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RESEARCH ARTICLE

Molecular Characterization and Phylogenetic Analysis of Canine Parvovirus Isolates in Palestine

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A B S T R A C T

Canine parvovirus (CPV) is a highly contagious viral disease and the most significant intestinal pathogens affecting dogs as well as puppies, making these infections a real danger on dog population worldwide. Therefore, this study was to elucidate and detect canine parvovirus strains circulating in Palestine. This was achieved by molecular analyzing of VP2 gene by polymerase chain reaction (PCR). In the current study, a total number of 25 dogs suffered from severe watery bloody diarrhea, vomiting and lethargy were examined serologically to confirm parvovirus antigens. Complete Blood count (CBC) was also involved to assess the effects of the virus on the hematological parameters of each dog. The PCR positive samples were evaluated by Sanger's sequencing method to characterize the virus and to obtain the essential information about the genotypes and nucleotide polymorphisms of CPV strains circulating in Palestine. The partial nucleotide sequences of VP2 gene were compared with reference VP2 gene sequences of CPV recorded in GenBank database. The phylogenetic analysis revealed that 24/25 (96%) of the sequences belonged to serotype CPV-2c and 1/25 (4%) belonged to serotype CPV-2b. The current obtained sequences were registered at the GenBank database under the following accession numbers: OQ924950- OQ924974. To our knowledge, this report is considered the first one to investigate the molecular characterization of CPV-2 in Palestine. This finding could be useful for commercial vaccine companies to select the suitable strains of CPV that include the prevalent antigenic types of the field virus, to enhance the immunity against CPV in dogs.

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INTRODUCTION

Canine parvoviruses (CPV) are among the most significant intestinal pathogens affecting dogs as well as puppies, making these infections a real danger on dog population worldwide (Decaro and Buonavoglia, 2011; Caddy, 2018). The causative agent is naked, single-stranded DNA virus, belonging to the Parvoviridae family, subfamily Parvovirinae, genus Protoparvovirus (Decaro and Buonavoglia, 2011; Hoang *et al.,* 2019). It was first identified in the late 1970s as a new and rapidly spreading disease in dogs, causing severe gastrointestinal distress and high mortality rates, particularly in puppies (Appel *et al*., 1978; Quintero-Gil *et al*., 2019). Since its emergence, CPV has evolved into several distinct strains, including CPV-2a, CPV-2b and CPV-2c, which vary in their antigenic properties and virulence (Zhou *et al*., 2017; Dema *et al*., 2023). These 3 variants differ from each other in capsid protein VP2 by specific amino acids at residue number 426, CPV-2 and CPV-2a: Asn; CPV-2b: Asp; CPV-2c: Glu (Decaro and Buonavoglia, 2011). The persistence and widespread nature of CPV make it a crucial subject for ongoing research and surveillance.

Canine parvoviruses are primarily transmitted through direct contact with infected dogs or indirectly through contact with contaminated feces, surfaces, or fomites

(Quintero-Gil *et al.*, 2019). This virus is extremely resistant to environmental conditions and can live for months in the environment, making it a formidable pathogen (Goddard and Leisewitz, 2010). Clinical signs, history, and laboratory tests such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and virus isolation by cell culture are used in diagnosis of CPV infections (Kurucay *et al*., 2023). Early and accurate diagnosis is crucial for prompt treatment and improved prognosis. There is no specific antiviral therapy for CPV; treatment focuses on supportive care, including fluid therapy, electrolyte balance, and antibiotics to prevent secondary bacterial infections. Early intervention and aggressive treatment can improve survival rates, particularly in puppies.

Knowledge about the genetic diversity and the genetic structure of CPV can help to understand origins and frequencies of introductions, identify the possible new emerging CPV strains, assess the risk of spread of disease and their transmission dynamics, and could help in the development of appropriate control strategies (Kaur *et al*., 2015; Timurkan and Oğuzoğlu 2015; Singh *et al*., 2021).

The CPV-2 infection and/or genetic diversity has never been studied in Palestine and many other countries around. In this report, the first partial VP2 gene sequences of Palestinian isolates of CPV-2 were determined, used for phylogenetic analysis and compared with other CPV-2 sequences of the same gene that retrieved from GenBank database.

MATERIALS AND METHODS

Sample collection: A total of 25 samples were collected from dogs brought to the Royal Care Veterinary Hospital at the city of Nablus. Most animals suffered from severe diarrhea, lethargy and fever and were suspected for CPV. Detailed clinical history was taken for each case. The stools of these cases were tested with the Canine Parvovirus Antigen (CPV Ag) Test (Asan Easy Test® PARVO, Asan Pharm Co., LTD). The stool samples from the positive cases were kept at -18°C at the laboratories of the Faculty of Agriculture and Veterinary Medicine until used. In addition, a complete blood count (CBC) was conducted for each case.

Extraction of genomic DNA: The manufacturer's instructions were strictly followed when utilizing the Macherey-NagelTM NucleoSpin® DNA Stool-extraction kit to extract genomic DNA from fecal samples collected from dogs with positive Canine Parvovirus Antigen (CPV Ag) test results.

Molecular diagnosis: In order to investigate CPV, PCR was carried out using previously reported primers (Buonavoglia *et al*., 2001; Fatima *et al*., 2017). The primer H-par-F (5'- CAGGTGATGAATTTGCTACA -3') targets the sequence of the VP2 gene of CPV-2 from position 3556 to 3575, while the primer H-par-R (5'- CATTTGGATAAACTGGTGGT -3') targets the sequence from position 4185 to 4166. The amplification give rise to a product of 630-bp. As positive control, DNA from commercial vaccine was used (Primodog, MERIAL) and a negative control of molecular biology grade water was used in this experiment.

Thermal conditions comprised of 95°C for 5 minutes as initial denaturation, 35 cycles of 94°C for 30 seconds as denaturation, 51.3°C for 1 minute as annealing, 72°C for 45 seconds as extension, and 72°C for 10 minutes as a final extension. The amplification conditions for DNA were achieved in a GenAmp PCR System 9700 (Applied Biosystems, Foster City, USA).

Separation of PCR products were carried out on 1.5% agarose gel at 110 V for 30 minutes. The PCR products were observed under UV light after staining with Ethidium bromide. Then, according the manufacturer's recommendations, the amplified products were further cleaned up employing the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel).

Sequencing and phylogenetic analysis: The cleaned up PCR products were Sanger-sequenced. The partial VP2 gene of CPV-2 sequences of the current study were assessed using BLAST (Basic Local Alignment Search Tool) and matched with other sequences of CPV-2 already available in the GenBank database.

Sequence homology and phylogenetic analysis: Using the BLAST system, the continuous partial VP2 gene sequences were compared to other previously published sequences of the same gene from CPV-2 genotypes deposited in NCBI (National Center for Biotechnology Information). The ClustalW in MEGA version 6 software was used to carry out multiple alignment of DNA sequences (Tamura *et al*., 2013). The degree of variation among CPV-2 genotypes obtained in this study was calculated via pairwise nucleotide sequence comparison with available sequence information (CPV-2a, CPV-2b, and CPV-2c) from the NCBI database. The phylogenetic analysis was depended on Clustal W alignments of a 561-bp segment of VP2 gene sequence. The phylogenetic tree was generated in MEGA version 6 software using the Neighbor-Joining program. The tree was drawn to scale, with length of branches corresponding to the evolutionary distances to construct the phylogenetic tree calculated by Kimura 2-parameter method (Kimura, 1980). The robustness of the groupings assessed with 1000 bootstrap resampling. The sequences of Turkey parvovirus USA (GU214705.1) and Human parvovirus 4 Ivory Coast (JN798193.1) were used as an out-group to study phylogenetic analysis for CPV-2 genotypes.

The CPV-2c and CPV-2b genotype sequences of partial VP2 gene and their products were multiple aligned with a references CPV-2c and CPV-2b sequences retrieved from GenBank to evaluate the variation in partial VP2 gene sequences and their products compared with references. In addition, the deduced amino acids of our CPV-2c genotype sequences in this study were aligned together to detect variations among these sequences.

RESULTS

Clinical signs: During clinical examination, variation in clinical manifestations of infection among infected dogs was noticed. 3 dogs (12.0%) displayed fever, anorexia, depression, bloody foul-smelling diarrhea, bloody vomiting and dehydration; 5 dogs (20.0%) had anorexia, bloody foul - smelling diarrhea and depression;

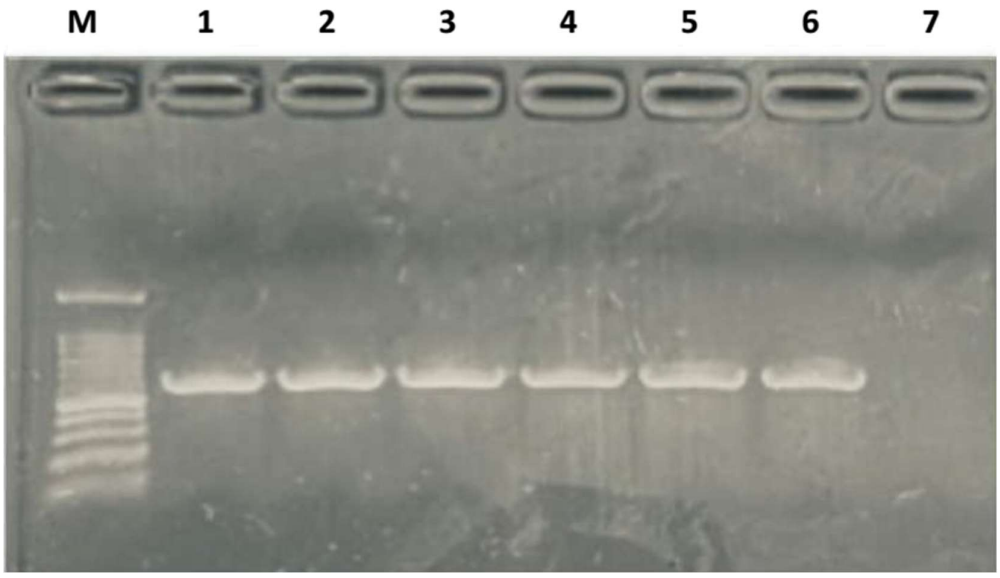

Fig. 1: PCR amplification products have been separated by electrophoresis using agarose gel concentration 1.5%. Lane M: 100-bp ladder DNA (Cibus), Lane 1: positive control (Primodog, Merial, France), Lanes 2-6: tested samples and Lane 7: negative control.

5 dogs (20.0%) suffered from anorexia, non-bloody diarrhea and dehydration; 5 dogs (20.0%) showed anorexia, bloody foul-smelling diarrhea and dehydration, and 7 dogs (28.0%) had only anorexia and depression.

Complete blood count (CBC): The CBC is a comprehensive assessment of the blood cell components including red blood cells (RBCs), white blood cells (WBCs), and platelets. In this study, the hematological analysis of the 25 cases infected with CPV-2, revealed that three cases (12.0%) had leukocytosis, and 14 cases (56.0%) had leukopenia. Additionally, 11 cases (44.0%) had lymphocytosis, while three cases (12.0%) had lymphopenia. Only two cases (8.0%) had neutrophilia and 18 cases (72.0%) had neutropenia. None of the cases showed erythrocytosis, while 7 cases (28.0%) had anemia. None of the cases showed hyperhemoglobinemia and 10 cases (40.0%) had hypohemoglobinemia. Four cases (16.0%) had thrombocytosis while three cases (12.0%) had thrombocytopenia.

PCR: All the 25 samples tested with the CPV-Ag Test (Asan Easy Test® PARVO, Asan Pharm Co., LTD), were positive with PCR using primers H-par-for and H-par-rev. The amplicon size was 630-bp length (Fig. 1)

Sequence homology and phylogenetic analysis: The genetic variety of CPV-2 genotypes circulating in Palestine was calculated using partial-length of VP2 gene sequences and their relationship with currently utilized vaccine strains. Sequence analyses of PCR amplicons were performed with same primer sets of conventional PCR (H-par-F and H-par-R primers). The obtained sequence results were compared to reference sequences retrieved from GenBank database (Fig. 2). Sequence analysis by NJ method of partial-length of VP2 gene sequences indicated there are two different variants of CPV2 circulating in Palestinian field. These include CPV-2c (residue 426 is Glu in CPV-2c) and COV-2b (residue 426 is Asp in CPV-2b). The strains from Palestine that grouped into a clade that has CPV-2c strains are the most common variant (96.0%) in Palestine, which are closely related to isolates from China, India and Nigeria. One strain (4.0%) from Palestine clustered into a clade that has CPV-2b strains, which is closely related to isolates from Australia and Japan. It is noteworthy that the variant CPV-2a did not exist in our research.

Analysis of CPV-2c VP2 sequences obtained in the current study from clinical samples showed more than 99.3% identical to each other (variation among CPV-2c sequences ≤0.7%). The CPV-2c sequences detected in this study were associated with 99.51%-100% identical to sequences isolated from India, Nigeria and China. However, CPV-2b VP2 sequence (OQ924960) obtained in this study had 99.84% identical to Australian CPV2b sequence (MN259054.1). Results of this study showed that 95.8% (23/24) of isolates of partial VP2 gene of genotype CPV2c sequences had a silent mutation at nucleotide position 1377 (C→T), which has amino acid N at amino acid position 459 in comparison with a reference MK518018 from China. This mutation is considered the most common in partial VP2 gene of genotype CPV2c sequences from Palestine. Analysis of the mutations in isolates of partial VP2 gene of genotype CPV2c sequences from Palestine showed that a total of 71% of mutations are silent mutations. Results of this study showed that 58.3% (14/24) of isolates of partial VP2 gene products of genotype CPV2c sequences had one or 2 amino acid substitutions compared to the reference isolate with accession number MK518018 from China (accession number for protein product of the gene is QCC20423). However, 41.7% (10/24) of isolates of partial VP2 gene products of genotype CPV2c sequences did not show amino acid substitutions. The isolate from Palestine with accession number OQ924960 which has a genotype CPV2b had nucleotide substitution at position 1252 A→C compared to the reference with accession number MN259054 genotype CPV2b from Australia. This led to amino acid substitution at position 418 I→L. Amino acid variations among partial VP2 sequences of CPV-2c genotypes (Fig. 3).

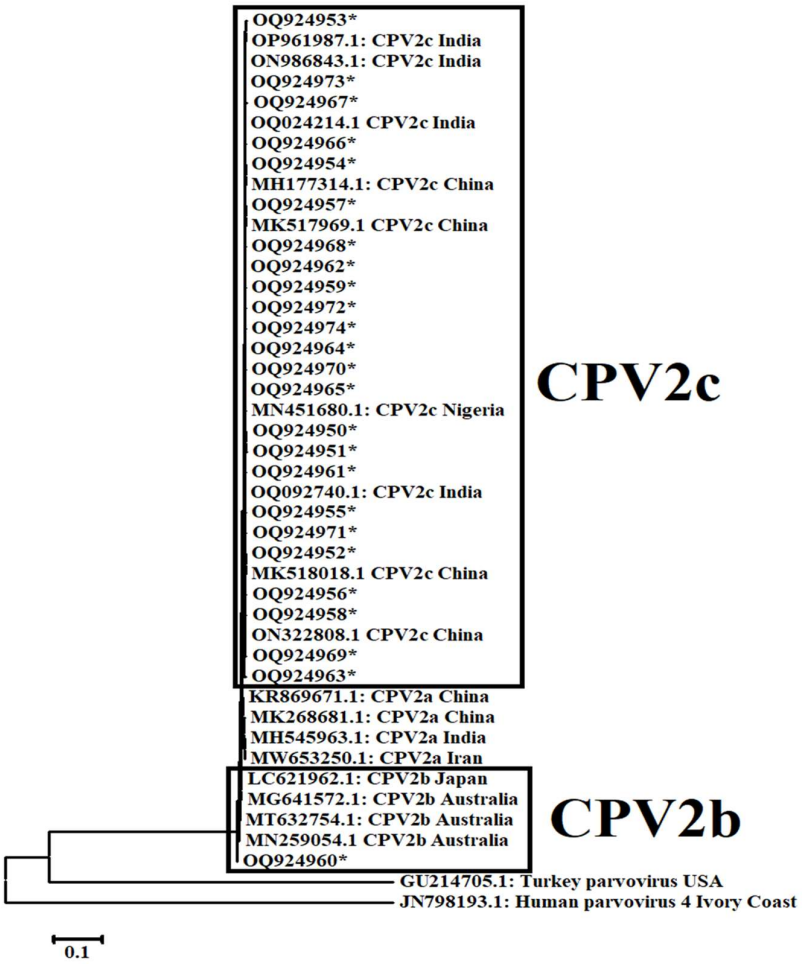

Fig. 2: Neighbor-Joining tree of 25 CPV2 in this study based on 561-bp of the VP2 gene sequence using Neighbor-Joining method. Sequences from Palestine for the VP2 gene of CPV2 denoted by asterisk and have accession numbers OQ924950- OQ924974. Reference sequences for VP2 gene of Canine parvovirus type 2 from different countries were retrieved from the GenBank database, including genotype CPV2a, CPV1b and CPV2c were used for phylogenetic analysis. The sequences of Turkey parvovirus USA (GU214705.1) and Human parvovirus 4 Ivory Coast (JN798193.1) were used as an out-group to study phylogenetic analysis.

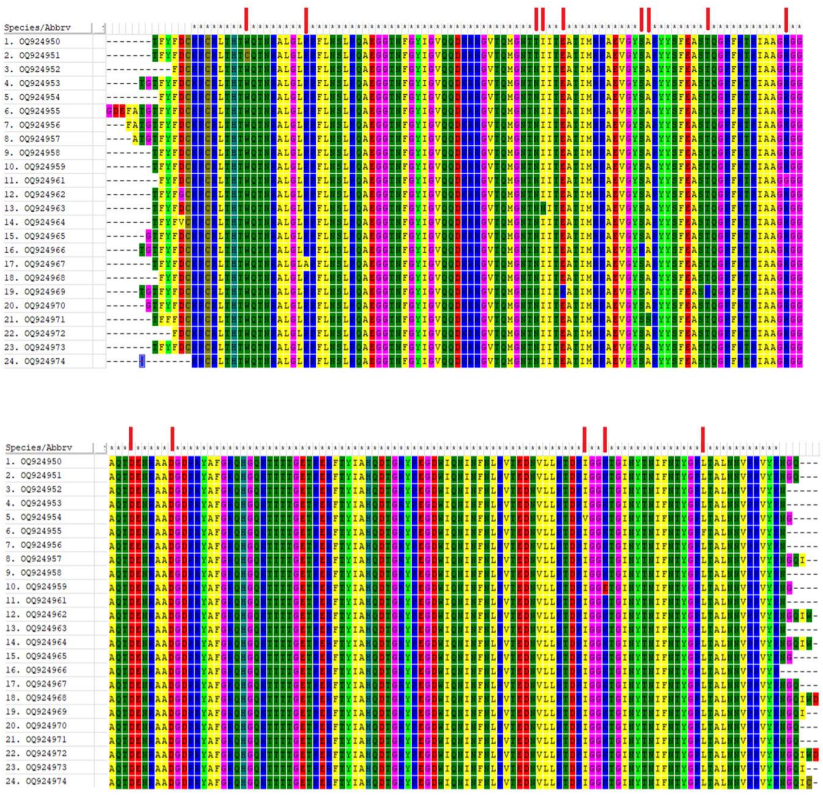

Fig. 3: The multiple amino acid alignments of the Palestinian sequences of CPV-2c antigenic types, the red lines showed the location of mutation in the CPV-2c gene product.

DISCUSSION

One of the main reasons for conducting this research is the lack of any information or data about CPV-2 infections in dogs in Palestine, and there is no study describing the molecular characteristics of CPV-2 variants in this country.

Clinical variability for CPV-2 disease progression has been reported previously (Lamm and Rezabek, 2008; Goddard and Leisewitz 2010; Calderón *et al*., 2011; Decaro and Buonavoglia, 2011; Quintero-Gil *et al*., 2019). Several factors have been responsible for the clinical variability for CPV-2 disease, including age, immune status, route of exposure, dose of virus, virulence of the strains and co-infection with other pathogens (Lamm and Rezabek, 2008; Decaro and Buonavoglia, 2011).

CBC is considered an important tool that can give information about the severity of illness, disease prognosis and treatment efficiency. Leukopenia is considered a major hematological abnormality in CPV-2 infected dogs (Mylonakis *et al*., 2016). This is due to the damage of the blast cells in bone marrow and lymphoid tissue atrophy, which leads to a deficiency in neutrophils and/or lymphocytes. However, only 56.0% of the infected dogs in this study showed leukopenia during examination, which is in accordance with other publications that reported 35.0-49.0% of the infected dogs with leukopenia (Mylonakis *et al*., 2016; Faz *et al*., 2017). However, leukopenia should not be used as a diagnostic test for CPV-2 infection, because it does not provide an indication for the virus infection (Faz *et al*., 2017). Other hematological abnormalities such as anemia, thrombocytopenia or thrombocytosis, pancytopenia, neutrophilic leukocytosis, neutropenia, lymphopenia, monocytosis and others may be observed in CPV-2 infected dogs (Mylonakis *et al*., 2016; Turley *et al.*, 2023). The clinical signs of the infected dogs with CPV-2c serotype have similar signs to other dogs infected with other genotypes of CPV-2a and CPV-2b, such as vomiting, diarrhea, fever, anorexia, depression, and leukopenia (De la Torre *et al*., 2018; Tuteja *et al.*, 2022).

The genotypes CPV-2a, CPV-2b, and CPV-2c have been detected in various proportions across several different countries (Nandi *et al*., 2009). The sequence analysis of VP2 gene sequences indicated that there are two different variants of CPV2 circulating in Palestine including CPV-2c as the major variant and CPV-2b. The results are consistent with other published epidemiological studies, which showed that the CPV-2c genetic variant is the major field strain in different parts of the world (Decaro and Buonavoglia, 2011; Castillo *et al*., 2020; Ogbu *et al*., 2020; Nguyen Manh *et al*., 2021). Data suggest that CPV-2c may be substituting CPV-2b as it was the most prevalent serotype (Meers *et al*., 2007) or these results suggest that the CPV-2c virus genotype may be introduced from China, India and Nigeria, while CPV-2b from Australia into Palestine through contaminated dogs and contaminated premises. However, these results are inconsistent with other results reported from different countries which showed that the CPV-2a or CPV-2b genetic variants are the major field strain infected the dogs (Timurkan and Oğuzoğlu, 2015; Amrani *et al*., 2016; Dei Giudici *et al*., 2017; Dinçer, 2017; Duque-García *et al*., 2017; Fatima *et al*., 2017; Sheikh *et al*., 2017; De la Torre *et al*., 2018; Polat *et al*., 2019; Etman *et al*., 2021; Ghajari *et al.*, 2021; Singh *et al.,* 2021; Abas *et al*., 2022).

Vaccination is an extremely important method used to protect and control CPV-2 infections in dogs (Qi *et al*., 2020). Both killed and attenuated live virus vaccines have been used for this purpose. According to Palestinian veterinary policy, dogs should be vaccinated at 2 months of age; the vaccines routinely used include variants of CPV-2, 2a, and 2b. The emergence of new antigenic variants has raised concerns about the efficiency of available vaccines because of the likelihood of vaccine protection is not achieved (Zhou *et al*., 2017).

The selection of vaccine must be carefully taken in consideration because, while protection against CPV-2a/2b serotypes using live attenuated vaccines has been stated to protect dogs for a maximum of 9 years (Litster *et al*., 2012), there is a strong inconsistency among researches dealing with the protection of these conventional vaccines against CPV-2c serotype (Hernández-Blanco and Catala-López, 2015; Miranda and Thompson, 2016; Yip *et al*., 2020). Vaccines should have the appropriate strains of CPV that includes the prevalent antigenic types of a field virus in order to provide a complete protection. So the findings of the current study's genotype analysis can be used to compare the vaccine genotypes and field genotypes molecularly, which may be useful for future research on vaccine development and production.

Conclusions: This study highlighted the importance of conducting molecular researches for the early detection and identification of emerged new genotypes of CPV-2. The mutations at DNA and amino acid levels suggest that the virus has evolved and emerged continuously. Since this virus is a DNA virus, its DNA substitution rate is significant, which leads to evolving new genotypes.

Additional and continuous molecular and epidemiological researches are needed in Palestine to evaluate the CPV infections as well as circulating genotypes and new genotypes evolved in dogs. This can help in selection the suitable vaccine that can protect dogs from infection. In case to provide a high or full protection, the used vaccines should involve all the circulating antigenic types of a field.

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Authors contribution: all authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

SA and GA: conceived the experimental design, contributed in sequencing and phylogenetic analysis of PCR final product in this experiment and preparation and reviewing of the research paper with statistical analysis; AD and MA: shared their experience in genetic analysis, review of gene primer design and linking it to genetic markers, drafted the manuscript and participated in DNA isolation and PCR; NK: participated in gel electrophoresis and review of the scientific paper after writing it; MA and TA: participated in sample collection, labeling and processing in the lab, which contributed greatly to the experiment.

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