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

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

3  
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27 **Abstract**

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

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

37 The results indicated the absence of non-specific amplification and cross-reaction with a  
38 range of other viruses belonging to the same taxonomic family, *i.e Rhabdoviridae*, as well as  
39 negative brain tissues from various host species. Analytical sensitivity ranged between 100 to  
40 10 standard RNA copies detected per reaction for N-gene and L-gene assays, respectively.  
41 Effective detection and high sensitivity of these assays on African isolates showed that they  
42 can be successfully applied in general research and used in diagnostic process and epizootic  
43 surveillance in Africa using a double-check strategy.

44

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

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## 59 1. Introduction

60

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

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

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

78 This fatal encephalitis is caused by virus members of the *Lyssavirus* genus (order  
79 Mononegavirales, family *Rhabdoviridae*) including *Rabies virus* (RABV). RABV is a  
80 neurotropic enveloped pathogen encompassing a negative single-stranded RNA that is around  
81 12 kb (kilobases) in size. As for all lyssavirus members, the RABV genome encodes five  
82 proteins with the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the  
83 glycoprotein (G) and the polymerase (L) separated by four non-coding intergenic regions  
84 (IGRS) of different lengths and surrounded by two untranslated regions (UTR) (5). In  
85 addition, RABV presents a wide range of animal host reservoir and a large genetic diversity,  
86 with particularly four phylogenetic groups circulating in Africa with Africa 1 and 4 lineages,  
87 and with Africa 2 and 3 clades (11, 12). Thus, diagnostic tools which are able to broadly  
88 detect RABV are required.

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

93 technique presents limits in the case of *ante-mortem* non-neural sample material or  
94 decomposed tissues. Furthermore, repeated sampling of skin biopsies is not practical for  
95 improvement of sensitivity of the FAT (10, 14, 15). To overcome the limits of FAT, several  
96 conventional and hemi-nested reverse transcription polymerase chain reaction assays (RT-  
97 PCR) targeting the N or L gene, have been developed (16, 17, 18, 19, 20, 21, 22) and widely  
98 applied for *the intra-vitam* diagnosis of human rabies routine diagnosis. Indeed, the N gene is  
99 the most conserved region among the RABV genome which is the reason for its frequent use  
100 as target for rabies virus diagnostic assays (23). Also, the L gene harbours highly conserved  
101 regions which have targeted for molecular detection (22, 24). Despite a higher sensitivity,  
102 hemi-nested RT-PCR presents some disadvantages in terms of workload, risk of  
103 contamination, and time. Thus, several real time molecular tests targeting the N or L gene  
104 have been developed to complement conventional diagnosis of rabies and rabies-related  
105 viruses (10, 21, 25, 26, 27, 28, 29, 30). However, none of them have been validated against  
106 African strains from a large diversity of geographical origins, except a recent study (31).  
107 In this paper, we describe the validation of two sensitive and specific real-time RT-PCR  
108 assays with TaqMan probes targeting conserved regions of the N and L genes, which were  
109 developed for broad detection of African RABV strains. The detection capacity on clinical  
110 specimens and sensitivity of our assays could suggest their suitable application in human  
111 diagnostic process and veterinary field, and their use as an added value and complement for  
112 already existing methods.

113

## 114 **2. Materials and methods**

115

### 116 **2. 1. Primers and probes design**

117 Initially, in order to design an assay capable of detecting African RABV isolates, full length  
118 genome sequences of two Africa 2 isolates available on GenBank database, corresponding to  
119 DRV-NG11 (GenBank Ac. No. KC196743) and CAR\_11/001h (GenBank Ac. No.  
120 KF977826), were used. Multiple alignments were carried out using clustalW algorithm  
121 implemented in the Mega 6.0 software (32). We selected conserved regions of the N gene and  
122 the L gene for development of two detection systems. Both primers and TaqMan probes were  
123 designed using Primer3web® software (version 4.0.0, Whitehead Institute for Biomedical  
124 Research). To avoid non-specific cross-reactions with others lyssaviruses, primers and probe  
125 were validated by BLAST analysis on NCBI. To ascertain their broad spectrum of detection,

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

129

## 130 **2. 2. Samples and viral RNA extraction**

131 A total of 16 RABV isolates from experimentally infected mouse brain tissues preserved in  
132 the archive of the national reference center for rabies virus (NRC-Rabies) at Institut Pasteur of  
133 Dakar, Senegal (NRC-Rabies IPD) and previously confirmed as rabies positive by FAT, was  
134 used in this study. In addition, 61 other RABV isolates from FAT-confirmed primary brain  
135 samples or experimentally infected mouse brain samples, provided by the NRC-Rabies at  
136 Institut Pasteur, Paris, France (NRC-Rabies IPP) (14), were also included. Furthermore, in  
137 order to assess the specificity of the two RABV detection assays, a panel of 20 primary brain  
138 specimens provided by NRC-Rabies IPP from a range of important animals for rabies virus  
139 transmission, and previously confirmed to be negative by FAT, was also tested. In addition,  
140 15 other major representatives of rhabdoviruses other than RABV and from the archive of  
141 NRC-Rabies IPD were also tested. Finally, 19 clinical specimens including skin biopsy, saliva  
142 and cerebrospinal fluid (CSF) samples received at NRC-Rabies IPP and collected from human  
143 patients suspected of rabies infection were evaluated with both of these molecular tools. All  
144 extractions from isolates provided by NRC-Rabies IPD were performed using the QIAamp®  
145 Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.  
146 The RNA was suspended in a final volume of 60 µl elution buffer and kept at -80°C until  
147 testing. For samples and isolates provided by NRC-Rabies IPP, extraction and storage were  
148 performed as previously described (31).

149

## 150 **2. 3. Real-time RT-PCR (RT-qPCR) assays**

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

159 Foster City, US) using the following temperature profile: 1 cycle of reverse transcription at  
160 50°C for 10 min, 1 cycle of denaturation at 95°C for 15 min, followed by 40 cycles of 95°C  
161 for 15 s and 60°C for 1 min. The RT-qPCR reactions were analyzed using the 7500 software  
162 (v2.0.1).

163

## 164 **2. 4. Specificity testing**

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

170

## 171 **2. 5. Analytical sensitivity assessment**

172

### 173 **2. 5. 1. Standard curves generation**

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

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

182

### 183 **2. 5. 2. Analytical testing**

184 The quantitative standards were used to evaluate the diagnostic assays performances such as  
185 limit of detection (LOD), the coefficient of correlation ( $R^2$ ) and the amplification efficiency  
186 (E) values. The LOD value represents the concentration where at least 50% of replicates of  
187 each dilution were detected. A linear model regression test was performed using R (version  
188 3.0.2, *The R Foundation for Statistical Computing*) to determine the value of  $R^2$ , indicating  
189 the goodness of regression. The E value was calculated with the formula  $E = 10^{(-1/\text{slope})} - 1$ .

190 Relative RABV genome copies in tested samples was calculated through the formula  $q =$

191  $10^{(Cq-b)/a}$ ; where **a** is the slope, **b** is the intercept from a standard curve of each specific  
192 target gene and **Cq** is the quantification cycle number. Furthermore, the Pearson correlation  
193 efficient where a coefficient of 1 represents a good correlation was also calculated.

194

### 195 **2. 5. 3. Determination of sensitivity**

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

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

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

212

### 213 **2. 5. 4. Repeatability and inter-laboratory concordance**

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



224 extracted by NRC-R IPP and storage at  $-80^{\circ}\text{C}$  until use. Details of this trial have been  
225 provided elsewhere (36).

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

230

### 231 **2. 5. 5. Comparison with conventional hemi-nested RT-PCR**

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

237

### 238 **2. 5. 6. Clinical sensitivity testing**

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

242

243

## 244 **3. Results**

245

### 246 **3. 1. Primers analysis**

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

253

### 254 **3. 2. Specificity**

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

257 different rhabdoviruses other than RABV species were tested in duplicate with both of the  
258 assays (**Table 2**). Fluorescent detection was obtained only with RABV isolates whereas non-  
259 targeted viruses were not detected. These results suggested that the designed primer pairs and  
260 probes exhibited no cross-reactivity with other species of lyssavirus (with Mokola and Lagos  
261 bat lyssaviruses) or other rhabdoviruses (**Table 2**). The amplicons size for some RABV  
262 isolates detected with each system was verified by 2% agarose gel electrophoresis and the  
263 amplified fragments were sequenced using the respective forward and reverse primers of the  
264 corresponding systems. A BLAST analysis of obtained sequences showed high identity rate  
265 with the target region of the RABV isolate CAR\_11/001h (GenBank Ac. No. KF977826)  
266 belonging to the Africa 2 phylogenetic clade. Then, these primers and probes were highly  
267 selective for RABV detection. In addition, we analyzed a panel of 20 FAT-negative primary  
268 brain samples from different vertebrate hosts important in RABV transmission. All were  
269 negative with both assays and confirmed that specificity was complete (Supplementary **Table**  
270 **A.2**).

271

### 272 3. 3. Analytical sensitivity

273 Diagnostic test performances of the new assays were assessed using several parameters.  
274 Analytical curves generated showed a LOD of 100 and 10 copies per reaction obtained for N-  
275 gene assay and L-gene assay, respectively with RABV isolate SA217695SEN (**Figure 1**).  
276 Regression line slope values were determined for N gene and L gene assays, with -3.08 and -  
277 3.1, respectively, which are close to the optimal slope value of -3.3. Significant respective  
278 linear regression p-values of 1.833e-09 and 6.977e-12 for N gene and L gene assays,  
279 respectively, and coefficients of correlation ( $R^2$ ) values higher than 0.99 indicated the  
280 goodness of the regression lines. Furthermore, high efficiency values (**E**) were obtained with  
281 both of these assays. A Pearson correlation test between the two regression curves revealed a  
282 coefficient of 0.99 ( $p < 0.0001$ ) suggesting that correlation between the Cq values obtained by  
283 both RT-qPCR methods was excellent (**Figure 1**).

284 To ascertain the broad detection capacity of established assays, various RABV isolates from  
285 different parts of the world, were tested. Considering detection of African RABV isolates, the  
286 L-gene system was able to detect all isolates tested while the N-gene assay detected only 90%  
287 of strains giving no-fluorescence signal with four isolates belonging to Africa-1 lineage,  
288 including isolates from Somalia (93002SOM and 93006SOM), Tanzania (96013TAN) and  
289 Morocco (87012MAR) (**Table 3a**). Furthermore, the L-gene system also detected 100% of

290 tested isolates from other countries of the world. Considering these isolates, the N-gene assay  
291 had a limited detection scale (55%) with no fluorescence signal with 9 isolates from Poland  
292 (96140POL), Germany (92001GER), Saudi Arabia (87001ARS), Turkey (94009TUR), Laos  
293 (99008LAO), Afghanistan (02052AFG), Russia (91041RUS) and one skunk isolate from  
294 USA (91004USA) (**Table 3b**) (38).

295 Results of all previous tests summarized in **Table 4** were used for sensitivity determination  
296 and ROC analysis as previously described (39). A sensitivity of 100% and 78.70% (CI 95%;  
297 68.40-89.00%) was found for the RABV L-gene assay and RABV N-gene assay, respectively,  
298 when compared to the reference technique. In addition, a PPV of 100% for each assay and  
299 NPVs of 100% and 72.91% (CI 95%; 56.76-82.79%) for L gene assay and N gene assays  
300 were obtained respectively (Fischer's exact test  $p < 0.0001$ ). All previously FAT- negative  
301 samples provided an undetermined mean Cq value after 40 cycles. Based on the results of the  
302 ROC analysis, we determined a Cq of 39.70 as the cut-off value below which reactions are  
303 positives for the L-gene assay and the N-gene assay, considering a 95% confidence interval.  
304 A Cq value above these cut-off values or an undetectable Cq was considered negative. An  
305 AUC of 1 was determined for each assay revealing that the tests have an excellent accuracy  
306 (**Figure 2**). Furthermore, Bland-Altman plot analysis also reported a non-significant bias  
307 (mean difference) of  $1.920 \pm 6.875$  (95% limits of agreement: -11.56 to 15.40), demonstrating  
308 that these two methods are approximatively equivalent (**Figure 3**).

309 Intra-run and inter-run CVs of 1.67 % and 2.20 %, respectively, were found for the RABV N-  
310 gene assay while the RABV L-gene assay showed intra-run and inter-run CVs of 1.08 % and  
311 2.16 %, respectively; indicating that assays are robust and highly repeatable(**Table 4**). In  
312 addition, the  $k$  of  $1 \pm 0.31$  (95% CI; 0.38-1.61) indicates that assays are accurate, detecting  
313 only RABV strains and giving 100% concordance to previous results obtained by the NRC-R  
314 IPP using a RT-qPCR (31) with the same samples ( $p = 0.001$ ), and concordant with the results  
315 expected for this inter-laboratory trial with RABV isolates (**Table 5**).

316 Simultaneously, both RT-qPCR assays and conventional hemi-nested RT-PCR were  
317 performed as previously described (22) on 13 ten-fold dilutions of the RABV isolate  
318 SA217695SEN with a titer of  $6.7 \cdot 10^7$  ID<sub>50</sub>/mL. The RABV L-gene assay detected until  
319  $0.00067$  ID<sub>50</sub>/mL, corresponding to 28 genome copies using the L-gene quantification  
320 equation. However, conventional hemi-nested RT-PCR and RABV N-gene assay presented a  
321 detection limit of  $6.7$  ID<sub>50</sub>/mL, corresponding to 75 copies of genome using N-gene  
322 quantification equation (**Table 6**).

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

334

335

#### 336 **4. Discussion**

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

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

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

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

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

388 We used ten-fold dilutions of the RABV isolate SA217695SEN with a titer of  $6.7 \times 10^7$   
389 ID<sub>50</sub>/mL and obtained with L-gene assay lower LOD value than with the hemi-nested RT-

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

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

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

408

#### 409 **Conflict of interest statement**

410

411 We declare that we have no conflict of interest.

412

#### 413 **Acknowledgements**

414

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418

#### 419 **References**

420

- 421 1. Hampson K, Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M, et al. Estimating the global burden of  
422 endemic canine rabies. PLoS Negl Trop Dis. 2015;9(4):e0003709.doi: 10.1371/journal.pntd.0003709.
- 423 2. Anderson, A., Shwiff, S.A., 2013. The cost of canine rabies on four continents. Transbound. Emerg.  
424 Dis., <http://dx.doi.org/10.1111/tbed.12168>.
- 425 3. Dietzschold B, Schnell M, Koprowski H. Pathogenesis of rabies. Curr Top Microbiol Immunol. 2005;  
426 292:45–56. [http://dx.doi.org/10.1007/3-540-27485-5\\_3](http://dx.doi.org/10.1007/3-540-27485-5_3).
- 427 4. World Health Organization, 2013. Expert Consultation on Rabies, Second report. World Health Organ.  
428 Tech. Rep. Ser. 982. pp. 1–150.
- 429 5. Delmas O, Holmes EC, Talbi C, Larrous F, Dacheux L, Bouchier C, et al. Genomic diversity and  
evolution of the lyssaviruses. PLoS One. 2008 Apr 30;3(4):e2057.

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487
6. Ross RS, Wolters B, Hoffmann B, Geue L, Viazov S, Grüner N, Roggendorf M, Müller T. Instructive even after a decade: Complete results of initial virological diagnostics and re-evaluation of molecular data in the German rabies virus "outbreak" caused by transplantations. *Int J Med Microbiol.* 2015 Oct; 305(7):636-43. doi: 10.1016/j.ijmm.2015.08.013.
  7. Vora NM, Orciari LA, Niezgodna M, Selvaggi G, Stosor V, Lyon GM 3rd, Wallace RM, Gabel J, Stanek DR, Jenkins P, Shiferaw M, Yager P, Jackson F, Hanlon CA, Damon I, Blanton JD, Recuenco S, Franka R. Clinical management and humoral immune responses to rabies post-exposure prophylaxis among three patients who received solid organs from a donor with rabies. *Transpl Infect Dis.* 2015 Jun; 17(3):389-95. doi: 10.1111/tid.12393.
  8. Vora NM, Basavaraju SV, Feldman KA, Paddock CD, Orciari L, Gitterman S, Griese S, Wallace RM, Said M, Blau DM, Selvaggi G, Velasco-Villa A, Ritter J, Yager P, Kresch A, Niezgodna M, Blanton J, Stosor V, Falta EM, Lyon GM 3rd, Zembower T, Kuzmina N, Rohatgi PK, Recuenco S, Zaki S, Damon I, Franka R, Kuehnert MJ; Transplant-Associated Rabies Virus Transmission Investigation Team. Raccoon rabies virus variant transmission through solid organ transplantation. *JAMA.* 2013 Jul 24;310(4):398-407. doi: 10.1001/jama.2013.7986.
  9. Jackson AC, Warrell MJ, Rupprecht CE, Ertl HC, Dietzschold B, O'Reilly M, et al. Management of rabies in humans. *Clin Infect Dis.* 2003; 36:60–3. <http://dx.doi.org/10.1086/344905>.
  10. Dacheux L, Wacharapluesadee S, Hemachudha T, Meslin FX, Buchy P, Reynes JM, et al. More accurate insight into the incidence of human rabies in developing countries through validated laboratory techniques. *PLoS Negl Trop Dis.* 2010;4(11):e765.
  11. Bourhy, H., Reynes, J. M., Dunham, E. J., Dacheux, L., Larrous, F., Huong, V. T., Xu, G., Yan, J., Miranda, M. E. & Holmes, E. C. (2008). The origin and phylogeography of dog rabies virus. *J Gen Virol* 89, 2673–2681.
  12. Talbi C, Holmes EC, de Benedictis P, Faye O, Nakouné E, Gamatié D, Diarra A, Elmamy BO, Sow A, Adjogoua EV, Sangare O, Dundon WG, Capua I, Sall AA, Bourhy H. Evolutionary history and dynamics of dog rabies virus in western and central Africa. *J Gen Virol.* 2009 Apr; 90(Pt 4):783-91. doi: 10.1099/vir.0.007765-0.
  13. WHO. Expert Consultation on Rabies—Second report. Geneva, Switzerland: World Health Organisation; 2013. 139 p
  14. OIE. Rabies. 2013. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016. Paris: World Organisation for Animal Health. Available from: <http://www.oie.int/en/international-standardsetting/terrestrial-manual/access-online/>
  15. Fooks, A.R., Johnson, N., Freuling, C.M., Wakeley, P.R., Banyard, A.C., McElhinney, L.M., Marston, D.A., Dastjerdi, A., Wright, E., Weiss, R.A., Müller, T., 2009. Emerging technologies for the detection of rabies virus: challenges and hopes in the 21<sup>st</sup> century. *PLoS Negl. Trop. Dis.* 3, e530.
  16. Heaton P. R, Johnstone P, McElhinney L. M, Cowley R, O'Sullivan E, and Whitby J. E. "Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses," *Journal of Clinical Microbiology*, vol.35,no.11,pp.2762–2766, 1997.
  17. Araújo DB, Langoni H, Almeida MF, Megid J. Heminested reverse-transcriptase polymerase chain reaction (hnRT-PCR) as a tool for rabies virus detection in stored and decomposed samples. *BMC Res Notes.* 2008 Jun 4; 1:17. doi: 10.1186/1756-0500-1-17.
  18. Panning M, Baumgarte S., Pfefferle S, Maier T, Martens A, and Drosten C, "Comparative analysis of rabies virus reverse transcription-PCR and virus isolation using samples from a patient infected with rabies virus," *Journal of Clinical Microbiology*, vol.48,no.8,pp.2960–2962,2010.
  19. De Benedictis P, de Battisti C, Dacheux L, et al., "Lyssavirus detection and typing using pyrosequencing," *Journal of Clinical Microbiology*, vol. 49, no.5, pp.1932–1938, 2011.
  20. Wacharapluesadee S, Phumesin P, Supavonwong P, Khawplod P, Intarut N, Hemachudha T. Comparative detection of rabies RNA by NASBA, real-time PCR and conventional PCR. *J Virol Methods.* 2011 Aug; 175(2):278-82. doi: 10.1016/j.jviromet.2011.05.007. Epub 2011 May 11.
  21. Coertse, J., Weyer, J., Nel, L.H., Markotter, W., 2010. Improved PCR methods for detection of African rabies and rabies-related lyssaviruses. *J. Clin. Microbiol.* 48 (11), 3949–3955.
  22. Dacheux L, Reynes JM, Buchy P, Sivuth O, Diop BM, Rousset D, Rathat C, Jolly N, Dufourcq JB, Nareth C, Diop S, Iehlé C, Rajerison R, Sadorge C, Bourhy H. A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. *Clin Infect Dis.* 2008 Dec 1; 47(11):1410-7. doi: 10.1086/592969.
  23. Kissi B1, Tordo N, Bourhy H. Genetic polymorphism in the rabies virus nucleoprotein gene. *Virology.* 1995 Jun 1;209(2):526-37.

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489  
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543  
544  
545  
546  
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24. Poch, O., Blumberg, B.M., Bouguelert, L., Tordo, N., 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *J. Gen. Virol.* 71, 1153–1162.
  25. Hayman, D.T., Banyard, A.C., Wakeley, P.R., Harkess, G., Marston, D., Wood, J.L., Cunningham, A.A., Fooks, A.R., 2011. A universal real-time assay for the detection of Lyssaviruses. *J. Virol. Methods* 177 (1), 87–93.
  26. Fischer M, Freuling CM, Müller T, Wegelt A, Kooi EA, Rasmussen TB, Voller K, Marston DA, Fooks AR, Beer M, Hoffmann B. Molecular double-check strategy for the identification and characterization of European Lyssaviruses. *J Virol Methods*. 2014 Jul; 203:23-32. doi: 10.1016/j.jviromet.2014.03.014.
  27. Hoffmann, B., Freuling, C.M., Wakeley, P.R., Rasmussen, T.B., Leech, S., Fooks, A.R., Beer, M., Müller, T., 2010. Improved safety for molecular diagnosis of classical rabies viruses by use of a TaqMan real-time reverse transcription-PCR “double check” strategy. *J. Clin. Microbiol.* 48 (11), 3970–3978.
  28. Wacharapluesadee S, Tepsumethanon V, Supavonwong P, Kaewpom T, Intarut N, Hemachudha T., Detection of rabies viral RNA by TaqMan real-time RT-PCR using non-neural specimens from dogs infected with rabies virus. *J Virol Methods*. 2012 Sep; 184(1-2):109-12. doi: 10.1016/j.jviromet.2012.05.013.
  29. Hayman DT, Johnson N, Horton DL, Hedge J, Wakeley PR, Banyard AC, Zhang S, Alhassan A, Fooks AR. Evolutionary history of rabies in Ghana. *PLoS Negl Trop Dis*. 2011 Apr 5; 5(4):e1001. doi: 10.1371/journal.pntd.0001001.
  30. Muleya W, Namangala B, Mweene A, Zulu L, Fandamu P, Banda D, Kimura T, Sawa H, Ishii A. Molecular epidemiology and a loop-mediated isothermal amplification method for diagnosis of infection with rabies virus in Zambia. *Virus Res*.2012Jan; 163(1):160-8. doi: 10.1016/j.virusres.2011.09.010.
  31. Dacheux L, Larrous F, Lavenir R, Lepelletier A, Faouzi A, Troupin C, Nourilil J, Buchy P, Bourhy H. Dual Combined Real-Time Reverse Transcription Polymerase Chain Reaction Assay for the Diagnosis of Lyssavirus Infection. *PLoS Negl Trop Dis*. 2016 Jul 5;10(7):e0004812.
  32. Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* 9, 299–306.
  33. Swets, J., 1988. Measuring the accuracy of diagnostic systems. *Science* 240,1285–1293.
  34. Karimollah Hajian-Tilaki. ROC curve analysis for medical diagnostic test evaluation. *Caspian J Intern Med* 2013; 4 (2): 627-635.
  35. Davide Giavarina. Understanding Bland Altman analysis. *Biochem Med (Zagreb)*. 2015 Jun; 25(2): 141–151. doi: 10.11613/BM.2015.015.
  36. European Union Reference Laboratory for Rabies (2015) Inter-laboratory test for rabies diagnosis. Proficiency test report Diag-06-2015-V1-EN.
  37. Reed and Muench. A simple method of estimating fifty per cent endpoints. *The American Journal of Hygiene*. Vol 27. May 1938.
  38. Troupin C, Dacheux L, Tanguy M, et al. Large-Scale Phylogenomic Analysis Reveals the Complex Evolutionary History of Rabies Virus in Multiple Carnivore Hosts. Parrish C, ed. *PLoS Pathogens*. 2016;12(12):e1006041. doi:10.1371/journal.ppat.1006041.
  39. OIE:Terrestrial Manual 2013. CHAPTER 1.1.5. PRINCIPLES AND METHODS OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES. May 2013.
  40. Farhadi; Saeede Salehi; Setare Adibzadeh; Farzaneh Aboulizadeh; Parniyan Alavi; Negin Nikouyan; Mohammad Ali Okhovat; Reza Ranjbaran; Gholam Reza Rafiei Dehbidi; Arash Shakibzadeh: Development of an In-House TaqMan Real Time RT-PCR Assay to Quantify Hepatitis C Virus RNA in Serum and Peripheral Blood Mononuclear Cells in Patients With Chronic Hepatitis C Virus Infection. *Hepat Mon*. 2015 August; 15(8): e28895. DOI: 10.5812/hepatmon.28895.
  41. Choudhary N, Wei G, Govindarajulu A, Roy A, Li W, Picton DD, Nakhla MK, Levy L, Brlansky RH: Detection of Citrus leprosis virus C using specific primers and TaqMan probe in one-step real-time reverse-transcription polymerase chain reaction assays. *J Virol Methods*. 2015 Nov; 224:105-9. doi: 10.1016/j.jviromet. 2015.08.022.
  42. Deubelbeiss A, Zahno ML, Zanoni M, Bruegger D, Zanoni R. Real-Time RT-PCR for the Detection of Lyssavirus Species. *J Vet Med*. 2014; 2014:476091. doi: 10.1155/2014/476091.
  43. World Health Organisation, 2013. Expert Consultation on Rabies, Second report. World Health Organ. Tech. Rep. Ser. 982. pp. 1–150.
  44. Kuo KW, Leung MF, Leung WC. Intrinsic secondary structure of human TNFR-I mRNA influences the determination of gene expression by RTPCR. *Mol Cell Biochem* 1997;177:1–6.
  45. Myers, T.W., Gelfand, D. H., Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochemistry* 1991, 30, 182–192.



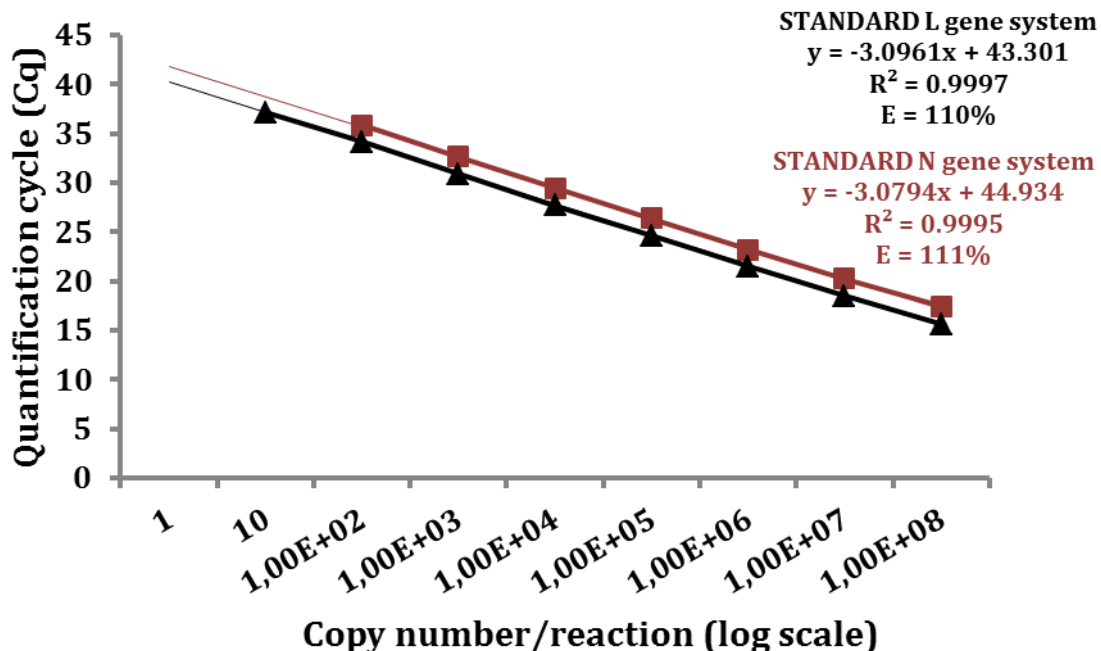
548 46. Fischer M, Freuling CM, Müller T, Wegelt A, Kooi EA, Rasmussen TB, Voller K, Marston DA, Fooks  
549 AR, Beer M, Hoffmann B. Molecular double-check strategy for the identification and characterization  
550 of European Lyssaviruses. J Virol Methods. 2014 Jul; 203:23-32. doi: 10.1016/j.jviromet.2014.03.014.  
551 47. McElhinney LM, Marston DA, Brookes SM, Fooks AR. Effects of carcase decomposition on rabies  
552 virus infectivity and detection. J Virol Methods. 2014 Oct; 207:110-3. doi:  
553 10.1016/j.jviromet.2014.06.024.  
554 48. Suin V, Nazé F, Francart A, Lamoral S, De Craeye S, Kalai M, Van Gucht S: A two-step lyssavirus  
555 real-time polymerase chain reaction using degenerate primers with superior sensitivity to the  
556 fluorescent antigen test. Biomed Res Int. 2014; 2014:256175. doi: 10.1155/2014/256175.  
557 49. Black EM, Lowings JP, Smith J, Heaton PR, McElhinney LM. A rapid RT-PCR method to differentiate  
558 six established genotypes of rabies and rabies-related viruses using TaqMan™ technology. Journal of  
559 Virological Methods 105 (2002) 25 – 35.

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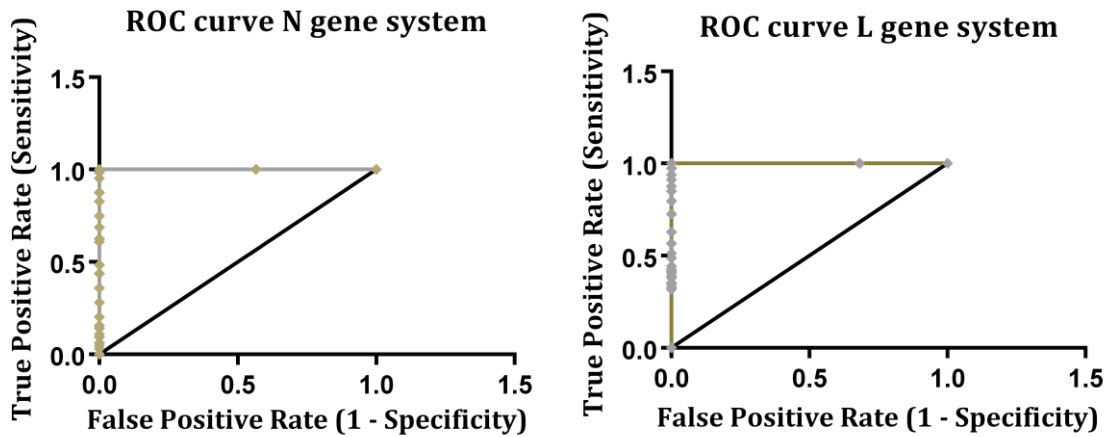
## 564 Highlights

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

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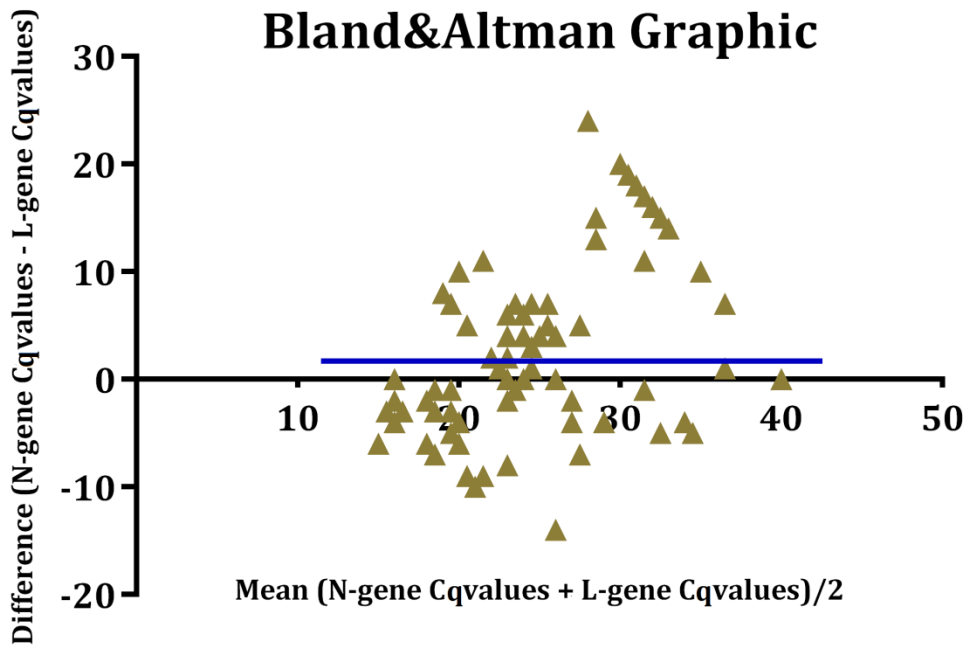


571 **Figure 1:** Standard curves of the developed TaqMan RT-qPCR assays using RABV-  
572 nucleoprotein gene-specific probe (N) and RABV-polymerase gene-specific probe (L). The  
573 slope equation, the correlation coefficient ( $R^2$ ) and the efficiency (E) of each linear regression  
574 curve are indicated in the figure. These standard curves were established using the RABV  
575 isolate SA217695SEN, and results are indicated as RNA copy number detected per reaction.  
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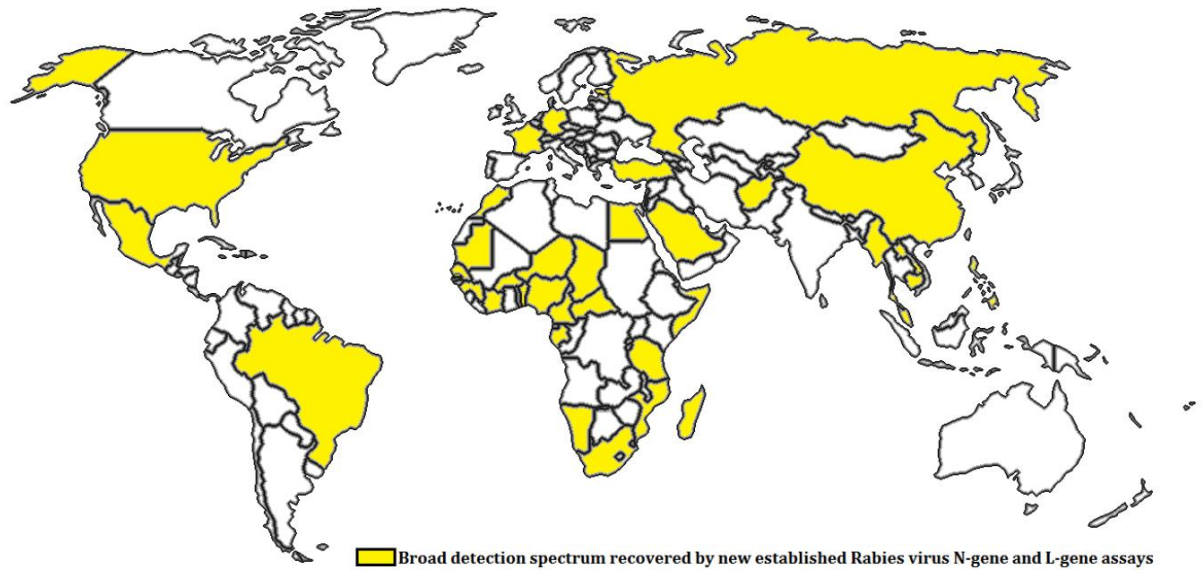
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**Figure 2:** ROC curves of the developed assays using XLSTAT. The AUC value of 1 indicates that the established assays are very powerful to detect RABV genome when samples are positives.



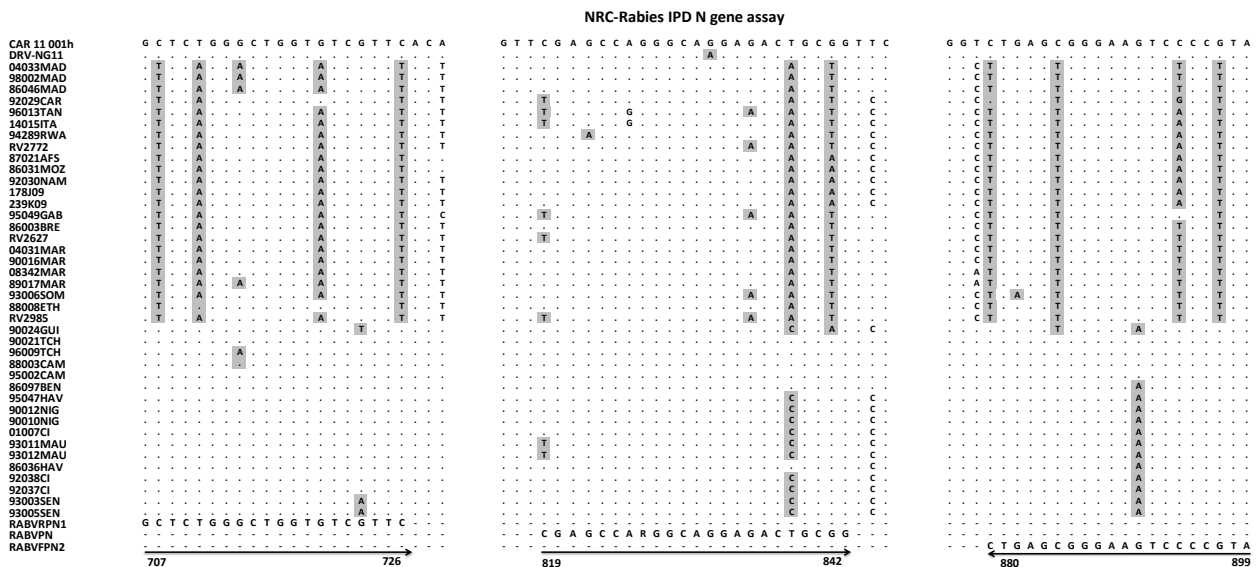
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**Figure 3:** Bland-Altman graphic of differences between N-gene and L-gene assays plotted against average Cq values of the two measurements. The bias of  $1.920 \pm 6.875$  (CI 95%; -11.56 - 15.40) units is represented by the gap between X axis (zero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

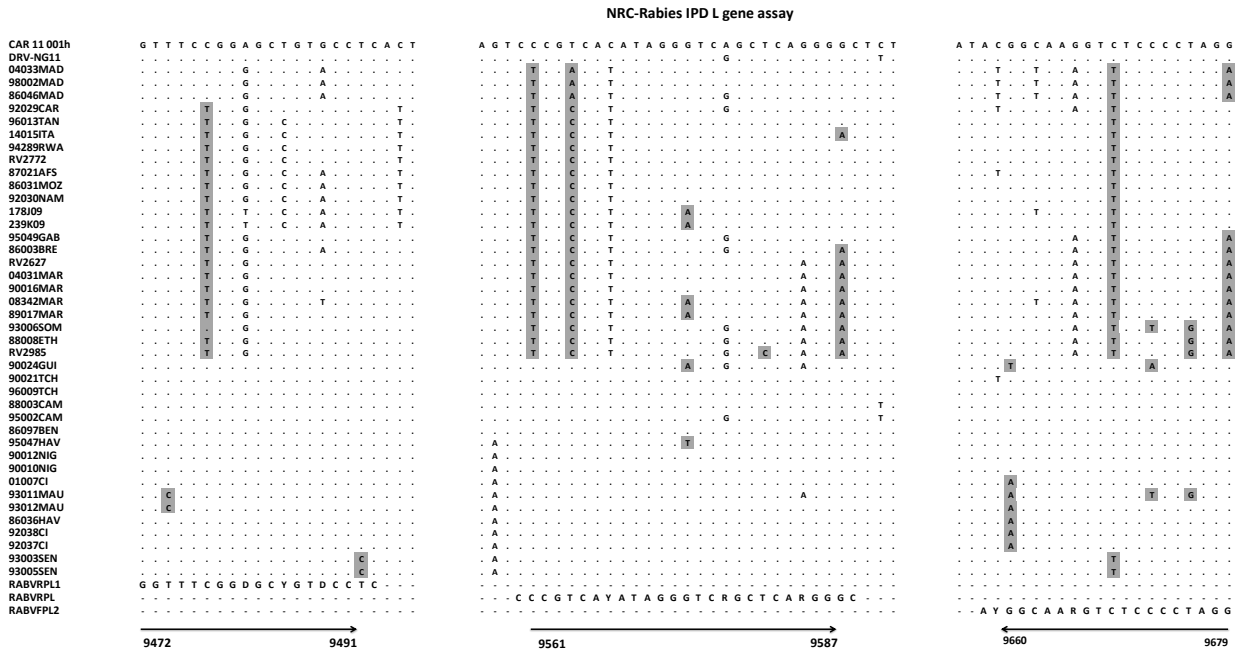


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 592 **Figure 4:** Broad detection spectrum of new RABV assays. The countries where tested strains  
 593 originated are colored in yellow, showing a significant detection of established RABV N-gene  
 594 and L-gene diagnostic assays.

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 597 **Figure A.1:** *In silico* multiple alignments of N gene primers and probe (NRC-Rabies IPD  
 598 N gene assay) with 44 partial nucleoprotein sequences from rabies virus isolates  
 599 belonging to lineage Africa1 and clade Africa2. Corresponding nucleotide positions of each  
 600 primer are indicated according to the reference sequence CAR\_11/001h (GenBank Ac. No.  
 601 KF977826). The arrows and the dots indicate the sense direction of oligonucleotides and  
 602 identity to the reference sequence CAR\_11/001h, respectively. Differences to primer  
 603 sequences are highlighted in gray. Description of RABV isolates used in this in silico analysis  
 604 is summarized in **Supplementary Table A.1.**



606

607 **Figure A.2:** *In silico* multiple alignments of L gene primers and probe (NRC-Rabies IPD

608 L gene assay) with 44 partial nucleoprotein sequences from rabies virus isolates

609 belonging to lineage Africa1 and clade Africa2. Corresponding nucleotide positions of each

610 primer are indicated according to the reference sequence CAR\_11/001h (GenBank Ac. No.

611 KF977826). The arrows and the dots indicate the sense direction of oligonucleotides and

612 identity to the reference sequence CAR\_11/001h, respectively. Differences to primer

613 sequences are highlighted in gray. Description of RABV isolates used in this in silico analysis

614 is summarized in **Supplementary Table A.1**.

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

Name	Type	Length	Sense	Sequence 5'-3'	Gene	Position <sup>a</sup>	GC (%)	T <sub>m</sub> (°C) <sup>b</sup>	Product Size (nt)
RABVRPN1	primer	20	Sense	GCTCTGGGCTGGTGTCTGTC	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-CCCGTCAYATAGGGTTCRGCTCARGGGC--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 <sup>a</sup>	SD	Mean Cq value <sup>a</sup>	SD
SA221203SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2011	<i>Mellivora capensis</i> (honey badger)	23.40	0.078	18.05	0.312
SA217694SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2011	<i>Canis lupus familiaris</i> (dog)	18.40	0.432	19.99	0.114
SA217695SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2011	<i>Canis lupus familiaris</i> (dog)	16.81	0.015	19.24	0.099
SA217750SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2011	<i>Canis lupus familiaris</i> (dog)	17.31	0.191	21.89	0.467
SA218152SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2011	<i>Canis lupus familiaris</i> (dog)	23.16	0.142	21.74	0.099
SH155966SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2001	<i>Homo sapiens</i> (human)	18.20	0.015	18.73	0.184
SH177846SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2005	<i>Homo sapiens</i> (human)	30.23	0.156	35.26	0.000
SA194858SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2008	<i>Canis lupus familiaris</i> (dog)	17.18	0.008	20.37	0.219
SA204014SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2010	<i>Canis lupus familiaris</i> (dog)	25.16	0.121	14.86	0.128
SA206776SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2010	<i>Canis lupus familiaris</i> (dog)	25.15	0.015	15.00	0.036
SA252888SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2013	<i>Canis lupus familiaris</i> (dog)	26.75	0.099	20.26	0.425
SA252913SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2013	<i>Canis lupus familiaris</i> (dog)	23.52	0.114	16.39	0.057
SA262037SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2013	<i>Canis lupus familiaris</i> (dog)	25.15	0.170	24.16	0.396
SA262503SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2014	<i>Canis lupus familiaris</i> (dog)	23.22	0.057	21.01	0.071
SA262518SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2014	<i>Canis lupus familiaris</i> (dog)	22.77	0.078	21.21	0.538
SA267115SEN	Rabies (RABV)	<i>Lyssavirus</i>	from CRORA database	Senegal	2014	<i>Canis lupus familiaris</i> (dog)	22.46	0.368	22.62	0.142
DakAnB1094	Kolongo (KOLV)	unassigned	JX276998	Central African Republic	1970	<i>Euplectes afra</i> (bird)	Neg	Neg	Neg	Neg
AnY1307	Mokola (MOKV)	<i>Lyssavirus</i>	NC_006429	Cameroon	1973	<i>Crocidura spp.</i> (shrews)	Neg	Neg	Neg	Neg
AnB373d	Sandjimba (SJAV)	unassigned	JX277024	Central African Republic	1970	<i>Acrocephalus schoenobaenus</i> (bird)	Neg	Neg	Neg	Neg
AnB4289	Nasoule (NASV)	unassigned	JX277012	Central African Republic	1973	<i>Andropadus virens</i> (bird)	Neg	Neg	Neg	Neg

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

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**SD:** standard deviation

**Neg:** negative; no cross-reactivity

**CRORA:** WHO Reference Centre for Arboviruses and Hemorrhagic 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 <sup>a</sup>	SD	Mean Cq value <sup>a</sup>	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
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**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 <sup>a</sup>	SD	Mean Cq value <sup>a</sup>	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	
<b>Intra-run CV</b>	<b>1.67 %</b>		<b>1.08 %</b>	
<b>Inter-run CV</b>	<b>2.20 %,</b>		<b>2.16 %,</b>	
	<b>Positive samples</b>	<b>Negative samples</b>	<b>Positive samples</b>	<b>Negative samples</b>
<b>Positive</b>	<b>64</b>	<b>0</b>	<b>77</b>	<b>0</b>
<b>Negative</b>	<b>13</b>	<b>35</b>	<b>0</b>	<b>35</b>
<b>TOTAL</b>	<b>77</b>	<b>35</b>	<b>77</b>	<b>35</b>
<b>Specificity</b>	<b>100%</b>		<b>100%</b>	
<b>Sensitivity</b>	<b>78.70% (CI 95%; 68.40-89.00%)</b>		<b>100%</b>	
<b>Positive Predictive Value (PPV)</b>	<b>100%</b>		<b>100%</b>	
<b>Negative Predictive Value (NPV)</b>	<b>72.91% (CI 95%; 56.76-82.79%)</b>		<b>100%</b>	

%: 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	Species	Origin	Year	Inter-laboratory trial	NRC-R IPP	NRC-R IPD	
						Expected results <sup>a</sup>	RT-qPCR	N-gene assay	L-gene assay
1	CVS 27 13-14	RABV (rabies virus)	<i>Fixed strain</i>	/	/	Positive	Positive	Positive	Positive
2	GS7 18-13	RABV (rabies virus)	<i>Vulpes vulpes</i>	France	1986	Positive	Positive	Positive	Positive
3	GREECE 03-15	RABV (rabies virus)	<i>Vulpes vulpes</i>	Greece	2012	Positive	Positive	Positive	Positive
4	BBLV 02-15	BBLV (Bokeloh bat lyssavirus)	<i>Myotis nattereri</i>	France	2012	Positive	Positive	Negative	Negative
5	DUVV 02-12	DUVV (Duvenhage virus)	<i>Homo sapiens sapiens</i>	South Africa	1971	Positive	Positive	Negative	Negative
6	EBL-1a 08-14	EBLV-1 (European Lagos bat virus 1)	<i>Eptesicus serotinus</i>	France	2002	Positive	Positive	Negative	Negative
7	EBL-2 01-15	EBLV-2 (European Lagos bat virus 2)	<i>Myotis daubentonii</i>	United kingdom	2004	Positive	Positive	Negative	Negative
8	Negative 17-13	/	<i>Vulpes vulpes</i>	France	2012	Negative	Negative	Negative	Negative
9	Negative 17-13	/	<i>Vulpes vulpes</i>	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 ID <sub>50</sub> /mL	L-gene RT-qPCR		N-gene RT-qPCR		RT-hemi-nested PCR
	Mean C <sub>q</sub> value <sup>a</sup>	SD	Mean C <sub>q</sub> value <sup>a</sup>	SD	Electrophoresis 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

**C<sub>q</sub>** : quantitative Cycle number

**a:** Mean C<sub>q</sub> 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<sup>7</sup> ID<sub>50</sub>/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**

<b>Patient Code</b>	<b>Origin</b>	<b>Hospital service</b>	<b>Sample</b>	<b>NRC-R IPP RT-qPCR</b>	<b>NRC-R IPD L-gene RT-qPCR</b>	<b>Final diagnostic</b>
H14-0004	France	CHU Mondor	Cerebro-spinal fluid	Negative	Negative	<b>RABV-positive patient</b>
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	<b>RABV-positive patient</b>
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	<b>RABV-negative patient</b>
H14-0008	France	CH Garches	Saliva	Negative	Negative	
H14-0009	France	CH Bastia	Skin biospy	Negative	Negative	<b>RABV-negative patient</b>
H14-0009	France	CH Bastia	Cerebro-spinal fluid	Negative	Negative	
H14-0010	France	CHU Cayenne	Skin biospy	Negative	Negative	<b>RABV-negative patient</b>
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 ( <i>Canis simensis</i> )	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**

<b>Isolate<sup>a</sup></b>	<b>Type of sample</b>	<b>Species</b>	<b>Origin</b>	<b>FAT</b>	<b>N-gene assay</b>	<b>L-gene assay</b>
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