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Evidence of bovine viral diarrhea virus in buffaloes (*Bubalus bubalis*) in Nineveh province

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Abstract

Investigating the sources of Pestivirus, family Flaviviridae, and bovine viral diarrhea virus (BVDV) infections is important in the epidemiology and control efficacy of this disease in large and small ruminants. This work is a preliminary attempt to investigate the prevalence of BVDV in buffalo by conducting a reverse transcriptase polymerase chain reaction in Nineveh province, Iraq. From November 2024 to the end of January 2025, a total of 158 blood samples were collected from buffaloes in 7 farms in various areas of Nineveh province, and from both sexes, different management, sole and interspecies rear, and no vaccination background against the disease. The molecular outcome objective 5' UTR gene region revealed a 7.59% total prevalence of BVDV, with a significantly higher prevalence of BVDV in buffaloes aged more than 5 years. Further, no statistical variability among buffaloes' sex, management, and study areas. Six procuring sequences in the current work are bookmarked in the GenBank database under identity numbers. Furthermore, the local sequences referenced were similar to those of Iran, Iraq, Mexico, China, and the USA (96.07%-100% identity). In conclusion, the bovine viral diarrhea virus was recorded in this work for the first time in Nineveh province, although the disease was reported earlier in cattle and sheep. Attention should be given to the role of buffalo in transmission and maintaining the virus. Hence, precise detection and differentiation of Pestivirus are crucial requirements for the elimination and evacuation efforts of BVDV in the animal population.

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Introduction

In bovine livestock, bovine viral diarrhea virus (BVDV) is one of the most predominant infectious diseases with obvious financial downsides and diverse clinical aspects. It is undergoing eradication programs worldwide (1,2). The virus is mostly infecting ruminants, including buffaloes, with a broad framework of domestic and wildlife animal populations (3,4). There are 2 known sorts of BVDV in terms of BVD1 and BVD2. Moreover, the HoBi-like emerging Pestivirus is being estimated as the third species, or BVD3, either through sharing 75% and 80% identity with BVD 1 and 2 genomes, furthermore displaying analogous symptoms in infected hosts (5,6). Based on the question of injury in cell culture, the virus is separated into cytopathic or noncytopathic biotypes (7-10). Both biotypes are able to manifest transient infections, which are characterized mostly by asymptomatic or temperate clinical disease signs of erosions of mucosal tissues, pneumonia, hemorrhagic enteritis, abortion, loss of fertility in females, and congenital deformity of fetuses (11). It is important to point out that the ability of noncytopathic strains to set up lifelong shedding immunotolerant persistently infected PI animals in early gestation is the main etiology of high prevalence recording of BVDV in many countries (12). Additionally, these PI animals are eligible for vertical transmission and are accountable for preserving circulation. Its reproduction and distribution are related to several circumstances, including

the mode of transmission, the immune condition, and the zone factor (13). Various regions in the BVDV genome have participated in the identification, differentiation, and study of the viral genetic diversity, such as the 5'untranslated region glycoprotein E2, Npro autoprotease, and NS3 protease (14,15). Depending on the nucleotide sequence, there were 21 subgenotypes of BVDV1 (a to u) and 4 subgenotypes of BVDV2 (A to B) referenced, with the non-cytopathic being the more common biotype in the animal globally (16,17). It has been known that the disparity is due to the subgenotypes not linked to the spectrum of manifestations but the differences in monoclonal antibody binding (18). Hence, the genetic diversity of the virus is not only a scientific concern but also a functional subscription for epidemiology, diagnosis, and vaccine preparation (19). Buffaloes (Bubalus bubalis) are domesticated bovines in most countries around the world (20,21). Buffaloes are also reared with cattle, which could be a potential risk problem when the pathogenic agents can be transmitted between both species. For this, accurate epidemiological monitoring of infectious diseases within buffalo herds is required (22).

Therefore, due to the absence of prior reports of the status of the disease in the study area, the aim of this study was to evaluate the molecular prevalence of BVDV in buffaloes in Nineveh province, Iraq.

Materials and methods

Ethical approve

Permission for this work has been obtained from the Institutional Animal Care and Use Committee UM.VET.2024.095.

Animal information and sampling

In this survey, 7 private local buffalo farms from three regions (Hawey-Alkaneesah, Al-Rahmaneyah, and Gogjalee) in Nineveh province were included. The numbers of animals in the flocks ranged from small herds (n= ≤12) and large herds (n= ≥ 30), both genders and ages from ≤ 2 years to ≥ 5 years. Some of these farms were interspecies structures with buffaloes, indoor and outdoor, reared for milk and meat production purposes. All farms had no history of protection against any Pestivirus vaccine. A total of 158 buffalo blood specimens under strict septic conditions were collected in sterile test tube gel for obtaining the serum that was further stored at -20°C till laboratory handling.

Viral genome reverse transcription polymerase chain reaction RT-PCR

The first step for molecular analysis was the extraction of the viral RNA genome from all individual serum samples (23,24), following the commercial (AddPrep viral nucleic acid extraction kit, Korea) protocol lines. Subsequently, reverse transcription polymerase chain reaction (RT-PCR) conducted employing universal Pestivirus primers, F: 5'-ATGCCCWT AGTAGGACTAGCA-3' and R: 5'-TCAACTCCATGTGCCATG TAC-3', which were obtained from (Macrogen Co.) set by Vilcek *et al.* (25). The primer magnifies the 288 bp of the 5'-UTR genome region of BVDV. The PCR cycling (T100 BioRad, USA) status was performed utilizing (ABM, one-step RT-PCR kit, Canada) directions with some modification, with a final volume of 25µl (26). The cycler program briefly cDNA Synthesisv45°C 45 min1, Initial Denaturation 94°C 2 min1, followed by 45 cycles of Denaturation 94°C 30 sec, Annealing 56°C 1 min, Extension 68°C 2 min, Final Extension 68°C 10 min. One BVDV +ve product was assigned as a positive control from an earlier study (27). All final reaction products were run on 1.5% agarose gel ethidium bromide-stained. The electrophoresis was carried out for 1 h. at 80 V. with a 300 MP power supply (BioRad, USA). 5 µl of DNA marker 100 bp (GeneDirex H3, Korea) as a standard molecular weight marker was used. In order to uncover the potential being of PI animals, the PCR-positive animals were retested in the next round after 21 days.

For genotyping, the 5'-UTR region of the BVDV genome was included in drawing the phylogenetic tree of the BVDV isolate. Phylogenetic analyses were created through the MEGA 11 software, neighbor-joining method. Furthermore, the evolutionary links were computed with 1,000 bootstrap replicates in the phylogenetic tree. The ClustalW program was conducted for multiple sequence alignments, and the data were retrieved from the references to viruses of BVDV in NCBI GenBank (28).

Statistical evaluation

Descriptive statistics was employed to establish the status of BVD in buffaloes, using the data from the current study analyzed in Excel 2010 on Windows 10. The Fisher Exact in the Epi-Info™ program was used to examine the odds ratio for the gender and area factors. Significant data were those with a P value < 0.05.

Results

The molecular analysis for serum samples of buffaloes in Nineveh province revealed that the total prevalence of bovine viral diarrhea was 12/158 (7.59%), and the finding documented no PI (0%) detection through re-run PCR to the positive samples the next 21 days (Table 1).

The result of this study observed significantly (P<0.028) higher prevalence among buffaloes aged over and equal to 5 years old was 11.45% (OR: 7.89, CI: 0.992 - 62.772) compared to buffaloes less than or equal to 2 years old which was 1.61%. Notably, there was no significant (P > 0.05) gap between the status of BVDV in male and female buffaloes, which were 5.71% and 7.84% respectively (Table 2). Also, there was no significant difference in the prevalence of BVDV between indoor and outdoor buffaloes, which were 5.26% and 11.11%, respectively (Table 2). Although the

prevalence was higher in Hawey-Alkaneesah at 10.29%, compared to the Gogjalee area at 6.06% and the Al-Rahmaneyah area at 5.26%, statistically, no significant differences were recorded (Table 2). This investigation indicates at the molecular base effective detection of viral RNA targeting the 5'-UTR region of the BVDV genome with an expected bp size of 288 on gel electrophoresis (Figure 1).

In this study, from all obtained sequences (n=12), six sequences of the BVD1 were deposited in the American NCBI GenBank with the accession numbers (PQ865901.1, PQ865902.1, PQ865903.1, PQ865904.1, PQ865905.1, and PQ865906.1). The similarity within and between the 5'-UTR region of the BVD1 genotype A and genotype B were 94.55-100, 99.47-100, and 81.62-100 alignment scores using the multiple sequence alignment online program (Table 3).

Table 1: The prevalence of BVDV in buffalo farms using RT- PCR technique

Farm	Total animal number	Number of Positive	Prevalence % / 1st round	Prevalence % / 2nd round
1	22	2	9.09	0.00
2	16	0	0.00	0.00
3	27	3	11.11	0.00
4	18	2	11.11	0.00
5	33	4	12.12	0.00
6	30	1	3.33	0.00
7	12	0	0.00	0.00
Total	158	12	7.59	0.00

Table 2: Bovine viral diarrhea infection in buffalo related to the sex and study area

Factors		Tested (n)	Positive [n(%)]	Odds ratio	Confidence of interval	P value
Age	≤ 2 years	62	1 (1.61) [a]	1		
	≥ 5 years	96	11 (11.45) [b]	7.89	0.992 - 62.772	0.028
Gender	Male	64	4 (6.25) [a]	1		
	Female	94	8 (8.51 %) [a]	1.39	0.401 - 4.844	0.76
Management	Outdoor	95	5 (5.26) [a]	1		
	Indoor	63	7 (11.11) [a]	0.44	0.134-1.468	0.223
Areas	Al-Rahmaneyah	57	3 (5.26) [a]	1		
	Gogjalee	33	2 (6,06) [a]	1.16	0.183 - 7.33	1.000
	Hawey-Alkaneesah	68	7 (10.29) [a]	1.95	0.506 - 8.386	0.343

Different letters (a, b) mean significant at $P < 0.05$.

Table 3: Alignment score within and between BVD1 sequences using the Multiple Sequence Alignment program

Sequences accession number	Genotypes	Alignment score
PQ865901.1A - PQ865903.1A	BVD1-A	94.55-100
PQ865904.1B - PQ865906.1B	BVD1-B	99.47-100
PQ865901.1A - PQ865906.1B	BVD1-A & BVD1-B	81.62-100

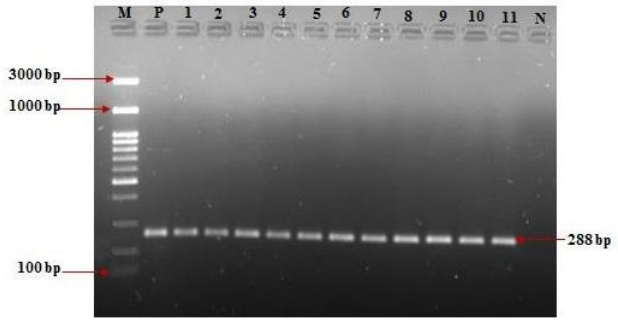

Figure 1: Gel image: Lane M) Mark 100-3000 bp DNA ladder; Lane 1-10) Conventional PCR technique detected BVD1 targeting the 5'-UTR gene in approximately band size 288 bp; Lane N) negative control.

Using the NCBI Blastn online program for the individual sequencing of the obtained sequences showed high relatedness (96.07% - 100% identity), with those sequences recorded in the GenBank of various countries such as Iran (AY954693.1 and EF210347.1), Iraq (MF347398.1 and MF347402.1), Mexico (MN811651.1), the USA (AF039178.1 and KP941587.1), and China (OR753412.1 and PP213451.1) (Table 4).

Following the 1000 nucleotide sequence reconstruction using MEGA 11 software and Bootstrap analysis, the tree was rooted with AY443026.1-BVD2-cattle, Argentina, which was used as an outgroup. The phylogenetic tree analysis showed two clades (genotypes A and B) of the obtained sequences for the 5'-UTR gene of BVD1 were closely linked (99.29%–100%) to those existing sequences of BVD1 mentioned above (Figure 2).

Table 4: Homology of BVD1 based on a partial 5'-UTR gene according to BLASTn in GenBank of NCBI

Accession number	Query Cover %	Identic Number %	GenBank Accession Number	Country	Genotypes
PQ865901.1A	100	100	AY954693.1	Iran	Genotype A
PQ865902.1A	100	100	EF210347.1	Iran	
PQ865903.1A	100	99.66	MF347398.1	Iraq	
	100	99.65	MN811651.1	Mexico	
	100	100	MF347402.1	Iraq	Genotype B
PQ865904.1B	100	99.48	AF039178.1	USA	
PQ865905.1B	100	97.06	KP941587.1	USA	
PQ865906.1B	100	96.57	OR753412.1	China	
	100	96.07	PP213451.1	China	

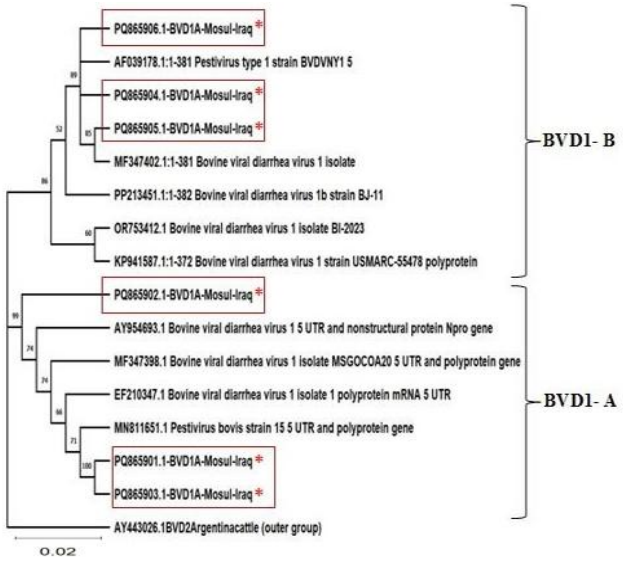

Figure 2: Phylogenetic tree of BVD1 strains (A and B) from Iraq (*). Partial DNA sequences of concatenated partial 5'-UTR gene were used as input.

Discussion

The distribution rate of BVDV in buffalo in the current survey was 7.59%. This observation indicates that the virus is circulating among buffalo farms in the study area. It has been recognized that the genus Pestivirus is publicized on a world level and studied for financial losses. Additionally, in the last few years, a newly developed number of the genus has been detected in various domestic and wild animals (29). Wahid and Al-Saad (30) revealed that the overall prevalence of BVDV was 17.14% in local buffalo calves in Basrah, Iraq. Other earlier reports of BVDV from different countries include: In Egypt, the prevalence was 40% and 23% for cattle and buffaloes, respectively (31). In Iran, it was 15.96% and 18.49% (32). In Argentina, it was 84.2% (8). In Southeast Brazil, the PCR-positive rate was 9% in buffaloes (4). In India, it was 32.49% (33).

As shown above, the differences in prevalence in this literature could be attributed to interspecies transmission, lack of vaccination, importation, and PI animals on a farm. These aspects align with (34-39). In Argentina, Craig *et al.* (8) revealed a high positive rate of BVDV 1 and 2 in buffalo herds, and the molecular basis approved the presence of PI buffalo animals that were formerly negative to the serological test, also natural co-infection with more than one BVDV 1a and 1b subtype in addition to BVDV-2. In another study, Medina-Gudiño *et al.* (24) announced the molecular detection of BVDV subtype 1b with CP biotype in buffaloes in Mexico.

The current result showed that the distribution rate of BVD1 was significantly higher among older buffaloes than younger buffaloes; this may be due to productivity regarding age by producer and herd size. In large herds, more animals are the primary source of production and are more economically attractive for the producers in Nineveh province. On the contrary, Wilson *et al.* (40) and Lotfy *et al.* (41) mentioned that there was no significant variation in BVD1 prevalence by age. The obtained result also showed no significant difference in the prevalence of BVD1 among male and female buffaloes. This finding was in agreement with Lotfy *et al.* (41). On the contrary, Wernicki *et al.* (42) stated that the prevalence of BVD disease was significantly higher among male bovines than among female bovines. Whereas Haji *et al.* (43) informed that higher prevalence of BVDV in female cattle than in males, this may be endorsed to the age of the animals.

The findings of our investigation indicate that the molecular method can precisely detect the viral genome depending on the 5'UTR gene mark at 288 bp, which gives meaning to the sensitivity and reality of the molecular technique used to detect the Pestivirus genus. Moreover, this site is highly conservative and could be performed for purpose sequence for diagnosing, epidemiology, genetic diversity, and phylogenetic analysis of Pestivirus significantly. This outcome is harmonic with the editing of previous references (39,44-46). It has been documented that based on the 5'UTR gene, diversified subgenotype isolates have been summarized. About 21 isolates belonged to BVDV1 (1a-1u) subgenotypes, and 3 isolates of BVDV2 have been referenced worldwide (47-49).

Different targeting genes have been used for the detection of BVD1 in buffaloes. However, in this study, the 5'UTR gene was used because it is more commonly used in epidemiology and genetic diversity studies (2,44). The conservation of these gene sequences at the species level exists in various copies in the genome, and the gene sequences are available in molecular databases (49). Moreover, regarding the sequencing and phylogenetic analyses of BVDV amplicons (n=12) of the 5'UTR gene for BVD1 obtained from cat serum samples, these genetic sequences were identified for the first time in Nineveh province, Iraq. Six sequences from these obtained sequences were found to have phylogenetic characteristics and have a very tight evolutionary relationship with the other BVD1 sequences included in the NCBI GenBank for various countries, including Iran (50,51), Iraq (52), Mexico (24), the USA (53), and China (54), with a highly related (96.07%-100% identity) after 1000 re-samplings using the Likelihood method on the Tamura-Nei model in MEGA11 software and Bootstrap analysis (28).

Conclusions

The current finding, for the first time, confirms the occurrence of BVDV in buffaloes, which could highlight the value of interspecies transmission of the virus between ruminant populations and the given necessity of vigilance selection and averting animal contact between herds as possible and identifying PI carrier ones to minimize and promote control infection in these species. Moreover, concentricity on good management and vaccination attempts is required. Genotyping and phylogenetic resolution of BVDV provide helpful support to the genetic links of these viruses, either found endemically in the area for a longer time or introduced recently or later.

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Conflict of Interest

All authors are satisfied with this article with no conflict.

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دلائل وجود فيروس الإسهال البقري الفيروسي في الجاموس في محافظة نينوى

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الخلاصة

إن التحقيق في مصادر فيروسات البستيفيرس من عائلة الفلافيريدي، ومن ضمنها الاصابة بفيروس الإسهال البقري الفيروسي، له أهمية في علم الأوبئة وفعالية السيطرة على هذا المرض في المجترات الكبيرة والصغيرة. هذا العمل هو محاولة أولية للتحقيق في انتشار فيروس الإسهال البقري الفيروسي في الجاموس من خلال إجراء تفاعل البلمرة المتسلسل في محافظة نينوى، العراق. من شهر تشرين الثاني ٢٠٢٤ إلى نهاية كانون الثاني ٢٠٢٥، تم جمع ١٥٨ عينة دم من الجاموس في ٧ حقول في مناطق مختلفة من محافظة نينوى، ومن كلا الجنسين، وبنظم إدارية مختلفة، وخلفية وحيدة وبين الأنواع، مع عدم وجود خلفية تطعيم ضد المرض. كشف هدف النتيجة الجزيئية لمنطقة الجين ٥ عن معدل انتشار كلي بنسبة ٧,٥٩٪ لفيروس الإسهال البقري الفيروسي في الجاموس، مع انتشار أعلى معنويا للفيروس في الجاموس الذي يزيد عمرها عن ٥ سنوات. فضلا عن انه لا يوجد اختلاف معنوي بين جنس الجاموس والإدارة ومناطق الدراسة. كما تم تسجيل ست تسلسلات في العمل الحالي في قاعدة بيانات بنك الجينات العالمي. كما وكانت التسلسلات المحلية المشار إليها مماثلة لتلك المسجلة في إيران والعراق والمكسيك والصين والولايات المتحدة وبنسبة تشابه ٩٦,٠٧-١٠٠٪. استنتجنا، إن تسجيل فيروس الإسهال البقري الفيروسي في هذا العمل لأول مرة في الجاموس في محافظة نينوى، وعلى الرغم من تسجيل المرض في وقت سابق في الأبقار والأغنام. لذا يجب الانتباه إلى دور الجاموس في نقل الفيروس والحفاظ عليه. وبالتالي، فإن الكشف الدقيق والتمييز بين فيروسات البستيفيرس من المتطلبات الحاسمة للقضاء على فيروس الإسهال البقري الفيروسي وإزالته من مجاميع الحيوانات.

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