



Evaluation of two molecular methods for the detection of *Yellow fever virus* genome

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Yellow fever virus (YFV), a member of the family *Flaviviridae*, genus *Flavivirus* is endemic to tropical areas of Africa and South America and is among the arboviruses that pose a threat to public health. Recent outbreaks in Brazil, Bolivia, and Paraguay and the observation that vectors capable of transmitting YFV are presenting in urban areas underscore the urgency of improving surveillance and diagnostic methods. Two novel methods (RT-hemi-nested-PCR and SYBR[®] Green qRT-PCR) for efficient detection of YFV strains circulating in South America have been developed. The methods were validated using samples obtained from golden hamsters infected experimentally with wild-type YFV strains as well as human serum and tissue samples with acute disease.

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1. Introduction

Yellow fever virus (YFV) is the prototype member of the genus *Flavivirus* in the family *Flaviviridae*, and is the etiologic agent of Yellow fever, an ancient infectious disease transmitted by *Culicidae* mosquitoes in Africa and South America. The YFV genome is single-stranded, positive-sense RNA approximately 11,000 nucleotides in length, encoding a polyprotein that contains three structural (5'-Capsid-C; Pre-Membrane-PrM; and Envelope-E-3') and seven non-structural (5'-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3') proteins (Fauquet et al., 2005).

The International Committee on the Taxonomy of Viruses (ICTV) recognizes 62 different virus species in the family *Flaviviridae*, many of them transmitted by mosquitoes (Fauquet et al., 2005). YFV and several other flaviviruses including *Dengue virus* (DENV), *West Nile virus* (WNV), *Japanese encephalitis virus* (JEV), *Saint Louis encephalitis virus* (SLEV) and *Rocio virus* (ROCV) are associated with human infection in South America (Monath, 1988).

The World Health Organization (WHO) estimates 200,000 annual YFV infections resulting in 30,000 deaths in endemic areas of Africa and South America (Monath, 2001; Robertson et al., 1996; WHO, 2010). In Brazil, the virus is responsible for jungle Yellow fever epidemics and epizootics with increasing morbidity and mortality rates. Viral transmission involves wild mosquito vectors of the *Sabethes* and *Haemagogus* genera (mainly the *Haemagogus janthinomys* species) and arboreal wild vertebrate hosts (mainly monkeys from the genera *Allouatta* [howlers], *Callythrix* [marmosets], and *Cebus* [calucho]). Humans become infected when they enter forest areas to harvest timber, explore for minerals, practice agricultural activities or pursue eco-tourism (Vasconcelos, 2003).

Despite the fact that urban Yellow fever has not been reported in Brazil since the early 1940s, substantial risk exists since *Aedes aegypti*, the anthropophilic mosquito related to YFV transmission, is present in urban areas of Brazil and other South American countries (Vasconcelos et al., 1999; WHO, 2010). Although RT-PCR assays are commonly employed for detection of several flaviviruses, including DENV, JEV, WNV and SLEV (Coimbra et al., 2008; Hull et al., 2008; Jeong et al., 2011; Lanciotti et al., 1992; Maher-Sturgess et al., 2008; Yeh et al., 2010), protocols for the detection of YFV are less well established. In this article, the development and validation of two molecular methods for detection of YFV are described and their utility with samples obtained from hamsters infected experimentally and infected humans is demonstrated.

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2. Materials and methods

2.1. Ethical aspects

All animal serum samples and virus strains used in this study were obtained from the collection of the Department of Arbovirology and Hemorrhagic Fevers at the Instituto Evandro Chagas, a World Health Organization Collaborating Centre for Research and Reference on Arboviruses. Animals used for the experiment were humanely handled in a biosafety level 2 facility and processed in a class II B2 safety cabinet. The study was approved by the Ethics Committee on Animal Research (CEPAN/IEC). Human samples (blood, sera and fresh and paraffin embedded tissues) were sent to the Instituto Evandro Chagas for diagnostic investigation during the 2009 Brazilian Yellow fever epidemic. Informed consent and epidemiological forms were filled out by patients before collection. Furthermore, all samples were included in the national database of the Brazilian Ministry of Health, which controls and enforces all ethical obligations established for the protection of human subjects.

2.2. Hamster specimens

A total of 51 young (2–3 weeks old) hamsters (*Mesocricetus auratus*) were separated into 17 groups of 3 each. Two hamsters from each group were infected with an YFV strain (Table 1); the third animal served as a negative control. Virus suspensions obtained from infected VERO cells were inoculated intraperitoneally with 6.9×10^6 PFU/mL of each strain. Animals were observed daily until day 3 post infection and then euthanized for sample (blood and viscera) collection. Presence of virus was confirmed by an immunofluorescence assay using an anti-YFV monoclonal antibody (Biomanguinhos, Fiocruz, Brazil). Serum samples were collected daily from 5 groups of infected hamsters to determine the sensitivity of the assays. A total of 200 serum samples were collected from days 0 through 7 post infection.

2.3. Sample collection

Four groups of samples were collected (total $n = 505$). Groups 1 and 2 ($n = 117$ each) were used for validation (Table 2). The Group 1 (YF) included blood, sera, fresh liver and paraffin-embedded liver tissues obtained from hamsters infected experimentally with dif-

Table 2
Samples used for composing the panel for the analyses of molecular methods.

Group	Type of sample	Source	Diagnostic evidence	No. of samples	Total		
YF	Sera	Infected hamster	Virus isolation, IF positive for YFV	34	117		
	Whole blood	Infected hamster	Virus isolation, IF positive for YFV	34			
	Liver fragment	Infected hamster	Virus isolation, IF positive for YFV	34			
	Paraffin embedded tissue	Human liver	Positive Immune Histochemistry for YFV	15			
Non YF	Sera	Non-infected hamster	Negative virus isolation, IF negative for flaviviruses including YFV	17	117		
	Blood	Non-infected hamster	Negative virus isolation, IF negative for flaviviruses including YFV	17			
	Liver fragment	Non-infected hamster	Negative virus isolation, IF negative for flaviviruses including YFV	17			
	Paraffin embedded tissue	Human liver	Negative Immune Histochemistry for YFV	15			
	DENV-1 RNA	C6/36 cell culture	Virus isolation, IF positive for DENV-1	15			
	DENV-2 RNA	C6/36 cell culture	Virus isolation, IF positive for DENV-2	15			
	DENV-3 RNA	C6/36 cell culture	Virus isolation, IF positive for DENV-3	16			
	DENV-4 RNA	C6/36 cell culture	Virus isolation, IF positive for DENV-4	1			
	ROCV RNA	VERO cell culture	Virus isolation, positive NT for ROCV	1			
	SLEV RNA	VERO cell culture	Virus isolation, positive NT for SLEV	1			
	BSQV RNA	VERO cell culture	Virus isolation, positive NT for BSQV	1			
	IGUV RNA	VERO cell culture	Virus isolation, positive NT for IGVU	1			
	Total						234

YFV: Yellow fever virus; DENV-1: Dengue virus type 1; DENV-2: Dengue virus type 2; DENV-3: Dengue virus type 3; DENV-4: Dengue virus type 4; ROCV: Rocio virus; SLEV: Saint Louis Encephalitis virus; BSQV: Bussuquara virus; IGVU: Iguape virus; NT: neutralizing test.

Table 1
Brazilian Yellow fever virus strains used for experimental study in hamsters.

Virus strain	Source of isolation	Year of isolation	Passage history ^b
BeAR378600	<i>Haemagogus</i> sp.	1980	1
BeH394880 ^a	Human	1981	2
BeH413820 ^a	Human	1983	1
BeH422973 ^a	Human	1984	1
BeH423602 ^a	Human	1984	2
BeH463676 ^a	Human	1987	1
BeAR513008	<i>Sabethes</i> sp	1992	2
BeH521244	Human	1993	1
BeH526722	Human	1994	1
BeH622205	Human	2000	1
BeH622493	Human	2000	1
BeH629290	Human	2000	1
BeAR630785	<i>Hg. Janthinomys</i>	2000	1
BeAR646536	<i>Hg. Leucocelaenus</i>	2001	1
BeH655417	Human	2002	1
BeAR678011	<i>Sa. Chloropterus</i>	2004	2
BeH686174	Human	2004	1

^a YFV strains used for analysis of correlation between genome detection and period post infection.

^b Number of passages in Vero cells after virus isolation in mice.

ferent YFV strains (Table 1). The second group (non-YF) group comprised blood, sera, fresh liver and paraffin-embedded liver samples from uninfected hamsters and RNA samples from supernatant of Vero or C6/36 cells infected with different flaviviruses (Table 2).

Group 3 was used to determine the sensitivity of the assays during experimental infections of hamsters. This group contained 200 serum samples of hamsters infected with wild type YFV strains collected from days 0 through 7 after infection (Table 1).

Group 4 contained 71 clinical samples (sera and viscera fragments) received during the 2008–2009 Yellow fever epidemic in Rio Grande do Sul, Minas Gerais and Mato Grosso States, Brazil (WHO, 2010). These samples were used to evaluate the clinical performance of the new molecular methods versus virus isolation in Vero cells or newborn mice.

2.4. RNA extraction

Viral RNA from serum, blood, fresh tissue fragments, and paraffin embedded tissues were extracted using the *QIAquick RNA extraction kit* (Qiagen, Hilden, Germany). Paraffin embedded tissue

samples were processed in xylol (Invitrogen, Carlsbad, CA, USA) prior to RNA extraction.

2.5. Primer selection

Primers were selected based on multiple sequence alignment implemented in Geneious 4.5 (Biomatters, Auckland, New Zealand). Alignments included both full-length and partial genome (E and NS5 genes) sequences available from GenBank. Conserved sequences (ranging from 150 nt to 500 nt in length) were selected as candidate regions. Subsequently, selected regions were used as a query in Genbank. All YFV sequences were then chosen for primer design using the Primer Seq software (Lasergene DNA Star Package, Madison, WI, USA). Specific primers were selected according with the lowest *E*-values (0.021) and highest query coverage score (100%). For the first round of the RT-hemi-nested-PCR the following primers were selected on the E gene region: forward (YFV 975F: 5'-CCTACTGGTCTTGGCTGTTGG-3') and reverse (YFV 1312R: 5'-AATGCTCCCTTCCCAAATA-3'). For the hemi-nested step the inner forward primer (YFV 995F: 5'-GACAGGGATTTCATTGAGG-3') was used in combination with the reverse primer YFV 1312R. For the qRT-PCR, primers on the NS5 region were selected (YFVNS5-F: 5'-CATGGTCGATTCATGGAAAG-3' and YFVNS5-R: 5'-CGCACAGCTTGTCTTGTCTC-3'). The primers blasted with 100% identity with 102 YFV sequences (GenBank) from around the world, including different geographic regions of Africa, South America (Brazil, Venezuela, Peru and Bolivia) and Central America (Trinidad).

2.6. Reverse transcription polymerase chain reaction (RT-PCR) and RT-hemi-nested-PCR assays

The RT-PCR method has been previously described by Vasconcelos et al. (2004). RT-PCR reactions contained 5 μ L (1–5 ng) of viral RNA, 200 μ M of each forward and reverse primer (Vasconcelos et al., 2004), 1 \times PCR buffer (50 mM Tris–HCl, 8.3 pH; 75 mM KCl), 50 mM MgCl₂, 0.1 mM dithiothreitol (DTT), 1 U/ μ L of RNasin RNase inhibitor, 200 μ M deoxynucleoside triphosphates (dNTPs), 1.125 U of Platinum Taq DNA polymerase, and 1 U of Superscript II Reverse Transcriptase, and water adjusted for 50 μ L. RNA samples, in a one step reaction were reverse transcribed for 1 h at 42 °C followed by 35 PCR cycles of 90 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min.

The RT-hemi-nested-PCR was based on the Deubel et al. (1997) protocol to enhance sensitivity of the RT-PCR described above. RT-PCR buffer and cycling conditions were similar to those described above except by the primers sets (see Section 2.5). For the hemi-nested PCR, RT-PCR products were diluted 1:100 in nuclease-free water, and the reactions were setup with 5 μ L of diluted products, 200 μ M of the forward and reverse primers, 1 \times PCR buffer (50 mM Tris–HCl, 8.3 pH; 75 mM KCl), 50 mM MgCl₂, 200 μ M deoxynucleoside triphosphates (dNTPs), 1.125 U of Platinum Taq DNA polymerase, and water adjusted to 50 μ L. PCR cycling conditions were identical to those described above. Positive (RNA extracted from YFV infected Vero cells) and negative (RNA extracted from uninfected Vero cells and RNase free water) controls were used. RT-PCR and hemi-nested-PCR products were visualized in a 3% agarose gel and inspected for appropriate size.

2.7. Quantitative real time PCR

For the quantitative real time RT-PCR (qRT-PCR), a commercial kit was used (SuperScript III Platinum SYBR[®] Green *On-step* qRT-PCR, Invitrogen, Carlsbad, CA, USA) and the reactions were run on an ABI Prism 7500 *Real time* PCR System (Applied Biosystem, Carlsbad, CA, USA). The method was adapted from the protocol described by

Dos Santos et al. (2008), using specific primers selected as described above. The reaction was performed in a final volume of 25 μ L containing 5 μ L of viral RNA (1–2 ng/ μ L), 0.5 μ L of SuperScript III RT Platinum Taq Mix, 0.2 μ M of each specific primer, 12.5 μ L of 2 \times SYBR Green fluorescent molecule, and 1 μ L of ROX. The amplification was carried out as follows: 50 °C for 20 min; 95 °C for 5 min; 45 cycles of 95 °C for 15 s, 55 °C for 40 s and 72 °C for 30 s. The melting curve was calculated during the incubation from 60 °C to 90 °C with a capture speed of 0.2 °C/s. The quantity of the qRT-PCR was expressed in PFU/mL based on the standard curve constructed from amplification values of RNA extracted from Vero E6 cells infected with the YFV strain BE H111 (stock titer 8.9×10^6 PFU/mL) diluted from 10^{-1} to 10^{-7} .

2.8. Determination of sensitivity and specificity indexes

Assay sensitivity (*S*) was determined using serial dilutions (10^{-1} to 10^{-7}) of supernatant of Vero cells infected with the YFV (strain BE H111) at an initial titer of 8.9×10^6 PFU/mL, as well as by testing the group 1 (YF) samples (Table 2). Assay specificity (*Sp*) for YFV versus other flaviviruses (Group 2; Non YF) was determined using RNA extracted from C6/36 cells infected with DENV 1, 2, and 3 serotypes.

The two new methods (RT-hemi-nested-PCR and qRT-PCR) were compared with the classical RT-PCR (Vasconcelos et al., 2004) and virus isolation methods. The classical RT-PCR described by Vasconcelos et al. is the current standard method used in Brazil for clinical diagnosis of YFV infections. This RT-PCR method amplifies a 1200 bp region of the envelope gene. The indexes of (*S*) and (*Sp*) were calculated using the following formula: $S = TP / (TP + FN) \times 100$ and $Sp = TN / (TN + FP) \times 100$, where TP is the number of true positive results; TN is the number true negative results; FP is the number of false-positive results; and FN is the number of false negative results (Table 2).

Assay performance was also assessed by testing 200 serum samples obtained from hamsters infected experimentally with the five YFV strains (Table 1) over days 0 through 7 post-infection for YFV genome (Group 3). Serum samples were tested for the presence of IgM antibodies against YFV using a protocol described previously (Kuno et al., 1987) replacing only the anti-human IgM with the anti-hamster IgM antibody (Mouse anti-hamster IgM, 1 mg/mL; RDI, Fitzgerald Industry, Concord, MA, USA).

2.9. Performance evaluation in clinical specimens

The methods were then compared with virus isolation and RT-PCR using 71 clinical samples (sera and tissue fragments, Group 4). Receiver Operating Characteristic curve analysis (ROC) (Fawcett, 2006; Zweig and Campbell, 1993) implemented in the BioStat software v.5.0 (Ayres et al., 2005) was used to determine the best procedure where (*S*) and (*Sp*) values closer to 1 and zero, respectively, represent ideal performance.

3. Results

3.1. RT-hemi-nested PCR and qRT-PCR product analyses

As a measure of specificity, classic assay products were visualized in agarose gels. Melting curve analysis was used in the SYBR[®] qRT-PCR assay. Amplicon sizes were 390 bp for the first round of amplification, and 370 bp for the hemi-nested step (Fig. 1a). The melting curve for the qRT-PCR assay is shown in Fig. 1b.

The limits of detection for the RT-PCR, RT-hemi-nested-PCR and qRT-PCR were, respectively, 8.9×10^3 , 8.9×10^1 and 8.9 PFU/mL (Fig. 2).

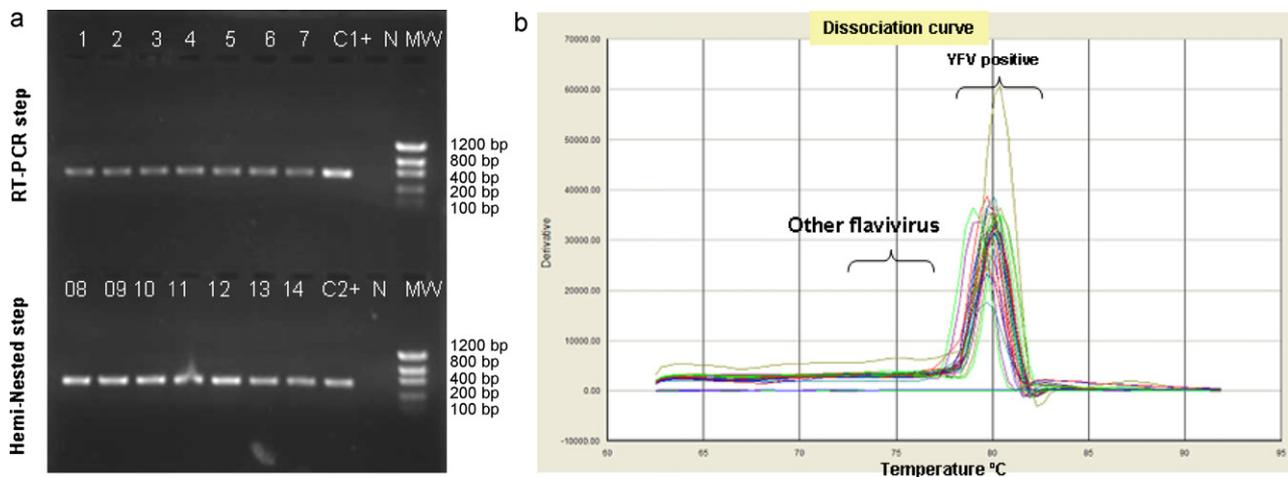


Fig. 1. Detection of YFV sequences in extracts of infected Vero cells. (a) Size fractionated, ethidium bromide labeled product ($4 \mu\text{L}$) of RT- (lanes 1–7) and RT-hemi-nested-PCR (lanes 8–14) steps (1% agarose gel). (b) The dissociation curve for the SYBR[®] qRT-PCR showing the melting temperature for positive YFV genome detection ranging from 79°C to 81°C (mean of 80°C). “C+1 and C+2” stand for positive controls used during the RT-PCR and hemi-nested-PCR steps, respectively. “NC” stands for negative control (RNA extracted from supernatant of uninfected Vero cells) and “MW” stands for molecular weight marker (100 bp DNA ladder, Invitrogen, Carlsbad, CA, USA).

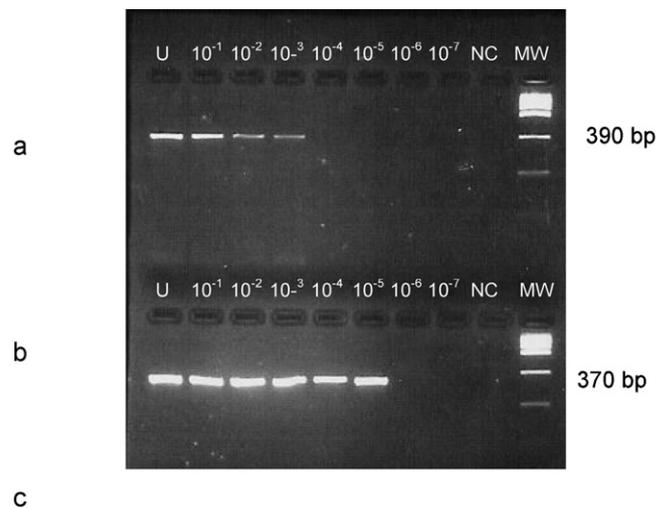
3.2. Sensitivity and specificity

Using the samples from the first and second groups, we calculated the S and S_p indexes and compared them to the “gold standard” virus isolation method. The values determined were: (a) RT-PCR: S : 52.1% (61/117); S_p : 100% (117/117); (b) RT-hemi-nested PCR: S : 70.9% (83/117); S_p : 98.2% (115/117); and (c) qRT-PCR: S : 92.3% (108/117); S_p : 100% (117/117).

Using the third group of samples, we determined that all methods were able to detect YFV during the first two days post infection (Table 3). By day 3, the hemi-nested-PCR and qRT-PCR outperformed RT-PCR. After day 5, qRT-PCR outperformed not only RT-PCR but also virus isolation. At days 6 and 7, hemi-nested PCR

also outperformed virus isolation. After day 3, virus isolation outperformed RT-PCR. The RT-PCR protocol was less sensitive ($S \leq 72\%$) than hemi-nested-PCR or qRT-PCR (Table 3). Among clinical samples, highest sensitivity was observed in liver samples and lowest in paraffin embedded samples, regardless of the method used. Overall, 100% specificity was observed for all methods except for sera and liver samples tested by the RT-hemi-nested-PCR method (Supplementary Table 1).

The number of positive specimens identified with each method for samples obtained during the 2009 YFV outbreak (group 4; $n=71$) were 9.8% ($n=7$), 19.7% ($n=14$), 29.5% ($n=21$) and 40.8% ($n=29$) for the standard RT-PCR, RT-PCR step (for the RT-hemi-nested-PCR), hemi-nested PCR step (for the RT-hemi-nested-PCR),



Method	Dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Viral load (PFU/mL)	8.9×10^5	8.9×10^4	8.9×10^3	8.9×10^2	8.9×10	8.9	0.8
RT-PCR	+	+	+	-	-	-	-
RT-hemi-nested-PCR	+	+	+	+	+	-	-
SYBR[®]qRT-PCR	+	+	+	+	+	+	-

Fig. 2. Detection of YFV sequences by RT-PCR and RT-hemi-nested-PCR and SYBR[®] qRT-PCR in serum from an infected hamster (panels a and b) and limits of detection using RT-PCR, RT-hemi-nested-PCR and SYBR[®] qRT-PCR methods (c). “+” stands for a positive result, “-” for a negative result, “U” for undiluted sample, “NC” for negative control (RNA extracted from VERO uninfected cells), and “MW” for molecular weight marker (Low DNA mass ladder, Invitrogen, Carlsbad, CA, USA).

Table 3

Performance of RT-PCR, RT-hemi-nested-PCR, qRT-PCR, classical RT-PCR and virus isolation in detection of YFV in infected experimentally hamsters.

Days post infection	Standard protocol Virus isolation	Current protocol RT-PCR ^(a/b)	New protocols RT-Hemi-Nested-PCR ^(a/b)			Serology MAC-ELISA positive ^(a/b)
			RT-PCR step	Nested-PCR step	qRT-PCR (a/b)	
Day 0	25/25	25/25, S=100%	25/25, S=100%	25/25, S=100%	25/25, S=100%	0/25
Day 1	25/25	25/25, S=100%	25/25, S=100%	25/25, S=100%	25/25, S=100%	0/25
Day 2	25/25	25/25, S=100%	25/25, S=100%	25/25, S=100%	25/25, S=100%	0/25
Day 3	25/25	20/25, S=80%	21/25, S=84%	25/25, S=100%	25/25, S=100%	0/25
Day 4	25/25	18/25, S=72%	21/25, S=84%	24/25, S=96%	25/25, S=100%	1/25
Day 5	23/25	13/25, S=52%	17/25, S=68%	21/25, S=88.8%	22/25, S=96%	3/25
Day 6	10/25	2/25, S=8%	5/25, S=20%	12/25, S= 48%	11/25, S=44%	10/25
Day 7	1/25	0/25, S=0%	0/25, S=0%	2/25, S= 8%	4/25, S=16%	25/25

a:positive; b:tested

	High sensitivity
	Medium sensitivity
	Low sensitivity
	Very low sensitivity

and qRT-PCR, respectively. Comparing virus isolation with these new methods revealed high concordance with the qRT-PCR showing an index of correlation of 97.2% ($n = 69$), where 25 were positive and 44 were negative by both methods. The two discordant results (2.8%) were both positive by the qRT-PCR and negative by virus isolation into Vero cells or in newborn mice.

3.3. Comparison of test performance

ROC analysis (Fig. 3) determined a d value of 0.63 for the RT-PCR (p cohort to $S = 0.37$; p cohort to $Sp = 1.0$), 0.33 for RT-hemi-nested PCR (p cohort to $S = 0.67$; p cohort to $Sp = 0.97$), and 0.03 for qRT-PCR (p cohort to $S = 0.67$; p cohort to $Sp = 0.97$).

4. Discussion

Yellow fever represents a serious public health concern in Africa and South America. Protocols for genome amplification

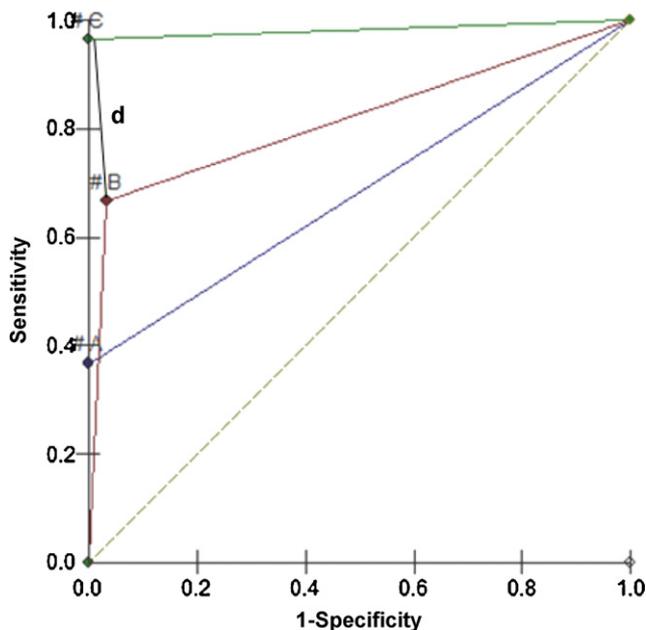


Fig. 3. Graphic representation of ROC (receiver operating characteristics) test for the RT-PCR (A), RT-hemi-nested-PCR (B) e SYBR[®] qRT-PCR. The best performance for each method is indicated by the line *d* (distance). In case of methods C and A, the line *d* coincides with the Y axis (p cohort to $Sp = 0$). “#” symbol represents the cohort point for each method.

and sequencing of YFV have been developed (Mutebi et al., 2001; Vasconcelos et al., 2004) and many partial and complete YFV sequences have been generated (Bryant et al., 2007, 2005). Recently, RT-PCR and other molecular protocols have been established for diagnostic and epidemiological surveillance (Deubel et al., 1997; Domingo et al., 2011; Mendez et al., 2007; Sanchez-Seco et al., 2006; Weidmann et al., 2010). However, none has been fully evaluated for use in endemic regions like South America.

The RT-hemi-nested-PCR and SYBR[®] qRT-PCR methods described here were developed and tested using well characterized samples from naturally infected humans, experimentally infected hamsters and infected cell lines. This approach enables evaluation of sensitivity, specificity and limit of detection of the assays compared to virus isolation, considered the “gold standard technique”. The current RT-PCR assay used for YFV genome detection in Brazil amplifies a fragment of 1200bp of the viral envelope and was originally adapted from a method described elsewhere (Vasconcelos et al., 2004). Application of that method to local clinical samples obtained during sylvatic Yellow fever outbreaks resulted in a high rate of false-negatives, consistent with low sensitivity indexes (approximately 75–80%). Notably, some RT-PCR negative samples were positive by virus isolation (Nunes et al., Instituto Evandro Chagas, data not shown). This failure may have resulted from suboptimal primer design due to lack of sequence information representing local strains. Another possibility is that the large size of the amplification product may amplify less efficiently and degrade more readily, thus lowering sensitivity. Therefore, the RT-PCR and RT-hemi-nested PCR protocols were developed with a smaller amplification product making them more sensitive in samples with low viral load such as clinical samples collected during the early phase of infection (less than 12 h after onset of symptoms), RNA obtained from degraded samples, and paraffin embedded samples. The sensitivity test results demonstrate that both new methods accomplish this goal. Although only samples from South America have been tested in this study, the sequence similarity of the primers to YFV strains from other parts of the world suggests they will amplify any YFV strain. Unfortunately, since the region where the primers are designed is high conserved, the methods cannot differentiate between post vaccine events and wild YF cases.

Whereas SYBR[®] qRT-PCR performed well in paraffin-embedded tissue samples, detecting signal in 13 of 15 samples, RT-PCR failed to detect signal in any samples and RT-hemi-nested PCR detected signal in only two samples. Whether these findings represent template degradation during processing into paraffin or the extraction of RNA from paraffin, or the presence of some external inhibitor

cannot be discerned from our data. Nonetheless, it is clear that qRT-PCR is the method of choice for surveillance of YFV sequences in paraffin embedded tissues.

The RT-hemi-nested PCR assay revealed a specificity of 98.2%. Although every effort to avoid contamination was undertaken (physical separation of RT-PCR and hemi-nested steps, separate pipette sets for each step of the protocol, and UV irradiation between tests), two false-positive results were obtained, presumably due to cross-contamination among samples when assay tubes were opened during the hemi-nested PCR step. The other methods (RT-PCR and qRT-PCR) were 100% specific with no false-positives. Contamination is a problem constantly associated with nested PCR methods and may be an important factor in final method selection. Another factor is sensitivity. The ROC curve test demonstrates the superior performance of qRT-PCR among the three evaluated molecular methods (RT-PCR, RT-hemi-nested PCR and qRT-PCR).

In developing countries cost is another important consideration in assay implementation. Although sensitive, specific assays have been developed with TaqMan™ (Applied Biosystems) and Plexor (Promega) probes, which are relatively expensive. In contrast, the SYBR® qRT-PCR approach offers the advantages of simplicity (one-step qRT-PCR), minimal risk of cross-contamination, sensitivity, and relatively low cost.

In conclusion, the new developed methods will be helpful in further YFV epidemics and can be used for the early diagnosis of infections caused by this important human pathogen.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jviromet.2011.02.025](https://doi.org/10.1016/j.jviromet.2011.02.025).

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