



## Prevalence of Bovine Viral Diarrhea Virus in Sheep in Nineveh Province

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### Abstract

NUMEROUS studies have been conducted at the global level to evaluate the prevalence of the bovine viral diarrhea virus (BVDV) in various animals, especially ruminants. It becomes clear that one of the most important steps in preventing viral infections, is to designing multiple programs for control, such as vaccination, the detection of vectors and the identification of predisposing factors to infection. This work is a first pioneering study designed to detect the infection rate of BVDV utilizing indirect ELISA and PCR technique, and to clarify the role of some risk related factors in sheep located in Nineveh province, Iraq. Between Dec. 2022 and July 2023, blood samples for this study were obtained from 184 sheep selected randomly represented 24 local and imported sheep flocks, from different regions of Nineveh province. The results were recorded that the infection rate of the disease in sheep was 16.84% and 2.71% based on the indirect ELISA and the conventional PCR technique respectively. Based on the multiplex PCR technique, three samples were BVDV-1 genotypes. Significantly the large herd size and older age showed higher risk to BVDV, with no significant difference observed between origin of animals. This study indicates that the evidence of BVDV in sheep and their role in the epidemiology of the disease in cattle and maintenance of the virus. Therefore, the study of epidemiology and genetic diversity of the virus in sheep and goats is recommended.

**Keywords:** BVDV, Prevalence, ELISA, PCR technique, Nineveh-Iraq.

### Introduction

Bovine viral diarrhea virus BVDV infect cattle, sheep, goat and pigs, resulting in wide range of clinical consequences such as hemorrhagic diarrhea, respiratory infection, abortion and stillbirth in which substantial financial impact to animal production may occur [1,2]. In regard to the Pestiviruses, a positive ss RNA of family Flaviviridae, the BVDV is belonged, with at least 19 species according to newly recorded known A to S. In addition to BVDV1 and 2, This genus includes the species of Classical swine fever

virus and Border disease virus [3,4]. As, novel pestiviruses were identified in different animals, like HoBilike virus in [5,6], antelope virus [7], Bungowannah virus from a pig [8], Aydin-like pestiviruses [9-11]. Moreover, BVDV-1 based on sequences analysis has several subspecies counting at least 21 subgenomes (BVDV1a-BVDV1u) and for BVDV-2 there are four subgenomes named (BVDV2a- BVDV2d). Bovine viral diarrhea viruses are also classified depending on their effects on the cell culture to cytopathic (CP) and non-cytopathic (NCP) biotypes [12]. The term persistently infected animals (PI) is one of the

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outcomes for BVDV infection usually occurred in fetuses that infected in the uterus during the first trimester of gestation producing an immune tolerance fetuses which if survive 1-2% they become persistently infected. These PI animals are often without detectable antibodies against the virus, and excrete the virus through all its secretions; therefore they consider one of the major provenances of BVDV infection, and being critical challenge for control of the disease [13-15]. Although cattle are the common host of BVDV; Pigs, sheep, goats and diverse other ruminants can also be infected, with the sheep and goats are of an important concern of BVDV disease, as they mostly reared with cattle, and playing important role in the disease transmission [6,16-18]. Diverse clinical details were recorded in cattle infected with bovine viral from various studies worldwide such as immunosuppression, mastitis, enteritis and respiratory, emaciation, mortality, infertility, congenital anomaly, mummification, abortion and stillbirth [1,19-25]. As in cattle, different ages of the small ruminants could be infected with BVDV, with acutely infected animals show intestinal and respiratory defects, and fetal anomalies or abortion in last trimester of pregnancy [26,27]. Typically the disease in caprine involve the reproductive system with very low chance for PI outstand [28]. The BVDV in sheep and goat farms were reported, the most outcomes of disease is reproductive problems, abortion and delivery of PI newborn [2,29,30].

In Nineveh province, the sheep farms are usually found in various size belonging to private own and mostly raised in close contact or some share the same farm with cattle, sheep reared for several purposes including the production of milk and meat and for trade purpose. Although the epidemiology of the BVDV has been studied in cattle in Nineveh province (31), in sheep this work is the first attempt to detect the prevalence and analysis of some related predispose conditions in sheep in Nineveh, Iraq.

## **Material and Methods**

### **Ethical approval**

The present work was licensed ethically from Committee of the College of Veterinary Medicine, University of Mosul (UM.VET.2022.079) on 11 November, 2022.

### **Animals, study area and sampling**

The Blood samples for this study obtained from 184 sheep selected randomly as 10%

for each farm, represented 24 local Iraqi and imported sheep, the age group were (8months to 4 years old), The herd size was small size <50 and large size >50 animal, from different regions in Nineveh province, Iraq between December 2022 and July 2023. The sheep farms usually close contact to cows and Feedlots calves flocks with mixed raised in some farms area. Generally, the animals were physically appear normal and some with backgrounds disorders in reproductive and respiratory system. According to history, no former vaccinations against pestivirus such as BVDV and BDV for these farms had equipped. Carefully, the blood was drawn by vein puncture into sterile vacutainers without anti-coagulant for serum obtaining and transported icebag and kept at -20°C until assays [32,33].

### **Laboratory analysis**

For estimating antibodies against BVDV in a serum samples, we used a commercial indirect ELISA kit (BVDV ELISA Ab, Bio-X Diagnostics, Rochefort, Belgium). The serum tested according to the manufacture instructions. The formula below was used for calculation:

$$\text{Value} = \frac{\text{Delta OD positive}}{\text{Delta OD Sample control}} \times 100$$

The samples analyzed based on the validation table in the protocol leaflet.

### **Antigen extraction and PCR amplification**

The genome of BVDV was debriefed from 184 sheep serum by the ( QIAamp® Viral RNA kit, France) following the protocol steps available in the kit guidelines. Thereafter, the conserved region 5' UTR gene of BVDV is targeted in a conventional PCR technique.

Amplification of 5'UTR in expected size 288 bp was achieved employing panpestivirus primers 324-F(5'ATGCCCTTAGTAGGACT AGCA3') and 326-R (5'-TCAACTCC ATGT GCCA TGTAC-3') referenced by Vilcek *et al.* [34]. Utilizing the supply (OneStep RT-PCR Kit, Qiagen, Germany) with final volume 25 µl of 5 µl of 5 x RT-PCR buffer, 1 µL enzyme, 1µM for every primer, and 2.5 µL extracted RNA. Amplification was performed in the thermocycler (Optimus 96G, United Kingdom) under the below conditions [35], (Table 1). Further, in order to detect BVDV and determine the genotype (BVDV1 and BVDV2) for positive samples from first PCR reaction, second run was performed

through using single forward primer BVD-F (5'-TGG AGA TCT TTC ACA CAA AGC-3') and two reverse primers for identifying BVDV1-BV1-R (5'-GGGAACCTAAGAACTAATC-3'), and BVDV2-BV2-R (5'GC TGTTTCACCCAGTT(A/G)TACAT3') at expected size 360 bp and 604 bp. respectively, which are designed by Gilbert *et al.* [36], using multiplex PCR technique. 2 control cDNA obtained from PI calves (accession numbers MF347399, MF491394) from earlier study [37] for BVDV1 and BVDV2 respectively used as positive control. Moreover, negative control for each PCR amplification excerpted from negative sheep applied. The reactions for Multiplex PCR was conducted similar to Nahed *et al.* [38] and Reshmi *et al.* [39]. At the end, 1.5% agarose gel electrophoresis (100V, 80Am 1 hour) and visualized by UV illuminator (Clinx Science, China) was used for interpretation.

#### Statistical analysis

The prevalence of BVDV in studied animals estimated using descriptive analysis using IBM SPSS Statistics for Windows, version 19 (IBM Corp., Armonk, N.Y., USA), and to investigate significance ( $P < 0.05$ ) for some linked data such as origin, age and herd size in the different sheep groups were analyzed the data statistically to assess the difference by Epi-Info TM 7, version 7 [40].

#### Results

In this study, the results show that the overall prevalence of BVDV in sheep was 16.84 % (31/184) based on the antibodies detected in serum samples using indirect ELISA (Table 2).

The finding of molecular assay in this work for a total of 184 serum samples of sheep tested by PCR technique revealed that 2.71% (5/184) of the samples were positive for BVDV antigen and the positive bands on gel electrophoresis were nearly 288 bp (Figure 1). The results of this study that based on multiplex PCR technique for amplification of cDNA fragments for both BVDV1 and BVDV2; indicates BVDV1 genotypes in 3 samples were the bands at approximately 360 bp and no BVDV2 was detected (Figure 2).

The results of this study revealed that the prevalence was significantly ( $P < 0.05$ ) higher in large herd size ( $> 50$  animal), (OR: 2.76, CI: 1.0706- 2.8833 ) comparing to small herd size ( $< 50$  animal) (Table 3). There was no significant

difference ( $p < 0.05$ ) between the prevalence of the disease between the local and imported animals, and finally the prevalence was significantly ( $P < 0.05$ ) higher in animals aged ( $> 1$  year) than the ( $< 1$  year) aged sheep (OR: 2.53, CI: 1.0287-6.2345) (Table 3).

#### Discussion

The BVDV has been documented in cattle population in Mosul, Iraq [31], In sheep, this study is the first preliminary work that aimed to predict the prevalence and to investigated some facilitating associated factors of this virus based on the serological detection of antibody in addition to the molecular assay for the virus genome by PCR technique in serum samples. From total of 184 sheep serum samples tested serologically and by PCR, the prevalence of BVDV were 16.84 % and 2.71% respectively. The serological rate was nearly close to what was reported by Al-Rubayie, [41] who was mentioned (21.48%) rate in sheep examined using indirect ELISA. The prevalence of BVDV in sheep has been recorded worldwide, in Turkey was 24.57% by ELISA [42], and 40.81% using PCR technique [28]. In Iran the seroprevalence of pestivirus was 75.9% [43]. In Switzerland was 13.5 % [44]. The disparity between the above mentioned infection rates could be clarified by reasons that may include the different management systems, animal ages, sensitivity of different laboratories analysis, herd composition, direct or close contact with the cattle in the farm, vaccination and control effort, sample size. The present results match those of Ince, [42]; Mani *et al.* [45] and Naouel *et al.* [46]. It has been revealed the presence of interspecies transmission of pestivirus between the cattle, sheep and goats as reported in earlier studies, and sheep and goats are similar to cattle can be infected with BVDV at all ages [2,11,26,47-49]. Also the current study found that the herds of  $> 50$  animals was considerably had greater risk compared to herds  $< 50$  animals. This result in the same line with what was mentioned by Hasan and Alsaad, [31] and Hassan *et al.* [50]. This data may be related to the purchase of newly animals, density, production, contamination and increased of PI source in the herd; large number of animals usually had higher probability to infection. The visions are in agreement with earlier literatures [51,52]. In the present study, no significant differences seropositivity appear between local and imported animal, which might be due to the fact that the absence of vaccination and control

programs ensure the circulating of pestivirus in these animals, moreover, the BVDV status has been documented in imported sheep countries such as Turkey [42] and Iran [43]. It has been suggested that the biosecurity deal to recent entry of new animals specially the PI animals, lack of adequate status and regular screening tests of the disease could be an important factors for viral distribution. This explanation is consistence with the findings of Ince, [42]. According to the seropositivity analysis data between the sheep age groups in this study, results indicated that prevalence of BVDV increased with age. A significant prevalence rate was detected in sheep aged >1 years compared to sheep aged <1 years, which is can be justified to their long-term exposure to pestiviruses, and the number of animals sampled. This outcome is parallel to previous studies [31,53,54]. It has been announced that the high seroprevalence rates observed in older animals can be clarified by the decrease in maternal antibodies and the boost probability of exposure to the virus compared to younger animal [31,42]. At the molecular level, successfully the current study detect the BVDV genome through targeting the conserved region 5' UTR gene of BVDV from positive animals samples indicating the sensitivity and accuracy of the technique and it can be depended for epidemiological studies, moreover its useful for genotyping and phyloanalysis of BVDV genotypes and sub genotypes. This result similar with Mao

*et al.* [55]; Deng *et al.* [56] and Evans *et al.* [57]. It has been known that the 5'UTR, Npro, E2, NS2-3 and NS5B are the common genomic sites used for classification and genotyping of BVDV [58-61]. Partial 5-UTR region generally applied for phylogenetic analyses and genotyping of BVDV isolates, then after by Npro and E2 codes, Moreover, nearly whole subgenotypes characterized recently were categorized utilizing these sequence regions [62].

### **Conclusion**

The current investigation was revealed the evidence of BVDV in sheep for the first time in Nineveh province at serological and molecular levels and could be highlighted the role of sheep as source of viral infection in cattle farms. Herd size and age considered in the virus prevalence. Future works might be necessary for investigation the epidemiology, phylogenetic and genetic diversity of BVDV in small ruminants including sheep and goats. Serious attempt should be applied to control the Pestiviruses in ruminant populations.

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### *Conflict of interest*

The authors claim no conflicts of interest.

**TABLE 1. Thermocycler program for 1<sup>st</sup> PCR technique**

Step	Temp.	Duration	Cycle
cDNA synthesis	50 °C	30 min	1
Initial denaturation of DNA	95 °C	15 min	1
Denaturation of DNA	94 °C	1 min	
Annealing of primers	50 °C	1 min	40
Extension	72 °C	1 min	
Final extension	72 °C	10 min	1

**TABLE 2. Prevalence of the BVDV in sheep based on indirect-ELISA and PCR technique**

Types of tests	No. Tested animals	No. Positive animals N / %	No. Negative animals N / %
ELISA	184	31 (16.8%)	152 (82.6%)
1 <sup>st</sup> PCR		5 (2.71%)	179 (97.2%)
2 <sup>ed</sup> PCR	5	3 (60%)	-----

TABLE 3. Odds ratio of the associated factors for BVDV in sheep .

Factors	No. of tested sample	No. of positive (%)	OR	CI	P value
<b>Herd size</b>					
<50	67	6 (8.95%) <sup>a</sup>	1		
>50	117	25 (21.36%) <sup>b</sup>	2.76	1.0706- 2.8833	0.03
<b>Origin</b>					
Local	92	13 (14.13%) <sup>a</sup>	1		
Imported	92	18 (19.56%) <sup>a</sup>	1.47	0.6771-3.2268	0.3
<b>Age</b>					
< 1 year	72	7 (9.72%) <sup>a</sup>	1		
> 1 year	112	24 (21.42%) <sup>b</sup>	2.53	1.0287-6.2345	0.004

OR: Odds ratio, CL: Confidence of interval, P: P value (P<0.05).

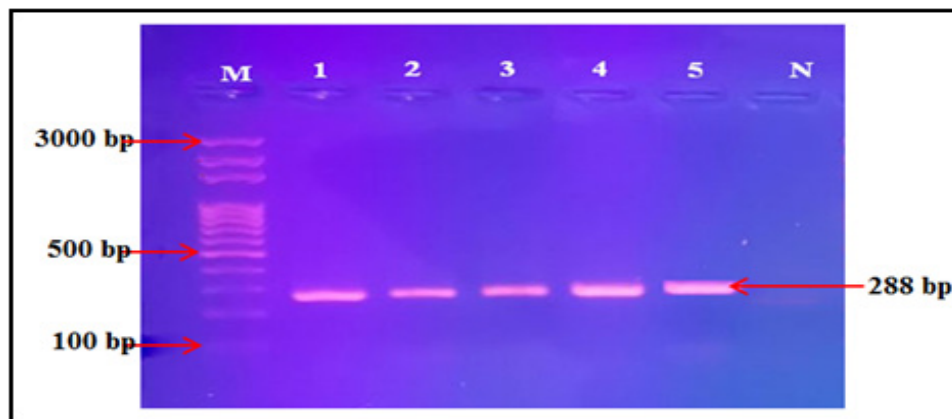


Fig. 1. Gel electrophoresis image: (Lane M) 100-3000bp “DNA ladder”; (Lane 1-5) BVDV positive samples in approximately 288bp.; (Lane N) cDNA the BVDV-free sheep.

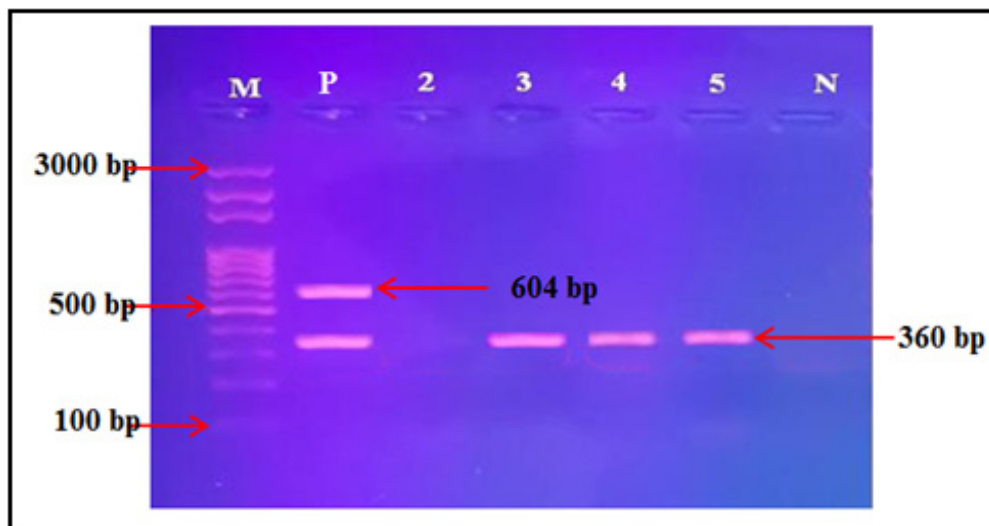


Fig. 2. Gel electrophoresis image: (Lane M) 100-3000bp DNA ladder; (Lane P) positive control for BVDV1 and BVDV2; (Lane 3-5) BVDV1 positive samples in approximately 360bp.; (Lane N) cDNA for negative sheep.



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### انتشار فيروس الاسهال الفيروسي البقري في الاغنام في محافظة نينوى

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### الخلاصة

أجريت العديدة من الدراسات على المستوى العالمي حول تقييم انتشار فيروس الاسهال الفيروسي البقري في الحيوانات المختلفة، وبخاصة المجترات، أصبح من الواضح أن من الخطوات المهمة لمنع الإصابة بالفيروس هي الاعتماد على برامج متعددة لمكافحة مثل التطعيم وتشخيص الناقل وتحديد العوامل المؤهبة للإصابة. تعد هذه الدراسة الاولى التي تهدف إلى الكشف عن مدى انتشار فيروس الاسهال البقري باستخدام تقنية الممتز المناعي للأجسام المضادة وتفاعل البلمرة المتسلسل، وتوضيح دور بعض العوامل المرتبطة بانتشار المرض في الاغنام في محافظة نينوى، العراق. من الفترة ما بين كانون الأول 2022 وتموز 2023، تم الحصول على عينات الدم لهذه الدراسة من 184 راسا من الاغنام، تم اختيارها عشوائياً مثلت 24 قطيعاً من الاغنام المحلية والمستوردة، من مناطق مختلفة من محافظة نينوى. تشير النتائج إلى أن معدل الانتشار الكلي لفيروس الاسهال الفيروسي البقري في الاغنام بلغ 16.84% و 2.71% اعتماداً على تواجد الأجسام المضادة لتفاعل الممتز المناعي وتفاعل البلمرة المتسلسل التقليدي على التوالي. واعتماداً على تقنية تفاعل البلمرة المتعددة، كانت هناك 3 عينات إيجابية للأنماط الجينية الاسهال الفيروسي البقري I- ، في حين لم يتم الكشف عن الاسهال الفيروسي البقري 2- في هذه الدراسة. أظهر حجم القطيع الكبير والعمر الأكبر وبشكل معنوي خطراً أعلى للإصابة بفيروس الاسهال البقري، مع عدم وجود فرق معنوي بين منشئ تلك الحيوانات. كما اشارت هذه الدراسة إلى ان وجود فيروس الاسهال الفيروسي البقري في الاغنام يمكن أن يساهم في وبائية المرض في الأبقار والحفاظ على الفيروس. لذلك نوصي بالدراسة الوبائية والتنوع الجيني لفيروس الاسهال البقري في المجترات الصغيرة (الاغنام والماعز).