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Isolation and Molecular Detection of Canine Distemper Virus from Infected Dogs

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Abstract

Canine distemper is a virus that affects dogs worldwide, is highly contagious, and has a high mortality rate. However, no research that focused on virus isolation in Iraq was conducted. The goal of this study was to isolate and genetically identify canine distemper virus in dogs. In this study, 50 samples (fecal, ocular and nasal swabs) were collected from dogs at different ages. These dogs were suspected to be infected with canine distemper disease having clinical signs such as eye discharge that was watery to pus like with fever, nasal discharge, gastrointestinal disease with vomiting and diarrhea. These samples were detected using rapid test. After that, the positive samples were propagated in fibroblast cell culture of chicken embryo to detect the cytopathic effect of the virus on cell culture for two passages. The isolated virus was also confirmed using real time PCR technique by amplification of the *N* gene. The results of cytopathic effects were rounded cells with giant cell formation of infected cells compared to non-infected cells. The isolated virus was also positive when detected by real time PCR. In conclusion, this study is the first study in Iraq to isolate virus in fibroblast cell culture of chicken embryo and the majority of canine distemper virus isolates caused a cytopathic effect.

Key words: Canine Distemper Disease, Rapid Test, Isolation, Real Time PCR, Iraq

Introduction

Canine distemper is extremely contagious and has a high fatality rate and this virus that affects dogs all around the world (1). Canine distemper virus A subclinical (asymptomatic) infection may lead to a clinical illness presentation (2). Fomites or the nasal aerosol pathway are believed to be the primary sources of effective infection secretion (3). The canine distemper virus infects the lymphatic system, then invades epithelial cells and sheds its virus, which allows the illness to spread to more puppies (4). This virus may also enter the central nervous system, most likely through the action of a third potential receptor termed GliaR found in glial cells (5,6).

The infection by canine distemper is multi-systemic disease that a one or more of the following symptoms respiratory conditions, such as pneumonia, eye discharge that may appear watery to pus like, the dog can develop fever, nasal discharge, illnesses of the gastrointestinal system that

cause nausea, vomiting, and diarrhea as well as seizures, twitching partial or complete paralysis, ataxia and tremors. In addition, lethargy reduced appetite hyperkeratosis and hyperplasia in teeth of puppies (7,8). It has been speculated that during the 17th century, Peru may have exported canine distemper to Spain. Antonio de Ulloa accurately characterized it in 1746; at the middle of the 18th century, the virus is a single-stranded RNA virus in the family of "*Paramyxoviridae*", which also contains viruses that cause mumps; measles, and rubella (9).

Dogs infected with Due to a reduction in maternally derived immunity, puppies aged 3 to 6 months are more susceptible to the virus than older dogs. Young puppies are protected by passive immunity, whereas the majority of adult dogs are protected by vaccination (4). Dogs of all breeds and ages, especially those who have not received vaccinations, can become infected by breathing in infectious aerosols from sick or asymptomatic animals or by handling contaminated food, brushes, water bowls, clothes, and other items (1,10).

The highly contagious canine distemper virus (CDV) can cause serious illness in young pups. The respiratory tract (nose, windpipe/trachea, and lungs), stomach and intestines, and brain are where the disease manifests itself most frequently. The virus impairs the immune system, making it challenging for afflicted dogs to combat other infections like parvovirus. and other secondary infection. Canine distemper virus CDV also known as canine *morbillivirus* sometimes termed footpad disease (2). The virus is an enveloped, non-segmented virion, and negative-sense RNA virus. The virus is pleomorphic, ranging in size from 150 to 300 nm, and has a spherical and filamentous form. The six genes that make up the genome encode six structural proteins: matrix (M), two transcriptase phosphoproteins (P) and polymerase (L), nucleocapsid protein (N), and glycoproteins, such as fusion protein (F) and hemagglutinine (H) (2,11). Moreover, two more nonstructural proteins, C and V, which are produced via alternative translation starts and RNA editing, respectively (4).

Veterinarians diagnose canine distemper on the basis of clinical appearance test. Symptoms of the disease may not appear clear except in advanced cases. Diagnostic tests include isolation of the virus on tissue culture, blood test, ELISA, immunofluorescence and reverse transcriptase-polymerase chain reaction test. Also, some viruses of RNA detected via RT PCR (12-19). In the last test, viral RNA can be detected in respiratory secretions, cerebrospinal fluid, feces and urine (20). In Iraq, CDV can causes a significant infection leading to death of infected dogs. Furthermore, many studies confirmed Iraq has the virus there. The disease was identified in Mosul by Saaed and Alsarhan, (21) who detected the CDV using microscopic examination of blood smear, rapid test and ELISA test. The virus was detected in Baghdad by Mansour and Hasso, (22) who confirmed this virus in domestic dogs using RT-PCR. In addition, the study of Mohammad et al., (23) confirmed the CDV in stray dogs using serological and molecular detection. However, there were no studies include the isolation of virus in Iraq. Therefore, the aim of this study is isolation and genetic detection of CDV in infected dogs.

Materials and Methods

Ethical approval: All protocols complied with the Animal Care and Use Committee's (ACUC) criteria and were authorized on November 6, 2023, under approval number PG/2476.

Sample collection and preparation: The samples (nasal, ocular and fecal swabs) were collected from 50 dogs at different ages suspected to be infected with canine distemper disease with clinical signs, such as watery or pus eye discharge, fever, nasal discharge, vomiting and diarrhea. These samples were diluted in 5 ml PBS. Then, the suspension was mixed using vortex mixer. After that is was left to stand at room temperature for 10 minutes. A cold centrifuge was used to centrifugal the suspension for ten minutes at 3500 rpm. To precipitate particles, the supernatant solution was transferred three times into sterile tubes. The suspension was also supplemented with 10 mg of streptomycin and 10,000 I.U. of crystalline penicillin per milliliter, and it was allowed to sit at room temperature for half an hour. Lastly, the suspension fluid was kept at -20 °C till use.

Rapid test: A commercial Kit (Rapid test kit, BIONOTE) was applied in this study according to manufacturer's protocol. The positive results were detected via formation of purple color that appeared across the center of the test device.

Preparation of fibroblast cell culture of chicken embryo: This technique was done at Microbiology Department / College of Veterinary Medicine / University of Baghdad, Iraq. These cells were prepared according to Baron *et al*., (1995).

Propagation of positive samples in fibroblast cell culture (FCC): Positive samples were inoculated in chicken embryo fibroblast cell culture (FCC). Before inoculation 0.3 ml of positive samples in complete monolayer CEFCC and the growth media discarded. Then, these samples were inoculated at 37 ℃ for 1 hour to virus adsorption occurred in cell culture. These cells received maintenance media added to them. Simultaneously, PBS was added to the control cell culture in the same way that the infected cells were. Finally, an inverted microscope was used every day to check the infected and control cell cultures for signs of cytopathic effect (CPE) induced by viruses in the cell culture. The isolated virus was propagated into CEFCC for two passages.

Molecular Diagnosis

Primers: The National Center for Biotechnology Information (NCBI) databases were utilized in this study's primer design for the amplification of the CDV *N* gene, as following at Table 1

Table 1: Primers designed for the *N* gene of CDV

Primer name		Sequence（5'--3'）	Product size
Glycoprotein 1 (*N* gene)	Forward	CAAAGACGTGTGGTCGGAGA	570
	Reverse	AGCTTCCTCCTTGGTGATGC	
Canis lupus familiaris B-Actin (reference gene)	Forward	CTTCCAGCCTTCCTTCCTGG	572
	Reverse	TGCTGTCACCTTCTCCGTTC	

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, 12µl of RNase Free dH2O, and 5 µl of RNA (sample).

Real time PCR: The PCR amplification program was done through the use of the SaCycler-96 of PCR system as follows: initial denaturation at 95°C for 60 sec for 1 cycle; denaturation on 95°C for 15 sec, annealing and extension on 60°C for 30 sec for 45 cycles; and melting curve on 60-95°C for 1 cycle.

Results and Discussion

Clinical observation: The clinical signs including pneumonia, eye discharge that may appear watery to pus like, the dog can develop fever, nasal discharge, gastrointestinal disease with vomiting and diarrhea (Figure 1).

Figure (1): Canine distemper virus infected dogs with periocular discharge

Results of rapid test: nine out of fifty samples (nasal, ocular and fecal swabs) were positive by Rapid test (Rapid test kit, BIONOTE) (Figure 2).

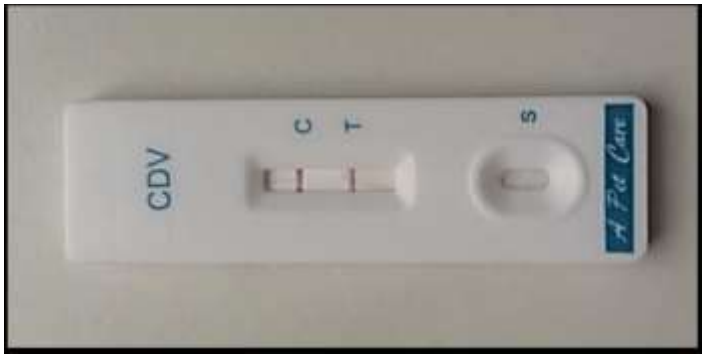


Figure (2): Canine distemper virus positive in infected dogs

Result of viral propagation on fibroblast cell culture of chicken embryo: The positive samples of rapid test, after preparation of the samples, were propagated in FCC for two passages. The cytopathic effects (CPE) were obvious on the first passage after 24 hours, and characterized by rounded cells, but after 48 hours they were characterized by rounded and patches of detached cells (Figure 3 A). The cytopathic effects (CPE) of second passage appeared after 24 hours and characterized by presence of rounded cells, giant cells and development of cell holes (Figure 3 B) along with compared with the control CEFCC (Figure 3 C).

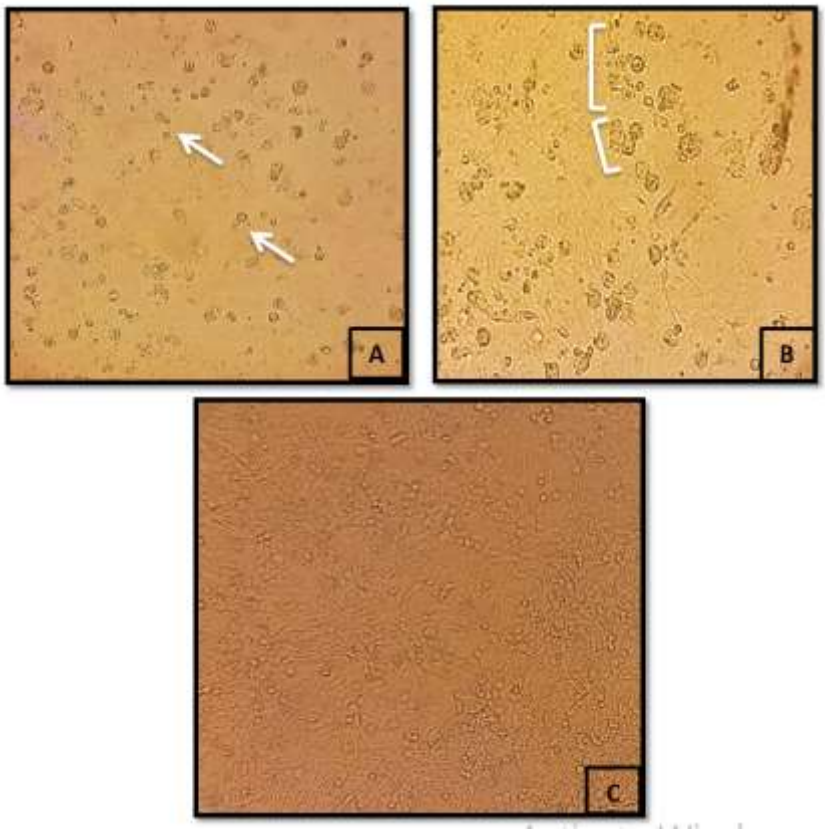


Figure (3): Cytopathic effect of canine distemper virus on fibroblast cell culture; A: 1st passage the CPE after 48h with rounded cell. B: 2nd Passage the CPE after 24h with giant cell (syncytia); C: control (non-infected)

Results of Real time PCR: Nine out of fifty samples (nasal, fecal swap) were positive (Figure 4).

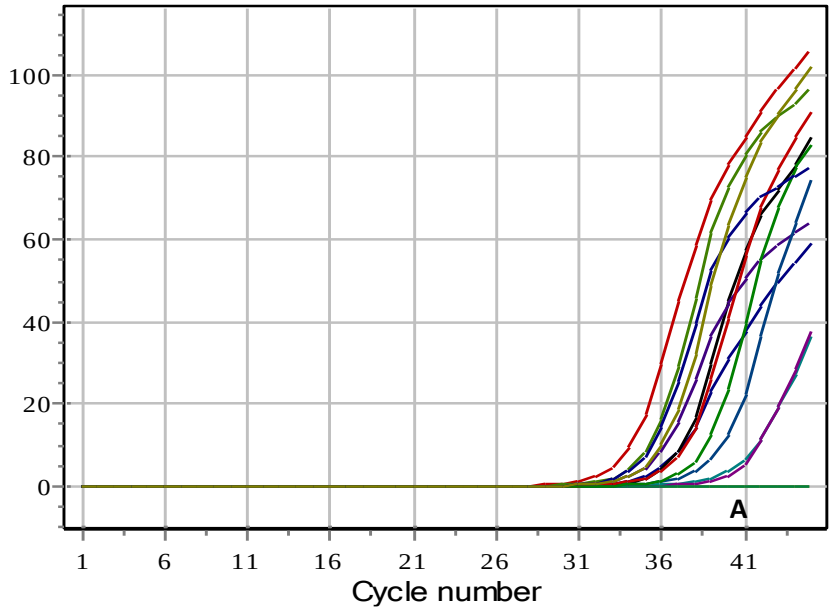
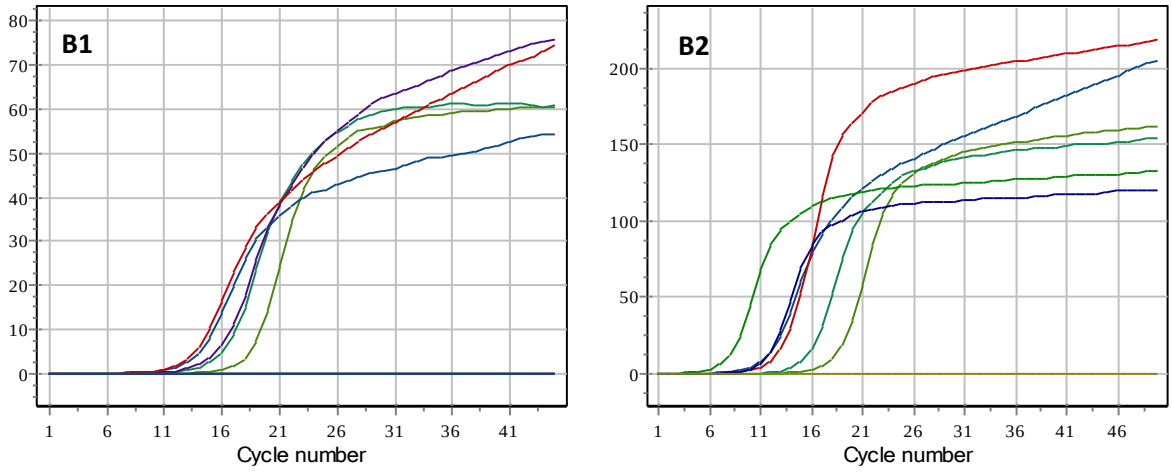


Figure 4: Plot of real time PCR amplification for detection of canine distemper virus via amplification of the *N* gene (positive sample), (B1,2 reference gene)



Discussion

Canine distemper disease (CDD) is a highly contagious and immunodepression disease of dog caused by *morbillivirus* (24). In this study, the clinical signs of CDV infection included pneumonia, nasal discharge, vomiting, diarrhea, and an ocular discharge that ranges from watery to pus-like, but the condition reaches neurological signs, which are limited to only a few including seizures, twitching partial or complete paralysis, ataxia and tremors. In addition, lethargy reduced appetite hyperkeratosis and hyperplasia in teeth of puppies these are reported by many authors (7,8). These findings agree with those mentioned by Karki et al., (25) who showed the disease is multisystemic infect the respiratory, gastrointestinal, and nervous systems of the animal. According to Anderson and von et al.'s (7) study, the pathophysiology and disease course of canine distemper are similar to those of a human measles virus infection. These signs and symptoms include fever, lymphopenia, respiratory problems, rash, and significant, widespread depletion of lymphoid organs and immunosuppression during the acute phase of the illness. Furthermore, the study of Mareri *et al*. (26) study revealed that a significant prevalence of neurological sequelae was associated with CDV infection.

Canine distemper disease virus was isolated on fibroblast cell culture (FCC) of chicken embryo which supports growth of CDV. It is therefore an effective technique for the initial isolation of the virus. The emergence of rounded, giant cells and the creation of holes in the cell were the primary cytopathic effects of virus growth on FCC, these findings agrees with Pawar et al., (27) who used cell line (B95a) for isolating the virus which caused CPE of canine distemper disease, and characterized by rounded cells (dead cell) and the cells were detached from the surface of culture flask, leaving vacuole in the cell monolayer due to destruction of the entire monolayer. Also, Seki et al., (28) who detected the CDV by using RT-PCR. Furthermore, the study of Pawar et al., (27) who found the syncytium produced on Vero, SLAM tag cell lines when infected with CDV after 5 passages. Also agreed with Tan et al., (29) who showed rounded and small syncytia on MDCK cells when infected with CDV (lung tissue) after 6 passages.

Conclusion

As a following of the results in this study, real-time PCR amplified the *N* gene in a highly quick and sensitive manner to identify CDV. Most CDV isolates is very virulent and caused cytopathic effect when isolated in chicken embryo fibroblast cell culture.

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Novelty Statement

The study's unique approach focuses on isolating the canine distemper virus from affected dogs and utilizing PCR to confirm the isolation.

Author contribution

These authors each contributed equally**.**

Conflict of Interest

The authors have declared no conflict of interest

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