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RESEARCH ARTICLE
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Antifungal effect of Neem (*Azadirachta indica*) Leaf Extract against *Alternaria* Pathogens isolated from Potato

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1. Introduction

Coronaviruses (CoVs) represent a broad group of enveloped, positive-sense single-stranded RNA viruses [1], known for their ability to infect humans and numerous animal species. They cause infections affecting vital systems including the respiratory tract, digestive organs, liver, and nervous system in a variety of hosts such as people, domestic animals, birds, bats, rodents, and wildlife [2]. Canine enteric coronavirus (CECoV) under the family Coronaviridae [1], specifically within the subfamily Orthocoronavirinae [3, 4]. Orthocoronavirinae encompasses four genera—Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus—of which CECoV is a member of Alphacoronavirus [5-7]. CECoV falls under the subgenus Tegacovirus (species Alphacoronavirus 1), which also includes significant pathogens of domestic animals such as transmissible gastroenteritis virus (TGEV) in pigs and feline coronavirus (FCoV) in cats [8]. Was first identified as a canine pathogen in 1971 during a gastroenteritis outbreak among military dogs [9]. CECoV possesses one of the largest known RNA virus genomes (~27.6–31 kb) [10], facilitating considerable genetic diversity through frequent recombination events [11], which facilitated by the RNA-dependent RNA polymerase (RdRp) [12]. It typically causes mild enteric illness, especially in young dogs and puppies [13–16], with fecal-oral transmission being the primary route [12, 19]. Infected dogs shed the virus for 6 to 9 days, sometimes longer without clinical symptoms, complicating infection control [17–19]. Due to similarities in clinical symptoms with other enteric pathogens such as canine parvovirus type 2, precise laboratory diagnosis is critical to differentiate CCoV infections [10, 20], utilizing techniques including rapid antigen detection tests, ELISA, PCR, electron microscopy, and viral culture [21]. In contrast, parvoviruses are small, non-enveloped, single-stranded DNA viruses notable for their minimal virion size of approximately 18–28 nm [22]. The family Parvoviridae infects a broad spectrum of invertebrate and vertebrate hosts, with canine parvovirus (CPV) representing a highly contagious member responsible for severe enteric disease in dogs [23, 24]. CPV emerged in the 1970s, likely through mutation and cross-species transmission from feline parvoviruses, and has since become a worldwide veterinary concern due to its high morbidity, rapid spread, and environmental persistence [25, 26]. CPV primarily targets actively dividing cells in the intestinal crypts and bone marrow, leading to acute gastrointestinal illness characterized by vomiting, hemorrhagic diarrhea, and leukopenia, especially in puppies and unvaccinated animals [27, 25]. Transmission occurs chiefly via the fecal-oral route, and the virus's environmental stability contributes significantly to its epidemiology [26]. Diagnosis relies on the detection of viral antigen in feces, molecular methods, and serology, while control strategies focus on vaccination, strict hygiene, and isolation of infected dogs [28]. The clinically similar presentations of CECoV and CPV infections pose diagnostic challenges, highlighting the need for sensitive, rapid, and specific diagnostic methods. Sandwich lateral flow immunochromatographic assays provide promising point-of-care tools to differentiate these infections quickly and accurately, facilitating improved clinical management and epidemiologic control. This study aims to determine the prevalence of canine enteric coronavirus (CECoV) and canine parvovirus (CPV) among dogs in Mosul using rapid antigen testing, and to assess the association of infection rates with host factors (such as age, breed, sex), environment, vaccination status, and dog origin (stray vs household). The findings will contribute to improved surveillance and targeted control strategies for canine enteric viruses in the region.

Materials and Methods

Animals Included in the Study

The research involved 100 dogs sourced from various areas across Nineveh Governorate, Iraq. These dogs represented a wide range of ages, genders, and breeds. Each animal exhibited clinical signs indicative of enteric viral infections, such as vomiting, diarrhea, weakness, dehydration, inappetence, and fever.

Sample Collection

Duplicate rectal swabs were taken from each dog. One swab was utilized for the detection of canine coronavirus (CCoV) antigens, while the second was employed to identify canine parvovirus (CPV) antigens, both through rapid diagnostic testing techniques.

Ethical Approval

The study was conducted under the approval of the Scientific Committee of the Department of Microbiology at the College of Veterinary Medicine, University of Mosul (Approval No. UM.VET.2024.033). All animal handling and sample collection procedures conformed to accepted scientific and ethical standards to ensure animal welfare.

Sandwich Lateral Flow Immunochromatographic Assay (Rapid Test)

This assay was employed to detect and differentiate antigens of canine coronavirus and canine parvovirus in rectal swab samples.

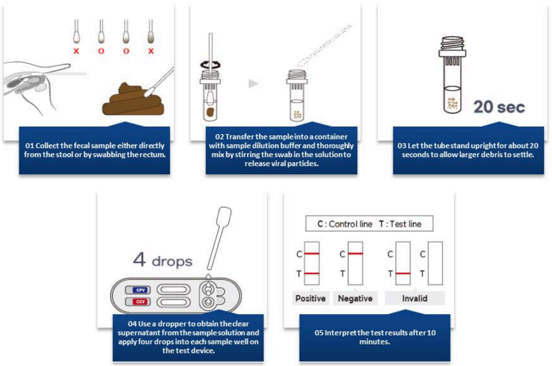
Kit Contents

The test kit (SUNLONGBIOTECH.COM, China) comprises the following components:

- 10 foil pouches, each containing:
  - ➔ A desiccant
  - ➔ A pipette
  - ➔ A test cassette
- 10 assay tubes prefilled with buffer
- 10 sterile swabs
- Manufacturer's instruction manual

Test Procedure

All materials, including specimens and test devices, were equilibrated to room temperature (15–25°C) prior to testing. The assay was performed strictly according to the manufacturer's guidelines provided with the kit.



Graphic 1. Test Procedure & Interpretation of the Antigen Rapid CCV Ag Test Kit

Interpretation of Results

- ➢ **Positive:** The test is considered positive when both the control line (C) and the test line (T) are visible, regardless of whether the test line is faint or clearly defined.
- ➢ **Negative:** A negative result is indicated when only the control line (C) appears and there is no visible test line (T).
- ➢ **Invalid:** If the control line (C) does not appear, whether or not a test line (T) is present, the test result is invalid and the test should be repeated.

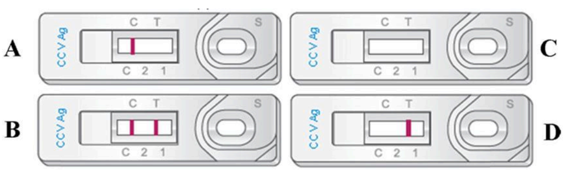


Figure 1. The results obtained from the Antigen Rapid CCV Ag Test Kit are illustrated graphically: (A) indicates a negative result, (B) shows a positive result, and (C) and (D) depict negative outcomes, as reported by [29].

Statistical Analysis

Statistical analysis was conducted using IBM SPSS version 19 [30].

Results

Results of Sandwich Lateral Flow Immunochromatographic Assay (Rapid Test)

A total of 300 rectal swab samples were obtained from 100 symptomatic dogs showing signs of gastrointestinal viral infections. Analysis using the sandwich lateral flow immunochromatographic assay (rapid test) identified the presence of canine enteric coronavirus (CECoV) and canine parvovirus (CPV) antigens as follows:

- ➔ 43% (43 dogs) were positive for canine coronavirus (CCoV), while
- ➔ 29% (29 dogs) tested positive for canine parvovirus (CPV).
- ➔ The remaining 28% (28 dogs) showed no evidence of either virus.

Note: Importantly, none of the dogs were found to be co-infected with both CCoV and CPV at the time of testing.

These results highlight the notable prevalence of CECoV and CPV among dogs exhibiting gastrointestinal clinical signs in the studied area, highlight the absence of mixed infections, and indicate the significance of rapid antigen testing for accurate diagnosis.

Infection Rates by Dog Category

Stray versus Household Dogs:

Infection prevalence was greater in stray dogs than in household pets; nonetheless, this difference did not reach statistical significance.

Sex:

Female dogs showed a higher infection rate than males; however, this difference was not statistically significant.

Age:

The highest infection rates were observed in dogs under one year of age, while dogs older than two years exhibited the lowest rates. Statistical evaluation revealed a significant difference between the youngest group and the older age categories, whereas no notable differences were found among the older groups themselves.

Breed Size:

Dogs of large breeds showed notably higher rates of infection compared to small breeds, which exhibited the lowest levels of infection.

Origin (Source) of Dogs:

Local/native breeds showed a markedly greater susceptibility to infection, particularly with the enteric form of canine coronavirus (CCoV), in comparison to imported breeds, which displayed the lowest infection rates.

Table 1. Infection Rates of Canine Coronavirus (CCoV) and Canine Parvovirus (CPV) Detected Utilizing Sandwich Lateral Flow Immunochromatographic (Rapid) Assay.

Factors	No. Tested Cases	No. Of Positive to CCV (%)	No. Of Positive to CPV (%)
Animals Management			
Household Dogs	61	22(36) [a]	12(19.6) [a]
Stray Dogs	39	21(53.8) [a]	17(43.5) [a]
Gender			
Females	41	14(34.1) [a]	1(2.4) [a]
Males	58	29(50) [a]	28(48.2) [b]
Age			
< 1 Year	44	28(63.6) [a]	15(34) [a]
≥ 1 Year – 2 Years	35	10(28.6) [b]	11(31.4) [a]
≥ 2 Years	21	5(23.8) [c, b]	3(14.2) [b]
Breed			
Small Breeds	59	18(30.5) [a]	13(22) [a]
Large Breeds	41	25(60.9) [b]	16(39) [b]
Source			
Native	56	38(67.8) [a]	19(33.9) [a]
Imported	44	5(11.4) [b]	10(22.7) [b]

Discussion

Enteric canine coronavirus (CCoV) is an important pathogen responsible for significant gastrointestinal disease in dogs, particularly young animals. The elevated morbidity and mortality rates seen in puppies highlight the critical need for prompt and precise diagnosis to ensure effective management and control of the disease. Timely and reliable differentiation of CCoV from other enteric pathogens, such as canine parvovirus (CPV), is critical in clinical decision-making, especially since clinical signs can overlap significantly. Rapid antigen detection tests have become widely favored because of their ease of use, quick results, and applicability in both fieldwork and clinical environments [31]. In the present study, a rapid test served as a preliminary screening method to differentiate canine coronavirus (CCoV) infections from canine parvovirus (CPV) in dogs showing clinical symptoms. Of the rectal swab samples analyzed in this study, canine coronavirus (CCoV) was detected in 43 dogs (43%), while canine parvovirus (CPV) was detected in 29 dogs (29%). Additionally, 28 dogs (28%) did not test positive for either virus, and no animals exhibited evidence of co-infection. These findings highlight the prominence of CCoV as an enteric pathogen in the examined canine population and emphasize the risk of misdiagnosis or overlooked infections if dedicated diagnostic testing is not utilized. The accuracy and practicality of rapid diagnostic tests for detecting CCoV and CPV have been supported by various earlier studies. For example, reference [29] found that rapid tests identified 54 out of 58 samples that were confirmed positive by nested PCR, demonstrating the suitability of rapid testing for field conditions. Similarly, study [32] conducted rapid testing on 109 dogs, detecting 5 CCoV-positive and 37 CPV-positive cases, with no evidence of co-infection—results that closely correspond to those observed in the current research. Several investigations further highlight the range of prevalence rates found with rapid tests: Reference [33] documented a canine parvovirus (CPV) prevalence of 37.7%, whereas reference [34] reported a higher prevalence of 50% utilizing the same lateral flow assay, illustrating consistent diagnostic performance across different studies.[35] identified CCoV in 8% and CPV in 50% of their cases, again illustrating that CPV often predominate in clinical presentations. Expanding on this, [36] carried out molecular surveillance and found that CCoV was the third most prevalent enteric virus at 20%. Their study also observed double and triple infections, noting that asymptomatic dogs actually harbored CCoV and canine distemper virus (CDV) more frequently than CPV-2, though the differences lacked statistical significance. A wider epidemiological view is offered by [37], whose meta-analysis found an overall canine coronavirus (CCoV) prevalence of 33%, with infections predominantly occurring in younger dogs. Interestingly, their analysis revealed that factors such as sex, season, and vaccination status did not have a significant impact on infection rates. Furthermore, CCoV-II was identified as the most prevalent serotype among dogs in China. Taken together, these findings endorse the use of rapid antigen detection as a practical first-line diagnostic tool for CCoV, especially in clinical or field environments where molecular testing is not available. Nonetheless, differences in prevalence reported across studies highlight the need to consider epidemiological background, the sensitivity of diagnostic methods, and the specific viral strains circulating locally when interpreting such data. Differences in infection rates reported across various studies and geographic locations can be explained by multiple factors. These include variations in diagnostic methodologies, where the specificity and sensitivity of the tests used differ. Additionally, the presence of diverse viral strains unique to specific regions affects prevalence findings. Apart from diagnostic variables, differences in healthcare standards and vaccination practices—particularly whether CCoV vaccination is deemed essential or optional—significantly influence observed infection rates. Moreover, factors such as the immune status of the dogs, environmental conditions, and management approaches also contribute to their vulnerability to infection. This study found a considerably higher occurrence of canine coronavirus (CCoV) and canine parvovirus (CPV) infections among younger dogs, especially those younger than three months, compared to older groups. This trend aligns with previous research. For instance, studies by [33] and [38] showed that 41.26% of puppies under three months tested positive for CPV, while infection rates decreased to 25% in puppies aged between three and six months, and further down to 20% in those aged six to twelve months. Although these differences are not always statistically significant, the evidence consistently suggests that puppies, particularly those younger than six months, are more susceptible to enteric viral infections due to immature immune defenses and delayed or incomplete vaccination protocols. Similarly, [39] found a CCoV infection rate of 26.67% in puppies aged three months or younger. More recently, [40] confirmed higher infection rates Younger dogs tend to be more susceptible to infection compared to their older counterparts. Interestingly, this higher vulnerability exists despite receiving better care and more comprehensive vaccination schedules, likely because young pups have lower levels of maternally derived antibodies—especially in regions where routine vaccination against CCoV is not common. On the other hand, some studies suggest that older dogs might also face increased risk due to declining immunity, as antibody levels against enteric canine coronavirus (ECCoV) fluctuate with age. However, this greater susceptibility in older animals may primarily result from the accumulated likelihood of exposure over time rather than a true weakening of their immune response. Although maternally acquired antibodies offer some early passive protection, evidence indicates that in populations without regular vaccination, this immunity alone is usually insufficient to fully guard against CCoV or canine parvovirus (CPV) infections. Moreover, there is a paucity of thorough research evaluating the durability of vaccine-generated antibodies over the long term, highlighting the need for more studies focused on the age-related immune responses to canine coronavirus. Together, these results highlight the vital need to establish early and comprehensive vaccination efforts, especially in areas where disease prevalence is high and maternal immunity alone fails to provide sufficient protection during puppies' most vulnerable early stages. The study's findings also reveal that stray dogs experience a notably greater rate of canine coronavirus (CCoV) infection compared to household pets. This observation is consistent with earlier studies which identify several factors contributing to the increased susceptibility of stray populations to enteric viral diseases. A key contributing factor is the lack of regular vaccination programs for stray dogs, who generally do not receive immunizations against enteric canine coronavirus (ECCoV) or other prevalent canine infections. This vaccination gap leaves stray dogs particularly prone to infection and positions them as significant reservoirs, facilitating ongoing viral spread within the broader dog population. Behavioral and environmental aspects further contribute to this increased risk. Stray dogs often roam freely and congregate in groups, facilitating the spread of pathogens through both direct contact and environmental contamination. In contrast, household dogs generally live indoors or in low-density settings, often limited to a single animal per household, which reduces their exposure to infectious agents. Household pets benefit from structured care that offers protective advantages. Even if dogs are not specifically vaccinated against canine coronavirus (CCoV), they often receive immunizations for other viruses, such as canine distemper virus and canine parvovirus. These vaccinations can trigger both targeted and broader immune responses, strengthening the dog's overall antiviral defense. The stimulation of humoral immunity along with non-specific cellular immune mechanisms may help pet dogs resist infections caused by different viruses, including CCoV. In contrast, stray dogs encounter increased environmental challenges—such as inadequate shelter, poor nutrition, and constant exposure to contaminated environments—that weaken their immune systems, making them more vulnerable to infections like enteric canine coronavirus (ECCoV). These combined factors contribute to the significantly higher infection rates observed in stray dog populations and highlight the urgent importance of expanded public health efforts. Implementing focused vaccination drives and community education programs aimed at stray dogs are crucial steps to control the transmission of canine coronavirus and other infectious diseases. The study found that imported dogs had a lower rate of canine coronavirus (CCoV) infection compared to local (native) breeds, which showed significantly higher infection levels. Although there is limited published research directly linking a dog's origin (imported versus local) with CCoV infection rates, practical observations and veterinary expertise provide some plausible explanations. Contrary to the common belief that imported dogs might be at greater risk due to stress from transportation, exposure to unfamiliar pathogens, or the introduction of new viral strains, the findings suggest that imported dogs actually had better protection. This increased resilience is largely credited to strict pre-import veterinary measures, including comprehensive vaccination protocols and detailed health screening. Examination of veterinary records revealed that many imported dogs had been vaccinated against CCoV either prior to or shortly after arrival, enhancing their immune defense and likely contributing to the reduced prevalence of infection. On the other hand, local breeds frequently experience inconsistent vaccination programs, particularly in areas where the CCoV vaccine is not considered essential and therefore often overlooked. This gap in preventive healthcare leads to greater susceptibility to enteric viral infections among native dogs. Vaccine-induced immunity is a multifaceted process affected by various factors, such as: Vaccine failure: Arising from improper vaccine storage, handling errors, incorrect administration, or the host's inadequate immune response. Suboptimal humoral immunity: Where cellular immune activation may occur but antibody production falls short of protective levels. Immunosuppression: The presence of other illnesses or the use of immunosuppressive treatments can weaken the host's immune response, thereby diminishing the effectiveness of vaccination. Antigenic mismatch: If there are considerable genetic variations between the vaccine strains and the viruses currently circulating in the field, this can hinder the immune system's ability to recognize the antigens properly, leading to reduced vaccine efficacy. These considerations underline the necessity for correct vaccination practices, careful post-vaccination monitoring, and ongoing reassessment of vaccine strain suitability relative to circulating viral variants. While imported dogs in this study demonstrated better immunological protection, the findings highlight the urgent need to enhance immunization coverage and vaccine strategy implementation among local dog populations to reduce CCoV transmission and disease burden. In this study, female dogs showed a higher rate of canine coronavirus (CCoV) infection compared to males. This finding differs from the results reported by [40], where males demonstrated a greater infection prevalence. The earlier research suggested that males' behaviors—such as increased roaming, territorial disputes, and more frequent interactions with other animals—might contribute to a higher risk of viral transmission. On the other hand, some experts believe that female dogs could be more prone to infection due to physiological stresses related to their reproductive cycles, such as estrus, pregnancy, and nursing. These factors can weaken the immune system, making females potentially more susceptible to infections like CCoV. Despite these observed differences in infection rates by sex, our statistical analysis found no significant association between gender and CCoV prevalence. This aligns with previous studies that reported no meaningful variation in infection rates between male and female dogs. The size of the dog breed has been recognized as an important factor influencing infection rates, with larger breeds showing a greater prevalence of infections compared to medium, small, and mixed-breed (mongrel) dogs. Several reasons might explain this pattern. Large dogs are often housed outdoors—such as on farms, in yards, or as guard dogs—where they have increased likelihood of contact with contaminated environments and more frequent interactions with other animals, including strays, which raises their chance of viral infection. On the other hand, small breeds tend to be kept indoors as pets, living in more controlled settings with limited exposure to outside pathogens. Their lower chances of coming into contact with infectious agents, along with generally better vaccination rates and closer medical care, likely contribute to their reduced infection rates. While breed size alone does not fully dictate infection risk, it works in conjunction with factors like environmental exposure, lifestyle, and the dog's role, influencing how infections spread. These observations highlight the need for focused health surveillance and preventive measures, especially for large breeds that are often kept outdoors or in communal environments where the likelihood of infection is higher. In this study, dogs that were infected often showed several symptoms associated with viral gastroenteritis occurring at the same time. The most common of these was watery diarrhea, indicating that it is a key sign of enteric canine coronavirus (CCoV) infection. This pattern matches previous findings, such as those by [41], who found that vomiting, diarrhea, and decreased appetite were the main symptoms seen with ECCoV. Interestingly, findings from [40] showed that healthy-appearing dogs actually tested positive for

ECCoV more often than those showing clinical symptoms. This highlights how widespread subclinical infections are and draws attention to the important role of dogs without symptoms in maintaining and transmitting ECCoV among canine populations. Together, these results emphasize that reliance on clinical signs alone is insufficient for accurate diagnosis. Laboratory confirmation is crucial, particularly during outbreak scenarios or when assessing vulnerable groups like young or unvaccinated animals, to ensure the effective detection and control of ECCoV infections.

Conclusions

This study highlights that enteric canine coronavirus (CECoV) is a major cause of gastrointestinal disease among dogs in Nineveh province, with a prevalence surpassing that of canine parvovirus. The findings underscore the need for continued surveillance, early diagnosis using rapid antigen detection tests, and improvement in immunization practices—especially among young, stray, and local dog populations. Implementing comprehensive public health strategies to increase awareness, vaccination coverage, and monitoring will be crucial to controlling CCoV and minimizing its impact on canine health.

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