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Original article

CLINICOPATHOLOGICAL FEATURES AND PHYLOGENETIC ANALYSIS OF RABIES INFECTION IN LOCAL IRAQI BREED CATTLE

M. H. HUSSAIN[1], KH. A. MANSOUR[1], S. A. A. AL-REDAH[2] & A. J. ABID[1]

[1]Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah; [2]Department of Anatomy and Histology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq

Summary

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Rabies is a lethal viral disease with no specific treatment caused by the *Lyssavirus*, which renders it dangerously hostile towards cattle. This study was conducted to score clinical and pathologic criteria for rabid dogs in the middle Euphrates of Iraq and link them to phylogenetic analysis. Eighty-one cattle were suspected to have rabies after a history of being attacked and having signs of rabies: restlessness, aggression, drooling saliva, hitting head to fences, inappetence, recurrent failed trials of defecation with an opened anus, and fever. Samples of the dead cattle cerebellum were referred for histopathology and molecular detection to confirm the diagnosis. The study showed that the most common clinical signs in the affected animals were behavioural changes (30% of all cattle), followed by a fever (25%), and ataxia (16%). The rest of the clinical signs appeared in varying proportions. With regard to histopathological evaluation, neurological lesions, inflammation, and gliosis scored 3 and were described as severe lesions, while the Negri bodies were moderately scored by 2 as an average number. The phylogenetic analysis revealed variations at a total of 24 places in multiple sequence alignments between two field rabies virus nucleotide sequences from the current investigation (>MW893685 and >MW893684) and 48 GenBank nucleotide sequences, including the reference strain (>NC_001542). Significant differences in relative locations were found between several field isolates and reference strains. Our study showed substitutions of phenylalanine, proline, histidine, lysine, serine and proline, threonine, and asparagine to serine, glutamine, arginine, leucine, serine, threonine, methionine, and valine at different sites. Furthermore, when building the phylogenetic tree, the current study isolates (Iraqi isolates) were mostly similar to Nigerian isolates. In conclusion, rabies in cattle is an endemic, life-threatening, transboundary disease that affects animals and public health in Iraq and needs more studies to explain the cause behind the recorded substitution mutations and the similarity of the Iraqi isolates with Nigerian isolates.

Key words: bovine, clinicopathological scoring, *Lyssavirus*, phylogenetic study, rabies

INTRODUCTION

Cattle, like other mammals, are highly vulnerable to rabies infection. The occurrence of rabies in cattle is variable according to the supervision scheme. The more stray dogs contact each other, the more infections occur. Infection causes hundreds of deaths annually, mainly in Asia and Africa. Worldwide, rabies causes a probable charge of 8.6 billion dollars per year. Forty percent of people attacked by suspected rabid dogs are children under 15 years of age. Dogs are the most significant reservoir for *Lyssa* viruses (Horton *et al.*, 2015) and the main cause of human rabies deaths; up to 99% of all rabies spreads to humans, due to the close relationship between them. Community education, early warning systems, and immunisation are all important components of a comprehensive health collaboration network. Together, "United Against Rabies" and the World Health Organization (WHO) are working to achieve "zero human deaths from dog-mediated rabies in 2030 (Bengoumi *et al.*, 2018).

An estimated 59,000 annual rabies-related deaths occur worldwide in over 150 countries, with Africa and Asia accounting for 95% of these fatalities (Mubashar *et al.*, 2023). The World Health Organization (WHO) reports 31,000 deaths in Asia, notably around 20,000 of these occurring in India alone (Kumar & Bakhru, 2022). Rabies has been documented in more than 150 countries across every continent except Antarctica (Grieve, 2022). Over three billion people reside in areas where rabies is a potential threat (Müller *et al.*, 2022). Some regions, including Australia, Nepal, Japan, and much of Western Europe, are fortunate to be free from canine rabies (Pantha *et al.*, 2020). Furthermore, rabies is classified as a neglected tropical disease (Guzman *et al.*, 2022), as it has essentially been eliminated on several islands. In the United States, animal rabies cases are primarily found in wildlife, accounting for over 90% of incidents, while domestic animals like dogs and cats make up just around 10% of the total cases. Among wildlife species, bats are responsible for approximately 70% of these cases (Ma *et al.*, 2022).

According to the World Health Organization (WHO), Iraq is considered a high-risk country for rabies transmission, with dogs being the primary source of infection. Prior investigations have yielded valuable insights into the molecular epidemiology of canine rabies within the Middle Eastern region. Notably, until this day, there has been a lack of available rabies isolates from Iraq for scientific scrutiny (David *et al.*, 2000; 2007). In an effort to enhance collaboration in rabies control, the Middle East and Eastern Europe Rabies Expert Bureau (MEEREB) network was established in 2010, substantially facilitating the exchange of information (Aylan *et al.*, 2011).

It is noteworthy that Iraq is currently not a participant in this network. Nevertheless, it is imperative to recognise that cases of rabies have been documented in neighbouring countries like Turkey and Iran, where dogs serve as the primary carriers of this disease, posing a threat to human populations (David *et al.*, 2007). In Iraq, there is an estimated population of approximately 800,000 dogs, a significant portion of which roam as stray animals or remain unvaccinated against rabies. Furthermore, wild animals such as foxes and jackals can also harbour the virus. The inaugural study on rabies in Iraq was conducted in 2013 (Horton *et al.*, 2013), in-

volving the collection of brain samples from domestic animals in the Baghdad province, which were subsequently verified for rabies. Employing state-of-the-art techniques, three out of forty samples tested positive through fluorescent antibody checks and hemi-nested RT-PCR, confirming the presence of *Lyssavirus* in the brain (Bengoumi *et al.*, 2018). The cattle frequently become infected with rabies because they come into close contact with the disease, potentially due to their interactions with infected wildlife or other contributing factors. Furthermore, Ismail and his team successfully identified the *Lyssavirus* strain in thirteen cases, which included eight cattle and five dogs, out of a total of fifty-three cases documented in Iraq (Ismail *et al.*, 2020).

Preventing rabies in Iraq involves a multifaceted approach, including pet vaccination, public education on disease risks, and prompt treatment for animal bite victims (Ismail *et al.*, 2020). Limited access to rabies vaccines and treatments in certain Iraqi regions hampers disease control efforts (Lankau *et al.*, 2014; Hashim *et al.*, 2021). The World Health Organization (WHO) collaborates with Iraqi health authorities to enhance prevention and control by expanding vaccine access and implementing awareness campaigns (Horton *et al.*, 2013; Hashim *et al.*, 2021).

Lyssavirus primarily spreads through dog bites, responsible for over 90% of rabies cases (Tekki *et al.*, 2014). Cattle are affected, especially in regions where vampire bats are present (ACHA, 1981). Dogs serve as significant carriers of Lyssavirus to humans and other farm animals in Africa (Moges, 2015). Additionally, jackals are reservoirs in Namibia, Botswana, and Zimbabwe, while bat-eared foxes play a role in southern and northern Africa (Warrell, 2010).

More recently, molecular diagnosis has shifted to real-time PCR detection, which asphyxiates the problems of conventional PCR and can also sense very small amounts of viral RNA. It has been revealed that real-time PCR may be up to 1,000 times more sensitive than nested PCR for the detection of RABV isolates (Warrell, 2010). The first real-time PCR assay developed for the detection and discernment of six *Lyssavirus* species required a separate reverse transcription step and a cocktail of seven primers to produce an amplicon of 500 bp (Black *et al.*, 2002). Brain tissue is the sample of choice for post-mortem diagnosis (Cohen *et al.*, 2007). Historically, the existence of Negri bodies (intracellular accumulations of RABV particles), first described in 1903, was considered indicative of rabies (Fooks *et al.*, 2009).

This investigation was designed to address the situation that arose in December 2018 when pet owners grew increasingly concerned about the possibility of rabies infecting their animals. This concern subsequently alerted farmers who practiced unrestricted livestock grazing, thereby elevating the risk of their animals falling victim to rabid dog attacks. As a result, there was a notable surge in infections among livestock. Hence, the primary objective of this study was to explore the clinicopathological characteristics and conduct a phylogenetic analysis of rabies infection in indigenous Iraqi cattle breeds.

MATERIALS AND METHODS

Ethical approval

The protocol of the study was accepted by the animal ethics committee of the Animal Science Study Programme, College of Veterinary Medicine, Al-Qadisiyah University, Iraq (Ref. No. 833).

Location and period of the study

This research was carried out in Al-Daghara, a suburban area within the Al-Diwaniyah Governorate, situated approximately 180 km to the south of Baghdad, Iraq, spanning from December 5, 2018 to December 5, 2019.

Animals

Out of a total of 2,000 cattle, 81 were suspected to have *Lyssavirus* infection following an attack by aggressive dogs. The included cattle were of different age, breed, and gender.

Clinical assessment and sample collection

The clinical assessment was multi-faceted, encompassing the following key stages: initial case reports submitted by farmers and animal owners; thorough documentation of case histories; hands-on clinical examinations conducted by the authors, which entailed observing nervous symptoms, aggressive behaviour, and inadequate nutrition in the affected cattle; detailed recording of clinical manifestations observed in the affected animals; and collection of laboratory samples, including cerebellum samples for histopathological analysis. Cerebellum samples were collected from dead cattle. Some samples were preserved in 10% formalin for histopathological examination, whereas others were transported promptly in cool-box containers for molecular analysis.

Histopathology

The formalin-fixed cerebellum tissue samples were transported to the Veterinary Pathology Laboratory. The cerebellar tissues were routinely cut and processed on a rotary microtome. Tissues were routinely fixed in paraffin wax, and 4–5 μm tissue slices were prepared and stained for histological examination using the standard haematoxylin and eosin stain (Bancroft & Stevens, 1982).

To evaluate the severity of the disease and track its development in cattle, a scoring system was established (Table 1). The extent to which the rabies virus has damaged the animal's nervous system and other organs can be estimated with the help of this grading system. The scoring system typically involves evaluating various histopathological and clinical features associated with rabies infection. Some of the key aspects considered in the scoring system included:

- Clinical signs: The severity of clinical signs in cattle suffering from rabies can be scored based on the presence and intensity of symptoms such as fever, behavioural changes, excessive salivation, difficulty swallowing, paralysis, and ataxia (loss of coordination).
- Histopathological changes: Histopathological examination of brain tissue samples from affected cattle can reveal characteristic lesions associated with rabies infection: Negri bodies (to assess the severity of the infection);

Table 1. Scores of pathological alterations observed in cattle affected by rabies.

Parameter	Score 0	Score 1	Score 2	Score 3
Clinical signs	–	–	–	Severe
Negri bodies	–	–	Moderate	–
Neuronal degeneration and necrosis	–	–	–	Severe
Inflammation	–	–	–	Severe
Gliosis	–	–	–	Severe

Table 2. PCR test conditions (steps/temperature, time, and cycles).

Step	Temperature (ºC)	Time	Cycle
Denaturation (initial)	94 ºC	120 s	1
Denaturation	94 ºC	30 s	
Annealing	58 ºC	45 s	35
Extension	72 ºC	60 s	
Final extension	72 ºC	10 min	1

neuronal degeneration and necrosis (based on the number of affected neurons and the severity of the damage); inflammation (the degree of inflammation in the brain tissue, characterised by the infiltration of inflammatory cells such as lymphocytes, plasma cells and macrophages, to evaluate the host immune response to the infection); gliosis (the proliferation of astrocytes and microglia in response to neuronal damage to assess the severity of the infection and the host response to the disease).

Each histopathological parameter recorded was scored on a scale of 0 to 3, with 0 indicating the absence of the pathological change and 3 representing the most severe manifestation of the change. The existence of Negri bodies, neuronal degeneration and necrosis, and gliosis were assigned a score of 3, while inflammation was gaven a score of 2.

Molecular diagnosis

Lyssavirus RNA was extracted using TRIzolTM1 in 10% of the cleared supernatant, as per the manufacturer's instructions. The reverse transcriptase-PCR test was performed with minor modifications to the protocol described by Heaton *et al.* (1997). Shortly after being extracted, 10 µL of the RNA was denatured for approximately 10 min at 75 ^{o}C, cooled, and reverse transcribed in 40 µL of the RT mixture that consisted of 2 µL of 10 µM primer: F(5' TGCATCCT TAGTCGGTC TGC3'), R(5' GAGGAG CACATGCAG CAATG 3'), 200 µL of superscript II Reverse Transcriptase, 1 µL of each dATP, dCTP, dTTP, dGTP, 10 U of RNAsin, 5 µL of 5× PCR buffer, 1 µL dithiothreitol and 1.5 µL of 50 mM magnesium chloride.

The RT mix has been incubated for sixty minutes in warm water at 42 ºC, boiled for five minutes, and then cooled on ice. Fifty µL of the amplified mix (PCR) comprising 1 µL of each dATP, dCTP, dTTP, dGTP, 2.5 U of Taq-Platinum polymerase, 5 µL of 10× PCR buffer, 1.5 µL of 50 mM magnesium chloride, and 4 µL of a mixture of 10 µM of the primers. After denaturation at 94 ºC for 120 sec, the PCR assay was finished according to the thermocycler programme (Table 2). Additionally, a 1.5% agarose gel was used to see and record the PCR amplicon using a Polaroid scheme gel Documentary 10002. The gel was stained with ethidium bromide (0.5 mg/mL). After electrophoresis, all samples yielded a positive result due to the existence of a distinct 596 bp band (Kwok & Higuchi, 1989; Beltrán *et al.*, 2014).

DNA sequencing and phylogenetic analysis

The sequence of the purified positive PCR products of a specific 596-bp segment gene made with the AB DNA sequencing instrument (Bioneer Company, Korea) was used to build the phylogenetic inves-

Table 3. Accession codes employed in this study to conduct the phylogenetic analysis

No.	Accession No	Source	No.	Accession No	Source
1	MW893685	Current study	26	EU853652	GenBank
2	MW893684	Current study	27	U22634	GenBank
3	EU038089	GenBank	28	MF537578	GenBank
4	EU718767	GenBank	29	MF537576	GenBank
5	EU0717867	GenBank	30	EU718767	GenBank
6	U22634	GenBank	31	EU718763	GenBank
7	EU038099	GenBank	32	EU718759	GenBank
8	EU038092	GenBank	33	EU718742	GenBank
9	MF537579	GenBank	34	EU718740	GenBank
10	MF537577	GenBank	35	EU718737	GenBank
11	MF537578	GenBank	36	EU718758	GenBank
12	MF537576	GenBank	37	EU718766	GenBank
13	KF022184	GenBank	38	EU718762	GenBank
14	KF022179	GenBank	39	EU038106	GenBank
15	EU038085	GenBank	40	MF537580	GenBank
16	EU718771	GenBank	41	EU478494	GenBank
17	KF022181	GenBank	42	E22636	GenBank
18	MF537579	GenBank	43	EU827272	GenBank
19	MF537577	GenBank	44	EU827270	GenBank
20	EU853652	GenBank	45	EU478520	GenBank
21	U22634	GenBank	46	EU478516	GenBank
22	MF537578	GenBank	47	EU478512	GenBank
23	EU853652	GenBank	48	EU478511	GenBank
24	MF537579	GenBank	49	EU478505	GenBank
25	MF537577	GenBank	50	NC_001542	Reference Clone-GenBank

tigation. The tree was constructed using the neighbour-joining method (MEGA v6) and the NCBI-Blast alignment methods (Table 3).

RESULTS

An outbreak was documented, involving 81 cattle displaying symptoms consistent with rabies, which accounted for 4.0% of a total of 2,000 cattle. The observed signs included restlessness, aggression, excessive salivation, head-banging against fences, and repeated unsuccessful attempts at defecation with an exposed anus. As the signs became more pronounced, the cattle exhibited aimless wandering and hoarse vocalisation. The suffering endured until their eventual demise, characterised by respiratory failure, marked emaciation, severe dehydration, and sunken eyes. It appears that all affected cattle exhibited the neurological form of infection (Table 4).

The onset of the disease depended on the location of the dog bites on the cattle; clinical signs manifested more rapidly when the bites were closer to the brain. If bitten on the head, signs appeared within 7–10 days, while bites on the legs resulted in signs emerging within 22–30 days. Age and gender did not appear to have any significant influence, as rabid dogs did not distinguish among their victims. Samples of the cerebellum from deceased cattle

were subjected to histopathological and molecular analysis to confirm the diagnosis.

Histologic changes in the cerebellum of rabid cattle revealed eosinophilic cytoplasmic inclusion bodies (Negri bodies) in Purkinje cells as characteristic features. Changes in Purkinje cells ranged widely from dark cytoplasm and nucleus, suggesting a start to degenerative changes, to others with chromatin condensation and shrinking with nucleus deformation (Fig. 1). Halo-like amorphous materials were present around degenerated cells. Gliosis also was shown. Other histological changes in the cerebellum also revealed oedema, gliosis, haemorrhage, perivascular cuffing, congestion, and neurosis, as shown on Fig. 2.

In conventional reverse transcription (RT-PCR), the examined samples, which had a band of approximately 563 bp, were considered positive. Using a primer set that amplified the N gene of *Lyssavirus*, all 81 brain tissue samples tested positive. The amplified gene's band intensity differed between samples (Fig. 3). RT-PCR, on the other hand, was able to detect the N

Table 4. Count (percentage) of 81 cattle with rabies, categorised according to clinical observations.

Clinical findings	Number of infected cattle	% of infected cattle
Fever	18/81	22.22%
Behavioural changes	25/81	30.86%
Excessive salivation	10/81	12.34%
Difficulty swallowing	8/81	9.88%
Paralysis	7/81	8.65%
Ataxia (loss of coordination)	13/81 undefined	16.05% undefined

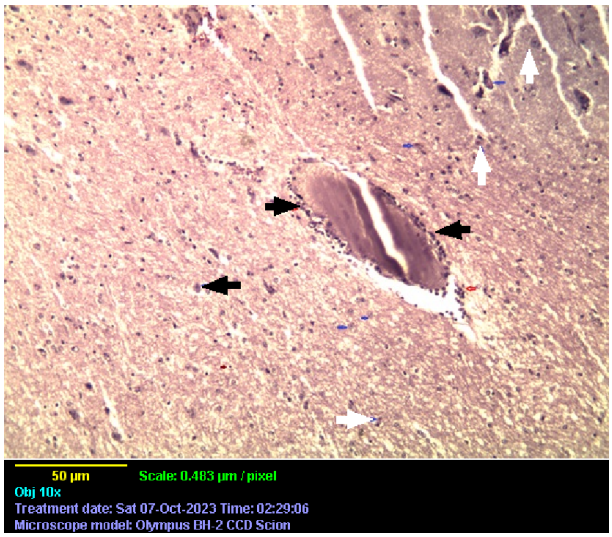

Fig. 1. Mild perivascular cuffing (black arrows) and slight mononuclear infiltration surrounding vascular thrombosis, eosinophilic intracytoplasmic inclusions bodies (Negri bodies) (white arrows). H&E staining, 100×.

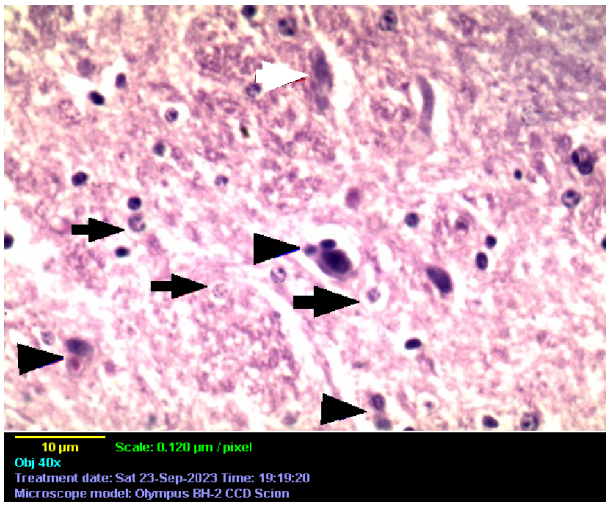

Fig. 2. Examination of the cerebellum in rabid cattle showing the presence of oedema, gliosis, and congestion. Additionally, Negri bodies were observed within Purkinje cells (black arrowheads). Surrounding the degenerated cells, there were halo-like amorphous materials (black arrows). Purkinje cells exhibited a ghost-like appearance, with bundles of lines around them, and the outline of the nucleus was indistinct (white arrow). H&E, 400×.

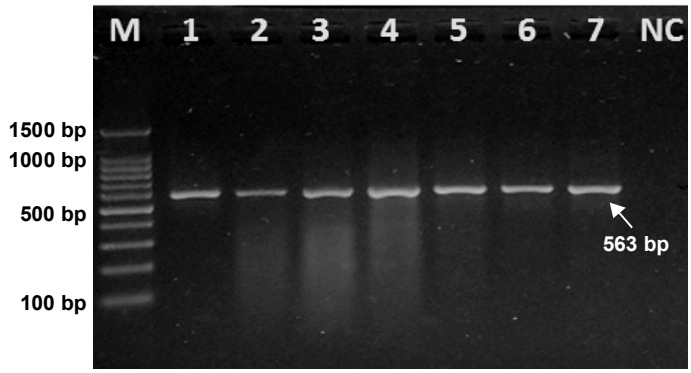

Fig. 3. Agarose gel electrophoresis showing RT-PCR detection of nucleoprotein gene in Lyssa virus. Lane M: ladder; Lanes 1 to 7: positive samples (563 bp); NC: negative control.

gene in 81 samples without any non-specific reactions at a specified melting temperature. The sequence has been submitted to the National Centre for Biotechnology Information (NCBI) under the accession numbers MW893685 and MW893684.

Variations at a total of 24 places were found in multiple sequence alignments between two field rabies virus nucleotide sequences from the current investigation (>MW893685 and >MW893684) and 48 GenBank nucleotide sequences, including the reference strain (>NC_001542). Significant differences in relative locations were found between several field isolates and reference strains. Our research strains displayed nucleotides (TCT) (serine) at

these codons, but other GenBank strains had nucleotides (TTT) (phenylalanine) at these positions. Our strains had the nucleotides (CAG, CGA, and CGT) for two amino acids (glutamine and arginine) replaced with the corresponding nucleotides (CCA, CAA, and CAT) for proline and histidine at codons (20, 67, and 168). Glutamine (CAA) was changed to proline (CCA), arginine (CGA), and lysine (AAA) at codons 21, 390, and 498, respectively. The leucine at positions 28 and 183 was changed to serine and proline, respectively. Serine and threonine replaced the asparagine at codons 48 and 55. The codons 50 and 62 positions of isoleucine were switched to threonine, while codons 65 and 76 of threonine were switched to serine and 76 to methionine, respectively. Asparagine replaced glycine at position 112 and valine at position 114. The alanine at codon 493 was changed to a valine (Tables 5 and 6).

The phylogenetic tree of the present study revealed that our two field rabies virus nucleotide sequences (>MW893685 and >MW893684) were most related to Nigerian isolates that were deposited at GenBank with the following accession numbers: >EU038087, >EU038106, >EU038084, >U22634, >MF537577, >MF537578, >MF537576, >EU853652, >EU853669, >EU853642, >EU853640, >EU853637, and >EU718766 (Fig. 4).

DISCUSSION

Two classes of rabies are clinically recognised; furious (also called encephalitic) and paralytic. The factors that determine the development of either form remain ill-depended (Laothamatas *et al.*, 2008). In this study, only the furious form has been recorded. Clinical signs in animals appeared after being attacked by dogs about thirteen to thirty six days later; the variation may depend on the site of the bite from which virus moves on to the nearest nerve to multiply and makes its pathogenicity. Besides, the general attitude of the attacked cattle may play a role as well as the age and breed, as reported in previous studies (Hemachudha *et al.*, 2013). Hydrophobia, opened anus and inappetence were the most common clinical signs beside the poor response to drugs.

In the winter of 2020, there were 81 presumed cases of rabies among a total of 2,000 cattle. During the survey, a spatial analysis of dog bite rates revealed that governorates in the middle and south of Iraq had the highest dog bite rates, with no evidence of a significant geographic distribution. The bite rates in the remaining governorates were consistently lower. In contrast, several governorates (Al-Muthanna, Maysan, and Saladin) have a high number of human cases and fewer cattle bites being reported (Ismail *et al.*, 2020).

The RT-PCR assay can be done in the laboratory the same day and is available for *Lyssavirus* from brain tissues in different species of animals. The attained outcomes have validated the RT-PCR test capacity as a diagnostic tool for identifying the *Lyssavirus*. Like other studies; the use of definite primers for the nucleoprotein site of *Lyssavirus* RNA increases the opportunity of success in identifying rabies infection (Heaton *et al.*, 1997).

Controlling rabies in dogs is crucial, aiming for the eventual elimination of dog-related cattle infections. Studies are needed to evaluate the effectiveness of these efforts, map disease prevalence to allocate resources, and set future goals. As part of a nationwide anti-rabies campaign,

Table 5. A total of 24 places variations found in multiple sequence alignment between two field rabies virus nucleotide sequences from the current investigation (>MW893685 and > MW893684) and 48 GenBank nucleotide sequences

Sequence	30	60	63	84	144	150	165	183	186	195	198	201	213	225	228	336	342	366	390	420	423	465	493	498	504	519
Codon	10	20	21	28	48	50	55	61	62	65	66	67	71	75	76	112	114	122	130	140	141	155	165	166	168	173
	TCT	CAG	CAA	TTG	AAC	ATA	AAC	CTA	ATT	ACC	ATA	CGA	TCT	ACA	ACG	GGC	TGT	AGC	CAA	TTC	AAG	ACT	GCG	GAA	CGT	CAG
EU038087	TTT	…	…	…	…	…	…	…	ACT	…	…	…	…		…	…	…	CGA	CGA	…	…	…	…	…	…	…	…
EU038106	TTT	…	…	...	…	…	…	…	…	AGT	ACA	CAA	…	ACC	…	…	…	…	CGA	…	…	…	…	…	…	…	…
EU038084	…	….	CCA	…	…	…	ACC	CCA	ACT	…	…	…	…	…	…	…	…	CGA	CGA	…	…	…	…	…	…	…	…
U22634	…	CCA	…	…	…	…	…	…	…	…	…	…	…	…	…	…	TAT	CGA	…	…	…	…	GTG	AAA		…	…
MF537577	…	…	…	TCG	…	…	…	…	…	…	…	…	TTT	…	…	…	…	CGA	…	…	…	…	…	…	CAT	…	…
MF537578	…	…	…	TCG	…	…	…	…	…	…	…	…	TTT	…	…	…	…	CGA	…	TTT	…	…	…	…	CAT	…	…
MF537576	…	…	…	TCG	…	…	…	…	…	…	…	…	TTT	…	…	…	…	CGA	CGA	TTT	…	ATT	…	…	CAT	…	…
EU853652	…	…	…	…	AGC	ACA	…	…	…	…	…	…	…	…	…	…	…	CGA	CGA	TTT	AGG	…	…	…	…	CCG	…
EU718767	…	…	…	…	AGC	ACA	…	…	…	…	…	…	…	…	…	GAC	…	CGA	CGA	…	AGG	…	…	…	…	CCG	…
EU718763	…	…	…	…	AGC	ACA	…	…	…	…	…	…	…	…	…	GAC	…	CGA	CGA	…	AGG	…	…	…	…	CCG	…
EU718759	…	…	…	…	AGC	ACA	…	…	…	…	…	…	…	…	…	GAC	…	CGA	CGA	…	AGG	…	…	…	…	CCG	…
EU718742	…	…	…	…	AGC	ACA	…	…	…	…	…	…	…	…	…	GAC	…	CGA	CGA	…	AGG	…	…	…	…	CCG	…
EU718737	…	…	…	…	AGC	ACA	…	…	…	…	…	…	…	…	…	GAC	…	CGA	CGA	…	AGG	…	…	…	…	CCG	…
EU718766	…	…	…	…	AGC	ACA	…	…	…	…	…	…	…	…	ATG	GAC	…	CGA	CGA	…	AGG	…	…	…	…	CCG	…

Table 6. Amino acids substitution mutations recorded in the study isolates [>MW893685 and > MW893684] compared to the GenBank Lyssa virus isolates

Sequence	30	60	63	84	144	150	165	183	186	195	198	201	213	228	336	342	366	390	423	465	493	498	504	519
Codon	10	20	21	28	48	50	55	61	62	65	66	67	71	76	112	114	122	130	141	155	165	166	168	173
	TCT	CAG	CAA	TTG	AAC	ATA	AAC	CTA	ATT	ACC	ATA	CGA	TCT	ACG	GGC	TGT	AGC	CAA	AAG	ACT	GCG	GAA	CGT	CAG
	Ser	Arg	Gln	Leu	Asn	Ile	Asn	Leu	Ile	Thr	Ser	Arg	Ser	Thr	Gly	Val	Ser	Gln	Lys	The	Ala	Gln	Arg	Gln
EU038087	Phe	…	…	…	…	…	…	…	Thr	…	…	…	…	…	…	…	Arg	Arg	…	…	…	…	…	…
EU038106	Phe	…	…	...	…	…	…	…	…	Ser	Thr	Gln	…	…	…	…	…	Arg	…	…	…	…	…	…
EU038084	…	….	Pro	…	…	…	The	Pro	The	…	…	…	…	…	…	…	Arg	Arg	…	…	…	…	…	…
U22634	…	Pro	…	…	…	…	…	…	…	…	…	…	…	…	…	Asp	Arg	…	…	…	Val	Lyc		…
MF537577	…	…	…	Ser	…	…	…	…	…	…	…	…	Phe	…	…	…	Arg	…	…	…	…	…	His	…
MF537578	…	…	…	Ser	…	…	…	…	…	…	…	…	Phe	…	…	…	Arg	…	…	…	…	…	His	…
MF537576	…	…	…	Ser	…	…	…	…	…	…	…	…	Phe	…	…	…	Arg	Arg	…	Ile	…	…	His	…
EU853652	…	…	…	…	Ser	Thr	…	…	…	…	…	…	…	…	…	…	Arg	Arg	Arg	…	…	…	…	Pro
EU718767	…	…	…	…	Ser	Thr	…	…	…	…	…	…	…	…	Asp	…	Arg	Arg	Arg	…	…	…	…	Pro
EU718763	…	…	…	…	Ser	Thr	…	…	…	…	…	…	…	…	Asp	…	Arg	Arg	Arg	…	…	…	…	Pro
EU718759	…	…	…	…	Ser	Thr	…	…	…	…	…	…	…	…	Asp	…	Arg	Arg	Arg	…	…	…	…	Pro
EU718742	…	…	…	…	Ser	Thr	…	…	…	…	…	…	…	…	Asp	…	Arg	Arg	Arg	…	…	…	…	Pro
EU718737	…	…	…	…	Ser	Thr	…	…	…	…	…	…	…	…	Asp	…	Arg	Arg	Arg	…	…	…	…	Pro
EU718766	…	…	…	…	Ser	Thr	…	…	…	…	…	…	…	Met	Asp	…	Arg	Arg	Arg	…	…	…	…	Pro

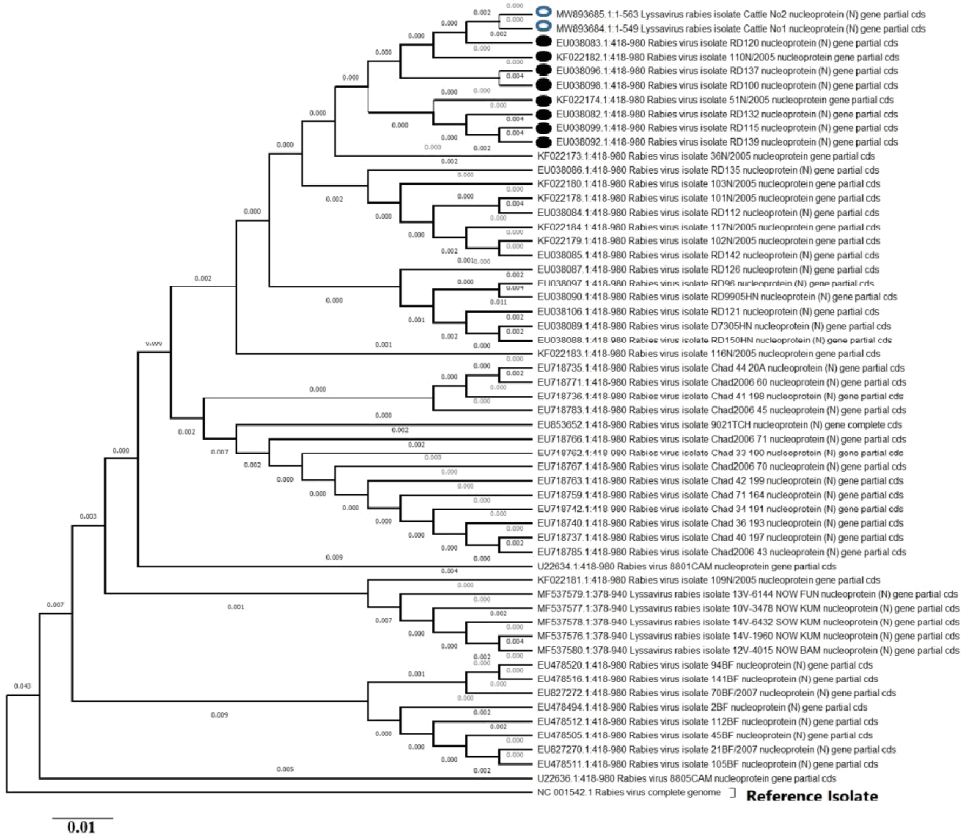

Fig. 4. Phylogenetic tree of *Lyssa* virus based on N protein nucleotide sequences from 48 GenBank and two Iraqi isolates using the Neighbor-joining (N-J) method and 1000 bootstrap replicates. The Iraqi isolates of the current study are marked with white dots and for the Genbank clones – with black dots.

researchers analysed dog tissue samples, finding a 9.5% rabies positivity rate out of 200 tested (Wallace *et al.,* 2015). Most reported cases were cattle (71%) (Horton *et al.,* 2013) which are considered a dead-end host, not contributing to onward transmission. Phylogenetic analysis revealed that *Lyssavirus* sequences from infected cattle (MW893685.1) were closely related to those in Nigerian dogs in 2013 (KC196743) (Zhou *et al.,* 2013), supporting previous findings in Iraq suggesting dogs as the primary source of cattle infection (Horton *et al.,* 2013).

Despite persistent efforts in Iraq since 2003, rabies control has not yielded desired results. Attempting to eliminate strays, regional police forces initiated operations necessitating collaboration among human, environmental, and veterinary health authorities (Thabit, 2012; Horton *et al.,* 2013). However, mass culling is not a sustainable solution due to potential disease transmission from carcasses (Lembo & Prevention, 2012; Othman, 2022). To address this zoonotic disease effectively, it is advisable to establish centralised laboratories for animal and human rabies

diagnosis in Iraq. These facilities should be linked to mandatory infection notifications, educational campaigns on responsible pet ownership, and widespread rabies vaccination initiatives.

This research, in agreement with previous ones (Ismail *et al.*, 2020), displayed significant influence of molecular approaches for *Lyssa*virus diagnosis and encourages the employment of the RT-PCR as a perfect policy in the detection of *Lyssavirus*. It is settled that the policy employed in this research should be successfully used in the routine identification because of its quick, precise, and highly sensitive results, paying positivity for the laboratory diagnosis of *Lyssa*virus in hot countries like Iraq (Dantas Jr *et al.*, 2004).

The recent phylogenetic investigation has identified substitutions in amino acids – specifically phenylalanine, proline, histidine, lysine, serine, threonine, and asparagine; for serine, glutamine, arginine, leucine, serine, threonine, methionine, and valine: at various locations (Betts *et al.*, 2003). These alterations suggest a potential impact on the severity of clinical signs, the behaviour of the virus, and its pathogenicity. For instance, the transformation of serine to arginine may modify the virus' engagement with host elements, influencing both virulence and the spread within the nervous system. Additionally, the substitution of phenylalanine with leucine could exchange hydrophobic amino acids, potentially affecting the structure of proteins and interactions with host cells. Moreover, the replacement of histidine with glutamine might influence receptor binding and replication within host cells, while a shift from lysine to arginine could impact protein interactions and immune evasion. The role of methionine in protein synthesis indicates that a threonine-to-methionine substitution may affect the efficiency of virus replication (Ünal *et al*., 2014). Understanding the functional implications of these amino acid changes is pivotal for unraveling the molecular mechanisms that underlie the pathogenicity and behaviour of the rabies virus. This suggests a sophisticated interplay between viral proteins and host factors, wherein even subtle alterations in amino acid composition can have profound consequences for the virus' ability to replicate, spread, and interact with the host immune system.

The latest analysis of the genetic relationships among rabies clades in Iraq demonstrated their origin from South Africa, distinguishing them from the clades observed in neighbouring countries such as Iran, where the origins are traced back to the Middle East and North Africa (Hosseini Heydarabadi *et al.*, 2020). Horton's extensive sampling efforts from 20 Middle Eastern countries between 1972 and 2014 documented the transboundary migrations of the rabies virus, establishing the existence of four genetically distinct clades among Middle Eastern nations (Horton *et al.*, 2013; Horton *et al.*, 2015). Previous research suggested the introduction of rabies from central and eastern Asia into the Middle East (David *et al.*, 2007). Thus, our study, along with Horton's earlier work (Horton *et al.*, 2013), represents one of the pioneering investigations into characterising rabies strains in Iraq. The authors argued that the three unique sequences in the studied region, forming the new Iraqi clade, have diverse origins, including Turkey, Iran, Syria, and Georgia, situated in northeastern and western Iraq. Notably, Turkey and Iran host rabies strains from various parts of the phylogenetic tree, coexisting with strains associated with Iraq. Our study diverges from Horton's research regarding

the strain origin; while the previous study linked it to the Middle East, possibly from eastern or western Iraq, our current findings suggest a closer resemblance to the African strain, specifically originating from Nigeria.

CONCLUSIONS

The current study showed that behavioural alterations were the most commonly seen clinical symptom in the affected animals, appearing in 30% of cases, followed by fever in 25% of cases, and ataxia in 16% of cases. The histopathological findings concluded that severe nerve lesions, inflammation, or gliosis were classified as score 3, while the presence of moderate Negri bodies was classified as score 2. Several field isolates were placed in significantly different positions relative to the reference strains, as determined by the phylogenetic analysis. Our research uncovered numerous amino acid swaps, including phenylalanine to serine, glutamine to arginine, leucine to serine, threonine to methionine, and asparagine to valine. In addition, when constructing the phylogenetic tree, the isolates from the current study (Iraqi isolates) showed the highest similarity to isolates from Nigeria. This study's findings suggest that the developed scoring system can be used to accurately categorise rabies-related lesions. It also aids in comprehending infected animal behaviour, allowing designing a programme to control the virus and easier dealing with affected animals.

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Correspondence:

Muthanna Hadi Hussain,
Department of Internal and Preventive Medicine, College of Veterinary Medicine,
University of Al-Qadisiyah,
Al-Diwaniyah, 58001, Iraq;
e-mail: Muthanna.Hussain@qu.edu.iq;
ORCID: 0000-0003-1605-6116