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Detection and Isolation of Bovine Parainfluenza Virus Type 3 (PIV-3) in Cattle in North Kordofan State, Sudan

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Abstract
Bovine parainfluenza virus type3 (BPIV-3) is a virus that causes respiratory infection in cattle worldwide. A total of 100 lung specimens condemned due to pneumonia were collected from Elobied slaughter house at North Kordofan state. Sandwich ELISA was used to detect BPIV-3 antigen in lung tissue specimens, Vero cells were used to isolate PIV-3 virus. Direct immunoflourcent antibodies test (FAT) was used to confirm the ELISA positive results of BPIV-3 antigen in lung tissue specimens as well as for the identification of the isolated virus. PCR was used for detection of BPIV-3 genome. PIV-3 antigen was detected in 20% of the 100 cattle lung tissue specimens tested by ELISA while it was detected in 25 (25%) cattle lung specimens tested by FAT. Using FAT, all ELISA positives for PIV-3 and 5 of the 80 ELISA negative specimens were found to be positive. One sample was isolated in Vero cells. PIV-3 genome was detected in all samples tested by PCR.
Keywords: Parainfluenza virus, Cattle, ELISA, FAT, Virus isolation, Sudan.

Introduction
Respiratory disorders are of major concern for Bovidae. They occur in all countries that practice intensive livestock farming. Bovine respiratory diseases (BRD) complex is a major cause of economic losses in the dairy cattle industry. Viruses and bacteria in combination with stress play a key role in triggering acute respiratory infections. It is generally accepted that viruses are the first pathogens to intervene, whereas bacteria act as the second invaders to worsen the ill animal's condition (Valarcher and Hägglund, 2006, Solís-Calderón *et al*., 2007). The most important viral agents causing respiratory infections are bovine viral diarrhoea virus (BVDV), bovine herpes virus type 1 (BHV-1), bovine respiratory syncytial virus (BRSV), parainfluenza virus type 3 (PIV-3) and bovine adenovirus (BAV) (Hägglund *et al.*, 2007).

Bovine parainfluenza virus -3 (BPIV3) is a long-recognized, currently underappreciated, endemic infection in cattle populations. Clinical disease is most common in calves with poor passive transfer or decayed maternal antibodies. It is usually mild, consisting of fever, nasal discharge, and dry cough. Caused at least partly by local immunosuppressive effects, BPIV3 infection

is often complicated by co infection with other respiratory viruses and bacteria, and is therefore an important component of enzootic pneumonia in calves and bovine respiratory disease complex in feedlot cattle (Ellis, 2012).

In Sudan, Eisa *et al*. (1979) reported the occurrence of antibodies to PIV-3 in sera of some domestic animals. Khalifa and Khallafallah (1974) reported that the mortality rate in bull and heifer calves of Kenana cattle at Ombenin livestock improvement center during 1964-1973 was 26.4% and 20.7%, respectively; the most common causes of death were pneumonia and digestive troubles. Since that time no attempt has been made to study BPIV-3 in cattle. However, Intisar *et al.* (2010) reported the first detection of Parainfluenza virus type 3 antigen and antibodies in camels. This study is intended to investigate the role of BPIV-3 in causation of respiratory infection in cattle in North Kordofan State through the detection of its antigen, antibodies and genome as well as the isolation of the virus.

Materials and Methods

Collection of specimens:

Cattle pneumonic lungs (n =100) were collected from Elobied central slaughter houses after the slaughter of apparently healthy animals and were put in sterile plastic bags and kept at-20º C until used.

BPIV3 antigen detection using ELISA:

Sandwich ELISA kits for detection of parainfluenza virus 3 (PIV3) antigen purchased from Bio-X Diagnostics (Jemelle, Belgium) were used. The test was carried out as instructed by the manufacturer.

Preparation of lung tissues:

One gram of lung tissue was put in sterile pestle and mortar with 2ml of lysis solution (provided with the kits). The lung tissue was cut into small pieces with a pair of sterile scissors, homogenized by grinding, and then transferred to a 15 ml sterile tube and centrifuged at 500×g for 10 min. The supernatant was collected in new sterile tube for testing.

Direct fluorescent antibody technique (FAT) for the detection of PIV3 antigen:

FAT was used to confirm the ELISA antigen positive results and to identify the isolated virus; the conjugate used was purchased from Bio-X Diagnostics (Jemelle, Belgium) and was used as instructed by the manufacturer.

Detection of BPIV-3 Genome:

Total RNA Extraction from Animal tissues using the QIAGEN RNeasy Kits:

The kits were used as instructed by the manufacturer.

RT/PCR:

Qiagen one step RT-PCR Kit was used, a pair of primers targeting HA gene were used, its sequence were PI3 A 5' TGTGCATGGTGAGTTCGCA 3', PI3 BR 5' ATTCAGCATCACGTGCCACTG 3', the expected amplicon size is 164 bp.

In a 0.2 ml eppendorf tube, 5µl RNA Template was added into 45 µl mastermix. The tubes were placed into Techne, TC-512 PCR thermocycler machine. The amplification conditiones were Reverse transcription in one step RT/PCR kit reagents at 50^{o}C for 30 minutes followed by 94^{o}C for 15 minutes this was followed by 40 cycles of PCR, denaturation at 94^{o}C for 30 seconds, annealing at 56^{o}C for 30 sec, and elongation of 72^{o}C for 30 sec, followed by final extension at 72^{o}C for 10 minutes.

Isolation of BPIV3 in tissue culture:

Three lung tissue specimens of sandwich ELISA positives for PIV-3 were inoculated into African green monkey kidney cells (Vero cells). Samples were prepared aseptically after the addition of antibiotics and antifungal.

Preparation of cattle lung tissue

The lung tissue fragments were placed in sterile pestle and mortar and cut with scissor and forceps into small pieces with little amount of phosphate buffer saline (PBS) and 1% antibiotics and antifungal were added. The homogenate was adjusted to 20% then transferred to 15 ml test tube and centrifuged at 800 g for 10 minutes, the supernatant was separated and collected in sterile tube and stored at – 20^{o} C until used.

Inoculation in Vero cells:

0.3 ml of the supernatant of homogenated lung tissue was used to inoculate the confluent monolayer in 25cm disposable tissue culture flasks after discarding the media, the inoculated cells were incubated at 37^{o} C for 2 hours. The inoculum was discarded and the cells were washed 3 times with sterile PD containing antibiotic and anti-fungal, unconsumed media without serum was added to the inoculated and control cells (un infected Vero cells). The flasks were observed daily under the inverted microscope for the appearance of cytopathogenic effect (CPE). Three blind passages were done before the sample was considered negative.

Identification of isolated viruses:

Tissue culture harvests were identified as PIV3 using ELISA, FAT and PCR.

Results

Detection of BPIV3 antigen:

Using ELISA, out of 100 lung tissue specimens tested, 20 were found to be positive (20%) for B PIV-3 antigen detection,13 of them were males and 7 of them were females, the details are presented in Table (1).

Table 1: Results of Sandwich ELISA test for BP1V-3 antigen detection in cattle lung tissue specimens collected from slaughter houses in Elobied, North Kordofan State.

Total tested	Males			Females			Total +ve
	Tested	+ve	%	Tested	+ve	%	
100	68	13	19.1%	32	7	21.9%	20

Detection of PIV3 antigen using Fluorescent antibody technique (FAT):

Fluorescent antibody technique (FAT) was used to detect PIV-3 antigen in lung tissues and to compare the results with that of ELISA. FAT was also used to identify the isolated virus in tissue culture. Using FAT, all ELISA positives for PIV-3 and 5 of 80 ELISA negative specimens were found to be positive. BPIV-3 antigen was detected in 20% of 100 cattle lung tissue specimens tested by sandwich ELISA while it was detected in 25 (25%) cattle lung specimens tested by FAT.

Results of FAT for PIV-3 antigen detection in cattle lung tissue specimens and the comparison between sandwich ELISA and FAT test is presented in Table (2). Positive and negative lung tissue specimens tested by FAT are shown in Figures (1a and 1b).

Table 2: Comparison between sandwich ELISA and FAT for PIV-3 antigen detection in cattle lung tissue specimens

FAT	ELISA		
	Positive	Negative	Total
Positive	20	5	25
Negative	-	75	75
Total	20	80	100

Figure 1a: Negative specimen with FAT for PIV-3 antigen detection.

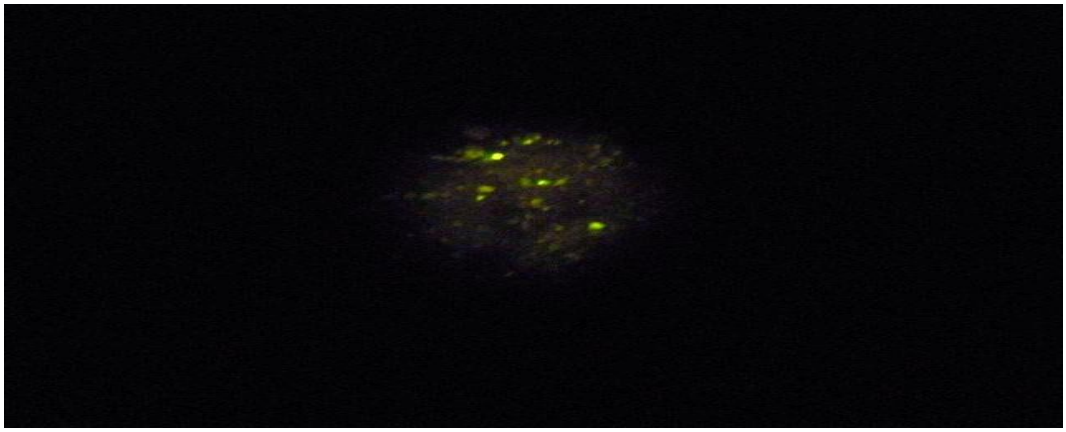

Figure 1b: Positive specimen with FAT for PIV-3 antigen detection

Isolation and identification of B PIV3 in tissue culture:

Three PIV-3 ELISA positive specimens were inoculated in Vero cells. After 2 passages, cytopathic effect (CPE) appeared after 3 days of inoculation as cells aggregation with rounding and floating cells, some multinucleated cells, elongation of the cells, cells sloughing from the intact sheet; the CPE reached more than 80% within 7-10 days post inoculation.

Uninfected Vero cells which were used as control is shown in Figure (2a) and cytopathic effect of PIV-3 in Vero cells is shown in Figure (2b).

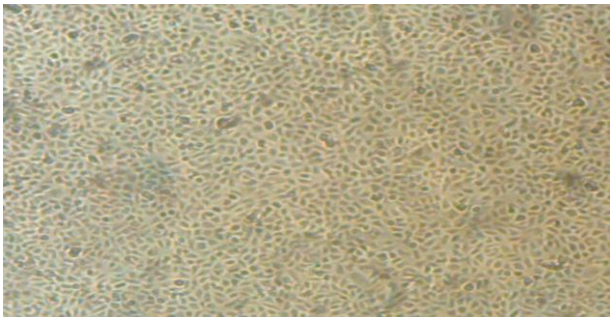

Figure 2a: Uninfected Vero cells used as control

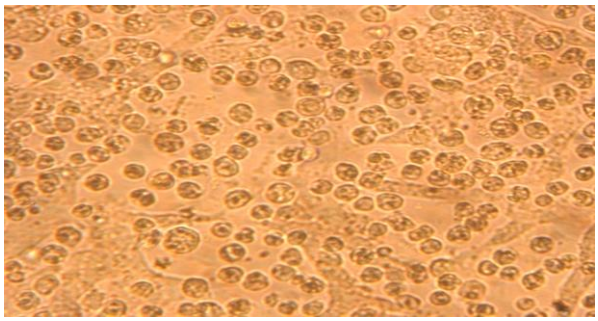

Figure 2b: Cytopathic effect of PIV-3 virus in vero cells

Reverse transcriptase polymerase chain reaction (RT-PCR):

When using RT-PCR test, (all) positive specimens by ELISA and FAT were found to be positive by RT-PCR. Gel electrophoresis for PIV-3 genome detected on 2 lung tissue specimens and isolated virus is presented in Figure (3).

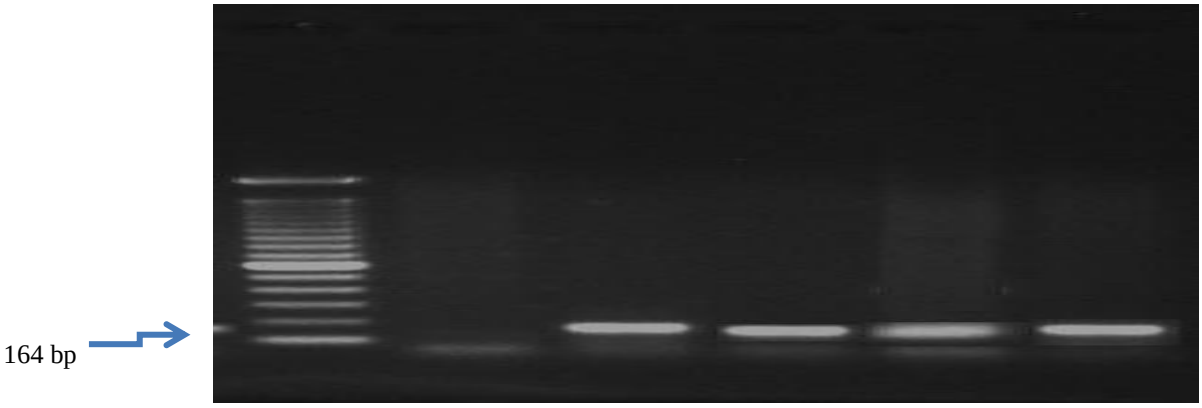

Figure 3: Gel electrophoresis of PCR product for the detection of PIV3 genome, expected amplicon size is 164 bp, M, 100 bp DNA ladder, Lane 1 negative control, Lane 2 positive control, Lanes 3, 4 tested samples, Lane 5, the isolated virus.

Discussion:

Parainfluenza virus3 is one of the viruses known to cause respiratory infection. The disease itself is not serious if there are no complications because the infection generally runs a clinical course of 3–4 days with complete recovery (Blood and Henderson, 1976, Murphy *et al.*, 1999). However infection with PIV-3 appears to predispose the host to secondary bacterial infection and it has also been shown to play a role in shipping fever. Furthermore, the strong active immunity that results from PIV3 infection is short lived and therefore does not protect the animal from repeated infections or from the threat of shipping fever if the animal is exposed to stress (Blood and Henderson, 1976, Murphy *et al.*, 1999). In Sudan, Abass *et al.* (1993) reported that respiratory disease was reported by 28.6% of camel pastoralists in Eastern Sudan.

In this study, Using ELISA, parainfluenza virus 3 antigen was detected in 20% of lung tissue specimens, 13% of them were males while 7% were females. Intisar *et al.* (2010) stated that the detection of PIV3 antigen in camel lung tissues in Sudan was reported for

the first time using sandwich ELISA; the highest prevalence of PIV3 antigen was observed in specimens from Eastern Sudan. The prevalence of PIV3 antigen detected in this study is far higher than that previously detected in camels (2.1%); this indicates the wide spread of this infection in cattle. In the present study, direct immunofluorescent technique used in this study detected PIV3 antigen in 25% of the samples compared with ELISA for detection of parainfluenza virus 3 antigen in lung tissue specimens, the virus antigen was detected in some specimens which were negative by ELISA meanwhile all ELISA positives were positive by FAT, this indicate the higher sensitivity of FAT over ELISA in detecting PIV-3 antigen. These results are in agreement with the previous reports stating the higher sensitivity of FAT over ELISA (Horacio *et al*., 1989, Intisar *et al*., 2010, Nada, 2012).

Many authors used FAT for viral antigen detection; in most of these reports FAT was found to have higher sensitivity over ELISA. Melvin *et al.* (1971) reported the use of FAT for rapid diagnosis of PIV-3 infections in children. FAT was used for testing nasal swabs collected from cattle showing respiratory disease, PIV-3 antigen was detected in these specimens (Alkan *et al*., 2000). Collin *et al.* (1996) reported that the confirmation of the identity of PIV-3 is usually achieved by FAT. Hornsleth *et al.* (1982) detected respiratory syncyatial virus (RSV) by ELISA in 37 (79%) and by FAT in 43 (91%) of 47 patients, he stated that FAT technique was found to be more sensitive than ELISA, especially for examination of specimens from nasopharyngeal secretions containing only small percentage of antigen positive cells.

In the present study, the isolation of PIV-3 from cattle lung specimens in Vero cell culture was adopted. Typical PIV-3 CPE have appeared; rounding refractile cells, cells elongation and sloughing with some syncytia formation, which are similar to that described by Henrickson (2003) and Intisar *et al.* (2010). The virus was identified by FAT and PCR. Isolation of PIV-3 in different cell cultures was documented. Van der Maaten (1969) compared three tissue culture systems for BPIV3 isolation. Sarkkinen *et al.* (1981) reported that PIV-2 and PIV-3 were grown in roller culture of Vero cells. Deborah *et al*. (1992) stated that bovine parainfluenza virus 3 that was isolated from natural occurring case of BRD was grown in Vero cells.

In this study RT-PCR was applied on PIV3 ELISA positive cattle lung specimens, the results of this test showed that PCR technique was rapid and highly sensitive and specific, currently it became alternative way for the diagnosis of PIV-3. Our results are in line with the previous reports, recently, the use of one- step multiplex real time PCR for PIV-3 in clinical samples from cattle has been done by Leenadevi *et al.* (2012), they reported that the RT-PCR assay was rapid, highly repeatable, specific and had a sensitivity of 97%. Rodrigo *et al.* (2008) applied RT-PCR in three Brazilian isolates and they reported the potential use of this technique for detection of bovine parainfluenza virus 3 in bovine clinical specimens.

This study showed the significant role of respiratory infection in health condition of cattle at North Kordofan as well as the wide spread of PIV-3 in cattle. Further work to characterize the virus is needed.

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كشف وعزل فيروس نظير الانفلونزا البقري من النوع 3 فى ولاية شمال كردفان السودان

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المستخلص

يعتبر فيروس نظير الانفلونزا من النوع 3 في الابقار من الفيروسات واسعة الانتشار، ويسبب مشاكل تنفسية في الابقار. جمعت 100 عينة من الرئات المعدمة في سلخانة الابيض والتي تظهر فيها علامات الالتهاب الرئوي . تم استخدام تقنية الاليزا للكشف عن انتيجين فيروس نظير الانفلونزا من النوع 3 في الابقار في انسجة الرئة. تم عزل فيروس نظير الانفلونزا في خلايا كلية القرد الافريقى الاخضر . تم استخدام اختبار التالق المناعى لتاكيد النتيجة الموجبة لاختبار الاليزا لانتيجين الفيروس فى عينات انسجة الرئة . تم الكشف عن الحامض النووى للفيروس باختبار البلمرة التسلسلى. انتيجين فيروس نظير الانفلونزا من النوع 3 تم اكتشافة في 20% من 100 عينة من عينات انسجة الرئات التى تم اختبارها بواسطة الاليزا بينما تم اكتشافها في 25% من عينات الرئات التي تم اختبارها بواسطة التالق المناعى . باستخدام تقنية التالق المناعى كل العينات التي كانت موجبة لاختبار الاليزا بالاضافة الي 5 عينات من 80 عينة سالبة لاختبار الاليزا وجدت موجبة للتالق المناعى .عينة واحدة تم عزلها في خلايا كلية القرد الافريقى الاخضر وايضا تم اكتشاف الحامض النووى للفيروس نظير الانفلونزا 3 باختبار البلمرة التسلسلى.