

Diagnostic Studies on Bovine Viral Diarrhea Infection in Cattle and Buffaloes with Emphasis on Gene Markers

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Abstract: A total number of 290 Friesian cattle and 210 buffaloes of different ages and sexes from different localities in Egypt were used in this study. The main clinical signs associated with infection with Bovine Viral Diarrhea Virus (BVDV) were drop in milk yield and reproductive disorders in cattle and persistent diarrhea and respiratory disorders in buffaloes. Enzyme Linked Immunosorbent Assay (ELISA) indicated 65.5 and 11.4%, while, Serum Neutralization Test (SNT) test indicated 58.61 and 25% positive cases using specific antibodies against BVDV in cattle and buffaloes, respectively. Indirect immunofluorescence test for pools of IgG positive and IgG negative sera clearly indicated that the intensity of green fluorescence was very strong in positive pools. Results of detection of BVDV antigen in serum by using commercial ELISA antigen kit clearly indicated that all animal's sera were uniformly negative for presence of BVDV. Electrophoresis on sera samples to study the genetic polymorphism failed to differentiate between infected and non infected animals.

Key words: Cattle · buffaloes · BVDV · diagnosis · ELISA · SNT · gene markers

INTRODUCTION

Bovine Viral Diarrhea (BVD) is a disease caused by BVDV. This virus presents in two genotypes [1-3] and its cultivation on tissue culture cells resulted in either cytopathic or noncytopathic biotype [4]. BVD is manifested clinically by diarrhea, respiratory affections, oral lesions, ocular symptoms and abortion. Severity of clinical affections depended upon multiple factors including immune status of animals, pregnancy state, strains of virus and environmental stress [5]. BVD infection raises the incidence of retained placenta, silent heat and mastitis [6]. In Egypt, the disease was firstly diagnosed by [7] and the virus was isolated from animals suffered from pneumoenteritis [8].

There were several directions that had been addressed to determine genetic variations among animals in response to infectious diseases as analysis of allelic variations. Electrophoresis is capable of differentiating minute, genetically controlled variations in proteins and provides a useful tool for detecting differences in charge and/or size among macromolecules. When combined

with specific enzyme-staining techniques, it permits visualization of differences present in very small quantities. Also, serum protein electrophoresis had been applied in many diseases as a diagnostic tool. Difference in protein profiles with obvious lower albumin, higher α_1 and γ globulins with lowest A/G ratio was evident in diseased sheep [9].

This study aimed to diagnose BVDV in cattle and buffaloes via detection of specific anti-BVDV-antibodies in sera by ELISA, SNT and IFAT as well as detection of BVDV antigen by using of ELISA antigen commercial kit. Investigation of the genetic polymorphism using Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE) of serum proteins to determine genetically controlled variations was another target.

MATERIALS AND METHODS

Animals: A total number of 290 Friesian cattle and 210 buffaloes of different ages and sexes, raised in different localities in Egypt, during the period extended from 2004 to 2005 was used in this study (Table 1).

Table 1: The total number of examined animals

Governorate	Cattle	Buffaloes
Beni-Suef	62	0
El-Fayoum	13	120
Damitta	215	30
Ismailia	0	60
Total examined animals	290	210

Blood and serum samples: Blood samples were collected from 87 cattle and 88 buffaloes suffering from different reproductive disorders as well as from some of apparently healthy animals and serum samples were separated.

Buffy coat: It was obtained by aseptic collection of blood with anti-coagulant, for detection of BVDV antigen, using lysis buffer method [10]

Virus strain: BVDV Iman strain is an Egyptian field strain (cytopathic), isolated from a calf with sever pneumoenteritis [8]. It was propagated in Madin Darby Bovine Kidney (MDBK) and used for detection of BVD antibodies by neutralization assay.

Tissue culture cells: MDBK cell line was used.

Tissue culture media: MEM (Minimum Essential Media) Eagles' modified with Earls salt and L-glutamine without sodium bicarbonate, was obtained from Sigma Aldrich. Inc.USA. The medium was prepared according to supplied instructions. It was used as growth medium (supplemented with 7% fetal calf serum gamma irradiated) or maintenance medium (supplemented with 2-3% fetal calf serum gamma irradiated). It was sterilized by filtration through a Seitz filter and kept at 4°C.

Serological examination for brucellosis: All serum samples from aborted animals were tested to exclude brucellosis by using of Rose Bengal Plate and Tube Agglutination Tests[11].

Enzyme Linked Immunosorbent Assay (ELISA): It was done by using of commercial IDEXX BVDV Antibody test kit [12, 13]. The test was performed according to manufacturer description.

Sandwich ELISA detection kits: BVDV antigen test kit/Serum plus. Catalog No.06-41124-04 was obtained from Herschel IDEXX Laboratories. IDEXX Scandinavia AB, Storrymningsvagen 5, SE-748 30 osterbybruk, Sweden [14].

Serum Neutralization Test (SNT): It was done according to [3,13].

Immunofluorescence test: It was carried out according to [15, 16].

Western blotting: It was carried out to detect BVDV in buffy coat [17].

SDS-PAGE: Sodium Dodecyle Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was done according to [18].

RESULTS

Clinical findings: The main clinical signs in examined animals were reproductive disorders in cattle and persistent diarrhea in buffaloes (Table 2).

Measurement of specific antibodies against BVDV in serum of animals by using ELISA commercial kit: Specific IgG reactivity of serum samples with BVDV antigens by ELISA coated plates indicated that 65.5 and 11.4% of examined cattle and buffaloes were positive, respectively (Table 3).

Table 2: The common clinical signs of examined animals

Species	Locality	Problems
Cattle (290)	Beni-suef	Sporadic cases of repeat breeding
	El-Fayoum cattle farm	Stunted growth, persistent diarrhea and respiratory distress
	Damitta cattle farm ¹	Abortion, reproductive insufficiency and birth of congenitally deformed calves
	Damitta cattle farm ²	Several cases of abortion, mastitis, endometritis in variable degrees with generalized various infections
Buffaloes (210)	El-Fayoum buffaloes farm	Diarrhea without response to antimicrobial treatment, Pneumonia, stunted growth and dermatitis associated with loss of hair
	Isamillia buffaloes farm	Abortion at different stages of pregnancy with negativity for brucellosis.
	Damitta buffaloes farm N° 1	They were reared within cattle farm for fattening and were newly purchased with signs of diarrhea, abortion and stunted growth

Table 3: The incidence (%) of specific antibodies against BVDV in cattle and buffaloes by ELISA

Species	Results		
	+ve	-ve	±
Cattle (n=87)	57 (65.5%)	26 (29.9%)	4 (4.6%)
Buffaloes (n=88)	10 (11.4%)	78 (88.6%)	- (0%)

Table 4: The incidence (%) of specific antibodies against BVDV in cattle and buffaloes by SNT

Species	Result	
	+ve	-ve
Cattle (n=87)	51 (58.60%)	36 (41.40%)
Buffaloes (n=88)	22 (25.00%)	66 (75.00%)

Table 5: Variation between ELISA and SNT for detecting BVDV antibodies in cattle and buffaloes

Species	Test	Result		
		+ve	-ve	±
Cattle (n=87)	ELISA	57 (65.5%)	26 (29.9%)	4 (4.6%)
	SNT	51 (58.6%)	36 (41.4%)	0
Buffaloes (n=88)	ELISA	10 (12.5%)	77 (87.5%)	0
	SNT	22 (25.0%)	65 (75.0%)	0

Table 6: BVDV antigen detection by ELISA

Species	Herd	ELISA	
		+ ve	- ve
Cattle (36)	Beni-Suef (23)	-	23
	Fayoum (6)	-	6
	Damitta cattle farm N° 1 (7)	-	7
Buffaloes (38)	Fayoum (38)	-	38

The percentage of BVDV positive cattle was 47.8, 69.5, 72.7 and 83.3% in Beni-Suef (farm No.1), Damitta (farm No.2), Damitta and El- Fayoum governorates, respectively. Meanwhile, the percentage of positive buffaloes was 26.3, 0.0 and 0.0% in El- Fayoum, Ismaillia and Damitta governorates, respectively.

Detection of antibodies in serum of examined animals by using of Serum Neutralization Test (SNT): The incidence of specific antibodies against BVDV in cattle and buffaloes by SNT was recorded in Table 4. Moreover, variations in the antibody detection results obtained by ELISA and SNT are shown in Table 5.

Detection of antibodies in animals by using of Indirect Fluorescent Antibody Technique (IFAT): Indirect immunofluorescence test showed strong intensity of green fluorescence in positive pool (Fig. 2). Unlikely, only weak background reaction was obtained by negative pool (Fig. 1). There was a correlation between IFAT results and both ELISA well as SNT.

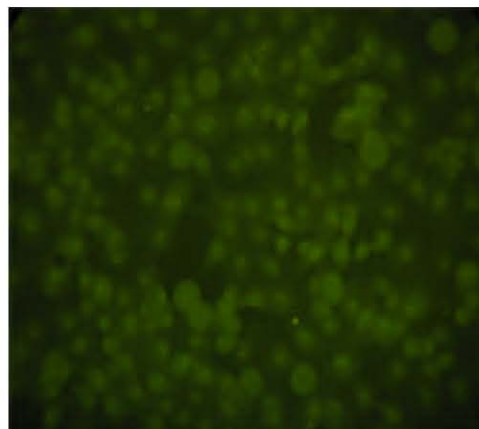


Fig. 1: Negative serum samples pool

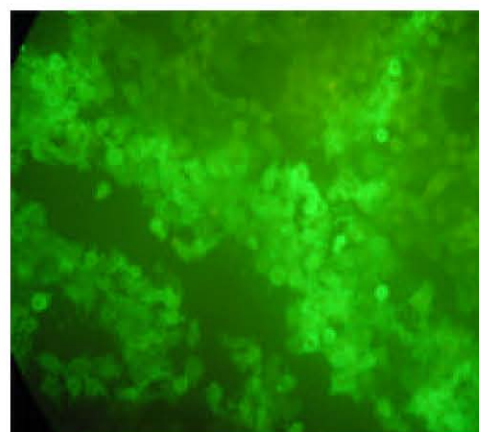


Fig. 2: Positive serum samples pool

Detection of BVDV antigen in serum samples by using of ELISA Sandwich commercial kit: All animal's sera were uniformly negative for the presence of viral antigen by using of such assay (Table 6).

Detection of variation in profiles of serum proteins by using of SDS-PAGE Cattle serum samples: Results of Fig. 3 showing:

- Protein band of molecular weight 97 kDa in both positive sera and in negative ones which was prominent by both silver and coomassie staining.
- At level of 75kDa there was sharp dense protein band shared in both positive sera while was faint in negative ones.
- In coomassie stained profile there was prominent band at level of 34kDa shared in two positive samples and one of negative samples.

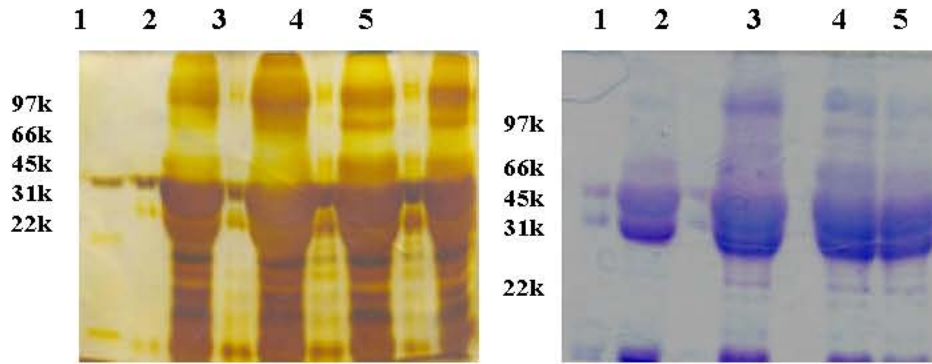


Fig. 3: A-Silver nitrate stained gel. individual serum samples diluted 1:4 in PBS. Wells No. 2 and 3 (negative individual samples) B- Coomassie brilliant blue stained gel of Well No. 1 (low molecular weight marker) Wells No. 4 and 5 (positive individual samples)

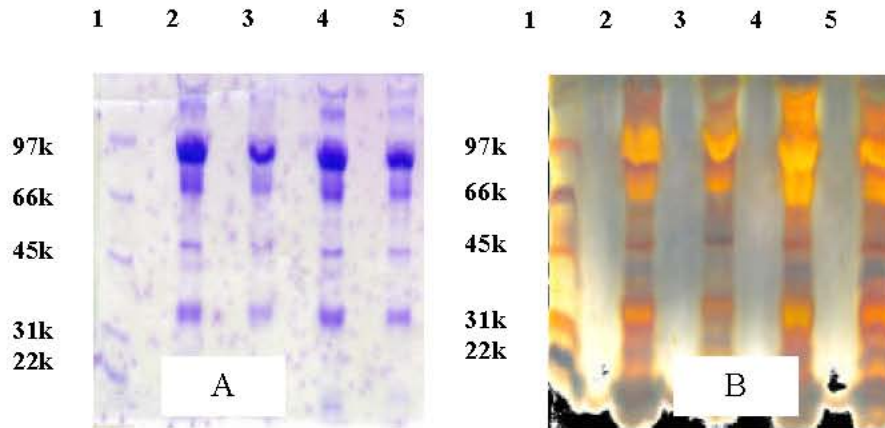


Fig. 4: A-Coomassie brilliant blue B-Silver nitrate stained gel

Well No. 1 (Lmw)
 Well No. 3 (negative sample diluted 1/32)
 Well No. 5 (positive sample diluted 1/32)

Well No. 2 (negative sample diluted 1/16)
 Well No. 4 (positive sample diluted 1/16)

Buffaloe serum samples: Results of Fig. 4 showing: Both silver and coomassie staining showed identity in electrophoretic profiles of positive and negative samples of same dilution (2&4) and(3&5) and fainting of profile intensity with increased dilution (2&3) and (4&5).

DISCUSSION

In last few decades great concern was directed towards BVDV as a primary pathogen affecting livestock reproductive health, immunosuppressive agent [19]. In the present study the prevalence of BVDV infection in five different governorates was carried out. Association of BVD infection with reproductive disorders was previously demonstrated [20]. Infection with

Two individual serum samples one positive and one negative each diluted in rate of 1/16 and 1/32 and loaded

in two gels, one stained by silver nitrate and the second by Coomassie brilliant blue against LMW

This virus induced in non suppurative oophoritis and necrosis of granulose cells in non pregnant cows and fetal death and abortion in pregnant animals [21, 22]. Results of detection of BVD specific antibodies by ELISA showed prevalence of antibodies in 47 to 83% of examined cattle samples with lower incidence in buffaloes. These results were in concordance with [23, 24] who demonstrated seroprevalence in cattle herds varied from 0.0 to 95.9%. Recently, [25] demonstrated high prevalence of BVD infection in dairy farms which (58 and 93%).

For results of BVDV antibodies prevalence in buffaloes, our records were in agreement with those of [26] who demonstrated prevalence of anti-BVDV antibodies in 24.67% by using of ELISA and 25.97% by

SNT. However, [27] demonstrated prevalence of anti-BVDV-antibodies in 52% in examined buffaloes. In addition to, [28] demonstrated prevalence of BVDV antibodies in 55.3% of examined Bison bison.

In cattle detection of antibodies against BVDV by ELISA was more sensitive than SNT while in buffaloes SNT was more sensitive than ELISA and that was in concordance with [26] who reported that seroprevalence of BVD infection in buffaloes was 24.67% by ELISA and 25.97% by SNT.

The difference between ELISA and SNT was minute except in Ismailia buffalo farm where prevalence of BVDV antibodies was 0% by ELISA and 21.9% by SNT. This can be explained as ELISA measurement done only for IgG and not IgM and assuming that sera from SNT positive IgG negative animals may had IgM due to recent infection.

Specificity of ELISA and SNT were further tested by detecting affinity of anti-BVDV-antibodies in sera to localize intracellular viral antigens in infected MDBK cells by indirect immunofluorescence test.

Virus isolation, antigen or nucleic acid detection are the only means for identifying and culling BVDV-PI animals. Measurement of serum antibodies to BVDV, though easier than virus isolation and antigen detection, is not practicable in the identification of BVDV PI-infected cattle for two reasons. First, PI animals are, by definition, immunotolerant to BVDV and may not develop anti-BVDV antibodies. Second, the prevalence of BVDV antibody-positive animals in the cattle population is high, typically about 70% [29, 30]. Previous studies have shown that serum is an excellent diagnostic specimen for BVDV because of high stability of the virus in serum and the ease of its collection, shipping and handling. Our findings were in agreement with those found by [24]. Who found that seroprevalence of BVDV, infection in five herds free from PI animals varied from 0.0 to 82.3% and these herds did not apply vaccination program against BVDV. In a similar study [25] demonstrated that prevalence of BVD infection in five dairy herds ranged from 58 to 93%. They failed to detect BVDV antigens in seronegative animals and this may be attributed to death of PI animals or soling of the blood sampling.

Unfortunately, there was lack of literature concerned with genetic resistance of cattle to BVDV. So in this study serum protein was used as marker of genetic makeup, especially that variation in genetic constitution that will be directly reflected on serum protein content. To detect serum protein contents, there are several biochemical

and molecular methods [31, 32] among which is electrophoresis that determines both quantitative and qualitative protein content. Although data concerning characterization of susceptibility and resistance to BVDV infection by protein electrophoresis or immunoelectrophoresis are very rare, yet this approach was considered in many other diseases to detect difference between diseased and healthy animals such as brucella infected and non infected camels[33]. However, in this study, electrophoresis of sera proteins failed to reveal prominent specific profiles for either BVD positive or negative animals. Therefore, this tool is insufficient for differentiating between infected and non infected animals and subsequently, can't identify resistance or susceptibility markers for BVD infection.

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