British Journal of Medicine & Medical Research
5(3): 396-403, 2015, Article no.BJMMR.2015.043
ISSN: 2231-0614

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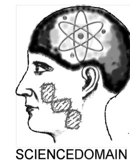

Evaluation of Molecular Detection for Viral Hemorrhagic Infection Cases of Yellow Fever and Dengue Fever in Côte d'Ivoire 2010-2012, West Africa

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Authors' contributions

This work was carried out in collaboration between all authors. Author NKES designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors NM and AV managed the analyses of the study. Authors SF and YA managed the technical analysis. Authors SA and DM were carried out in administration proof. All authors read and approved the final manuscript.

Article Information

DOI:10.9734/BJMMR/2015/12582
Editor(s):
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(4) Anonymous, Anna University-BIT Campus, India.
Peer review History: http://www.sciencedomain.org/review-history.php?iid=662&id=12&aid=6087

Short Communication

Received 8th July 2014
Accepted 22nd August 2014
Published 13th September 2014

ABSTRACT

Background: Viral hemorrhagic fevers are emergent and endemic in Africa and in South America. In Côte d'Ivoire, Yellow fever cases were reported yearly and the distribution of mosquitoes in the country are the main factors for high incidence of Flaviviruses. The poorly reporting of viral hemorrhagic fever cases in some regions, the lack of international interest land and the underestimation of molecular surveillance method contribute to increase the risk for public health.
Objectives: To evaluate the performance of molecular diagnostic methods in national surveillance

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of two emergent Flaviviruses, Yellow fever virus and Dengue virus in Côte d'Ivoire.
Study Design: 63 sera from suspected cases in 2010-2011 of viral hemorrhagic fevers were analyzed to detect viral RNA of Flaviviruses and to compare the results in three different methods.
Results and Conclusion: The Flavivirus RT-PCR has showed the high molecular detection by12% and 6% for real time PCR. The methods are specific and high sensitive for the screening of tick and mosquito-borne Flaviviruses in clinical samples. This study confirms the high circulation of Flaviviruses and the introduction of Dengue virus in Côte d'Ivoire. The combination of real time PCR and the Flavivirus RT-PCR contribute to ameliorate the detection panel of molecular detection in Côte d'Ivoire and was a suitable method for the National Laboratory Reference.

Keywords: Hemorrhagic fever; flaviviruses, molecular detection; RT-PCR; dengue; yellow fever; West Africa.

1. INTRODUCTION

Flaviviruses are arthropod-transmitted viruses and responsible for considerable morbidity and mortality and may cause severe encephalitic, hemorrhagic, hepatic, and febrile illness in vertebrates, including humans [1-2]. Yellow fever virus (YF), Dengue viruses (DEN-1-4), Tick-borne encephalitis virus (TBE), Japanese encephalitis virus (JE), St. Louis encephalitis virus (SLE), and West Nile encephalitis virus (WN) belongs to genus of Flavivirus [3-4].

Since 2004, the World Health Organization (WHO) reported 414,785 cases of Yellow fever worldwide, of these, 200,000 cases were from South America and Africa [5-6]. In Côte d'Ivoire, cases of Yellow fever were reported each year and some imported cases from West Africa in Europa have been most reported [7-10]. In 2011, the national surveillance of YF in Côte d'Ivoire has reported 10 deaths and the first cases of dengue hemorrhagic fever and the cases are been confirmed in Regional Reference Laboratory [11-12]. Most cases of hemorrhagic fever are likely to be large underestimates, such that Flavivirus infections may still represent a significant risk for the public health in Africa [13].

Because there are no specific clinical symptoms for infection by a determined virus, and because different Flaviviruses could be present in the same area, a genus diagnosis by PCR would be a useful first-line diagnostic method. The clinical diagnosis of Flavivirus infections is not unambiguous due to unspecific symptoms varying from mild, febrile illness to viral hemorrhagic fever.

Conventional Flaviviruses diagnosis recommended by the WHO is based on serology tests screening for the presence of virus-specific YF-IgM antibodies in the patient serum. The exceptional sensitivity of the PCR method allowed rapid detection and identification of Flaviviruses in mosquitoes and clinical samples [14-20], in which virus culture is difficult or time-consuming and when early diagnosis is necessary for clinical treatment and has implications for vaccination and mosquito control. Most current molecular assays for Flaviviruses use highly specific primers, which may only amplify from one species, or a range of closely related species [17,21-28]. The NS5 region is the most amplified gene for Flaviviruses detection and has the most conserved region in genome [25,27,28-31].

In this study, the performance of molecular methods for detection of both Flaviviruses: Yellow Fever and Dengue Fever will be evaluate for clinical samples from the National Program Surveillance in Côte d'Ivoire.

2. MATERIALS AND METHODS

2.1 Clinical Samples

63 Blood sera were collected in 2010-2011 from suspected cases of hemorrhagic fever and were stored at -20ºC before treatment. The samples were collected from National Surveillance Program for viral hemorrhagic fevers of Côte d'Ivoire and were transferring tothe National Reference Laboratory in Pasteur Institute Abidjan. The sera were collected between 1-7 days after the clinical symptoms. Epidemiological data of the patients were collected for the National Surveillance Program in CNR Arbovirus (Data not shown). Informed written consent was obtained from all patients.

2.2 RNA Extraction

200 µl of sera were used in RNA extraction protocol by magnetic extraction [32]. Briefly, after

the lysis incubation, RNA was captured by silica and the solution was washed in magnetic stirring rotation. RNA was eluted and stored at -20ºC or used immediately for molecular analysis. Positive strains controls were strain IvoryC1999, strain 17D (Gift of Robert Koch Institut) and Vaccine anti-amaril 17D (Sanofi Aventis) were extracted by the same RNA extraction method. Serial dilutions of RNA from the control strains were applied and each sample was tested in triplicate for molecular tests.

2.3 Hemi-nested Flavivirus RT-PCR

The molecular detection of viral hemorrhagic fever was performed by the protocol of Scaramozzino et al. [24] with minor modifications for the detection of Flaviviruses using NS5gene.The primers were listed (Table 1). The first RT-PCR was involved with 8 µl of extracted RNA, 0.2 µM of each primer CFD2 and MAMD, 1X Reaction puffer (Invitrogen) and 0.2 µl of Reverse Transcriptase-Taq Polymerase (In vitrogen). The second hemi-nested was performed with the primers FS778 and CFD2. The reaction has included 5µl of amplicon from the first RT-PCR, 1X Go-Taqbuffer (Promega), 0.4 mM of dNTPS, 0.5 µM of each primer FS778 and CFD2 and 1Unit Go-Taq Flexi DNA Polymerase (Promega). The PCR reactions were running by using the 9700 Thermocycleur with the same conditions described by Scaramozzino et al. [24]. 10 µl amplicon of hemi-nested Flavivirus PCR were identified by their molecular weights analyzed by electrophoresis in a 1.5% agarose gel, and the separated fragments were stained with ethidium bromide and visualized under UV light trans-illumination.

2.4 Real Time RT-PCR for Yellow Fever/ Dengue Fever

5µl of extract RNA was amplified in 20µl reaction volume containing 1X Reaction buffer (Invitrogen), 0.3 µM of each primer (Table 1), 0.6 µM Probe, 1 Unit Enzyme-mix (Invitrogen) and 0.5 µl of ROX dye (Promega). The amplification was running in 7500 Real time PCR (Applied Biosystems) by the follow conditions: Reverse Transcription: 50ºC, 2 min, initial denaturation: 95ºC, 15 min: (40 cycles) 95ºC, 15 sec, 60ºC, 1 min. Positive controls RNA wereviral RNA from the vaccine YF 17D and positive strains YF and negative controls were performed by RNase free water in each amplification run.

2.5 Sensitivity of Molecular Methods

The viral vaccine YF17D was diluted in 500 µl sterile PBS1X and the RNA was extracted by magnetic extraction (32). Serial dilutions of RNA were performed in RNase-free water from 10^{-1}-10^{-8} to evaluate the limit detection of viral load in hemi-nested Flavivirus PCR and in real time RT-PCR. Each 5µl of viral RNA was tested triplicate in both methods as the same above conditions.10 µl amplicon of Flavivirus PCR were identified by their molecular weights analyzed by electrophoresis in a 1.5% agarose gel, and the separated fragments were stained with ethidium bromide and visualized under UV light trans-illumination.

3. RESULTS

3.1 Sensitivity of Molecular Methods by Serial Dilution of RNA

The detection limit of molecular methods was evaluated by using the vaccine strains YF17D as positive template. Real time RT-PCR showed 100 fold sensitive (2 log10) than hemi-nested Flavivirus PCR ($p < 0.001$, Mc Nemar's test). In addition, 74.5% (20/27) of RNA dilutions were been tested positive by real time PCR and only 51% (14/27) were tested positive in hemi-nested Flavivirus PCR (Table 2). The real time PCR has showed a good correlation of target dilution with linear increasing of the threshold cycle (Ct). The high detection limit of those methods is suitable for detect clinical sera of suspected cases of Flaviviruses.

3.2 Detection of Flaviruses by Molecular Methods in Clinical Sera

This study was performed the capacity of quick molecular detection in suspected cases from 2010 and 2011. The hemi nested Flavivirus PCR (Method 1) has showed high detection by 12% (8/63) of suspected cases while real time PCR (Method 2) has detected 6% (4/63) of samples (Table 3). The 220 bp of amplified product of Flavivirus PCR was showed in Fig 1. Our results were confirmed by the performance of the International Reference Laboratory (Method 3) which has tested 20% (13/40) positive by molecular analysis with the broad range of primers by Flaviviruses.

The real time PCR was shown 4 positive samples (6%) for dengue group-specific assay

and no positive sample for Yellow fever virus and confirmed the introduction of Dengue fever virus in Côte d'Ivoire in 2011 (Table 3). The quality (storage condition) and the low quantity of the samples were poorly and affect the molecular detection because 36.5% (23/63) of samples cannot be analyzing. These results confirm the previous results for the high sensitivity of the method 1 for Flaviviruses detection.

The hemi-nested Flavivirus was a good indication for general screening of Flaviviruses and been used for mosquito national surveillance. These results confirm the circulation of Flaviviruses in West Africa, and the need to implement the molecular detection in National Reference Laboratory.

Table 1. List of used oligonucleotides and probes for molecular detection

Name	Sequence (5'-3')	References	Gene/ Location
CFD2	5'-GTGTCCCAGCCGGCGGTGTCATCAGC-3'	[23]	NS5 9232 - 9258
MAMD	5'-AACATGATGGGRAARAGRGARAA-3'	[24]	NS5 9006-9029
FS778	5'-AARGGHAGYMCDGCHATHTGGT-3'	[24]	NS5 9044- 9066
YFS3-F	5'-AGGTCCAGTTGATCGCGGC-3'	[33]	NS3 4857-1875
YFS3-R	5'-GAGCGACAGCCCCGATTTCT-3'	[33]	NS3 4961-4942
YFS3-probe	5'-FAM-TGGTCAACGTCCAGACAAAACCGAGC-3'-TAMRA	[33]	NS3 4893-4921
DEU-F	5'-AAGGACTAGAGGTTAKAGGAGACCC-3'	[25]	3'UTR 10589-10613
DEU-R	5'-CGWTCTGTGCCTGGAWTGATG-3'	[25]	3'UTR 10699-10677
DEU-Probe	5'-FAM-TCTGGTCTTTCCCAGCGTCAATATGCTGTT-3'-TAMRA	This study	3'UTR 10610–10636

Table 2. Sensitivity of molecular tests in this study

Viral RNA dilution	Heminested flavivirus PCR	Real time PCR (YF)	
	Samples detected/total	Samples detected/total	Threshold cycle (Ct)
10^{-1}	3/3	3/3	17
10^{-2}	3/3	3/3	20
10^{-3}	2/3	3/3	23
10^{-4}	1/3	2/3	26
10^{-4}	2/3	2/3	29
10^{-5}	2/3	2/3	31
10^{-6}	1/3	3/3	35
10^{-7}	0/3	2/3	37
10^{-8}	0/3	0/3	-
Total	14/27	20/27	

Table 3. Comparison of the molecular detection methods

Detection results	Method 1 Hemi-nested PCR/ tested (%)	Method 2 Real-time detection yellow fever or dengue detection/tested (%)	Method 3 International reference labor detection/tested (%)
Positive	8/63 (12.70)	4/63 (6.35)	13/40 (20.63)
Negative	55/63 (87.30)	59/63 (93.65)	27/40 (42.85)
Not tested	0	0	23 (36.50)
Total	63/63 (100)	63/63 (100)	63/63 (100)

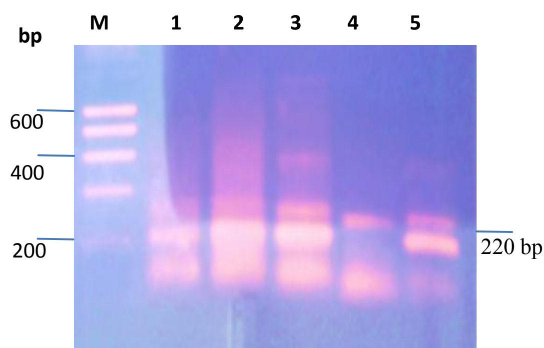

Fig. 1. Molecular detection by hemi-nested Flavivirus PCR

M: Molecular weight marker;1: Positive Strain 17D, 2-3: positive strains; 4: clinical negative sample; 5: positive clinical serum SH7

4. DISCUSSION

Molecular assays have showed to be more sensitive than virus isolation or serological tests. This is because; serological assays were the first screening method and show the limitation by cross reactivity and their consequences. The mass campaign of Yellow Fever vaccination in Côte d'Ivoire can reduce the incidence but the mosquito control was not effective in the country and the introduction of new variants can promise the serological surveillance recommended by WHO [34]. Our approach was to evaluate the molecular Flaviviruses diagnosis methods in clinical sera and subsequently to compare with the results from International Reference Laboratory.

In this study, we have tested the molecular detection for sera suspected cases from 2010 and 2011 and compared the sensitivity and specificity. The hemi-nested Flavivirus PCR has showed 12%, while real time RT-PCR for dengue group-specific has detected 6% of the samples. The results of the Reference laboratory have higher by 20% and confirm the circulation of Flaviviruses in Côte d'Ivoire. The use of generated oligonucleotides is proven to ameliorate the detection by variable region occurs from mutations. This results shows that the NS5 gene and 3'UTR region are the most conserved region of Flaviviruses [24,25,28,35]. Scaramozzino et al. [24] have reported the detection of 30 different tick-and mosquito-borne Flaviviruses by using the hemi-nested method with generated primers for broad detection. Due to the establishment of a sentinel network for viral hemorrhagic fever surveillance, which includes blood collections from febrile cases for virus detection, it was possible to detect early the introduction of new strains of Flaviviruses into the human population. Traditional serological methods based on neutralization and fixed cell ELISA have proven effective for identifying Flaviviruses and must be confirmed by molecular methods. The using of generated primers by method detection allows the broad detection of the five distinct genotypes of YF strains in Africa, dengue viruses and new variants strains of Flaviviruses.

Our results have demonstrated similar high sensitivity of the molecular test in serial dilution of extracted viral RNA with the previous results [28,31] using the same target sequence in pan-flavivivirus detection RT-PCR.

In this study, the hemi-nested Flavivirus has demonstrated similar sensitivity as first described in previous studies using degenerate primers with internal consensus region of NS5 region of all Flaviviruses [24,27]. In our study, the hemi-nested method cannot discriminate Yellow Fever virus and Dengue virus because the positive amplicon were not being sequencing because limiting of the laboratory. The high detection limit of real time PCR (2log) was significant and confirm the sensitivity by 2-10 PFU/ml corresponding of 6 genomes copies/reaction using real time RT-PCR in several studies for the detection of Yellow fever and Dengue fever [16,28,36].

The high number of not tested samples in Laboratory could be due to (i) the long transport of samples by not optimal freezing conditions from patient to the laboratory (ii) the small quantity of some sera for all available tests. The accessibility for molecular tests was necessary for rapid specific screening of hemorrhagic

Flaviviruses surveillance in Côte d'Ivoire. However, the real-time PCR is also used to quantitate the viral load in blood samples, making it a useful tool to investigate the role of viremia in pathogenesis of hemorrhagic Flaviviruses [36].

The confirmation of molecular tests in the International Reference Laboratory contributes to the quality control in Institut Pasteur, Côte d'Ivoire for detection of viral hemorrhagic fevers. The integration in external quality control compendium for molecular tests for Flaviviruses was very necessary in West Africa [20,34].

5. CONCLUSION

The clinical diagnosis of Flaviviruses is particularly difficult because the symptoms are quite similar to those of a wide range of diseases, including hemorrhagic viral diseases, leptospirosis, viral hepatitis, and malaria. The circulation of Flaviviruses is a public heath treat in Côte d'Ivoire and in West Africa.

This study confirms the detection by 12% of Flaviviruses in 2010-2012 and the detection of 6% of Dengue virus in molecular tests. Flavivirus PCR and the real time PCR for Yellow Fever or Dengue Fever could be used as a first-line rapid diagnostic PCR screening test for both viruses. A definitive identification or confirmation obviously requires both complete sequencing and the appropriate expertise in Flaviviruses identification in Reference laboratories. Since, it would take only a few hours, PCR detection of a Flaviviruses directly from patient samples could help the physician choose the appropriate first-line treatment. The use of a single-step RT-PCR can shorten the reaction time for early recognition of the hemorrhagic Flaviviruses in patients and appropriate for molecular and entomological surveillance in African endemic countries. The circulation of Flaviviruses is endemic in Côte d'Ivoire and the integration of the molecular surveillance is essential and can be supported by the international network monitoring of Flaviviruses.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGMENTS

This work was supported by research grants from the Ministère de la Santé by the national WHO, the CDC and RIIP. We thank the Robert Koch Institut for providing of positive strains and effective collaboration for the establishment of molecular techniques and the Department of Epidemic Viruses in Pasteur Institute, Abidjan.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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