

Original Article

Ante-mortem Diagnosis of Human Rabies Cases Using SYBR Green Real-Time PCR

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Abstract

Background: Rabies, as the most important zoonotic disease, is transmitted through a bite or scratch by an infected domestic or wild carnivores and bats or contact of open wound with infected saliva. The fluorescent antibody test (FAT) is the “gold standard” diagnostic method for suspected brain samples. For close monitoring of unknown encephalitis, rabies surveillance, and also the limitations for post-mortem diagnosis of rabies in human and performing fast prophylactic measures for other individuals in contact with rabid patients, ante-mortem diagnosis based on molecular methods such as real-time polymerase chain reaction (PCR) seems to be more reliable. In this study, we detected 2 positive rabid cases using SYBR Green real-time PCR for the first time in Iran.

Methods: In this study, 3 saliva samples at intervals up to 6 hours were collected from any of the nine suspected patients with nonspecific symptoms between March 2016 and March 2017. Total RNA extraction, cDNA synthesis and real-time PCR were performed along with confirmed negative and positive controls. Then, we tracked the patients for follow-up and understanding of their status. On brain samples of patients who died, FAT and MIT (mouse inoculation test) were performed to obtain definitive results.

Results: In this study, the patients were 4 females and 5 males, between 8 and 80 years old from different geographical areas of Iran. The ante-mortem saliva samples of 2 out of nine patients who died were positive by SYBR Green real-time PCR. Positive results of FAT test on these samples confirmed the presence of rabies virus infection in their brains and also the ante-mortem diagnosis results.

Conclusion: The results of this study suggest that SYBR Green real-time PCR technique on saliva sample can be used as an applicable method for ante-mortem diagnosis of rabies to avoid infection of other people such as the treating medical staff or family members of the patient.

Keywords: Diagnosis, Human, Rabies, Real-time polymerase chain reaction (PCR)

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Introduction

Rabies, as one the most important zoonotic diseases in domestic and wild animals, is a potentially life-threatening agent for humans.¹ Rabies causes acute and fatal viral encephalitis that definitely leads to death. This neurotropic virus is a single stranded negative sense RNA virus that belongs to the *Lyssavirus* genus of the *Rhabdoviridae* family.² The presence of virus in saliva is the most important factor in disease transmission.³ Actually, the disease is transmitted through a bite or scratch by infected domestic or wild carnivores and bats.² Over 55 000 people annually die worldwide due to rabies, and more than 99% of deaths occur in Africa, Asia and South America.¹ According to the World Health Organization (WHO), every year, 10 million people receive post-exposure prophylaxis (PEP) worldwide.⁴ Available data shows that dogs are the main cause of rabies deaths in the world.² Rabies is an endemic disease in Iran; however, most cases of rabies in Iran are reported from the northeast and southwest of the Caspian sea.^{1,5}

There are few diagnostic techniques to detect rabies. So far, the use of traditional methods is very limited for diagnosis of rabies in developed countries because it only can diagnose through brain tissue in post-mortem samples. The fluorescent antibody test (FAT) is the “gold standard” diagnostic method for rabies that is confirmed by animal and human tissue samples before or after death with detection of rabies virus nucleocapsid by immunofluorescence. FAT is a sensitive method but it is a difficult technique and requires expensive fluorescence microscopy.⁶ The mouse inoculation test (MIT) is an animal experimentation diagnostic method for rabies that requires a large number of laboratory animals, environmental and ethical issues in animal experiments, skilled/trained staff and it is also time consuming. Moreover, genetic variability in rabies virus could not be identified by this technique.⁷ Promoting genomic identification and characterization of virus-based amplification and detection of nucleic acids as NATs in less time can be used for help to experiment or even as a unique process for detection before

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and after death.^{8,9} Some molecular methods such as real-time polymerase chain reaction (PCR) seem to be reliable compared to FAT as a traditional method.¹⁰ Real-time PCR, to detect rabies virus and other Lyssavirus species, is widely used from 2004. This is a highly sensitive method to detect virus genome which overcomes the limitations of other molecular methods, and detects capable rabies virus from the samples even by small amounts.¹¹

In this study, the saliva samples from suspected rabies patients before death, sent to the WHO Collaborating Center for Reference and Research on Rabies, were examined using SYBR Green real-time PCR. It is hoped that this method, in the future, will be used for rapid diagnosis of rabies death in human samples sent to the WHO Collaborating Center for Reference and Research on Rabies, Pasteur Institute of Iran given the high cost of other molecular methods such as real-time quantitative reverse transcription PCR (real-time qRT-PCR).

Materials and Methods

Study Sample

In this study, 3 saliva samples were collected from any of the 9 suspected patients at intervals up to 6 hours during a period of time between March 2016 and March 2017. Inclusion criteria were saliva samples collected from the oral cavity of suspected rabies patients with both specific and unspecific clinical symptoms such as irritability, headache, vomiting, mild fever, muscle aches, fatigue, retreat, and in some cases photophobia with/without bite by animal suspected of rabies history. All patients had not received PEP measures. Exclusion criteria was the saliva samples of suspected rabies patients that after death, their brain samples were not sent to the WHO Collaborating Center for Reference and Research on Rabies, Pasteur Institute of Iran. Another exclusion criterion was aspiration of sputum and throat. Also, the samples that were not sent to the laboratory in compliance with the cold chain storage were not acceptable. Clinical data were abstracted from those recorded in the form filled in by physicians and submitted along with the samples. The saliva samples were collected by a nurse on admission to the hospital via suction using a sterile syringe or a plastic dropper. Therefore, before collecting the samples, it is important that all sampling procedures and how to write them were carefully trained.

All methods, RNA extraction, cDNA synthesis and real-time PCR standard were already controlled by using positive and negative animal saliva.

RNA Extraction

RNA extraction was performed immediately after receiving samples. Initially, 400 μ L of saliva added to 200 mL proteinase K and incubated for 30 minutes at 56°C; the samples were placed in a vortex every 5 to 10 minutes. The

total RNA was extracted using TRIzol[®] reagent (Invitrogen) according to the manufacturer's instructions and stored at -80°C until use. Quality of RNA extracted was studied by *GAPDH* gene.

cDNA Synthesis

Reverse transcription of RNA was performed to produce cDNA by SuperScript[®] III kit (Invitrogen) and random hexamer primer in accordance with the kit guideline. Before starting reaction, reaction Mix A (containing dNTP, random hexamer, water double distilled) and reaction Mix B (containing RT buffer 10X, MgCl₂ (25mM), DTT (0.1M), RNaseOUT (40 u/ μ L), RT superscript III and DMSO) prepared to the required extent. The cDNA synthesis protocol by using of thermocycler (bio-Rad, USA) was carried out respectively by incubation at 70°C for 5 minutes, putting on ice for one minute, mixture for 10 minutes at room temperature and incubation at 95°C for 5 minutes. For quality control of work, the RNA and cDNA concentrations were measured using the Nanodrop (Biotek, USA) in ng/ μ L and a wavelength of 260 to 280 nm.

SYBR Green Real-Time PCR Assay

Each run of PCR has been confirmed with the negative control (a saliva sample from a healthy person) and positive control (rabid animal saliva sample that was checked by FAT method on its brain sample).

The SYBR Green real-time PCR assay was carried out in 20 μ L PCR mixture volume consisting of 10 μ L of SYBR Green qPCR mix (2x) (Qiagen, USA), 0.5 μ L N165-146 (10 pmol/ μ L) primer, 0.5 μ L JW12 (10 pmol/ μ L) primer, and 5 μ L cDNA; primer sequences are shown in Table 1. Amplification was carried out at 95°C for 10 minutes, followed by 45 cycles in 2 steps: 95°C for 15 seconds, 55°C for 1 minute. Melting curve analysis consisted of a denaturation step at 65-95°C, rising by one degree for each step, waiting for 90 seconds of pre-melt conditioning, waiting for 5 seconds for each step afterward. The reactions were carried out in the Rotor-Gene 6000-QIAGEN. The primers were designed to recognize conserved regions between the N genes of lyssaviruses.

Supplementary Tests

In this study, we tracked the patients for follow-up and understanding of their status and also performed FAT on the brain of patients who died; specimens from all brains with negative FAT results were submitted for MIT.

Direct Fluorescent Antibody Test

This technique was carried out according to the described method by the WHO and the World Organization for Animal Health (OIE). The expansion of the sample was prepared to the approximate diameter of 16 mm glass slide

Table 1. Details of Primers Used in the Study

| Nucleotide Sequence (5'-3') | PCR | Primer Name | Position | Ref. |
|-----------------------------|-------------|-------------|----------|------|
| ATGTAACACCYCTACAATG | SYBER GREEN | JW12 | 55-73 | 12 |
| GCAGGGTAYTTRTACTCATA | | N165-146 | 165-146 | 12 |

placed on the upper part, then monolayer filter paper was put for 30 minutes in cold acetone to establish the sample. After removing the slide, it was air-dried and then 25 µL fluorescein-labelled monoclonal anti-rabies immunoglobulin (Bio-Rad, USA) was spilled on the slide and incubated at 37°C for 1 hour in a humid chamber. Further, it was washed with phosphate buffered saline (PBS) in 3 successive washes for 5–10 minutes. The slide was rinsed with distilled water, air-dried and mounting buffered glycerol applied. It was visualized under an immunofluorescent microscope (Nikon, Japan) at 400× magnification; bright/dull/dim apple-green or yellow-green, round to oval intracellular accumulations were observed. Both of positive and negative controls were run beside the other specimens.^{13,14}

Mouse Inoculation Test

In order to confirm the negative results of molecular tests and FAT, MIT was done by the following the procedure. For each test, 10 mice of Swiss albino strain, the age of 3 to 4 weeks (weighing 12 to 14 g) were used, and 0.03 mL of 10% suspension prepared from brain tissue or saliva samples suspected to rabies virus were inoculated into the brains of mice, then the mice health problems were examined daily for 30 days. Usually, rabies symptoms (include hair stand on end, squat, tremors, and paralysis) appear after 5 to seven days in case of infection by the virus.¹⁵

Results

We studied nine patients with a history of bite by a suspected rabid animal. The patients were 4 females and 5 males, between 8 to 80 years old from different geographical areas of Iran (Table 2).

Five (50.5%) patients were bitten by dogs; among them 3 patients did not have any history of bite by suspected animals but were admitted as unknown encephalitis patients along with unspecific rabies clinical symptoms. Among all these patients, 4 patients died, and the results of the postmortem diagnosis analysis showed 2 of them certainly died of rabies (Table 3).

The present data show the ante-mortem saliva samples of 2 dead patients were positive by SYBR Green real-time PCR. After death of patients, FAT test was certainly positive which showed the presence of rabies virus infection in their brains (Figure 1). MIT test, as a sensitive method, confirmed the negative results of the molecular method and FAT in 2 brain samples after 28 days (Table 3).

Discussion

Rabies causes acute fatal encephalitis that leads to death of many people worldwide each year. Unfortunately, most of the encephalitis of unknown etiologies were reported annually due to lack of rapid and sensitive diagnostic methods for rabies in developing countries. Therefore, timely diagnosis and treatment of rabies can begin to take preventive measures in public health and to avoid unnecessary treatments for patients. In recent years, nucleic acid amplification techniques for molecular diagnosis of rabies have been identified as a great revolution in early diagnosis of rabies before death.¹⁶ These methods, such as RT-PCR, hemi-nested RT-PCR and Real-time PCR can be used with sensitivity and accuracy for ante-mortem diagnosis of rabies.⁴

In our study, we analyzed the saliva samples of nine patients using SYBR Green Real-Time PCR technique for

Table 2. Patients Demographic Information

| Province of Patient | Age | Cause of Referral | Bite Date | Bite Site | The Final Status of the Patient |
|---------------------|-----|-------------------|------------|---------------------------------|---------------------------------|
| Kerman | 10 | Dog bite | 15/06/2016 | Hips-finger | Discharged |
| Hormozgan | 80 | Mongoose bite | Unknown | Head | Death |
| Tehran | 25 | Dog bite | 21/09/2016 | Leg | Discharged |
| Mazandaran | 8 | Dog bite | 11/07/2016 | Hips-finger-hand | Death |
| Qom | 50 | Fox bite | 04/05/2016 | Head and face- left hand-tonnes | Death |
| Qazvin | 49 | Dog bite | 14/05/2016 | Unknown | Discharged |
| Fars | 32 | Wolf bite | 19/01/2016 | Finger on left foot | Discharged |
| Hamedan | 16 | Dog bite | 31/01/2016 | Unknown | Discharged |
| South Khorasan | 29 | Unknown | 01/03/2016 | Unknown | Death |

Table 3. The Comparison of Rabies Diagnostic Techniques in 9 Patients

| Number Patient | Ante-mortem Diagnosis for Saliva (SYBR Green Real-Time PCR) | Status | Postmortem Diagnosis for Brain | |
|----------------|---|--------|--------------------------------|-----|
| | | | FAT | MIT |
| 1 | - | Alive | ... | ... |
| 2 | + | Dead | + | ... |
| 3 | - | Alive | ... | ... |
| 4 | - | Alive | ... | ... |
| 5 | + | Dead | + | ... |
| 6 | - | Dead | - | - |
| 7 | - | Alive | ... | ... |
| 8 | - | Dead | - | - |
| 9 | - | Alive | ... | ... |

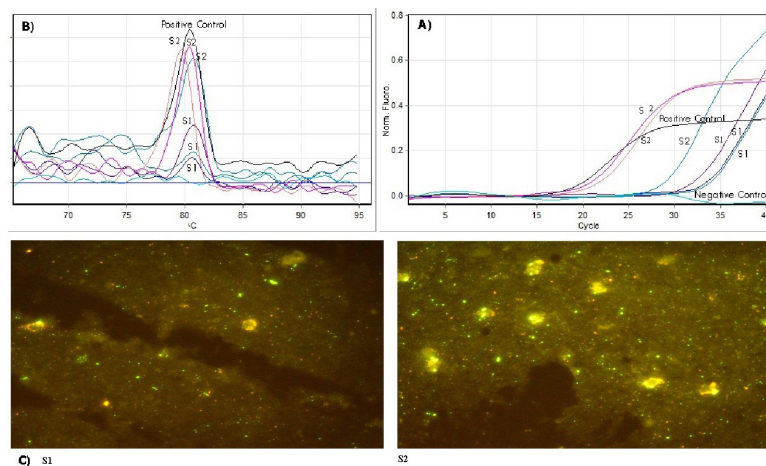


Figure 1. SYBR Green Real-Time PCR (A & B) and FAT (C) results of a rabid case in this study. The amplification plot for the analysis of saliva samples (A) and their melting spike (B); primer-dimer formation is represented on the left where the lines follow no clear pattern. Dissociation curves for 3 samples with different CT values are seen. Melting spikes of positive control are reflecting melting temperature of about 81°C. (C) The FAT test confirmed the results obtained by Real-Time PCR. S1: Patient No. 2, S2: Patient No.5.

the presence of rabies virus. These samples were collected during 2-3 days from patients with suspected rabies at different disease stage. Saengseesom et al showed that molecular techniques can be a useful tool in diagnosis before death. They used SYBR green real-time PCR technique on saliva and cerebrospinal fluid (CSF) samples of 15 rabid dogs. Thirteen (87%) saliva samples and 4 CSF samples were positive for the rabies virus.¹⁷ Also in the study performed by Dacheux et al on 51 neck skin biopsy specimens, the presence of rabies virus was confirmed in 43 cases by RT-hnPCR techniques.¹⁸ Mani et al, during the study for detection of rabies virus RNA by the real-time qRT-PCR technique showed that in ante-mortem diagnosis of rabies, 45.5% of CSF samples, 60% of nuchal skin biopsies and 85.7% of saliva samples were positive.¹⁹ Crepin et al reported presence of rabies virus in saliva and nuchal skin biopsy specimens from nine out of 28 suspected patients by the RT-PCR technique.²⁰ According to these studies, it seems that saliva samples are very suitable for rapid tests.

Nagaraj et al showed that among the 24 suspected patient saliva samples tested by RT-PCR and real-time PCR techniques, 18 cases were detected as positive by Real-time PCR and 6 cases by RT-PCR technique (3 saliva samples were positive in both tests). This study showed that real-time PCR, with a sensitivity of 75%, is an accurate and sensitive method for the diagnosis of rabies virus using saliva samples within a few hours.⁷ In our investigation, 2 out of 9 samples were positive for the presence of the virus in SYBR Green real-time PCR. After death, the diagnoses were confirmed at autopsy by direct FAT on their respective brain tissue samples.

Coertse et al tested the brain of mice which were injected with rabies virus from various species of Lyssavirus. They found that, in terms of sensitivity, real-time PCR and heminested PCR methods were both able to identify RNA viruses of the same dilution; however, in this study, it has been shown that Real-Time PCR was more rapid than Heminested PCR.²¹ Kaw et al showed that test sensitivity

was 71.4% for SYBR green Real-Time PCR and 57% for Nested RT-PCR on 11 skin biopsies of patients with clinical symptoms referred to health centers.⁶ In another study, Kaw et al found that among 12 saliva samples from animals suspected of rabies by using SYBR Green real-time PCR, 5 samples were positive with a sensitivity of 62.5% and 3 of them were positive with a sensitivity of 37.5% by using the nested RT-PCR.³ Recent studies have shown that real-time PCR based on SYBR Green, as a sensitive and rapid technique, is a useful tool for rabies diagnosis in human biological samples such as saliva and it can be a universal technique for the detection of Lyssavirus.^{7,21}

It was found that timing of sample collection is critical and detection of viral nucleic acid in saliva sample for the ante-mortem diagnosis of human rabies is not possible more than 5 hours after sample collection.²² The results of this study suggest that SYBR Green real-time PCR technique on saliva sample can be used for rapid diagnosis of rabies to avoid infection of other people such as the treating medical staff or family members of the patient. Since rabies in Iran is reported in nearly all of the provinces,²³ using a rapid test is necessary.

Limitations

In this study, due to lack of adequate training, sometimes, sampling was not done correctly and aspiration of sputum and throat was sent to the laboratory. As another problem, in some cases, samples were sent to the lab without maintaining proper temperatures. To solve this problem, we informed all hospitals of correct sampling through the Zoonosis Control Department, Center for Communicable Diseases Control, Ministry of Health and Medical Education.

Authors' Contribution

BK performed all of the experiments and wrote the primary draft. MA done the RNA extraction of all samples. RB and AG supported the molecular experiments. FJ performed the statistical analysis. MRS collected all samples. MF designed and theorized of the study and edited the manuscript.

Conflict of Interest Disclosures

The authors have no conflicts of interest.

Ethical Statement

Human brain samples were obtained from routine diagnostic activities at the WHO collaborating center for reference and research on rabies, Pasteur Institute of Iran. The center repository has been registered for research purposes and declared, in accordance with acquiring a license from the Reference Health Laboratories Research Center, Ministry of Health and Medical Education, Tehran, Iran. Also, with aim of accurate rabies surveillance, human saliva samples were sent to the WHO Collaborating Center for Reference and Research on Rabies, Pasteur Institute of Iran for ante-mortem diagnosis of rabies for the first time in Iran.

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