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Protocols

Development and validation of a real-time RT-PCR assay for the quantification of rabies virus as quality control of inactivated rabies vaccines

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ABSTRACT

Rabies is an infectious viral disease, characterized as a neglected zoonosis, responsible for nearly 60,000 deaths annually. The virus is transmitted mainly by dogs in Africa and Asia, and wildlife in Europe and the Americas, to all mammals' species, causing severe encephalitis almost always fatal after the onset of neurological symptoms. Human rabies can be prevented through extensive vaccination of dogs and pre/post-prophylaxis treatments in humans with inactivated rabies vaccines. The vaccine manufacture involves a series of quality control assays using laboratory animals, which are mandatory to exclude the presence of viable residual virus. The quality controls must be carried out in various steps during the vaccine production, which demands the use of a large number of animals. In this study, we standardized a real-time quantitative RT-PCR duplex assay to be used during intermediate stages of the vaccine production. This assay was done for the quantification of vaccine strain rabies virus, targeting rabies nucleoprotein, and β-actin mRNA of BHK-21 cells as an internal endogenous control. The results showed specific amplification, with the analytical sensitivity ranged from 10^1 to 10^6 $TCID_{50}$/mL with high repeatability rate for the quantification of rabies virus in inactivated vaccine samples. Global organizations are engaged to develop new approaches to determine viable residual virus, and this assay can be applied in combination with traditional *in vitro* methods for the release of intermediate batches of vaccines during the production process, keeping the *in vivo* tests only for final release.

1. Introduction

Rabies is an infectious viral endemic disease spread throughout more than 150 countries worldwide (WHO, 2017). It is responsible for nearly 60,000 deaths annually, mainly in Africa and Asia, with 40% of them occurring in children under 15 years old (WHO, 2017). Rabies virus is a bullet-shaped, negative-stranded RNA virus member of the *Rhabdoviridae* family, and its genetic material encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-directed RNA polymerase (L) (Davis et al., 2015). The virus is transmitted by bites, scratches or licks and affects all mammals, including humans. Therefore the virus is classified as a major zoonosis, characterized as severe encephalitis, which makes it almost always fatal after the onset of neurological symptoms (Balaram et al., 2016; Jackson, 2013).

Human rabies can be prevented through extensive vaccination of dogs and prevention of dog bites, since these animals are responsible for roughly 99% of all rabies transmissions to humans (Dürr et al., 2017; Velasco-Villa et al., 2017; WHO, 2017). Also, oral immunization programs of wildlife had major breakthroughs in rabies control in various European countries and the United States (Mähl et al., 2014; Maki et al., 2017). Moreover, human rabies can also be dealt with vaccination in pre/post-prophylaxis treatments (Takayama-Ito et al., 2014).

Since the development of the first rabies vaccine by Pasteur in the 1880s, several other vaccine types are available for veterinary and human immunization, such as the inactivated virus used in humans and domestic animals and the live attenuated for wildlife animals (Bruckner et al., 2003; Zhu and Guo, 2016). Several international organizations determine the production standards that are necessary to certify the quality and safety of these vaccines in the course of the production as

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internal controls, as well as for the final products before their release (Bruckner et al., 2003). The safety, inactivation, potency and pyrogenicity controls are some of these standards, which demand the use of large quantities of animals (Bruckner et al., 2003). Reports from USA and Europe manufactures indicate that approximately 70,000 mice per year are used exclusively for the potency assay (Stokes et al., 2012). With this in mind, several organizations engaged with animal welfare have motivated the development of alternative methods following the Three Rs principles (Reduce, Replace, Refine) (Bruckner et al., 2003). Thus, in order to reduce the number of animals, many studies have been done to develop alternative *in vitro* cell culture based assays. Some of these studies were already accepted by the European Pharmacopeia and other agencies as standard for the quality control of inactivated veterinary and human rabies vaccines (Stokes et al., 2012).

Numerous assays applying techniques of molecular biology such as RT-PCR have been developed in recent years to enhance the diagnosis of human and animal rabies (Dacheux et al., 2016; Faye et al., 2017; Wadhwa et al., 2017). However, not much has been done regarding the application of molecular tools for the improvement of quality control methods for anti-rabies vaccines.

Thus, the aim of this study is to standardize and validate a quantitative RT-PCR assay to be used in the determination of viable residual virus in inactivated rabies vaccines during the intermediate steps of the manufacture.

2. Materials and methods

2.1. Samples and viral RNA extraction

Samples of viable and inactivated rabies virus were obtained from the Institute of Technology of Paraná (TECPAR, Curitiba, Brazil), which produces inactivated rabies vaccine for dogs and cats. Rabies virus (PV – Pasteur virus) was propagated in Baby Hamster Kidney (BHK-21) cells, incubated with Dulbeccos's Modified Eagle's medium (D-MEM, Sigma-Aldrich, USA) and HAM F-12 (Sigma-Aldrich, USA) medium supplemented with 0,055% sodium pyruvate (Sigma-Aldrich, USA), 2,44% sodium bicarbonate (Merck, USA), 1,5% D-(+)-glucose (Sigma-Aldrich, USA), 0,05% gentamicin sulfate (Inlab, BR) and 3% fetal bovine serum (Laborclin, BR). Cells were cultured in T75 flasks for up to 3 days until a confluent monolayer was reached, inoculated with PV virus and incubated for up to 5 days at 37 °C. Viral suspension was collected and inactivation was done with 0.02 volumes of beta-propiolactone at 2–8 °C with continuous shaking for 48 h, then stored at −80 °C until use. Virus titers were determined by titration in BHK-21 cells as previously described (Inoue et al., 2003; Reed and Muench, 1938). The virus titer of the working stock was 1.0×10^6 $TCID_{50}$/mL.

A total volume of 200 u L of sample was processed to isolate rabies virus RNA using PureLink Viral RNA/DNA Mini Kit (Invitrogen, US), following the manufacturer's instructions. Viral RNA was eluted in a final volume of 50 µL RNase free water and stored at – 80 °C until testing.

2.2. Primers and probes design

Primers and probes used in this study are described in Table 1. The nucleoprotein gene, RABVgp1 (GenBank Ac. No. 001542.1) was selected as the target for the assay, since this is the most conserved region in the RABV genome, frequently used in diagnosis assays (Kissi et al., 1995). Primers and hydrolysis probes were designed with the aid of the Primer Designer™ Tool (version 1.2.4, Applied Biosystems, US), and were validated by BLAST analysis available on the NCBI database. A second assay, targeting specifically β-actin mRNA in BHK-21 cells, was also validated as an internal endogenous control (Zhang et al., 2015). Primers and probes were manufactured by Paraná Molecular Institute of Biology (IBMP, Curitiba, Brazil).

2.3. Real-time RT-PCR (RT-qPCR) assays

The duplex RT-qPCR was performed using the TaqMan One Step RT-PCR Master Mix kit (Applied Biosystems, US). The final reaction mixture of 15 µL contained 200 nM primers, 100 nM hydrolysis probes and 5 µL sample in RNase-free water. Positive controls containing master mix with standard RNA and negative controls consisting of master mix with sterile RNase-free water were both included in each run. The amplification was carried out by ViiA™ 7 Real-Time PCR System (AB Applied Biosystems, US) with the following reaction conditions: 1 cycle of reverse transcription at 50 °C for 30 min; 1 cycle of denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.4. Validation parameters

2.4.1. Specificity

A panel of 7 viruses commonly found in encephalitis cases was used to determine the specificity of the assay. Clinical samples of cerebrospinal fluid (CSF) previously tested as positive for cytomegalovirus (CMV), enterovirus non-polio (EV), herpes virus 2 (HSV2), varicella zoster virus (VZV), herpes virus 1(HSV1), erithrovirus B19 (EB19), human herpes virus 6 (HHV6) and Epstein-Barr virus (EBV), obtained from the Universitary Hospital of Federal University of Paraná (HC-UFPR, Curitiba, Brazil), underwent the same procedure described above.

2.4.2. Standard curves generation

Samples obtained from viable rabies virus cultures were diluted in RNAse-free water from 10^6 to 1 $TCID_{50}$/mL, to create a serial 10-fold dilutions of extracted RNA to be used in all assays as a standard curve. Quantitative standard curves were then generated by ViiA™ 7 Real-Time PCR System (AB Applied Biosystems, US) with the mean Cq values obtained from triplicates in each assay.

2.4.3. Linearity

The linear dynamic range was determined from the highest to the lowest quantifiable $TCID_{50}$/mL value and was done through a calibration curve that covered at least 6 $\log_{10}$ concentrations.

Table 1
Oligonucleotide sequences of primers and probes used in this study.

Name	Type	Length	Sense	Sequence 5'-3'	Gene	Position[a]	Tm (°C)[b]	Product size (nt)
RABV-RN1	Primer	23	Forward	5'-GAAGAGATCGCACATACGGAGAT-3'	Rabies Virus Nucleo-protein	1260-1282	58	82
RABV-FN1	Primer	22	Reverse	5'-TGTTTAGAAACTCGGCGAATGA-3'		1342-1321	58	
RABV-PR1	Probe	30	Forward	5'-6FAM-AGTCAGTTCCAATCATCAAGCTCGTCCAAA-BBQ -3'		1290-1319	69	
ACTB-R	Primer	25	Forward	5'-CAGCACCATGAAGATCAAGATCATT-3'	BHK-21 cells B-actin	1083-1107	60	131
ACTB-F	Primer	22	Reverse	5'-CGGACTCATCGTACTCCTGCTT-3'		1213-1192	60	
ACTB-P	Probe	25	Forward	5'-VIC-TCACTGTCCACCTTCCAGCAGATGT–BBQ -3'		1159-1183	65	

6FAM, 6-carboxyfluorescein; VIC,2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein; BBQ, blackberryquencher; nt, nucleotides.
[a] Corresponding nucleotide positions of RABVgp1 (GenBank Ac. No. 001542.1), and of Mesocricetus auratus b-actin mRNA (GenBank Ac. No. AJ312092).
[b] Melting temperature (°C).

2.4.4. Analytical testing

The variables used to evaluate the RT-qPCR assay were the coefficient of correlation (R^2) which indicates the goodness of regression, the amplification efficiency (E) through the formula $E = 10^{(-1/slope)} - 1$, and the limit of detection (LOD). The LOD test was chosen since its concentration can be detected with 95% of certainty.

2.4.5. Repeatability

For this test, various RT-qPCR reactions were done with one sample in the same run (intra-assay) and in different runs (inter-assay), and the SD of the Cqs as well as the coefficients of variation (CVs) were later calculated.

2.5. Inactivated virus sensitivity testing

After the RT-qPCR was validated, samples of inactivated rabies virus were analyzed to determine the sensitivity of the assay with this particular template. It must be established that this assay recognizes the damages in the RNA arising from the process of chemical inactivation in order to quantify the inactivated virus.

To further evaluate the applicability of the RABV-ActB duplex assay, inactivated virus suspensions (SVI) of rabies virus obtained from TECPAR were spiked with log10 dilutions of viable rabies virus, and tested according to the *in vitro* assay developed by Takayama-Ito et. al. (2014) for the detection of viable residual rabies virus in inactivated vaccines with minor modifications. Spiked SVI samples were incubated for 2 h in 96 well plates with BHK-21 cells, replaced by fresh media supplemented with 2,5% bovine fetal serum and incubated for 72 h at 36 °C with 5% CO_2. Culture media was then removed and cells were stained with the Direct Immunofluorescent Assay (DIFA) method, using fluorescein isothiocyanate (FITC)-labeled anti-rabies mAb (BioRad, FR) diluted 1/20 in Evans Blue solution (Sigma-Aldrich, USA) for the quantification of positive wells, meaning viable virus. Samples (200 µL) were taken at the moment of incubation, as well as at the end of the incubation period. RNA was extracted as described above and quantified by the RABV-ActB duplex assay.

3. Results

3.1. Primers and probes analysis

Primers and probes designed for this study underwent BLAST analysis and were proven to be highly homologous to target regions of RABV sequences available at GenBank database. Furthermore, *in silico* analysis revealed no mismatches to sequences from various rabies virus available at NCBI.

3.2. Validation parameters

The proposed assay for the quantification of vaccine strain rabies virus was assessed using several parameters. In order to determine the linear dynamic range, a calibration curve with 7 log10 concentrations was built. As shown in Fig. 1, the reaction is linear from 10^1 to 10^6 TCID50/mL.

The limit of quantification (LOQ) of the resulted analytical curve was 10^1 TCID50/mL per reaction obtained for the RABV N-gene duplex assay, with a slope value of −3.246, close enough to the optimal value of −3.3. Additionally, the coefficient of correlation (R^2) and the efficiency value (E) obtained for the targeted RABV N-gene in the duplex assay were 0.99 and 103%, respectively, indicating a good regression line (Fig. 1).

The sensitivity of the method was assessed using a 2-fold dilution series of standard cultured virus, with 20 replicates per dilution level. The LOD for the RABV N-gene duplex assay was 10^{-1} TCID50/mL. Intra-run and inter-run CVs of 9,2% and 6,4%, respectively, were found for the RABV N-gene duplex assay, indicating the high robustness and repeatability of this assay (Table 2).

3.3. Specificity

In order to ensure the specificity of the new system and to evaluate the occurrence of non-specific cross-reactivity, an isolate previously characterized as RABV and 7 clinical CSF samples positive for other virus capable of causing encephalitis were tested. As expected, each assay amplified and detected only the internal control, since human and rodent β-actin are homologues (Table 3). Cq value of 33,41 was determined as the Limit of Detection of the duplex assay, so it was established as the cut-off value for positive results. Cq values higher than the LOD (Cq > 33.41) were considered negative.

3.4. Evaluation of inactivated virus

The analysis of inactivated rabies virus samples was done after the assay proved to be robust. Viable and inactivated viruses were analyzed in the same run, and it was observed that both samples had positive amplification results, with Cq mean of 16.020 and 16.949, respectively, confirming that the duplex system is still able to identify the rabies' damaged RNA (Fig. 2).

From the combination of the RT-qPCR assay with the *in vitro* method for the quantification of inactivated rabies virus, it was detected (Table 4) that even when there was identification of positive wells by the DIFA method, quantification results from samples spiked with concentrations lower than 10^1 $TCID_{50}$/mL of viable rabies virus did not differ more than 0.5 log, this shows that there is no actual difference in the viral load of samples.

4. Discussion

Despite various attempts to reduce the numbers, refine the conditions of *in vivo* methods and even replace the use of laboratory animals necessary for quality controls assays needed to ascertain the safety and efficacy of inactivated rabies vaccines, little has been done specifically regarding the inactivation assay (Stokes et al., 2012). Development of an *in vitro* assay remains necessary in order to replace the large amount of animals, especially mice, required for the residual live virus assay demanded for the release of all batches of inactivated rabies vaccines worldwide.

In this study, we developed a TaqMan real-time duplex assay for the quantification of rabies virus in vaccines samples to be used in combination with an *in vitro* assay for the detection of residual live virus from inactivated rabies vaccines.

The new rabies vaccine virus quantification assay resulted in a high coefficient of determination (R^2) and outstanding efficiency (E = 103%), which demonstrates that this assay can be successfully applied to quantitative analysis of viral loads in rabies vaccines samples. Furthermore, the evaluation of analytical sensitivity proved that the assay can detect low amounts of RABV RNA, with LOD of 10^{-1} $TCID_{50}$/mL of RNA target per reaction, which indicates that the assay is highly sensitive, similar to previously reported assays for viral RNA using real-time RT-PCR (Dacheux et al., 2016; Faye et al., 2017; Hue et al., 2011; Stokes et al., 2012; Wadhwa et al., 2017).

During the specificity assessment and its selectivity to detect RABV, the new oligonucleotides designed in this study showed a high specific rate and no cross detection of other tested encephalitis-causing viruses. Nevertheless, there was the detection of human β-actin by the primer pair and probe used for the assay's internal control. This event occurred due to the 100% similarity between *Homo sapiens* and *Mesocricetus auratus*β-actin mRNA.

Achieving a sensitive duplex assay able to quantify rabies RNA with β-actin as an internal control opens a wide range of possibilities to expand this method to clinical diagnostic. This is possible due to the assay being a reliable quantitative tool for *intra-vitam* rabies diagnosis in humans and *post-mortem* diagnosis in animals, with much further analysis.

After the validation of the RABV RT-qPCR, in order to prove its applicability in the aid of *in vitro* determination of residual live virus in

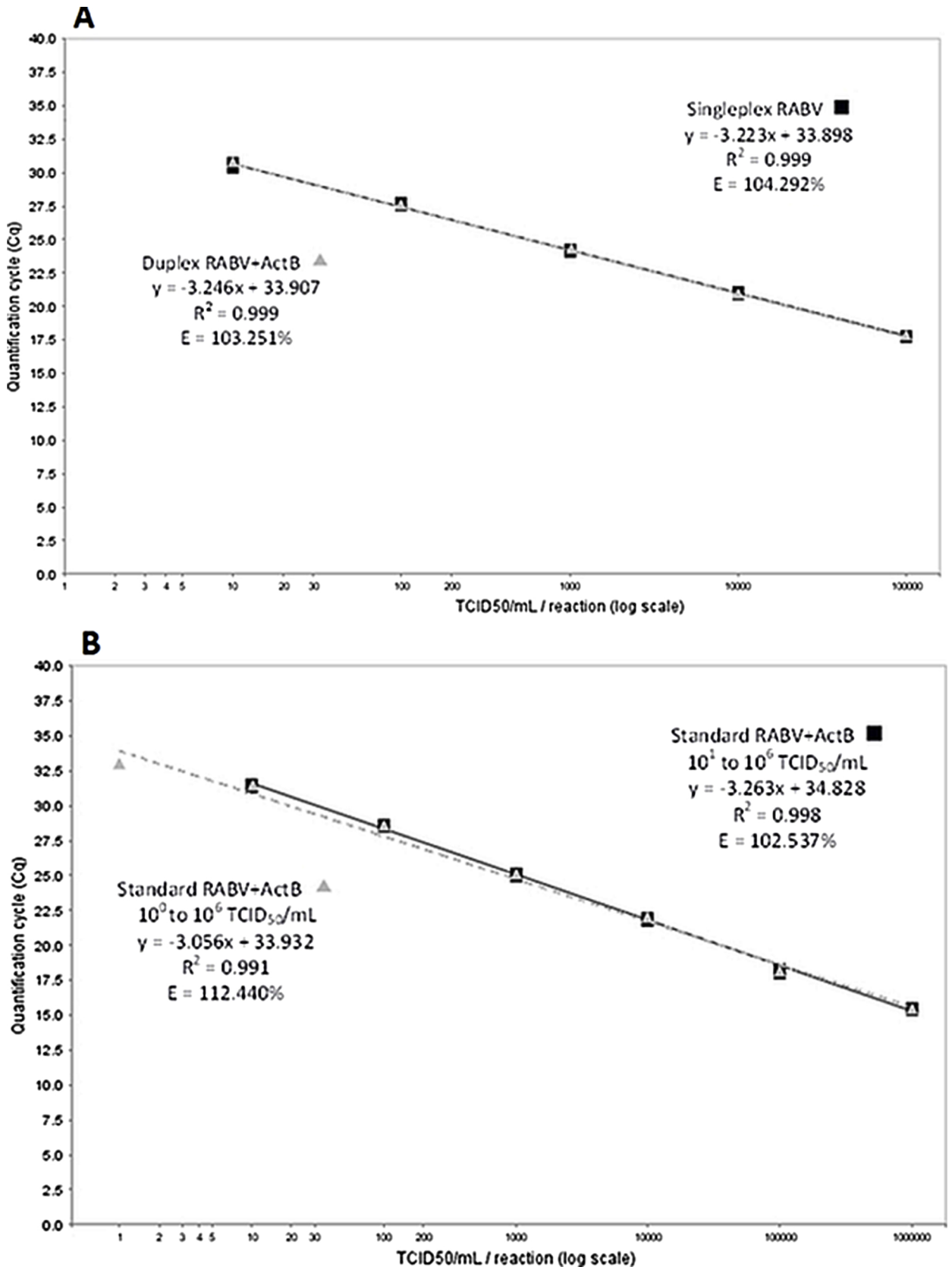

Fig. 1. Construction of standard curve for the duplex system RABV nucleoprotein gene and BHK-21 b-actin gene. (A) Evaluation of duplexing the RABV quantification system with the identification of ActB internal control, with no difference in efficiency in the duplex assay. (B) Linear Dinamic Range of the duplex assay RABV-ActB. The slope equation, the correlation coefficient (R^2) and the efficiency (*E*) of the linear regression curve are shown in the figure.

Table 2
Results of repeatability, reproducibility test and LOD.

Intra-run					Inter-run				
$TCID_{50}$/mL	RABVgp1		ActB		$TCID_{50}$/mL	RABVgp1		ActB	
	CqMean	%CV	CqMean	%CV		CqMean	%CV	CqMean	%CV
1,00E + 00	31.88	9.2	37.20	2.4	1.00E + 00	30.49	6.4	37.26	0.2
1,00E-0,5	32.65	2.9	37.41	3.6					
1,00E-01	33.41	2.5	37.42	3.0					

%: percentage, Cq: quantification cycle, CV: coefficient of variation, ActB: BHK-21 B-actin gene, RABVgp1: rabies virus nucleoprotein.

Table 3
Specificity results of the RABV-AtcB duplex assay.

	RABVgp1 Cq value	ActB
RABV	14.92	30.30
BHK-21	37.70	21.53
Enterovirus non-polio	36.77	24.61
Cytomegalovirus	37.06	32.82
Herpes virus 2/ Varicella zoster virus	37.73	21.63
Herpes virus 1	und.	36.48
Erithrovirus B19	38.40	32.42
Human Herpes virus 6	37.41	27.39
Epstein-Barr virus	und.	24.21

Cq: quantification cycle, ActB: BHK-21 B-actin gene, RABVgp1: rabies virus nucleoprotein, und.: undetermined. RABV: vaccine strain rabies virus, BHK-21: baby hamster kidney cell without rabies virus infection.

Table 4
Evaluation of RT-qPCR in combination with *in vitro* method.

TCID50/mL	DIFA % +	RT-qPCR RABV					
		0 hr			72 hr		
	Mean	Cq	TCID50/mL	log10	Cq	TCID50/mL	log10
10^1	11	12.27	226654.625	5.36	16.96	6887.523	3.84
10^0	6	12.92	139307.141	5.14	19.41	1108.811	3.04
10^-1	4	11.99	277676.625	5.44	18.82	1719.762	3.24
10^-2	0	13.11	120904.281	5.08	18.03	3088.186	3.49
10^-3	0	13.20	113306.367	5.05	18.61	2006.937	3.30
10^-4	0	11.96	286159.938	5.46	18.16	2802.671	3.45
SVI	9	12.78	155091.438	5.19	19.07	1424.467	3.15

Cq: quantification cycle, RT-qPCR RABV: quantification assay for rabies virus nucleoprotein. SVI: inactivated viral suspension of rabies virus, DIFA% +: percentage of positive wells identified with direct immunofluorescent assay method.

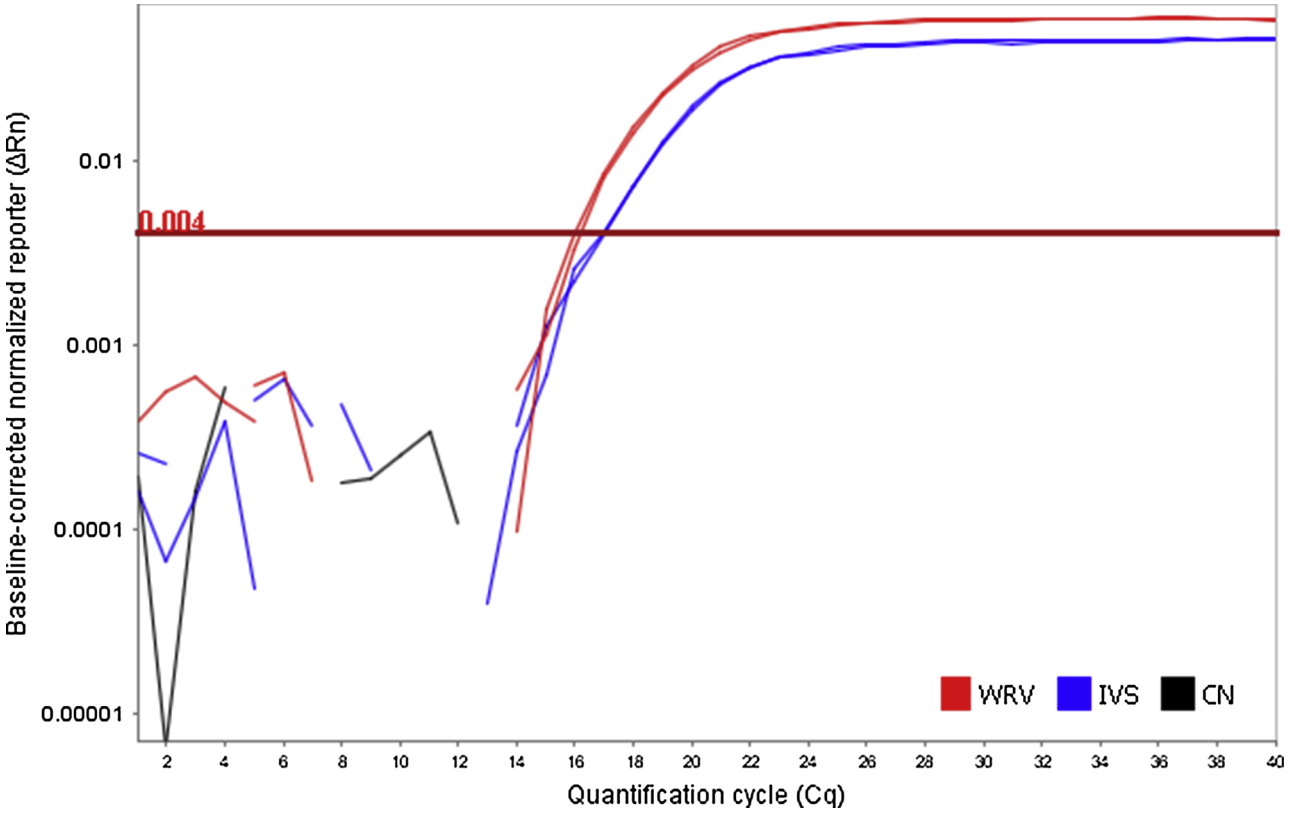

Fig. 2. Comparison of amplification of viable and inactivated rabies virus. WRV: working rabies virus, IVS: inactivated viral suspension, CN: negative control of the reaction (RNase-free H_2O).

inactivated vaccines, samples of inactivated rabies virus were analyzed. In the production of inactivated rabies vaccine, the chemical agent beta-propiolactone is used for viral inactivation and acts by alkylating mainly purine residues of cell DNA and viral RNA alike, causing intrastrand and interstrand associations, which results in errors in replication. These effects culminate in alterations of structure and capability of viral RNA to be used as template by various polymerases, thus inactivating the deadly virus (Perrin and Morgeaux, 1995). Therefore, considering the mechanisms by which the agent damages viral RNA, we established whether the developed assay would be able to recognize inactivated rabies virus in order to quantify it. As shown in Fig. 2, the assay was still capable of detecting inactivated virus and, in preliminary evaluations of the RT-qPCR assay combined with the *in vitro* method (Table 4), it was possible to observe that amplification of live virus could only be detected by the molecular method in concentrations above 10^1 $TCID_{50}$/mL. It was noticed that, in lower concentrations, log10 values do not vary more than 0.5 log.

There are already cell culture in-vitro assays accepted for the determination of residual live virus in rabies vaccines, available in the European Pharmacopeia 8.2, (2014), for example, whose results take 21 days.

Our group developed a cell culture in-vitro assay that can answer this question in 4 days (manuscript is being written). We believe that the molecular assay developed here can be used in combination with the cell culture assay with the objective of increasing the certainty of the test result. While the cell culture assay will assess the samples inactivation, the molecular assay will quantify the virus present and both results combined will be used to ascertain that the sample is in fact inactivated.

The molecular assessment would be done by comparing the RABV quantification in the beginning of incubation of the sample in the cell culture with the quantification in the end, and the sample would be considered inactivated with a final quantification lower than the initial.

In Brazil, according to the country's law, manufactures must perform the in-vivo residual live virus assay in all final batches of inactivated rabies vaccines. Although this test is obligatory for final batches, bulk products are also analyzed by the in-vivo method. The implementation of an in-vitro assay combining the two techniques in the bulk product analyzes would result in the reduction of 2/3 of mice used for this end, which in our line of production would result in a reduction of the use of approximately 5 thousand mice per year.

In conclusion, the RABV-ActB quantification system developed by our group shows a high specificity, sensitivity and repeatability rate for the quantification of vaccine strain rabies virus in inactivated vaccine samples. Moreover, it can be used combined with traditional *in vitro* methods for the development of new approaches aiming the determination of residual live virus in inactivated rabies vaccines, which must be validated to be used during the intermediate steps in the vaccine manufacturing process.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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References

Balaram, D., Taylor, L.H., Doyle, K.A.S., Davidson, E., Nel, L.H., 2016. World rabies day – a decade of raising awareness. Trop. Dis. Travel Med. Vaccines 2, 19–28. https://doi.org/10.1186/s40794-016-0035-8.

Bruckner, L., Cussler, K., Halder, M., Barrat, J., Castle, P., Duchow, K., Gatewood, D.M., Gibert, R., Groen, J., Knapp, B., Levis, R., Milne, C., Parker, S., Stünkel, K., Visser, N., Volkers, P., 2003. Three Rs approaches in the quality control of inactivated rabies vaccines: the report and recommendations of ECVAM workshop 48. ATLA Altern. Lab. Anim. 31, 429–454.

Dacheux, L., Larrous, F., Lavenir, R., Lepelletier, A., Faouzi, A., Troupin, C., Nourlil, J., Buchy, P., Bourhy, H., 2016. Dual combined real-time reverse transcription polymerase chain reaction assay for the diagnosis of lyssavirus infection. PLoS Negl. Trop. Dis. 10, 1–18. https://doi.org/10.1371/journal.pntd.0004812.

Davis, B.M., Rall, G.F., Schnell, M.J., 2015. Everything you always wanted to know about rabies virus (but were afraid to ask). Annu. Rev. Virol. 2, 451–471. https://doi.org/10.1146/annurev-virology-100114-055157.

Dürr, S., Fahrion, A.S., Knopf, L., Taylor, L.H., 2017. Editorial: towards elimination of dog mediated human rabies. Front. Vet. Sci. 4, 142–143. https://doi.org/10.3389/fvets.2017.00142.

Faye, M., Dacheux, L., Weidmann, M., Diop, S.A., Loucoubar, C., Bourhy, H., Sall, A.A., Faye, O., 2017. Development and validation of sensitive real-time RT-PCR assay for broad detection of rabies virus. J. Virol. Methods 243, 120–130. https://doi.org/10.1016/j.jviromet.2016.12.019.

Hue, K.D.T., Tuan, T.V., Thi, H.T.N., Bich, C.T.N., Anh, H.H.Le, Wills, B.A., Simmons, C.P., 2011. Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma. J. Virol. Methods 177, 168–173. https://doi.org/10.1016/j.jviromet.2011.08.002.

Inoue, K.I., Shoji, Y., Kurane, I., Iijima, T., Sakai, T., Morimoto, K., 2003. An improved method for recovering rabies virus from cloned cDNA. J. Virol. Methods 107, 229–236. https://doi.org/10.1016/S0166-0934(02)00249-5.

Jackson, A.C., 2013. Rabies: Scientific Basis of the Disease and Its Management, 3rd ed. Academic Press, Oxford, UK.

Kissi, B., Tordo, N., Bourhy, H., 1995. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology 209, 526–537.

Mähl, P., Cliquet, F., Guiot, A., Niin, E., Fournials, E., Saint-jean, N., Aubert, M., Rupprecht, C.E., Gueguen, S., 2014. Twenty Year Experience of the Oral Rabies Vaccine SAG2 in Wildlife : A Global Review. pp. 1–17. https://doi.org/10.1186/s13567-014-0077-8.

Maki, J., Guiot, A.L., Aubert, M., Brochier, B., Cliquet, F., Hanlon, C.A., King, R., Oertli, E.H., Rupprecht, C.E., Schumacher, C., Slate, D., Yakobson, B., Wohlers, A., Lankau, E.W., 2017. Oral vaccination of wildlife using a vaccinia – rabies - glycoprotein recombinant virus vaccine (RABORAL V - RG ®): a global review. Vet. Res. 48–57. https://doi.org/10.1186/s13567-017-0459-9.

Perrin, P., Morgeaux, S., 1995. Inactivation of DNA by beta-propiolactone. Biologicals 23, 207–211. https://doi.org/10.1006/biol.1995.0034.

Reed, L., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497. https://doi.org/10.1016/S0166-0934(02)00249-5.

Stokes, W., McFarland, R., Kulpa-Eddy, J., Gatewood, D., Levis, R., Halder, M., Pulle, G., Kojima, H., Casey, W., Gaydamaka, A., Miller, T., Brown, K., Lewis, C., Chapsal, J.M., Bruckner, L., Gairola, S., Kamphuis, E., Rupprecht, C.E., Wunderli, P., McElhinney, L., De Mattia, F., Gamoh, K., Hill, R., Reed, D., Doelling, V., Johnson, N., Allen, D., Rinckel, L., Jones, B., 2012. Report on the international workshop on alternative methods for human and veterinary rabies vaccine testing: State of the science and planning the way forward. Biologicals 40, 369–381. https://doi.org/10.1016/j.biologicals.2012.07.005.

Takayama-Ito, M., Nakamichi, K., Kinoshita, H., Kakiuchi, S., Kurane, I., Saijo, M., Lim, C.K., 2014. A sensitive invitro assay for the detection of residual viable rabies virus in inactivated rabies vaccines. Biologicals 42, 42–47. https://doi.org/10.1016/j.biologicals.2013.11.003.

Velasco-Villa, A., Escobar, L.E., Sanchez, A., Shi, M., Streicker, D.G., Gallardo-Romero, N.F., Vargas-Pino, F., Gutierrez-Cedillo, V., Damon, I., Emerson, G., 2017. Successful strategies implemented towards the elimination of canine rabies in the Western Hemisphere. Antiviral Res. 143, 1–12. https://doi.org/10.1016/j.antiviral.2017.03.023.

Wadhwa, A., Wilkins, K., Gao, J., Condori Condori, R.E., Gigante, C.M., Zhao, H., Ma, X., Ellison, J.A., Greenberg, L., Velasco-Villa, A., Orciari, L., Li, Y., 2017. A pan-lyssavirus taqman real-time RT-PCR assay for the detection of highly variable rabies virus and other lyssaviruses. PLoS Negl. Trop. Dis. 11, e0005258. https://doi.org/10.1371/journal.pntd.0005258.

WHO, 2017. Rabies - Fact Sheet n° 099.

Zhang, Y., Wu, S., Song, S., Lv, J., Feng, C., Lin, X., 2015. Preparation and characterization of a stable BHK-21 cell line constitutively expressing the Schmallenberg virus nucleocapsid protein. Mol. Cell. Probes 29, 244–253. https://doi.org/10.1016/j.mcp.2015.05.010.

Zhu, S., Guo, C., 2016. Rabies control and treatment: from prophylaxis to strategies with curative potential. Viruses 8, 279–302. https://doi.org/10.3390/v8110279.