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First Immunohistochemical Characterization of Bovine Rabies in Al Jabal Al Akhdar, Libya: A Case Report

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Immunohistochemistry; Rabies; Bovine; Libya.

A B S T R A C T:

Abstract: This study aimed to apply immunohistochemistry (IHC) for detecting the rabies virus (RABV) in formalin-fixed, paraffin-embedded (FFPE) brain tissues from native cattle in Al Jabal Al Akhdar, Libya. A brain tissue sample, previously diagnosed with rabies via histopathology, was retrieved from the archives of the Department of Pathology and Clinical Pathology, Faculty of Veterinary Medicine, Omar Al-Mukhtar University. IHC was performed using the monoclonal antibody N/P Seren Dr Finke N161/5 (RABV) α-rabbit. Distinct granular viral antigen deposits were observed in the cytoplasm of Purkinje cells in the cerebellum, confirming rabies infection. No immunoreactivity was detected in the negative control. These findings validate and highlight the potential of IHC as a reliable complementary diagnostic tool in resource-limited settings where fresh tissue samples are unavailable, reinforcing its utility for retrospective studies and outbreak investigations. This work also serves as a follow-up confirmation of Libya's first reported bovine rabies case in the same region, emphasizing the need for enhanced diagnostic infrastructure to support rabies surveillance and control programs in endemic areas.

أول توصيف مناعي نسيجي كيميائي لداء الكلب البقري في منطقة الجبل الأخضر، ليبيا: تقرير حالة

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المستخلص:

هدفت هذه الدراسة إلى استخدام تقنية الكشف المناعي النسيجي (IHC) في تشخيص فيروس السعار (داء الكلب) في أنسجة دماغية مُثبتة بالفورمالين ومضمَّنة بالبارافين من أبقار محلية بمنطقة الجبل الأخضر في ليبيا. تم تحليل عينة دماغية من أرشيف قسم الباثولوجيا الإكلينيكية بكلية الطب البيطري بجامعة عمر المختار، والتي سبق تشخيصها بالسعار بواسطة الفحص النسيجي التقليدي. استُخدمت تقنية IHC مع جسم مضاد أحادي النسيلة (N/P Seren Dr Finke N161/5 (α-rabbit للكشف عن مستضدات الفيروس. أظهرت النتائج ترسبات حبيبية واضحة للمستضدات الفيروسية في خلايا بركنجي بالمخيخ، مع غياب أي تفاعل في العينة الضابطة السالبة. يؤكد هذا الكشف صحة التشخيص السابق ويسلط الضوء على أهمية استخدام IHC كأداة تشخيصية تكميلية في المناطق محدودة الموارد، حيث يصعب نقل العينات الطازجة أو تخزينها. تُبرز الدراسة أهمية IHC في تحسين المراقبة الوبائية للسعار، خاصةً في المناطق التي تعتمد على العينات المُثبتة، وتؤكد على الحاجة إلى تعزيز البنية التحتية التشخيصية في ليبيا. تُعد هذه الدراسة تأكيدًا لحالة سابقة نُشرت من المنطقة ذاتها، مما يعزز مصداقية النتائج ويدعم تطبيق التقنية في السياقات المماثلة.

الكلمات المفتاحية:

الكشف المناعي النسيجي؛ السعار؛ الأبقار؛ ليبيا.

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INTRODUCTION

Rabies is an acute, progressive, and zoonotic viral encephalitis classified as a notifiable disease under international health regulations. With historical records tracing its impact on human and animal populations for over five millennia (Rupprecht et al., 2002; Velasco-Villa et al., 2017), the disease is caused by highly neurotropic RNA virus species within the genus Lyssavirus (family Rhabdoviridae) (Rupprecht et al., 2017). While Chiroptera (bats) and Carnivora (e.g., foxes, raccoons) serve as primary ecological reservoirs, domestic dogs remain the most significant global vector for human exposure (Hampson et al., 2015). The virus persists in enzootic cycles among domestic, feral, and wildlife populations, with transmission to humans occurring predominantly through percutaneous inoculation of infected saliva via bites, scratches, or mucosal contact (World Health Organization [WHO], 2018; Franco-Molina et al., 2021). Rabies virus transmission primarily occurs through percutaneous inoculation, most commonly via the bite of an infected host. Following entry, virions undergo retrograde axonal transport, migrating along peripheral nerves and synapses to reach the central nervous system (CNS) (Hemachudha et al., 2005). Subsequent viral replication within the CNS leads to widespread neuronal dissemination, followed by centrifugal spread to highly innervated sites such as the salivary glands, enabling viral shedding in saliva (Fooks & Jackson, 2020). Notably, current diagnostic modalities lack sensitivity during the prolonged incubation period (typically 1–8 weeks), as neither viral antigens nor RNA are reliably detectable in peripheral tissues at this stage (World Health Organization [WHO], 2018). Consequently, a definitive diagnosis requires postmortem analysis of brain tissue (e.g., hippocampus, brainstem) or trigeminal ganglia (Mani & Madhusudana, 2013). Rapid and accurate diagnosis in suspected animal cases is critical to inform timely post-exposure prophylaxis (PEP) in humans, thereby preventing fatal (Hampson et al., 2015).

Rabies virus infection is histopathologically defined by non-suppurative encephalitis accompanied by intracytoplasmic Negri bodies in neuronal cells. However, while classically pathognomonic, these eosinophilic inclusions are inconsistently observed and absent in 20–60% of confirmed cases , significantly limiting their diagnostic reliability (World Health Organization [WHO], 2018)(Jogai et al., 2000). Furthermore, reliance on fresh tissue samples for conventional diagnostic methods (e.g., direct fluorescent antibody test, dFAT) poses substantial biosafety risks due to potential viral aerosolization and environmental contamination (Rupprecht & Salahuddin, 2019). In resource-limited settings or delayed diagnostic scenarios, formalin-fixed paraffin-embedded (FFPE) tissues often remain the only available material for post-mortem analysis. This necessitates the adoption of advanced techniques capable of overcoming the antigenic alterations caused by prolonged fixation (Organization, 2018). Immunohistochemistry (IHC) and indirect FAT have emerged as critical tools in this context, offering enhanced sensitivity and specificity through targeted antigen-antibody interactions. These methods enable precise visualization of viral antigens even in immunogenically compromised FFPE samples, thereby bridging the diagnostic gap left by conventional histopathology (Ramos-Vara, 2005). For instance, IHC has demonstrated superior accuracy in detecting rabies virus nucleocapsid proteins in archived brain tissues, particularly in cases where Negri bodies are absent or equivocal. Consequently, integrating IHC into rabies surveillance protocols not only improves diagnostic confidence but also aligns with biosafety best practices by minimizing hazardous tissue handling (Achkar et al., 2019; Dacheux et al., 2008; Farahtaj et al., 2019; Rupprecht & Salahuddin, 2019). In Libya, where diagnostic infrastructure remains constrained, the confirmation of rabies poses significant challenges. The first documented case of animal rabies in the country a calf from the Al-Jabal Al-Akhdar region (Sharif et al., 2021) underscores the urgent need for accessible and reliable diagnostic tools. This study aims to implement immunohistochemistry (IHC) for rabies virus detection in formalin-fixed brain tissues from this case, marking the first application of this technique in

Libya's diagnostic framework. By prioritizing IHC, we address two critical gaps: (1) the limitations of conventional histopathology, which often fails to identify Negri bodies in up to 60% of infections, and (2) the biosafety risks associated with handling fresh specimens in resource-limited settings. The validation of IHC in this context not only enhances diagnostic accuracy but also establishes a scalable model for rabies surveillance in regions with comparable logistical constraints. This approach aligns with global recommendations for decentralized, low-cost rabies diagnostics (Lembo et al., 2006)(WHO, 2023) and demonstrates the feasibility of advanced molecular techniques in resource-scarce environments.

MATERIALS AND METHODS

Cases

A formalin-fixed, paraffin-embedded (FFPE) brain tissue specimen was retrieved from the archival repository of the Department of Pathology and Clinical Pathology, Faculty of Veterinary Medicine, Omar Al-Mukhtar University (Al-Bayda, Libya). This specimen originated from a suspected rabies case in a bovine subject that had received a histopathologically confirmed rabies diagnosis in a prior study (Sharif et al., 2021). The FFPE block served as the primary material for comparative diagnostic evaluation in this investigation.

Histopathology and Immunohistochemistry (IHC)

For histopathology, 4μm brain tissue sections were deparaffinized in xylene (3×5 min), rehydrated through graded ethanol (100%→70%), and rinsed. Nuclei were stained with Harris' s hematoxylin (8 min), differentiated in 1% acid alcohol (30 sec), and blued in tap water (5 min). Cytoplasmic/extracellular components were counterstained with eosin (3 min). Slides were dehydrated (70%→100% ethanol), cleared in xylene, and mounted with DPX medium. For IHC, the tissue specimens derived from the paraffin-wax-embedded block were meticulously sectioned to a thickness of 2 μm, subsequently affixed onto positively charged slides, and then subjected to thermal treatment in an oven maintained at 60 °C for 30 minutes. The tissue sections underwent a process of deparaffinization and hydration through a systematic immersion of the slides in progressively decreasing concentrations of ethanol (100%, 95%, and 70%) for intervals of 3 minutes each, followed by a thorough washing in distilled water for 5 minutes. Tissue samples were processed through sequential treatments: 98–100% formic acid (CHEM KING; 5 min; tap water rinse), 3% H_2O_2 (City Pharma; 30 min; Tris-buffered saline [TBS] wash), equilibration in 1× PK buffer (5 min), and enzymatic digestion with Proteinase K (BIO Basic Canada Inc; 0.1–0.5 mg/mL; 10 min at 38°C). Samples were incubated with primary antibody (N/P Seren Dr Finke N161/5 (RABV) α-rabbit; Friedrich-Loeffler-Institut [FLI], Germany) diluted 1:2000 in 10% goat serum/Tris-buffered saline (TBS) for 2 hours at room temperature (RT), followed by TBS washes. Subsequent steps included: incubation with anti-rabbit IgG secondary antibody (VECTOR Laboratories, USA; 1:200 in 10% goat serum/TBS, 2 hours at RT), application of VECTOR ABC avidin-biotin-peroxidase complex (30 minutes, RT), and development using a VECTOR DAB chromogen kit (10 minutes, RT), with TBS washes between each step. Following distilled water rinsing (5 min), tissue slides were counterstained with Mayer's Hematoxylin (30 sec), dehydrated through a graded ethanol series (70%, 90%, 100%; three sequential 1-min immersions per concentration), cleared in xylene (three 3-min immersions), and mounted with DPX.

RESULTS

Routine H&E staining revealed characteristic histopathological features of rabies encephalitis, including perivascular cuffing by lymphocytes and microgliosis in the cerebellar cortex. Negri bodies, eosinophilic cytoplasmic inclusions pathognomonic for rabies (Figure 1, A–B), were observed in Purkinje neurons. For IHC, tissues were stained with N/P Seren Dr Finke N161/5

(RABV) α-rabbit monoclonal antibody specific for rabies viral. The IHC examination demonstrates a large amount of distinct, granular viral antigen deposits of variable sizes (brown chromogen, DAB) within the cytoplasm of Purkinje cells of the cerebellum, confirming rabies infection (Figure 1, C-E). The negative control shows no immunoreactivity (Figure 1, F).

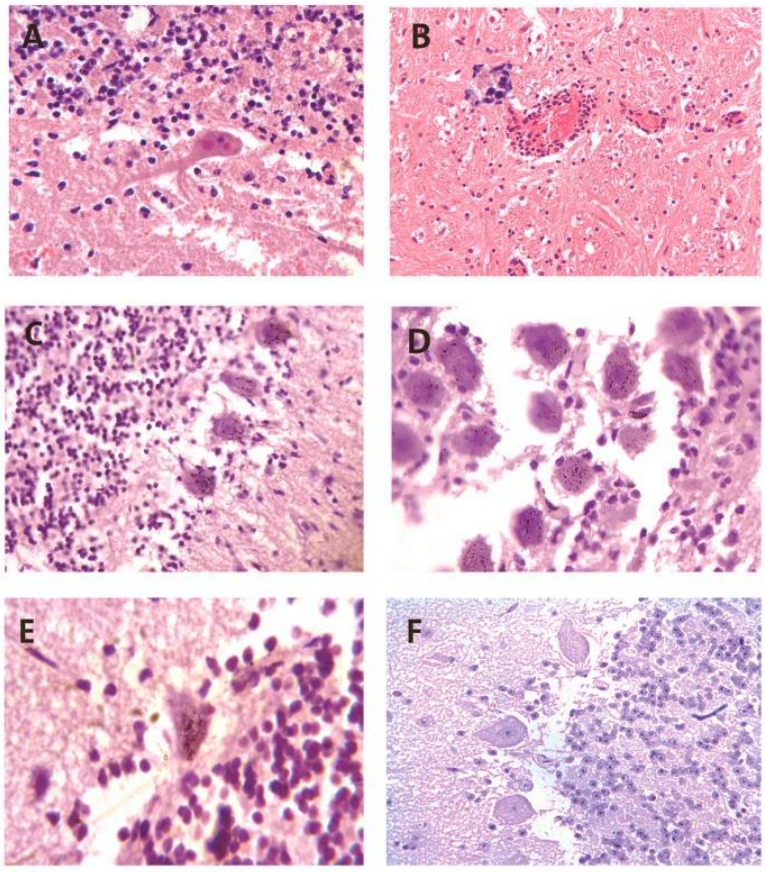

Figure 1. Histopathological and immunohistochemical features of Rabies encephalitis in the cerebellar cortex of a Calf. (A–B): Histopathological features using hematoxylin and eosin (H&E) staining. (A) Intracytoplasmic Negri bodies within Purkinje neurons, displaying eosinophilic staining with basophilic granular cores. (B) Perivascular lymphocytic cuffing and microglial nodules. (C–F): Immunohistochemical (IHC) detection of rabies virus nucleoprotein. (C-E) Viral antigen localization (brown chromogen, DAB) within cytoplasmic Negri bodies in Purkinje neurons, exhibiting granular to globular morphology. (F) The negative control section was processed without primary antibody, showing no immunoreactivity. Counterstained with Mayer's hematoxylin (400x).

DISCUSSION

The present study demonstrates the successful application of immunohistochemistry (IHC) for the detection of rabies virus (RABV) antigens in formalin-fixed, paraffin-embedded (FFPE) brain tissues from a histopathologically confirmed bovine rabies case in Al Jabal Al Akhdar, Libya. This marks the first documented use of IHC in Libya's diagnostic framework for rabies surveillance, offering a critical advancement in a resource-limited setting where fresh tissue samples are often inaccessible. The granular viral antigen deposits observed in Purkinje cells of the cerebellum, coupled with the absence of nonspecific immunoreactivity in controls, validate IHC as a reliable complementary tool for rabies diagnosis. These findings align with global efforts to decentralize diagnostic capabilities while addressing the limitations of conventional histopathology and biosafety concerns associated with handling fresh specimens.

The diagnostic challenges posed by rabies in regions with constrained infrastructure, such as Libya,

are multifaceted. Conventional histopathology, while instrumental in identifying hallmark Negri bodies, fails to detect these pathognomonic inclusions in 20–60% of confirmed cases, as highlighted by the World Health Organization (WHO, 2018). This study corroborates these limitations, as the initial histopathological diagnosis required subsequent IHC confirmation due to equivocal Negri body visibility. By contrast, IHC enabled precise localization of RABV nucleocapsid proteins even in FFPE tissues, overcoming antigenic alterations caused by prolonged formalin fixation. This aligns with prior studies demonstrating IHC's superior sensitivity in detecting RABV antigens in archived samples (Achkar et al., 2019; Jauregui et al., 2019).

Moreover, the biosafety risks inherent to fresh tissue handling—particularly aerosolization of infectious virions during processing for direct fluorescent antibody testing (dFAT)—are mitigated by IHC, which utilizes inactivated FFPE samples. This advantage is critical in settings lacking biosafety level 3 facilities, as emphasized by (Rupprecht et al., 2017). The compatibility of IHC with archival materials also facilitates retrospective studies and outbreak investigations, as evidenced by this case, which revisits Libya's first reported bovine rabies infection (Sharif et al., 2021).

While the direct fluorescent antibody test (dFAT) remains the gold standard for rabies diagnosis, its reliance on fresh or chilled tissues renders it impractical in regions with logistical constraints. IHC, however, requires no specialized equipment beyond standard histopathology laboratories, making it a scalable alternative. The monoclonal antibody (N/P Seren Dr Finke N161/5) employed here exhibited high specificity, with no cross-reactivity observed in controls. This specificity is pivotal for differentiating rabies from other neurotropic pathogens, a common challenge in areas where differential diagnoses such as bovine spongiform encephalopathy or Listeriosis may confound clinical assessments (Fooks & Jackson, 2020). The utility of IHC extends beyond diagnostics; it enhances epidemiological surveillance by enabling antigenic mapping within neural tissues. For instance, the preferential localization of viral antigens in Purkinje cells observed here mirrors patterns reported in equine and canine rabies cases (Achkar et al., 2019; Tasioudi et al., 2015). Such findings underscore the neurotropism of RABV and its conserved pathogenesis across species, reinforcing IHC's role in comparative pathology.

A key limitation of this study is its focus on a single archived case, which precludes broader statistical validation of IHC's sensitivity and specificity in Libyan contexts. Future studies should expand sample sizes to include confirmed and suspected rabies cases, incorporating molecular techniques like RT-PCR to establish concordance rates. Additionally, the availability of monoclonal antibodies in resource-limited regions remains a logistical hurdle. Collaborative initiatives with international reference laboratories like the Friedrich-Loeffler Institute could bolster local capacity through reagent sharing and technical training. Libya's status as a rabies-endemic region necessitates urgent improvements in diagnostic infrastructure. This study demonstrates that IHC can bridge existing gaps, providing a cost-effective, biosafe method for rabies confirmation. By integrating IHC into national surveillance protocols, Libyan authorities could enhance case detection accuracy, streamline outbreak responses, and align with WHO recommendations for decentralize diagnostics (WHO, 2018). Furthermore, retrospective application of IHC to archived samples may uncover undiagnosed cases, refining historical incidence data and informing vaccination strategies.

In conclusion, this work underscores the transformative potential of IHC in rabies-endemic regions. By validating its diagnostic efficacy in Libya's first confirmed bovine case, we advocate for its adoption as a routine complementary tool, particularly where logistical and infrastructural barriers impede conventional methods. Such advancements are pivotal to achieving the global goal of zero human rabies deaths by 2030, as outlined by the WHO.

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Author contributions

Fawzia Mohamed conducted laboratory tests and preparations and wrote the manuscript. Monier Sharif supervised the research, conducted the laboratory examination, and reviewed the final version of the manuscript.

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