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ANTIGENIC AND MOLECULAR IDENTIFICATION OF SOME VIRUSES CAUSING RESPIRATORY DISORDER IN CALVES

(With 4 Tables and 4 Photos)

By

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التعريف الأنجينى والجزيئى لبعض الفيروسات المسببة للاعراض التنفسية فى العجول

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يعتبر فيروس البارا-انفلونزا-٣ البقرى والفيروس التنفسى المتضخم البقرى من الفيروسات المنتشرة انتشارا كبيرا بين الابقار وتتسبب فى احداث مشاكل تنفسية جسيمة ونظرا لان تلك الفيروسات تكون دائما متشابهة الاعراض فان الحاجة الى التشخيص المعملى الدقيق والسريع اصبحت ملحة. لذلك تم تجميع عدد ١٨ عينة من عجول عليها اعراض تنفسية (عدد ١١ عينة مسحات انفية وعدد ٧ عينات من نسيج رئوى) من احدى المزارع بمحافظة كفر الشيخ. تم فحص هذه العينات للكشف عن فيروس البارا-انفلونزا-٣ باستخدام اختبار ادمصاص الدم واختبار الفلورسنت المناعى المشع وايضا تم الكشف عن الفيروس التنفسى المتضخم فى نفس العينات باستخدام اختبار الفلورسنت المناعى المشع واختبار الاليزا. تم استخدام اختبار الاستنساخ العكسى لاختبار تفاعل انزيم البلمرة المتسلسل للتاكد من وجود كل من الفيروسين فى العينات. اظهرت النتائج وجود فيروس البارا-انفلونزا-٣ فى ١٧ عينة بنسبة ٩٤،٤% باستخدام اختبار الاستنساخ العكسى لاختبار تفاعل انزيم البلمرة المتسلسل مقابل١٣ عينة ايجابية بنسبة ٧٢،٢% باستخدام اختبار الفلورسنت المناعى. وكذلك بالنسبة للفيروس التنفسى المتضخم تم التاكد من وجوده باستخدام اختبار الاستنساخ العكسى لاختبار تفاعل انزيم البلمرة المتسلسل فى ١٦ عينة بنسبة ٨٨،٩% مقابل ١٤ عينة بنسبة ٧٧،٨% باستخدام اختبار الاليزا. ونظرا للحساسية العالية لاختبار الاستنساخ العكسى لاختبار تفاعل انزيم البلمرة المتسلسل التى اثبتت من النتائج السابقة يوصى باستخدام ذلك الاختبار كطريقة مثلى لتشخيص فيروسى البارا-انفلونزا-٣ والفيروس التنفسى المتضخم.

SUMMARY

Bovine parainfluenza virus Type 3 (BPI3) and bovine respiratory syncytial virus (BRSV), are ubiquitous respiratory pathogens of cattle, which contribute to causation of bovine respiratory disease complex. As these respiratory viral pathogens cause very similar clinical signs, laboratory diagnosis of these pathogens becomes important. A total of 18 respiratory samples (11 nasal swab samples collected from diseased calves and 7 lung tissue specimens from dead calves) were collected from a farm in Kafer El-Sheikh Governorate. All specimens were analyzed for BPI-3 by heamadsorption and direct immunofluorescent techniques. The same samples were investigated for the presence of BRSV using indirect fluorescent antibody technique and ELISA. RT-PCR technique was used to confirm the presence of both viruses. BPI-3 virus was detected by RT-PCR assay in 17 of 18 (94.4%) samples tested versus to 13 of 18 (72.2%) were detected by FAT. Also, BRSV was detected by RT-PCR in 16 of 18 (88.9%) specimens tested versus to 14 of 18 (77.8%) were detected by ELISA technique. Considering the higher sensitivity of RT-PCR assay that revealed from previous data, it can be recommended as the method of choice in diagnosis of both BPI-3 and BRSV.

Key words: *Calves, bovine parainfluenza, respiratory diseases.*

INTRODUCTION

Respiratory diseases have had a major impact on the overall health of cattle and continue to be of great importance even today. Many of the diseases that have been shown to impact the respiratory tract of cattle have been grouped into an overall category known as bovine respiratory disease (BRD) complex. This includes shipping fever syndrome, enzootic calf pneumonia, acute respiratory distress syndrome, and atypical interstitial pneumonia (Baker 1995; Ames 1997; Apley 2006; Bolte, *et al.*, 2009). These diseases are considered the major cause of economic losses in cattle, sheep and goat industry (Zaki, *et al.*, 2000; Ellis, 2001). Pathogens that have been implicated in the causation of this complex include microbial and viral pathogens. In terms of microbial pathogens, the most common are *Pasteurella species* (Apley, 2006). Studies have shown that the major viral pathogens that contribute to BRD are: bovine parainfluenza – 3 virus (BPI-3), and bovine respiratory syncytial virus (BRSV) (Lehmkuhl and Gough, 1977; Gagea, *et al.*,

2006). These pathogens, along with stress and other environmental factors, have been shown to have a synergistic effect on each other so that the severity of the disease is worse with concurrent infections than with an individual pathogen (Brodersen and Kelling 1998; Godinho, *et al.*, 2007). In addition, viral pathogens have been show to weaken the host's immune response making the host more susceptible to opportunistic pathogens (Potgieter, 1995). BPI-3 is one of the most important agents associated with upper respiratory disease of cattle allover the world. The uncomplicated respiratory infection caused by PI-3 virus run a benign clinical course of 3-4 days with complete recovery. However, the true importance of the infection in cattle drives from its role in endemic pneumonia.

The term parainfluenza virus was originally coined because some of disease symptoms are influenza-like and because the virus particle, like that of influenza in having haemagglutination and neuraminidase activities (Field, *et al.,* 1996). BPI-3 was originally isolated from cattle with shipping fever (SF) and designated as SF_4 (Reisinger, *et al.,* 1959). Since then BPI-3 virus was incriminated in many respiratory disorders, in calves and other animal species (Kite, *et al.,* 1994; Steinhagen and Hubent, 1995). In Egypt BPI-3 virus was recorded by Hamdy, 1966; Atta and Singh 1967; *Baz, et al.,* 1986).

Parainfluenza-3 virus belongs to genus Respirovirus in the family Paramyxoviridea of order Mononegavirals, the non segmented negative single stranded RNA viruses (Pringle, 1991). The genomic RNA has approximately 15,462 nucleotides and composed of six genes (Field, *et al..,* 1996).

Bovine respiratory syncytial virus (BRSV) can cause severe lower respiratory tract infection in cattle. Clinical disease occur most often in young calves but adult cattle can develop severe disease as well (Schrijver, *et al.,* 1997).

RSV was named for the characteristic merging of cells it causes, which forms multinucleated masses of protoplasm called syncytial (Baker and Frey, 1985; Zeidan, *et al.,* 2000). BRSV was 1st isolated from cattle with respiratory disease in Switzerland in 1970 by Paccaud and Jacquier, 1970 and was reported in 1974 from cattle in United States (LeBlanc, *et al.,* 1991). The first isolation in Egypt from cattle was conducted by Saber, *et al.,* 1996. Seroepidemiologic surveys for antibodies to BRSV have indicated that the virus is wide spread, seropositivity up to 80% (Collins, *et al.,* 1988; Bastawecy, *et al.,* 2002). BRSV is a single- stranded negative-sense RNA virus which belongs to

the Pneumovirus genus, a member of the paramyxoviridae family. Its genome has approximately 15,140 nucleotides. The viral genome is transcribed into 10 subgenomic $mRNA_s$ which encode for 11 different proteins (Huang and Wertez, 1982; Arns, *et al.*, 2003).

Although there is a large list of pathogens that contribute to BRD, the clinical signs of infection are very similar. Typical signs include rapid respiration, anorexia, nasal and /or ocular discharge, depression, fever, interstitial pneumonia and reproductive failure (Brock 2004; Solis-Calderon, *et al.,* 2005; Apley 2006). Because of the similarities in clinical presentation, so the aim of the current study is to develop methods for quick and accurate diagnosis of the cause of infection.

MATERIALS and METHODS

Samples:

A total of 11 nasal swab samples were collected from diseased calves suffering from respiratory manifestation and 7 lung tissues were collected from dead calves on a farm in Kafer El-Sheikh Governorate.

Cells: MDBK cell (Madien Derby Bovine kidney) obtained from Virology Department, Animal Health Research Institute, Dokki Cairo used for blind passages of the samples

Viruses:

- Local Egyptian BPI-3 virus strain45 of a titer $10^{6.2}$ $TCID_{50}$/ml was kindly supplied from Rinder Pest like disease Department at Veterinary Serum and Vaccine Production Institute, Abbassia, Cairo
- Local Egyptian strain of BRSV was supplied by Animal Health Research Institute Dokki, Cairo. The virus titer $10^{5.8}$ $TCID_{50}$/ml. Each virus was calculated according to Reed and Muench (1938).

Antisera:

Locally prepared in rabbit in Virology Department Animal Health Research Institute according to Grist (1979).

Conjugates:

Anti rabbit FITC conjugate was supplied by Sigma immunochemical used in indirect FAT.

- Rabbit antibovine Horse raddish peroxidase diluted at 1:1000 in diluting buffer used in ELISA.
- Standard anti bovine PI-3 serum conjugated with FITC used in direct FAT.

Identification of BPI-3 virus using heamadsorption: The test was applied according to Elizabeth, *et al.* (1997) on infected MDBK cell and examined under inverted microscope.

Fluorescent antibody technique for detection of BPI-3 & BRSV: The test was conducted according to Coons (1956) using frozen cryostat section from the seven diseased lung tissue samples at 5-8u and for samples inoculated on MDBK in order to detect viral antigen of BPI-3 by direct FAT according to Vander Hide (1971) and indirect FAT used for detection of the BRSV was applied according to Vilcek, *et al.* (1994).

Solid phase ELISA for detection of BRSV: The test was conducted according to Voller, *et al.* (1979) the calculation and determination of cut off value were according to Peterfy, *et al.* (1983).

Reverse transcriptase polymerase chain reaction (RT-PCR):

Nine positive BPI-3 and BRSV nasal swab samples as well as the 5 positive BPI-3 and 4 positive BRSV of lung tissue samples were pooled independently for each virus to be identified using RT-PCR. The negative specimens (5 samples for BPI-3 & 4 samples for RSV) were also investigated independently by RT-PCR technique for confirmation.

RNA extraction:

The viral RNA_s were extracted according to Chomczynski and Sacchi (1987) using acid guanidinium thiocyanate-phenol-chloroform extraction. The RNA_s were precipitated in isopropanol and the pellet washed in cold ethanol and centrifuged, then the pellet was air dried and redissolved in 50ul diethylpyrocarbonated-treated water (DEPC) (Sigma). The samples (1ul) of these total RNA_s were reverse transcribed and the resulting cDNA were used as templets for PCR reactions using specific oligonucleotide primers listed in Table (1). The oligonucleotide primers were designed according to the published sequence of BPI-3 by Lyon, *et al.* (1997) and the published sequence of BRSV by Van der poel, *et al.* (1997). The primers define a 400bp and 204bp segments of BPI-3 and BRSV genomes respectively in the regions encoding the fusion (F)-protein.

Table 1: Primers used in RT- PCR reactions of BPI-3 and BRSV

Target	Name (strand)	Primer sequence
BPI-3	PS1873 (F)	5'-CATTGAATTCATACTCAGCAC-3'
	PAS2273 (R)	5'-AGATTGTCGCATTT(AG)CCTC-3'
BRSV	(P751) (F)	5'-GTGCATTAAGAACTGGATGG-3'
	(P752) (R)	5'-GCAAAAAGAGGGATACCAGAGT-3'

PCR amplification:

Briefly, the templates and primers were mixed with Taq polymerase in a volume of 50ul of amplification buffer. The RT-PCR reaction was performed in a programmable DNA thermal cycler according to the cycling protocols listed in Table (2).

Table 2: Cycling protocols for amplification of F gene of both BPI-3 and BRSV

Target	Amplicon size	Cycling condition			No. of cycle
		Step	Temp.	Time	
BPI-3 F gene	400 bp	Initial denaturation	94°C	2 min.	1 cycle
		Denaturation	94°C	45 sec.	35 cycles
		Annealing	51°C	45 sec.	
		Extension	72°C	1 min.	
RSV F gene	204 bp	Denaturation	94°C	1 min.	38 cycles
		Annealing	58°C	1 min.	
		Extension	72°C	1 min.	

Analysis of PCR products using agar gel electrophoresis:

The analysis were carried out according to Sambrook, *et al.* (1989). Briefly 10ul of each PCR product was loaded on agarose gel (1.5% for PI-3 and 1% for RSV), containing 1ul/ml ethidium bromide in Tris-acetate buffer. Positive control viruses as well as DNA-Marker Ladder were also included.

RESULTS

The collected samples propagated on MDBK cell. Results of heamadsorption are shown in Photo (1-A). It demonstrates number of G. pig RBC_s adsorbed on the surface of monolayer of infected MDBK cell while there is no RBC_s adsorbed on the sheet of normal non infected cells Photo (1-B). The results were positive in 12 of 18 (66.7%) specimens tested as shown in Table (3).

Direct FAT used for detection of BPI-3 virus revealed observed intracytoplasmic fluorescence in 13 out of 18 (72.2%) tested slides of infected MDBK cell as shown in Photo (2-A). Also the indirect FAT used for detection of BRSV revealed positive fluorescence in 11 of 18 (61.1%) tested slides as shown in Photo (2-B). The ELISA technique

detected a higher percentage of BRSV positive samples (77.8%) than the FAT did. The detailed data are shown in Tables (3&4).

The immunofluorescence test performed on sections of the lung tissue of seven dead animals revealed 4 positive lung tissue samples of either BPI-3 or BRSV.

Detection of BPI-3 and BRSV using RT-PCR are shown in photo 3 and 4 respectively.

The detection of BPI-3 or BRSV by different used methods are presented in Tables (3 & 4)

Table 3: The detection of BPI-3 by different used methods

Type of sample	No. of sample	Diagnostic method of used					
		Heamadsorption		FAT		RT-PCR	
		No. of +ve	No. of -ve	No. of +ve	No. of -ve	No. of +ve	No. of -ve
Nasal swabs	11	8	3	9	2	10	1
Lung tissues	7	4	3	4	3	7	0
Total	18	12	6	13	5	17	1
%		66.7%	33.3%	72.2%	27.8%	94.4%	5.6%

Table 4: The detection of BRSV by different used methods

Type of samples	No. of samples	Diagnostic method of used					
		FAT		ELISA		RT-PCR	
		No. of +ve	No. of -ve	No. of +ve	No. of -ve	No. of +ve	No. of -ve
Nasal swabs	11	7	4	9	2	9	2
Lung tissues	7	4	3	5	2	7	0
Total	18	11	7	14	4	16	2
%		61.1%	38.9%	77.8%	22.2%	88.9%	11.1%

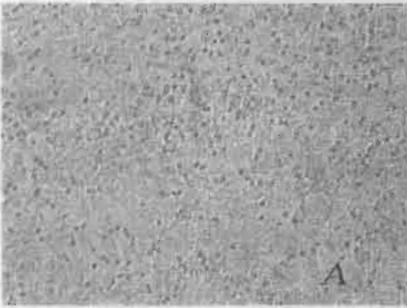

Photo (1-A) +ve heamadsorption

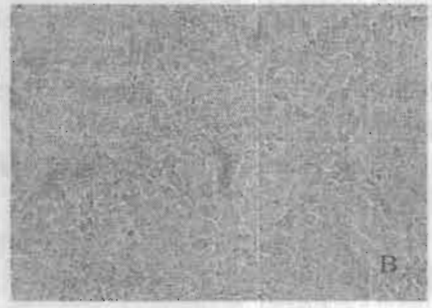

Photo (1-B) normal MDBK cell

Photo (2-A) Shows observed intracytoplasmic fluorescence of PI-3V

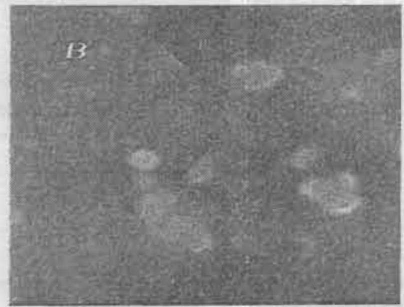

Photo (2-B) Shows intracytoplasmic fluorescence with some syncytia of BRSV

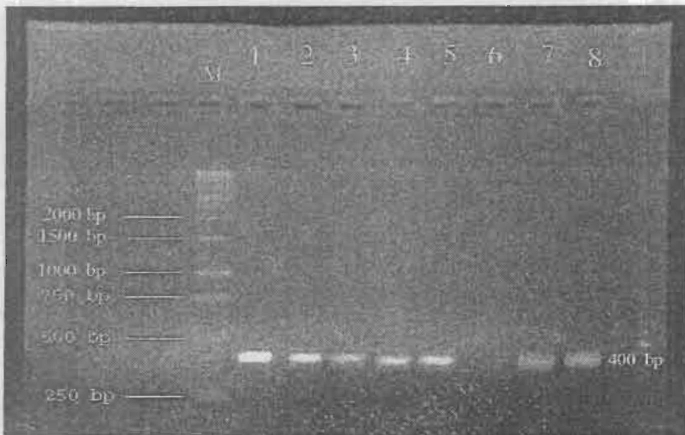

Photo 3: Shows ethidium bromide stained 1.5% agarose gel electrophoresis of PCR product of BPI-3 virus. Lane M: 1KB DNA ladder. Lane 1: control +ve. Lane 2&3: +ve samples from N.S & lung tissue Lane 4 to 8: five –ve samples that even could not detected by FAT.

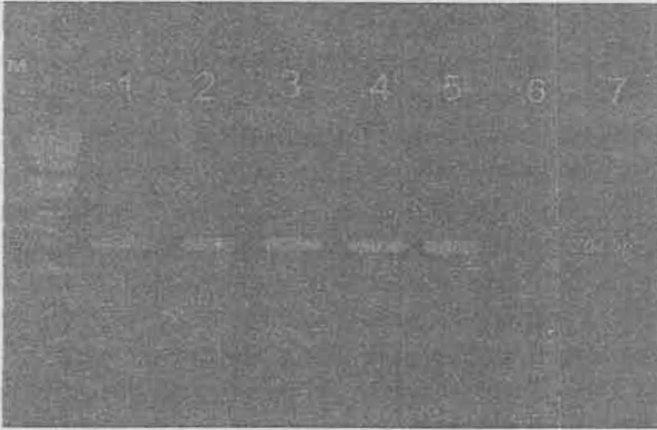

Photo 4: Shows ethidium bromide stained 1% agarose gel electrophoresis of PCR product of BRSV. Lane M: 100bp DNA ladder. Lane 1: control +ve. Lane 2 &3: +ve samples from N.S & lung tissue Lane 4 to 7: the four –ve samples that even could not detected by ELISA.

DISCUSSION

World wide, many preceeding studies have demonstrated a negative impact of bovine respiratory disease complex on cattle industry. Recently, BRSV has been reported to be responsible for 14 to 71% of the respiratory diseases (Ames, 1997). This virus is responsible for a high morbidity (60 to 80%) and a mortality that can reach 20%. However, BPI-3 infections cause less serious disease than BRSV (Verhoeff and van Nieuwstadt, 1984) which has a worldwide distribution and high serum antibody prevalence in adult animals (Bryson, 1990). Although the role of PIV-3 remains ambiguous as it seems, in some cases, responsible of respiratory signs but in other it is clinically unapparent or causes only mild disease (Graham *et al.,* 1999) it nevertheless significantly correlated with respiratory diseases in cattle (Stott *et al.,* 1980). The virus is thought to have a predisposing role in shipping fever and enzootic pneumonia. The predisposing role of BPIV-3 in bovine respiratory diseases is probably correlated to its immunosuppressive effects. (Adair *et al.,* 2000).

Because of the similarities in clinical presentation, it is important to develop methods for quickly and accurately differentiation of the cause of infection. Therefore, our article planed to fulfill towards the

application of some of antigenic and molecular methods with special reference to RT-PCR technique for identification of these two viruses.

Immunoflurescence (IF) test Performed in this work on sections of lung provide a rapid diagnosis for both BPI-3 virus and BRSV, but its limitation are obvious when dealing with epizootics of respiratory disease with low mortality (Kimman, *et al.,* 1986). As well the specimen integrity and the number of intact cells present in sample may be crucial for a reliable direct immunofluorescent assay (Reina, *et al.,* 1995). Also this direct antigen testing often lacks sensitivity and require confirmation by indirect antigen testing following specimen culture (Fan and Henrickson, 1996) for this reason we propagated all samples on tissue culture for three blind passages and detected by FAT the results revealed that the percentages of positive samples were (61.1%) and (72.2%) for BRSV and PI-3 virus, respectively. These results agree with previous results of (Vilcek, *et al.,* 1994).

Virus isolation was not attempted, since it is not considered to be a practical diagnostic method of choice (Vilcek, *et al.,* 1994) as the viral isolation is complicated by the high sensitivity of the agent and the fact that several passages are necessary before CPE develops (Valentova and kovarcik, 2003). Also the isolation is a laborious procedure with unpredictable results because animal that develop the disease are not the best of choice as in most cases of BRSV or BPI-3 is obtained during isolation procedures for other viral pathogens rather than procedure specifically carried out for those viruses (Arns, *et al.,* 2003). Moreover, tissue samples containing high concentrations of those viral antigens frequently do not reproduce the virus in cell cultures (Dubovi, 1993).

BPI-3 virus was detected in 12 of 18 (66.7%) samples tested by haemadsorption technique on infected MDBK cell culture. The test considered good indicator system for the presence of BPI-3 virus beside it proved to be more rapid and sensitive than waiting till the cytopathic change appearance. This finding is similar to that described previously by St.George, (1969). Also, this, agreed with the results of Toth and Jankura (1990) who diagnosed BPI-3 by both heamadsorption technique and immunofluorescence where both techniques match with each other for demonstration of PI-3 virus in cell culture however the haemadsorption offers several advantages over fluorescent antibody technique as it is more easy to read and does not require especial equipment for observation.

BRSV was detected in 14 out of 18 (77.8%) total specimens' analyzed using ELISA technique this results agreed with previous results

of West, *et al.* (1998) Graham, *et al.* (1999) Hazari, *et al.* (2002). They concluded that ELISA technique has been shown to be an invaluable test for determining the presence of BRSV in clinical samples. ELISA has been shown to be highly sensitive, specific, rapid, Alternative methods such as virus isolation and immunohistochemistry are time-consuming and laborious and results may be difficult to interpret whereas ELISA technology lends itself to large-scale testing of a greater number of samples (Hill, *et al.,* 2007). However, it requires use of viral protein specific antibodies for detection.

Molecular methods for the diagnosis of viral infection are now well established in routine virology laboratory and have replaced conventional techniques, such as viral isolation by cell culturing or detection of a virus-specific antibody response, approaches which, in comparison, are slow or lack sensitivity. (Kalvatchev, *et al.,* 2004). The wide spread use of PCR has improved the laboratory diagnosis and understanding of viral etiology of different clinical syndromes.

RT-PCR assays that performed in our study were standardized to amplify fragments of 400bp and 204bp corresponding to parts of (F) gene of both BPI-3 and BRSV genomes respectively. The (F) gene was chosen as the target for the RT-PCR assay to become of wide detection range because it is one of the most conservative genes in the family Paramyxoviridae and consequently, represent a good alternative to be used for virus detection in calves with unknown history about those viruses (Renata, *et al.,* 2004) therefore, this gene is a good choice for our study.

Our result of BPI-3 (F) gene RT-PCR assay (PCR-F) revealed the detection of viral RNA in 17 of 18 (94.4%) samples tested comparing with 72.2% detected by FAT. Also respiratory syncytial viral RNA was detected in 16 of 18 (88.9%) sample tested comparing with 77.8% detected by ELISA technique indicating with no doubt that (PCR-F) assay is more sensitive than the conventional methods in diagnosis of both BPI-3 and BRSV. This result agreed with the published data of Vilček, *et al.* (1994) West, *et al.* (1998) and Renata, *et al.* (2004) who indicated that RT-PCR technique is much more sensitive than the conventional virological methods.

Many of previous studies employed successfully fusion protein gene of BPI-3 virus in RT-PCR assays (Karron, *et al.,* 1993; Lyon, *et al.,* 1997; Maria *et al.,* 1998).

Several RT-PCR for RSV detection based on the fusion (F) gene (Vilček, *et al.,* 1994; Van der poel, *et al.,* 1997 and Valentova, *et al.,*

2003) have been developed and evaluated in studies involving numerous field specimens. Therefore RT-PCR is potentially useful for improving the sensitivity of RSV detection.

In conclusion the respiratory disease-complex considers one of the most important causes of economic impact in livestock especially in calves so the rapid and accurate diagnosis of the viruses causing this disease is important. The use of sensitive and rapid techniques as FAT and ELISA was beneficial but the use of RT-PCR technique proved to be much more reliable, quick, sensitive and easy method of detection of virus. So it is considered a gold standard test for diagnosis of bovine parainfluenza-3 and bovine respiratory syncytial virus.

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