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Serological and molecular evidence of respiratory viral mixed infection in sheep and goats

Yahia Hassan Ali[1]*, Intisar Kamil Saeed[1], Muaz Magzob Abdellatif[1], Alaa Mustafa[2], Hind AbdelMajeed Rikabi[3], Husham Mohammed AlHassan Attaalfadeel[4]

[1] Department of Biological Sciences, College of Science, Northern Border University, Arar, Saudi Arabia; [2] Department of Mathematic, College of Science and Arts, Northern Border University, Arar, Saudi Arabia; [3] Department of Pharmacy Practice, College of Pharmacy, Northern Border University, Arar, Saudi Arabia; [4] Department of Mathematic, College of Science, Northern Border University, Arar, Saudi Arabia.

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Abstract

Respiratory infections are considered within the major constraints of animal production; viruses are the major causing pathogens. This study aimed to elucidate the prevalence of parainfluenza virus-3 (PIV-3), bovine viral diarrhea virus, and respiratory syncytial virus (RSV) in sheep and goats and the existence of co-infections. A total of 270 sheep and 220 goat pneumonic lung tissues were collected from slaughterhouses in four different areas. Enzyme-linked immunosorbent assay was used to detect the antigen of the three viruses, fluorescent antibody technique and polymerase chain reaction confirmed enzyme-linked immunosorbent assay positive results. Prevalence detected for PIV-3 was 11.10% in sheep and 9.50% in goats, pestivirus was 10.40% in sheep and 7.70% in goats, and RSV was 17.80% in sheep and 5.00% in goats. Detected co-infections were 5.60% for PIV-3 and pestivirus in sheep and 4.00% in goats and pestivirus and RSV was observed only in goats (1.40%). Co-infection of the three viruses was detected in only one goat sample (1.00%). The existence of the three viruses in sheep and goats was confirmed. To our knowledge, this is the first report of the co-infections of PIV-3, pestivirus, and RSV in sheep and goats in the studied areas.

Introduction

Respiratory infections are the second most significant health problems facing sheep and goats, leading to major losses. Respiratory diseases of sheep and goats were found to be about 6.00% of the total small ruminant disease.[1] An abattoir study in Ethiopia, revealed the existence of pneumonia lesions in 18.00% of examined sheep and goat lungs.[2] Similar study showed pneumonia in 9.00% of sheep lungs in Ghana and 13.00% in Nigeria.[3] The main viruses encountered in this syndrome are parainfluenza virus-3 (PIV-3), respiratory syncytial virus (RSV), pestiviruses, bovine viral diarrhea virus (BVDV), border disease virus (BDV), and bovine herpes virus-1. The PIV-3 is a non-segmented, negative sense, enveloped, and single-stranded RNA virus classified in the *Respirovirus* genus of the *Paramyxoviridae* family.[4] The association of PIV-3 with respiratory infections in sheep and goats is well documented globally.

The PIV-3 was found in pneumonic sheep lungs in Ghana, Nigeria,[3] Türkiye,[5] Algeria,[6] and China[7]. In Sudan, respiratory infections associated with PIV-3 were reported in sheep, goats and camels.[8,9] Like sheep, goats are susceptible to respiratory viral infections; PIV-3 infection was proved in goats in China.[10]

Pestiviruses are positive sense, single stranded RNA viruses in the *Flaviviridae* family, including classical swine fever virus and BVDV. The BVDV is known to be one of the most encountered viruses in respiratory infections in small and large ruminants. The BVDV antigen was found in sheep and goats in Türkiye,[11] and in sheep in Greece.[12] The BDV was also found to cause high mortality in lambs in Spain.[13] In Sudan, BVDV was detected in camels,[14] cattle,[15] sheep, and goats.[16]

Respiratory syncytial virus is one of the major causes of respiratory infections. The RSV is classified in the *Pneumovirus* genus, *Pneumovirinae* subfamily, and *Paramyxoviridae* family, containing human RSV, bovine RSV, ovine RSV, caprine RSV, murine pneumovirus, and

***Correspondence:**

Yahia Hassan Ali. PhD
Department of Biological Sciences, College of Science, Northern Border University, Arar, Saudi Arabia.
E-mail: Yahia.Ali@nbu.edu.sa

canine pneumovirus.[17] The identification of the virus as a causal agent of respiratory infections in large and small ruminants was thoroughly studied. A histopathological study of pneumonic sheep lungs in Saudi Arabia suggested RSV as a cause for these cases.[18] The RSV antigen was detected in sheep lungs in Ghana and Nigeria,[3] and in goat lungs in Türkiye.[19] Existence of RSV in goat nasal swabs was reported in China[10] and Nigeria.[20] In Sudan, work on RSV is scarce, and viral antigen was detected in camels,[21] cattle, sheep, and goats.[22]

This study aimed to elucidate the prevalence of PIV-3, RSV, and BVDV in pneumonic sheep and goats beside the existence of viral co-infection in Sudan.

Materials and Methods

Sample size determination. The sample size was estimated following the published formula,[23] with expected prevalence of 10.00% as previously reported by Saeed *et al.*[8] and 95 confidence intervals at five required absolute precision;

$$n = \frac{1.96^2\, p\,(1-p)}{d^2}$$

where, p is the predictable occurrence, d is the desired precision, and n is the necessary sample size. Replacing each value equals n = 137. The sample size was increased to 490 to cover sheep and goats and improve accuracy.

Sample collection. A total of 270 sheep and 220 goat pneumonic lung tissue samples were collected randomly from slaughterhouses in four areas in Sudan, including Atbara at the River Nile State (150 sheep and 100 goats), Wad Medani at the Gezira State (50 sheep and 50 goats), Rabak at the White Nile State (50 sheep and 50 goats), and the Khartoum State (20 sheep and 20 goats). Samples were put on ice and sent to the Central Veterinary Research Laboratory in Khartoum, Sudan, where they kept frozen till examined.

Antigen detection. Samples were tested for PIV-3, BVDV, and RSV antigens using enzyme-linked immunosorbent assay (ELISA) Kits (BIO X Diagnostics, Rochefort, Belgium). The kits were sandwich ELISA based on coating the plates with specific antibodies against each virus to be tested, and used according to the instructions of the manufacturer.

Co-infection of PIV-3, BVDV, and RSV viruses. Results of samples tested for PIV-3, BVDV, and RSV using ELISA were analyzed to investigate the existing co-infections.

Fluorescent antibody technique (FAT). Direct FAT was used to confirm ELISA antigen reactive samples. After fixing the sample on the glass slide, specific antibodies against specific virus conjugated with fluorescein isothiocyanate were added. The conjugate was purchased from Bio-X Diagnostics and used according to the protocol provided.

Polymerase chain reaction (PCR). Reverse transcription (RT)-PCR was employed to confirm selected ELISA-positive tissues (n = 9) for each virus tested.

RNA extraction. Lung tissue samples (30.00 mg) were used to extract RNA using RNeasy Kits (Qiagen, Germantown, USA), as directed by the manufacturer. The RNAs were extracted from nine ELISA positive samples for each virus tested.

Synthesis of cDNA. The Moloney murine leukemia virus reverse transcriptase was used to synthesize the cDNA. In a final amount of 20.00 µL, the solution included 4.00 µL of 5X RT-enzyme buffer (ABgene House, Epsom, UK), 0.40 µL of dNTPs (10.00 mM μL^{-1}; Promega, Madison, USA), and 0.50 µL of RNasin (40.00 U μL^{-1}; Promega), and 50.00 pM of primers and 0.50 µL of Moloney murine leukemia virus (250.00 U μL^{-1}; AB gene, Cheshire, UK) were added to 5.00 µL of purified genome and the reaction was carried out as per instructions of the manufacturer.

Detection of PIV-3 RNA. The reaction was done using Reverse-it TM One Step RT-PCR Kit (AB gene) following the protocol described previously.[24] Briefly, Taq polymerase (1.50 U; Eurobio Scientific, Les Ulis, France) was added to cDNA and primers including sense: CATTGAATTCATACTCAGCAC and anti-sense: AGATTGTCGCATTT(AG)CCTC with expected product size of 400 bp in a total volume of 50.00 µL of amplification buffer ($MgCl_2$: 1.50 mM and 5.00 mM for PIV3 primers, 40.00 pmol of each deoxyribonucleotide triphosphate, 16.60 mM (NH_4), SO_4, 67.00 mM Tris/HCl with pH of 8.80, and 0.01% Tween 20. Thermal conditions included initial denaturation at 95.00˚C for 10 min, denaturation at 95.00˚C for 45 sec, annealing at 45.00˚C for 45 sec, and extension at 72.00˚C for 45 sec for 35 cycles, ended by a final extension at 72.00˚C for 10 min.

Detection of BVDV RNA. The PCR was performed as described formerly[25] utilizing designed primer sequences[26] including sense: ATGCCCATAGTAGGACTA CCA and anti-sense: TCAACTCCATGTGCCATGTAC with expected product size of 188 bp. Briefly, the cDNA was mixed with 5.00 µL of 10X Taq polymerase buffer (AB gene), 0.50 µL of dNTPs (10.00 mM μL^{-1}; Promega), 3.00 µL of $MgCl_2$ (25.00 mM μL^{-1}; AB gene), and 0.50 µL of Taq (5.00 U μL^{-1}; AB gene). Cycling was made as follows: 94.00 ˚C for 1min, denaturation (94.00 ˚ C for 1 min), annealing (56.00 ˚C for 1 min), extension (72.00 ˚C for 1 min) for 35 cycles, and a final extension at 72.00 ˚C for 7 min.

Detection of RSV RNA. The virus nucleic acid was amplified according to a previous report[27] in two rounds; in the first run primers targeting N coding region of the RSV genome were used (sense: CATCTCAATAAGTTGTG TGG and anti-sense: TCTACAACCTGTTCCATTTC) with expected product size of 731 bp.[27] The program was set as follows: 1st denaturation at 94.00 ˚C for 12 min, 35 cycles of denaturation at 94.00 ˚C for 60 sec, annealing at 58.00 ˚C

for 60 sec, and elongation at 72.00 ˚C for 90 sec. For the second PCR, 10.00 µL of the 10-fold diluted product was used as a template with internal N region primers. The 2nd run amplified 731 nucleotides through the following temperature: 94.00 ˚C for 12 min followed by 35 cycles of denaturation at 94.00 ˚C for 45 sec, annealing at 49.00 ˚C for 60 sec, and elongation at 72.00 ˚ C for 60 sec, ending with a final elongation for 10 min.

Gel electrophoresis. After completion of the amplification cycles, the products were separated in agarose gel (1.00%); positive and negative controls, as well as 100 bp DNA marker were included.

Statistical analysis. Pearson's chi-squared test was used to investigate the correlation between location, species, and the prevalence of PIV-3, BVDV, and RSV. Statistical significance was determined at a 99.00% confidence level ($p < 0.01$). Analysis was performed using SPSS Software (version 27.0; IBM Corp., Armonk, USA).

Results

Detection of PIV-3 antigen. The overall detected prevalence of PIV-3 in sheep and goats was 10.40%; in sheep, it was 11.10% and the highest prevalence (40.00%) was found in the Khartoum State. The prevalence in goats was 9.50% and the highest prevalence (50.00%) was also found in the Khartoum State (Fig. 1; Table 1).

Detection of BVDV antigen. Prevalence of BVDV in both species was found to be 9.20%; out of 270 sheep lung tissues examined, 10.40% tested positive and the highest prevalence (15.00%) was seen in the Khartoum State. Prevalence of the virus in goats was 7.70% and the highest prevalence (45.00%) was observed in the Khartoum State as well (Fig. 1; Table 1).

Table 1. Prevalence of bovine viral diarrhea virus (BVDV), parainfluenza virus-3 (PIV-3), and respiratory syncytial virus (RSV) according to the state as detected by enzyme-linked immunosorbent assay. Data are presented as No. (%).

Viruses	Species	River Nile	Gezira	White Nile	Khartoum
PIV-3	Sheep	10 (6.70)	9 (18.00)	3 (6.00)	8 (40.00)
	Goats	5 (5.00)	3 (6.00)	3 (6.00)	10 (50.00)
BVDV	Sheep	10 (6.70)	7 (14.00)	8 (16.00)	3 (15.00)
	Goats	5 (5.00)	2 (4.00)	1 (2.00)	9 (45.00)
RSV	Sheep	39 (26.00)	0 (0.00)	2 (2.50)	0 (0.00)
	Goats	5 (5.00)	2 (4.00)	3 (6.00)	1 (2.00)

Detection of RSV antigen. In sheep and goats, prevalence of RSV was 10.60%; out of 270 sheep lung tissue samples examined for RSV antigen detection, 15.20% were tested positive and the highest prevalence (26.00%) was detected in the River Nile State. Detected antigen in goats was 5.00% and the highest prevalence (6.00%) was seen in the White Nile State (Fig. 1; Table 1).

Co-infection of PIV-3, BVDV, and RSV. Co-infection of PIV-3 and BVD in both species was 4.90%; in sheep, it was seen in 15 samples (5.60%) and the highest prevalence (10.00%) was found in the Khartoum State. The PIV-3 and BVD co-infection in goats was detected in nine samples (4.10%) and the highest prevalence (5.00%) was observed in the Khartoum and River Nile States. Co-infection of BVD and RSV was detected only in three samples (1.40%) of goats and the highest prevalence (5.00%) was seen in the Khartoum State. Existence of the three viruses was detected in only one goat sample (1.00%) in the River Nile State (Fig. 2; Table 2).

Pearson's chi-squared test showed that state appeared to have a significant effect ($p < 0.01$) on the frequency of BVDV, PIV-3, and RSV. Species correlated with the incidence of RSV. The BVDV was related to PIV-3 (0.470).

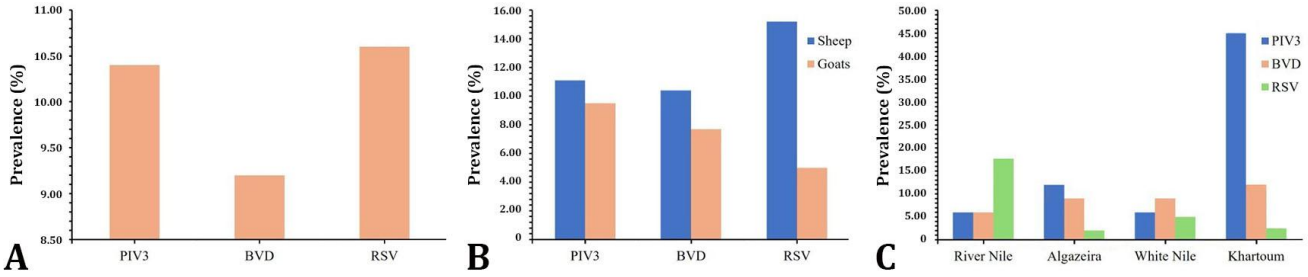

Fig. 1. Prevalence of BVD, PIV3 and RSV as detected by ELISA, **A)** overall, **B)** species wise, and C) according to the state investigated.

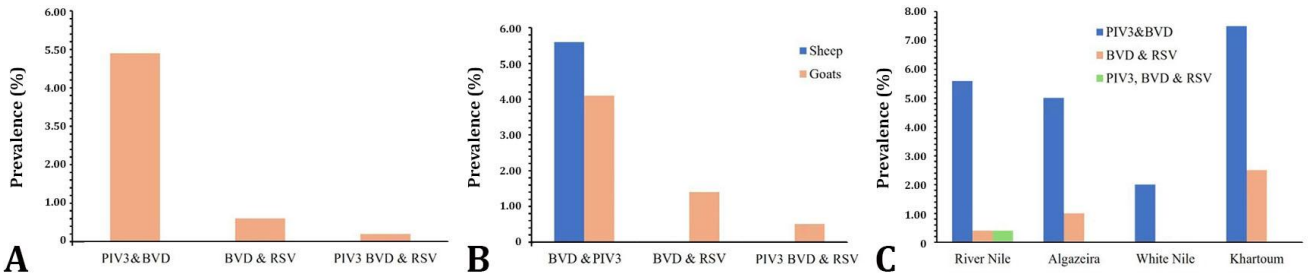

Fig. 2. Prevalence of mixed virus infections as detected by ELISA, **A)** overall, **B)** species wise, and **C)** according to the state investigated.

Table 2. Prevalence of mixed bovine viral diarrhea virus (BVDV), parainfluenza virus-3 (PIV-3), and respiratory syncytial virus (RSV) infections according to the state as detected by enzyme-linked immunosorbent assay. Data are presented as No. (%).

Viruses	Species	River Nile	Gezira	White Nile	Khartoum
BVDV and PIV-3	**Sheep**	9 (6.00)	3 (6.00)	1 (2.00)	2 (10.00)
	Goats	5 (5.00)	2 (4.00)	1 (2.00)	1 (5.00)
Total		14 (5.60)	5 (5.00)	2 (2.00)	3 (7.50)
BVDV and RSV	**Sheep**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
	Goats	1 (1.00)	1 (2.00)	0 (0.00)	1 (5.00)
Total		1 (0.40)	1 (1.00)	0 (0.00)	1 (2.50)
BVDV, PIV-3 and RSV	**Sheep**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
	Goats	1 (1.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total		1 (1.40)	0 (0.00)	0 (0.00)	0 (0.00)

Fluorescent antibody technique. All ELISA positive samples for the three viruses were reactive to FAT. The BVDV antigen was detected in all 45 ELISA positives (28 sheep and 17 goats) and all 51 PIV-3 antigen ELISA positives (30 sheep and 21 goats) were found to be positive. The RSV antigen was detected using FAT in all 52 ELISA reactive samples (41 sheep and 11 goats).

Reverse transcription PCR. No amplification product was detected when control negative was used as a template, while the control genome gave positive result. All tissues under test (n = 9) gave positive results. Clear bands were visualized on ethidium bromide-stained gel, corresponding exactly to the expected band size for PIV-3 (~400 bp), BVDV (~288 bp), and RSV (~731 bp), (Fig. 3).

Discussion

The PIV-3 is nominated as one of the main causing agents of respiratory infections in animals. In this study, the prevalence of the virus detected in sheep lungs was 11.00%, which is very close to the previous report (10.00%)[8] and lower than that reported in cattle in Sudan (20.00%),[28] but higher than the results obtained in camels (2.00%).[9] Similar results (11.00%) were reported in Algeria,[6] and Ghana and Nigeria (14.00%).[3] Higher prevalence of PIV-3 antigen in sheep lungs was reported in Türkiye (19.00 - 40.00%),[5,20] and China (21.00%). Within states, our results showed the highest prevalence in sheep in Khartoum State, which is in line with the previous report.[8]

Prevalence of PIV-3 antigen in goats in this study was (9.00%), which is much lower than the results presented previously in Sudan (48.00%),[8] Nigeria (23.00%),[21] Türkiye (50.00%),[20] and Romania.[29] The Highest prevalence was observed in the Khartoum State which agreed with the previous report.[30] The detected PIV-3 prevalence in sheep and goats is comparable as previously noted in different reports.[20] Pestivirus is considered to have significant role in respiratory infections; in Sudan, BVDV antigen was found in camel and cattle lungs.[14,15] Antibodies to the virus in sheep and goats were found;[31] later, an outbreak of pestivirus in sheep in Sudan was reported.[16] In this study, the viral antigen was found in 10.00% of sheep lungs; this is almost like the previous report (10.50%) in Sudan.[32] Similar results (10.00%) were found in Greece[12] and reviewed reports from 24 countries (11.00%).[33] Our results are higher than reported prevalence in different countries, including 6.00% in Italy,[33] 5.00% in Türkiye,[34] and 1.00% in Iraq.[35] However, far more prevalence (58.00%) was reported in sheep and goats in Türkiye.[11] The BDV antigen was detected in India,[36] while the genome was detected in 16.00% of sheep in Iraq.[35] The highest prevalence was detected in the Khartoum State; the same observation was reported previously.[31] In goats, pestivirus antigen was detected in 5.50% of samples in this study; this is considered a low prevalence compared to the previous report in Sudan (12.00%);[31] nonetheless, it is higher than some reports, such as 3.00% in Türkiye.[34] The BDV genome was found in 3.00% of tested goats in Iraq.[35] Variable higher results

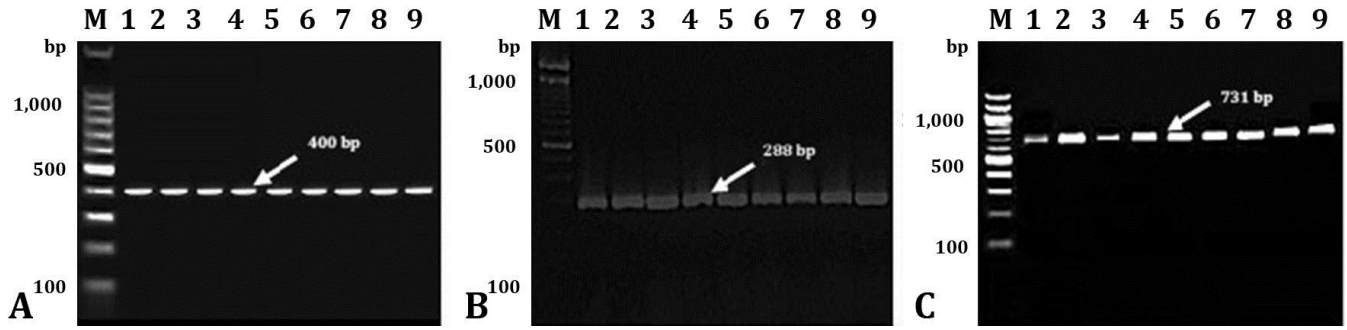

Fig. 3. Ethidium bromide-stained agarose gel. The RT-PCR was carried out on ribonucleic acid purified from lung tissues using specific primers for **A)** BVD, **B)** PIV3 and, **C)** RSV. Lane M: 100 bp molecular weight marker, Lanes 1-9: ELISA-positive samples.

were detected, like 9.00% in reviewed reports from 24 countries,[32] 33.00% in Italy,[37] and 50.00% in Egypt.[38] The highest prevalence in goats was also observed in the Khartoum State. Pestivirus prevalence was noticed to be higher in sheep than goats; this observation was reported elsewhere.[34] The RSV is one of the main causative agents of respiratory infections in humans and different animal species. In the present study, viral antigen was detected in 18.00% of sheep lungs; this is higher than reported prevalence in Sudan (5.00%),[22] and slightly higher than the report from Ghana and Nigeria (14.00%).[3] Higher prevalence was detected molecularly in Iraq (23.00%),[39] and more higher antigen detection was reported in Türkiye (50.00%).[19] Within states, the highest prevalence of RSV was noticed in the River Nile State (26.00%), unlike the previous report where the highest prevalence was found in the White Nile State (6.00%).[22] Prevalence of RSV antigen was detected in 5.00% of goat lungs in this study; the same result was reported previously in Sudan[22] and Türkiye,[40] and higher prevalence was detected in Nigerian goats (10.00%)[20] and goat lungs in Türkiye (54.00%).[19] Unlike the results in sheep, the highest prevalence of RSV in goats was detected in the White Nile State. Prevalence of RSV was found to be far higher in sheep than goats, unlike the previous report in Sudan where the prevalence in sheep and goats was almost the same.[22]

Co-infection of respiratory viruses is frequently occurring and known to increase the disease syndrome in different animal species. In this work, PIV-3 and pestivirus co-infection was seen in 6.00% of sheep and 4.00% of goat samples; association of PIV-3 and BVDV in respiratory infections in cattle was documented previously.[41] Serological evidence for PIV-3 and BVDV, and other respiratory viruses co-infection as dual infection was observed as 34.50%, while triple viral antibodies were detected in 31.00% of sheep and goats in Türkiye,[42] as well as 0.40% of cattle in Brazil.[43] Serological evidence of PIV-3 and BVDV infections in sheep and goats were reported in Bulgaria.[44]

In this work, co-infection of pestivirus and RSV was detected in only three goat samples (1.40%) and the highest prevalence (5.00%) was seen in the Khartoum State. Co-infection of PIV-3 and RSV was not found in our study; however, antigens of both viruses were detected in sheep and goat lungs in Türkiye.[19] Existence of PIV-3 (56.00%) and RSV (6.50%) in goat nasal swabs was reported in China,[10] and PI3 and RSV co-infection was found in 8.00% of tested Nigerian goats.[20]

Multiple viral co-infections were observed through antigen detection in sheep lung tissues in Nigeria. Ten cases (14.30%) were positive for peste des petits ruminant's virus (PPRV), PIV-3, and RSV; five (7.20%) were dual, including two PIV-3 and PPRV, two RSV and PPRV, and one PIV-3 and RSV.[3] The PPRV, PIV-3, and RSV antigens were detected in 81.00% of tested goat lungs in Nigeria; 79.00% as single, where co-infections were 11.00 dual and two triple.[45] In the present study, co-infection of the three viruses was detected in only one goat sample (1.00%) in the River Nile State. Antibodies to PIV-3, RSV, and BVDV were reported in 3.00% of sheep in Spain; 12.00% for PIV-3 and RSV, 1.00% for PIV-3 and pestivirus, and 1.00% for RSV and pestivirus;[46] antibodies to the three viruses were detected only in 0.50% of animals, while antibodies to RSV and BVDV were found in 55.00% of Brazilian sheep.[47]

It was concluded from the results of this study that PIV-3, BVDV, and RSV infections are circulating in sheep and goats in different areas of Sudan. To our knowledge, this is the first report of the existence of dual and triple infections of these viruses in sheep and goats in Sudan. The results of this study can lead to promotion in health and the breeding of herds, as well as the production of local vaccines against these diseases in the country.

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Conflict of interest

The authors declare that there is no conflict of interest.

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