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Title: Development and validation of sensitive real-time RT-PCR assay for broad detection of rabies virus

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Abstract

Rabies virus (RABV) remains one of the most important global zoonotic pathogens. RABV causes rabies, an acute encephalomyelitis associated with a high rate of mortality in humans and animals and affecting different parts of the world, particularly in Asia and Africa. Confirmation of rabies diagnosis relies on laboratory diagnosis, in which molecular techniques such as detection of viral RNA by reverse transcription polymerase chain reaction (RT-PCR) are increasingly being used.

In this study, two real-time quantitative RT-PCR assays were developed for large-spectrum detection of RABV, with a focus on African isolates. The primer and probe sets were targeted highly conserved regions of the nucleoprotein (N) and polymerase (L) genes.

The results indicated the absence of non-specific amplification and cross-reaction with a range of other viruses belonging to the same taxonomic family, *i.e Rhabdoviridae*, as well as negative brain tissues from various host species. Analytical sensitivity ranged between 100 to 10 standard RNA copies detected per reaction for N-gene and L-gene assays, respectively. Effective detection and high sensitivity of these assays on African isolates showed that they can be successfully applied in general research and used in diagnostic process and epizootic surveillance in Africa using a double-check strategy.

Keywords: Rabies virus (RABV), real-time RT-qPCR assays, molecular techniques, broad detection, Africa

1. Introduction

Rabies is a lethal and neglected zoonotic disease with significant public health impact in many parts of the world, especially in developing countries (1, 2). Rabies can affect almost all mammals, including humans (3). Indeed, rabies causes at least 59,000 human deaths annually worldwide, with 36.4% of them occurring in Africa alone (1, 4).

Human exposures occur mainly through dog bites and children are the most affected by the disease, with 4 out of every 10 deaths occurring in children under the age of 15 (3, 5). However, in some industrialized countries, rabies virus transmission was also reported through transplantation of organs from donors whose rabies infection had not been recognized. These cases of rabies virus transmission by tissues transplantation were mostly due to a long incubation period in the donor (6, 7, 8).

Rabies is an incurable disease and clinical presentation in humans can be sometimes difficult to distinguish from encephalitis symptoms caused by other viral infections (9). In this context, laboratory diagnosis is essential to confirm the diagnosis (10). Moreover, the diagnosis of rabies is often confirmed late in the course of the disease or *postmortem* because prognosis depends on history of exposure and clinical findings, and timely and reliable diagnosis may be very important for the prevention of rabies in the relatives of the patient and the healthcare workers.

This fatal encephalitis is caused by virus members of the *Lyssavirus* genus (order Mononegavirales, family *Rhabdoviridae*) including *Rabies virus* (RABV). RABV is a neurotropic enveloped pathogen encompassing a negative single-stranded RNA that is around 12 kb (kilobases) in size. As for all lyssavirus members, the RABV genome encodes five proteins with the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the polymerase (L) separated by four non-coding intergenic regions (IGRS) of different lengths and surrounded by two untranslated regions (UTR) (5). In addition, RABV presents a wide range of animal host reservoir and a large genetic diversity, with particularly four phylogenetic groups circulating in Africa with Africa 1 and 4 lineages, and with Africa 2 and 3 clades (11, 12). Thus, diagnostic tools which are able to broadly detect RABV are required.

Currently, the “gold standard” technique for confirming *postmortem* cases is the direct fluorescent antibody test (FAT) (13, 14). For *intra-vitam* diagnosis, this method can detect viral nucleocapsid antigens in tissues section of skin biopsies. However, viral antigens are often only detectable at the final phase of the disease and not always by the FAT. Also, this

technique presents limits in the case of *ante-mortem* non-neural sample material or decomposed tissues. Furthermore, repeated sampling of skin biopsies is not practical for improvement of sensitivity of the FAT (10, 14, 15). To overcome the limits of FAT, several conventional and hemi-nested reverse transcription polymerase chain reaction assays (RT-PCR) targeting the N or L gene, have been developed (16, 17, 18, 19, 20, 21, 22) and widely applied for *the intra-vitam* diagnosis of human rabies routine diagnosis. Indeed, the N gene is the most conserved region among the RABV genome which is the reason for its frequent use as target for rabies virus diagnostic assays (23). Also, the L gene harbours highly conserved regions which have targeted for molecular detection (22, 24). Despite a higher sensitivity, hemi-nested RT-PCR presents some disadvantages in terms of workload, risk of contamination, and time. Thus, several real time molecular tests targeting the N or L gene have been developed to complement conventional diagnosis of rabies and rabies-related viruses (10, 21, 25, 26, 27, 28, 29, 30). However, none of them have been validated against African strains from a large diversity of geographical origins, except a recent study (31).

In this paper, we describe the validation of two sensitive and specific real-time RT-PCR assays with TaqMan probes targeting conserved regions of the N and L genes, which were developed for broad detection of African RABV strains. The detection capacity on clinical specimens and sensitivity of our assays could suggest their suitable application in human diagnostic process and veterinary field, and their use as an added value and complement for already existing methods.

2. Materials and methods

2. 1. Primers and probes design

Initially, in order to design an assay capable of detecting African RABV isolates, full length genome sequences of two Africa 2 isolates available on GenBank database, corresponding to DRV-NG11 (GenBank Ac. No. KC196743) and CAR_11/001h (GenBank Ac. No. KF977826), were used. Multiple alignments were carried out using clustalW algorithm implemented in the Mega 6.0 software (32). We selected conserved regions of the N gene and the L gene for development of two detection systems. Both primers and TaqMan probes were designed using Primer3web® software (version 4.0.0, Whitehead Institute for Biomedical Research). To avoid non-specific cross-reactions with others lyssaviruses, primers and probe were validated by BLAST analysis on NCBI. To ascertain their broad spectrum of detection,

all primers and probes were analyzed *in silico* with sequences of a large panel of isolates from different parts of Africa. Primers and probes were synthetized by TIBMol-Biol (Berlin, Germany).

2. 2. Samples and viral RNA extraction

A total of 16 RABV isolates from experimentally infected mouse brain tissues preserved in the archive of the national reference center for rabies virus (NRC-Rabies) at Institut Pasteur of Dakar, Senegal (NRC-Rabies IPD) and previously confirmed as rabies positive by FAT, was used in this study. In addition, 61 other RABV isolates from FAT-confirmed primary brain samples or experimentally infected mouse brain samples, provided by the NRC-Rabies at Institut Pasteur, Paris, France (NRC-Rabies IPP) (14), were also included. Furthermore, in order to assess the specificity of the two RABV detection assays, a panel of 20 primary brain specimens provided by NRC-Rabies IPP from a range of important animals for rabies virus transmission, and previously confirmed to be negative by FAT, was also tested. In addition, 15 other major representatives of rhabdoviruses other than RABV and from the archive of NRC-Rabies IPD were also tested. Finally, 19 clinical specimens including skin biopsy, saliva and cerebrospinal fluid (CSF) samples received at NRC-Rabies IPP and collected from human patients suspected of rabies infection were evaluated with both of these molecular tools. All extractions from isolates provided by NRC-Rabies IPD were performed using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was suspended in a final volume of 60 µl elution buffer and kept at −80°C until testing. For samples and isolates provided by NRC-Rabies IPP, extraction and storage were performed as previously described (31).

2. 3. Real-time RT-PCR (RT-qPCR) assays

A similar RT-qPCR protocol was used for the detection of both N and L genes. All samples were tested in duplicate and RT-qPCR was performed using the Quantitect Probe RT-PCR kit (Qiagen, Hilden, Germany) in a 20 µl reaction mixture containing 2x QuantiTect probe RT-PCR master mix, 0.25 µl of QuantiTect RT mix, 400 nM PCR primers and 200 nM TaqMan probes and 5 µl of 1:10 diluted RNA in RNase-free water. Positive controls containing master mix with standard RNA of each system and negative controls consisting of master mix with sterile RNase-free water were included in each run. The reaction was carried out on a 7500 Fast Real Time system cycler or on a 7500 Real Time system cycler (Applied Biosystems,

Foster City, US) using the following temperature profile: 1 cycle of reverse transcription at 50°C for 10 min, 1 cycle of denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The RT-qPCR reactions were analyzed using the 7500 software (v2.0.1).

2. 4. Specificity testing

The specificity, representing the probability that the assay gives a negative result without presence of the targeted nucleotide region, was calculated by the formula Sp = a / (a + c); where **a** is the number of true negatives samples and **c** is the number of false positives. The specificity was determined using a panel of FAT negative samples and a panel of non-RABV rhabdoviruses.

2. 5. Analytical sensitivity assessment

2. 5. 1. Standard curves generation

Plasmids were generated by inserting the amplified RT-qPCR amplicons into pCRII (Life Technologies, GmbH, Darmstadt) and standard RNAs were synthetized with T7 RNA-polymerase by TIB MolBiol (Berlin, Germany) according to manufacturer's recommendations.

Serial 10-fold dilutions of standard RNAs, from 10^8 to 1 molecule, were prepared in RNase-free water. Corresponding mean Cq (quantification cycle) values obtained per triplicates per standard dilution were then plotted proportionally to the logarithm of the input copy numbers to generate a quantitative standard.

2. 5. 2. Analytical testing

The quantitative standards were used to evaluate the diagnostic assays performances such as limit of detection (LOD), the coefficient of correlation (R^2) and the amplification efficiency (E) values. The LOD value represents the concentration where at least 50% of replicates of each dilution were detected. A linear model regression test was performed using R (version 3.0.2, *The R Foundation for Statistical Computing*) to determine the value of R^2, indicating the goodness of regression. The E value was calculated with the formula $\mathbf{E = 10^{(-1/slope)} - 1}$. Relative RABV genome copies in tested samples was calculated through the formula **q=**

$\mathbf{10^{(Cq-b)/a}}$; where **a** is the slope, **b** is the intercept from a standard curve of each specific target gene and **Cq** is the quantification cycle number. Furthermore, the Pearson correlation efficient where a coefficient of 1 represents a good correlation was also calculated.

2. 5. 3. Determination of sensitivity

The sensitivity is defined as the probability that an assay give positive results in the presence of specific target. This was calculated by the formula **Se = d/ (d+b)**; where **b** represents the number of false negatives and **d** is the number of true positives. Positive and negative predictive values were also calculated by formulas **PPV= d / (d+c)** and **NPV= a/ (b+a),** where **a** is the number of true negatives samples and **c** is the number of false positives**.**

Moreover, a **ROC** («Receiver Operating Characteristic») analysis was performed, using the statistical software XLSAT (Version 2015.5.01.23654, *Copyright Addinsoft 1995-2015*) to identify the threshold value below which the test should be considered positive. Accuracy of assays was measured by the area under the ROC curve **(AUC)** with 95% confidential interval (CI) of AUC. An area higher than 0.9 represents an excellent test and a value of 0.5, a worthless test (33, 34).

Finally, the comparability between Cq values given by these two quantitative assays was assessed using a Bland-Altman analysis where the agreement between these two measurements was quantified by using a graphical approach. Statistical limits of agreement are calculated by using the mean and the standard deviation of the differences between assays with 95% CI (35).

2. 5. 4. Repeatability and inter-laboratory concordance

The inter-assay and intra-assay repeatability were determined by coefficients of variation (**CVs**) of Cq value. Indeed, RNA of the RABV isolate SA217695SEN was extracted and amplified 10 times in the same run, for the assessment of intra-assay repeatability and in 10 different runs by the same operator, in the same laboratory to evaluate inter-assay repeatability. Inter-laboratory concordance was evaluated using a range of anonymous samples which have been initially received, extracted and analyzed at the NRC-R IPP in the framework of its participation in 2015 in an inter-laboratory trial organized by the European Union reference laboratory for rabies, which is located in Nancy, France (36). The test panel tested with the two newly developed RT-qPCR consisted of nine anonymous samples, either uninfected or infected with various lyssavirus species and which have been previously

extracted by NRC-R IPP and storage at -80°C until use. Details of this trial have been provided elsewhere (36).

Results obtained with both of these tools were compared to previous RT-qPCR results of the NRC-R IPP in terms of Cohen's kappa coefficient ***(k)***, which is measure of the agreement between two raters. Statistical analysis was performed using XLSAT where the statistical significance was set to $p < 0.05$.

2. 5. 5. Comparison with conventional hemi-nested RT-PCR

Suckling newborn-mices were inoculated experimentally by the RABV isolate SA217695SEN and a virus titer of 6.7 10^7 ID_{50}/mL was obtained according to the method of Reed and Muench (1938) (37). Ten-fold serial dilutions of this virus stock were used to compare the sensitivity of the two RT-qPCR methods with that of a conventional hemi-nested RT-PCR (RT-hnPCR) in the conditions which have been previously described (22).

2. 5. 6. Clinical sensitivity testing

Testing of a collection of nineteen RABV-positive or negative specimens including CSF, saliva and biopsy skin, was also carried out in duplicate to confirm the reliability of the new L-gene RT-qPCR.

3. Results

3. 1. Primers analysis

BLAST analysis of designed primers and probes showed high homology with target regions of RABV sequences available from GenBank database. *In silico* analysis also revealed limited number of mismatches for primers and probes with sequences from other parts of Africa belonging to lineage Africa 1 and clade Africa 2 (Supplementary **Table A.1**) (Supplementary **Figures A.1 and A.2**). The TaqMan probes were labeled 5' FAM (6-carboxyfluorescein) / 3' BlackBerry Quencher (BBQ) and details are listed in **Table 1**.

3. 2. Specificity

In order to ensure the specificity of the new systems and to evaluate the occurrence of non-specific cross-reactivity, 16 isolates previously characterized RABV field samples and 15

different rhabdoviruses other than RABV species were tested in duplicate with both of the assays (**Table 2**). Fluorescent detection was obtained only with RABV isolates whereas non-targeted viruses were not detected. These results suggested that the designed primer pairs and probes exhibited no cross-reactivity with other species of lyssavirus (with Mokola and Lagos bat lyssaviruses) or other rhabdoviruses (**Table 2**). The amplicons size for some RABV isolates detected with each system was verified by 2% agarose gel electrophoresis and the amplified fragments were sequenced using the respective forward and reverse primers of the corresponding systems. A BLAST analysis of obtained sequences showed high identity rate with the target region of the RABV isolate CAR_11/001h (GenBank Ac. No. KF977826) belonging to the Africa 2 phylogenetic clade. Then, these primers and probes were highly selective for RABV detection. In addition, we analyzed a panel of 20 FAT-negative primary brain samples from different vertebrate hosts important in RABV transmission. All were negative with both assays and confirmed that specificity was complete (Supplementary **Table A.2).**

3. 3. Analytical **sensitivity**

Diagnostic test performances of the new assays were assessed using several parameters. Analytical curves generated showed a LOD of 100 and 10 copies per reaction obtained for N-gene assay and L-gene assay, respectively with RABV isolate SA217695SEN (**Figure 1**).
Regression line slope values were determined for N gene and L gene assays, with -3.08 and -3.1, respectively, which are close to the optimal slope value of -3.3. Significant respective linear regression p-values of 1.833e-09 and 6.977e-12 for N gene and L gene assays, respectively, and coefficients of correlation (**R^2**) values higher than 0.99 indicated the goodness of the regression lines. Furthermore, high efficiency values (**E**) were obtained with both of these assays. A Pearson correlation test between the two regression curves revealed a coefficient of 0.99 ($p < 0.0001$) suggesting that correlation between the Cq values obtained by both RT-qPCR methods was excellent (**Figure 1**).
To ascertain the broad detection capacity of established assays, various RABV isolates from different parts of the world, were tested. Considering detection of African RABV isolates, the L-gene system was able to detect all isolates tested while the N-gene assay detected only 90% of strains giving no-fluorescence signal with four isolates belonging to Africa-1 lineage, including isolates from Somalia (93002SOM and 93006SOM), Tanzania (96013TAN) and Morocco (87012MAR) (**Table 3a**). Furthermore, the L-gene system also detected 100% of

tested isolates from other counties of the world. Considering these isolates, the N-gene assay had a limited detection scale (55%) with no fluorescence signal with 9 isolates from Poland (96140POL), Germany (92001GER), Saudi Arabia (87001ARS), Turkey (94009TUR), Laos (99008LAO), Afghanistan (02052AFG), Russia (91041RUS) and one skunk isolate from USA (91004USA) (**Table 3b**) (38).

Results of all previous tests summarized in **Table 4** were used for sensitivity determination and ROC analysis as previously described (39). A sensitivity of 100% and 78.70% (CI 95%; 68.40-89.00%) was found for the RABV L-gene assay and RABV N-gene assay, respectively, when compared to the reference technique. In addition, a PPV of 100% for each assay and NPVs of 100% and 72.91% (CI 95%; 56.76-82.79%) for L gene assay and N gene assays were obtained respectively (Fischer's exact test $p < 0.0001$). All previously FAT- negative samples provided an undetermined mean Cq value after 40 cycles. Based on the results of the ROC analysis, we determined a Cq of 39.70 as the cut-off value below which reactions are positives for the L-gene assay and the N-gene assay, considering a 95% confidence interval. A Cq value above these cut-off values or an undetectable Cq was considered negative. An AUC of 1 was determined for each assay revealing that the tests have an excellent accuracy (**Figure 2**). Furthermore, Bland-Altman plot analysis also reported a non-significant bias (mean difference) of 1.920±6.875 (95% limits of agreement: -11.56 to 15.40), demonstrating that these two methods are approximatively equivalent (**Figure 3**).

Intra-run and inter-run CVs of 1.67 % and 2.20 %, respectively, were found for the RABV N-gene assay while the RABV L-gene assay showed intra-run and inter-run CVs of 1.08 % and 2.16 %, respectively; indicating that assays are robust and highly repeatable(**Table 4**). In addition, the ***k*** of 1±0.31 (95% CI; 0.38-1.61) indicates that assays are accurate, detecting only RABV strains and giving 100% concordance to previous results obtained by the NRC-R IPP using a RT-qPCR (31) with the same samples ($p= 0.001$), and concordant with the results expected for this inter-laboratory trial with RABV isolates (**Table 5**).

Simultaneously, both RT-qPCR assays and conventional hemi-nested RT-PCR were performed as previously described (22) on 13 ten-fold dilutions of the RABV isolate SA217695SEN with a titer of 6.7 10^7 ID_{50}/mL. The RABV L-gene assay detected until 0.00067 ID_{50}/mL, corresponding to 28 genome copies using the L-gene quantification equation. However, conventional hemi-nested RT-PCR and RABV N-gene assay presented a detection limit of 6.7 ID_{50}/mL, corresponding to 75 copies of genome using N-gene quantification equation (**Table 6**).

The RABV L-gene RT-qPCR assay was selected as the most sensitive technique for detection of RABV and was thus applied for the screening of human clinical samples from patients suspected of rabies infection. The clinical detection of RABV RNA was analyzed in duplicate using a panel of human samples from the archive of the NRC-R IPP and which were previously tested using a RT-qPCR assay (31). Compared to the latter technique considered as the reference test, all 4 previous RABV-positive samples were correctly detected with RABV-L TaqMan probe assay, whereas any cross-reactivity was detected with all 15 RABV-negative specimens. Thus, the proposed RABV-L gene assay gave a 100% concordance to the previous diagnosis results obtained with the RT-qPCR assay performed by NRC-R IPP (31), demonstrating its effectiveness to detected RABV in clinical specimens with high sensitivity, and its suited use as diagnostic tool in human RABV cases (**Table 7**).

4. Discussion

Despite rapid and sensitive RABV methods developed in recent years (27, 28, 30, 31, 42), only a few of them have been validated for the detection of African RABV strains with a large diversity of origin (21, 30, 31). Development of rapid and suitable molecular diagnostic tools for large-scale detection RABV isolates is important for routine diagnostic testing and epidemiological surveillance, particularly in Africa where rabies remains endemic. In this study, we developed two TaqMan real-time assays for broad detection of African RABV isolates in samples and for *intra-vitam* diagnosis in humans and *post-mortem* diagnosis in animals. These assays are based on conserved regions on N and L RABV genes. Oligonucleotide sequences for each assay were designed for wide detectability of African RABV isolates (**Table 1,** Supplementary **Figures A.1** and **A.2**) (22). Regarding the specificity assessment and their selectivity to detect RABV on a large range of samples, the new primers pairs and probes showed a high specific rate and no cross detection of other tested rhabdoviruses and none of the 6 other lyssaviruses species tested. These results were also confirmed by sequencing a selected number of amplicons obtained from positive results.

Moreover, evaluation of the analytical sensitivity proved that assays can detect a low amount of RABV RNA with estimated LODs ranging from 100 to 10 copies of RNA target per reaction, which indicates that our assays are highly sensitives, similar to previously reported assays for viral RNA detected by TaqMan real-time RT-PCR (21, 31, 40, 41, 42). In addition, the high coefficients of determination (**R^2**) and very good efficiencies (**E** >100%) demonstrate

that they can both be successfully applied to quantitative analysis of viral loads in tested samples infected with RABV.

However, with the high genetic diversity of RABV, development of a single sensitive real time assay covering all widely known phylogroups remains a challenge (43, 27). During the validation of the N-gene and L-gene based RT-qPCR assays, the broad detection spectrum was assessed through a large panel of RABV isolates representing different continents, with a focus on African isolates. Indeed, African strains were detected by both assays with a high coverage rate, mainly with the L gene-based assay. Thus our assays offer good performances on detection of African isolates and could be a useful complement for a recent published method (31) which presents some limits in detection of RABV isolates from Senegal belonging to the Africa 2 clade. Evaluation of the broad detection performance of our assays on isolates from other regions of the world displayed an excellent coverage level of the RABV L-gene RT-qPCR, whereas the RABV N-gene assay was limited with important non-African RABV strains. The reduced sensitivity of the N-gene assay despite good *in silico* profiles and performance demonstrated by the assays could be explained with the presence of secondary structure formation of the target area which may make it less accessible for the N-gene oligonucleotides during the RT-step (44, 45). All the RABV isolates tested (61 isolates belonging to the phylogenetic lineage cosmopolitan and clades Africa 2 and 3) were successfully detected, mainly with the L gene-based assay which provides a test with a large spectrum of RABV detection.

Although in the comparative analysis, the L gene-based assay offered better performances than the N-gene assay regarding sensitivity and predictive values, it seems reasonable to perform a double-check strategy on tested samples, using both assays in order to increase reliability during use in routine rabies identification or epidemiological surveys as previously reported for others lyssaviruses (46, 31). Moreover, high AUC value found in ROC analysis predicted that both assays are useful for accurate detection of RABV in positive samples. Furthermore, the assays showed a good agreement between measurements as shown by the Bland-Altman analysis results, confirming their ability to be used efficiently for RABV detection. Additionally, inter-assay and intra-assay variability below 3% and strong inter-laboratory concordance results ensure the repeatability and the RABV specificity of the two assay systems. Our assays, particularly the L-gene based assay, could be used accurately in different geographical contexts for RABV detection.

We used ten-fold dilutions of the RABV isolate SA217695SEN with a titer of 6.7 10^7 ID_{50}/mL and obtained with L-gene assay lower LOD value than with the hemi-nested RT-

PCR (22), although it has to be confirmed with a larger panel of isolates. The L-gene based assay was able to detect RABV with as low as 6.7 10^{-4} ID_{50}/mL and could be successfully applied in RABV diagnostic process, particularly on decomposed material or samples with low viral load (15, 47). Furthermore, compared to the hemi-nested RT-PCR, the established RT-qPCRs offer some important advantages in reduction of workload and run time, while providing a higher sensitivity compared to the conventional RT-PCR.

Based on its better performances, the L gene-based assay was chosen for determination of the detection capacity with clinical specimens in comparison with a previously existing RT-qPCR (31) considered as the reference technique. We evaluated the usefulness of the L-gene based assay for the *intra-vitam* diagnosis of rabies in human using a collection of positive and negative specimens including skin biopsy, saliva and cerebro-spinal fluid. Compared to the RT-qPCR assay from the NRC-R IPP (31) considered as the reference test, our L-gene based assay provided a strong concordance and the sensitivity was also high. This technique could be useful in clinical diagnostic as reliable quantitative tools for *ante-mortem* rabies diagnosis in humans (22, 31).

Finally our assays, particularly the L-gene based assay, could be a complement for existing methods for rabies diagnosis (21, 31, 46, 48, 49) with a high specificity, sensitivity and repeatability and more suitable for broad detection of African RABV strains.

Conflict of interest statement

We declare that we have no conflict of interest.

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Highlights

- Two reliable real-time quantitative RT-PCR assays were developed for RABV detection.
- RT-qPCRs were targeted to highly conserved regions in nucleoprotein and polymerase.
- Analytical sensitivity ranged between 100 to 10 RNA copies per reaction for assays.
- Assays can be successfully applied on African isolates for research and diagnostic.

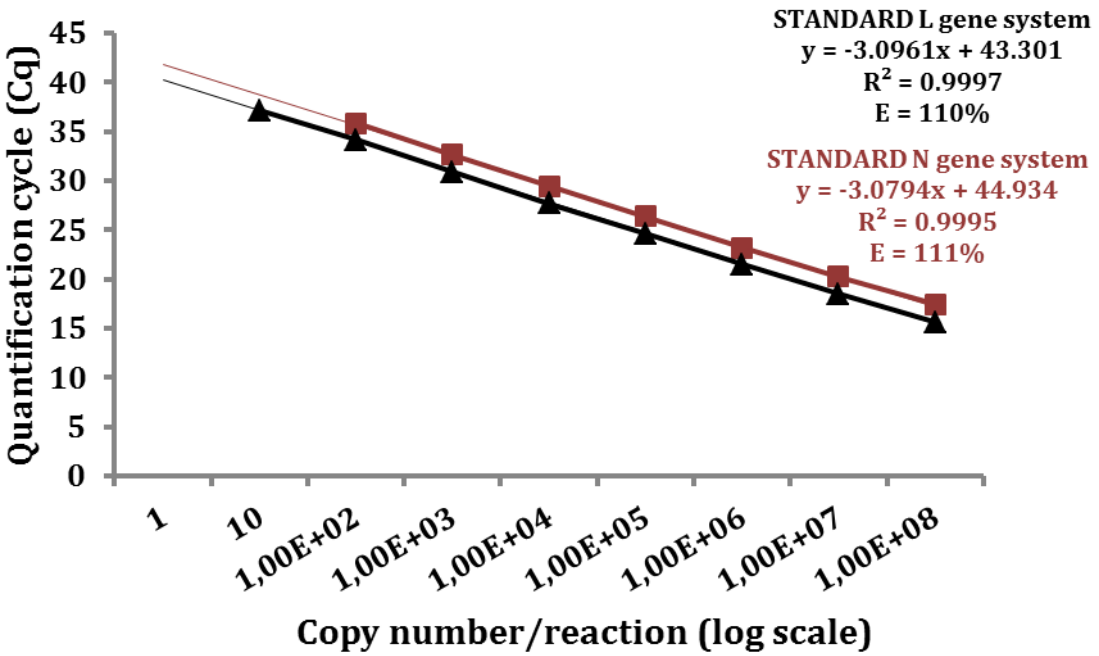
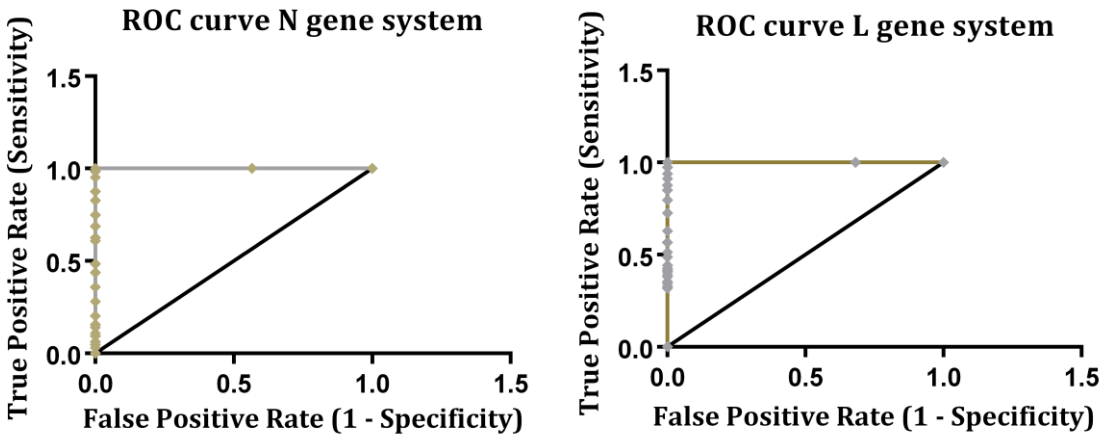
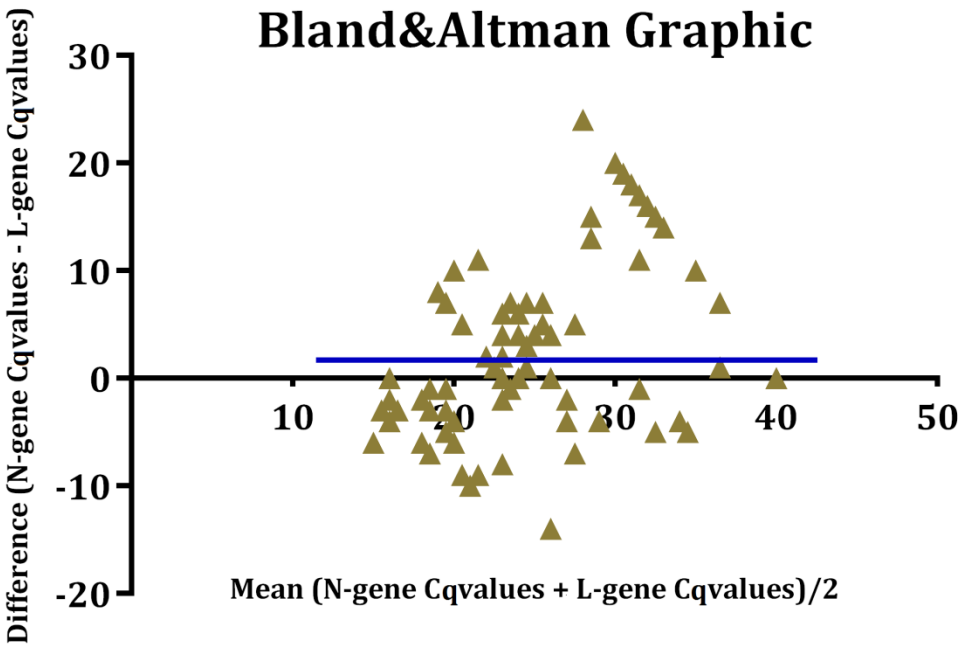


Figure 1: Standard curves of the developed TaqMan RT-qPCR assays using RABV-nucleoprotein gene-specific probe (N) and RABV-polymerase gene-specific probe (L). The slope equation, the correlation coefficient (R^2) and the efficiency (E) of each linear regression curve are indicated in the figure. These standard curves were established using the RABV isolate SA217695SEN, and results are indicated as RNA copy number detected per reaction.



<u>Figure 2</u>: ROC curves of the developed assays using XLSTAT. The AUC value of 1 indicates that the established assays are very powerful to detect RABV genome when samples are positives.



<u>Figure 3</u>: Bland-Altman graphic of differences between N-gene and L-gene assays plotted against average Cq values of the two measurements. The bias of 1.920±6.875 (CI 95%; -11.56 - 15.40) units is represented by the gap between X axis (zero differences) and the line of equality (blue) parallel to the X axis.

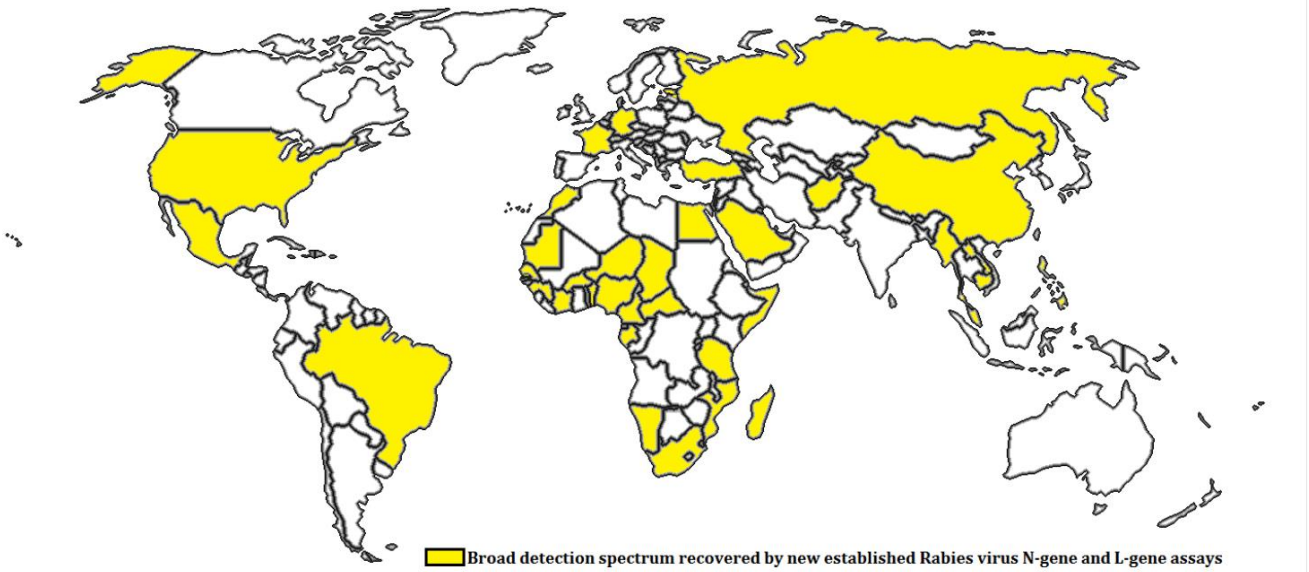


Figure 4: Broad detection spectrum of new RABV assays. The countries where tested strains originated are colored in yellow, showing a significant detection of established RABV N-gene and L-gene diagnostic assays.

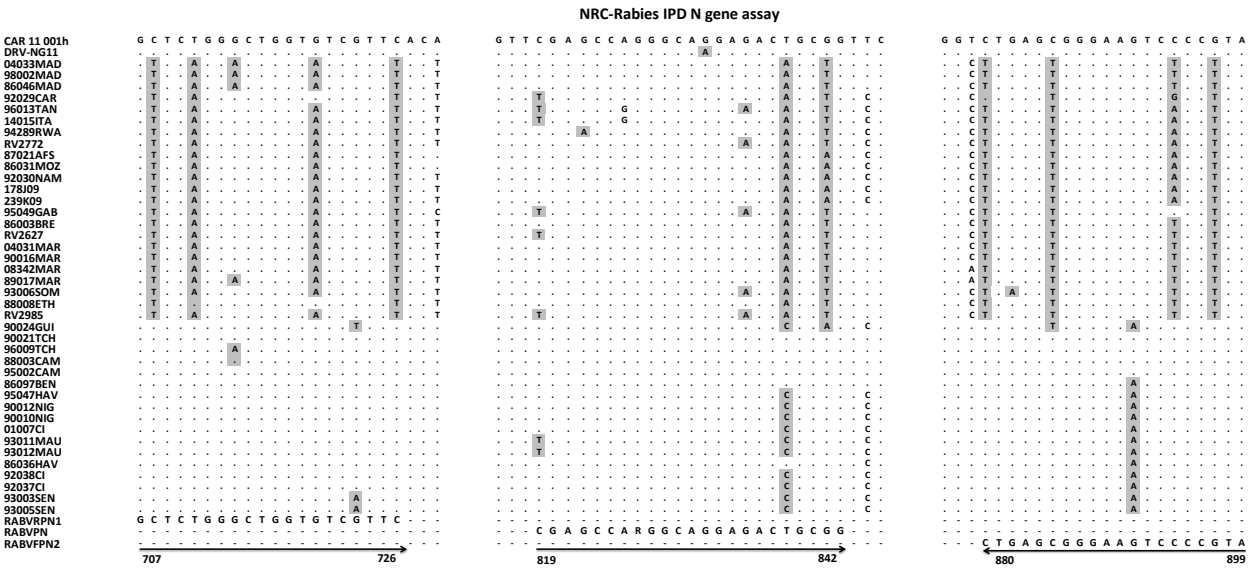
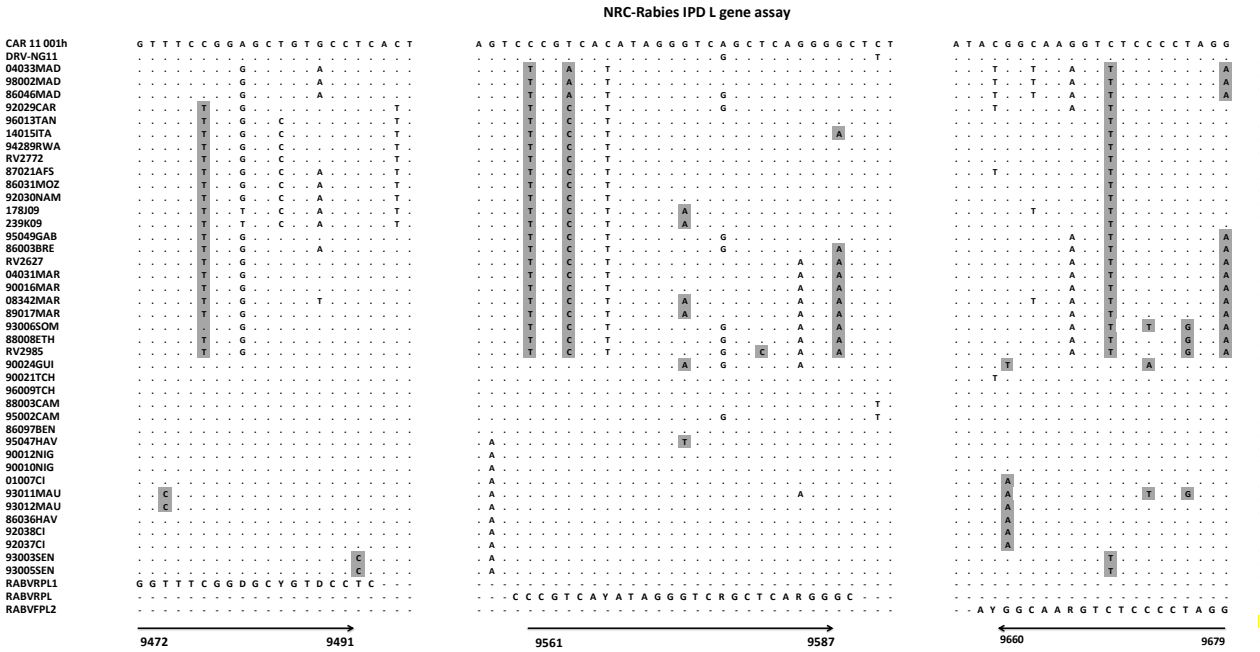


Figure A.1: ***In silico*** **multiple alignments of N gene primers and probe (NRC-Rabies IPD N gene assay) with 44 partial nucleoprotein sequences from rabies virus isolates belonging to lineage Africa1 and clade Africa2.** Corresponding nucleotide positions of each primer are indicated according to the reference sequence CAR_11/001h (GenBank Ac. No. KF977826). The arrows and the dots indicate the sense direction of oligonucleotides and identity to the reference sequence CAR_11/001h, respectively. Differences to primer sequences are highlighted in gray. Description of RABV isolates used in this in silico analysis is summarized in **Supplementary Table A.1.**



<u>Figure A.2</u>: *In silico* multiple alignments of L gene primers and probe (NRC-Rabies IPD L gene assay) with 44 partial nucleoprotein sequences from rabies virus isolates belonging to lineage Africa1 and clade Africa2. Corresponding nucleotide positions of each primer are indicated according to the reference sequence CAR_11/001h (GenBank Ac. No. KF977826). The arrows and the dots indicate the sense direction of oligonucleotides and identity to the reference sequence CAR_11/001h, respectively. Differences to primer sequences are highlighted in gray. Description of RABV isolates used in this in silico analysis is summarized in **Supplementary Table A.1.**

Table 1: Oligonucleotide sequences of primers and probes designed in this study

Name	Type	Length	Sense	Sequence 5'-3'	Gene	Position[a]	GC (%)	Tm (°C)[b]	Product Size (nt)
RABVRPN1	primer	20	Sense	GCTCTGGGCTGGTGTCGTTC	Nucleoprotein	707-726	65.00	58.87	
RABVPN	probe	23	Sense	6FAM-CGAGCCARGGCAGGAGACTGCGG--BBQ	Nucleoprotein	819-841	73.91	67.74	193
RABVFPN2	primer	20	Antisense	TACGGGGACTTCCCGCTCAG	Nucleoprotein	880-899	65.00	59.14	
RABVRPL1	primer	20	sense	GGTTTCCGGDGCYGTDCCTC	Polymerase	9472-9491	65.00	58.94	
RABVPL	probe	27	sense	6FAM-CCCGTCAYATAGGGTCRGCTCARGGGC--BBQ	Polymerase	9561-9587	66.66	67.36	208
RABVFPL2	primer	20	Antisense	CCTAGGGGAGACYTTGCCRT	Polymerase	9660-9679	65.00	59.02	

FAM, fluorescein amidite; **BBQ**, blackberry quencher;

a: corresponding nucleotide positions of RABV strain CAR_11/001h (GenBank Ac. No. KF977826)

b: melting temperature (°C)

nt: nucleotides

Table 2: Description and results of rhabdoviruses isolates used in this study for Specificity assessment

Isolates	Virus	Genus	References	Place of isolation	Year of isolation	Species	Rabies virus N-gene assay		Rabies virus L-gene assay	
							Mean Cq value[a]	SD	Mean Cq value[a]	SD
SA221203SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2011	*Mellivora capensis* (honey badger)	23.40	0.078	18.05	0.312
SA217694SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2011	*Canis lupus familiaris* (dog)	18.40	0.432	19.99	0.114
SA217695SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2011	*Canis lupus familiaris* (dog)	16.81	0.015	19.24	0.099
SA217750SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2011	*Canis lupus familiaris* (dog)	17.31	0.191	21.89	0.467
SA218152SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2011	*Canis lupus familiaris* (dog)	23.16	0.142	21.74	0.099
SH155966SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2001	*Homo sapiens* (human)	18.20	0.015	18.73	0.184
SH177846SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2005	*Homo sapiens* (human)	30.23	0.156	35.26	0,000
SA194858SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2008	*Canis lupus familiaris* (dog)	17.18	0.008	20.37	0.219
SA204014SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2010	*Canis lupus familiaris* (dog)	25.16	0.121	14.86	0.128
SA206776SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2010	*Canis lupus familiaris* (dog)	25.15	0.015	15.00	0.036
SA252888SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2013	*Canis lupus familiaris* (dog)	26.75	0.099	20.26	0.425
SA252913SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2013	*Canis lupus familiaris* (dog)	23.52	0.114	16.39	0.057
SA262037SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2013	*Canis lupus familiaris* (dog)	25.15	0.170	24.16	0.396
SA262503SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2014	*Canis lupus familiaris* (dog)	23.22	0.057	21.01	0.071
SA262518SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2014	*Canis lupus familiaris* (dog)	22.77	0.078	21.21	0.538
SA267115SEN	Rabies (RABV)	*Lyssavirus*	from CRORA database	Senegal	2014	*Canis lupus familiaris* (dog)	22.46	0.368	22.62	0.142
DakAnB1094	Kolongo (KOLV)	unassigned	JX276998	Central African Republic	1970	*Euplectes afra* (bird)	Neg	Neg	Neg	Neg
AnY1307	Mokola (MOKV)	*Lyssavirus*	NC_006429	Cameroon	1973	*Crocidura spp.* (shrews)	Neg	Neg	Neg	Neg
AnB373d	Sandjimba (SJAV)	unassigned	JX277024	Central African Republic	1970	*Acrocephalus schoenobaenus* (bird)	Neg	Neg	Neg	Neg
AnB4289	Nasoule (NASV)	unassigned	JX277012	Central African Republic	1973	*Andropadus virens* (bird)	Neg	Neg	Neg	Neg

AnD42443	Lagos Bat (LBV) 3	*Lyssavirus*	NC020807	Senegal	1985	*Eidolon helvum* (fruit bat)	Neg	Neg	Neg	Neg
AnB672	Lagos Bat (LBV) 2	*Lyssavirus*	from CRORA database	Central African Republic	1974	*Micropteropus pusillus* (fruit bat)	Neg	Neg	Neg	Neg
An K 6909	Lagos Bat (LBV) 4	*Lyssavirus*	from CRORA database	Guinea	1985	*Nycteris gambiensis* (Gambian Slit-faced Bat)	Neg	Neg	Neg	Neg
LBVNIG1956	Lagos Bat (LBV) 6	*Lyssavirus*	EF547431	Nigeria	1956	*Eidolon helvum* (fruit bat)	Neg	Neg	Neg	Neg
DakHD763	Le Dantec (LDV)	*Ledantevirus*	AY854650	Senegal	1965	*Homo sapiens* (human)	Neg	Neg	Neg	Neg
DakAnD5314	Keuraliba (KEUV)	*Ledantevirus*	JX276996	Senegal	1968	*Tatera kempi* (gerbil)	Neg	Neg	Neg	Neg
ArD89384	Chandipura (CHNV)	*Vesiculovirus*	from CRORA database	Senegal	1992	Phlebotomus sp. (sandflies)	Neg	Neg	Neg	Neg
SudAr1275	Obodhiang (OBOV)	*Ephemerovirus*	HM856902	Sudan	1963	*Mansonia uniformis* (mosquitoes)	Neg	Neg	Neg	Neg
ArY31-65	Nkolbisson (NKOV)	*Ledantevirus*	JX277015	Cameroon	1965	*Eretmapodites leucopus* (mosquitoes)	Neg	Neg	Neg	Neg
DakAnB439	Garba (GARV)	unassigned	KM204982	Central African Republic	1970	*Corythornis cristata* (bird)	Neg	Neg	Neg	Neg
SAAr1995	Mossuril (MOSV)	*Hapavirus*	KM204993	Mozambique	1959	*Culex sitiens* (mosquitoes)	Neg	Neg	Neg	Neg

SD: standard deviation

Neg: negative; no cross-reactitvity

CRORA: WHO Reference Centre for Arboviruses and Hemorragic Fevers; Institut Pasteur, BP 220, Dakar, Senegal

a: Mean Cq value from duplicates

Table 3a: Evaluation of detection spectrum in Africa

Isolates	Origin	Species	Year of isolation	Reference	Phylogenetic clade - subclade	N-gene assay		L-gene assay	
						Mean Cq value[a]	SD	Mean Cq value[a]	SD
93012MAU	Mauritania	dog	1993	KX148237	Africa-2	27.35	0.685	31.39	0.049
93011MAU	Mauritania	dog	1993	KX148236	Africa-2	16.83	1.180	26.30	0.615
91031MAU	Mauritania	dog	1991	NRC-Rabies IPP	Africa-2	16.21	1.660	25.22	0.289
90024GUI	Guinea	dog	1990	KX148244	Africa-2	17.28	0,000	23.32	0.028
92038CI	Ivory Coast	dog	1992	KX148233	Africa-2	21.56	0.098	23.93	0.148
90003CI	Ivory Coast	dog	1990	NRC-Rabies IPP	Africa-2	17.82	0.042	22.24	0.113
90026CI	Ivory Coast	dog	1990	NRC-Rabies IPP	Africa-2	16.32	0.487	26.03	0.926
01007CI	Ivory Coast	dog	2001	KX148235	Africa-2	14.85	0.289	22.06	1.385
92037CI	Ivory Coast	dog	1992	KX148232	Africa-2	23.23	0.120	24.46	1.046
95047HAV	Burkina Faso	dog	1995	KX148230	Africa-2	15.88	0.070	16.30	0.572
86036HAV	Burkina Faso	dog	1986	KX148234	Africa-2	11.78	0.127	17.65	0.615
90012NIG	Niger	dog	1990	KX148229	Africa-2	19.045	0.289	20.18	0.014
90010NIG	Niger	dog	1990	KX148231	Africa-2	14.235	0.388	17.35	0.247
86070NGA	Nigeria	dog	1986	NRC-Rabies IPP	Africa-2	26.4	0.028	23.31	1.520
86003BRE	Nigeria	dog	1986	KX148201	Cosmopolitan – AF1a	26.15	0.226	22.41	0.763
95002 CAM	Cameroon	dog	1994	KX148242	Africa-2	15.41	0.197	16.75	0.028
88003CAM	Cameroon	dog	1987	KX148243	Africa-2	17.93	0.028	19.14	0.077
88006CAM	Cameroon	dog	1987	NRC-Rabies IPP	Africa-2	18.38	1.350	21.46	0.622

96009TCH	**Chad**	**dog**	**1996**	**KX148241**	**Africa-2**	**13.96**	**0.063**	**18.06**	**0.275**
92018TCH	**Chad**	**dog**	**1992**	**NRC-Rabies IPP**	**Africa-2**	**24.36**	**0.296**	**23.78**	**0.544**
97138TCH	**Chad**	**dog**	**1997**	**NRC-Rabies IPP**	**Africa-2**	**15.07**	**0.707**	**20.9**	**0.098**
90021TCH	**Chad**	**dog**	**1990**	**KX148240**	**Africa-2**	**15.49**	**0.021**	**17.70**	**0.968**
86097BEN	**Benin**	**Cat**	**1986**	**KX148107**	**Africa-2**	**15.10**	**0.021**	**17.23**	**0.056**
94289RWA	**Rwanda**	**dog**	**1994**	**KX148205**	**Cosmopolitan – AF1b**	**28.74**	**0.021**	**22.23**	**0.134**
92028CAR	**Central African Republic**	**dog**	**1992**	**NRC-Rabies IPP**	**Cosmopolitan – AF1b**	**26.42**	**0.197**	**28.1**	**0.395**
92029CAR	**Central African Republic**	**dog**	**1992**	**KX148208**	**Cosmopolitan – AF1b**	**23.68**	**0.544**	**21.72**	**1.046**
86031MOZ	**Mozambique**	**dog**	**1986**	**KX148203**	**Cosmopolitan – AF1a**	**26.73**	**0.346**	**16.29**	**0.268**
87021AFS	**South Africa**	**Human**	**1981**	**KX148103**	**Cosmopolitan – AF1b**	**27.69**	**0.346**	**21.12**	**0.197**
92030NAM	**Namibia**	**dog**	**1992**	**KX148204**	**Cosmopolitan – AF1b**	**22.91**	**0.806**	**14.66**	**0.339**
86092EGY	**Egypt**	**Human**	**1979**	**KX148101**	**Cosmopolitan – AF4**	**25.90**	**0.021**	**26.36**	**0.063**
95049GAB	**Gabon**	**dog**	**1995**	**KX148202**	**Cosmopolitan – AF1a**	**30.14**	**0.417**	**25.02**	**0.155**
93002SOM	**Somalia**	**dog**	**1993**	**KX148198**	**Cosmopolitan – AF1a**	**Neg**	**Neg**	**25.47**	**0.311**
96013TAN	**Tanzania**	**dog**	**1996**	**KX148206**	**Cosmopolitan – AF1b**	**Neg**	**Neg**	**19.75**	**2.008**
93006SOM	**Somalia**	**Jackal**	**1993**	**KX148199**	**Cosmopolitan – AF1a**	**Neg**	**Neg**	**22.40**	**0.120**
87012MAR	**Morocco**	**dog**	**1987**	**NRC-Rabies IPP**	**Cosmopolitan – AF1a**	**Neg**	**Neg**	**33.39**	**0.141**
88008ETH	**Ethiopia**	**dog**	**1988**	**KX148200**	**Cosmopolitan – AF1a**	**28.11**	**0.240**	**23.99**	**0.113**
04031MAR	**Morocco**	**dog**	**2004**	**KX148195**	**Cosmopolitan – AF1a**	**26.13**	**0.007**	**20.09**	**0.113**
90016MAR	**Morocco**	**dog**	**1990**	**KX148196**	**Cosmopolitan – AF1a**	**27.78**	**0.219**	**22.62**	**0.240**
98002MAD	**Madagascar**	**Human**	**1998**	**KX148210**	**Cosmopolitan – AF1c**	**24.77**	**0.162**	**28.52**	**0.155**
15005AFS	**South Africa**	**Mongoose**	**2014**	**NRC-Rabies IPP**	**Africa-3**	**23.78**	**0.601**	**31.34**	**0.049**

15002AFS	**South Africa**	**Mongoose**	**2014**	**KX148221**	**Africa-3**	**25.24**	**0.014**	**21.06**	**0.728**

Neg: negative or no fluorescence signal

Cq : quantitative Cycle number

a: Mean Cq value from duplicates

SD: standard deviation

Determination of clades and subclades was done according to Troupin C, *et al.*, 2016 (38)

NRC-Rabies IPP: National Reference Renter for rabies virus database at Institut Pasteur, Paris, France

Table 3b: Evaluation of detection spectrum in other continents

Isolates	Origin	Species	Year of isolation	Reference	Phylogenetic clade - subclade	N-gene assay		L-gene assay	
						Mean Cq value[a]	SD	Mean Cq value[a]	SD
91047FRA	France	Fox	1991	KX148127	Cosmopolitan – WE	27.11	0.162	22.73	0.155
96002FRA	France	Fox	1996	KX148126	Cosmopolitan – WE	27.38	0.077	20.41	1.301
96140POL	Poland	Raccoon dog	1993	KX148120	Cosmopolitan – CE	Neg	Neg	16.31	0.106
92001GER	Germany	Fox	1991	KX148135	Cosmopolitan – WE	Neg	Neg	22.68	0.289
93039EST	Estonia	Raccoon dog	1991	KX148149	Cosmopolitan – NEE	35.35	0.353	22.33	0.049
86054YOU	Bosnia and Herzegovina	Wolf	1986	KX148145	Cosmopolitan - EE	37.08	0.353	25.54	0.007
87001ARS	Saudi Arabia	Fox	1987	NRC-Rabies IPP	Cosmopolitan – ME1a	Neg	Neg	33.08	0.183
94009TUR	Turkey	Dog	1993	KX148165	Cosmopolitan – ME2	Neg	Neg	26.22	0.000
94272PHI	Philippines	Dog	1994	KX148259	Asian – SEA4	31.25	0.084	32.22	0.127
99009BUR	Burma	Dog	1999	NRC-Rabies IPP	ND	19.15	0.007	33.4	0.183
99008CBG	Cambodia	Dog	1999	KX148252	Asian – SEA3	32.05	0.007	36.51	0.014
99010LAO	Laos	Dog	1999	KX148255	Asian – SEA3	Neg	Neg	29.60	0.459
02043CHI	China	Dog	ND	NRC-Rabies IPP	Asian – SEA2a	36.55	0.070	36.11	1.096
02045CHI	China	Dog	ND	NRC-Rabies IPP	Asian – SEA2a	32.28	0.905	35.91	0.100

91014MEX	**Mexico**	**Dog**	**1991**	**KX148110**	**Cosmopolitan – AM2a**	**Neg**	**Neg**	**21.32**	**0.353**
86001BRE	**Brazil**	**Dog**	**1986**	**KX148216**	**Cosmopolitan – AM3a**	**19.04**	**0.007**	**27.42**	**0.063**
91001USA	**USA**	**Skunk**	**1982**	**KX148213**	**Cosmopolitan – AM1**	**35.61**	**0.339**	**20.81**	**0.141**
91004USA	**USA**	**Skunk**	**1991**	**KX148224**	**Arctic-related – A**	**Neg**	**Neg**	**23.64**	**0.480**
02052AFG	**Afghanistan**	**Dog**	**2002**	**KX148225**	**Arctic-related – AL1b**	**Neg**	**Neg**	**22.40**	**0.028**
91041RUS	**Russia**	**Fox**	**1991**	**NRC-Rabies IPP**	**Cosmopolitan – CA1**	**Neg**	**Neg**	**23.18**	**1.011**

Neg: negative or no fluorescence signal

Cq : quantitative Cycle number

a: Mean Cq value from duplicates

SD: standard deviation

Determination of clades and subclades was done according to Troupin C, *et al.*, 2016 (38)

NRC-Rabies IPP: National Reference Renter for rabies virus database at Institut Pasteur, Paris, France

ND: not determined

Table 4: Repeatability test and summary of results used for analytical specificity and analytical sensitivity determination

	N-gene assay		L-gene assay	
Intra-run CV	**1.67 %**		**1.08 %**	
Inter-run CV	**2.20 %,**		**2.16 %,**	
	Positive samples	**Negative samples**	**Positive samples**	**Negative samples**
Positive	**64**	**0**	**77**	**0**
Negative	**13**	**35**	**0**	**35**
TOTAL	**77**	**35**	**77**	**35**
Specificty	**100%**		**100%**	
Sensitivity	**78.70% (CI 95%; 68.40-89.00%)**		**100%**	
Positive Predictive Value (PPV)	**100%**		**100%**	
Negative Predictive Value (NPV)	**72.91% (CI 95%; 56.76-82.79%)**		**100%**	

%: percentage

All samples were previously tested using the reference technique, FAT.

CV: coefficient of variation

Table 5: Description of isolates used and results of inter-laboratory concordance

Identification	Isolates	virus	Spieces	Origin	Year	Inter-laboratory trial	NRC-R IPP	NRC-R IPD	
						Expected results[a]	RT-qPCR	N-gene assay	L-gene assay
1	CVS 27 13-14	RABV (rabies virus)	*Fixed strain*	/	/	Positive	Positive	Positive	Positive
2	GS7 18-13	RABV (rabies virus)	*Vulpes vulpes*	France	1986	Positive	Positive	Positive	Positive
3	GREECE 03-15	RABV (rabies virus)	*Vulpes vulpes*	Greece	2012	Positive	Positive	Positive	Positive
4	BBLV 02-15	BBLV (Bokeloh bat lysssavirus)	*Myotis natterreri*	France	2012	Positive	Positive	Negative	Negative
5	DUVV 02-12	DUVV (Duvenhage virus)	*Homo sapiens sapiens*	South Africa	1971	Positive	Positive	Negative	Negative
6	EBL-1a 08-14	EBLV-1 (European Lagos bat virus 1)	*Eptesicus serotinus*	France	2002	Positive	Positive	Negative	Negative
7	EBL-2 01-15	EBLV-2 (European Lagos bat virus 2)	*Myotis daubentonii*	United kingdom	2004	Positive	Positive	Negative	Negative
8	Negative 17-13	/	*Vulpes vulpes*	France	2012	Negative	Negative	Negative	Negative
9	Negative 17-13	/	*Vulpes vulpes*	France	2012	Negative	Negative	Negative	Negative

a: Expected results by European Union Reference Laboratory for Rabies during inter-laboratory trial 2015 (36).

Assays detected only RABV strains and gave a 100% concordance to previous results obtained with the RT-qPCR (31) from NRC-R IPP, used as reference technique. A Cohen's kappa coefficient (k) of 1±0.31 (95% CI; 0.38-1.61) (p= 0.001) was obtained.

Assays were also concordant with expected results of inter-laboratory trial on the same samples.

Table 6: Detection limits of established RT-qPCR assays and conventional RT-hemi-nested PCR

TITERS ID50/mL	L-gene RT-qPCR		N-gene RT-qPCR		RT-hemi-nested PCR
	Mean Cq value[a]	SD	Mean Cq value[a]	SD	Electrophorese gel results
6.7E+07	11.00	0.141	15.61	0.820	(+++)
6.7E+06	12.74	0.212	18.08	0.049	(+++)
6.7E+05	15.63	0.346	21.23	0.007	(+++)
6.7E+04	19.57	1.378	24.61	0.60	(++)
6.7E+03	24.58	0.516	28.55	0.381	(++)
6.7E+02	28.51	0.636	31.79	0.622	(++)
6.7E+01	31.74	0.919	36.82	0.021	(+)
6.7	33.51	0.487	39.16	0.487	(+)
0.67	36.63	0.084	Neg	Neg	Neg
0.067	37.44	0.084	Neg	Neg	Neg
0.0067	38.07	0.035	Neg	Neg	Neg
0.00067	38.83	0.254	Neg	Neg	Neg
0.000067	Neg	Neg	Neg	Neg	Neg

Neg: negative or no fluorescence signal
Cq : quantitative Cycle number
a: Mean Cq value from duplicates
(+++): highest band intensity
(++): average band intensity
(+): lowest band intensity
Ten-fold serial dilutions of RABV isolate SA217695SEN with an initial virus titer of 6.7 10^7 ID_{50}/mL were tested with new assays. The reference technique was the RT-hnPCR previously described (22).

Table 7: Clinical sensibility assessment using *intra-vitam* human specimens

Patient Code	Origin	Hospital service	Sample	NRC-R IPP RT-qPCR	NRC-R IPD L-gene RT-qPCR	Final diagnostic
H14-0004	France	CHU Mondor	Cerebro-spinal fluid	Negative	Negative	RABV-positive patient
H14-0004	France	CHU Mondor	Skin biospy	Positive	Positive	
H14-0004	France	CHU Mondor	Saliva day 1	Negative	Negative	
H14-0004	France	CHU Mondor	Saliva day 2	Negative	Negative	
H14-0004	France	CHU Mondor	Saliva day 3	Negative	Negative	
H14-0004	France	CHU Mondor	Saliva day 4	Negative	Negative	
H14-0004	France	CHU Mondor	Saliva day 6	Negative	Negative	
H14-0004	France	CHU Mondor	Saliva day 8	Negative	Negative	
H14-0007	Italia	CHU Milan	Skin biospy	Positive	Positive	RABV-positive patient
H14-0007	Italia	CHU Milan	Cerebro-spinal fluid day1	Positive	Positive	
H14-0007	Italia	CHU Milan	Cerebro-spinal fluid day2	Positive	Positive	
H14-0008	France	CH Garches	Skin biospy	Negative	Negative	RABV-negative patient
H14-0008	France	CH Garches	Saliva	Negative	Negative	
H14-0009	France	CH Bastia	Skin biospy	Negative	Negative	RABV-negative patient
H14-0009	France	CH Bastia	Cerebro-spinal fluid	Negative	Negative	
H14-0010	France	CHU Cayenne	Skin biospy	Negative	Negative	RABV-negative patient
H14-0010	France	CHU Cayenne	Saliva	Negative	Negative	

H14-0011	**France**	**CHU Cayenne**	**Biospy skin**	**Negative**	**Negative**	**RABV-negative patient**
H14-0011	**France**	**CHU Cayenne**	**Saliva**	**Negative**	**Negative**	

RABV: rabies virus

NRC-R IPD L-gene assay gave a 100% concordance to the previous diagnostic results obtained with the combo RT-qPCR (31) from NRC-R IPP, used as reference technique.

Table A.1: Description of African RABV isolates used for *in silico* analysis of developed assays

Origin	Isolate	Species	Year	Phylogenetic clade - subclade	Accession number
Benin	86097BEN	Cat	1986	Africa-2	KX148107
Burkina Faso	86036HAV	Dog	1986	Africa-2	KX148234
Burkina Faso	95047HAV	Dog	1995	Africa-2	KX148230
Cameroon	88003CAM	Dog	1987	Africa-2	KX148243
Cameroon	95002CAM	Dog	1994	Africa-2	KX148242
Central African Republic	92029CAR	Dog	1992	Cosmopolitan – AF1b	KX148208
Central African Republic	CAR_11_001	Human	2011	Africa-2	KF977826
Chad	90021TCH	Dog	1990	Africa-2	KX148240
Chad	96009TCH	Dog	1996	Africa-2	KX148241
Ethiopia	88008ETH	Dog	1988	Cosmopolitan – AF1a	KX148200
Ethiopia	RV2985	Ethiopian wolf (*Canis simensis*)	2014	Cosmopolitan – AF1a	KP723638
Gabon	95049GAB	Dog	1995	Cosmopolitan – AF1a	KX148202
Guinea	90024GUI	Dog	1990	Africa-2	KX148244
Ivory Coast	01007CI	Dog	2001	Africa-2	KX148235
Ivory Coast	92037CI	Dog	1992	Africa-2	KX148232
Ivory Coast	92038CI	Dog	1992	Africa-2	KX148233
Kenya	14015ITA	Human	2014	Cosmopolitan – AF1a	KX148207
Madagascar	04033MAD	Dog	2004	Cosmopolitan – AF1c	KX148209
Madagascar	86046MAD	Dog	1986	Cosmopolitan – AF1c	KX148211
Madagascar	98002MAD	Human	1998	Cosmopolitan – AF1c	KX148210
Mauritania	93011MAU	Dog	1993	Africa-2	KX148236
Mauritania	93012MAU	Dog	1993	Africa-2	KX148237
Monzambique	86031MOZ	Dog	1986	Cosmopolitan – AF1a	KX148203
Morocco	04031MAR	Dog	2004	Cosmopolitan – AF1a	KX148195
Morocco	08342MAR	Dog	2008	Cosmopolitan – AF1a	KX148193

Morocco	89017MAR	Dog	1989	Cosmopolitan – AF1a	KX148194
Morocco	90016MAR	Dog	1990	Cosmopolitan – AF1a	KX148196
Morocco	RV2627	Cow	2009	Cosmopolitan – AF1a	KF155001
Namibia	92030NAM	Dog	1992	Cosmopolitan – AF1b	KX148204
Namibia	178J09	Jackal	2009	Cosmopolitan – AF1b	JX473838
Namibia	239K09	Namibian kudu	2009	Cosmopolitan – AF1b	JX473840
Niger	90010NIG	Dog	1990	Africa-2	KX148231
Niger	90012NIG	Dog	1990	Africa-2	KX148229
Nigeria	86003BRE	Dog	1986	Cosmopolitan – AF1a	KX148201
Nigeria	DRV_NG11	Dog	2011	Africa-2	KC196743
Rwanda	94289RWA	Dog	1994	Cosmopolitan – AF1b	KX148205
Senegal	93003SEN	Dog	1991	Africa-2	KX148238
Senegal	93005SEN	Dog	1992	Africa-2	KX148239
Somalia	93006SOM	Jackal	1993	Cosmopolitan – AF1a	KX148199
South Africa	87021AFS	Human	1981	Cosmopolitan – AF1b	KX148103
Tanzania	96013TAN	Dog	1996	Cosmopolitan – AF1b	KX148206
Tanzania	RV2772	Dog	2009	Cosmopolitan – AF1b	KF155002

Determination of clades and subclades was done according to Troupin C*, et al.*, 2016 (38)

Table A.2: Analytical specificity of established assays using RABV-negative samples

Isolate[a]	Type of sample	Species	Origin	FAT	N-gene assay	L-gene assay
03-0816FRA	P	Bat	France	Neg	Neg	Neg
09-0907FRA	P	Dog	France	Neg	Neg	Neg
09-0923FRA	P	Red fox	France	Neg	Neg	Neg
09-0934FRA	P	Cat	France	Neg	Neg	Neg
09-0945FRA	P	Dog	France	Neg	Neg	Neg
09-0957FRA	P	Cat	France	Neg	Neg	Neg
09-0960FRA	P	Dog	France	Neg	Neg	Neg
10-0016FRA	P	Badger	France	Neg	Neg	Neg
10-0084FRA	P	Bovine	France	Neg	Neg	Neg
10-0438FRA	P	Red fox	France	Neg	Neg	Neg
10-0498FRA	P	Sheep	France	Neg	Neg	Neg
10-0527FRA	P	Mongoose	France	Neg	Neg	Neg
10-0533FRA	P	Cat	France	Neg	Neg	Neg
10-0534FRA	P	Dog	France	Neg	Neg	Neg
10-0560FRA	P	Dog	France	Neg	Neg	Neg
10-0615FRA	P	Cat	France	Neg	Neg	Neg
10-0621FRA	P	Cat	France	Neg	Neg	Neg
10-0793FRA	P	Bovine	France	Neg	Neg	Neg
10-0814FRA	P	Dog	France	Neg	Neg	Neg
10-0913FRA	P	Dog	France	Neg	Neg	Neg

a: All samples were tested at the NRC-R, Institut Pasteur, Paris.

P: primary brain sample

Neg: negative

FAT: direct fluorescent antibody test