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Rapid Diagnostic Technique for Rabies Virus Detection

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ABSTRACT

Precise diagnosis is crucial for implementing suitable and timely treatment strategies, especially when dealing with life-threatening infections like rabies. Rapid diagnosis of rabies is crucial for preventing transmission to the patient's immediate contacts and medical staff, thereby controlling the further spread of the virus. Conventional methods for detecting rabies are either time-consuming or necessitate expensive equipment operated by trained professionals. This paper presents a novel rapid diagnostic method for rabies. This innovative approach leverages an isothermal reaction within a single platform, offering results within an hour and eliminating the need for specialized equipment. This method uses Cas13a enzyme, buffer, three guide RNAs, and a probe to detect rabies virus RNA using a lateral flow chromatography technique in samples suspected of rabies virus. After doing the test, the results are indicated by the presence of the control (C) and test (T) lines on the strip. Preliminary evaluations demonstrate that this rapid method correlates well with established diagnostic standards, showing promising sensitivity and specificity values.

INTRODUCTION

Rabies, a viral infection, is responsible for hundreds of deaths globally each year. Iran, like many other countries, also reports cases of rabies. Since the beginning of 2023, rabies has resulted in at least 16 deaths in Iran, according to preliminary unpublished data. The causative agent of this disease is a virus from the *Rhabdoviridae* family and the *Lyssavirus* genus. Rabies virus is an RNA virus featuring a negative-sense single-stranded genome encompassing five genes: *M*, *N*, *P*, *L*, and *G* [1]. The rabies virus displays diverse strains and reservoirs worldwide, with each country harboring strains endemic to its region. Iran has identified at least four endemic wild strains: KX148.185, KX148.190, KX148.186, and JX987734 [1]. To our knowledge, no types of bat rabies viruses have been observed in Iran to date [2]. The rabies virus is transmitted to animals or humans with the saliva of rabid animals when someone is bitten. Furious rabies leads to symptoms such as irritability, hallucinations, incoordination, hydrophobia, and aerophobia. Death occurs due to cardiac and respiratory arrest after a few days. Paralytic rabies is responsible for about 20% of human cases. This form progresses over a more extended period compared to the furious type; muscle paralysis begins at the site of the wound, followed by the gradual onset of coma, leading eventually to death.

Vaccination and post-exposure prophylaxis play crucial roles in reducing rabies mortality rates [3]. The WHO-recommended standard diagnostic test for rabies is a time-intensive immunohistochemical assay conducted postmortem on brain tissue samples [4].

Given the lethal and contagious nature of rabies, prompt non-invasive antemortem diagnosis is of utmost importance.

CONVENTIONAL DIAGNOSTIC TESTS FOR RABIES

Various diagnostic methods have been developed to detect the rabies virus in samples. However, these methods are often time-consuming and require expensive equipment operated by specialized personnel. The World Health Organization (WHO) recommends the Fluorescent Antibody Test (FAT) as the gold standard for rabies virus diagnosis, which requires at least three hours to yield results [4]. Additionally, the FAT is limited to postmortem analysis of brain tissue. It requires rigorous sample preparation under controlled conditions, a fluorescent microscope, and a FITC-conjugated anti-

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nucleocapsid antibody. Furthermore, the test demands an incubator, specific reagents, and a skilled diagnostician. The sensitivity of FAT for early-stage rabies warrants careful evaluation, particularly when viral titers are low. At these reduced concentrations, test accuracy can be compromised. Also, inadequate washing or improper application of conjugated antibodies may result in false positivity, mainly when performed by inexperienced technicians.

Polymerase Chain Reaction (PCR) is a versatile technique applicable to both antemortem and postmortem samples. In PCR, viral RNA is extracted from the specimen and reverse-transcribed into complementary DNA (cDNA). Using specific primers and a master mix, DNA polymerase enzymes amplify the cDNA in a series of thermal cycles. The amplified PCR fragments are then separated by agarose gel electrophoresis. The presence of rabies virus genes is confirmed by visualizing the bands under UV light with a Gel Documentation System. The PCR technique encompasses potential pitfalls, including sample loading errors, improper concentrations of templates or primers, and contamination that can skew results, leading to false positives or negatives. Results of PCR can be obtained within a minimum timeframe of about two hours [5].

Loop-mediated Isothermal Amplification (LAMP) is another technique for detecting the rabies virus. This method functions at a steady temperature between 60-65 °C, necessitating only a simple heat source or thermal block, thereby being adaptable for field use in diverse climates. LAMP requires multiple primer sets, typically 4-6 pairs, targeting short DNA sequences within the gene. Designing these primer sets is complex, with the potential for primer-dimer interactions that may interfere with the amplification. The exclusive reliance on Bsm DNA polymerase for the amplification process post-transcription is a notable limitation of LAMP. LAMP reactions generate a turbid byproduct, magnesium pyrophosphate, which can be quantified using a turbidimeter. The LAMP assay requires at least 90 min to yield results. Yet, optimizing the primer sets and reaction conditions is time-intensive [6]. To expedite detection, some researchers recommend combining the RT-LAMP assay with the Cas12a enzyme, a method referred to as DETECTR [7].

In the real-time quantitative PCR (qPCR) method, viral RNA is transcribed into complementary DNA (cDNA). qPCR is conducted in a thermocycler equipped with optics to illuminate each sample with light of a specific wavelength. A DNA-binding dye intercalates with the double-stranded DNA during qPCR, resulting in an enhanced fluorescence signal. Thus, as the DNA product accumulates with each cycle, the fluorescence intensity increases correspondingly and is measured in real time. This technique is highly quantitative and can amplify larger DNA fragments. Nonetheless, the required apparatus is costly, and the entire procedure, including data analysis, generally exceeds three hours, even when executed by an experienced operator [6].

Enzyme-linked Immunosorbent Assay (ELISA) variants, including direct and sandwich formats, are utilized to test for rabies infection in samples. Enzyme-conjugated mono- or polyclonal antibodies target a rabies virus antigen, and a spectrophotometer measures the resulting colorimetric change in the substrate. Nonetheless, ELISA presents challenges; unsuitable antibody sources, incorrect reagent concentrations, insufficient washing, and suboptimal incubation times may compromise accuracy. It is important to note that ELISA duration can vary according to the assay's specific protocol and could take less or more than four hours [8]. Moreover, ELISA typically targets the rabies virus nucleoprotein due to its diagnostic relevance.

RABIES DIAGNOSIS via LATERAL FLOW CHROMATOGRAPHY

In prokaryotes, RNA-guided enzymes serve various defensive roles against mobile genetic elements such as viruses and plasmids. These enzymes are central to systems like Argonaute proteins, CRISPR, and OMEGA, which are involved in genetic regulation and immunity. This capability to cleave single-stranded or double-stranded DNA or RNA underpins the development of novel diagnostic techniques. Recently, lateral flow chromatography, an advancement in virus detection methods, has been developed [9,10]. This method, portable and with an assay time of about one hour at 37°C, utilizes Cas13, an RNA nuclease from the CRISPR system, to target and cleave single-stranded RNA.

Cas13, upon binding to its associated CRISPR RNA (crRNA) or guide RNA (gRNA), catalyzes both the maturation of crRNA and the degradation of the target RNA. The gRNA or crRNA consists of a short-engineered RNA with a stem-loop architecture—including a Direct Repeat (DR) sequence for binding to Cas13 and an adjoining sequence that anneals to the target RNA (refer to Figure 1A). Cas13 displays a phenomenon termed 'collateral activity,' whereby, upon target recognition, its RNase activity extends beyond the target, nonspecifically slicing nearby RNA molecules, such as the reporter RNA. Each variant of the Cas13 enzyme (designated as a, b, c, or d), hailing from distinct bacterial origins, exclusively binds to and cleaves its corresponding DR sequence, a unique signature of 24-36 nucleotides. Simply, the crRNA (or gRNA) must begin with a specific DR sequence recognizable by the pertinent Cas13 enzyme variant S. The sequence designed to hybridize with the target RNA is then appended to this DR (details in Table 1).

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Table 1. Direct repeat (DR) of Cas13 protein. It should be noted that these sequences are DNA and must be translated into RNA, then added to the beginning of gRNA (crRNA).

No.	Cas protein	Species	DR sequence
1	LwCas13a	*Leptotrichia wadei*	GATTTAGACTACCCCAAAAACGAAGGGGACTAAAAC [9]
2	RxCas13d	*Ruminococcus flavefaciens XPD3002*	CCCCTACCAACTGGTCGGGGTTT [13]
3	PspCas13b	*Prevotella sp.*	GTTGTGGAAGGTCCAGTTTTGAGGGGCTATTACAAC [17]

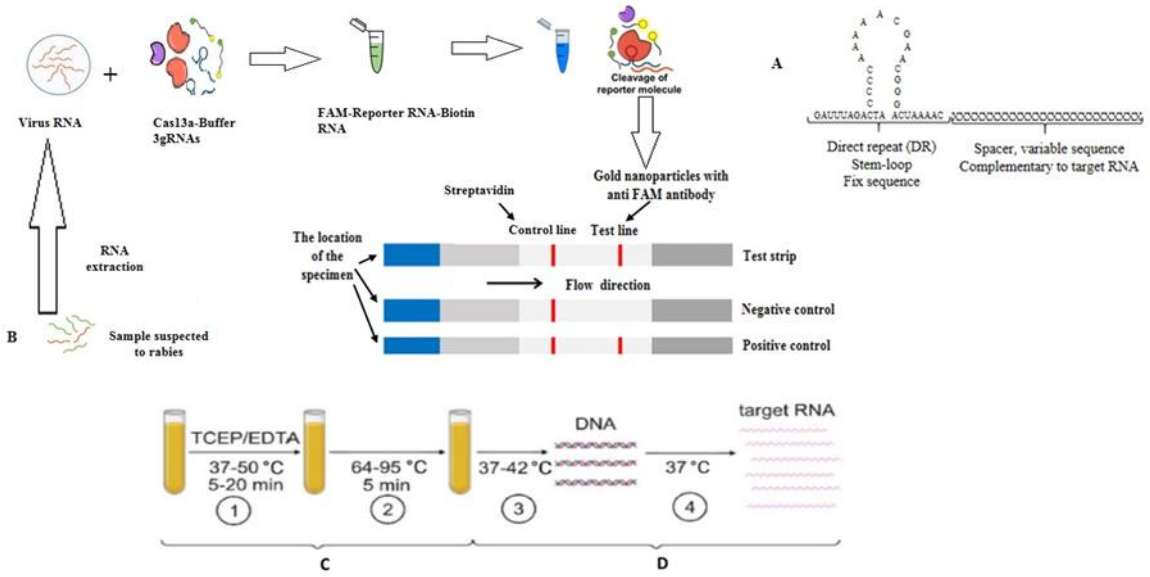


Fig. 1. A) Direct Repeat (DR) sequence for binding to CRISPR enzyme. B) Procedures of rapid virus detection in suspicious samples by lateral flow assay. C) Heating Unextracted Diagnostic Samples to Obliterate Nucleases (HUDSON) assay which follows by D) Specific High-sensitivity Enzymatic Reporter unLocking (SHERLOCK)

Each test requires several components besides the Cas13 enzyme and its accompanying buffer. This assay involves the addition of three guide RNAs (gRNAs), target RNA isolated from the sample, and a fluorescently labeled detection probe. The design of the probe entails a fluorophore (FAM) at the 5' end, biotin at the 3' end, and a linker composed of poly-uracil sequences. The first step involves activating the Cas13 enzyme in a reaction tube by incubation at 37˚C for 20 min. Subsequently, the template RNA and detection probe are introduced to the mixture and incubated at 37˚C for an additional 30 min; afterward, the reaction mixture is diluted tenfold and applied to a sample pad on a pre-coated lateral flow strip. After a two-minute incubation period, if the gRNAs can bind to their complementary region on the target RNA, the Cas13 enzyme will cleave first the target and then the probe. Then, the biotin end of the cleaved probe binds to streptavidin attached to gold nanoparticles. The FAM end binds to an anti-FAM antibody; both pre-coated on the strip. A positive test will display both the control (C) and test (T) lines on the strip, corresponding to the biotin and FAM, respectively. Conversely, if the gRNAs fail to bind the target RNA, Cas13 remains inactive, and neither the target RNA nor the probe will be cleaved. As a result, the probe will bind to streptavidin from its biotin side, and only the control line (C) will be visible, indicating a negative test result (Figure 1B).

Indeed, some researchers have introduced combined platforms that utilize Recombinase Polymerase Amplification (RPA) and CRISPR technologies [11]. RPA pre-amplifies the viral RNA in low concentrations within the initial sample to levels suitable for detection by various assays. For RNA virus detection, double-stranded DNA (dsDNA) templates are constructed from sequences obtained from GenBank using specific primers. The forward primer includes a T7 promoter sequence at its 5' end to facilitate the transcription of single-stranded RNA (ssRNA) from the dsDNA template using the HiScribe™ T7 High Yield RNA Synthesis Kit. Subsequently, the specific DR sequence required for recognition by the targeted Cas13 enzyme variant is incorporated at the beginning of both forward and reverse primers used to amplify the virus gene of interest. This enables the subsequent synthesis of gRNAs utilizing the HiScribe™ T7 High Yield RNA Synthesis Kit. The gRNA sequences must be reverse complements of the target sites within the viral RNA to ensure accurate base pairing. The resulting reaction mixture is ultimately applied to a designated lateral flow assay strip for visual diagnosis, as depicted in Figure 1B.

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An additional technique for directly detecting RNA virus genomes in liberated virions is termed Heating Unextracted Diagnostic Samples to Obliterate Nucleases (HUDSON), followed by Recombinase Polymerase Amplification (RPA) and CRISPR-based detection [12]. Initially, unprocessed diagnostic samples are heated at a moderate temperature range of 37-50˚C to deactivate endogenous nucleases and subsequently at a higher thermal range of 64-95˚C to inactivate the viruses, a step referred to as HUDSON (see Figure 1C) [13]. After HUDSON treatment, the contents of the microtube are utilized in the RPA-CRISPR assay for virus detection (illustrated in Figure 1B).

A CRISPR-based diagnostic assay has been developed that integrates nucleic acid pre-amplification with the specificity of CRISPR–Cas enzyme systems to identify DNA or RNA sequences. Named Specific High-sensitivity Enzymatic Reporter unLocking (SHERLOCK), this technique enables the detection of RNA or DNA molecules at high sensitivity in clinical samples [14]. SHERLOCK can identify single-stranded RNA (ssRNA) targets at minuscule concentrations, around two attomoles per liter (10^{-18} M), surpassing the detection sensitivity of Cas13a, which detects targets in the femtomolar range (10^{-15} M) [15]. The isothermal procedure first involves treating the sample with the HUDSON method, followed by raising the temperature to 37-42 ˚C to ensure that only the DNA strands are left intact. Subsequently, an RNA polymerase enzyme synthesizes numerous RNA copies from these DNA templates at 37˚C (Figure 1D) [16]. Finally, the amplification products obtained by SHERLOCK are utilized in the RPA-CRISPR assay (illustrated in Figure 1B).

CONCLUSION

Conventional rabies virus detection methodologies are time-intensive and dependent on costly equipment and specialized technical expertise. Implementing these detection facilities in high-risk infection zones—such as border checkpoints, ports, airports, and rural areas—is often unfeasible. Moreover, the sensitivity of these methods is inadequate for detecting low viral concentrations, which has catalyzed the development of innovative techniques.

Traditional Recombinase Polymerase Amplification (RPA) carries a risk of non-specific amplification, mandating the evaluation of different primer sets to identify the most appropriate pairs for accurate detection. Alternatively, RPA can be combined with exonuclease-probe-based strategies to enhance detection specificity. Essential modifications to the probes, such as incorporating fluorophores or quenchers, substantially elevate the cost of these assays.

The LAMP technique has certain drawbacks, but the higher test temperature (65 °C) can reduce nonspecific results. Yet, the high test temperature requirement of 65°C and the necessity of an electric photometer limit LAMP's application in off-grid or field settings, where access to reliable electrical power might be scarce.

The requirement to design four distinct primer sets for LAMP increases its operational complexity, restricting its practicality for some users.

The PCR and qPCR require specialized personnel and equipment, often rendering them impractical under resource-limited financial conditions. Additionally, PCR and qPCR may lack the necessary targeting precision and sensitivity to detect certain diseases efficiently (Table 2).

Table 2. Workflow comparison of qPCR and different CRISPR based assays for Rabies virus detection.

Methods	RNA extraction (copy number) copy/µL	Reverse transcription	Need Apparatus	qPCR	Detection
Current method	4-10	by thermal cycler 80 minutes	Yes	40 minutes	ct based
SHERLOCK	10-100	RT-RPA 25 minutes	No	Cas13a 30 minutes	Strip based 2 minutes
AIOD-CRISPR	10-100	RT-RPA 25 minutes	Yes	Cas12a 10 minutes	LED/UV based
DETECTR	70-300	RT-LAMP 20minutes	Yes	Cas12a 10 minutes	Strip based 2 minutes

Recent methods employing Recombinase Polymerase Amplification (RPA), such as SHERLOCK, HUDSON, and AIOD-CRISPR, leverage amplification of RNA from initial samples to improve the detection of low viral concentrations in the cerebrospinal fluid and salivary samples. However, amplification may increase the likelihood of mutations occurring within the RNA sample. Accumulation of mutations over multiple replication cycles could result in altered RNA sequences that gRNAs no longer recognize. These techniques require several preparatory and reaction setup steps, necessitating the expertise of trained personnel. In contrast, lateral flow assays are more user-friendly, do not require complex equipment or amplification processes, and yield reliable results with minimal sample volumes.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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