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EVALUATION OF REVERSE TRANSCRIPTION-PCR PROTOCOLS BASED ON THE FUSION GENE FOR DIAGNOSIS OF BOVINE RESPIRATORY SYNCYTIAL VIRUS INFECTIONS

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Abstract: Bovine respiratory syncytial virus (BRSV) is a pneumovirus in the family paramyxoviridae, is an important cause of acute respiratory disease in postweaning calves and feedlot cattle. The real-time reverse transcriptase PCR protocols were developed to detect BRSV infection in infected animals. The sensitivity of RT-PCR protocols based on fusion gene were evaluated using different Mastermixes such as Qiagen One Step RT-PCR (Qiagen) for conventional RT-PCR, Superscrip probe (Invitrogen) and QuentiTec probe (Qiagen) for real-time RT-PCR with and without internal control. The detection limit of different RT-PCR protocols using serial dilutions from BRSV plasmid and based on different probes was 10 RNA copies/ml. Furthermore, the specificity of real-time RT-PCR was evaluated using different bacterial and viral strains which can be isolated from respiratory infected animals. In another side, the real-time RT-PCR in combination with ß-actin and conventional RT-PCR showed detectable Ct-values only with BRSV strain.

Keywords*:* BRSV, real-time RT-PCR, conventional PCR, internal control.

Introduction

Bovine respiratory syncytial virus (BRSV) is a fragile RNA, a single-stranded negative-sense RNA virus which belongs to the pneumovirus genus, a member of the Paramyxoviridae family. Its genome has approximately 15140 nucleotides and encodes 10 different mRNA molecules (*Buchholz et al., 1999; Grubbs et al., 2001*)

These viruses may cause respiratory tract infection and disease but often predispose the cattle to the bacterial pathogens. Additional viruses associated with

bovine respiratory disease. BRSV appears to be an important virus in the bovine respiratory disease complex because of its frequency of occurrence, predilection for the lower respiratory tract, and its ability to predispose the respiratory tract to secondary bacterial infection (*Ames, 1993*).

The virus is transmitted when an animal that is infected sheds the virus in secretions such as nasal discharge and a common example of this would be nose to nose contact (*Knight et al., 2001*). BRSV is transmitted horizontally by direct contact with respiratory secretions (aerosol infection). Infection is facilitated by crowding during the milking process and when animals are housed during the winter months. Newly acquired calves should be isolated and monitored for the presence of infection to prevent contamination of uninfected herds. BRSV infection causes severe respiratory signs in young cattle and frequently leads to the death of the infected animal (*Baker et al., 1997*).

The isolation of BRSV from clinically affected animals using conventional cell culture is challenging because of fragility of BRSV even in optimally stored samples. Thus, virus isolation attempts are often unsuccessful (*Kimman et al., 1986*). Therefore, the diagnosis of BRSV infection is more commonly performed through the detection of specific antibodies by sero-diagnostic methods such as complement fixation test and ELISA and indirect immunofluorescence test (IIF) as described by (*Westenbrink et al., 1987*).

IIF technique uses only a small portion of the entire organ, the infected area can be missed and requires special equipment (*Valarcher et al., 1999*). In contrast, diagnostic methods such as ELISA, virus isolation or real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) that uses organ homogenates extends the analysis to larger parts of the sample.

The BRSV genome encodes three glycoproteins: Nucleoprotein protein gene (N), the large attachment protein (G) and the fusion protein (F). The G and F proteins mediate binding of virus to cells and F is also responsible for fusion of viral and cell membranes. The fusion (F) protein coding region is less variable and therefore a more suitable target for the design of diagnostic tests (*Eleraky et al., 2003).*

Classical methods of diagnosis based on BRSV antigen detection or virus isolation from lung samples and nasal swab demonstrated poor sensitivity due to low viral titers shedding (*Kimman et al., 1989a*). Consequently, several authors were developed different RT-PCR assays based on nucleoprotein gene (*Boxus et al., 2005)*, against the fusion gene (*Hakhverdyan et al., 2005; Larsen et al., 1999*) and nested RT-PCR directed against the nucleoprotein gene (*Valarcher et al., 1999).*

In present study, a real-time RT-PCR assay with and without ß-actin internal control which targeting fusion gene was described, its sensitivity and specificity were evaluated using field infected samples. The sensitivity of this real-

time RT-PCR assay was compared with cell culture and conventional reverse transcriptase PCR.

Material and methods

Virus strains and culture

The bovine respiratory virus strain (RVB-017) was propagated on diploid cell line from primary calves lung cells containing 10% fetal calve serum, 1% glutamine and 1% nonessential amino acid. The virus also was maintained in MEM (Eagle's minimum essen medium containing 1 % gentamycin without fetal calve serum). The virus isolated from trachea of diseased cattle showed respiratory manifestation. The virus titer was determined as 50 % tissue culture infective dose ($TCID_{50}$), and inoculated the cell culture with 10^4 $TCID_{50}$ /ml. The cell culture was incubated at 37°C for 4 days. The virus was harvested then showing 90-100 % cytopathic effect. The infected cell was scrapped and the supernatant was clarified through centrifugation, liquated and stored at -70°C or RNA extracted with RNeasy mini kit for RT-PCR assay.

Preparation of BRSV plasmid

PCR products of primer set of RT-PCR that covering the entire sequence of the BRSV fusion protein (F) were cloned in the pCDNA3 plasmid (Invitrigen) leading to the pN constructs. pN plasmid was linearized with the restriction enzyme XbaI (Roche Diagnostics GmbH, Mannheim, Germany). N control RNAs was transcribed from the T7 promoter with T7/SP6 RNA transcription kit (Roche Diagnostics GmbH, Mannheim, Germany) as recommended by the manufacturer. A purification step was performed using the RNeasy mini Kit (Qiagen, Hilden, Germany) to remove non-incorporated nucleotides.

Extraction of viral RNA

BRSV-RNA was extracted using RNeasy mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. 30 mg tissue or nasal swab transport medium or 600 µl cell pellets (5 x 10^6 cells) lysed in 600 µl RLT buffer and then homogenized. Ethanol is added to the lysate to provide ideal binding conditions. The lysate is then loaded onto the RNeasy silica membrane. RNA binds and all contaminants are efficiently washed away. Pure and concentrated RNA is eluted in water.

Conventional PCR

Conventional PCR was carried out in 25 µl reaction volume. PCR mixtures contained 0.75 µl working solution of both forward primer (BRSV S1, 20 pmol/µl) and reverse primer (BRSV S2, 20 pmol/µl) described by *(Oberst et al. 1993)*, 14,4

µl Nuclease-free water (Qiagen, Hilden, Germany), 5.0 µl PCR buffer (Qiagen® 5x), 1.0 µl dNTP (10 mM of each dATP, dCTP, dGTP,and dTTP; Qiagen), 0.1 µl RNase inhibitor and 1.0 µl RT-Mix (Qiagen, OneStep RT-PCR). Finally 2.0 µl RNA template were added. All primers were delivered by TIB MOLBIOL GmbH (Berlin, Germany) as can be seen Table 1.

The PCR reaction was performed on Thermocycler (MasterCycler, Eppendorf, Hamburg, Germany) as follow: a reverse-transcription reaction temperature at 50°C for 30 min and initial PCR activation of Taq-polymerase for 15 min at 95°C followed by 49 cycles of 30 sec at 94°C, 30 sec at 64°C, 1 min at 72°C and finally one extension cycle at 72°C for 10 min.

The PCR product was detected by electrophoresis through a 2 % gel stained with ethidium bromide and visualized under UV light.

Protocol of single real-time RT-PCR assay

The single real-time RT-PCR reaction was performed in 20 µl reaction volume. PCR mixtures contained 0.8 µl working solution of both primer BRSV-sdl-R and BRSV-sdl-R, 0.8 µl BRSV-probe (1pmol/µl), 10.0 µl QuantiTect probe RT-PCR Mastermix (Qiagen, Hilden, Germany) and 0.2 µl QuantiTect probe RT-Mix (Qiagen, Hilden, Germany). Finally 5.0 µl template was added. The primer concentration in working solutions was 10 pmol/µl. All primers were delivered by TIB MOLBIOL GmbH (Berlin, Germany) as can be seen in Table1.

The PCR reaction was performed in Stratagene thermocycle with the following programe: RNA is reverse-transcribed at 50°C for 30 min initial PCR activation of Taq-polymerase for 15 min at 95°C followed by 45 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 60°C.

The single real-time RT-PCR was performed also using another MasterMix. PCR mixture was contained 3.5 RNase free water, 0.5 µl of each BRSV-sdl forward and reverse primers, 2.5 µl BRSV TaqMan probe, 0.5 µl RT-mix (Superscript III RT/ Platinum-Mix, Invitrogen), 12.5 µl PCR buffer. Finally 5.0 µl template was added. The primer concentration in working solutions was 10 pmol/µl. All primers were delivered by TIB MOLBIOL GmbH (Berlin, Germany), see Table1.

The PCR reaction was performed in Stratagene thermocycle with the following programe: RNA is reverse-transcribed at 50°C for 30 min initial PCR activation of Taq-polymerase for 2 min at 95°C followed by 55 cycles of 30 sec at 94°C, 30 sec at 58°C, 30 sec at 68°C and finally cooling at 37°C for 30 sec.

Protocol of Duplex real-time RT-PCR assay

The duplex real-time RT-PCR reaction was performed in 20 µl reaction volume. PCR mixtures contained 2 µl of BRSV mix-1 [200 µl mix prepared from 20 µl of BRSV-sdl forward and reverse primer 2.5 µl BRSV-FAM probe and 157.5 µl 0.1 x tris EDTA buffer (pH 8)], 2 µl ß-actin mix-2 [200 µl mix 2 prepared from

5 µl of ACT forward and reverse primer, 2.5 ACT-HEX probe, 187.5 µl 0.1 x tris EDTA buffer (pH 8)], 0.8 µl RNase free water, 10 µl QuentiTect Probe RT-PCR MasterMix and 0.2 µl QuantiTect Probe RT-Mix. Finally, 5.0 µl template was added. The primer concentration in working solutions was 100 pmol/µl. All primers were delivered by TIB MOLBIOL GmbH (Berlin, Germany) as can be seen in Table 1.

The PCR reaction was performed in Stratagene thermocycle with the following program: RNA is reverse-transcribed at 50°C for 30 min initial PCR activation of Taq-polymerase for 15 min at 95°C followed by 45 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 60.

Table 1. Primers and probes for BRSV-detection

Application	Primer	Sequence	Acc-No	Position	Product Size (bp)	Reference
real-time RT-PCR	BRSV-sdl-F	5′-ACA CCC CCT GTT GGA AAC TAC A-3′	FJ543092	914-935	66	This study
	BRSV-sdl-F	5′-AAA AGA CAC AGA GCC TGC ATT GTC AC-3`		1038-1013		
	BRSV-TaqMan	Cy5-ACC ACC CAC GAT CTG TCC TAG TTA AGC A-BBQ		1009-982		
Conventional RT-PCR	BRSV-S1	5′- TTA CCA CAC CCC TCA GTA CA-3′	M58350	741-760	381	This study
	BRSV-S2	5′- CAT TGT GTC ACA GAA CAC TC-3′		1123-1104		
Internal control (ß-actin)	ACT-1005-F	5′- CAG CAC AAT GAA GAT CAA GAT CAT C-3′	DQ838049	966-990	129	This study
	ACT-1135-R	5′- CGG ACT CAT CGT ACT CCT GCT T- 3′		1096-1075		
	ACT-1081-TaqMan	HEX- TCG CTG TCC ACC TTC CAG CAG ATG T- BHQ1		1042-166		

Determination of analytical sensitivity

The sensitivity of conventional Qiagen one step RT-PCR and single real-time RT-PCR assay was evaluated using Superscript and QuantiTect MasterMix probe and duplex real-time RT-PCR with ß-actin was evaluated with serial dilution from BRSV control virus ranged between10^7 to10^1 RNA-copies/ml and also with ten-fold serial dilution of BRSV plasmid ranged between between10^7 to10 RNA-copies/ml.

Analytical specificity

The specificity of both PCR assays was evaluated by reference control of etiological cause of different diseases agents either viral or bacterial organism can cause respiratory manifestation and misdiagnosis with BRSV or cause secondary infection to BRSV infection. These agents such as bovine herpes virus1 and 4,

bovine viral diarrhea, bovine para-infulenza 3, Malignant catarrhal fever, bovine respiratory syncytial virus and bacterial causes such as *Brucella abortus, Chlamydian psittacii, Coxiella burnettii, Campylobacter fetus, E. coli, Leptospira spp., Listeria monocyotgenes, Mycoplasma bovis, Neopsora caninum and Mycobacterium avium subsp. paratuberculosis.*

Diagnostic sensitivity

We examined 87 naturally infected animals through collected nasal swabs from animals showed respiratory symptoms. These samples were extracted with RNeasy mini kit (Qiagen) and examined with conventional PCR in parallel to single and duplex real-time RT-PCR to determine the diagnostic ability of different examined PCR assays to field infection.

Assessment of PCR efficiency

Assessments of the amplification efficiency and the precision of the assay under optimized conditions were performed by serial dilutions of reference virus strain and BRSV-cloning plasmid. Standard curve construction was performed for real-time RT-PCR and the slopes were used for the calculation of amplification efficiency (E) by using the equation $E=10^{(-1/slope)}-10$

The robustness of the assay was investigated as follows. Duplicates of the serial dilutions of reference virus strain were inoculated on cell culture and analysed with conventional and real-time RT-PCR assay under optimized concentration of PCR reagents.

In addition, the influence of different MasterMix PCR reagents were investigated as Qiagen® One step RT-PCR kit (Qiagen) for conventional Rt-PCR, QuantiTect probe RT-PCR Master mix Kit (Qiagen) Superscript III RT/ Platinum –Mix kit (Invitrogen, California, USA) for real-time RT-PCR assay.

Results and Discussion

Analytical sensitivity

BRSV control virus

The results of analytical sensitivity based on serial dilution of virus control and type of RT-PCR assay were differed. The detection limit of single real-time RT-PCR protocol using QuentiTec (Qiagen) and superscript (Invitrogen) probe and duplex real-time RT-PCR using QuentiTec probe (Qiagen) based on serial dilution of virus PK 1/06 was 10^2 RNA copies but higher than the detection limit of conventional RT-PCR which was performed using QuentiTec one-step PCR probe (Qiagen), it was 10^3 RNA copies as can be seen in Table 2.

PCR efficiency of real-time RT-PCR was evaluated with QuantiTect probe and with Superscript probe based on serial dilution of virus control strain PK 1/06 and for duplex real-time RT-PCR with QuentiTec probe.

In principles, the correlation coefficients (r^2) of real time RT-PCR with the two probes and duplex real-time RT-PCR with QuentiTec probe assay were exceed 0.9866 and showed linear relationship between Ct-values and the corresponding serial dilution of BRSV virus.

Table 2. Analytical sensitivity of different PCR protocols based on serial dilution of BRSV control strain and different Mastermix probe.

PCR assay	RT-PCR	Real-time RT-PCR		Duplex real-time RT-PCR	
Mastermix Probe	Qiagen one-step PCR	QuantiTec (Qiagen)	Superscript (Invitrogen)	QuantiTec probe(Qiagen)	
Virus (PK 1/06)				Ct-Values	ß-actin
1.00E-00	positive	24.0	25.7	21.9	35.4
1.00E-01	positive	27.8	28.3	24.0	36.4
1.00E-02	positive	30.5	31.6	27.3	37.3
1.00E-03	positive	33.4	34.9	30.5	32.3
1.00E-04	negative	36.0	37.9	33.9	34.6
1.00E-05	negative	40.0	39.9	37.0	33.5
Regression coefficient	-	0.9957	0.9866	0.9866	

BRSV plasmid

The sensitivity of different RT-PCR protocols was evaluated base on serial dilution of BRSV plasmid.

Table 3. Analytical sensitivity of PCR protocols based on serial dilution of BRSV Plasmid and different Mastermix probe.

PCR assay	Conventional RT-PCR	Real-time RT-PCR		Duplex real-time RT-PCR	
Mastermix Probe	Qiagen One step	QuantiTec (Qiagen)	Superscript (Invitrogen)	QuantiTec probe(Qiagen)	
BRSV plasmid				Ct-Values	ß-actin
1.00E+08	positive	14.2	16.5	14.5	38.3
1.00E+07	positive	17.0	20.9	18.1	38.2
1.00E+06	positive	20.7	24.7	21.3	36.3
1.00E+05	positive	23.9	27.3	25.1	37.5
1.00E+04	positive	27.7	29.9	27.9	36.1
1.00E+03	positive	31.1	33.9	31.7	35.8
1.00E+02	positive	35.0	36.6	34.7	38.2
1.00E+01	positive	38.2	36.9	38.2	37.3
1.00E+00	positive	No Ct	No Ct	No Ct	37.2
Regression coefficient		0.9994	0.9963	0.9992	

The results revealed similar sensitivity between conventional RT-PCR and real-time RT-PCR either with QuentiTect (Qiagen) or Superscript (Invitrogen) Probe and duplex real-time RT-PCR based on QuentiTec probe (Qiagen) that was about 10 RNA copies/PCR and results showed detectable ct-values of ß-actin with all dilutions as can be seen in Table 3.

There was linear relationship between threshold cycle and the concentration of BRSV plasmid. Regression coefficient (r^2) was calculated that was in case of real time RT-PCR assay 0.999 and 0.996 with QuentiTec and Superscript probe respectively and 0.999 in duplex real-time RT-PCR assay as can be seen in Figure 1 and Table 4. Consequently, the PCR efficiency of real-time RT-PCR with serial dilution of BRSV plasmid was evaluated 94.54% and 109.60, 98.99% in QuentiTec and Superscript probe of single real-time RT-PCR assay and duplex real-time RT-PCR assay respectively as shown in Table 4.

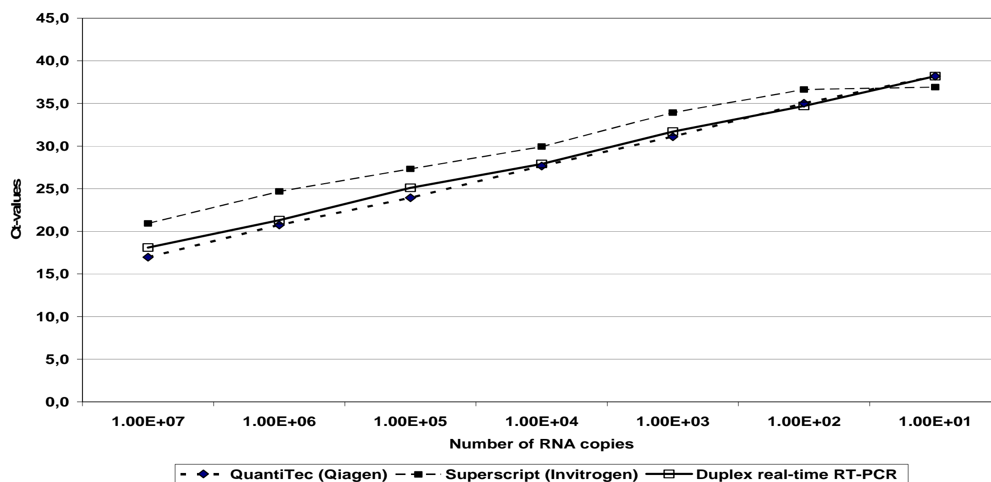

Figure 1. Relationship between Ct-values and the log-concentration of BRSV plasmid.

Table 4. Data of determination of analytical PCR sensitivity according to BRSV plasmid.

Method	MasterMix Probe	Dilution of BRSV plasmid (according to table 3)		
Parameter		r^2	PCR-efficiency	Detection limit
Real-time RT-PCR	Superscript	0.996	109.60%	10
	QuantiTec	0.999	90.54%	10
Duplex real-time RT-PCR	QuantiTec	0.999	98.99%	10
Conventional RT-PCR	Qiagen one step			10

Analytical specificity

The spectrum of detection of conventional RT-PCR and real-time RT-PCR was varied by amplifying RNA and DNA extracted from different bacteria and viruses associated with respiratory disease and can cause cross reaction with BRSV. The conventional RT-PCR showed defined PCR product only with BRSV and has never any detectable product with other microbial species. In another hand, the single real-time RT-PCR revealed detectable Ct with BRSV and was negative with other microbial species but showed weak positive signal with 38.5 Ct values with *Mycoplasma bovis* strain which extracted from milk sample. In repetition with the same *Mb.bovis* strain and the result revealed negative result which mean presence of cross contamination in first assay with BRSV. Furthermore, the duplex real-time RT-PCR showed only detectable Ct with BRSV strain and hasn't got any cross reaction with other tested species and combined with detectable Ct-values of ß-actin internal control.

Diagnostic sensitivity

After examination of 87 field samples of respiratory disease animals, the conventional RT-PCR showed 70 positive samples from 87 totals. In contrast, the single real-time RT-PCR showed 73 positive compared to 69 positive in case of duplex real-time PCR which has got detectable Ct-values of ß-actin internal control in all samples even with false negative result.

BRSV is one of the most important respiratory viruses due to its ability to cause infection to respiratory tract and its ability to predispose the respiratory tract to secondary bacterial infection (*Ames et al., 1993*). Presumptive diagnosis of BRSV can be determine depending on clinical feature of disease in infected animal or epidemiological occurrence of disease in herd. Lack of definite BRSV diagnosis because of inadequate viral isolation using cell culture and due to fragile nature of the virus. Consequently, direct detection of BRSV antibodies was performed using the serological test has become the standard method for BRSV diagnosis. In the present, several authors have used PCR as more sensitive test for diagnosis such as nested PCR with two round of amplification to detect the virus in clinical samples (*Belak and Thoren, 2001*) but the nested PCR have several disadvantage such as it is a bit complex due to it needs two round of amplification and a gel elctrophoresis which lead to high risk of contaminations.

These lead to important need to developed new simple and reliable diagnostic method for BRSV detection in clinical samples as quantitative RT-PCR which can be performed in a single-step and in closed-tube with fluorescence TaqMan detection probe. Several real-time PCR assays were developed according to nucleoprotein gene of BRSV (*Valacher et al., 1999; Boxus et al., 2005*). In another side, there is other studies were developed according to Fusion gene due to its stabile nature and less variable as (*Hakhverdyan et al., 2005; Vilcek et al., 1994; Larsen et al., 1999*).

In order to obtain highly sensitive PCR assay, we developed a real-time RT-PCR assay and the sensitivity of this assay was performed includes ß-actin internal control and without internal control using varied BRSV sources such as BRSV control virus isolated from clinical samples and cloning plasmid virus. The results of real-time RT-PCR assays was also compared with conventional PCR. The sensitivity analysis with BRSV reference strain revealed that the detection level of both conventional and real-time RT-PCR assays was similar at 10 RNA-copies/ml. As far as, the detection limit of real-time RT-PCR assay using different MasterMix probe according to BRSV control strain was higher sensitive than conventional RT-PCR and similar to duplex real-time RT-PCR with ß-actin table 2. Furthermore, the detection level of the three PCR assays was similar in case of using serial dilution that prepared from virus plasmid, it was 10 RNA-copies/ml.

Our data corresponding to other studies which have about similar detection range such as *Hakhverdyan et al.* (2005) developed fluorogenic reverse transcription PCR depend on F-gene and its detection level was 10 RNA-copies/ml, in nested PCR depend on F and G gene was 0.1 $TCID_{50}$ (*Vilcek at al., 1994*). Furthermore our sensitivity detection level of both conventional and real-time RT-PCR was higher than another assay as detection level of conventional RT-PCR was 10^5 RNA copies/ml (*Larsen et al., 1999*) and 10^3 RNA copies/ml of real-time RT-PCR assay depend on nucleoprotein gene (*Boxus et al., 2005*).

The specificity of our developed PCR assay was evaluated with different viral and bacterial species which can cause respiratory disease in animal and make misdiagnosis to BRSV or cause secondary infection to BRSV infection. Our result of conventional RT-PCR and duplex real-time RT-PCR assays was revealed detectable Ct only with BRSV control virus which mean highly specific test to BRSV diagnosis. In contrast real-time RT-PCR was revealed weak Ct-value with Mycoplasma bovis strain which isolated from milk sample but in repetition this sample back to be negative which mean presence of contamination.

In our practical use to the three PCR assays for detection of BRSV in clinical samples of natural infected animal revealed that the real-time RT-PCR assay is most reliable and rapid test for BRSV diagnosis. The result of real-time RT-PCR assay showed 73 positive samples against 70 and 69 positive sample from 87 totals in conventional and duplex real-time RT-PCR, respectively. This result showed bit of competition between the primer of BRSV and ß-actin that lead to low sensitive assay and also conventional RT-PCR has still risk of electrophoresis contamination and handling complex.

Conclusion

The result revealed that real-time RT-PCR is most a rapid, sensitive and specific assay for detection of BRSV in clinical samples and easily applied in routine diagnosis.

Ocena protokola reversne transkripcije PCR na bazi fuzionog gena za dijagnozu infekcije respiratornim sincicijalnim *virusom kod goveda*

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Rezime

Respiratorni sincicijalni *virus kod goveda* (BRSV), pneumovirus iz porodice *paramyxoviridae* je važan uzrok akutnog respiratornog oboljenja teladi nakon odbijanja i goveda u tovilištu. PCR protokoli reverzne transkriptaze u realnom vremenu su razvijeni da otkriju BRSV infekciju u zaraženim životinjama. Osetljivost RT-PCR protokola na osnovu spajanja gena su ocenjeni korišćenjem različitih Mastermixes kao što su Qiagen One Step RT-PCR (Qiagen) za konvencionalnu RT-PCR, Superscrip probe (Invitrogen) i QuentiTec probe (Qiagen) RT-PCR u realnom vremenu, sa i bez unutrašnje kontrole. Granica detekcije različitih RT-PCR protokola koji koriste serijski rastvore iz BRSV plazmida i zasnovane na različitim probama, bilo je 10 RNK kopija / ml. Osim toga, specifičnost RT-PCR u realnom vremenu je ocenjen korišćenjem različitih bakterijskih i virusnih sojeva koji mogu biti izolovani kod životinja sa respiratornim zaraznim oboljenjima. S druge strane, RT-PCR u realnom vremenu u kombinaciji sa ß-actin i konvencionalnom RT-PCR, pokazala je uočljive CT-vrednosti samo sa BRSV sojem.

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