

A Field Contribution on the Relation Between Reproductive Disorders and Bovine Viral Diarrhea Virus Infection in Buffalo-Cows

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Abstract: A total number of 1850 female buffaloes reared at Lower Egypt was gynecologically examined by ultrasonography and blood samples were collected during a period of four years (2004- 2008). Out of these animals 1262 (68.22%) were suffering from reproductive disorders. The recorded reproductive disorders were ovarian inactivity (56.97%), endometritis (14.10%), delayed puberty (10.30%), mastitis (7.37%), repeat breeding (4.60%), retained placenta (3.25%); abortions (1.51%), vaginal/uterine prolepses (1.03%) and cystic ovaries (0.87%). Bovine viral diarrhea virus (BVDV) was detected by RT-PCR in 46.29% of the total examined samples; the incidence was high in animals having genital disorders (61.60%) as compared with healthy animals (8.0%). Nested RT-PCR indicated that all animals were infected with BVDV-1 genotype. It could be concluded that a tight association is found between the occurrence of reproductive disorders and instantaneous infections with BVD virus.

Key words: BVD • Buffaloes • Reproductive disorders • RT-PCR-Nested RT-PCR

INTRODUCTION

Bovine Virus Diarrhea (BVD) is one of the most imperative worldwide diseases in domestic and wild ruminants. It causes substantial damage in infected herds as well as an extensive consequence on animal health and productivity in dairy cattle [1].

The disease is caused by Bovine Virus Diarrhea Virus (BVDV), which belongs to the genus Pestivirus of the family Flaviviridae. The genome of BVDV is a single stranded (+) RNA of about 12.5 kb in length without a poly (A) tail [2- 4]. BVDV isolates comprise 2 different genotypes; BVDV-1 and BVDV-2, both may infect cattle, sheep and less frequently, also other ruminants and pigs [5]. This virus shared antigenicity with border disease and classical swine fever [6].

Infections with BVDV have different consequences, such as drop in milk yield, fertility problems, immunosuppression, diarrhoea, thrombocytopenia and frequently, inapparent courses [7-9]. The severity of clinical affects depends upon the immune status of the host, strain of the virus, reproductive status and environmental stress [9,10].

Reproductive losses usually occur in BVD infected animals as a result of ovarian dysfunction [11], anoestrus [1], retained placenta [12], endometritis [13], repeat breeding [14], reduced conception rate [15] and direct damage to the embryo [16]. Also, BVD caused obvious decrease in ova/embryo recovery, partial or complete absence of the preovulatory LH surge, testicular lesions and poor semen quality. The semen produced by persistently infected bulls may contain virions that can be transmitted either by natural or artificial insemination [17,18]. In pregnant animals, transplacental infection can lead to abortion, stillbirth, malformation and persistent infection of the offspring. Persistently infected animals shed the virus for the rest of their life and play a key role in the epidemiology of infection [1,19,20].

Serological procedures and virus isolation are the preferred methods for diagnosis of BVDV infected animals. However, negative serological results must be interpreted with caution since persistently infected animals have no neutralizing antibodies or antibodies only at low titers and may appear healthy. The detection of BVDV-1 by PCR amplification is an alternative to conventional tests. RT-PCR method is being used for

detection of viral nucleic acid as a specified method which is fast enough to detect the virus at a maximum of 8 h [20].

This investigation intended to correlate between the occurrence of reproductive disorders and infection with BVDV in female buffaloes. First BVDV was detected by PCR amplification of the viral RNA using RT-PCR as it would allow rapid identification of BVDV. Then nested-PCR was conducted to detect infection by BVDV-1 since this genotype has main effect on reproductive system in an attempt to diagnose and help in the elimination of this disease.

MATERIALS AND METHODS

Animals: A total number of 1850 female buffaloes (2-9 years), reared at Lower Egypt was used to execute this investigation throughout a period of 4 consecutive years (2004-2008). These animals were kept in smallholder farms and fed mainly on green fodder (*Trifolium alexandrinum*) with an inadequate amount of concentrate mixture and no regular system of vaccination or veterinary intervention.

A complete data on case history, owners complain, clinical examination and reproductive status were recorded for each animal. All buffalo-cows were subjected to rectal examinations using Ultra sound apparatus (Pia Medical Falcs e'Saote, the Netherlands) with an endorectal linear array 6-8 MHz transducer and reproductive status and/or disorders were recorded.

Blood sampling: Two blood samples were collected through the jugular vein puncture from each of examined buffaloes; a sample was collected on EDTA as anticoagulant to obtain whole blood sample. The other sample was collected without EDTA, kept to clot, centrifuged at x1500g for 20 minutes, sera were separated and kept at -20°C until analyzed. Sera were screened for brucellosis using Rose Bengal test [21].

Virological Tests: A total number of 175 random representative samples was used for virological examination. A similar volume of ficol (Sigma, ST. Louis, MO, USA) was added to the whole blood sample, centrifuged at 1500 rpm for 15 minutes and the buffy coat was collected [22].

Virus isolation and propagation: The virus was isolated in 12-well flat-bottomed micro plates previously cultured

by Madin Darby Bovine Kidney cells (MDBK cells, VACSERA, Agoza, Egypt). The virus was then titrated in 96-well tissue culture plate where MDBK cells were added to all the wells of the micro plates (100 µl per well, 3×10^5 cells per ml) and observed daily until confluency. Then, 50 µl per well of pooled serum samples or tissue suspensions sequential 10-fold dilutions were added to eight wells per dilution. The plates were cultured for 3 days at 37°C in a humid atmosphere containing 5% CO₂. The plate was frozen and thawed three times and the contained tissue and fluid were centrifuged at 5000 rpm for 5 minutes and the supernatant fluid was used for RT-PCR. The method of isolation and identification was done as outlined by [20,23].

Rapid RT-PCR: Rapid RT-PCR was performed on both buffy coat and virus isolated from tissue culture of a titer 10^3 and also from the isolated buffy coats directly using a special kit (One -Step RT-PCR, QIAGEN, GmbH, Germany). The primers Oligonucleotide primers from 5'UTR of virus genome (PF1 5'-ATGCCCTTAGTAGGACTAGC-3' and PR1 5'-ACTCCATGTGCCATGTACAG-3') were used for RT reaction and subsequent PCR amplification [24].

Nested RT- PCR (nRT-PCR): In order to specifically amplify a part of the 5'UTR of the virus genome, oligonucleotide primers to be used in nested-PCR were chosen from sequence data of the PCR product. The primers used in this study were Pesti-N-F 5'-GATGGCTTAAGCCCTGAGTA-3' and Pesti-N-R 5'-GCTGTATTCGTAACAGTCGG-3' [24]. After completion of the PCR cycle, 5 ml of the RT-nPCR products were separated by 1.5% agarose gel electrophoresis. Ethidium bromide staining allowed visualization of the RT-nPCR product using an ultraviolet transilluminator.

The incidence of detection of BVD virus in female buffaloes either in apparent healthy animals or in those having reproductive disorders was recorded. Also, the prevailing strain of the virus was detected by nested RT-PCR.

RESULTS

Incidence of reproductive disorders: Out of 1850 female buffaloes, examined in Lower Egypt during a period of 4years (2004-2008), 1262 (68.22%) suffered from reproductive disorders (Table 1). The main recorded reproductive disorders were ovarian inactivity (56.97%),



Fig. 1: Agarose gel electrophoresis of the amplification RT-PCR products generated from buffalo-cow using primers specific for BVDV for rapid diagnosis



Fig. 2: Agarose gel electrophoresis of the amplification nRT-PCR products generated from buffalo-cow using primers specific for BVDV

Table 1: Incidence of reproductive disorders in examined female buffaloes (%)

Animals	No.	Percent
Healthy	588	31.78
Reproductive disorders	1262	68.22
Ovarian inactivity	719	56.97
Endometritis	178	14.10
Delayed puberty	130	10.30
Mastitis	93	7.37
Typical Repeat breeders	58	4.60
Retained placenta	41	3.25
Abortions	19	1.51
Vaginal/uterine prolapse	13	1.03
Cystic ovaries	11	0.87
Total number	1850	100.00

Table 2: Incidence of BVD in female buffaloes in relation to reproductive disorders (%)

Animals	No. of examined animals	BVD negative		BVD positive	
		No.	%	No.	%
Healthy	50	46	92.00	4	8.00
Reproductive disorders	125	48	38.40	77	61.60
Ovarian inactivity	35	13	37.14	22	62.86
Endometritis	27	13	48.15	14	51.85
Delayed puberty	15	6	40.00	9	60.00
Mastitis	10	7	70.00	3	30.00
Typical Repeat breeders	12	5	41.67	7	58.33
Retained placenta	8	3	37.50	5	62.50
Abortions	6	2	33.33	4	66.67
Vaginal/uterine prolapse	6	5	83.33	1	16.67
Cystic ovaries	6	3	50.00	2	33.33
Total number	175	94	53.71	81	46.29

endometritis (14.10%), delayed puberty (10.30%), mastitis (7.37%), repeat breeding (4.60%), retained placenta (3.25%), abortions (2.3%). However, Rose Bengal test indicated that all of these animals were negative reactors for brucellosis.

Prevalence of BVDV infection: Examination of 175 representative random samples using RT-PCR for detection of BVD virus demonstrated a band at 294 bp (Fig.1) in 46.29% of the total examined samples (Table 2). The incidence of BVDV infection was higher in female buffaloes suffering from reproductive disorders (61.60%) as compared with healthy animals (8.0%). Moreover, nested RT-PCR demonstrated the presence of 160 bp band (Fig. 2) characteristic for infection with BVDV-1 genotype of the virus.

Prevalence of BVD in relation to reproductive disorders: The percentage of buffaloes with reproductive disorders and simultaneously positive for BVD was 61.60%. Meanwhile, the percentage in buffaloes showing no reproductive disorders was 8.0% (Table 2). The incidence(%) was high in animals suffering from ovarian inactivity (62.86), Endometritis (51.85), delayed puberty (60.0), typical repeat breeders (58.33), retained placenta (62.50) and abortion (66.67) and was low in animal having mastitis (39.78), vaginal/uterine prolapsed (16.67) and cystic ovaries (33.33).

DISCUSSION

Out of the world 160 million heads of buffaloes, 3.9 million heads are found in Egypt and produced 65% of meat and milk used by local population. Despite these animals are bred under harsh socioeconomic conditions [25].

In the current study, case history, owner complain, clinical examination and ultrasonographic scanning of the genital organs of 1850 female buffaloes, revealed that 68.22% suffering from reproductive disorders; including ovarian inactivity (56.97%), endometritis (14.10%), delayed puberty (10.30%), mastitis (7.37%), repeat breeding (4.60%), retained placenta (3.25%), abortions (1.51%), vaginal/uterine prolapse (1.03%) and cystic ovaries (0.87%). In this respect, it was reported that buffaloes suffering from a lot of problems, mainly reproductive disorders which cause great economic losses, especially in animals kept in small holder farms and exposed to stressful conditions such as mal-nutrition, bad hygiene, parasitism and pollution [25]. However, it was not aimed here to discuss these problems, but intention was directed to investigate the possible correlation between the occurrence of these disorders and infection with BVDV in buffaloes.

BVDV causes obvious economic losses in cattle worldwide and has recently been targeted for eradication in several national programs. In addition to gastrointestinal disease, BVDV causes reproductive and respiratory disorders and persistent infections [1,26]. Because carriers are constantly viremic and continually shed and maintain the virus in the environment, their identification and removal from the herd is an essential component of programs for the control and eradication of BVDV [27].

In the present investigation, BVDV was detected by RT-PCR in 46.29% of the total examined samples; the incidence was high in animals having genital disorders (61.60%) as compared with healthy animals (8.0%). In buffaloes, [28] demonstrated prevalence of anti-BVDV-antibodies in 52% of examined samples. However, [9] and [29] demonstrated the prevalence of anti-BVDV antibodies in 24.67 and 12.50% by ELISA and 25.97 and 25.0% by SNT, respectively.

Detection of BVDV in animals is based on virus isolation or by measuring specific antibodies using complement fixation test, ELISA, immunoperoxidase and immunofluorescence assays [30]. Molecular techniques

have also been used to detect the presence of virus in blood and serum samples [7, 31]. Comparison of RT-PCR with ELISA and cell culture immunoperoxidase tests for the detection of ruminant pestivirus infections revealed that RT-PCR is more sensitive than the other tests [32].

In this study, nRT- PCR which can be used to type BVDV from infected cell cultures and blood as well as the buffy coat was described. Previously, several PCR-based assays for typing BVDV were reported [33]. Typing with one of these assays, however, was indirect and involved restriction endonuclease digestion of PCR products for typing[34]. A second assay used the specific amplification of BVDV2 for typing, with a negative result indicating BVDV1 [26].

Buffaloes in this study were not vaccinated against BVD, negative for brucellosis and showed no symptoms of disease at the time of examination. However, in similar cases in non vaccinated cows, BVDV was present in 50.9-79.2% of the cases [35-38]. The immunosuppressive effect of BVD virus may be a triggering factor for activation of other diseases in latently infected animals [1, 38].

Nested-PCR was used herein and it indicated the presence of BVDV-1 genotype in the present infected female buffaloes. In this respect, it was reported that this technique is a sensitive method and it could be used for detection of serotype of BVDV in bovine [39].

In this study, a clear association was observed between the increased incidences of BVD infections and the occurrence of reproductive disorders in the examined buffaloes. In cattle, field and epidemiological studies revealed that BVDV has an adverse impact on reproductive performance in the form of infertility, significant drop in conception rate, abortions, endometritis, repeat breeding as well as testicular lesions and poor semen quality [16,18,40-42]. Also, [1,9] added that anoestrus, repeat breeding and abortion are the main reproductive disorder in BVD positive buffaloes. Early reproductive losses occurred as a result of ovarian dysfunction [18], endometritis [13] and direct damage to the embryo [16, 43].

The existence of apparently healthy animals persistently infected with BVDV emphasizes the need for rigorous screening of all animals to identify the animals carrying the infectious agent. Serological procedures and virus isolation are the preferred methods

for diagnosis of BVDV infected animals. However, negative serological results must be interpreted with caution since persistently infected animals have no neutralizing antibodies or antibodies only at low titers and may appear healthy with acceptable semen quality. The detection of BVDV-1 by PCR amplification is an alternative to conventional tests. RT-PCR method is being used for detection of viral nucleic acid as a specified method which is fast enough to detect the virus at a maximum of 8 h [20].

It could be concluded that a tight association is found between the occurrence of reproductive disorders and instantaneous infections with BVD-1 virus in buffaloes. Rigorous screening of all animals using recent technique is required to identify persistently infected animals using an accurate techniques as RT-PCR and nRT-PCR.

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