square test was performed for statistical significance analysis.

However, among 54 cows screened for BVDV Ag based on serum samples collected from the same cows, 50 tested positive (92.59%) (Supplementary table 1). Considering the age groups, BVDV Ag positivity was observed in 94.7% of cows aged ≤7 years, while cows in the >7 years age category exhibited a BVDV antigen positivity rate of 87.5% (Supplementary Table 1). Regarding pregnancy status, 84.6% (22/26) of pregnant cows were BVDV antigen-positive, whereas 100% (14/14) of non-pregnant cows tested positive. Decreased milk production was observed in 30 cows, out of which 86.7% (26 out of 30) tested positive for BVDV antigen. Conversely, only 10 cows had a history of congenital defects, and all of these were BVDV antigen positive (Supplementary Table 1).

DISCUSSION

BVDV infection in cattle typically causes mild or no symptoms in non-pregnant, healthy animals. However, infections during pregnancy can result in various reproductive complications, including failure to conceive, abortion, stillbirth, congenital deformities, and the birth of persistently infected calves [22]. Acute BVDV infection is associated with immunosuppression, which increases the risk of secondary complications like mastitis and respiratory diseases in affected animals or herds [23, 24]. These combined reproductive and immunosuppressive effects lead to significant economic losses, establishing BVDV as a major viral pathogen affecting cattle worldwide [25]. This study examined 54 cows with reproductive issues, and discrepancies were observed between serum and milk sample results. These findings highlight the importance of considering both sample types for effective BVDV screening in suspected animals [26]. The observed discrepancies in antigen positivity between serum and milk samples emphasize the need for a comprehensive testing approach to minimize false-negative results [27]. Animals that tested negative in one sample type but positive in the other suggest a localized viral presence or variations in shedding patterns [9]. The differences in ELISA titers between serum and milk samples suggest variations in viral dynamics within different bodily fluids [22, 28]. Future research should investigate factors influencing variations in viral load between serum and milk.

Older cows were more likely to have been exposed to BVDV multiple times throughout their lives, and the findings of this study align with previous reports [29, 30]. The study also found significantly higher BVDV antigen positivity in pregnant cows. Accordingly, BVDV prevalence is higher in pregnant cows compared to non-pregnant cows, probably due to immunosuppression, fetal infection, abortion, calving difficulties, etc. [29]. However, several limitations should be noted, including the sample size and geographic constraints, the lack of longitudinal data, and the absence of molecular confirmation. Therefore, future investigations should include larger, geographically diverse populations and integrate molecular diagnostics, such as PCR, to improve accuracy. Longitudinal studies and deeper analyses of viral shedding, tissue tropism, and herd-level risk factors are essential for a better understanding of BVDV dynamics and for enhancing control strategies.

CONCLUSIONS

In conclusion, the study reveals a significant prevalence of BVDV antigen in both milk and serum samples, with a notable discrepancy in positivity rates between the two types of samples collected from the same animals. The findings emphasize the importance of a comprehensive approach, advocating for the testing of both serum and

milk samples to effectively screen for BVDV in suspected animals. The observed variations in antigen positivity between sample types suggest the potential for localized viral presence or shedding pattern variations. Risk factor analysis indicates associations between BVDV positivity and pregnancy.

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AUTHOR CONTRIBUTIONS

MGH conceived the study. MAU, SMNH, and JZ collected samples; MGH and MAU conducted ELISA, analyzed data, and wrote the draft of the manuscript. MRI, SA, and SS reviewed the whole manuscript. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

SUPPLEMENTARY MATERIALS

Supplementary Table 1. ELISA value variations in BVDV suspected cows (Supplementary materials).

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