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MOLECULAR DETECTION OF BOVINE VIRAL DIARRHEA VIRUS IN WHOLE BLOOD AND ORAL SWABS IN CATTLE AND BUFFALOES

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ABSTRACT

Bovine viral diarrhea (BVD), a common viral disease, can affect large ruminants, both domesticated and wild. In Assiut Governorate, there is little information about BVD despite their financial losses. Thus, the aim of this investigation was to study the current situation of BVDV infection among large ruminants in Assiut Governorate. In this study, 39 cattle and 11 buffaloes were obtained from various villages in the Assiut Governorate, Egypt. Whole blood and oral lesions swabs were collected for laboratory investigation using RT-PCR. Nine cattle and one buffalo were found to have BVDV-RNA. The clinical findings accompanied by BVDV infection in examined animals were fully discussed. The species, age, and sex of the animals that underwent molecular testing had no significant effect on the BVDV infection rate ($P<0.05$). Meanwhile, Assiut Governorate's climatic conditions were found to have a significant influence ($P<0.001$) on the BVD infection rate, in which the prevalence of BVDV infection was highest in the summer season. Emphasizing the importance of putting effective prevention and control measures in place throughout Egypt is recommended in order to minimize the prevalence of BVDV.

Keywords: BVD, whole blood, oral swabs, RT-PCR, Risk factors, Assiut

INTRODUCTION

Bovine viral diarrhea (BVD) is one of the most dangerous diseases in the world that spreads widely and causes large financial losses to the livestock sector (Tian *et al.,* 2021). BVD is caused by the BVD virus (BVDV) that belongs to the genus *Pestivirus* within family *Flaviviridae* (Chang *et al.,* 2021). An 11.3–13.1 kb single-stranded positive-sense RNA is present in pestiviruses that are enveloped viruses (Porto *et al.,* 2021). Genus *Pestivirus* includes several important genotypes such as BVDV-1 (*Pestivirus* A), BVDV-2 (*Pestivirus* B), classical swine fever (*Pestivirus* C), border disease (*Pestivirus* D), and HoBi-like (*Pestivirus* H) (Afify *et al.,* 2022). The BVDV genome has a lengthy open reading frame (ORF) that is surrounded by a highly conserved untranslated region (UTR) for replication and translation controls at the 5' and 3' termini, respectively

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(Abd El-Hafeiz *et al.,* 2022). The BVDV's cytopathic (Cp) and non-cytopathic (NCp) biotypes can be identified by their genetic diversity and in vitro cell culture features (Zhang *et al.,* 2022). When cattle or buffaloes are infected with BVDV, they may suffer from thrombocytopenia, immuno-suppression with subsequent infections, respiratory problems, gastrointestinal troubles, and reproductive failure (Al-Rubaye and Hasso, 2012; Kučer *et al.,* 2022). Several endemic countries, in particular Egypt, have initiated BVDV control or eradication programs due to the economic consequences of BVD (Atwa *et al.,* 2019). In 1972, BVDV was initially discovered in Egypt in a calf that had acute enteritis (Hafez, 1972). Most Egyptian BVDV studies depend mainly on the detection of viral antibodies and/or virus isolation (Soltan *et al.,* 2015). Reverse transcriptase polymerase chain reaction (RT-PCR) has been demonstrated to be a simpler and faster way to detect BVDV than virus isolation, with great specificity and sensitivity (Youssef *et al.,* 2023). BVD is not widely recognized in Assiut Governorate, so the current investigation aimed to molecularly detect an extremely preserved region of the BVDV genome's 5' UTR in whole blood and oral lesion swabs using RT-PCR and investigate the relation-ship between certain risk variables, including species, sex, age, seasonal fluctuation, and BVD infection. Additionally evaluating the results of whole blood versus oral swabs samples in detection of the viral RNA.

MATERIALS AND METHODS

1. Ethical approval

All animals that were employed in this investigation were dealt ethically by the Research Ethical Committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, which approved the study 06/2024/0253.

2. Animals

A total of 39 cattle and 11 buffaloes of all ages and sexes from various villages in Assiut Governorate were admitted to the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University, between September 2023 and July 2024. A clinical evaluation of the diseased cattle and buffaloes was performed in compliance with Jackson and Cockcroft (2002). The animals under study showed a range of signs, with varying degrees of severity, suggesting that they may be infected with BVDV. The commonly noticed signs were fever, oral lesions, diarrhea, respiratory signs (cough and nasal discharge), swelling of superficial lymph nodes, corneal opacity, and skin exanthema. Samples of oral swabs and whole blood were obtained from each diseased animal.

3. Sampling

Using a sterile cotton swab soaked in phosphate buffer saline (PBS, pH 7.4), oral swabs were obtained from oral lesions in each diseased cattle and buffalo. Additionally, 2 ml of whole blood were collected from the jugular vein into sterile vacutainer tubes containing ethylene diamine tetra acetic acid (EDTA). These samples were kept at -20°C in order to extract the RNA later.

4. Molecular testing

4.1. Viral RNA extraction

Fifty samples from both whole blood and oral swabs were utilized to extract viral RNA using the EasyPure® Simple Viral DNA/RNA extraction kit (TransGen Biotech, China), following the directions provided by the producer.

4.2. Primers

The current investigation assessed the specificities of the primers (Metabion International AG, Germany), which were selected for the 5'UTR of BVDV based on previous study (Weinstock *et al.,* 2001). Table 1 shows the primer sequences and their locations within the viral genome.

Table 1: The nucleotide sequences, locations of the primers utilized in the detection BVDV genome's 5' UTR region, and the sizes of the products obtained via RT-PCR

Primer	Nucleotide sequences	Position 5' - 3'	Product size bp
Forward: 103	5'- TAG CCA TGC CCT TAG TAG GAC -3'	103 - 124	290
Reverse: 372	5'- ACT CCA TGT GCC ATG TAC AGC -3'	372 - 392	

4.3. RT-PCR to identify the BVDV 5'UTR region

Potential of a particular RT-PCR to amplify the BVDV 5'UTR's highly conserved region. With the ABT 2X RT mix kit (Applied Biotechnology, Egypt), the extracted RNA was reverse transcribed to cDNA in accordance with the manufacturer's guidelines, and it was stored at -20°C until it was required. cDNA fragments of 290 bp length were amplified using primer sets 103 forward and 372 reverse. DNTPs and polymerase enzyme were obtained from the ABT red master mix (2X) (Applied Biotechnology, Egypt) for the present work. PCR was conducted using a PCR thermocycler (Peqlab, Germany), and the subsequent reagents were utilized: a final volume of 16 µl that includes 8 µl ABT red master mix (2X), 1 µl of each primer 103 and 372 (10 pmol), 3 µl cDNA sample, and 3 µl RNase-free water. The thermal cycling settings were, in short, a 5-minute initial denaturation at 95°C (40 cycles of denaturation at 94°C for 1 minute, 59°C for 1 minute for the annealing phase, and 72°C for 1 minute for extension), and a final 10 minute extension at 72°C.

4.4. PCR product evaluation and identification

Seven microliters of amplified PCR products were loaded for reaction observation. Before being examined using a gel UV transilluminator (Syngene, United Kingdom), the amplicons were subjected to a 60-minute gel electrophoresis procedure at 90 V and 155 mA in a 1.5% agarose gel with ethidium bromide (10 mg/ml) staining. The size of the amplicons was assessed using size ladder DNA of 100 bp.

5. Statistical evaluation

The molecular and epidemiological results were obtained and analyzed using the Chi-square of independence (2007) utilizing the statistical package for the social sciences (SPSS) version 16 software.

RESULTS

1. Prevalence of BVDV in investigated animals by RT-PCR

cDNA samples were subjected to a PCR in order to produce the required bands at 290 bp due to the BVDV 5'UTR (Figure 1). The prevalence of BVD infection in diseased animals was shown in Table (2).

Table 2: Prevalence of BVD infection in investigated animals by RT-PCR.

Animal	No. of examined animals	RT-PCR		P-value
		Positive No. (%)	Negative No. (%)	
Cattle	39	9 (23.08)	30 (76.92)	0.3
Buffalo	11	1 (9.09)	10 (90.91)	
Total	**50**	**10 (20)**	**40 (80)**	

No significant variation at $p<0.05$

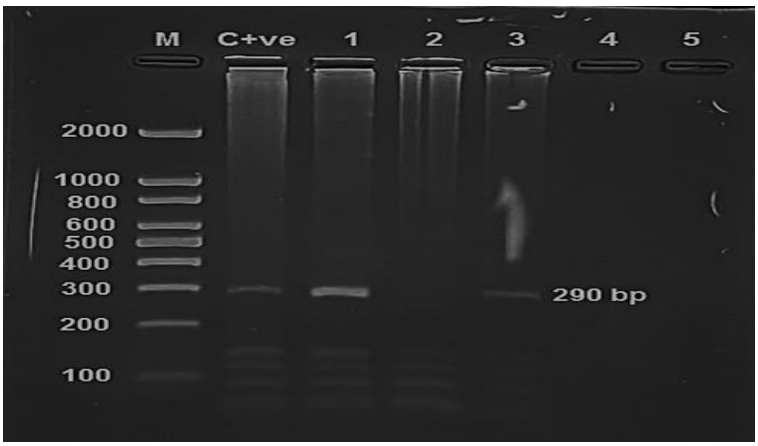

Figure 1: The electrophoresis of agarose gel of RT-PCR following 5'UTR of BVDV amplification in diseased animals. Lane M: DNA ladder 100 bp, lane C+ve: Control positive sample; lanes 1and 3: Positive samples and lanes 2,4 and 5: Negative samples.

2. Clinical findings of examined cases

The recorded clinical signs in the studied cases were summarized in Table 3. Animals infected with BVD in this study suffered oral lesions (erosions and ulcers in gum, palate, papillae, and tongue), beside ulcers in commissure, muzzle, and nostrils, fever, diarrhea, respiratory signs, and enlarged superficial lymph nodes in some cases.

Table 3: Clinical signs of BVD in studied animals.

Clinical findings	No. of assessed diseased animals	BVD	
		Positive No. (%)	Negative No. (%)
Gum lesions and diarrhea	5	1 (20%)	4 (80%)
Gum lesions and fever	10	2 (20%)	8 (80%)
Gum lesions, diarrhea and fever	6	0 (0%)	6 (100%)
Gum, commissure, muzzle & nostrils lesions, respiratory signs and fever	4	2 (50%)	2 (50%)
Gum lesions, respiratory signs, fever and enlarged superficial lymph node	9	2 (22.22%)	7 (77.78%)
Gum lesions, respiratory signs, fever and corneal opacity	3	0 (0%)	3 (100%)
Gum lesions, diarrhea, respiratory signs, fever and enlarged superficial lymph node	3	0 (0%)	3 (100%)
Gum lesions, respiratory signs, fever, enlarged superficial lymph node and lameness	1	0 (0%)	1 (100%)
Gum and papillae lesions, respiratory signs and enlarged superficial lymph node	3	1 (33.33%)	2 (66.67%)
Gum, palate, muzzle & nostrils lesions, diarrhea, respiratory signs, fever, corneal opacity and skin exanthema	3	0 (0%)	3 (100%)
Tongue & palate lesions, respiratory signs and fever	3	2 (66.67%)	1 (33.33%)
Total	50	10 (20%)	40 (80%)

3. Potential risk factors

This study was dealt with some risk factors such as age and sex susceptibility and seasonal variation that influence the prevalence of BVD infection (Table 4). The age and sex of the infected animals with BVD had no significant effect on the infection rate, while the prevalence of BVDV infection was significantly highest in the summer season (Table 4).

Table 4: Association between BVD infection in examined diseased animals and possible risk factors based on findings of RT-PCR.

Variant		No. of animals investigated	RT-PCR		P-value
			Positive No. (%)	Negative No. (%)	
Age	3 months-1.5 year	16	6 (37.50)	10 (62.50)	0.1
	>1.5-3 years	20	2 (10)	18 (90)	
	>3 - 5years	14	2 (14.29)	12 (85.71)	
	Total	**50**	**10 (20)**	**40 (80)**	
Sex	Male	20	6 (30)	14 (70)	0.15
	Female	30	4 (13.33)	26 (86.67)	
	Total	**50**	**10 (20)**	**40 (80)**	
Season	Summer	7	6 (85.71)**	1 (14.29)	0.000
	Autumn	22	1 (4.55)	21 (95.45)	
	Winter	14	1 (7.14)	13 (92.86)	
	Spring	7	2 (28.57)	5 (71.43)	
	Total	**50**	**10 (20)**	**40 (80)**	

No significant change at $p<0.05$ **Highly significant increase at $p<0.001$ (0.000).

4. Comparison between results of whole blood and oral swabs samples in infected animals with BVD.

The findings indicated that oral swab samples were superior, as viral RNA was identified in 10 animals, whereas only one animal tested positive using whole blood samples, which also yielded a positive result with oral swabs (Table 5).

Table 5: Comparison between whole blood and oral swabs samples results in detection of BVD infection in examined animals.

		Whole blood		Total
		Positive	Negative	
Oral swab	Positive	1	9	10
	Negative	0	40	40
Total		1	49	50

DISCUSSION

BVD infection is among a number of diseases that pose a serious risk to animal producers (Abd El-Hafeiz *et al.*, 2022). By employing RT-PCR, the prevalence of BVD was established at 23.08% (9/39) in cattle, where in buffaloes the prevalence was 9.09% (1/11) with no statistically significant difference between the studied species. Our result concurred with Dehkordi (2011); Soltan *et al.* (2015) and Sharawi *et al.* (2016), who concluded that there was no discernible variation in BVD infection by species of examined animals. Our result would imply that cattle and buffaloes are equally prone to BVD infection. The 5'UTR was found in 10 (20%) of the 50 examined animals that were molecularly identified as suffering from BVDV infection in Assiut Governorate. A lower prevalence of

BVDV infection was observed by El-Bagoury *et al.* (2014); Soltan *et al.* (2015) and Youssef *et al.* (2023), who recorded that 8.4%, 10.4%, and 14% of the studied animals detected BVDV infection in Qaluobia, Ismailia, and Assiut Egypt, correspondingly. On the other hand, a greater infection rate was discovered by Atwa *et al.* (2014), who reported that 23.08% of investigated animals had BVDV infection in Damietta, Egypt. The differences in BVDV infection prevalence rates between previous studies may be caused by variations in sample numbers, sanitary environments, ambient circumstances, and the use of different diagnostic procedures.

Clinical signs, including oral lesions [ulcers in gum, papillae, palate, and tongue], commissure, muzzle, and nostril lesions, diarrhea, respiratory signs [nasal discharge and cough], fever, and swelling of peripheral lymph nodes, linked to suspected clinical cases of BVD in the current study were identical to those described in previous investigations published by Al-Rubaye and Hasso (2012); Soltan *et al.* (2015); Atwa *et al.* (2019); Kučer *et al.* (2022) and Youssef *et al.* (2023).

Some risk factors were explored for their interaction with the BVDV infection rate, including age and sex of examined animals and seasonal variations. The information obtained indicates that not every one of these aspects might have a substantial effect on the BVDV infection rate, even though it is still necessary to investigate the precise ways in which these factors interact biologically with BVDV infection (Youssef *et al.*, 2023). Concerning age susceptibility, the rate of BVDV infection did not differ statistically significantly among the examined age groups of the animals under investigation. This outcome supported the findings of Youssef *et al.* (2023), who reported that the prevalence of BVDV infection was diagnosed in animals at the ages of 3 months-1 year, >1-3 years, and >3-5 years with no significant variation. Our findings suggest that the animals under study are similarly vulnerable to contracting BVD. Regarding sex susceptibility, the rate of BVDV infection did not differ statistically significantly between male and female studied animals. Our findings were consistent with those of Wilson *et al.* (2016) and Youssef *et al.* (2023), who found no discernible variations in BVDV infection rates by animal sex. Our findings may suggest that both male and female animals are equally prone to BVD, which is a non-sex-related disease; however, this is dependent on a variety of ecological and handling circumstances; animals of both sexes may contract BVD at different times in their lives. These variables include the level of stress and viral exposure (Youssef *et al.*, 2023). Studying seasonal variations and the frequency of BVDV infection, there was a significant increase in the prevalence of BVDV infection in the summer season (85.71%) compared to the autumn (4.55%), winter (7.14%), and spring (28.57%) seasons. Our results were in accordance with those of Atwa *et al.* (2012), who observed that the summer season had a significantly higher prevalence of BVDV infection (93.7%) than the spring, winter, and autumn seasons (50%). The reason for our results could be explained by BVDV's increased ability to survive in warmer climates (Youssef *et al.*, 2023).

Nine diseased animals exhibited BVDV infection in oral swab samples exclusively, while one case had BVDV infection in both whole blood and oral swab samples. This finding indicates the superiority of oral lesion samples over whole blood samples to detect BVD infection in animals.

CONCLUSION

The current investigation found that cattle and buffaloes in Assiut Governorate, Assiut, Egypt, were infected with BVDV. Oral swabs are better than whole blood samples for diagnosis of BVD. These findings have an impact on the economy and emphasize the need for effective prevention and control measures to be put in place across Egypt to reduce the frequency of BVD. Furthermore, laboratory testing is required to validate any clinical suspicions of BVD in order to rule out diseases that share clinical similarities with BVD, such as foot-and-mouth disease, vesicular stomatitis, blue tongue, malignant catarrhal fever, bovine papular stomatitis, and infectious bovine rhinotracheitis.

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الكشف الجزيئي لفيروس الإسهال الفيروسي البقري في الدم الكامل والمسحات الفموية في الأبقار والجاموس

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الإسهال الفيروسي البقري (BVD)، مرض فيروسي شائع، يمكن أن يؤثر على المجترات الكبيرة، سواء المستأنسة أو البرية. هناك القليل من المعلومات عن مرض الإسهال الفيروسي البقري على الرغم من خسائره الاقتصادية في محافظة أسيوط. وبالتالي، كان الهدف من هذا البحث دراسة حالة الإصابة بفيروس الإسهال الفيروسي البقرى بين المجترات الكبيرة في محافظة أسيوط حيث تم الحصول على عينات من ٣٩ رأسًا من الأبقار و١١ جاموسة من قرى مختلفة من محافظة أسيوط، مصر. تمثلت العينات من الدم ومسحات من الآفات الفموية لإجراء الفحص المعملي باستخدام تفاعل البلمرة المتسلسل العكسي. تم اكتشاف الحمض النووي الريبوزي لفيروس الإسهال الفيروسي البقري في تسعة أبقار وجاموسة واحدة. تمت مناقشة النتائج الاكلينيكية المصاحبة للإصابة بفيروس الإسهال الفيروسي البقري في الحيوانات التي تم فحصها بشكل كامل. عند دراسة مدى تأثر معدل الإصابة بفيروس الإسهال الفيروسي البقري بنوع وعمر وجنس الحيوانات التي خضعت للاختبار الجزيئي، لم يكن هناك فرق كبير (P <٠,٠٥). في الوقت نفسه وجد أن الظروف المناخية لمحافظة أسيوط لها تأثير معنوي (P<٠,٠٠١) على معدل الإصابة بفيروس الإسهال الفيروسي البقري حيث كان معدل انتشار عدوى بفيروس الإسهال الفيروسي البقري أعلى في فصل الصيف. يوصى بالتشديد على أهمية وضع تدابير فعالة للوقاية والسيطرة في جميع أنحاء مصر من أجل تقليل انتشار فيروس الإسهال الفيروسي البقري.