Scientific Bulletin. Series F. Biotechnologies, Vol. XX, 2016
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

STUDIES CONCERNING THE OPTIMISATION OF REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION TECHNIQUE OF PAN-SIMBU VIRUS GROUP

Maria Rodica GURAU[1], Stelian BARAITAREANU[1], Marius Andrei MANESCU[1], Mihaela Cristiana POPP[1], Doina DANES[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: doruvet@yahoo.com

Abstract

Several molecular methods have been developed for diagnostic or surveillance of those agents of emerging infectious diseases, including for the Schmallenberg-Simbu group viruses. Serological surveillance of the Schmallenberg-Simbu group viruses in Romania revealed the presence of positive ruminants and it rise up the question about the presence of virus into the environment. In this frame, the paper has described preliminary studies concerning the optimisation of classical RT-PCR of pan-Simbu virus group. We used the OneStep RT-PCR Kit and made minor changes as follows. For one reaction were used 5 µl 5x OneStep RT-PCR Buffer, 1.5 µl dNTP 10 mM, 1.5 µl OneStep RT-PCR Enzyme Mix, 4 µl primer panOBV-L-2959 F 10 µM, 4 µl primer panOBV-L-3274R 10 µM and 9 µl RNase-free water. Into reaction tubes were transferred 25 µL master mix + 10 µL sample. Thermal cycling program consisted of one cycle of 50°C - 30 min and one cycle of 95°C - 15 min, followed by 42 cycles of 95°C - 30 s, 55°C - 30 s, 72°C - 30 s and 72°C - 10 min. All results obtained by real time RT-PCR (virotype SBV RT-PCR Kit) and classical RT-PCR were correlated with the quantity of estimated RNA by fluorometry. The sensitivity of classical RT-PCR was lower than sensitivity of real time RT-PCR, the positive result being acquired at a minimum of 3.91 ng/µl RNA per sample. The specificity of methods was the same, without non-specific electrophoretic bands detection. Therefore, our classical RT-PCR protocol can be a useful tool in evaluation of virus circulation in countries with or without history of associated Simbu disease in livestock, or with reported seroconversion.

Key words*: PCR, diagnostic, real-time RT-PCR, Orthobunyavirus, Schmallenberg virus.*

INTRODUCTION

Simbu serogroup viruses are arthropod-borne bunyaviruses antigenically related (Horne and Vanlandingham, 2014), some of them involved in livestock's pathological disorders (Coverdale et al., 1978; Jagoe et al., 1993; Hoffmann et al., 2012).

Several molecular methods have been developed for diagnostic or surveillance of those agents of emerging infectious diseases (Baraitareanu and Danes, 2014), including for the Schmallenberg-Simbu group viruses (Hoffmann et al., 2012).

Since October 2011, when a novel orthobunyavirus of the Simbu serogroup, subsequently named Schmallenberg virus (SBV) was first identified in German cows, several European groups of researchers have developed molecular diagnostic tools able to identify SBV or Simbu serogroup (PSV) (Fischer et al., 2013; Hoffmann et al., 2012; Afonso et al., 2014; Balenghien et al., 2014; Schulz et al., 2015). Also, the retrospective studies or meta-analyses concerning molecular tools used in the diagnostic of orthobunyavirus, are already available (Afonso et al., 2014; Balenghien et al., 2014; Manescu et al., 2015; Schulz et al., 2015).

The PCR optimization strategies aim to correct one or more parameters, in order to enhance specificity and sensitivity at an optimal confidence level (Roux, 2009).

The confidence of diagnostic method can be quantified by interlaboratory comparison of results. For this reason, Schulz et al. (2015) conducted the European interlaboratory comparison of real-time RT-PCR for Schmallenberg virus (SBV) detection on experimental and field samples. In this study, they identified that the confidence of the results can affected by the method of extraction of

RNA-SBV from semen samples (Schulz et al. 2015).

Fischer et al. (2013) developed a pan-Simbu real-time reverse transcriptase PCR able to detect several viruses of Simbu serogroup (Aino virus, Akabane virus, Douglas virus, Oropouche virus, Peaton virus, Sabo virus, Sango virus, Sathuperi virus, Schmallenberg virus, Shamonda virus, Shuni virus, Simbu virus, Thimiri virus, Tinaroo virus), Bunyamwera serogroup (Batai virus, Bunyamwera virus, Ngari virus) and, probably, California serogroup (Tahyna virus, Chatanga virus, La Crosse virus, Jamestown Canyon virus, Snowshoe hare virus, Inkoo virus). This broad molecular tool for screening allows the identification of targeted viruses both in mammalian samples and in the samples of the vector insect (Fischer et al., 2013), which recommends it as an excellent method of epidemiological surveillance.

Serological surveillance of the Schmallenberg-Simbu group viruses in Romania has revealed the presence of positive ruminants (Danes et al., 2014) and it rises up the question about the presence of virus into the environment. In light of these circumstances, the paper described the preliminary studies concerning the optimisation of reverse transcription polymerase chain reaction technique of pan-Simbu virus group.

MATERIALS AND METHODS

In order to optimise the RT-PCR technique for the detection of pan-Simbu Virus Group (PSV) RNA, the following materials were used:
(1) Five dilution series of positive control RNA of the Schmallenberg virus (Friedrich-Loeffler-Institut, Greifswald–Insel, Riems, Germany);
(2) Qubit RNA HS Assay Kit and Qubit 3.0 Fluorometer (Invitrogen, Canada);
(3) *virotype* SBV RT-PCR Kit (Qiagen, Germany);
(4) SmartCycler and Life Science 2d SmarCycler software (Cepheid, USA);
(5) OneStep RT-PCR Kit (Qiagen, Germany);
(6) Agarose + TAE/TBE (1x) + Ethidium bromide (10 mg/ml).
Forward and revers primers for amplification of L-Segment are presented in table 1.
RNA quantification of the dilution series of SBV-RNA were in accord with the recommendations of the manufacturer (Invitrogen, Canada).

Table 1. Sequence of forward and revers primers used for amplification of L-Segment and size of expected PCR product (Fischer et al., 2013)

Primer name	Sequence	Product size
panOBV-L-2959 F	5'-TTGGAGARTATGARGCTAARATGTG-3'	279 bp
panOBV-L-3274R	5'-TGAGCACTCCATTTNGACATRTC-3'	

Detection of RNA from Schmallenberg virus by real-time RT-PCR was performed in accord with the protocol described in *virotype* SBV RT-PCR Kit Handbook (Qiagen, Germany). Briefly, preparation of reaction mix was performed in 25 µL/sample (20 µL master mix + 5 µL sample), and the real-time RT-PCR protocol consisted in one cycle of 45°C - 10 min and one cycle of 95°C - 10 min, followed by 40 cycles of 95°C - 15 s, 56°C - 30 s, 72°C - 30 s.
Classical RT-PCR for RNA-SBV detection was performed by adapting a previously described RT-PCR protocol developed for the detection of pan-Simbu Viruses (Fischer et al., 2013). We used the OneStep RT-PCR Kit (Qiagen, Germany) and made minor changes as follows.
For one reaction were used 5 µl 5x OneStep RT-PCR Buffer (Qiagen, Germany), 1.5 µldNTP 10 mM (Qiagen, Germany), 1.5 µl OneStep RT-PCR Enzyme Mix (Qiagen, Germany), 4 µl primer panOBV-L-2959 F 10 µM (Fischer et al., 2013), 4 µl primer panOBV-L-3274R 10 µM (Fischer et al., 2013) and 9 µlRNase-free water. Into reaction tubes were transferred 25 µL master mix + 10 µL sample. Thermal cycling program consisted of one cycle of 50°C - 30 min and one cycle of 95°C - 15 min, followed by 42 cycles of 95°C - 30 s, 55°C - 30 s, 72°C - 30 s and 72°C - 10 min.
Agarose electrophoresis was performed to visualize the PCR products (0.9 g agarose, 60 ml TAE/TBE 1x, 5 µl ethidium bromide; 10 mg/ml; 100V; 1,5A; 35 min).

RESULTS AND DISCUSSIONS

RNA quantification of the dilution series of SBV-RNA (provided by Friedrich-Loeffler-Institut) have been done with Qubit RNA HS Assay Kit in Qubit 3.0 Fluorometer. The quantities of total RNA in each dilution are presented in table 2.

Table 2. Dilution series of positive control RNA of the Schmallenberg virus

Dilution	RNA quantity (ng/µl)*
10^{-2}	4.92
10^{-3}	3.91
10^{-4}	2.46
10^{-5}	1.36
10^{-6}	0.84

* RNA quantification with Qubit RNA HS Assay Kit in Qubit 3.0 Fluorometer

Real-time RT-PCR technique provided quantification cycle (Cq±SD) 17.19±0.141 for dilution 10^{-2}, 21.55±0.140 for dilution 10^{-3}, 24.16±0.164 for dilution 10^{-4}, 28.61±0.139 for dilution 10^{-5}, and 30.93±0.113 for dilution 10^{-6} (figure 1).

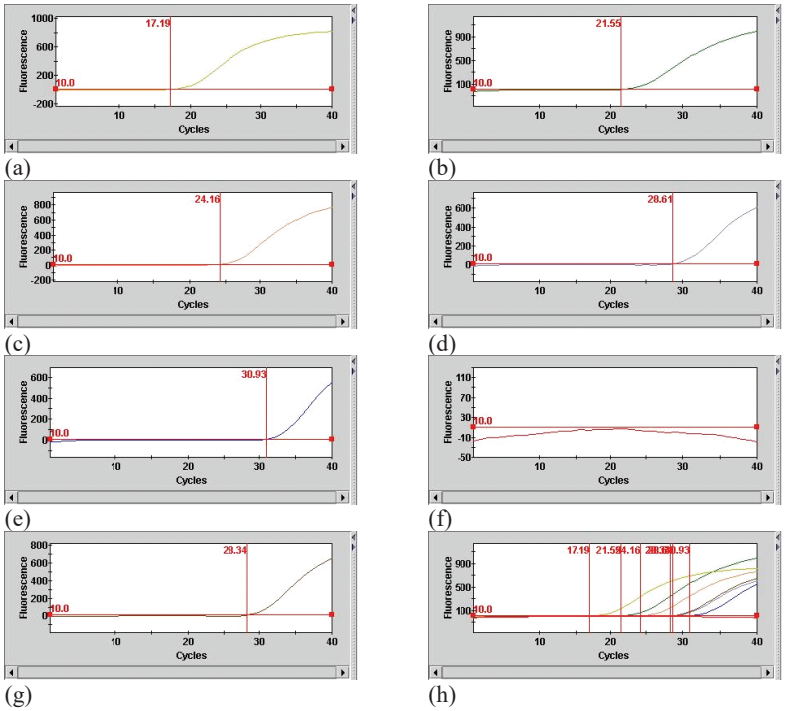
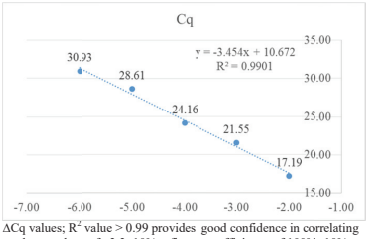


Figure 1. Quantification cycle (Cq) values obtained by Real-time RT-PCR for five dilutions of positive control RNA of the Schmallenberg virus (Friederich-LoefflerInstitut, Germany), positive and negative controls of *virotipe* SBV RT-PCR Kit (Qiagen, Germany). (a) Cq value of dilution 10^{-2}; (b) Cq value of dilution 10^{-3}; (c) Cq value of dilution 10^{-4}; (d) Cq value of dilution 10^{-5}; (e) Cq value of dilution 10^{-6}; (f) Cq value of negative control supplied by *virotipe* SBV RT-PCR Kit; (g) Cq value of positive control supplied by *virotipe* SBV RT-PCR Kit; (h) Cq values of all amplification curves (analysis with Smart Cycler Life science software 2.0d)

Also, Cq value of positive control supplied by *virotipe* SBV RT-PCR Kit validated the reaction (Qiagen, Germany). All dilutions were analysed in triplicate, in three runs. The efficiency of real-time RT-PCR technique was evaluated by slope (-3.454) and R^2 value (0.9901) and the precision by standard deviation (<0.164) of all Cq values obtained in each dilution (figure 2).

The results of classical RT-PCR for RNA-SBV protocol are synthesized in table 3.

All results obtained by real time RT-PCR and classical RT-PCR were correlated with the quantity of estimated RNA by fluorometry.



ΔCq values; R^2 value > 0.99 provides good confidence in correlating values; a slope of ~3.3±10% reflects an efficiency of 100%±10%.

Figure 2. Linear regression, R^2 value and slope analysis of results for five serial decimal dilutions

The sensitivity of classical RT-PCR was lower than real time RT-PCR, the positive result being acquired only for dilutions 10^{-2} and 10^{-3}.

Table 3. Result of classical RT-PCR for RNA-SBV

Dilution	Results*
10^{-2}	++
10^{-3}	+
10^{-4}	±
10^{-5}	-
10^{-6}	-

*++ obviously band, + clear band, ± weak band, - no band

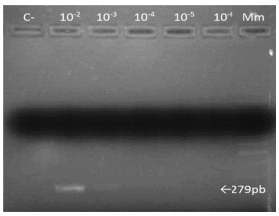


Figure 3. PCR electrophoresis results in classical PCR

Based on the recorded data the sensitivity of classical RT-PCR was estimated at a minimum of 3.91 ng/µl RNA per sample. The specificity of methods was identical, without the detection of nonspecific electrophoretic bands (figure 3). The results were similar with those of Fischer et al. (2013).

CONCLUSIONS

A classical RT-PCR assay was optimised to provide a rapid and sensitive molecular method for the surveillance of orthobunyaviruses' Simbu serogroup.

The assay can be a useful tool in evaluation of virus circulation in countries with or without history of associated Simbu disease in livestock, or with reported seroconversion.

However, to obtain reliable results using our classical RT-PCR protocol, the sample should contain minimum amount of 3.91 ng/µl of RNA.

ACKNOWLEDGEMENTS

The dilution series of RNA-SBV were kindly provided by Dr. Bernd Hoffmann from Friedrich-Loeffler-Institut (Greifswald–Insel, Riems, Germany).

REFERENCES

Afonso A., Abrahantes J.C., Conraths F., Veldhuis A., Elbers A., Roberts H., Van der Stede Y., Méroc E., Gache K., Richardson J., 2014. The Schmallenberg virus epidemic in Europe-2011-2013, Prev. Vet. Med., 116(4):391-403.

Balenghien T., Pages N., Goffredo M., Carpenter S., Augot D., Jacquier E., Talavera S., Monaco F., Depaquit J.,Grillet C., Pujols J., Satta G., Kasbari M., Setier-Rio M.L., Izzo F., Alkan C., Delécolle JC, Quaglia M, Charrel R, Polci A, Bréard E., Federici V., Cetre-Sossah C., Garros C., 2014. The emergence of Schmallenberg virus across Culicoides communities and ecosystems in Europe, Prev. Vet. Med., 116(4):360-369.

Baraitareanu S., Danes D., 2014. Pathology related with "novel" emerging infectious agents in livestock, Scientific Works. Series C. Vet. Med., LX(1):41-46.

Coverdale O.R., Cybinski D.H., St George T.D. 1978. Congenital abnormalities in calves associated with Akabane virus and Aino virus. AustVetJ.54(3):151-2.

Danes D., Baraitareanu S., Gurau M.R., Dan M., Bartoiu I.A., Moldovan H., Danes M., 2014. Preliminary Results of Schmallenberg virus Serosurveillance in Romania. Advances in Environmental Technology and Biotechnology. Energy, Environmental and Structural Engineering Series 26:112-116.

Fischer M., Schirrmeier H., Wernike K., Wegelt A., Beer M., Hoffmann B., 2013. Development of a pan-Simbu real-time reverse transcriptase PCR for the detection of Simbu serogroup viruses and comparison with SBV diagnostic PCR systems, Virol. J., 10:327.

Hoffmann B., Scheuch M., Höper D., Jungblut R., Holsteg M., Schirrmeier H., Eschbaumer M., Goller K.V., Wernike K., Fischer M., Breithaupt A., Mettenleiter T.C., Beer M., 2012. Novel Orthobunyavirus in Cattle, Europe, 2011, Emerging Infectious Diseases. 18:469-472.

Horne K.M., Vanlandingham D.L., 2014. Bunyavirus-vector interactions. Viruses. 6(11):4373-97.

Jagoe S., Kirkland P.D., Harper P.A., 1993. An outbreak of Akabane virus-induced abnormalities in calves after agistment in an endemic region. Aust Vet J.70(2):56–58.

Manescu M.A., Baraitareanu S., Gurau M.R., Danes D., 2015. Molecular tools available for the detection of Schmallenberg-Simbu group viruses. 4th ISAA. Proc. Rom. Acad., Series B, Supplement 1, 120-123.

Roux K.H., 2009. Optimization and troubleshooting in PCR. Cold Spring HarbProtoc. 2009(4):pdb.ip66. http://cshprotocols.cshlp.org/content/2009/4/pdb.ip66.long

Schulz C., van der Poel W.H.,Ponsart C., Cay A.B., Steinbach F.,Zientara S., Beer M., Hoffmann B., 2015. European interlaboratory comparison of Schmallenberg virus (SBV) real-time RT-PCR detection in experimental and field samples: The method of extraction is critical for SBV RNA detection in semen. J.Vet.Diagn.Invest.27(4):422-30.

MISCELLANEOUS