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EPIDEMIOLOGICAL ASPECT OF BOVINE VIRAL DIARRHEA VIRUS- II INFECTION IN DAIRY CATTLE

(With 2 Figures)

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السمة الوبائية لفيروس الإسهال البقرى -٢ فى الأبقار الحلوب

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تهدف هذه الدراسة إلى توصيف الخواص الإكلينيكية والفيرولوجية لإصابة الأغشية المخاطية والترمبوسيتوبنك للأبقار الحلوب بفيروس الإسهال البقرى. تشتمل خطوات الدراسة على فحص الحيوانات المصابة إكلينيكيا، تجميع عدد ٣١ عينة سيرم تشمل جميع الأبقار المصابة، ٨ عينات لبن من الأبقار المصابة، ٨ مسحات مهبلية من الأبقار التى تعانى من نزيف ومن الأبقار المجهضة. تم إستخدام العترة المرجعية العالمية NADL كعترة ممثلة للنوع الوراثى الأول والعترة المحلية Behera-CP 58/99 والتى سبق عزلها وتوصيفها جينيا وأنتيجينيا ممثلة للنوع الوراثى الثانى. تم عزل فيروس الإسهال البقرى من جميع المسحات المهبلية (٨) ، ومن (٨/٥) من عينات اللبن. جميع المعزولات من النوع الغير سيتوباثولوجى حيث لا يوجد أى تأثير سيتوباثولوجى للفيروس على الخلايا النسيجية المحقونة. بإستخدام الأجسام المناعية الآحادية الخاصة بالنوع الوراثى الثانى والتى تعمل على الجليكوبروتين ٥٣ ، تم الكشف على الفيروس داخل السيتوبلازم بإستخدام إختبار الصبغة الفلورسنتى والأمينوبيروكسيداز. بإستخدام إختبار الإليزا، تم الكشف عن الأجسام المناعية المضادة للفيروس فى جميع عينات السيرم حيث كانت العيارية تتراوح ما بين ≤ ١٢٨/١ إلى ≤ ٥١٢/١. من ذلك نخلص إلى أن فيروس الإسهال البقرى النوع الوراثى الثانى موجود ويصيب العديد من قطعان الأبقار مسببا أعراض مرضية متعددة لذا فمعرفة السمة الوبائية للفيروس على المستوى الجزيئى له أولوية. تحسين الخطط الإستراتيجية فى التشخيص والتحكم فى المرض لازمة لتقليل الخسائر الناتجة عن الإصابة بالفيروس.

SUMMARY

The purpose of this study was to characterize the clinical and virologic features of the mucosal and thrombocytopenic BVDV infection in infected dairy cattle. Strategy of examination included clinical examination of diseased animals, serum samples (n=31) represented all diseased cattle, milk samples (n=8) represented clinically diseased cattle and vaginal swabs (n=8) from the hemorrhagic diseased and aborted cattle were tested. An international reference strain (NADL: National Animal Disease Laboratory) and local cytopathic BVDV genotype-II strain (Behera-CP 58/99) were used as positive controls. The virus was isolated from the 8 vaginal swabs and 5 out of the 8 milk samples. All the isolates were ncp that no CPEs were noticed over the 3 passages. By specific BVDV genotype -II monoclonal antibodies (MAbs) against gp53, the viral antigen was identified using Fluorescence isothiocyanate (FITC)-conjugated anti-bovine IgG (specific intra cytoplasmic fluoresce granules) and horseradish peroxidase (HRP)-conjugated anti-bovine IgG (specific intra cytoplasmic brown granules) were detected. By enzyme linked immunosorbant assay (ELISA) technique, all serum samples were positive against the BVDV that had neutralizing antibodies titer ranges from $\geq$ 1/128 to $\geq$ 1/512. In conclusion, BVDV type -II do exist in cattle population and the understanding the molecular epidemiology is fundamental. Improved diagnostic and control strategies are essential to reduce losses inflected by BVDVs infection.

Key words: *Bovine viral diarrhea virus- II, Epidemiological aspect, isolation, immunohistochemistry.*

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a complex pathogen of ruminants. The high prevalence of BVDV in combination with its negative effects on reproduction and the general health condition in affected herds result in significant economic losses to the cattle industry globally (Houe, 2003). The viral pathogenicity is related to its broad tissue tropism in the infected animal, its capacity to elicit damaging host responses, and most probably, an as yet incompletely defined direct mechanism of virulence (Potgieter, 1997; Brock, 2004).

According to the eighth report of the International Committee on Taxonomy of Virus (ICTV), there are two BVDV genotypes, BVDV-1

and BVDV-2, together with border disease (BD) virus, classical swine fever (CSF) virus and Giraffe virus, constituting the genus *Pestivirus* of the family *Flaviviridae* (Fauquet, *et al.*, 2005). Bovine viral diarrhea virus strains are recognized as either cytopathic (cp) or noncytopathic (ncp), according to their effect in cell culture (Harding *et al.*, 2002). As the overwhelming majority of BVDV isolates are noncytopathic (90%), infections can easily go unnoticed (Bezek *et al.*, 1994). Cattle persistently infected (PI) with the ncp-BVDV are the main reservoir within the herds and play the most important role in spreading of the disease (Bolin, 1990). There is a predominance of studies showing that the prevalence of PI animals ranges from 0.5% to 2% (Houe, 1999).

Based on genetic and pathogenic properties, the low virulent classical BVDV strains, is present in either genotype and the hyper virulent strains, responsible for the hemorrhagic syndrome, are belong to genotype II (Pellerin *et al.*, 1994; Ridpath and Bolin, 1995). The mechanism of increased virulence with some isolates of BVDV genotype II is currently unknown. However, the ability of BVDV genotype II to cause severe clinical disease and death is due, at least in part, to thrombocytopenia and the resulting hemorrhagic syndrome (Sandvik, 2005).

In Egypt, the sero-prevalence of BVDV infection in the rural localities is 51.8% (Abd El-Hafeiz, *et al.* 2010). Both genotypes were isolated from the different clinical samples and were characterized genetically and antigenically (Abd El-Hafeiz, 2002, 2005; Abd El-Hafeiz *et al.* 2009).

The purpose of this study was to characterize the clinical and virologic features of the mucosal and thrombocytopenic BVDV infection in infected cattle.

MATERIALS and METHODS

BVDV-infection herd-status:

Herd-statuses concerning BVDV-infection were defined based on the physical examination of the herds. Strategy of examination included clinical examination of diseased animals and a possible carry-over effect of BVDV-infection. Pyrexia (rectal temperature 39.4 °C) was observed in the infected cows with bloody diarrhea for 48 hrs and hemorrhage on the mucosal membrane, reduction of the milk production and abortion within the first 3 months of gestation.

Field samples:

Milk samples (n=8) represented clinically diseased cattle were tested against the BVDV. Milk somatic cells from each milk sample were prepared and purified as described by Radwan *et al.* (1995). Briefly, 25 ml of each sample was centrifuged at 1000 *xg*, 4°C for 15 minutes to pellet the somatic cells. The cell pellet was re-suspended in 15 ml phosphate buffered saline (PBS) and centrifuged at 200 *xg*, 4°C for 15 minutes. Second washing of cell pellet was applied in 5 ml PBS and centrifuged at 200 *xg*, 4°C for 15 minutes. The supernatant was removed and the purified milk somatic cells (PMSC) were re-suspended in 0.5 ml of PBS and storied at –70 °C till inoculated on the tissue culture (TC).

Vaginal swabs (n=8) from the hemorrhagic diseased and aborted cattle were collected and transport in transport medium and inoculated on the TC as the standard method.

Serum samples (n=31) were tested against BVDV-antibodies with indirect solid-phase enzyme linked immunosorbant assay (ELISA) techniques.

Virus strain:

An international reference strain (NADL: National Animal Diseases Laboratory) as genotype I was used as positive control on TC. Also, local cytopathic BVDV genotype-II strain (Behera-CP 58/99) that was isolated from the PMSC and identified genetically and antigenically previously (Abd El-Hafeiz, 2002) was used as coating antigen in ELISA technique.

Virus isolation:

The samples were prepared and cultured in Madin Darby bovine kidney (MDBK) cells (tested against latent infection with BVDV and mycoplasma). The inoculated MDBK was cultured with minimum essential medium (MEM; Life Technologies, Grand Island, NY), supplemented with 2% fetal bovine serum (FBS, Biowest, France), penicillin–streptomycin-fungizone as 100 IU, 100 µg and 25 µg per ml medium respectively (Sigma–Aldrich, St. Louis, MO) and incubated under the standard culture conditions (37 °C, 5% CO_2 and 85% RH). The inoculated MDBK was daily examined for the cytopathic effects (CPEs) development along 5-7 days for 3 passages (Schweizer and Peterhans, 1999).

Immunofluorescence antibody (IFA) technique:

From the 3rd passage and after 48 hrs post inoculation (Po.I.) of the 4th passage, the viral agent was identified using immunofluorescence antibody (IFA) technique as outlined by (Bolin *et al.*, 1991). A specific

BVDV type-II monoclonal antibodies (MAbs) against gp53 and Fluorescence isothiocyanate (FITC) conjugated anti bovine IgG (VMRD, INC. Pullman, WA, USA) were used to identify the positive samples that examined by an inverted epifluorescence phase-contrast trinuclear microscope (Nikon ECLIPSE-TS100, Japan) with 20X plan a chromatic lens and a digital camera DS-U2 with NIS elements software.

Immunoperoxidase technique:

As discussed briefly in (Abd El-Hafeiz *et al.*, 2011), cultured MDBK cells on coverslips and 48 hrs Po.I. of samples from the 3rd passage, the inoculated samples were tested against the viral antigen using the specific BVDV type -II MAbs against gp53 (VMRD, INC. Pullman, WA, USA) and horseradish peroxidase (HRP) conjugated anti-bovine IgG (Bethyl laboratories, INC, Germany) at the recommended concentration ($1/10^3$) in PBS pH 7.2. The chromogen, Diaminobenzidine tetrahydrocloride (DAB), was prepared as 5 mg of DAB in 10 ml of 0.05 M Tris-HCl pH 7.4 (6.1 gm Tris-base, 50 ml deionized water and 37 ml of 1N HCl) and filtered through filter paper before addition of 150 µl of freshly prepared 3% H_2O_2. To each well, 100 µl of chromogen was added and incubated at room temperature for 5 minutes before washing the coverslips thoroughly by distilled water and examined using an inverted epifluorescence phase-contrast trinuclear microscope (Nikon ECLIPSE-TS100, Japan) with 10 X plan a chromatic lens and a digital camera DS-U2 with NIS elements software.

Indirect enzyme linked immunosorbant assay (ELISA):

By using the concentrated and purified local cytopathic BVDV genotype-II strain (Behera-CP 58/99) as described by Chu and Zee (1984); Kelling *et al.* (1990) and at antigen concentration 11.2 µg per well, the ELISA test was done that described briefly by Crowther, (2001).

RESULTS

Physical finding:

Nine of the cattle met the criteria for euthanasia during the study period. In the infected herds, appetite remained normal, but diarrhea, characterized by blood and mucosal casts, was a consistent finding in all infected cases. Pyrexia (rectal temperature 39.4 °C), hemorrhage with erosion on mucosal membrane and abortions within the first 3 months of gestation were finding. In post mortem of dead cattle, severe hemorrhage on the internal organs specially the digestive tract with depletion of the lymph nodes.

Virologic and serological findings:

BVDV was isolated from the 8 vaginal swabs and 5 out of the 8 milk samples. All the isolates were ncp that no CPEs were noticed over the 3 passages. By specific BVDV type -II MAbs against gp53, the viral antigen was identified using FITC-conjugated anti-bovine IgG (specific intra cytoplasmic fluoresce granules, Figure 1) and HRP-conjugated anti-bovine IgG (specific intra cytoplasmic brown granules, Figure 2) were detected.

By ELISA technique, all serum samples were positive against the BVDV that had neutralizing antibodies titer ranges from ≥ 1/128 to ≥ 1/512.

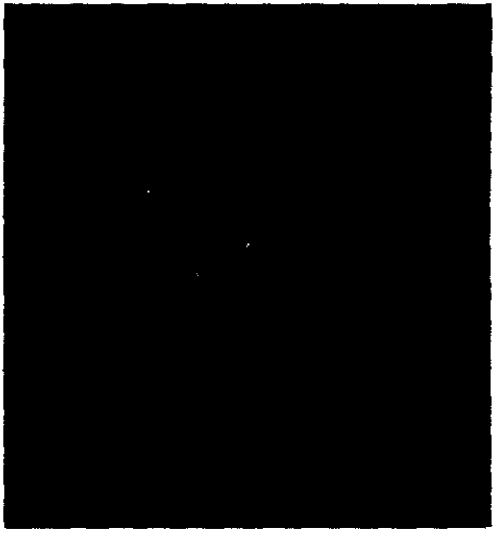

Figure 1: After 48 hrs Po.I. in the 4th passage, the viral agent was identified using a specific BVDV type -II MAb against gp53 and Fluorescence isothiocyanate (FITC) conjugated anti bovine IgG, specific intra cytoplasmic fluoresce granules were observed (X 200).

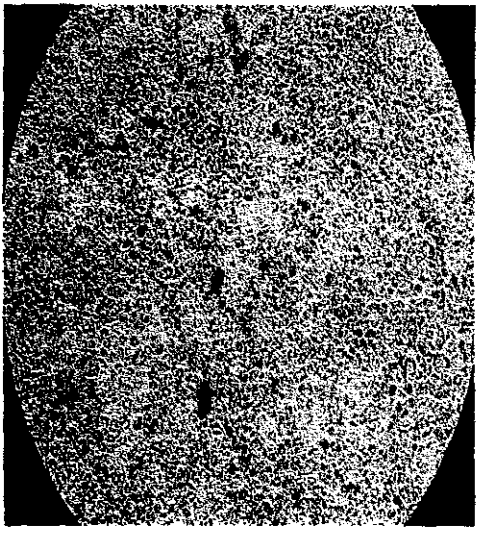

Figure 2: After 48 hrs Po.I. in the 4th passage, the viral agent was identified using a specific BVDV type -II MAb against gp53, HRP conjugated anti-bovine IgG and DAB (Diaminobenzidine tetrahydrocloride) as chromogen, specific intra cytoplasmic brown granules were observed (X 100).

DISCUSSION

Infections with BVDV are endemic in cattle populations in most parts of the world. BVDV-seropositive cattle are allegedly virus free (Houe, 1995). Although bovine viral diarrhea (BVD) can be suspected from the clinical signs, the wide range in both diversity and severity makes them at best unreliable for diagnostic investigations.

Outbreaks of severe, peracute disease in adult cattle and calves were reported in 1993 and 1995, for the first time in the United States and Canada, respectively (Pellerin *et al.*, 1994; Drake *et al.*, 1994; Carman *et al.*, 1998). These outbreaks, which involved beef, dairy and veal operations, were characterized by fever, diarrhea, abortion, leukopenia, thrombocytopenia, and death. Thrombocytopenia, regardless of the underlying etiology, results from only 3 basic mechanisms: decreased production, accelerated destruction, or abnormal sequestration of platelets (Warkentin and Kelton, 1994). The underlying mechanisms are complex, multifactorial, and incompletely understood.

Diagnostic assays that can be scaled up for testing of large number of samples are needed. They need to be as sensitive and specific as possible, but since no test is able to operate at 100% for both parameters, a choice of a deliberate trade-off in either sensitivity or specificity is a more realistic option, and then combined with a different set of back-up assays (Sandvik, 2005).

Except for in special cases, infectious virus is investigated by inoculation of bovine cell cultures. Several virus isolation (VI) protocols have been developed, using different cell culture formats, periods of incubation and serial passage of the inoculum, to meet the demands for different purposes. Virus isolation is an essential back-up and reference test for other indirect methods for identification of BVDV, and should be available to all laboratories using other tests to detect the BVDV indirectly (Sandvik, 2005). Since most field isolates of BVDV are ncp, the inoculated cells are routinely fixed after 3–5 days of the 2-3rd passage on TC and examined for presence of BVDV antigens either by immunofluorescence or immunoperoxidase staining (Anonymous, 2004). Here, BVDV was isolated from the 8 vaginal swabs and 5 out of the 8 milk samples. All the isolates were ncp that no CPEs were noticed over the 3 passages. By specific BVDV type -II MAbs against gp53, the viral antigen was identified using FITC-conjugated anti-bovine IgG (specific intra cytoplasmic fluoresce granules, Figure 1) and HRP-

conjugated anti-bovine IgG (specific intra cytoplasmic brown granules, Figure 2) were detected.

For testing of large series of serum samples, ELISAs have many advantages. They are independent of cell cultures and challenge viruses, give a test result within a few hours, are relatively inexpensive both to establish and run, and are suitable for automation. In principle, three kinds of antigen can be used, which each influence the diagnostic properties of the assay. In the indirect ELISAs, BVDV harvested from infected cell cultures is used, which allows viral nonstructural proteins to be included. Thus, the antibodies assayed will be against the full spectrum of immunogenic proteins encoded by the virus (Beaudeau *et al.*, 2001). In this format, viral antigen is immobilized on the solid phase, onto which specific antibodies and subsequently detecting enzyme-conjugated antiglobulins bind. A positive reaction is recognized by color development in the substrate solution, which is read optically and reported as optical density (OD) values (Tijssen, 1985; Schrijver and Kramps, 1998). In this study, all serum samples were positive against the BVDV that had neutralizing antibodies titer ranges from $\geq$ 1/128 to $\geq$ 1/512.

In conclusion, BVDV type -II do exist in cattle population and the understanding the molecular epidemiology is fundamental. Improved diagnostic and control strategies are essential to reduce losses inflected by BVDVs infection.

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