Vet.Med.J.,Giza. Vol.48, No.2. (2000): 227-238.

EVALUATION OF A RECOMBINANT ELISA ANTIGEN FOR SERODIAGNOSIS OF BOVINE VIRAL DIARRHEA IN IMMUNE AND INFECTED CATTLE SERA IN EGYPT

A. A. EL-KHOLY*; H. M. GHALY*; SAMIRA SAID * and M.M. EL-SABBAGH*

* Veterinary Serum & Vaccine Research Institute, ARC, P.O.Box 131, Abassia, Cairo, Egypt.

Received: 17.7.1999.
Accepted: 7.11.1999.

SUMMARY

The recombinant protein (r1448) in crude lysate of insect cells infected with a previously developed recombinant baculovirus, expressing two structural proteins, the nucleocapsid protein (p14) and the envelope glycoprotein (gp48) of the NADL strain of bovine viral diarrhea virus (BVDV), was evaluated as a coating antigen in a standard enzyme-linked immunosorbent assay (ELISA) for detection of antibody against homologous and heterologous strains of BVDV. The ELISA was compared to standard virus neutralization test (VNT) for detection of antibody against the local Iman strain of BVDV in sera of calves immunized with the locally produced Pneumo-3 inactivated vaccine, experimentally infected calves and contact controls. Antibody titers estimated by ELISA were closely correlated with antibody titers estimated by VNT (average r= 0.917). Nevertheless, the recombinant protein (r1448) was an effective and inexpensive ELISA antigen for detection of antibody against homologous and heterologous strains of both genotypes of BVDV (BVDV1 and BVDV2) in reference hyperimmune cattle sera. In conclusion, the recombinant antigen (r1448) is a reliable candidate for diagnostic tests of BVDV infections in Egypt.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a small enveloped RNA virus currently classified in the genus Pestivirus, which also includes hog cholera virus (HCV) of swine and border disease virus (BDV) of sheep, in the family Flaviviridae (Francki et al., 1991). The mature viral proteins encoded by BVDV are : a nonstructural autoprotease (p20), followed by the nucleocapsid protein

(p14), the three mature viral envelope glycoproteins (gp48, gp25, and gp53), and the non-structural proteins : p125/p54-p80, p10, p32 and p133/p58-p75 (Donis and Dubovi, 1987 and Collett, 1992).

BVDV is associated with a variety of devastating disease manifestations, causing a worldwide economic impact and a considerable threat to the livestock industry (Duffell et al., 1986; Radostits and Littlejhons, 1988; Baker, 1990; Dubovi, 1994 and McGowan and Krikland, 1995).

Two biotypes of BVDV exist in nature, cytopathic (cpBVDV) and non cytopathic (ncpBVDV), that can be differentiated by their effects on cell cultures. The cpBVDV induces cytoplasmic vacuolation and cell death in susceptible cell culture (Gillespie et al., 1960). while, the ncpBVDV has a little effect in cell cultures (Gillespie et al.,1962). Either separately or in combination, both viral biotypes induce diseases that range from clinically mild to fatal disease (Bolin et.al.,1985 and Baker, 1990). Primary post-natal infections with BVDV, termed bovine viral diarrhea (BVD), are usually subclinical but may result in fever, lymphopenia, inappetance, diarrhea and drop in milk yield (Radostits and Littlejohns, 1988). The ncpBVDV is usually isolated from field outbreaks of the disease. However, certain ncpBVDV strains induce a clinically severe form of acute BVD that is characterized by fever, lymphopenia, thrombocytopenia, diarrhea, thrombocytopenic purpura and death (Corapi et.al., 1990 and Bolin and Ridpath, 1992). Primarily, BVDV genotype 2 has been associated with these outbreaks of acute BVD (Pellerin et.al.,1994 and Ridpath et.al.,1994). Persistently infected cattle serve as reservoirs for maintaining and spreading the BVDV within a herd. Upon superinfection with certain cpBVDV, persistently infected animals succumb to fatal mucosal disease (MD) or chronic BVD (Bolin et.al.,1985). Most naturally occurring outbreaks of mucosal disease appear to be induced by antigenically matched pairs of noncytopathic and cytopathic BVDV (Howard et.al 1987 and Corapi et.al., 1990). However, antigenically distinct viruses also pair to induce mucosal disease (Ridpath and Bolin 1991).

Viral neutralization is the standard test for serodiagnosis of BVD. Other diagnostic assays that likely detect more viral proteins are also used such as whole virus ELISA, fluorescent antibody and immunoperoxidase staining. All of these assays require tissue culture systems, use of live virus, are time-consuming, expensive and difficult to apply in large scale screening of animals (Howard et al., 1985). The nucleocapsid protein of several viruses expressed in baculovirus has been established to be an appropriate ELISA antigen for diagnosis of many infectious diseases of animals (Reid-Sanden et.al.1990; Ahmad et al., 1991 and Ismail et al., 1995). Morcover, the envelope glycoproteins of BVDV were found useful as ELISA antigens for convalescent serodiagnosis

BVDV infection (Kwang et al., 1995). In the present study, use of a previously developed recombinant baculovirus expressing the nucleocapsid protein (p14) and the envelope glycoprotein (gp48) of the NADL strain of BVDV (El-Kholy, 1997) is evaluated for detection of anti-BVDV antibody in sera of locally vaccinated and infected calves. That could be of significant importance in epidemiological studies of BVDV infections in Egypt.

MATERIALS AND METHODS

Viruses and cells:

The cytopathic Iman strain of BVDV (Baz, 1975) which is the local vaccinal strain, was propagated and titrated on BVDV-free Madin-Darby bovine kidney (MBDK) cell culture (Baz, 1975). The MBDK cells were grown at 37°C, in a 5% CO_2 humidified atmosphere, in minimum essential medium with Earle's salts (MEME) supplemented with 1% antibiotic-antimycotic solution and 10% fetal calf serum. Spodoptera frugiperda (Sf9) insect cells (Invitrogen, San Diego, CA, USA) were maintained at 27°C in EX-cell 401 medium supplemented with 10% fetal calf serum. Sf9 cells were used for propagation of the recombinant baculovirus expressing two structural proteins p14 and gp48 of the NADL strain of BVDV, was constructed in a previous study (El-Kholy, 1997).

Vaccination of calves:

Nine cross breed apparently healthy male calves (Fresian X local), about 6-9 months old, an average body weight of 150 kilograms, were used in this study. Calves were housed individually in an isolation facility on arrival at the Veterinary Sera & Vaccines Researches Institute (VSVRI), Abbassia, Cairo, Egypt. Before experimental studies, serum samples were collected for titration of antibody against Iman strain of BVDV. Nasal swabs and buffy coats were obtained for BVD viral isolation. Calves were randomly assigned to three groups each of 3 animals. In the first group (G1), each calf was immunized with intramuscular 5 ml of the locally produced inactivated Pneumo-3 vaccine (BVD, Para-infleunza-3 and Infectious Bovine Rhinotracheitis) then, received a booster vaccinal dose 14 days after the initial dose. The second group (G2) was experimentally infected with BVDV and the third group (G3) was kept as non-vaccinated, non-infected contact to G2.

Viral exposure:

Calves of G2 were inoculated intranasally with approximately 10^6 tissue culture infective dose fifty ($TCID_{50}$) of Iman strain of BVDV in 10 ml of cell culture medium.

Serum samples:

Sera were harvested from tubes of clotted blood on days: 0, 3, 7, 14, 21, 28, 45, 60, 90, 120, 150, and 180 after the initial vaccination (G1); 0, 3, 7, 10, 14, 21 and 28 days after viral exposure (G2 and G3).

Reference sera:

Four reference hyperimmune sera against different strains of BVDV genotypes 1 & 2, and three negative control sera were kindly offered by Dr. Steven R. Bolin, Acting Director, at the National Animal Disease Center, Ames, Iowa, USA.

All serum samples and reference sera were tested for the presence of antibodies against BVDV by the viral neutralization test (VNT) using the Iman strain of BVDV and by ELISA using recombinant protein (r1448).

Preparation of the recombinant antigen :

Log-phase Sf 9 cells (2.5 X 10^6 cells/ml), seeded in EX-cell 401medium containing 10% fetal calf serum, were inoculated with the recombinant virus at an MOI of 5 and incubated at 27°C for 2-3 days or until all cells appeared infected. The cells were harvested by centrifugation at 1000 x g for 5 min, washed in 0.5 ml of phosphate buffered saline (PBS, pH 7.4), and resuspended in 10 volumes of cell lysis buffer (150 mM sodium chloride; 50 mM Tris HCl, pH 7.4; 0.01% sodium dodecyl sulfate; 1µg/ml Aprotinin and 0.5% sodium deoxycholate). Cell lysate was stirred for 1 hour at 4°C, clarified by centrifugation at 14,000 x g for 5 min and stored at -20°C as the crude recombinant antigen.

Enzyme - linked immunosorbent assay (ELISA):

An indirect ELISA was conducted after some modifications in methods described by (Ahmad et.al., 1993). Several conditions were evaluated to optimize the ELISA test and to provide a minimal background. Lysate from recombinant baculovirus-infected Sf9 cells, diluted 1:100 in PBS containing 0.001% Triton X-100, was found to be the optimal antigen (r1448) dilution. The 96- well microtitration ELISA plates (Dynatech) were coated with 100 µl/well of r1448 antigen and kept at 4°C overnight. The plates were decanted, washed 3 times in PBS, pH 7.4 containing 0.5% Tween 20; blocked by adding 50 µl/well of blocking buffer (PBS, pH 7.4 containing 5% non-fat milk) and incubated at 37°C for 1 hour. The plates were decanted, washed three times and dried as before. Each bovine serum sample was diluted to 1:10 in PBS and inoculated at 50 µl/well in duplicates. Each plate included a positive and a negative sera as well as a blank control. The plates were incubated at 37°C for one hour, then decanted and washed. The conjugate, horse radish peroxidase-labeled goat anti-bovine IgG diluted 1:800 in PBS buffer, was added at 50 µl/well and the plates were incubated at 37°C for one hour, decanted, washed then, 100 µl/well of the TMB ELISA substrate (Gibco) was added, and the plates were agitated until the color developed (3-5 minutes). The reaction was then halted by adding 50 µl/well of the sulfuric acid (1.25 M).

The plates were read using a spectrophotometric computer-assisted microplate reader at wavelength 450 nm. The mean absorbance values were

converted automatically by the computer program (Dynatech P.C. Software) into mean ELISA titers based on the control positive and negative values. An absorbance value of 0.5 was determined as the cut off point.

Viral neutralization test (VNT) :

The VNT in microtitration plates, as described by (Coggins, 1964), was conducted in parallel with ELISA in order to evaluate the developed ELISA antigen in detecting antibody against BVDV in bovine sera. Serial four fold dilutions of each serum sample in serum free MEM medium were added in quadruplicate to wells of 96-well flat bottomed tissue culture microtitration plates. Each well was inoculated with 100 $TCID_{50}$ of the Iman strain of BVDV and the plates were incubated at 37°C for 1 hour then, MEM containing 10^4 of MBDK cells was added to each well. The microtitration plates included one column of wells as non-infected cell control, and another one as virus control. The microtitration plates were incubated at 37°C for 2-3 days and examined microscopically for the presence of cytopathic effect (CPE). The virus neutralizing (VN) titer of the sera was expressed as the reciprocal of end point dilution that protected cells in ≥50% of the wells.

RESULTS

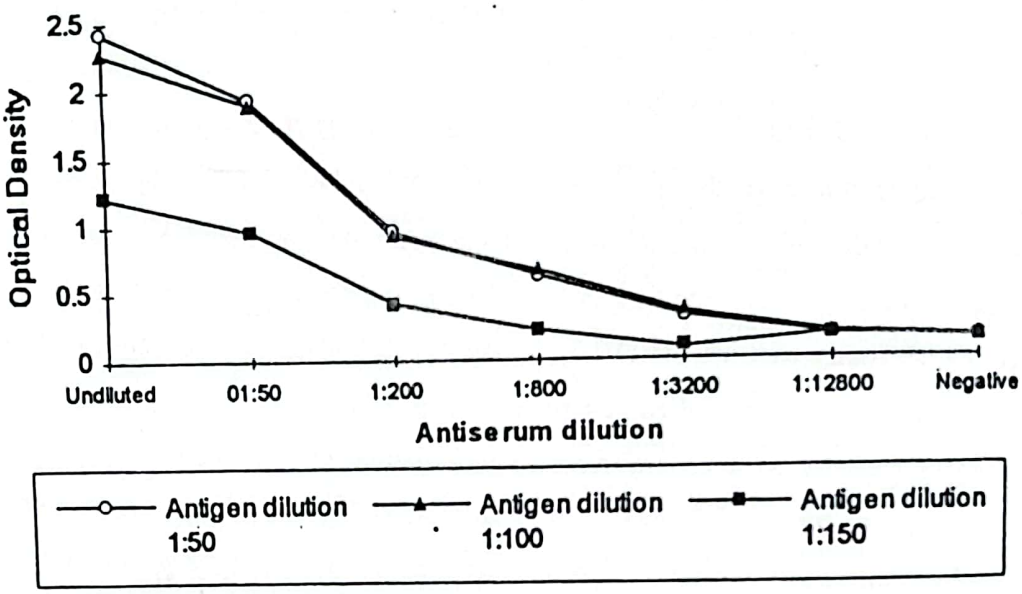
Indirect ELISA and VNT:

Figure (1) shows a typical titration curve of the recombinant antigen (r1448) with the reference anti-BVDV hyperimmune serum. Higher dilutions of the antigen gave progressively lower A_{450} values whereas, with the negative sera (fetal calf serum) there was no significant influence of the antigen dilutions on the A_{450} values.

Sera from BVDV-infected and immune calves as well as anti-BVDV reference hyperimmune sera reacted variably to optimum dilution of the recombinant antigen (r1448) in a standard indirect ELISA. The serum sample with an ELISA titres of < 1000 equivalent to VN-antibody titre less than 8 was considered negative. As shown in table (I), the positive Iman VN-Ab titers as estimated by VNT ranged between 8 and < 128 in sera of vaccinated calves (G1), whereas, in sera of experimentally infected calves (G2) VN-Ab titres ranged between > 8 and > 512 (Table II). The positive anti-r1448 ELISA titers ranged between 1430 and 6882 in sera of G1 calves (Table I) and between 1475 and 7240 in sera of G2 calves (Table II). Neither Iman VN-Ab nor anti-r1448 ELISA titers could be estimated in serum samples of control contact calves (G3). In reference anti-BVDV antisera the Iman VN-Ab titres ranged between 512 and 4096 while, anti-r1448 ELISA titres ranged between 4922 and 9756 (Table III).

The ELISA as shown in Tables I II, and III gave an excellent agreement with the VNT in detection of both seropositive and seronegative animals for BVDV. A highly significant correlation was found between the anti-r1448 antibody ELISA

Fig. (1): Optimization of NADL r1448 recombinant antigen dilution versus anti-BVDV antiserum for ELISA



Antiserum dilutions	Optical density (OD) at wavelength 450 nm		
	Antigen dilution 1:50	Antigen dilution 1:100	Antigen dilution 1:150
Undiluted antiserum	2.421	2.271	1.215
Antiserum 1:50	1.921	1.886	0.957
Antiserum 1:200	0.947	0.916	0.419
Antiserum 1:800	0.655	0.616	0.216
Antiserum 1:3200	0.349	0.311	0.084
Antiserum 1:51200	0.188	0.166	0.171
Negative serum	0.156	0.147	0.142

Table (I); Anti-recombinant B.VD viral antigen (NADL r1448) ELISA titres compared to viral neuralizing antibody (VN-Ab) titres against BVDV - Iman strain in sera of calves vaccinated with Pneumo-3 local vaccine (group 1)

Days Post vaccination	Calf No.						Average	
	Calf "1"		Calf "2"		Calf "3"			
	Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre
0	0	0	0	0	0	0	0	0
3	0	18	0	26	0	12	0	18.7
7	0	27	0	32	0	15	0	24.7
14 (booster dose)	≤4	<1000	≥2	<1000	≤2	<1000	2.7	<1000
21	≥8	1430	16	1480	8	1650	10.7≥	1520
28	≥16	2600	≥16	2650	≥16	2835	16	2659
42	32	2545	≥64	2885	≥32	4322	42.7	3250.7
60	≥64	55546	≤128	5316	≥64	5356	85.3	5408.7
90	64	4286	≥64	4560	≥64	6882	64	5242.7
120	32	3160	≥32	3005	≥32	2664	32	2943
150	16	1880	≥16	2800	≥8	1652	13.3	2110.7
180	≥8	1600	≥8	1600	≥8	1486	8	1562

Titre is expressed as the reciprocal of the end point serum dilution.

Table (II); Anti-recombinant BVD viral antigen (NADL r1448) ELISA titres compared to viral neuralizing antibody (VN-Ab) titres against BVDV - Iman strain in sera of experimentally infected (group 2) and contact control (group 3) calves.

Animal group	Calf No.	Days aftr viral exposure													
		0 day		3 days		7 days		10 days		14days		21 days		28 days	
		Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre	Iman VN-Ab titre	r1448 ELISA titre
Group (2)	1	0	0	≥8	1475	≥16	2280	≥64	3980	128	4065	256	5066	512	7032
	2	0	0	≥8	1730	32	2490	128	3460	256	4560	≥256	5742	≥512	7240
	3	0	0	≥8	1850	≥16	2495	≥64	3389	512	6960	≤512	6585	≥512	7123
Mean		0	0	8	1685	21.3	2421.6	85.3	3609.7	298.7	5195	341.3	5797	512	7123
Group (3)		Neither Sn-Ab titre nor ELISA titre could be detected in contact control calves through out the experimental peiod													

Titre is expressed as the reciprocal of the end point serum dilution.

Table (III): Comparison of anti-NADL-r1448 ELISA titres with Anti-BVAV / Iman virus neutralizing antibody (VN-Ab) titres in reference and local anti-BVDV antisera.

BVDV antisera	Genotype BVDV	Anti-BVDV-Iman VN-Ab-titre	Anti-NADL-r1448 ELISA titre
Singer	1	4096	9756
TGAC	1	1024	6177
890	2	512	7703
5390	1		4922
Iman	2	512	7211

Titre is expressed as the reciprocal of the end point serum dilution.

titres and the Iman VN-Ab titres against BVDV-Iman strain (r=0.923 in G1; r=0.935 in G2; r=0.895 in reference antisera with an average correlation of r=0.917).

DISCUSSION

The existence of immunotolerant and persistently infected animals along with the wide-spread distribution of the non-cytopathic (NCP) biotype, genotype 2 and the genetic diversity among the strains/isolates of BVDV present a problem in diagnosis, prevention and control of the disease (Gillespie et al.,1962; Radostits and Littlejohns, 1988; Ridpath et al., 1994 and Paton, 1995).

The diagnostic utility of crude lysate from infected insect cells, containing the recombinant protein (r1448), was determined by standard indirect ELISA. The r1448 was used as a coating antigen in ELISA plates. Dilution of the antigen in PBS containing 0.001% Triton X-100 and the overnight blocking using 5% non-fat milk in PBS at 4°C, were found to be optimal conditions for detection of the anti-BVDV antibodies in cattle sera. That might be related to the nature of Triton X-100 as a nonionic detergent that has been known to bind preferentially to hydrophobic proteins and it breaks up aggregations of the expressed proteins in crude lysate (Pryde, 1986, and Ahmad et al., 1993). The p14 and gp48 of BVDV are mostly hydrophilic (Silva-Krott et al., 1994).

In the present study, the local Iman strain of BVDV was used as an antigen in virus neutralization test (VNT), to verify the relative efficacy of the developed recombinant antigen. The crude lysate from Sf 9 cells infected with the recombinant baculovirus, containing the recombinant protein (r1448) has proved to be sensitive and efficient ELISA antigen for detection of anti-BVDV antibodies in sera of cattle immunized against and infected with the local Iman strain as well as in hyperimmune sera raised against both genotypes 1 and 2 of heterologous BVDVs. The anti-BVDV Singer antiserum scored higher values of antibody titre, compared to other reference antisera used in this study. This finding could be explained by that Singer strain is the most related BVDV to NADL strain, based on speculations given by Ridpath et al. (1994) and Pellerin et al. (1995) who grouped Singer and NADL strains in

the same subdivision (1a) within genotype 1 of BVDV. Besides, cross-reactivity between genotypes 1 and 2 of BVDV is relatively low and viruses could escape neutralization by antibody raised against heterologous variants (Pellerin et al., 1994). Also, the high correlation estimated between the anti-r1448 ELISA titers and anti - Iman serum neutralizing titers, $r = 0.816$, supported that the developed ELISA is relatively sensitive and specific. Moreover, it indicated that the expressed p14-gp48 protein is high proportionally conserved among BVDV strains. The use of nucleocapsid protein expressed in baculovirus has been established to be an appropriate ELISA antigen for diagnosis of many infectious diseases of animals and humans (Reid-Sanden et al., 1990; Ahmad et al., 1993 and Ismail et al., 1995). Moreover, the envelope glycoproteins of BVDV were found to have certain neutralizing activity and to be good ELISA antigens for serodiagnosis (Donis et al., 1988 and Kwet al., 1995). That would strongly support the objectives proposed as well as the results of this study to use the recombinant p14-gp48 viral protein as a novel diagnostic tool for BVD-MD. The relatively higher range of antibody titre estimated in sera of G2 calves versus G1 calves could be attributed to methods of BVDV inactivation used in killed vaccines that may affect the elicited immune response against some viral proteins (Bolin and Ridpath, 1990).

Other ELISAs have been developed using the putative BVDV as a coating antigen, after different treatments, and proved that ELISA is technically superior to the serum neutralization test for routine detection of anti-BVDV antibodies in cattle sera (Howard et al., 1985 and Cho et al., 1991).

However, the risk of handling and using the putative virus with the costs encountered in propagation and purification of the virus stocks would increase its disadvantages for safety and economic concerns. Moreover, upon the development of an effective recombinant vaccine for BVD-MD, the putative virus will not distinguish between the vaccinated and infected animals in the field.

Production of the recombinant protein was inexpensive since a 75 cm_2 flask containing (1.5 X 10^5 / ml Sf 9 cells) provided antigen adequate for coating 10 standard 96-well plates, testing 440 serum samples in duplicates beside the controls. The crude lysate used for coating the ELISA plates showed neither interference nor loss in sensitivity and a low background suggested that purification of the recombinant protein was not required.

In conclusion, the results obtained in this study proved that the recombinant BVDV p14-gp48 protein provided an effective, sensitive and inexpensive ELISA antigen for indirect diagnosis of BVD by detecting antibody against p14-gp48 protein of BVDV in cattle sera without the need for the hazardous handling of the putative virus and expensive costs of the tissue culture systems. The

obtained results initiate a potential for future work concerning the immunological importance of the p14 and gp48 regions of the pestiviruses in diagnosis of infected animals and development of effective recombinant vaccines as well.

REFERENCES

Ahmad, S.; Bassiri ,M.; Banerjee, A.K.; and Yilma, T. (1993) : Immunological characterization of Vesicular Stomatitis Virus nucleocapsid (N) protein expressed by recombinant baculovirus in Spodoptera exigua larvae : Use in differential diagnosis between vaccinated and infected animals. Virology; 192 : 207-216 .

Baker, J.C. (1990) : Clinical aspects of bovine viral diarrhea virus infection. Rev. Sci. Tech. Off. Int. Epiz.; 9 (1) : 25-41.

Baz, T.I. (1975) : Isolation, characterization and serological studies on BVD-MD virus in Egypt. A Ph.D. Thesis presented to the Microbiology Dept., Fac. Vet. Med., Cairo Univ.

Bolin, S.R.; McClurkin, A.W.; Cutlip, R.C., and Coria, M.F. (1985) : Severe clinical disease induced in cattle persistently infected with non-cytopathic bovine viral diarrhea virus by superinfection with cytopathic bovine viral diarrhea virus. Am. J. Vet. Res., Vol.46, No.3, pp. : 573-576.

Bolin, S.R., and Ridpath, J.E. (1990) : Range of viral neutralizing activity and molecular specificity of antibodies induced in cattle by inactivated bovine viral diarrhea virus vaccine. Am.J.Vet.Res.,.51 (5), pp. : 703-707.

Bolin, S.R., and Ridpath, J.E. (1992) : Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. Am.J.Vet.Res.,.53 (11), pp. : 2157-2163.

Cho, H.J.; Masri, S.A.; Deregt, D.; Yeo, S-G, and Elizabeth, J. Thomas (1991) : Sensitivity and specificity of an enzyme-linked immunosorbent assay for the detection of bovine viral diarrhea virus antibodies. Can.J.Vet.Res.; 55 : 56-59.

Coggins, L. (1964) : Standardization of virus-neutralization test for bovine virus diarrhea. Am. J. Vet. Res.; 25 : 103 - 107.

Collett, M.S. (1992) : Molecular genetics of pestiviruses. Comp. Immunol. Microbiol. Infect. Dis.; Vol.15, No.3, pp. : 145-154.

Corapi, W.V.; Elliot, R.D.; French, T.W.; Arthur, D.G.; Bezek, D.M., and Dubovi, E.J. (1990): Thrombocytopenia and hemorrhages in veal calves infected with bovine viral diarrhea virus. J. Am. Vet. Med. Assoc.; 169 : 590-596.

Donis, R.O.; Corapi, W., and Dubovi, E.J. (1988) : Neutralizing monoclonal antibodies to bovine viral diarrhea virus bind to the 56-58 KDa glycoprotein. J. Gen. Virol.; 69 : 77-86.

Donis, R.O. and Dubovi, E.J. (1987) : Glycoproteins of bovine viral diarrhea-mucosal disease virus in infected bovine cells. J. Gen. Virol.; 68 : 1607-1616.

Dubovi, E.J. (1994) : Impact of bovine viral diarrhoea virus on reproductive performance in cattle. Vet. Clin. North Am. Food Anim. Pract., 10 (3) : 503 - 514.

Duffell, S.J; Sharp, M.W, and Bates, D. (1986) : Financial loss resulting from BVD-MD virus infection in a dairy herd. Vet. Rec.; 118 : 38-39.

El-Kholy, A.A. (1997) : Molecular and immunological studies on bovine viral diarrhoea virus infections in cattle. Ph.D. Thesis presented to Int. Med., Infect. Dis. and Fish Dept., Fac. Vet. Med., Cairo Univ., P. 39 - 79.

Francki, R.I.B; Fauquet, C.M; Knudson, D.L. and Brown, F. (1991) : Classification and nomenclature of viruses: Fifth report of the international committee on taxonomy of viruses. Arch. Virol., Supplement 2, pp.: 223-233.

Gillespie, J.H.; Baker, J.A., and McEntee, K. (1960) : A cytopathogenic strain of virus diarrhea virus. Cornell Vet.; 50, pp. : 73-79.

Gillespie, J.H.; Madin, S.H., and Darby, N.B. (1962) : Cellular resistance in tissue cultures induced by non-cytopathogenic strains to a cytopathogenic strain of virus diarrhea of cattle. Proc.Soc.Exp.Biol.Med.; 110 : 248-250 .

Howard, C.J.; Brownlie, J. and Clarke, M.C.(1987) : Comparison by the neutralization assay of pairs of noncytopathogenic and cytopathogenic strains of bovine virus diarrhoea virus isolated from cases of mucosal disease. Vet. Microbiol.; 13 : 361-369.

Howard, C.J.; Clarke, M.C. and Browlie, J. (1985) : An enzyme linked immunosorbant assay (ELISA) for the detection of antibodies to bovine viral diarrhoea virus (BVDV) in cattle sera. Vet. Microbiol.; 10 : 359 - 369.

Ismail, T.M.; Yamanaka, M.K.; Saliki, J.T.; El-Kholy, A.; Mebus, C. and Yilma, T. (1995) : Cloning and expression of the nucleoprotien of Peste des Petits Ruminants virus in baculovirus for use in serological diagnosis. Virology; 208 : 776-778.

Kwang, J.; Bolin, S.R. and Littledike, E.T. (1995) : Bovine viral diarrhea serologic diagnostic reagents prepared from bacterially expressed recombinant proteins. J. Vet. Diagn. Invest.; 7 (1): 143-145.

McGowan, M.R. and Kirkland, P.D. (1995) : Early reproductive loss due to bovine pestivirus infection. Br. Vet. J.; 151 (3) : 263-270.

Paton, D.J. (1995) : Pestivirus diversity. J. Comp. Pathol. 112 (3) : 215 - 236.

Pellerin, C.; Van Den Hurk; Lecomte, J. and Tijssen, P. (1994) : Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology.; 203 : 260 - 268.

Pellerin, C.; Moir, S.; Lecomte, J. and Tijssen, P. (1995) : Comparison of the p125 coding region of bovine viral diarrhoea viruses. Vet. Microbiol., 45 (1) : 45 - 57.

Pryde, J. (1986) : Triton X-114 : a detergent that has come in from the cold. Trends Biochem. Sci.; 11 : 160 - 163.

Radostits, O.M. and Littlejohns, I.R. (1988) : New concepts in the pathogenesis, diagnosis and control of diseases caused by bovine viral diarrhea virus. Can. Vet. J.; 29 : 513-528.

Reid-Sanden, F.L.; Sumner, J.W.; Smith, J.S.; Fekadu, M.; Shaddock, J.H, and Bellini, W.J (1990): Rabies diagnostic reagents prepared from a rabies N gene recombinant expressed in baculovirus. J. Clin. Microbiol.; 28 : 858-863.

Ridpath, J.F. and Bolin, S.R. (1991) : Hybridization analysis of genomic variability among isolates of bovine viral diarrhea virus using cDNA probes. Mol. Cell Probes : 291-298.

Ridpath, J.F.; Bolin, S.R and Dubovi, E.J (1994) : Segregation of bovine viral diarrhea virus into Genotypes. Virology; 205 : 66-74.

Silva-Krott, I.U; Kennedy, M.A. and Potgieter, L.N. (1994) : Cloning, sequencing, and in vitro expression of glycoprotein gp48 of a noncytopathogenic strain bovine viral diarrhea virus. Veterinary Microbiology 39 : 1-14.