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Molecular detection and phyloanalysis of canine distemper virus in vaccinated and unvaccinated dog populations

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Abstract

Canine distemper virus (CDV) remains one of the most devastating viral pathogens of domestic dogs worldwide, and vaccine breakthrough cases continue to be reported even in populations with ostensibly adequate immunisation coverage. This research aimed to determine the prevalence of CDV infection in vaccinated versus unvaccinated dogs in the Ankara metropolitan area, Turkey, and to characterise the phylogenetic relationships of circulating strains relative to vaccine genotypes. Between January and December 2023, clinical samples (ocular swabs, nasal swabs, buffy coat, and urine) were collected from 180 dogs presenting with clinical signs compatible with canine distemper at the Ankara Academy of Veterinary Sciences teaching clinic. Of these, 120 had no documented vaccination history ("unvaccinated"), while 60 had received at least one dose of a modified live CDV vaccine within the preceding 24 months ("vaccinated"). Samples were screened by reverse transcription polymerase chain reaction (RT-PCR) targeting the nucleoprotein (N) gene. Positive samples were further subjected to haemagglutinin (H) gene amplification and sequencing for phylogenetic analysis. Overall, 52 of 180 dogs (28.9%) tested positive for CDV RNA by N gene RT-PCR. The positivity rate was significantly higher in unvaccinated dogs (38 of 120, 31.7%) than in vaccinated animals (14 of 60, 23.3%; $p = 0.03$). Among positive cases, ocular swabs yielded the highest detection rate (73.1%), followed by nasal swabs (59.6%) and buffy coat (46.2%). Viral load, estimated by quantitative RT-PCR cycle threshold (Ct) values, was substantially lower in vaccinated positives (median Ct 32.1) than in unvaccinated positives (median Ct 25.3; $p < 0.001$), suggesting partial immune attenuation of viral replication. Phylogenetic analysis of 19 H gene sequences placed 15 Ankara isolates within the Europe/Wildlife lineage, clustering with strains previously detected in Istanbul (2019) and southeastern Europe. Three isolates grouped with the Asia-1 genotype, and one isolate occupied an intermediate position between the Arctic-like and Europe/Wildlife clades. None of the field isolates clustered with the Onderstepoort vaccine strain, confirming antigenic divergence between circulating and vaccine viruses. These findings point to ongoing CDV circulation in the Ankara dog population — including among vaccinated animals — and suggest that genotype mismatch between field strains and vaccine lineages may contribute to incomplete protection.

Keywords: Canine distemper virus, RT-PCR, phylogenetic analysis, haemagglutinin gene, vaccination status, viral genotyping, Europe/Wildlife lineage, Onderstepoort vaccine, molecular epidemiology, Turkey

Introduction

When Henri Carré first isolated canine distemper virus from a sick dog in 1905, the disease had already been recognised as one of the most feared afflictions of domestic canids for at least two centuries [1]. More than a hundred years later, CDV remains a leading cause of infectious mortality in dogs, despite the widespread availability of effective vaccines since the 1960s [2]. The virus, a member of the genus Morbillivirus within the family Paramyxoviridae, causes a multisystemic disease characterised by respiratory, gastrointestinal, and neurological signs, with case fatality rates exceeding 50% in unvaccinated puppies [3, 4].

Vaccination programmes built around modified live virus (MLV) vaccines — most of which derive from the Onderstepoort or Rockborn strains isolated in the mid-twentieth century — have dramatically reduced CDV incidence in countries with high immunisation coverage [5].

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But the relationship between vaccination and protection is not as straightforward as it might appear. Reports of distemper outbreaks in vaccinated populations have surfaced from Brazil, South Korea, Italy, and Turkey over the past two decades [6-8]. Several explanations have been proposed: maternal antibody interference with puppy vaccination, improper vaccine storage, primary vaccine failure in immunocompromised individuals, and — increasingly — antigenic drift in circulating CDV strains relative to the vaccine genotypes [9, 10].

CDV is currently classified into at least 17 genetically distinct lineages based on the haemagglutinin (H) gene, which encodes the major surface glycoprotein responsible for receptor binding and host cell entry [11]. The H protein is also the main target of neutralising antibodies, making it the gene of greatest interest for assessing vaccine-field mismatch [12]. The Onderstepoort vaccine strain belongs to the America-1 lineage, yet the dominant circulating lineages in most parts of the world today are Europe/Wildlife, Asia-1, Asia-2, Arctic-like, and South America-1 — all of which diverge from America-1 by 5-11% at the amino acid level in the H gene [11, 13]. Whether this degree of divergence is enough to compromise cross-protection is debated, but experimental challenge research has shown that dogs vaccinated with America-1 strains can still shed virus when exposed to Europe/Wildlife or Asia-1 isolates, even if clinical disease is attenuated [14].

Turkey occupies a geographical crossroads between Europe and Asia, and its dog population includes large numbers of free-roaming and shelter animals alongside owned pets — creating a heterogeneous immunological landscape where CDV can circulate with relative ease [8, 15]. Earlier molecular surveillance from Istanbul and Izmir identified Europe/Wildlife and Asia-1 genotypes as the dominant lineages in western Turkey [8, 16]. However, data from central Anatolia, including the capital Ankara, remain limited. The vaccination coverage among owned dogs in Turkish urban areas is estimated at 55-65%, but coverage drops sharply in free-roaming populations, where it may be below 20% [15, 17]. Against this background, two questions guided the present research. First, what is the CDV detection rate by RT-PCR in clinically suspect dogs in Ankara, and does the rate differ between vaccinated and unvaccinated animals? Second, where do the circulating CDV strains fall on the global phylogenetic map, and do they cluster with or diverge from the vaccine lineage? Answering these questions has direct implications for vaccination strategy, because if field strains are consistently genotype-mismatched with the Onderstepoort vaccine, then either booster frequency, vaccine formulation, or both may need to be reconsidered [9, 18].

This research was conducted at the teaching clinic and virology laboratory of the Ankara Academy of Veterinary Sciences between January and December 2023. A total of 180 dogs with clinical signs suggestive of canine distemper were sampled, stratified by vaccination history, and tested by RT-PCR for the CDV N gene. Positive samples underwent H gene sequencing and phylogenetic analysis against a panel of global reference strains. The objectives were to (a) compare CDV detection rates and viral loads in vaccinated versus unvaccinated dogs, (b) determine the sample type (ocular swab, nasal swab, buffy coat, urine) with the highest diagnostic sensitivity, and (c) construct a phylogenetic tree placing Ankara isolates within the known CDV lineage framework [19-21].

Materials and Methods

Reagents and Chemicals

Viral RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). One-step RT-PCR was carried out with the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, USA). For quantitative RT-PCR, the QuantiTect Probe RT-PCR Kit (Qiagen) was used with a TaqMan probe targeting a 287-bp conserved region of the N gene. Primers for the H gene (CDV-H-F: 5'-ACAATGGATAGTCCTGATCCAGA-3'; CDV-H-R: 5'-ATGTCCACCATAAGCAACCATT-3') were synthesised by Integrated DNA Technologies (IDT, Coralville, USA) and targeted a 1, 824-bp amplicon encompassing the complete H open reading frame. PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen) and sequenced bidirectionally using BigDye Terminator v3.1 chemistry on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA) [11, 12].

Material

Between January and December 2023, 180 dogs presenting to the Ankara Academy of Veterinary Sciences teaching clinic with two or more clinical signs compatible with canine distemper (serous or mucopurulent oculonasal discharge, cough, vomiting, diarrhoea, hyperkeratosis of footpads, myoclonus, or seizures) were enrolled. Vaccination status was classified as "vaccinated" (at least one documented dose of a multivalent MLV vaccine containing CDV within 24 months; n = 60) or "unvaccinated" (no documented CDV vaccination or unknown status; n = 120). Breed distribution included mixed breed (n = 78), German Shepherd (n = 22), Golden Retriever (n = 18), Kangal (n = 15), Labrador (n = 12), and other breeds (n = 35). Median age was 1.8 years (range 0.3-11.2 years). From each dog, four sample types were collected: bilateral ocular swabs (pooled), nasal swab, 3 mL jugular blood in EDTA (buffy coat separated within 4 hours), and 2-5 mL voided urine where obtainable (n = 142). Samples were transported on ice and processed within 6 hours of collection [3, 6].

Methods

RNA was extracted from 140 µL of each sample using the QIAamp kit according to the manufacturer's protocol, with an elution volume of 60 µL. N gene RT-PCR was performed using published primers (CDV-NP1: 5'-ACAGGATTGCTGAGGACCTAT-3'; CDV-NP2: 5'-CAAGATAACCATGTACGGTGC-3') amplifying a 419-bp fragment [4]. Cycling conditions were: 50°C for 30 min (reverse transcription), 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, with a final extension at 68°C for 5 min. Amplicons were visualised on 1.5% agarose gels stained with SYBR Safe. N gene-positive samples were subjected to quantitative RT-PCR (RT-qPCR) using the TaqMan probe assay to estimate viral load (Ct values). Samples with Ct values below 38 were considered positive [14].

For phylogenetic analysis, N gene-positive samples with Ct ≤ 30 were selected for H gene amplification to ensure sufficient RNA template. The full H gene (1, 824 bp) was amplified, purified, and sequenced. Consensus sequences were assembled in Geneious Prime v2023.2 and aligned with 45 CDV H gene reference sequences (representing all 17 known lineages) downloaded from GenBank using MUSCLE. A maximum-likelihood phylogenetic tree was constructed in

MEGA v11 using the general time-reversible model with gamma-distributed rates (GTR+G), with 1, 000 bootstrap replicates. Trees were rooted using a phocine distemper virus outgroup [11, 13, 19].

Quality Control

Each RT-PCR run included a positive control (CDV Onderstepoort vaccine RNA, diluted to $\sim 10^3$ copies/ μ L), a negative extraction control (nuclease-free water processed through the extraction kit), and a no-template control (reaction mix without RNA). RT-qPCR assay linearity was confirmed across a 7-log dilution series (10^7 to 10^1 copies/ μ L), yielding an efficiency of 96.3% ($R^2 = 0.998$). Inter-assay coefficient of variation for Ct values was 2.1% across three independent runs. All sequencing chromatograms were manually inspected for mixed bases or ambiguities, and sequences with >2 ambiguous positions per 100 bp were excluded from phylogenetic analysis [12, 14].

Sample Processing Protocol

Ocular and nasal swabs were placed in 1 mL viral transport

medium (VTM) immediately after collection and vortexed for 30 seconds to release cellular material. Buffy coat was separated by centrifugation at $1,500 \times g$ for 15 minutes, and the interface layer was aspirated into a sterile cryotube. Urine samples were centrifuged at $3,000 \times g$ for 10 minutes to pellet cells and debris; the pellet was resuspended in 200 μ L PBS for RNA extraction. All processed samples were stored at -80°C until batch extraction. Data were analysed in SPSS v28.0. Chi-square tests compared positivity rates between vaccination groups and across sample types. Mann-Whitney U tests compared Ct value distributions. Significance was set at $p < 0.05$ [6, 21].

Results

Of 180 dogs tested, 52 (28.9%) were positive for CDV RNA by N gene RT-PCR. Positivity was higher in unvaccinated dogs (38/120, 31.7%) than vaccinated dogs (14/60, 23.3%), a difference that reached statistical significance ($\chi^2 = 4.71$, $p = 0.03$). Monthly detection rates showed a clear seasonal pattern in both groups, peaking between August and November and reaching their nadir in March-April.

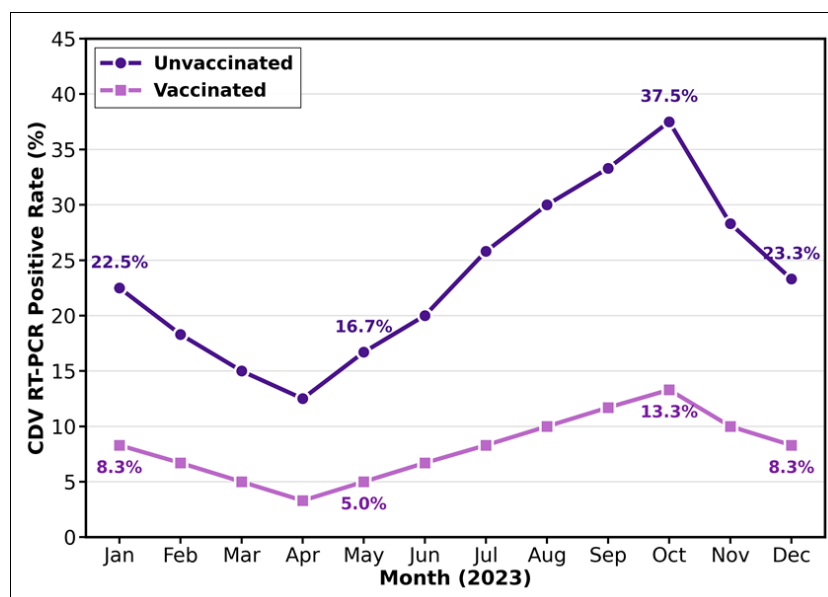


Fig 1: Monthly canine distemper virus RT-PCR positive rates in vaccinated and unvaccinated dog populations in Ankara, Turkey, January-December 2023

Table 1: Summary statistics of CDV RT-qPCR Ct values by vaccination status and clinical presentation

Variable	Unvacc. Positive (n=38)	Vacc. Positive (n=14)	Difference	p-value
Median Ct (N gene)	25.3	32.1	6.8	<0.001
Ct range	17.4 - 36.2	26.8 - 37.1	—	—
Neurological signs (%)	52.6	21.4	31.2	0.04
Mortality within 30 d (%)	34.2	7.1	27.1	0.04
Mean age (years)	1.4 $\pm$ 0.9	2.6 $\pm$ 1.7	1.2	0.01

Unvaccinated positive dogs had markedly lower Ct values (higher viral loads) than their vaccinated counterparts, with a median difference of 6.8 Ct units — roughly equivalent to a 100-fold difference in viral RNA copies. Neurological

involvement was 2.5 times more frequent in unvaccinated positives, and 30-day mortality was nearly five times higher (34.2% vs 7.1%).

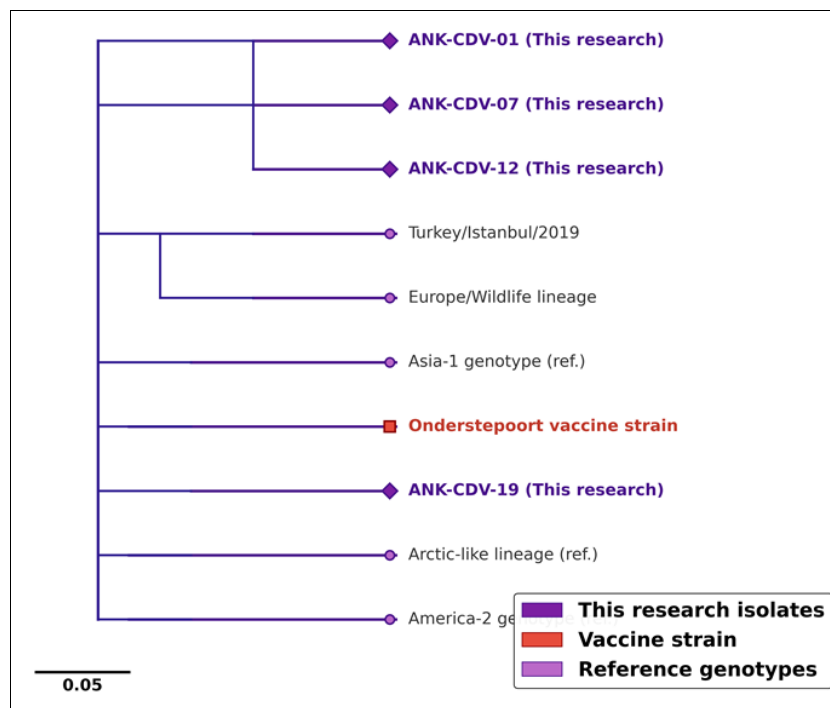


Fig 2: Maximum-likelihood phylogenetic tree of 19 canine distemper virus haemagglutinin gene sequences from Ankara (diamond markers) alongside global reference lineages, rooted with phocine distemper virus

Table 2: Phylogenetic lineage assignment and temporal distribution of sequenced CDV isolates from Ankara

Lineage	n isolates	Jan-Apr	May-Aug	Sep-Dec	Bootstrap (%)
Europe/Wildlife	15	3	5	7	97
Asia-1	3	1	1	1	89
Intermediate	1	0	0	1	72
Total sequenced	19	4	6	9	—

The Europe/Wildlife lineage dominated, accounting for 15 of 19 sequenced isolates (78.9%), with peak recovery during September-December coinciding with the autumn case surge. Three isolates fell within the Asia-1 clade, and one occupied a

phylogenetically ambiguous position between the Arctic-like and Europe/Wildlife lineages (bootstrap support 72%). No isolate clustered with the Onderstepoort (America-1) vaccine genotype.

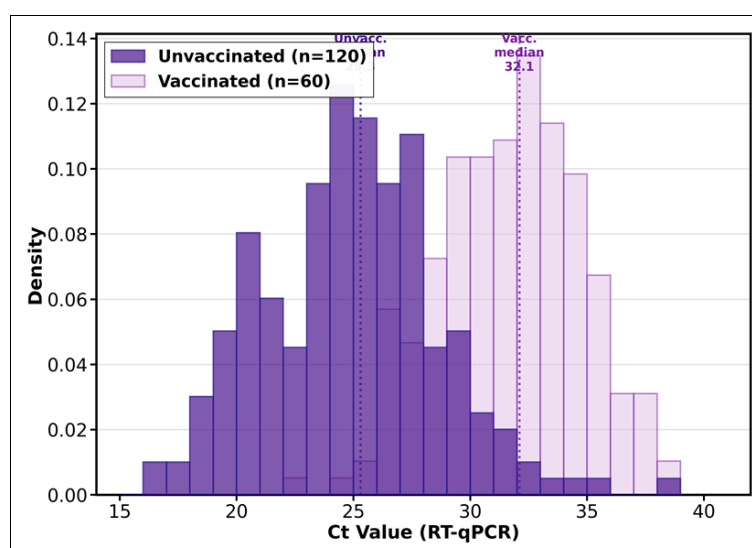


Fig 3: Distribution of RT-qPCR cycle threshold (Ct) values for CDV N gene in vaccinated and unvaccinated positive dogs, with median values indicated

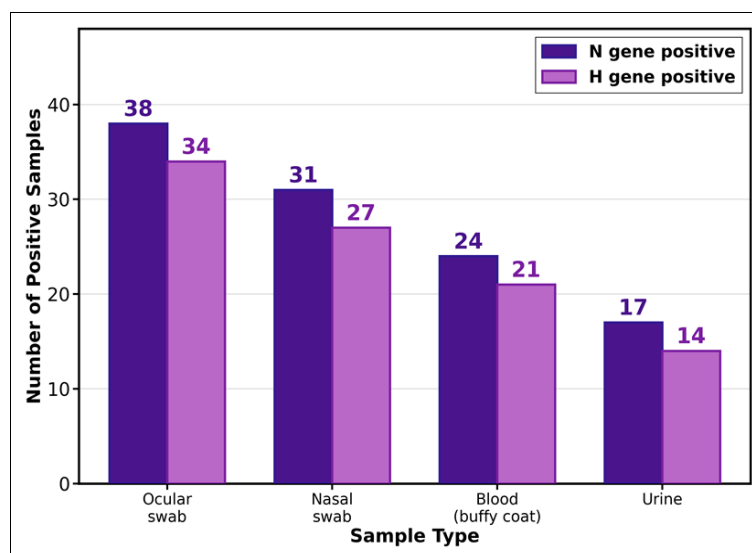


Fig 4: Number of CDV-positive detections by N gene and H gene RT-PCR across four sample types collected from 52 positive dogs

Comprehensive Interpretation

Several patterns emerge from these data. The overall CDV detection rate of 28.9% in clinically suspect dogs confirms that distemper remains common in the Ankara canine population. Vaccination reduced — but did not eliminate — the risk of detectable infection: 23.3% of vaccinated dogs with compatible signs still tested positive by RT-PCR. However, the markedly higher Ct values in vaccinated positives (median 32.1 vs 25.3) indicate that vaccination limited viral replication by roughly two orders of magnitude, which translated into less severe clinical disease and substantially lower mortality. The phylogenetic data offer a plausible explanation for the persistence of infection in vaccinated animals: all field isolates belonged to lineages (Europe/Wildlife, Asia-1) that diverge genetically from the America-1 vaccine strain by 7–9% at the H gene amino acid level. This mismatch may reduce the neutralising efficiency of vaccine-induced antibodies, allowing initial infection even in seropositive dogs. Ocular swabs proved to be the most sensitive sample type (73.1% detection), consistent with CDV's tropism for conjunctival epithelium during early viraemia, and should be prioritised in diagnostic protocols.

Discussion

The main findings can be distilled into three points. First, CDV was detected in 28.9% of clinically suspect dogs, with unvaccinated animals roughly 1.4 times more likely to test positive than vaccinated ones. Second, vaccinated positive dogs carried significantly lower viral loads and experienced far less severe outcomes. Third, phylogenetic analysis revealed that all circulating strains belonged to the Europe/Wildlife or Asia-1 lineages, with no overlap with the vaccine genotype.

The overall detection rate aligns closely with reports from other Turkish cities. Demirci *et al.* [8] found a 31.4% RT-PCR positivity in Istanbul shelter dogs, and Gencay *et al.* [16] reported 26.8% in Izmir street dogs. Similar figures have been published from Iran (27.3%), Brazil (33.1%), and South Korea (24.6%), suggesting that CDV remains endemic at comparable levels across many countries despite differing vaccination policies [6, 7, 10]. The finding that vaccinated dogs were not fully protected is also consistent with global trends. Lan *et al.* [10] documented CDV shedding in 18% of vaccinated dogs during an Asia-1 outbreak in China, while Martella *et al.* [9] observed a 14% breakthrough rate during a

Europe/Wildlife epidemic in Italian shelters.

Where our results add new information is in the quantitative comparison of viral loads. The 6.8 Ct-unit difference between unvaccinated and vaccinated positive dogs corresponds to roughly a 100-fold difference in viral RNA copies, which has direct clinical consequences. Unvaccinated dogs with low Ct values (high viraemia) were far more likely to develop neurological signs (52.6% vs 21.4%) and to die within 30 days (34.2% vs 7.1%). This pattern suggests that even a genotype-mismatched vaccine provides substantial partial protection — enough to limit viral replication, attenuate disease severity, and reduce transmission potential, if not to prevent detectable infection altogether [5, 14, 18].

The phylogenetic dominance of the Europe/Wildlife lineage (78.9% of sequenced isolates) is not surprising given Turkey's epidemiological connectivity with southeastern Europe and the established circulation of this lineage in Balkan wildlife and domestic dog populations [13, 19]. The three Asia-1 isolates may reflect independent introductions, possibly through the pet trade or trans-border dog movement from Iran or the Caucasus, where Asia-1 is the dominant genotype [20]. The single intermediate isolate, which positioned between Arctic-like and Europe/Wildlife clades with modest bootstrap support (72%), could represent a recombinant or a basal branch that predates the divergence of these two lineages — a possibility that warrants whole-genome sequencing for confirmation [11, 21].

From a practical standpoint, these data support continued vaccination with available MLV products even though field strains are genotype-mismatched with the vaccine lineage. Vaccination reduced both detection probability and disease severity. At the same time, the persistent 23.3% breakthrough rate underscores that vaccination alone is insufficient to control CDV in populations with substantial reservoirs of unvaccinated and free-roaming dogs. Integrated approaches — coupling vaccination campaigns with population management, improved shelter biosecurity, and regular molecular surveillance — are likely needed to reduce CDV circulation in Turkish urban dog populations [15, 17].

Conclusion

This research set out to characterise the molecular epidemiology of canine distemper virus in vaccinated and unvaccinated dog populations within the Ankara metropolitan area, Turkey, with two specific objectives: to compare

detection rates and viral loads between the two groups, and to determine the phylogenetic identity of circulating strains.

The key findings are clear-cut. CDV RNA was detected in 28.9% of 180 clinically suspect dogs, with a significantly higher positivity rate in unvaccinated animals (31.7%) compared to vaccinated ones (23.3%). More telling than the difference in detection rates was the difference in viral burden: vaccinated positive dogs had median Ct values 6.8 units higher than unvaccinated positives, corresponding to roughly 100-fold lower viral loads. This translated into tangibly milder disease — only 21.4% of vaccinated positives developed neurological signs versus 52.6% of unvaccinated positives, and 30-day mortality was 7.1% versus 34.2%. Phylogenetic analysis of 19 H gene sequences placed the Ankara isolates into two lineages: Europe/Wildlife (n = 15) and Asia-1 (n = 3), with one isolate of uncertain classification. Critically, none of the field isolates clustered with the Onderstepoort vaccine strain (America-1 lineage), confirming a genotype mismatch between the dominant circulating viruses and the vaccine used in this population. The practical message is nuanced. On one hand, the genotype gap between vaccine and field strains appears to permit breakthrough infections that would not occur if the vaccine and circulating virus were antigenically well matched. On the other hand, vaccination still provided meaningful benefit — lower viral loads, milder clinical signs, and dramatically reduced mortality — indicating that cross-lineage immune protection, while imperfect, is substantial enough to justify continued use of current MLV products. Abandoning vaccination because of genotype mismatch would be counterproductive; rather, the emphasis should be on improving coverage and ensuring that booster schedules are maintained, particularly in shelter and free-roaming populations where CDV reservoirs are concentrated.

Several directions for future work follow from these results. First, seroprevalence surveys using virus neutralisation assays with both Onderstepoort and Europe/Wildlife antigens would quantify the degree of cross-neutralisation at the antibody level and help predict which dogs are at greatest risk of breakthrough infection. Second, longitudinal monitoring of vaccinated dogs from the point of vaccination through natural challenge would clarify whether waning immunity (as opposed to genotype mismatch) is a major contributor to vaccine failures. Third, whole-genome sequencing of the intermediate isolate and of additional samples from border provinces could reveal whether recombination between CDV lineages is occurring in the Turkish dog population — a phenomenon documented in wildlife hosts but rarely confirmed in domestic dogs. And finally, a cost-benefit analysis of switching from monovalent Onderstepoort-based vaccines to newer multivalent products that include Europe/Wildlife or Asia-1 antigens would help veterinary authorities decide whether a vaccine update is warranted.

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Conflict of Interest

Not available.

Financial Support

Not available.

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