SHORT COMMUNICATION

Adaptation of a real-time RT-PCR assay for the detection of Schmallenberg virus

Carmen L. Perera*, Liliam Rios, María T. Frías, Lester J. Pérez

Animal Virology Group, National Center for Animal and Plant Health (CENSA), Apartado 10, San José de las Lajas, CP 32 700, Mayabeque, Cuba. E-mail: claura@censa.edu.cu

ABSTRACT: Schmallenberg virus was first detected in Germany in October 2011, associated with congenital malformations in cattle, sheep and goats. This novel emergent agent causes mild disease in cattle with decreased milk production, fever and diarrhea. In March 2012, the German Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, reported the development and validation of a real-time RT-PCR for the diagnosis of this new virus. The Animal Virology Laboratory at the National Center for Animal and Plant Health in Cuba has adapted the protocol previously reported on the LightCycler platforms using two different mix conditions. In all cases, amplification curves obtained were specific and all the dilutions tested showed an increase in the Ct-values. Nevertheless, the sensitivity of the test was not affected. Thus, the test for Schmallenberg virus detection is enabled for the possible emergency of this agent in Cuba.

Key words: Schmallenberg virus, real-time RT-PCR, emergent.

Adaptación de un ensayo de RT-PCR en tiempo real para la detección del virus de Schmallenberg

RESUMEN: El virus de Schmallenberg fue diagnosticado por primera vez en octubre de 2011 en Alemania, asociado a malformaciones congénitas en bovinos, ovinos y cabras. Este nuevo agente emergente causa enfermedad ligera en bovinos, con disminución de la producción de leche, fiebre y diarreas. En marzo de 2012 se reportó el desarrollo y validación de un RT-PCR en tiempo real para el diagnóstico de este nuevo virus por el grupo alemán del Institute of Diagnostic Virology, Friedrich-Loeffler-Institut. El Laboratorio de Virología Animal del Centro Nacional de Sanidad Agropecuaria, en Cuba, adaptó el protocolo previamente reportado a la plataforma de LighCycler usando dos condiciones de mezcla diferentes. En todos los casos las curvas de amplificación obtenidas fueron específicas y todas las diluciones evaluadas mostraron un incremento en los valores de Ct. A pesar de esto, la sensibilidad de la técnica no se afectó. Por lo tanto, la técnica para la detección del virus de Schmallenberg está disponible ante la posible emergencia de este agente en Cuba.

Palabras clave: Virus de Schmallenberg, RT-PCR en tiempo real, emergente.

In August 2011, an unidentified disease syndrome was first reported in dairy cattle in Germany. Fever and decreased milk yielding were the main clinical signs observed (1). The duration of the clinical signs was between 2-3 weeks even though some individual affected animals recovered over a few days (2,3). This disease syndrome was associated with a new virus which was provisionally named Schmallenberg virus (SBV). This agent has been phylogenetically related to Shamonda, Aino and Akabane viruses, all they members of Simbuserogroup included into the genus *Orthobunyavirus* of the family *Bunyaviridae*. This

*Corresponding autor: Carmen L. Perera. Animal Virology Group, National Center for Animal and Plant Health (CENSA), Apartado 10, San José de las Lajas, CP 32 700, Mayabeque, Cuba. Tel.:+5347863206; Fax:+5347861104. E-mail addresses: claura@censa.edu.cu.

relationship was found based on similarities between the small (S), medium (M) and large (L) genes (4). Nevertheless, a final classification for this agent has not yet been established by the International Committee on Taxonomy of Viruses.

From results obtained in experimental reproductions, SBV has also been associated with fetal deformities in sheep, goats, and cattle. In naturally infected pregnant small ruminants, SBV has been found causing stillbirths and births of newborns with one or more defects (3). An important aspect to take into account is the fact that viruses of the genus *Orthobunyavirus* are arthropod-borne viruses, transmitted by mosquitoes and/or Culicoides biting midges. Therefore, it is possible to assume that SBV could also be transmitted by similar-type vectors (3). This transmission route increases the potential risk of SBV's spread worldwide. To date, this emergent viral agent has been reported in eight Member States of the European Union, the Netherlands, Germany, Belgium, France, the UK, Italy, Luxembourg and Spain (5).

The development of new diagnostic techniques to establish early detection of the SBV is an important task for the diagnosis laboratories. However, to our knowledge, only one assay has been reported for this purpose (6).

The most important task for the Animal Virology Laboratory at the National Centre for Animal and Plant Health in Cuba is the diagnosis of emerging and re-emerging transboundary animal diseases. Within the area of assay design, relevant issues include chemistry, target selection, cycling conditions and thermocycling platform selection (7). Chemistry and platform are the most variable aspects between the different laboratories (8). Regarding platform, each instrument has inherent characteristics that must be addressed as an essential step in the process of assay validation (8). Hence, the adaptation of the different diagnosis protocols to each condition into each laboratory is a very relevant aspect. The current work was aimed to accomplish the adaptation of the real-time RT-PCR (rRT-PCR) developed by Bilk *et al.* (6) on the LightCycler platforms.

The rRT-PCR assay developed by Bilk *et al.* (6) was optimized on ABIPrism7500 platforms (AP7500) (Applied Biosystems, CA, USA). For the adaptation of the assay, the primer pair (SBV-S-382F: 5'-TCAGATTGTCATGCCCCTTGC-3', SBV-S-469R: 5'-TTCGGCCCCAGGTGCAAATC-3'), probe (SBV-S-408FAM: 5'-FAM-TTA AGG GAT GCA CCT GGG CCG ATG GT-BHQ1-3') and serial dilutions of positive control RNA SBV (10^2-10^6 copies/µl) were used. The reagents above mentioned were kindly provided by Dr.

Bernd Hoffman, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Riems Island, Germany.

On the LightCycler 1.5 and 2.0 platforms (Roche Applied Science, Mannheim, Germany), the detection of SBV genome was performed using two different mixes. In all cases, a water control was included. The final conditions for each mix were the following:

Mix I: The synthesis of cDNA was performed by random priming and using M-MLV reverse transcriptase, as described previously by Díaz de Arce *et al.* (9). The reagent/primer final concentration was 1xQuantitect-SYBR-Green PCR kit/0.4 µM, with each sample in a 20 µL total reaction volume that included 5 µL of cDNA template. The thermal profile used was the following: 15 min at 95°C (inactivation reverse transcriptase/activation *Taq* polymerase), followed by 52 cycles of 10 s at 95°C (denaturation), 5 s at 56°C (annealing) and 20 s at 72°C (elongation). After the PCR cycles, a melting curve was generated (15 s at 95°C, 1 min at 65°C, 15 s at 95°C) to discriminate between specific amplicons and non-specific amplification products (in all cases the ramp time was 1°C per second).

Mix II: The mixture contained 3.2 µL RNase-free water, 4 µL 5x QIAGEN OneSetp RT-PCR buffer, 4 µL 5x Q-Solutions, 1 µL dNTP Mix (containing 10 mM of each dNTP), 1 µL QIAGEN OneSetp RT-PCR Enzyme Mix, 0.25 µL RNase inhibitor, 2 µL SBV specific primer/probe-mix (10 mM SBV-specific primers + 1.875 mM SBV-specific probes) for one reaction and 5 µL RNA template was added. For reverse transcription and amplification, the following temperature profile was used: 30 min at 50°C (reverse transcription), 15 min at 95°C (inactivation reverse transcriptase/activation Taq polymerase), followed by 52 cycles of 10 s at 95°C (denaturation), 20 s at 55°C (annealing) and 30 s at 72°C (elongation).

On LightCycler 1.5 and 2.0 platforms, specific amplification curves for SBV detection were obtained. In addition, a linear-range was observed when serial dilutions of the SBV were assessed (Fig. 1 and 2). On the other hand, amplification curves were not observed when negative controls were evaluated. The Cycle threshold values (Ct-values) were not different for the serial dilutions assessed when Mix I or Mix II were used. Therefore the same degree of sensitivity was obtained for both mixes of reaction.

Nevertheless, it is important to highlight the fact that the curves obtained on LightCycler 1.5 and 2.0 platforms showed an increase in the Ct-values compared with the results obtained by Bilk *et al.* (6). However, the adapted assay was able to detect the same RNA viral
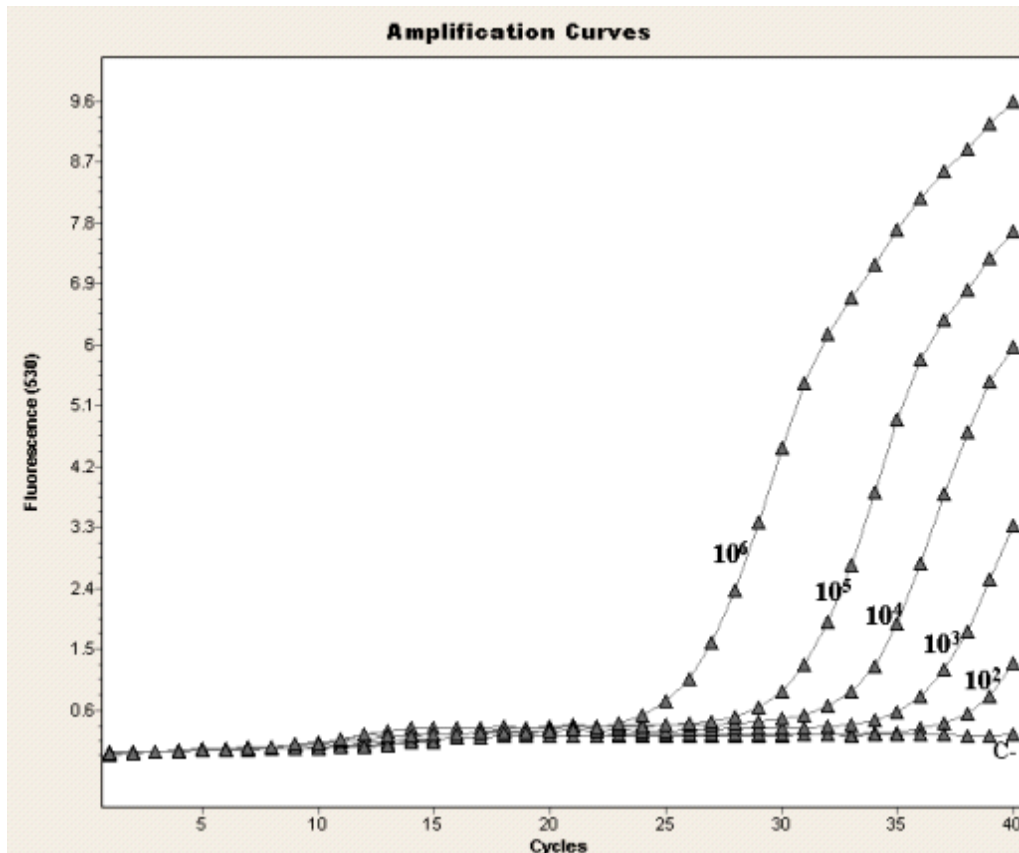
FIGURA 1. Evaluation on LightCycler platforms of the serial dilutions of RNA copies obtained from Shmallenberg virus using Mix I./ *Evaluación en plataformas de LightCycler de las diluciones seriadas de las copias de ARN obtenidas de virus de Shmallenberg usando la Mezcla I.*

copies that the previously reported by Bilk *et al.* (6) between 10^2 - 10^6 copies/μl. Thus, even though a displacement in the Ct-values on LightCycler 1.5 and 2.0 platforms was observed, the sensitivity of the test was kept.

In the current work, an rRT-PCR assay for the detection of the new emergent virus named SBV was adapted on LightCycler 1.5 and 2.0 platforms.Viral diseases emergence is the major concern in public and animal health (10). The recent incidences of emerging and re-emerging transboundary animal diseases have led to very heavy losses all over the world. Several examples in the last years have been the outbreaks caused by foot-and-mouth disease on three continents (Africa, Asia and South America), classical swine fever (Africa, Asia and Europe), rinderpest (Africa and Asia), or highly pathogenic avian influenza (Africa, Asia and Europe) (11).

The most recent episode regarding a viral diseases emergence was caused by SBV in the European Union during the last half of 2011 (1). The SBV has been associated with congenital malformations in cattle, sheep and goats, with a high impact on production leading to economic losses (12). On the other hand, the transmission route of SBV enhances the potential risk of worldwide dissemination (2).

The early warning systems and the rapid and highly specific detection of the agents are major tasks, considering that the timely recognition of such viral infections would prevent the spread of the diseases to large animal populations. Therefore, the development of novel and powerful diagnostic assays is today a basic issue in veterinary research and animal healthcare. Molecular virology offers a range of new methods, which are able to accelerate and improve the diagnosis of infectious diseases in animals (11).

The results obtained in this study showed the flexibility of the rRT-PCR developed by Bilk *et al.* (6). This assay was adapted to other platforms and two different mix conditions. The adapted assay maintained the specificity and sensitivity of the original protocol despite the increase of the Ct-values.
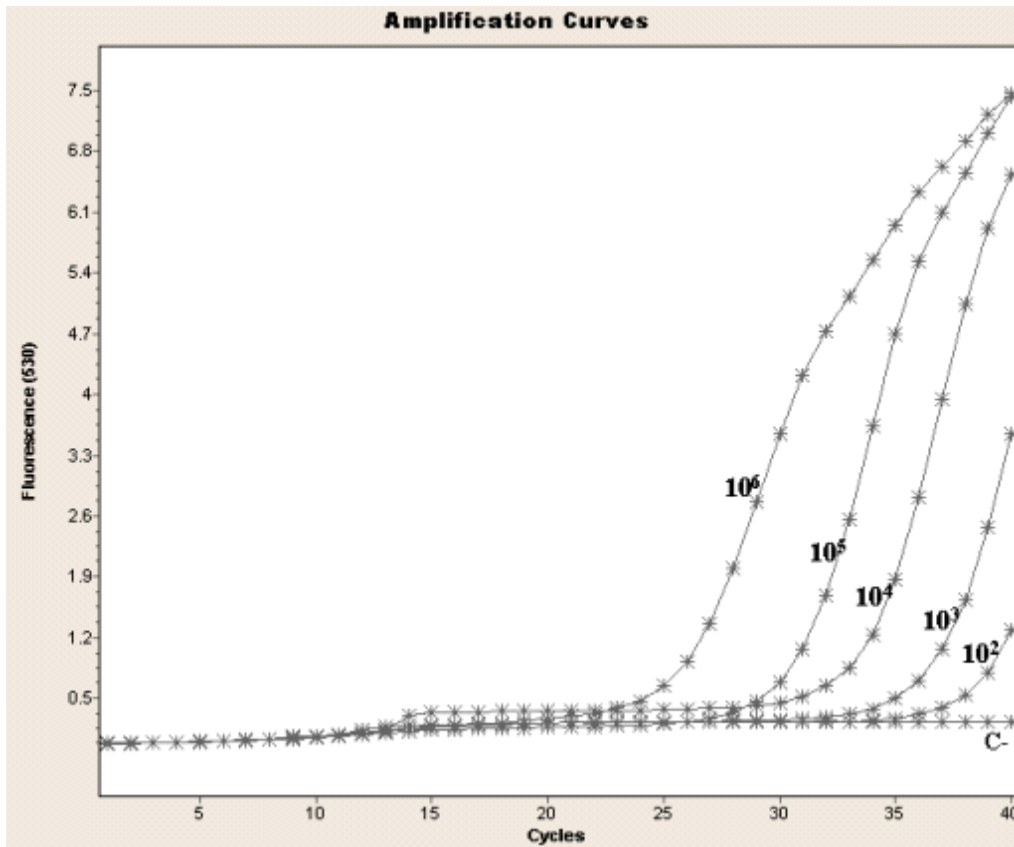
FIGURA 2. Evaluation on LightCycler platforms of the serial dilutions of RNA copies obtained from Shmallenberg virus using Mix II./ *Evaluación en plataformas de LightCycler de las diluciones seriadas de las copias de ARN obtenidas de virus de Shmallenberg usando la Mezcla II.*

The displacement in the Ct-values showed by the LightCycler 1.5 and 2.0 platforms compared with AP7500 platforms could be related to two different aspects. The first one could be explained by the effect of the ramps between different platforms of real time which was previously reported by Perez *et al.* (13). This issue is due to the fact that the temperature transition rate recommended for the LightCycler 1.5 instrument is 20ºC per second, while the rate suggested for the AP7500 platform is 1ºC per second; the time it takes to get from one temperature to the next one in the heating/cooling cycle is longer in the AP7500 than in the LightCycler 1.5 and 2.0 instruments. Consequently, the Ct-values could be displaced (13). The second one could be due to the possible degrading of RNA during the transport of the positive controls from the Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Riems Island, Germany to the Animal Virology Laboratory, CENSA. This final aspect is nowadays one of the most important concerns for the sample sending between different laboratories of diagnosis.

In conclusion, an rRT-PCR for new emergent SBV was adapted on LightCycler 1.5 and 2.0 platforms. Additionally, two different mixes of reaction were assessed to determine the flexibility of the test. Finally, this assay for SBV detection can be used for a possible emergence of this agent in Cuba.

ACKNOWLEDGMENTS

REFERENCES

1. FLI. Update from the Friedrich Loeffler Institute on Schmallenberg virus 4. (2012). Disponible en: http://www.fli.bund.de/no_cache/de/_startseite/aktuelles/tierseuchengeschehen/schmallenbergvirus.html (15.02.12).

2. Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, Schirrmeier H, et al. Novel orthobunya virus in Cattle. Emerg Infect Dis. 2011;18:469-472.

3. Muskens J, Smolenaars AJ, van der Poel WH, Mars MH, van Wuijckhuise L. Nederlandse melkbedrijven door het Schmallenberg virus. Tijdschr. Diergeneeskd. 2012;137:112-115.

4. Lievaart-Peterson K, Luttikholt SJM, Van den Brom R, Vellema P. Schmallenberg virus infection in small ruminants - First review of the situation and prospects in Northern Europe. Small Ruminant Research. 2012;106(2):71-76.

5. Sanidad Animal. INFO. Actualización epidemiológica de la enfermedad de Schmallenberg. 2012. Disponible en: http://www.sanidadanimal.info/sanidadanimal/es/actividades/emergentes-online/virus-schmallenberg-vsb/137-actualizacion-epidemiologica-schmallenberg.html .

6. Bilk S, Schulze C, Fischer M, Beer M, Hlinak A, Hoffmann B. Organ distribution of Schmallenberg virus RNA in malformed newborns. Vet Microbiol. 2012;159(1-2):236-238.

7. Hayden RT, Hokason KM, Punods SB, Bankowski MJ, Belzer SW, Carr J, et al. Multicenter comparison of different real-time PCR assays for quantitative detection of Epstein-Barr virus. J Clin Microbiol. 2008;46(1):157-163.

8. Bentley HA, Belloni DR and Tsongalis GJ. Parameters involved in the conversion of real-time PCR assays from the ABI prism 7700 to the Cepheid SmartCycler II. Clin Biochem. 2005;38:183-186.

9. Díaz de Arce H, Nuñez JI, Ganges L, Barreras M, Frias MT, Sobrino F. An RT-PCR assay for the specific detection of classical swine fever virus in clinical samples. Vet Res. 1998;29:431-44.

10. Domingo E. Mechanisms of viral emergence. Vet Res. 2010: 41, 38 DOI: 10.1051/vetres/2010010.

11. Belák S. Molecular diagnosis of viral diseases, present trends and future aspects. A view from the OIE Collaborating Centre for the Application of Polymerase Chain Reaction Methods for Diagnosis of Viral Diseases in Veterinary Medicine. Vaccine. 2007;25:5444-5452.

12. Garigliany M-M, Bayrou C, Kleijnen D, Cassart D, Jolly S, Linden A, et al. Schmallenberg virus: A new Shamonda/Sathuperi-like virus on the rise in Europe. Antiviral Res. 2012. Disponible en: http://dx.doi.org/10.1016/j.antiviral.2012.05.014 .

13. Pérez LJ, Díaz de Arce H, Tarradas J, Rosell R, Perera CL, Muñoz M, et al. Development and validation of a novel SYBR Green real-time RT-PCR assay for the detection of classical swine fever virus evaluated on different real-time PCR platforms. J of Virological Methods. 2012;174:53-59.