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One-Step RT-qPCR assay for detection and quantification of equine infectious anemia virus *in-vitro*

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ABSTRACT. Equine infectious anemia virus (EIAV) infection often results in an initial febrile response, followed by recurrent cycles of the disease and finally, a prolonged asymptomatic period. These variations in clinical signs are due to a number of factors, including virus strain, equid species and differences in susceptibility among animals. As a consequence of the close relation between viral strain and disease, studies about *in-vitro* replication and fitness of EIAV in macrophages, which are the target cells of the virus, depend on accurate measurement of viral load throughout the infection period. We developed a method to quantify EIAV *in-vitro* using a one-step RT-qPCR system from a control RNA synthesized for this purpose. Designed primers amplified a 520 base pair fragment from the gag gene region that was inserted into a pGEM-T Easy Vector plasmid and propagated in *Escherichia coli* DH5-α. The bacteria with the construct were propagated and sufficient quantities of the template DNA were produced. The RNA was synthesized *in-vitro* from the plasmid linearization product and was used for standardization of a one-step RT-qPCR system with a minimum detection limit of 10 to

15 molecules. The efficiency of the reaction was 101%, with r^2 equal to 0.997. This new method can be used for the determination of virus titer in EIAV replication studies *in-vitro*.

Key words: EIAV; viral load; RNA; synthesis

INTRODUCTION

Equine Infectious Anemia (EIA) is caused by the Equine infectious anemia virus (EIAV), classified within the family *Retroviridae*, subfamily *Orthoretrovirinae* and genus *Lentivirus*. EIA is one of the main diseases that act as obstacles to the development and expansion of the equine industry. It is a transmissible, difficult to control and incurable disease that affects *Equidae*, with cosmopolitan distribution and mandatory reporting in many countries, requiring negative serological results for participation in animal events, local/regional transit and international trade.

The clinical course of EIAV infection occurs in three distinct phases: acute phase, evidenced by viremia and thrombocytopenia (Sellon, 1993); chronic phase, characterized by recurrent cycles of anemia with weight loss, viremia, edema, thrombocytopenia (Leroux et al., 2004) and an unapparent phase, with absence of clinical signs and undetectable viremia (Dong et al., 2012). This varied manifestation of clinical signs is a result of various factors, including virulence of the viral strains, equine species and differences in susceptibility between animals. (Cook et al., 1998; Cook et al., 2001; Cook et al., 2002)

To understand the dynamics of virus infection, it is useful to elucidate the pathogenesis and persistence of viruses, allowing prediction of disease progression, and evaluating the effects of antiviral therapy as well (Iwami et al., 2013). In general, virus isolation techniques or polymerase chain reaction (PCR) along with mathematical models are useful to determine certain aspects of *in-vitro* virus infection that are usually associated with disease severity, such as: sites of infection, used for detection of *Zika virus* (ZIKV) in amniotic fluids of microcephaly fetuses (Calvet et al., 2016), target cells, for identification of alveolar macrophages and dendritic cells such as *Measles virus* (MV) primary target, when it is transmitted by aerosol inhalation in non-human primates (Lemon et al., 2011), and viral gene functions, such as in the *Human immunodeficiency virus* (HIV) Vif gene, which is capable of inhibiting APOβEC class restriction factors expression, ensuring viral replication. (Hultquist et al., 2011)

Cells susceptible to replication by EIAV *in vitro* and which are mainly used for isolation of field isolates include monocyte-derived macrophages, which are difficult to maintain *in-vitro* (Cook et al., 2001; Malmquist, 1973) and immortalized fibroblast cell lines; however, the virus do not develop cytopathic effects in these cell lines. Some cells are used for cell-adapted viral strains such as the equine dermal (ED) cells and canine fibroblasts. Additionally, some EIAV clones that were constructed for viral fitness studies were also adapted for growth in the same cell types used for EIAV field strains (Whetter et al.; 1990; Cook et al., 1998), However, these cells do not develop alterations in cell morphology or growth kinetics; as a result, it is difficult to estimate the capacity of replication of different viral strains in the different cell types.

Therefore, the studies described so far for the detection of EIAV infection in tissues and organs, viral fitness, identification of infected cells and quantification of viral load were

performed by PCR for proviral DNA, RT-PCR for detection of viral RNA or by *in situ* hybridization. (Dong et al., 2012). However, the use of these techniques is restricted by the need for primers and probes with targets in highly conserved regions of the virus, which so far is not possible in regions where EIAV field samples have not been isolated and the genome sequenced. In these cases, there are limitations for primer design that can be used in PCR. (Craigo et al., 2013). Even EIAV gene regions such as *gag*, which are predicted to be conserved, have some genetic variability (Quinlivan et al., 2007; Cappelli et al., 2011). In addition, some inapparent carriers maintain viral loads below the detection limit of the currently available PCR-based assays (Cook et al., 2013).

Taking into account all of the difficulties involved in the cultivation, detection and quantification of EIAV *in vitro* and considering the importance to determine the viral fitness that is associated with pathogenesis, persistence and progression of the disease, we standardized a real-time *OneStep RT-qPCR* system from a control RNA produced in our laboratory, with the objective of improving accuracy and simplifying methods of detection of EIAV *in-vitro*.

MATERIAL AND METHODS

Primers and probes

The primers ($EIAV_{PV1458}$: TTC AGA ACG CAA ATG AGG AA and $EIAV_{PV1988}$: TGT TAC TAC CAC AAA CTG TCC A) were designed for the EIAV gene region called *gag* using the Primer3Plus (Untergasser et al., 2007), based on the nucleotide sequence described for the virulent strain $EIAV_{PV}$ (Rwambo et al., 1990; Cook et al., 2002) generating a 520 base pair fragment of the infectious molecular clone $EIAV_{UK}$, since it has complete homology with the consensus sequence of the surface glycoprotein of the $EIAV_{PV}$. (Cook et al, 1998).

The TaqMan probe (FAM ACG GGA AGC AAG GGG CTC AAG GGA GGC CBHQ-1) was previously described by Cook et al., (2002), which labeled the 5 'terminus with the 6-carboxyfluorescein fluorochrome reporter and the 3' terminus with the Black-Hole 1 fluorescence quenching molecule (Biosearch Technologies, Novato, CA).

Preparation for the RNA positive controls

The fragment of interest from the $EIAV_{UK}$ molecular clone was amplified by PCR using GoTaq® Flexi DNA Polymerase System (Promega). The reactions (25µL) consisted of 0.4µM primers, 3.0µM MgCl2 and 10µM de dNTP, under the following conditions: 30 cycles of denaturation at 95° C for 30s, annealing at 56° C for 30 sec and extension at 72° C for 1 min.

After amplification, the *gag-520* fragment was digested with the *EcoRI* enzyme, linked into the *pGEM-T easy vector* (Promega), using the enzyme *T4 ligase* (Promega) and propagated in bacteria *Escherichia coli* DH5-α, with subsequent midiprep (Midi Kit, Qiagen) using the high copy plasmids protocol. 50ng of pGEM-*gag*520 DNA (as suggested by the manufacturer) was subjected to linearization with the enzyme *PSTI* (Promega) and 100ng of the linearization product was used as a template for *in-vitro* transcription using the *T7 Ribo-MAX* ™ Express Large-Scale RNA Production System (Promega), according to the manufacturer's instructions.

The integrity of the transcribed RNA was monitored by analysis on 1.0% agarose gels stained with ethidium bromide. The newly synthesized RNAs were treated with DNase (Ambiom) according to the manufacturer's recommendations. Supernatants containing the synthetic RNAs were purified using the RNeasy mini kit (Qiagen) and the number of copies of

RNA generated after the entire process was quantified on the Qubit Fluorometric Quantitation (Thermo Fisher Scientific) equipment according to the manufacturer's instructions.

Construction of the qPCR standard curve

The One-Step RT-qPCR for the EIAV was standardized using the Real Time Ready RNA virus master mix kit (Roche), adding 0.6µM MgCl2, 0.4µM primers and a 125nM probe. The Rotor Gene (Qiagen) thermal cycler was run under the following temperature conditions: 50° C for 8 min, 95° C for 30s, 40 cycles of 95° C for 30s and 1 min at 60° C. Once submitted to real-time PCR, the fluorescence was recorded and the results analyzed by the software Rotor-Gene Q 2.3.1.49 in order to construct a standard curve and calculate the efficiency of the qPCR.

RESULTS

Obtaining the insert of interest *gag 520*

Initially a standard PCR reaction using the designed primers amplified a 520 base pair fragment of the $EIAV_{UK}$ molecular clone, which was visualized on 1.0% agarose gel. The generated fragment was extracted and purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega) following the manufacturer's recommendations. An aliquot was sent for sequencing, which proved to be the fragment of interest from the EIAV *gag* gene region. The remaining product after being bound to the plasmid vector and transformed into *E. coli* DH5-α bacteria, was divided into four aliquots and propagated in 25 mL of LB medium. Subsequently, the resulting product was quantified with a Low DNA Mass Ladder (Thermo Fisher Scientific) (Figure 1).

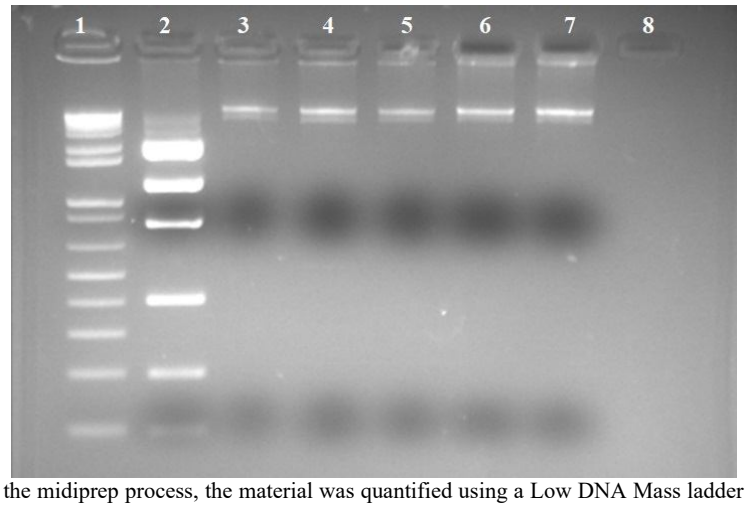


Figure 1: After the midiprep process, the material was quantified using a Low DNA Mass ladder (2). Of the four bacterial culture aliquots containing plasmid *pGEM-gag 520* DNA (3 to 6), only aliquots 6 and 7 obtained the minimum amount of 50 ng required to proceed with the linearization process of the plasmid. The negative control was ultrapure water (8) and the molecular weight marker 1kb plus (1).

Only two aliquots obtained the minimum amount of 50 ng required to continue the linearization steps. The efficiency of plasmid linearization and the availability of the genetic material of interest were confirmed by 1% agarose gel electrophoresis. (Figure 2).

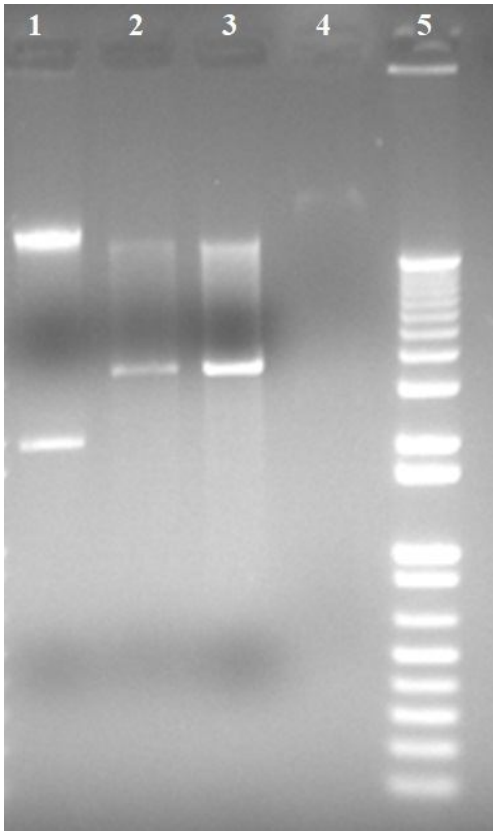
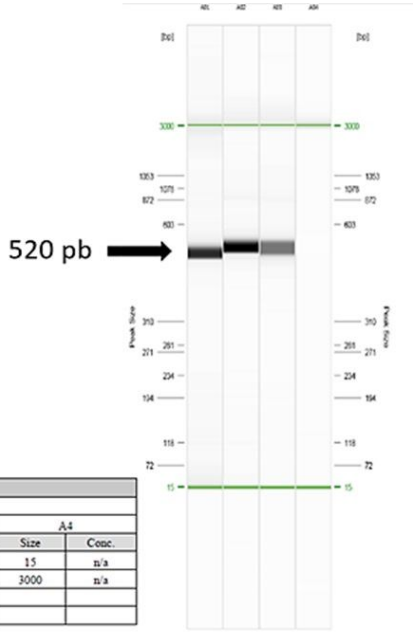


Figure 2: Preparation of the template DNA to RNA synthesis. The band that ran the slowest corresponds to the linearized plasmid (2 and 3) and the band that ran fastest corresponds to the plasmid in its circular form (1). Negative control with ultrapure water (4) and molecular weight marker 1kb plus (5).

The next step consisted of the synthesis of RNA from DNA. The concentrations of the control RNA generated after the synthesis were 164 ng /uL for aliquot 1, 63 ng/uL for aliquot 2 and 10ng/uL of the aliquot of the *pGEM-gag 520*.

Integrity analysis of the synthetic RNAs

To check for possible failures in the reverse transcription process of the synthesized RNAs, a RT-PCR was performed using the OneStep RT-PCR kit (Qiagen) and a capillary electrophoresis, which showed agreement between the height of the bands of the synthesized product when compared to the positive control *pGEM-gag 520*, indicating that the reverse transcription capacity of the produced RNAs remained intact (Figure 3). The integrity of RNA is a key element for the successful application of RT-qPCRs. (Fleige and Pfaffl, 2006).



Overall Result Table							
2015-11-26							
A1		A2		A3		A4	
Size	Conc.	Size	Conc.	Size	Conc.	Size	Conc.
15	n/a	15	n/a	15	n/a	15	n/a
509	4.03	527	11.87	521	4.26	3000	n/a
517	6.12	3000	n/a	538	3.94		
3000	n/a			3000	n/a		

Figure 3: Image generated by the Qiaxcel equipment after the capillary electrophoretic run of the 520-bp target RT-PCR products to test the newly synthesized EIAV RNAs. A1 corresponds to the synthetic EIAV RNA of aliquot 1; A2 corresponds to the synthetic EIAV RNA of aliquot 2; A3 corresponds to the positive control, plasmid pGEM-*gag* 520 and A4 refers to the negative control of the reaction with ultrapure water.

Standardization of the control curve

The standard curve construction was prepared in serial dilutions (log_{10}) from 10^{-7} to 10^{-3} of the *pGEM-gag 520* construct (Figure 4). In parallel, the amplification test was performed on the synthetic RNA aliquots that were produced (Figure 5).

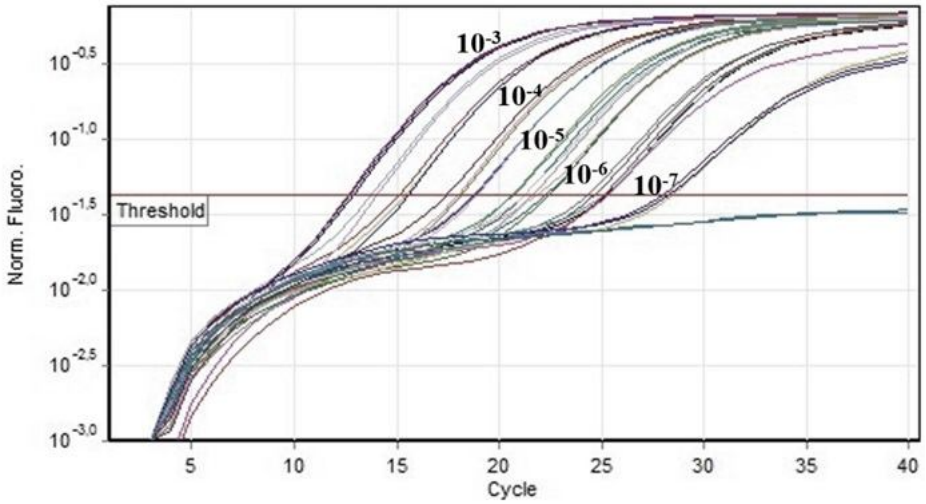


Figure 4: Construction of the control curve. Representation the amplification curves of the standard plasmid DNA curve.

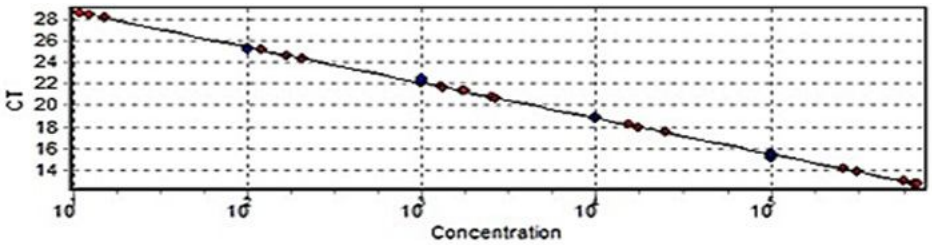


Figure 5: Schematic representation of the standard curve containing the log10 dilution points of plasmid DNA (blue) and tested dilution points of the two synthetic EIAV RNAs (red), which amplified within the linearity of the standard curve.

The minimum detection limit for the standardized system was between 10 and 15 molecules. The efficiency of the reaction was 101%, with r^2 equal to 0.997.

DISCUSSION

In this article a real-time *One Step qRT-PCR* system was standardized for direct quantification of the EIAV. For this purpose, we synthesized a control RNA and constructed a standard curve. This system consists of performing reverse transcription and PCR steps in a single tube. This ensures RNA integrity, a key element for the successful application of RT-qPCRs. (Fleige and Pfaffl, 2006) and increases detection sensitivity (Schroeder et al., 2006), as well as reducing sample handling, time to obtain the result and the number of false-positive results, since the two-step process in a single closed system avoids potential cross-contamination during sample preparation (Bao et al., 2008).

The standardization of the real time *One Step qRT-PCR* system was performed in triplicate. The efficiency of the reaction was 0.997, which is quite good because it indicates that the products generated practically double every cycle. (Fonseca JR et al., 2013). The minimum limit of detection was between 10 and 15 molecules, with a detection range of 10^4 logarithms. The quantification of the EIAV by competitive RT-PCR and analysis of gel electrophoresis stained with ethidium bromide generated a minimum detection limit of 100 molecules with a detection range varying from 10^4 to 10^5 logarithms (Cook et al., 2001). This indicates that, although the detection intervals between the two systems are similar, the real-time *One Step qRT-PCR* system showed a sensitivity 10 times higher than competitive RT-PCR.

Increasingly, One-Step RT-qPCR assays have been standardized for the detection and early diagnosis of viral diseases, as in the study by Bao et al. (2008) with Pest des petits ruminants virus; their system was able to detect 8.1 copies of RNA and obtained an increase in detection rate from 46.7 to 73.3% when compared to conventional PCR. The same method was validated for the detection of Dengue viruses in sera from individuals diagnosed as positive, demonstrating that sensitivity of detection can be one infectious particle (Leparc-Goffard et al., 2009), which provides a reliable and sensitive quantitative system.

Cook et al. (2002) standardized a real-time Multiplex-PCR for the quantification of EIAV RNA in equine plasma, using a control RNA from the *gag* gene region with 1459 base pairs to construct the standard curve. In this article, the RNA control synthesized to

construct the standard curve came from the same gene region and generated a fragment with 520 base pairs. The qPCRs using smaller amplicons have the shortest cycles, generating reliable results in a much faster way (Fonseca JR et al., 2013). In addition, the standard *One-Step RT-qPCR* system uses only one pair of primers, different from the reaction described by Cook et al., (2002), which used seven primers, making the process more laborious and expensive.

Here, we standardized a One Step RT-qPCR in real time using a positive control synthesized in our laboratory for the construction of the standard curve. This technique minimizes reagent costs, requires less time to generate a result, reduces the risk of contamination, avoids use of agarose gels stained with ethidium bromide, a highly carcinogenic substance, guarantees RNA viability and provides high sensitivity, decreasing the number of equivocal results. Thus, the synthesized RNA along with the standard curve will be useful for prospective studies of EIAV replication *in-vitro*.

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CONFLICT OF INTEREST

The authors declare they do not have any conflict of interests.

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