VacciMonitor 2025;34:e053425
www.vaccimonitor.finlay.edu.cu
Original Article

Preliminary evaluation of humoral immune response for rabies vaccine using a developed lateral flow immunochromatographic test

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In this study, a nanogold lateral flow immunochromatographic test was developed for the detection of rabies antibodies using a panel of well-characterized clinical and experimental serum samples. The lateral flow immunochromatographic rabies virus antibody test underwent a comprehensive evaluation, including an assessment of its limit of detection, cross-reactivity, interference from potential substances, and overall performance. Sensitivity evaluation revealed a limit of detection of 0.5 IU/mL, indicating a positive result. When compared with ELISA using different sera samples, the lateral flow immunochromatographic rabies virus antibody test exhibited robust performance with a sensitivity of 91.1 %, specificity of 92 %, and an overall accuracy of 91.5 %. These results suggest that the lateral flow immunochromatographic rabies virus antibody test could be a suitable tool for evaluating antibody levels in vaccinated animals. Moreover, it provides an alternative approach for assessing the efficacy of inactivated rabies virus vaccines.

Keywords: rabies virus; rabies vaccines; immunochromatographic assays; sensitivity and specificity.

Introduction

Rabies is attributed to lyssaviruses, with the rabies virus (RABV) serving as the primary causative agent of human rabies. Once symptoms manifest, the disease becomes incurable, leading to inevitable death due to encephalomyelitis. Annually, around 59,000 individuals succumb to rabies, with a staggering 95 % of these fatalities occurring in developing countries across Asia and Africa.(1) Over the past few decades, there has been a significant reduction in human rabies cases transmitted by dogs in the western hemisphere. This decline is attributed to the successful implementation of widespread dog vaccination programs, coupled with effective control measures to manage the dog population.(2,3)

Mitigating the global burden of human rabies is best achieved through the effective control of canine rabies.(4) The World Health Organization, the Food and Agriculture Organization of the United Nations, the World Organization for Animal Health and the Global Alliance for Rabies Control are currently collaborating to intensify their efforts towards eliminating dog-mediated rabies by 2030. Together, they are joining forces to provide comprehensive support to countries in achieving this goal.(5) In the initial phase of this initiative, mass dog vaccination has taken precedence as

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the most cost-effective strategy for controlling and ultimately eliminating rabies. To implement widespread vaccination in resource-limited countries, it is imperative to devise a vaccination program that is not only effective, but also cost-efficient.[6] The establishment of robust surveillance systems to detect animal rabies is a crucial component in controlling the spread of rabies. These systems play a vital role in clarifying the disease burden and monitoring the effectiveness of control measures, such as mass dog vaccination.[7]

The immune response to rabies involves both cell-mediated and humoral immunity. However, it is acknowledged that virus-neutralizing antibodies (VNAs) play a crucial role in providing protection against RABV infection.[8] Enzyme-linked immunosorbent assays (ELISA) are now widely accepted for detecting anti-rabies glycoprotein antibodies. Numerous studies have demonstrated a strong correlation between these assays and VNA tests, specifically relying on the detection and measurement of anti-G protein antibodies.[9]

Serological tests present several drawbacks, including challenges in standardization and the necessity of using live RABV, especially in VNA tests. This requires specialized containment facilities and skilled professionals, among other considerations. Consequently, there is a pressing need to explore alternative techniques for evaluating rabies control, surveillance, and vaccine efficacy. The lateral flow immunochromatographic assays (LFIs) have been created for the swift detection of RABV antigens. These devices present significant advantages, including rapidity, user-friendliness, absence of a requirement for additional equipment,[10] and cost-effective approach for detecting various infectious diseases (antigens/ antibodies) in humans and veterinary medicine.[11] The use of gold nanoparticles (AuNPs), which are nanoparticles of gold typically ranging in size from 1 to 100 nanometers, conjugated to specific antigens has improved the sensitivity and specificity of detection methods like LFIs; this method can detect antibodies in samples, generating visible bands on a test strip. Nevertheless, the sensitivity and specificity of LFIs may fluctuate depending on factors such as the targeted antigenic protein antibodies, assay format, and the quality of reagents used.[12]

The performance of LFIs for detecting rabies antibodies continues to be a subject of ongoing investigation, and studies have produced inconsistent results. Some reports have shown high sensitivity and specificity compared to conventional methods.[13] On the other hand, additional studies have indicated lower sensitivity, particularly in samples with minimal antibody content.[14]

The objective of this study was to assess the sensitivity of LFI in detecting rabies antibodies using a panel of well-characterized clinical and experimental sera samples, comparing the results with ELISA. Our findings aim to offer valuable insights into the potential use of LFI as a rapid test for detecting rabies antibodies in veterinary practice and as an initial method for evaluating rabies vaccine efficacy.

Materials and Methods

Serum samples

Clinical and experimental serum samples (n = 200) were supplied by the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) and various pet clinics in Cairo; the experimental serum samples (n = 150) had been selected for this study and were previously evaluated with satisfactory results by CLEVB.

Reference rabies antibody (n = 1) was provided by Strain Bank Department at CLEVB (2.39 $\log_{10}$ $TCID_{50}$/ mL) (4.5 IU/mL); it was used for assessment the limit of detection (LOD) of the lateral flow immunochromatographic rabies virus (LFI-RABV) antibody test.

Ethical approval

The current study followed the Animal Research Reporting of *In-Vivo* Experiments (ARRIVE) guidelines. All procedures involving animal use strictly adhered to the guidelines established by the Institutional Animal Care and Use Committee at the Agricultural Research Center (ARC-IACUC). Ethical approval for this study was obtained from the committee (ARC-IACUC) approval No (ARC-CLEVB-24-24). The

manuscript is considered compliant with bioethical standards in good faith.

No anesthesia or euthanasia protocols were employed for the animals involved in this study, as all animal-dependent methodological procedures were categorized as either no or low-pain procedures that can be ethically performed on a conscious and alive animal.

Rabies virus glycoprotein G

RABV glycoprotein G (strain CVS-11), His Tag (RAG-V55H5) was expressed from baculovirus-insect cells. It contains AA Lys 20 - Lys 458 (Accession # ADJ29911.1) and was purchased from ACROBiosystems, USA.

Rabbit anti-dog IgG

Anti-dog IgG (whole molecule) antibody produced in rabbit (Cat # D7407) was purchased from Sigma-Aldrich, USA.

Preparation of nanogold particles of 40 nm diameter size[11,15]

To synthesize AuNPs, the following procedure was undertaken: initially, 50 mL of ultra-pure water was brought to a vigorous boil with stirring using a hot plate stirrer. Concurrently, sodium citrate at a concentration of 0.01 % (w/v) was introduced into the boiling water. Subsequently, 1 mL of a 1 % gold (III) chloride (HAuCl4) solution was added to the boiling water. As the reaction progressed, the color of the solution turned red, indicating the formation of AuNPs. Following this, sodium azide at a concentration of 0.02 % (w/v) was incorporated into the solution, that was allowed to cool, and the diameter of the resulting nanogold particles, falling within the range of 400-600 nm, was confirmed using a spectrophotometer.

Characterization of prepared nanogold particles

UV-Vis absorption spectra of the synthesized AuNPs were acquired using a UV–Vis spectrophotometer (Shimadzu UV-3600, Japan) within the wavelength range of 200 to 800 nm.

Scanning electron microscopy (SEM)

The analysis was conducted using a field emission environmental scanning electron microscope (FE-SEM) model (Quattro S, Thermo Scientific USA) to examine the surface properties of synthesized (AuNPs) with an acceleration voltage of 15 kV. To guarantee accurate investigation and particle size determination, the samples were scanned with coatings onto carbone grids using STEM unit. ImageJ software was used for image analysis using more than 50 random images for determining the Au mean particle size.

Conjugation of nanogold particles with rabies virus glycoprotein G[16]

The pH of the nanogold particles was adjusted to 8.5 using a 0.02 M K2CO3 solution. With gentle stirring, 1 mL of RABV glycoprotein G at a concentration of 1 mg/mL was mixed with 100 mL of the prepared nanogold particles. The mixture was lightly shaken for 15 min. To block any unreacted sites, 1 % (w/v) polyethylene glycol (PEG-20,000) was added to the mixture with gentle stirring for an additional 15 min. Afterward, the mixture underwent centrifugation at 12,000 rpm for 1 h. The resulting conjugated RABV glycoprotein G with nanogold particles were then suspended in 1 mL of a dilution buffer containing 3 % (w/v) sucrose, 20 mM Tris, 1 % (w/v) bovine serum albumin, and 0.02 % (w/v) sodium azide. The suspension was stored at 4 ˚C for further use.

Dispensing of conjugated rabies virus glycoprotein G with nanogold particles, non-conjugated rabies virus glycoprotein-specific rabbit IgG, and rabbit anti-dog IgG on nitrocellulose membrane and conjugation pad[11,17]

The sample pad consisted of glass fiber (Ahlstrom 222) was pretreated with a buffer solution at pH 8.5. The buffer solution was prepared using ultrapure water and included the following components: 1 % (w/v) polyvinylpyrrolidone (PVP), 2 % (w/v) titron X100, 3.81 % (w/v) Borax, 0.1 % (w/v) casein sodium salt, 0.15 % (w/v) sodium dodecyl sulfate, 0.5 % (w/v) sodium cholate, and 0.02 % (w/v) sodium azide. Following pretreatment, the sample pad was dried at 37 ℃ to eliminate any remaining moisture.

Conjugation pad: the glass fiber (Ahlstrom 8964) was pretreated with a conjugation-treated buffer solution prepared at pH 7.4. This buffer solution consisted of 20 mM phosphate buffered saline, 2 % (w/v) bovine serum albumin, 2.5 % (w/v) sucrose, 0.3 % (w/v) PVP, 1 % (w/v) Triton X-100, and 0.02 % (w/v) sodium azide. Following pretreatment, the conjugation pad was dried at 37 ℃ to eliminate any remaining moisture. Subsequently, the pretreated conjugation pad was saturated with RABV glycoprotein G-conjugated nanogold particles. It was then dried at 37 ℃ for 1 h and stored in a dry condition for further use.

Nitrocellulose (NC) membrane (mdi CNPF-PD31) was used. Utilizing an Iso-flow dispenser, two lines were deposited onto the NC membrane, measuring 300 mm × 25 mm. The test line was dispensed with purified mouse monoclonal antibody specific to RABV glycoprotein G (0.1 mg/1 mL) at a volume of 1 µL per 1 cm line. Simultaneously, the control line was dispensed with rabbit anti-dog IgG (3 mg/mL) at a volume of 1 µL per 1 cm line. Following the dispensing process, the loaded NC membrane was dried at 37 ℃ for 4 h and then stored in a dry condition. To assemble the components, a polyvinyl chloride backing card was employed to adhere the treated sample pad, conjugated pad, loaded NC membrane, and absorbent pad together. The assembled structure was subsequently cut into a 4 mm width, as illustrated in Figure 1.

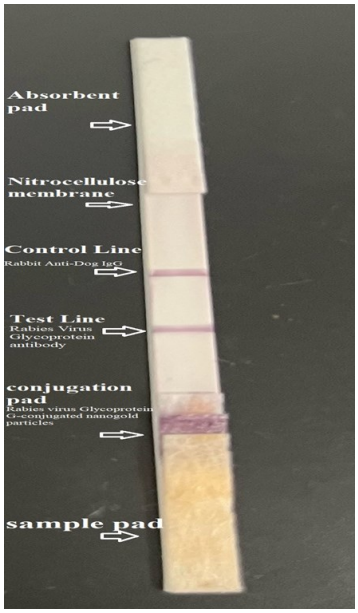

Fig. 1. Description of the prepared LFI-RABV antibody strip indicating the incorporation of distinct components: a sample pad, conjugation pad, nitrocellulose membrane, test line, control line, and an absorption pad.

Analytical specificity testing using other viral strains

The LFI-RABV antibody test was assessed using antibodies against various canine viruses, such as canine parvovirus, canine adenovirus, canine parainfluenza, and canine distemper.

Cross-reactivity and interfering substances

The LFI-RABV antibody test underwent evaluation for potential cross-reactivity with various bacterial strains (10^7 CFU/0.1 mL), including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Streptococcus pyogenes*, *Bordetella bronchiseptica* and *Clostridium perfringens*. Additionally, the test was examined for reactivity with interfering substances, such as whole blood, dexamethasone, and phenylephrine.

Analytical sensitivity testing of developed LFI-RABV antibody

The limit of detection of the LFI-RABV antibody test was established by testing threefold serial dilutions of the reference rabies serum, ranging from 0.48 $\log_{10}$ $TCID_{50}$/mL (0.056 IU/mL) to 2.39 $\log_{10}$ $TCID_{50}$/mL (4.5 IU/mL). The dilutions assessed using the developed LFI-RABV antibody test were compared with the results obtained from ELISA kit (BioPro Rabies ELISA, cat# RAB01-02, BioPro, Prague, Czech Republic).[17]

Performance of the developed LFI-RABV antibody test compared to ELISA

Sensitivity, specificity, and accuracy of the LFI-RABV antibody test were assessed by comparing it to ELISA. Two hundred clinical and experimental serum samples were subjected to testing with both the LFI-RABV antibody test and ELISA. Samples were classified as positive if ELISA yielded a positive result, and negative if ELISA indicated a negative result.[11]

Results

Gold nanoparticles structure verification

Figure 2 exhibits the morphological structure and particle size distribution of AuNPs using FE-SEM investigation with different magnifications. FE-SEM micrographs offer the AuNPs aggregation onto coated carbon grids with an average particle diameter size ranging from 30-40 nm as counted by ImagJ software. These findings were further evidenced by UV-Absorbance analysis; the Figure 3 shows the absorption spectra of prepared AuNPs around λ 527.3 nm as a peak of Au absorbance indicating a diameter size that ranged 40 nm.

Analytical sensitivity testing

The minimal antibody titer (IU/mL) detectable by the LFI-RABV antibody test was 0.5 IU/mL, unequivocally indicating a positive result, as depicted in Figure 4. Sensitivity testing, validated through ELISA, further corroborated the positive detection capability of the LFI-RABV antibody test at a dilution of 1/27(1.43 $\log_{10}$/ $TCID_{50}$/mL) (0.5 IU/mL), as outlined in Table 1.

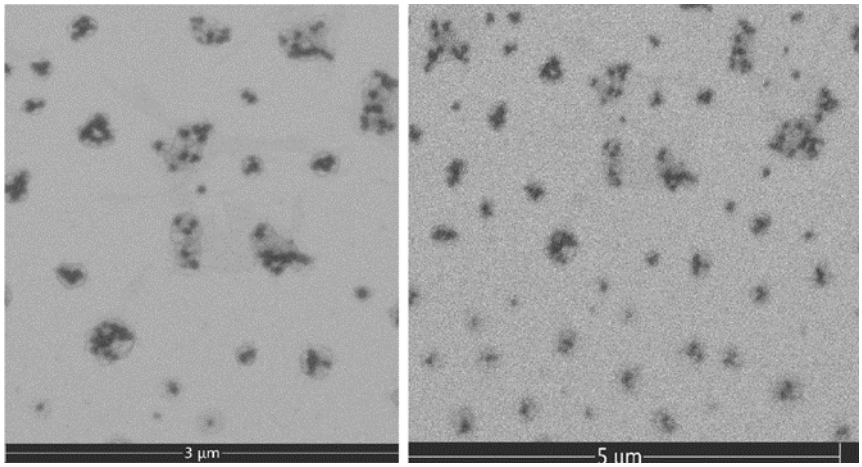

Fig. 2. FE-SEM micrographs of AuNPs with scaling 3 and 5µm and original magnification 30000X, 16000X; respectively at 15 Kv.

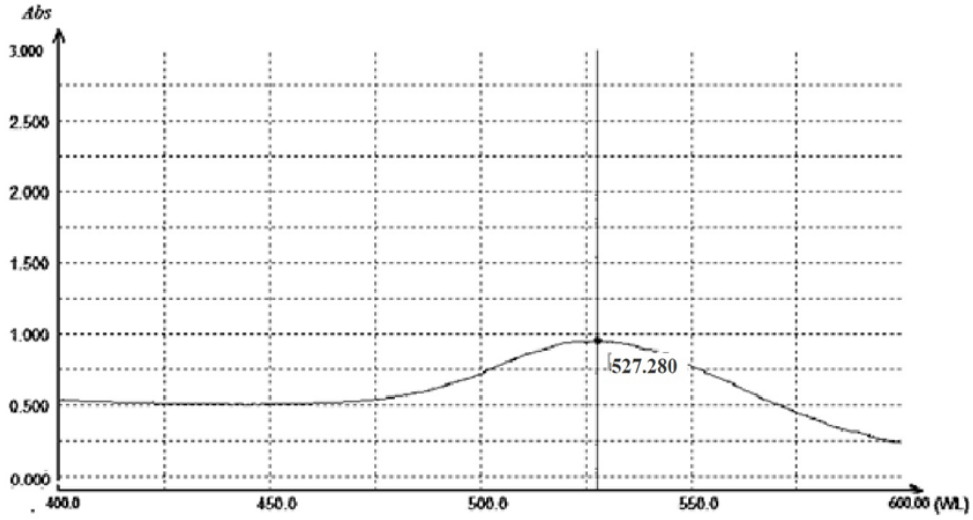

Fig. 3. The spectrophotometric profile of prepared AuNPs reveals a pronounced absorption peak at approximately 527.3 nm.

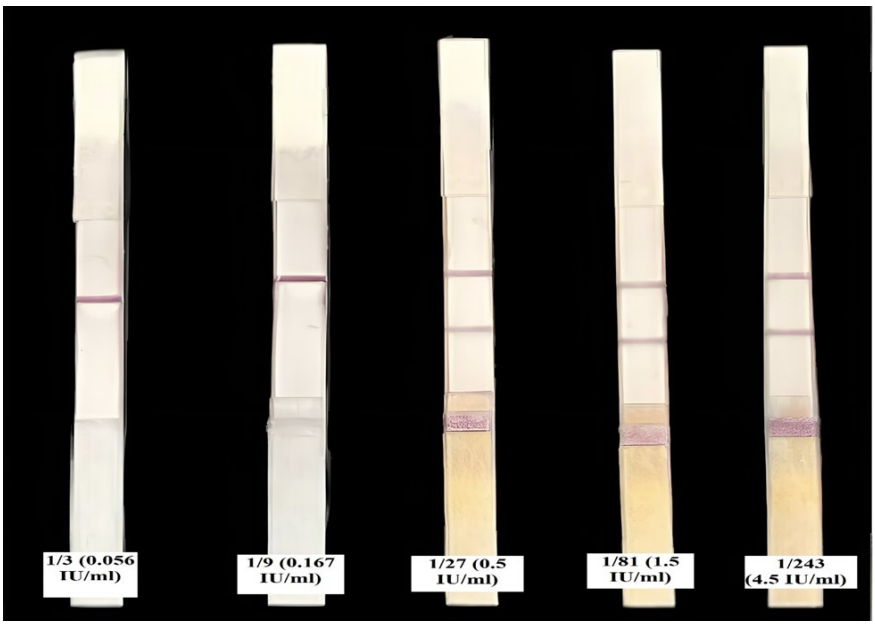

Fig. 4. Limit of detection (LOD) of the prepared LFI-RABV antibody test determined through serial dilutions of phosphate buffer saline spiked with rabies antiserum. This systematic approach allowed the precise characterization of the test's analytical sensitivity and detection threshold.

Table 1. Comparison of the LFI-RABV antibody test and ELISA for detecting rabies virus antibodies at various serum dilutions.

* Serum dilution	Reference rabies virus antibody titer (IU/mL)	** LFI-RABV antibody test	*** ELISA (% positivity)
1:243	4.5	+ve	+ve (97.01 %)
1:81	1.5	+ve	+ve (81.7 %)
1:27	0.5	+ve	+ve (71.5 %)
1:9	0.167	−ve	−ve (42.4 %)
1:3	0.056	−ve	−ve (15.7 %)

*+ve: positive detection of antibodies. Both the test line and the control line were clearly visualized on the test strip. −ve: negative detection of antibodies, only the control line was visualized on the test strip, with no test line appearing. **Serum dilutions (e.g., 1:243) indicate the fold dilution of the test serum. *** Based on the manufacturer's guidelines, the test serum is classified as positive if it shows an inhibition of 40 % or higher when compared to the negative controls. Furthermore, an inhibition level of 70 % corresponds to a concentration of 0.5 IU/mL.

Analytical specific testing

The LFI-RABV antibody test showed a positive result for RABV antibodies and negative results for antibodies against canine parvovirus, canine adenovirus, canine parainfluenza, and canine distemper.

Cross-reactivity and interfering substances

The LFI-RABV antibody test demonstrated no cross-reactivity or interference with various bacterial strains (10^7 CFU/0.1 mL), including *Escherichia coli, Staphylococcus aureus, Salmonella* Typhimurium*, Streptococcus pyogenes*, *Bordetella bronchiseptica, and Clostridium perfringens*. Moreover, the test exhibited no interference from substances such as whole blood, dexamethasone, and phenylephrine.

Table 2. Relative sensitivity, specificity, and accuracy of the developed LFI-RABV antibody test compared with the ELISA method.

A*				
Method	**ELISA**			**Total Results**
	Results	**Positive**	**Negative**	
LFI-RABV antibody	Positive	160 (True positive)	2 (False positive)	
	Negative	15 (False negative)	23 (True negative)	
Total results		175	25	200

B			
Sample	**Sensitivity (%)**	**Specificity (%)**	**Accuracy (%)**
Rabies sera	91.1 %	92 %	91.5 %

*A: True positive (LFI-RABV antibody test and ELISA yield positive results). False positive (LFI-RABV antibody test gives a positive result, while ELISA gives a negative result). True negative (both LFI-RABV antibody test and ELISA give negative results). False negative (LFI-RABV antibody test gives a negative result, while ELISA gives a positive result). B: performance metrics of the developed LFI-RABV antibody test; sensitivity, specificity and precision were evaluated.

Evaluation of the developed LFI-RABV antibody test compared to ELISA

The sensitivity, specificity, and accuracy of the developed LFI-RABV antibody test, in comparison to the ELISA test, were 91.1 %, 92 %, and 91.5 %, respectively, as illustrated in Table 2.

Discussion

This study presents a novel approach for assessing antibodies against the RABV using the LFI-RABV antibody test. The findings offer valuable insights into the potential application of LFI as a rapid test for detecting rabies antibodies in veterinary practice, serving as an initial method for evaluating the efficacy of rabies vaccines. The test was designed to detect antibodies against rabies glycoprotein G in canine sera, specifically from canines vaccinated with the inactivated rabies vaccine. The RABV glycoprotein G was procured and conjugated with AuNPs for this purpose. Additionally, a commercial RABV glycoprotein antibody and an anti-dog IgG[13] were employed in the development of a LFI-RABV antibody test. The conjugate prepared with 40 nm AuNPs exhibited satisfactory stability and demonstrated immunological reactivity towards rabies antibodies. Interestingly, similar study supports the utilization of 20-40 nm AuNPs for conjugate preparation in immunochromatographic tests.[15]

The data presented here confirm that the average diameter obtained for AuNPs verifies the production of nanoparticles in the nanoscale structure, with a mean diameter size of 40 nm. This was validated using FE-SEM micrographs showing the AuNPs aggregation onto coated carbon grids with an average particle diameter size ranging from 30-40 nm as counted by ImagJ software and UV-Absorbance analysis, which indicated a distinct spectrophotometric profile for the prepared AuNPs. The profile revealed a pronounced absorption peak, representing the Au absorbance fingerprint, observed at approximately 527.3 nm; this finding is consistent with a similar published research.[17]

The evaluation of the analytical sensitivity of the developed LFI-RABV antibody test unveiled a LOD of 0.5 IU/mL, indicative of a positive result. Additionally, analytical sensitivity testing assessed through ELISA, substantiated the positive detection capability of the LFI -RABV antibody test at a dilution of 1/27 (corresponding to 1.43 $\log_{10}$/$TCID_{50}$/mL) and 0.5 IU/ mL. Interestingly, a study indicated that sera exhibiting virus-neutralizing antibody titers of ≥ 0.5 IU/mL

effectively neutralized the RABV, resulting in the absence of a test line. Conversely, sera with titers < 0.5 IU/mL did not inhibit the formation of the test line when assessed using the RABV G detection kit.[13]

To assess the analytical specificity of the developed LFI -RABV antibody test, its performance was evaluated for detecting RABV antibodies and differentiating them from antibodies against other canine viruses: canine parvovirus, canine adenovirus, canine parainfluenza, and canine distemper. The test consistently and accurately identified rabies antibodies while yielding negative results for all other tested antibodies. Furthermore, the LFI-RABV antibody test demonstrated no cross-reactivity with various bacterial strains, including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Streptococcus pyogenes*, *Bordetella bronchiseptica*, and *Clostridium perfringens*. Additionally, the test showed no interference from substances such as whole blood, dexamethasone, and phenylephrine.

Sera obtained from subjects who were either unvaccinated or had received other vaccines such as canine distemper, canine parvovirus, canine adenovirus, canine coronavirus, and canine parainfluenza did not yield false-positive results when subjected to immunochromatographic test. This outcome underscores the specificity of the immunochromatographic test for detecting sera containing antibodies against the RABV.[16] Interestingly, in study using RAPId Neutralizing Antibody (RAPINA) test based on the principle of immunochromatography to evaluate RABV in unvaccinated subjects, no false-positive results were obtained, demonstrating a test specificity of 100 %. These individuals had previously received vaccinations for polio, tetanus, diphtheria, pertussis, BCG, measles, and hepatitis B. This observation suggests that the RAPINA test did not cross-react with antibodies induced by other viruses.[14]

The performance metrics of the developed LFI-RABV antibody test, in contrast to the ELISA test, yielded a sensitivity of 91.1 %, specificity of 92 %, and an overall accuracy of 91.5 %. In comparison to the Rapid Fluorescent Focus Inhibition Test (RFFIT), the RAPINA displayed sensitivity, specificity, and accuracy values of 88.7 %, 91.9 %, and 90.4 %, respectively.[14] In other separate study, RAPINA proved suitable for detecting RABV G protein-specific antibodies even in undiluted serum samples. Out of the 57 samples utilized without dilution in both the RFFIT and the RAPINA, 25 of the 27 samples that tested positive in the RFFIT, also tested positive in the RAPINA, resulting in a sensitivity of 92.6 %. The specificity was determined as 96.6 % (29/30). An assessment of 772 samples obtained from veterinary hospitals in Japan revealed a sensitivity of 97.6 % (659/675) and a specificity of 91.8 % (89/97).[18] In a subsequent study, the second version of RAPINA demonstrated a sensitivity, specificity, and concordance rates of 100 %, 98.34 %, and 98.6 %, respectively, when compared with RFFIT.[19] The RAPINA test proves to be a promising diagnostic tool due to its ability to deliver results for rabies antibodies within 15 min, at a low cost, and with easy execution. It boasts long-term stability across a wide range of environmental conditions and eliminates the need for prior neutralization with live virus. These advantages signify that immunochromatographic test represent an ideally useful test for clinical laboratories without specialized equipment and for field diagnosis, particularly by personnel with less specialized training, enabling the rapid detection of RABV-specific antibodies.[20]

Conclusion

The developed LFI-RABV antibody test is appropriate for evaluating antibody levels in vaccinated animals, providing an alternative approach for the assessment of inactivated RABV vaccine efficacy.

Conflict of interest

The authors declare that there is no conflict of interest.

Author's contributions

Mohamed Samy Abousenna: Conceptualization, methodology, formal analysis, investigation, data curation, writing-original draft preparation, writing-review and editing.

Sara El Sawy Ahmed: methodology, validation, and investigation.

Darwish Mahmoud Darwish: methodology, formal analysis, and investigation.

Fady Abd El-Mohsen Shasha: methodology, formal analysis, investigation.

Amal Abd El-Moneim Mohamed: methodology, validation, and formal analysis.

Heba A. Khafagy: methodology, formal analysis, and data curation.

Mohamed Mahmoud Youssef: formal analysis, and data curation.

Nermeen Gouda Shafik: validation, and investigation.

All authors read and approved the final manuscript.

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Evaluación preliminar de la respuesta inmune humoral a la vacuna antirrábica empleando la prueba inmunocromatográfica de flujo lateral

Resumen

En este estudio se desarrolló una prueba inmunocromatográfica de flujo lateral basada en nanopartículas de oro para la detección de anticuerpos contra la rabia, utilizando un panel de muestras clínicas y experimentales de suero bien caracterizadas. Este ensayo se sometió a una evaluación exhaustiva, que incluyó una valoración de su límite de detección, reactividad cruzada, interferencia potencial de sustancias y rendimiento general. La evaluación de la sensibilidad reveló un límite de detección de 0,5 UI/mL para esta prueba inmunocromatográfica de flujo lateral desarrollada, lo que indica un resultado positivo. Cuando se comparó con el ensayo de ELISA, utilizando diferentes muestras de suero, la prueba inmunocromatográfica de flujo lateral para anticuerpos del virus de la rabia mostró un sólido rendimiento con una sensibilidad del 91,1 %, una especificidad del 92 % y una precisión global del 91,5 %. Estos resultados sugieren que la prueba inmunocromatográfica de flujo lateral para anticuerpos contra el virus de la rabia podría ser una herramienta adecuada para evaluar los niveles de anticuerpos en animales vacunados. Además, constituye un método alternativo para evaluar la eficacia de las vacunas inactivadas contra el virus de la rabia.

Palabras clave: virus de la rabia; vacunas antirrábicas; inmunoensayo; sensibilidad y especificidad.

Received: October 20, 2024 *Accepted: February 18, de 2025*