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Laboratory Confirmation of Human Rabies by RT-PCR and qRT-PCR Bamako: Report of a Case in a 4-Year-Old Girl at the Mali Hospital

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Abstract

Rabies is a zoonotic disease of viral origin transmitted mainly to humans by biting or licking the injured skin of sick animals. It is an untreatable disease that is fatal once it has been declared. Worldwide, 59,000 cases of rabies-related deaths are recorded each year. The diagnosis of rabies is clinical, biological, and anatomopathological. The aim was to establish the laboratory confirmation capacity for human rabies in Mali. The patient, a Malian female, aged four years, was bitten by a dog around her house in district sixth of Bamako near national road number six in 2020. The patient visited the doctor after a two-month bite. However, she was neither referred to the health center for an effective therapeutic measure nor vaccination (post-exposure prophylaxis). The patient was presented with psychomotor excitation, hypersalivation, aerophobia, and hydrophobia symptoms and visited a Malian hospital named "Hospital of Mali". The patient was admitted with a history of animal bites and symptoms of rabies, and the patient was classified as a probable rabies case. The patient was then referred to an infectious disease physician and hospitalized. After referring the patient to the infectious disease physician, the physician sent oral swab and cerebrospinal fluid (CSF) samples to confirm to the Laboratory of Applied Molecular Biology. Human rabies was confirmed (RT-PCR) by the zoonotic unit of the Laboratory of Applied Molecular Biology. Diagnosis and therapy of human rabies without post-exposure management after a dog bites were still challenging. It was possible to con-

firm the human rabies case in Mali by RT-PCR and qRT-PCR.

Keywords

Dog Bite, Human Rabies, Diagnosis, Mali

1. Introduction

Human rabies is an acute fatal disease that an infected animal produces. It is a zoonotic viral disease [1]. About 59 000 people die by year through 150 countries where Africa (36.4%) and Asia (59.6%) were more affected with 95%. Children are at high risk, particularly those under 15 years old [1]. In Africa, it was estimated 21 476 human deaths per year due to dog-mediated rabies (36.4% of global human deaths), with a loss of 1.34 million each day [2].

Further, the WHO classifies rabies disease into two clinical categories, furious and paralytic rabies [2]. According to WHO criteria for diagnosing diseases, unless an infectious disease is confirmed through laboratory testing for epidemiological and surveillance purposes, the disease will be included in the possible or probable categories. Although the post-mortem diagnosis of human rabies using a brain sample and direct immunofluorescence is almost 100% specific and sensitive, the ethical problem could arise in some countries. Lyssavirus RNA can be detected and amplified from many biological fluids and tissue samples (e.g., saliva, CSF, tears, skin, concentrated urine, and hair follicles). The highest test's sensitivity is seen with skin biopsies (including hair follicles) and saliva [2].

Thus, using RT-PCR on an oral swab or cerebrospinal fluid could be used for the human post-mortem diagnosis. The advent of new molecular techniques, particularly PCR and its modifications, may increase the specificity and sensitivity of the ante-mortem diagnosis of human rabies.

Laboratory methods such as fluorescence antibody testing (FAT) on corneal smears, frozen section skin biopsy, isolation of virus from CSF or saliva, and detection of antibodies in CSF and serum vary in sensitivity depending on the stage of clinical disease and the expertise within each laboratory [3]. Crepin *et al.* [4] study showed encouraging results with saliva samples tested from suspected rabies patients by RT-PCR.

However, there is a lack of human rabies diagnosis in developing countries like Mali, where human rabies ranks highest among fatal encephalitis cases. The objective of this case report was to establish an RT-PCR and Real-Time PCR (SYBER Green I) protocol for use in laboratory confirmation diagnostic, a sample including oral swab and cerebrospinal fluid (CSF).

2. Case Report

The four years old female patient (HD) with a history of a dog bite, bitten on the left shoulder with scars still visible two months ago, visited the hospital. It should

be noted the patient did not receive any treatment after the dog bite. She was admitted to the hospital (named Hospital of Mali) on August twenty-four, 2020. After the clinical observations, we noted psychomotor excitation with hypersalivation, aerophobia, hydrophobia symptoms, and then the patient was classified as a probable case of rabies. The patient was referred to the infectious disease physician; he made an oral swab a CSF and sent those to confirm human rabies by the Laboratory of Applied Molecular Biology. The patient was put under sedation, and the laboratory established a rabies case the same day. The patient died the next day.

The CSF was centrifuged at 5000 rpm for 5 minutes at 4˚C, and oral swab was vortex a few seconds at the laboratory before RNA extraction was processed. RAN extraction was performed using the QIAamp®Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with 200 µl of CSF pellet and 200 µl of the oral swab, respectively. The RNA was suspended in a final volume of 60 µl elution buffer and kept at −80˚C until testing. For quality control of work, the RNA and cDNA concentrations were measured using the Bio Photometer (Eppendorf, Germany) in µg/µL to a wavelength of 260 to 280 nm.

The RNA positive control was provided by the Central Laboratory veterinary from a dog brain sample positive by immunofluorescence technique confirmed by RT-PCR (http://erepository.uonbi.ac.ke/handle/11295/153668) and the negative control oral swab from the patient without rabies history.

Reverse transcription of RNA was performed to produce cDNA by GoScript® Reverse Transcriptase kit (Promega, USA) and the N gene primer (Table 1) in accordance with the kit guideline. The primers were designed to recognize conserved regions N genes of lyssaviruses (Table 1). The first reaction was carried out with mix A containing (total RNA, primer, nuclease-free water) and second with mix B containing (GoScript 5X reaction buffer, 25 mM of $MgCl_2$, 10 µM of dinucleotide triphosphate (dNTPs), GoSript RT, and nuclease-free water) prepared according to the manufacturer's instructions. The cDNA synthesis was performed using the thermocycler PTC 200 (MJ Research, USA), 1] incubated mix A at 70˚C for 5 minutes, followed by one minute on ice, and 2] mix of this one plus the mix B was incubated for 5 minutes at 25˚C (annealing) followed by 42˚C for 1 hour (extend) and incubated at 70˚C for 15 minutes.

Amplification was carried out using the thermocycler PTC 200 (MJ Research, USA) with the specific Nucleoprotein gene primer (Table 1). The total reaction

Table 1. The oligonucleotide sequences used in this report.

Name	Type	Length	Sense	Sequence 5' – 3'	Gene	Posistion[1]	Tm (˚C)[2]	Product size (nt)
S3DB2	Primer	20	Sense	AATGCAACTCTTTGAAGGGA	Nucleoprotein	231 - 250		182
S3DB3	Primer	20	Antisense	GAGCAGACCGACTAAAGATG	Nucleoprotein	313 - 432		

[1]Corresponding nucleotide positions of RABV strain 1557-2IZSVe (GenBank Ac. No. MK471246.1); [2]Melting temperature (˚C); Nt. Nucleotides.

volume was 25 µl containing 1 µl cDNA, 1x Buffer (Mg^{2+} free), 3 mM of $MgCl_2$, 0.4 mM of dNTPs, 0.4 µM of each primer, and 0.025 U of *Taq* polymerase (Promega, USA). The program was conducted according to 1 cycle of denaturation at 95˚C for 10 minutes followed by 40 cycles of 95.0˚C for 10 seconds (denaturation), 50˚C for 10 seconds (annealing), and 65˚C for 15 seconds (extension). The product was visual on electrophoresis gel 1.5% with ethidium bromide (**Figure 1**).

The RT-qPCR was performed using the Luna Universal Probe qPCR Master Mix kit (BioLabs, New England). The SYBR Green method real-time PCR assay was carried out in 20 µL mixture volume consisting of 10 µL of Luna Universal Probe qPCR Master Mix (1×) (Biolabs,), 0.4 µL of each primer (**Table 1**), 1 µL cDNA. Amplification was carried out at 95˚C for 20 seconds 1 cycle, followed by 40 cycles in 2 steps, 95˚C for 03 seconds, 60˚C for 30 seconds. The reactions were carried out on a StepOnePlus (Applied Biosystems, USA). The qRT-PCR results corroborate with those obtained by RT-PCR (**Figure 2**).

3. Discussion

Early declaration of animal bites and diagnosis of rabies cases are essential for initiation of specific prevention and/or therapy. The patient was not declared after a dog bite. The patient in the present case was visited the health center with some probable symptoms of rabies. Generally, the incubation period of the disease following a rabid animal bite is between 14 - 90 days [5]. It was reported that the amount of virus is inversely proportional to the incubation period; this incubation is long for bites in the peripheral region compared to the head and

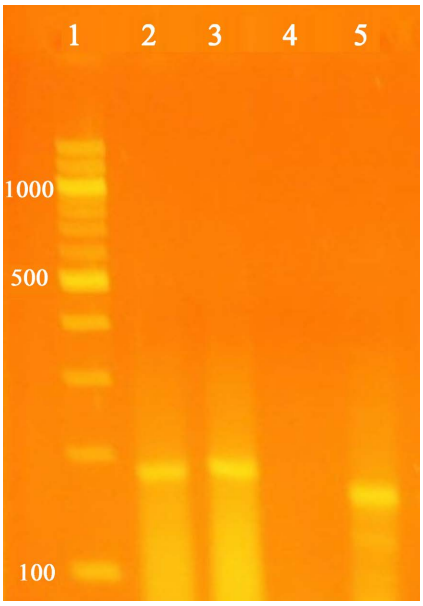

Figure 1. The results of RT-PCR of oral swab and cerebrospinal. The 182 bp product of the nucleoprotein of the rabies virus was amplified in the RT-PCR assay. Lane 1 - 100 bp DNA ladder, lane 2 - 3 human oral swab and cerebrospinal fluid samples, lane 4 – negative control, lane 5 – positive control.

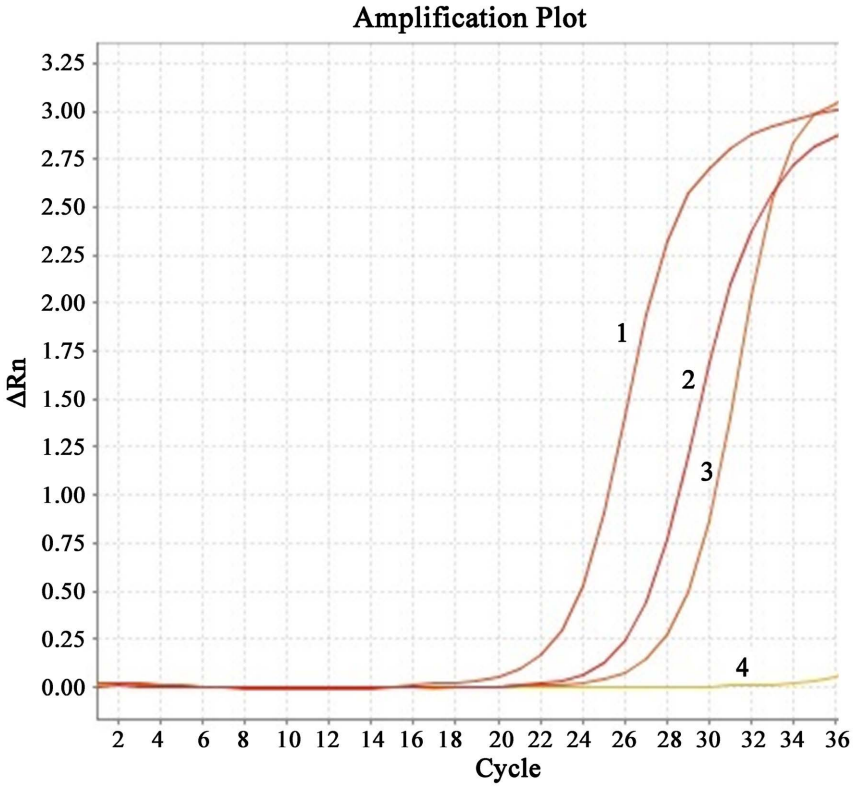

Figure 2. The results of qRT-PCR of oral swab and cerebrospinal fluid. Curve 1 and 3 are human oral swab and cerebrospinal fluid samples, respectively, 3 positive control and 4 negative control.

neck [6]. The patient in the present case was confirmed using RNA extraction, RT-PCR, and qRT-PCR. The case of [5] was confirmed by RT-PCR. The limitation of this report is that there was no information on the biter dog and the prophylactic measures applied after the dog bite. Our patient died one day after hospitalization compared to four, five days for Tuğba *et al.*, 2017 [5] and Hosseinalipour *et al.* [7], 2019, respectively.

Diagnosis of human rabies is based on two distinct forms recognized, furious and paralytic (WHO). The classical furious (encephalitic) form constitutes about 80% of human cases based on instinctive clinical signs and symptoms. Although the diagnosis rarely poses a difficulty, laboratory assistance may be required in some cases where some clinical signs are lacking, like aerophobia and hydrophobia. The second form (paralytic or atypical) constitutes about 20%, and usually, it's diagnostic poses a dilemma because they are often clinically indistinguishable from other diseases like neuroparalytic complications like Guillain-Barre Syndrome (GBS) [7] [8] [9] [10].

Rapid diagnosis of rabies is vital for initiating clear and appropriate infection control and public health management measures [11]. Early diagnosis can obviate the need for unnecessary treatment laboratory tests and reduce the time of medical management.

Although human rabies is known to be almost 100% fatal, a survival case is

reported who developed rabies following a bat bite in the USA by Willoughby *et al.* in 2005 [12] and has revived interest in the medical community to attempt experimental therapeutic approaches.

Carried on the surveillance and laboratory confirmation in clinically suspected cases (ante and post mortem) of rabies are imperative in all countries, even some are free in human rabies cases. Hence, this is working towards rabies elimination in the near future [2]. Geographical boundaries cannot restrain the rabies virus; as long as foci of wildlife or canine rabies exist everywhere, and international travel and global trade of livestock, pets, and wildlife continue, the threat of reintroduction of rabies exists even in countries that have been rabies-free for many years [11].

BLAST analysis of sequence primers showed high homology with target regions of RABV sequences available from the GenBank database.

Despite the world global health engagement, the human rabies diagnosis is unless practices and also lack of data available in the developing country are the challenge for the objective zero human rabies death. Rabies is acute and fatal among viral encephalitis diseases. Rabies is also among the five zoonotic diseases priorities under surveillance in Mali, but there is a lack of human cases confirmed by laboratory diagnosis.

The true disease burden and public health impact due to rabies remain underestimated due to the lack of simple, sensitive, and cost-effective laboratory methods for rabies diagnosis. This may be one of the important reasons why rabies remains a neglected zoonotic disease in many developing countries in Asia and Africa [13] [14]

Furthermore, several advances made in understanding the behavior of the virus and accurate laboratory tests to diagnose rabies during the ante-mortem period may not be routinely available in clinical practice in each country. The accuracy of a post-mortem diagnosis remains high, probably due to the larger amount of tissue and organs that may be sampled. [15].

The cross-reactivity with other viruses can influence the test outcome during the ante-mortem period. Goldwasser *et al.* [16] reported virus concentration variation in post mortem salivary gland samples by immunofluorescence in different areas. Detection of rabies by fluorescent antibody technique (FAT) is limited by the unpredictable nature of the distribution of viral antigens in samples during the early stages. Antigens may also be absent in saliva or CSF samples due to the presence of neutralizing antibodies [17]. One case report demonstrated the success of hnRT-PCR where all other tests, including FAT, were negative [18]. Serum neutralizing antibodies against the virus usually appear after the tenth day of symptoms [19], and therefore, serological techniques are reserved for assessing antibody response following vaccination rather than for making a laboratory diagnosis. The antibodies in the CSF appear two to seven days after their appearance in the serum. In one series, it was detected in less than 50% of patients after the ninth day of symptoms [20].

Most of the conventional techniques used for post-mortem analysis of the brain are of limited value to support the intravitam diagnosis of rabies [21] [22].

Obtaining a post-mortem brain (biopsy, autopsy) remains a challenge due to religious, cultural, and other factors, with decreased rates of an autopsy being a common problem in both developed as well as developing countries. Availability of methods of ante-mortem diagnosis of rabies in samples other than brain tissue is critical and would contribute to the control and elimination of rabies [11].

To avoid significant mismatches due to rabies virus genetic diversity, we used oligonucleotides (**Table 1**) that recognize specific and highly conserved sequences on the N protein. We were able to detect by amplification of nucleoprotein gene the rabies viral RNA from oral swab and cerebrospinal fluid using RT-PCR (**Figure 1**.)

Crepin *et al.* [4] tested saliva samples from 28 patients by conventional RT-PCR and confirmed the presence of rabies virus nucleic acid among these samples by post-mortem examination. They recommended this assay along with a direct immunofluorescence test on skin biopsy specimens as a simple testing protocol for intravitam diagnosis of rabies. Nagaraj *et al.* [23] also reported rabies virus RNA detection from saliva samples. A load of virus and the duration of virus shedding in the saliva are of paramount importance for rabies diagnosis. Conventional RT-PCR has been reported to be a reliable test for ante-mortem diagnosis in two other separate case reports by Smith *et al.* [18] and Fooks *et al.* [24].

However, a significant limitation of these assays is the need for stringent quality control measures to avoid false positive results and the lack of international standards and universal protocols to be used for diagnosis. Currently, molecular assays are not recommended for routine post-mortem diagnosis of rabies; if brain tissue is available FAT should be performed. However, they can be used for ante mortem diagnosis of human rabies and epidemiological surveys in laboratories with strict quality control procedures and with experience and expertise in using such techniques [2].

Rabies still continues to be one of the most neglected zoonotic diseases worldwide. The low level of commitment to rabies control is partly attributable to a lack of accurate and extensive surveillance data to indicate the disease burden, frequent misdiagnosis of rabies, and an absence of intersectoral coordination [11].

RNA detection tests can be performed on a range of biological samples such as CSF, saliva, tears, urine, skin biopsy, extracted hair follicles, and brain tissue for ante-mortem and post-mortem diagnosis of human rabies. These assays have been found beneficial for the diagnosis of rabies in decomposed and archival samples [25] [26] [27] [28] and have an essential role in retrospective diagnosis and epidemiological studies.

To contribute to the world's global health engagement, we have established this laboratory confirmation protocol with an oral swab and cerebrospinal fluid

sample. Though financial and logistical barriers may prevent the routine use of molecular diagnostic assays, the cost/benefit ratio should still be measured. However, the rapid reduction in diagnostic time and cost with the current molecular methods complementing the conventional indicates that they will soon become a viable technology in diagnostic and reference laboratories globally [29] [30].

Nucleic acid amplification and detection techniques have been evaluated as an adjunct to conventional tests for ante mortem and post mortem rabies diagnosis. Thus, most of them target the highly conserved rabies viral nucleoprotein gene [11].

We observe that the amplification product size of the positive sample from a dog is different than the human oral swab and cerebrospinal fluid sample (**Figure 1**). To verify this specificity of the sequences primer, we carried out an alignment of this primer with the rabies nucleoprotein sequences available in the Genbank. We found the complete homology with the primer.

To date, this is the first report in Mali highlighting the importance of molecular methods in the ante mortem diagnosis of rabies using oral swab samples and cerebrospinal fluid by conventional RT-PCR and SYBR Green real-time PCR assays.

Real-time PCR based assays allow the detection and quantification of genome copies; hence a reduction in cross-contamination is achieved due to the "closed-tube" nature of these assays [11]. The results of two-step RT—Real-time PCR based on Syber green I assay allow us the viral load between oral swab and cerebrospinal fluid and also the positive control which is from dog infected brain biopsies (**Figure 2**). These viral loads are known through the different threshold values. When it is low, that means genome copies number is high.

Nagaraj *et al.* [23] reported that the Real-time PCR using the SYBR Green is more sensible than the conventional RT-PCR on saliva samples for ante mortem rabies diagnosis and Hayman *et al.* [31] reported that it may be used as a universal real-time assay for the detection of Lyssaviruses.

Mani *et al.* [11], for their ante mortem study, show the results of real-time TaqMan PCR for viral RNA was more sensitive with saliva samples 6/7 (85.7%) than other samples from clinically suspected rabies patients. They fund combined with rabies virus neutralizing antibody detection in CSF, and ante mortem rabies diagnosis was achieved in all samples.

The Syber Green I results allow us that the oral swab sample have the Ct value low than positive control and the cerebrospinal fluid sample respectively. Thus, we show capability and implementation of the human rabies diagnosis by RT-PCR and Syber Green in Mali.

4. Conclusion

Rabies remains a public health problem; this is the first report of human rabies case confirmed by the laboratory in Mali. Rabies remains the infectious disease

with the highest fatality rate, and there is no specific therapy for encephalopathy symptoms. Diagnostic confirmation became a challenge in the low endemic areas country like Mali. The accessibility of the molecular tool in this context could be used for the documentation of cases.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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