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Isolation of Bovine Viral Diarrhea Virus from Calves using Rabbits and Comparative Detection of both Viral Antigen and Antibody for BVDV Genotypes I & II in Experimentally Inoculated Rabbits

Ahmed Abd El Samie Hassan Ali*

Bovine viral diarrhea virus (BVDV) is a world wide pathogen infects a wide variety of ruminants. A random 42 field pooled calf samples were collected and inoculated simultaneously intranasal, oral and intraperitoneal into 6 *New Zeeland rabbits*. The rabbits were observed for 25 day, slaughtered then tissue harvests from spleen, lung, lymph nodes and intestine were pooled, homogenized; the supernatant was clarified and concentrated for second inoculation. A another *16* BVDV sero-negative *New Zeeland rabbits*, classified into 4 groups, each of 4 designated for second closed inoculation with clarified supernatant of rabbit tissue harvests of first inoculation in comparison to reference BVDV type-1-NADL, BVDV type-2-890 and control negative. Clinical scores and samples included nasal & rectal swabs and sera from rabbits were collected on days, **3, 5, 7, 10, 15**, and **21** post inoculations for isolation and detection of its antigens on MDBK cells using outgrowth ELISA and immunofluorescence as well as measurement of BVDV antibodies by serum neutralization test. Virus antigen detection was reported on days 5 & 7 while the antibodies were initially detected on day

* *Department of Virology, Faculty of Veterinary Medicine. Zagazig University, Egypt*
Email: abdelsamie35@yahoo.com
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7 with titers of 0, 8, and 8 of groups 1, 2 & 3 and continued variably up to the end of sampling time day 2[1st] with titers reached 10, 24 & 40 respectively. This study documented the existence of BVDV in the samples and susceptibility of rabbits to infection.

Key words:

.INTRODUCTION

BVDV is a pathogen of cattle and of world wide distribution **(Baker, 1995))**. It is responsible for severe illness, persistent infection and deaths among free living and captive ruminants (**Doyle and Heuschele, 1983; Nettleton and Enterican, 1995**). In addition, BVDV affects broad range of hosts including domestic rabbits (**Baker *et al.*, 1954; Nettleton, 1990**). BVDV includes many genotypes with subtypes that are classified within the ***Genus Pestiviruses***, ***Family Flviviridae*** along with the flavi and human hepatitis C viruses. (**Francki *et al.*, 1991, Ridpath *et al.*, 1994**). BVDV infection is acquired through inhalation of infective virus particles with subsequent viral replication in respiratory epithelial and endothelial cells. Dissemination of the virus occurs as a result of a cell mediated viremia in lympocytes and monocytes **(Carpenter, 1986))**. Rabbit is considered a small sized host, useful for the laboratory diagnosis of viral diseases. The present study was directed to evaluation of rabbit's susceptibility to BVDV infection, the isolation of BVDV, detection of its antigens; antibodies from bovine samples into rabbits in comparison to reference BVDV biotypes in experimentally inoculated rabbits.

MATERIAL AND METHODS

Reference BVDV: BVDV genotype-1-CP- *[Cytopathic-NADL]*, genotype-2-NCP *[None Cytol.Jathic-890]* of a closed titer to 2x 10^6 $TCID_{50}$/ml, that were used before **(Ahmed Abd El-Samie, 1998)** and obtained from ATCC, USA.

Rabbits and its inoculation: Twenty (22) BVDV sero-negative New Zeeland non pregnant rabbits, 6-7 weeks old, were conventionally housed and maintained separately in an isolation facility during the course of the experiment. A group of 6 rabbits were used for first inoculation with the prepared, pooled bovine field samples. Another 16 rabbit were randomly assigned to 4 groups, 4 rabbits in each for second inoculation in comparison to reference BVDV biotypes.

Bovine field Samples: Pooled 42 field samples included nasal, rectal swabs, necropsied tissues and blood samples were collected from affected non vaccinated calves. The samples were prepared, clarified by centrifugation and the resultant supernatants used for virus isolation. The samples inoculated intranasal, oral, intraperitoneal into 6 rabbits, observed for 25 day. Later, the rabbits were necropsied and sera, nasal & rectal swabs, tissues from spleen, lymph nodes, lung, intestine were pooled, homogenized, clarified then used for second closed inoculation into rabbits in comparison to reference BVDV biotypes. The 16 rabbit groups were identified as second inoculation of harvest from first inoculation, BVDV type-1 & BVDV type-2 and control. Each appropriate group was inoculated with 1 ml of $2x10^6$ $TCID_{50}$ of reference virus biotypes and 2 ml of first tissue harvests. The inoculation was proceeded alternatively intraperitoneal, intranasal and oral. Clinical scores of temperature, respiratory and/or diarrhea were reported. Blood/sera were collected immediately at 0-day and at 3, 5, 7, 10, 15, 21 days after inoculation. Eventually, the rabbits were slaughtered and spleen, lymph nodes, intestine were harvested and prepared for virus isolation and detection of its antigen into MDBK cells using outgrowth ELISA and immunofluorescence as well as measurement of BVDV antibody by serum neutralization test.

Detection of BVDV and its antigen using outgrowth ELISA: MDBK cell culture grown in complete EMEM (Gibco, USA) of antibiotic mixtures and equine serum [Sigma] (to avoid the adventions BVDV contamination. A micro titer cell culture monoclonal antibody ELISA was applied to detect BVDV in rabbit

serum samples (**Saliki, 1994; Ahmed Abd El Samie, 1998**). The assay depends on the replication of BVDV from the rabbit sera in MDBK cells under optiumun conditions of temperature and **CO2 5%.** The cells were dried, fixed with aceton 80%, stained with BVDV monoclonal antibody (Dubovi) and peroxidase and then examined under ordinary microscope **x 10.**

Detection of BVDV viral antigens by immunofluorescence (IF): Another groups of inoculated MDBK cells, were fixed in cold acetone 80% and stained with BVDV polyclonal flourecenisothiocyanate (FITC) conjugated antibody **(NVSL, Ames, Iowa, USA)** diluted 1:40. The cells were mounted with glycerol mounting fluid, cover slipped, and examined under fluorescent microscopy at x40 &100 **(Xue and Minocha, 1996).**

Measurement of BVDV antibody using serum neutralization test (SNT): Using 2 fold serum dilutions cell culture SNT was used to assess neutralizing antibody to BVDV types (**Fulton *et al.*, 1997**). All sera were heated at **56°C** for 30 minutes before use. The antibody titers were expressed as the reciprocal of the highest serum dilutions capable of neutralizing the virus and inhibition of cytopathogenecity.All sera were tested twice and the mean titer was calculated.

REULTS

Detection of BVDV antigen Outgrowth ELISA: BVDV was detected in the samples collected from closed second experimentally inoculated rabbits on days 5, 7 post inoculations. Prescence of viral antigens were represented by intracytoplasmic pink color after staining, figure (1).

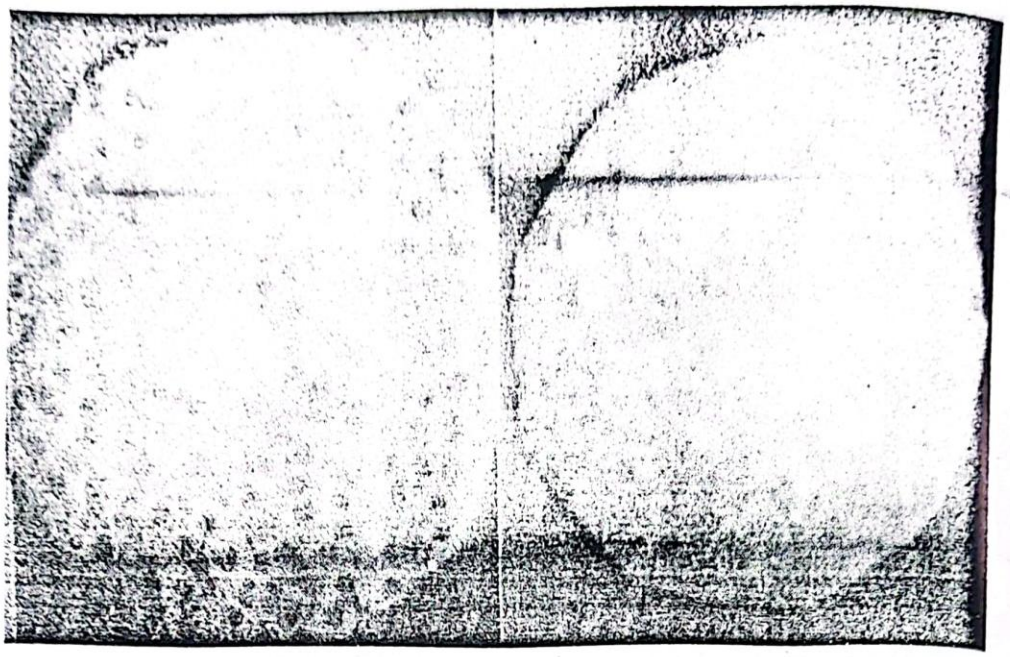
Detection of BVDV antigen by immunofluorescence: BVDV from rabbit organs collected post second closed inoculation was detected in cell culture and expressed by intra-cytoplasmic bright yellow illumination, figure (2).

A- B-

Figure (1): Showing detection of BVDV and antigen by outgrowth ELISA and includes:

A-An MDBK cells, stained with BVDV monoclonal antibodies, showing no intra cytoplasmic pink coloration.

B-Infected MDBK cells, stained with BVDV monoclonal antibodies, showing intra cytoplasmic pink coloration as result of BVD viral antigens.



A- **B-**

Figure (2): Detection of BVDV antigens by immunofluorescence:
A- Normal MDBK cells stained with BVDV polyclonal antibodies labeled with FITC, showing no fluorescence.
B- Infected MDBK cells stained with BVDV polyclonal antibodies labeled with FITC, showing intracellular bright yellow fluorescence.

Measurement of BVDV antibody: antibody in sera considered indicative of infection and will be referred to as positive if they had antibody titers of > 4 to one or more of the BVDV strains. BVDV neutralizing antibody started to develop on 7th day in all rabbit groups inoculated with either first inoculation or reference biotypes. The initial means of antibody titers were 0, 8, & 8 of groups 1, 2, & 3 respectively. The corresponding titers increased with relatively variable significant levels of 10, 24, & 40 throughout the post inoculation sampling time day 21; no BVDV antibodies were detected in control group, figure (3).

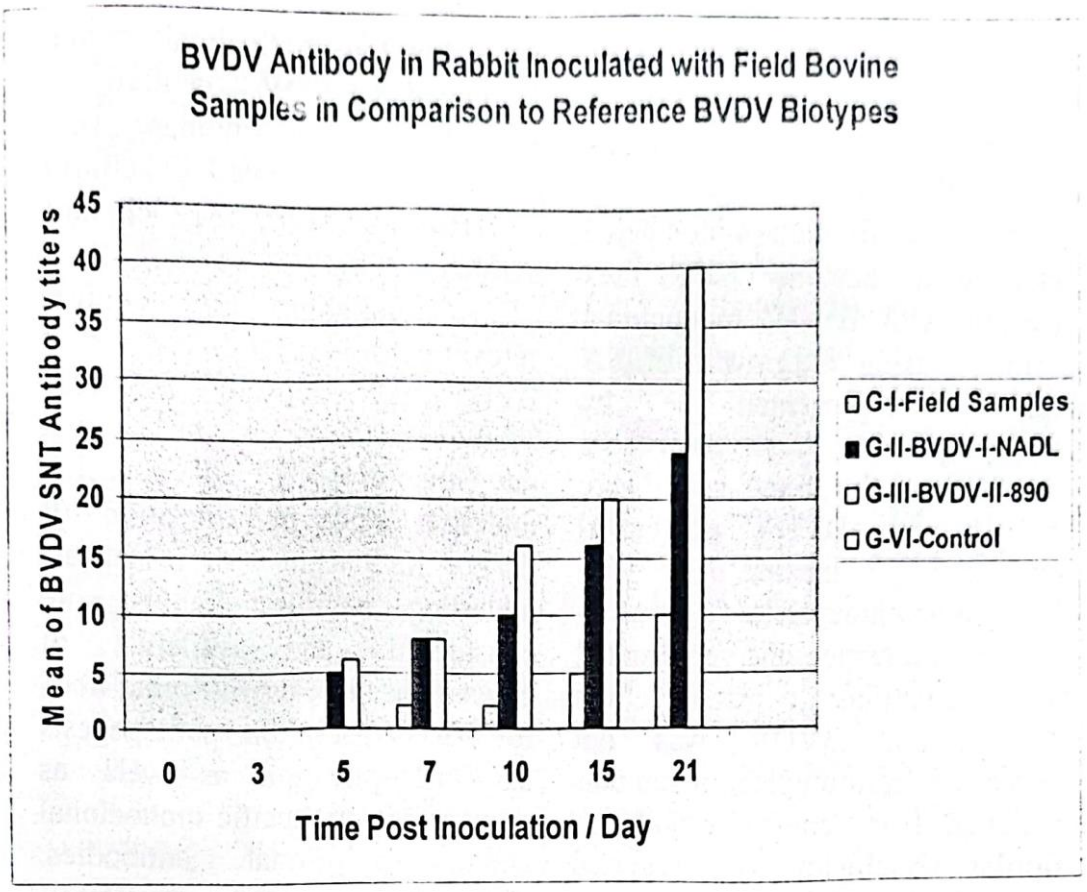


Figure (3) BVDV antibody in rabbit inoculated with bovine field samples in comparison to reference BVDV biotypes.

DISCUSSION

BVDV is an RNA virus within the *Family Flaviviridae* and BVDV infects a wide variety of hosts. The virus has two genotypes and each has two sub biotypes (**Ridpath, 1994**). BVDV was detected and recovered from field bovine samples by inoculation into rabbits post two closed inoculation. This documents the interspecies transmission and cross infection that can occur frequently and has been achieved experimentally (**Terpstra and Wensvoort, 1988; Carllson, 1991).**

BVDV and its antigens of inoculated rabbit's harvests in comparison to reference types were

demonstrated on 5 & 7 post inoculation. Although there was no typical BVDV cytopathic effects on MDBK but its antigens were detected after fixation of inoculated cells with acetone 80% then staining with BVDV monoclonal antibody using outgrowth ELISA and represented by intracytoplasmic pink coloration. In addition, the fixed cells were stained with BVDV polyclonal antibody labeled with flourecenisothiocyanate using immunoflouresnce and represented by intracytoplasmic bright yellow illumination. BVDV was not recovered from any serum samples obtained from control rabbits. A similar conclusion was reported **(Fernelius et al., 1969; Enterican *et al.*, 1995; Dubovi, 1996; Fulton *et al.*, 1997)**. The authors found that following acute BVDV infection, the virus is usually shed in most excretions and secretions from day 4 to 10, but may be excreted for a longer period in some individuals.

As shown in Figure (3), there were differences in the onset and duration of the detectable specific antibody, which depended upon the involved, inoculated virus harvests, biotypes, routes of inoculation and the rabbit's susceptibility. Although it was low but relatively all rabbits developed BVDV neutralizing antibody. These findings were previously documented by **(Bolin and Ridpath, 1989; Frolich and Streich, 1998)**.

Conclusion and clinical relevance: this is the first report of BVDV like exposure in rabbits. Moreover, the study documented the uncontrolled pathogenecity, infection and multiplication of BVDV in a wide variety of hosts including rabbits. Rabbits are considered an available of convenient size, useful living host, for BVDV isolation, pathogenesis and multiplication as well as preparation of specific monoclonal and/or polyclonal antibodies. Alternative adaptation of BVDV from calf to rabbit and vice versa to may produce an immunizing virus. Further study requires for demonstration the role of rabbits as may carriers for BVDV.

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