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Sequencing Analysis of the *N* gene of Canine Distemper Virus from Infected Dogs in Baghdad City

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A B S T R A C T

The primary aim of this study was to analyze the nucleocapsid (N) gene of the Canine Distemper Virus (CDV) in infected canines within Baghdad, Iraq. A total of 50 samples, encompassing nasal swabs and fecal specimens, were obtained from canines of various ages and breeds. Utilizing a Rapid Test, nine of 50 samples tested positive for CDV. Subsequently, the RNA from the positively tested samples was extracted and subjected to Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for cDNA synthesis. The synthesized cDNA was further amplified through conventional PCR targeting the N gene. Six amplified products were submitted to Macrogen, South Korea, for sequencing. Sequence analysis revealed multiple silent and missense point mutations within the N gene. Phylogenetic analysis indicated a 99% similarity of the N gene of the Iraqi isolates with a known strain from the USA and India strains. Overall, this study identifies point mutations in the N gene of CDV in infected canines in Iraq and establishes that the circulating strain exhibits a high genetic resemblance to the USA and India strains. Therefore, it is imperative to formulate and implement high-efficacy vaccines and comprehensive control strategies. This represents the first study in Iraq to focus on the N gene analysis of CDV in infected dogs.

Keywords: canine distemper disease, conventional RT-PCR, *N* gene, sequencing, Iraq

INTRODUCTION

There are several viruses called Morbilliviruses that started acting as causative agents as well. Canines are susceptible to the fatal canine distemper disease, which is extremely dangerous and contagious worldwide (1). The disease is brought on by a single-stranded, encapsulated, negative-sense RNA virus known as canine distemper virus (CDV) belongs to genus morbilliviruses, which are members of the Paramyxoviridae family. The nucleocapsid (N), phospho (P), large (L), matrix (M), hemagglutinin (H), and fusion (F) proteins are the six structural proteins of the canine distemper virus. Two additional non-structural proteins (C and V), which were found as extra transcriptional units within the P gene, are also present. It shares this characteristic with other paramyxoviruses (2). The lympho-, neuro-, and epitheliotropism of CDV usually results in a systemic infection of almost all organ systems, including the respiratory, digestive, urinary, lymphatic, endocrine, cutaneous, skeletal, and central nervous system (CNS) (3). It has been established that the nucleoprotein (N), the most common structural viral protein, regulates transcription, replication, and the encapsidation of the RNA genome into an RNase-resistant nucleocapsid (4). It is also the structural viral protein that is most prevalent. There is some variation in the N gene among CDV isolates even though this protein makes up the conserved portion of the CDV genome (5). Genetic sequencing and subsequent phylogenetic analysis are procedures that assist in comprehending the antigenic variations between diverse biological samples, in addition to revealing the evolutionary

relationship between samples taken from various geographic regions (6). Nucleic acid sequencing, which reveals the nucleotide sequence contained in the gene under study, can be used to anticipate the structure and function of the protein that the gene codes for (7).

Clinical signs are used by veterinarians to determine the diagnosis of canine distemper. Except in severe situations, the disease's symptoms might not be readily apparent. The virus can be diagnosed by either isolation using tissue culture, blood testing, enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and RT-PCR tests (8–15). Many studies revealed the viral RNA was found in urine, feces, cerebrospinal fluid, and respiratory secretions (16). In Iraq, CDV can result in a serious infection that kills infected dogs. Many studies about the virus were performed in Iraq (17-19). The study of Saaed and Alsarhan (17) used a microscopic examination of a blood smear, a rapid test, and an ELISA test to detect the virus in Mosul. While the study of Mansour and Hasso (18) who diagnosed the virus in Baghdad used RT-PCR to validate it in domestic dogs. Additionally, the study of Mohammed et. al. (19) used molecular and serological approaches to confirm the CDV in stray dogs. But the amplification of nucleocapsid (*N*) gene sequencing had not been studied before at least in Iraq; therefore, this study aimed to investigate the N gene of CDV by sequencing as this gene may be responsible for the virulence of this virus detected in Baghdad, Iraq.

MATERIALS AND METHODS

Ethical Approval

All procedures in this study were reviewed and approved by the local Ethics Committee at the College of Veterinary Medicine, University of Baghdad (P.G. 2476) issued on 6/11/2023.

Sample Collection

Nasal swabs and fecal samples were collected from 50 dogs aged 5-8 months suspected to have canine distemper infection as they displayed clinical symptoms of the disease, including watery or pus-filled eye discharge, fever, nasal discharge, vomiting, and diarrhea. These samples were collected from different small animal clinics in Baghdad, Iraq. Then, these samples were placed in test tubes and stored at -20 °C till use.

Phytochemical Screening

The objective of the phytochemical analysis was to determine the biochemical constituents present in both extracts of Iraqi and American *L. camara* by observing their color reactions with various reagents. The usual methodology was employed to analyze each extract for the presence of alkaloids, glycosides, tannins, diterpenes, flavonoids, carbohydrates, proteins, steroids, and saponins (12-14).

Rapid Test

A commercial Kit (Rapid test kit, BIONOTE) was applied in this study according to the manufacturer's protocol. The presence of a purple color across the center of the test device was an indicator of the positive result.

Conventional RT-PCR

The primers used for the amplification of the *N* gene of CDV were designed in this study according to the National Center for Biotechnology Information (NCBI) databases, as follows:

Table 1. Primers designed for the N gene of CDV

Primer		Sequence (5'--3')	Start-stop	Size bp
Glycoprotein 1 (*N* gene)	F	CAAAGACGTGTGGTCGGAGA	604-623	570
	R	AGCTTCCTCCTTGGTGATGC	1173-1154	

F= Forward, R= Reverse

Genomic RNA was extracted from nasal swabs and fecal samples using the TRI reagent/Promega, USA. Then, the extracted RNA was converted into complementary DNA (cDNA) using LunaScript Reverse Transcriptase/Biolabs/England. The reverse transcription reaction was done at a total volume of 20 µL, which included: 2 µL of LunaScript RT SuperMix, 1 µL of random primers, 12 µL of RNase Free dH_2O, and 5 µL of RNA). Then, the PCR reaction components were prepared including 10 µL of Maxter Mix, 0.5 µL of each primer pair (Table 1), 5 µL of cDNA and RNase-free water up to 20 µL. The PCR amplification program was done through the use of the Applied Biosystem of the PCR system as follows: initial denaturation at 95°C for 60 sec for 1 cycle; denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, extension at 72 °C for 45 sec for 45 cycles; and extra extension on 72°C for 10 min for 1 cycle. The PCR products were subjected to electrophoresis of 2% agarose gel at 10 volts for 40 min. Then, the UV- trans-illuminator was used to visualize DNA bands, and the photos were captured using a special camera.

Sequencing

The PCR products of six cDNA samples already amplified by RT-PCR targeting the N gene of CDV as well as the forward primer were submitted to the sequencing service (Macrogen, Korea) to perform Sanger sequencing. Then, the sequences were analyzed using BLAST of NCBI, and Molecular Evolutionary Genetics Analysis (MEGA) software version 6 was used for phylogenetic tree construction. After that, the N protein was analyzed using Raptorx software.

RESULTS

Conventional RT-RT PCR

The agarose gel electrophoreses revealed bands of the partially amplified N gene with the expected band size of 570 bp in 9 out of 50 samples (Figure 1).

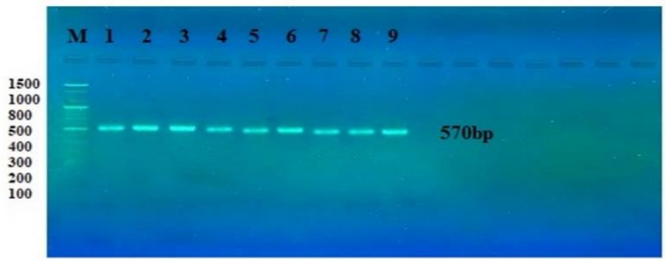

Figure 1. Agarose gel electrophoresis (2%) shows DNA bands of 570 bp expected to be the N gene of CDV partially amplified by conventional RT-PCR. Lane M= DNA size marker (100-1500 bp), Lane 1-9= positive samples

Sequencing Analysis

The sequence analysis showed the presence of alterations in various regions of the *N* gene nucleotide sequence with varying percentages in comparison to other CDVs that exist worldwide. Either a transition or transversion, missense or silent mutations were reported. The CDV was distinct from other isolates in Iraq according to these nucleotide alterations that compared with ID: AF378705.1 (Table 2).

Table 2. Genetic variation at the *N* gene of Canine Distemper Virus

Sample No.	Type of substitution	Location	Nucleotide	Nucleotide codon	Amino acid change	Predicted effect	Identities (%)
1	Transversion	871	G/T	TGT/TTT	C(Cysteine)/F(Phenylalanine)	Missense	99
2	Transition	874	A/G	GAT/GGT	D(Aspartic acid)/G(Glycine)	Missense	99
	Transition	956	T/C	GCT/GCC	A(Alanine)/A(Alanine)	Silent	99
3	Transversion	966	C/A	CAT/AAT	H(Histidine)/N(Asparagine)	Missense	99
4	Transversion	1050	A/T	ATC/TTC	I(Isoleucine)/F(Phenylalanine)	Missense	99
5	Transition	958	T/C	CTT/CCC	L(Leucine)/P(Proline)	Missense	99
	Transition	959	T/C	CTT/CCC	L(Leucine)/P(Proline)	Missense	99
	Transversion	1083	G/C	GCA/CCA	A(Alanine)/ P(Proline)	Missense	99

The N protein analysis

The protein analysis showed the secondary structure of the N viral protein of the canine distemper disease virus. Figure 2 shows that this protein is made up of an alpha helix, beta sheet and coil. The viral protein revealed unexpected structure including amino acid leading to slight differences in viral proteins.

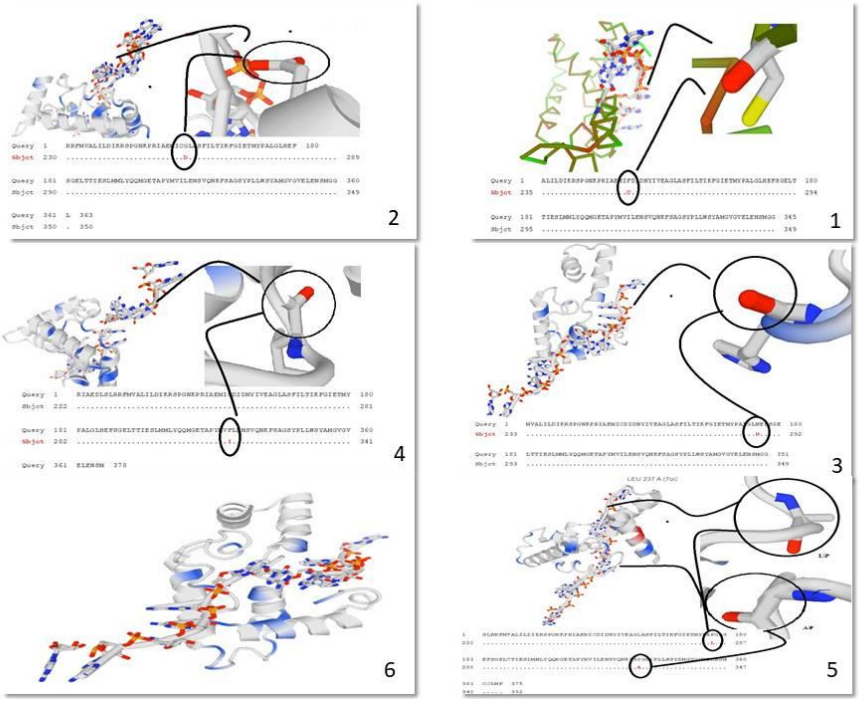

Figure 2. Nucleocapsid amino acid analysis of Canine Distemper Virus (CDV) shows presence of helixes, β-Sheets and coils within the structures

Phylogenic Tree of CDV Based on the *N* gene

The nucleotide sequences of the N gene of the Iraqi CDV isolates were successfully registered in NCBI under the accession numbers: OR492277.1, OR492278.1, OR492281.1, OR492280.1, OR492279.1 and OR492282.1. The isolates of this study were found to be comparable to the USA, China, South Africa, Australia, India, UK, Kazakhstan and Canada strains (AF378705.1, MF926604.1, KY971530.1, OL441602.1, MZ926744.1, AF305419.1, HM063009.1 and GU138403.1), respectively with 99% homology and 96% homology with strains ID: EU716337.1, ID: LC159587.1, ID: AF164967.1, ID: MK431532.1 ,ID: MW535268.1 ,ID: OQ442787.1, ID: EU072200.1 as indicated in Table 3 and Figure 3

Table 3. Iraqi CDV compared with other registered global viral isolates based on the *N* gene

Accession Number	Country	Isolation Source	Compatibility
AF378705.1	USA	Canine	99
MF926604.1	China	Dog	99
KY971530.1	South Africa	Wild-type canine	99
OL441602.1	Australia		99
MZ926744.1	India		99
AF305419.1	UK		99
HM063009.1	Kazakhstan	Mink	99
GU138403.1	Canada		99
EU716337.1	USA: Chicago	Canis familiaris	97
LC159587.1	Viet Nam	Canis lupus familiaris	96
AF164967.1	Switzerland		96
MK431532.1	Taiwan	Dog	96
MW535268.1	China: Jiangsu	Red panda	96
OQ442787.1	Kazakhstan	Pusa caspica	96
EU072200.1	Hungary		96

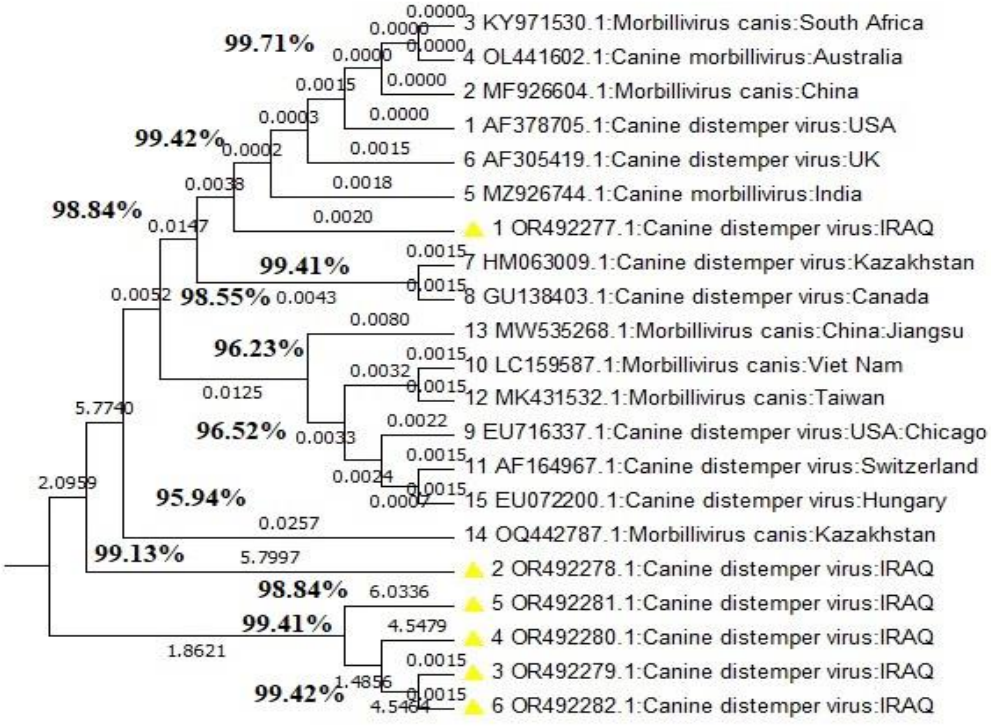

Figure 3. Phylogenic tree analysis of the Iraqi CDVs based on the N gene sequence registered in the NCBI under the accession numbers OR492277.1, OR492278.1, OR492281.1, OR492280.1, OR492279.1 and OR492282.1 compared to other global CDV strains

DISCUSSION

Canine distemper is a highly contagious disease causing immunodepression in dogs due to canine morbillivirus infection (20). The clinical manifestations of canine distemper virus (CDV) infection include pneumonia, ocular discharge (which may manifest as watery or purulent), and the dog may have fever, nasal discharge, gastrointestinal

illness characterized by vomiting and diarrhea. Additionally, the condition might progress to neurological symptoms. In addition, lethargy, reduced appetite, hyperkeratosis and hyperplasia in the teeth of puppies were also reported by many authors (21, 22).

The substitutions nucleotides in the N gene were found in CDV in this study. According to Table 2, the N gene of this virus showed the following substitutions: Alanine, showed silent mutation, while Cysteine/Phenylalanine, Aspartic acid/Glycine, Histidine/Asparagine, Isoleucine/Phenylalanine, Leucine/Proline, Alanine/Proline had missense mutations. These nucleocapsid changes may result in a high degree of antiviral resistance and/or serve a supporting role in the formation of high levels of pathogenicity in CDV or vice versa.

Antiviral resistance can occur as a consequence of alterations in RNA binding, modifications in the N-terminal or C-terminal regions of the NP protein, disruptions in protein-protein interactions, and modifications in the surface of the NP protein that enable the virus to evade detection by the host immune system. This can result in persistent infections and reduced efficacy of vaccines. This might enhance the virus's capacity to evade immune-based antiviral therapies (23).

Mutations in the *NP* gene may enhance pathogenicity by optimizing its binding to vRNA or interaction with other viral proteins, resulting in more effective viral replication and larger viral loads. This might lead to exacerbated clinical symptoms and heightened viral transmission. Moreover, modifications in the NP might possibly impact the virus's capacity to attach to and invade certain cell types, so broadening its tropism and directing its focus towards previously unaffected regions by the original virus. This has the potential to result in a broader spectrum of clinical manifestations and perhaps more perilous sequelae. Furthermore, the NP interacts with proteins inside the host cell and may interfere with several biological processes. Genetic alterations in the NP may exacerbate these disturbances, resulting in heightened cellular harm and inflammatory reactions, so contributing to a more severe illness (24).

According to the protein data inferred from sequencing analysis, the CDV N protein's secondary structure is made up of helical, β-sheet, and coil or ribbon models. An essential factor in assessing the structural model of viral proteins is the conservation of secondary structure (25). These mutations which involved transition or transversion substitutions (nonsense or missense), resulted in changing the order of amino acids. Adenine to Guanine or Guanine to Adenine are examples of purine nucleotide changes that occur during the transition, while Cytosine to Thymine or Thymine to Cytosine are examples of pyrimidine nucleotide changes that occur during the transition, approximately two of every three single nucleotide alterations (26). The transversion is a spontaneous point mutation that transforms the pyrimidine (one ring) nucleotide (Thymine or Cytosine) to the purine (2 rings) nucleotide (Adenine or Guanine) and vice versa (27). These point mutations which can be either nonsense mutations (synonymous replacements) or missense mutations (nonsynonymous substitutions) occur within a gene's protein-coding domain. Missense mutations, which change the amino acid sequence of a protein, produce changed amino acids, whereas mutations which do not change the amino acid sequence, are known as silent mutations (28).

The point mutation might potentially lead to diverse repercussions for the virus, mostly affecting its secondary structure. Modifications in the RNA binding domain of the N protein may impact its capacity to engage with the viral RNA, possibly causing disturbances in viral replication or assembly. If the mutation impacts the areas responsible for protein-protein interactions, it has the potential to disrupt the essential interactions necessary for viral replication, assembly, or nucleocapsid transport. The N protein also engages in interactions with proteins found in the host cell. Modifying its structure might potentially impact these interactions, so altering viral entrance, replication, or immune evasion. The mutation's impact on the virus's virulence may vary, potentially resulting in either heightened or diminished pathogenicity. This, in turn, may lead to a broader spectrum of clinical manifestations or heightened disease severity (23, 24).

The phylogenic tree analysis showed that the N gene of CDV of the Iraqi isolates (OR492277.1, OR492278.1, OR492281.1, OR492280.1, and OR492279.1) was similar to that found in the USA, China, South Africa, Australia, India, UK, Kazakhstan and Canada strains (AF378705.1, MF926604.1, KY971530.1, OL441602.1, MZ926744.1, AF305419.1, HM063009.1 and GU138403.1), with 99% homology. In addition, the N gene of the same virus was similar to that of Viet Nam, Switzerland, Taiwan, China, Kazakhstan and Hungary strains under accession number (LC159587.1, AF164967.1, MK431532.1, MW535268.1, OQ442787.1 and EU072200.1) with 96% homology. Furthermore, the Iraqi isolate (OR492282.1) showed 100% homology without any mutation with the USA strain (AF378705.1).

Since the *N* gene is conserved and widely used to detect CDV, conventional PCR proved to be a very precise and accurate approach for the detection of this virus (29). The study of Farancesco et al. (29) revealed that domestic dogs with CDV infections inhabit with Marsican brown bears, that live in Central Italian protected areas. Sabatino et. al., (30) showed six amino acid alterations and substitutions in the viral protein encoded by the gene sequences, in contemporary Italian dog strains that are part of the European wild dogs. Additionally, this study is in agreement with that of Mansour and Hasso, (18) who found the N gene of CDV was closely related to that of China, the

USA, and the UK strains, while other isolates in Iraq were closely related to Hungary, Switzerland, Germany, and Japan strains.

In conclusion, this investigation serves as an important study in Iraq focusing on the molecular characterization of the CDV. Utilizing RT-PCR for the amplification of the N gene has proven to be an effective methodology for the rapid and precise identification of CDV. Notably, the sequencing data revealed the presence of point mutations within the N gene, enriching our understanding of the virus genetic diversity. The phylogenetic analysis further indicated that the CDV isolates circulating in Iraqi dogs had few genetic variations relative to strains identified in a range of countries, including the United States, China, South Africa, Australia, India, the United Kingdom, Kazakhstan, and Canada. These findings collectively underscore the necessity for the development and implementation of vaccines with high efficacy, as well as the formulation of comprehensive control strategies to mitigate the impact of this highly virulent and internationally dispersed strain.

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N/A

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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التحليل الجيني لجين *N* لفيروس طاعون الكلاب من الكلاب المصابة في مدينة بغداد

اية محمود جبر، رواء صلاح الدين جمعة

فرع الأحياء المجهرية، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

الهدف الأساسي من هذه الدراسة هو تحليل جين (*N*) لفيروس طاعون الكلاب (CDV) في الكلاب المشتبه اصابتها في بغداد، العراق. حيث تم جمع ٥٠ عينة تشمل مسحات الأنف وعينات البراز، من مختلف الأعمار والسلالات. باستخدام الاختبار السريع، تسع من ٥٠ عينة كانت إيجابية لفيروس CDV. بعد ذلك، تم استخلاص الحمض النووي الريبي (RNA) من العينات التي تم اختبارها بشكل إيجابي وإخضاعها للنسخ العكسي - تفاعل البوليميراز المتسلسل (RT-PCR) لتخليق الحمض النووي الريبوزي (cDNA). تم تضخيم [cDNA] بشكل أكبر من خلال PCR التقليدي الذي يستهدف الجين N. تم ارسال ستة من هذه العينات المضخمة إلى شركة ماكروجين، كوريا الجنوبية، لإجراء تسلسلها. تم كشف تحليل التسلسل عن عدة طفرات صامتة ونقطية في الجين N. اشار التحليل الوراثي الى تشابه بنسبة ٩٩٪ للجين N للعزلات العراقية مع السلالة المعروفة من السلالات الامريكية والهندية. بشكل عام، حددت هذه الدراسة الطفرات النقطية في الجين N لفيروس CDV في الكلاب المصابة في العراق وأثبتت أن السلالة المنتشرة تظهر تشابهًا جينيًا عاليًا مع سلالات الولايات المتحدة الأمريكية والهند. ولذلك، فمن الضروري يوصى بتطوير جودة تطبيق اللقاح واستراتيجيات المكافحة الشاملة. تمثل هذه الدراسة الأولى في العراق التي تركز على تحليل الجين N لفيروس CDV في الكلاب المصابة.

الكلمات المفاحية: طاعون الكلاب المعدي، تفاعل انزيم البلمرة التقليدي، تحليل التسلسل، المورث *N*، العراق