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Research Article

How Can a Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Protocol Be Proposed for the Detection of Canine Coronavirus?

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Abstract

Coronaviruses (CoVs) comprise a group of viruses widely distributed globally that primarily affect the enteric and respiratory systems of various animal species. Canine Coronavirus (CCoV) causes a common infection, particularly in animals housed in large groups, such as in kennels or shelters. Although traditionally associated with mild clinical signs, CCoV has emerged as a highly significant pathogen in veterinary medicine. In 2005, a virulent variant known as pantropic canine coronavirus was identified for the first time; it is capable of causing systemic disease and high mortality rates in puppies. The virus is primarily transmitted via the fecal-oral route, and clinical signs of the enteric form include lethargy, anorexia, vomiting, and diarrhea. However, the most common presentation is asymptomatic, where the animal shows no signs but sheds the virus into the environment. In cases of pantropic disease, severe signs such as profound lymphopenia, ataxia, and seizures also occur. Definitive diagnosis is complex and not routinely performed, as the clinical presentation resembles that of other agents, such as canine parvovirus. While techniques like ELISA and electron microscopy exist, more precise methods are required. Given that the disease is underrecognized and difficult to diagnose based solely on clinical signs, this project proposes a conventional RT-PCR molecular protocol using *in silico-designed* primers targeting the M gene (membrane protein) to provide an alternative diagnostic option.

Background

Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses classified within the family *Coronaviridae*, order *Nidovirales*. At the ultrastructural level, they are characterized by a crown-like appearance due to spike proteins protruding from the virion surface, a feature that gives rise to their name [1]. These viruses are primarily associated with enteric and respiratory diseases affecting both mammals and birds [2]. The coronavirus particle envelope is composed of four main structural proteins-Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N)-encoded by ORF2, ORF4, ORF5, and ORF6, respectively [3,4]. CoVs were initially classified into three antigenic groups (I, II, and III) based on phylogenetic and antigenic analyses. Group I included canine coronavirus, along with feline coronavirus types 1 and 2, porcine transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine epidemic diarrhea virus, and human coronaviruses 229E and NL63 [2]. However, current classification establishes that the *Coronaviridae* family is divided into the subfamilies *Coronavirinae* and *Torovirinae*, which share similarities in morphology, genomic organization, and gene

expression mechanisms. In turn, the *Coronavirinae* subfamily is subdivided into four genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus* [5,6]. Due to the close genetic relationship between some of these viruses-evidenced by greater than 96% amino acid identity in key domains of the replicase 1ab-porcine Transmissible Gastroenteritis Virus (TGEV), Canine Coronavirus (CCoV), and Feline Coronavirus (FCoV) are no longer considered independent viral entities, but rather variants with distinct host ranges belonging to the same species, designated *Alphacoronavirus-1* [7].

Canine Coronavirus (CCoV) is widely distributed worldwide [7]. This virus is host-specific, affecting only domestic and wild canids [8] two main genotypes are recognized-CCoV type I and CCoV type II-both associated with enteric disease, with the simultaneous circulation of both frequently observed. Furthermore, CCoV type II is subdivided into types a and b, comprising classical CCoV and TGEV-like strains, respectively [7]. Additionally, Canine Respiratory Coronavirus (CRCoV) has been described; it differs genetically and antigenically from the aforementioned viruses and belongs to the betacoronavirus group [2].

The M gene

The M gene is used because M proteins are the most abundant proteins in any coronavirus virion and are among the most conserved and constrained viral structural proteins. They play a key role in viral assembly, budding, and immunomodulation [8].

Pathogenesis

It begins when the virus enters the host and invades the enterocytes at the tips of the intestinal villi, causing destruction, shortening, atrophy, and loss of the brush border, as well as subsequent generalized mild gastroenteritis-primarily in puppies [9]. Diarrhea ensues, followed by viral shedding into the environment; the viral load is very high at the onset of the first clinical signs. Fecal shedding occurs over a period of 6 to 9 days but may be prolonged in some puppies [10].

Clinical presentation

Clinical signs of canine coronavirus disease with enteric manifestations include the sudden onset of diarrhea; vomiting, anorexia, and lethargy-with or without fever-may also be present [9]. An asymptomatic form of CCoV also exists; although damage to the small intestine and viral shedding in feces occur, there are no clinical signs, yet the disease can still spread to susceptible puppies or adult dogs.

CCoV infection is highly prevalent in shelters and kennels, where the enteric form is characterized by high morbidity and low mortality-the latter being related to the age at infection, exposure to the virus, and the level of maternal antibody transfer [9]. However, fatal cases are often associated with mixed infections involving canine coronavirus and other viral pathogens, such as canine parvovirus type 2, canine adenovirus type 1, or canine distemper virus [11].

Diagnosis

Clinical diagnosis is complex because clinical signs can be confused with those of other diseases, such as parvovirus and certain parasitic infections [9], consequently, diagnostic testing is routinely performed [3].

Diagnosis can be achieved through the detection of viral antigens and antibodies using the following techniques:

Direct Immunofluorescence: Can only be performed post-mortem using frozen histopathological sections or small intestine impression smears [12].

Electron Microscopy: Useful for detecting the virus in feces. If the sample contains a low viral load, it may yield false-negative results [12].

Cell Culture Isolation: For *in vivo* diagnosis, the best sample is feces [12]. It should be noted that isolating CCoV type I in cell cultures is impossible, which hinders the acquisition of information regarding its pathogenic role [2].

ELISA: Ideal for processing large numbers of samples due to its rapid execution and high automation potential [8].

Reverse-Transcriptase PCR: Offers high sensitivity and specificity, in addition to providing a faster diagnosis than conventional methods. It can be used on fecal samples even when the virus is inactive or the number of virions is low [12].

Serum Neutralization: Although neutralizing antibody levels against CCoV are low, this test is the most specific method for quantifying the serological response [8].

Indirect Immunofluorescence: Available as immunoenzymatic that detect the presence of antibodies against CCoV; They are commercially available and easy to use [12].

Immunohistochemistry: Monoclonal antibodies are used to detect highly pathogenic CCoV antigen in tissues such as the lungs, kidneys, and liver [9].

Treatment

In cases of enteric presentation, intravenous fluid and electrolyte replacement is suggested; antiemetics, intestinal mucosal protectants, appetite stimulants, and B-complex vitamins may also be prescribed [9]. There are no antiviral drugs available for the treatment of CCoV infections [3].

Prevention

Cleaning and disinfecting areas where dogs infected with CCoV have been housed, along with the application of temperatures above 65°C (for 40 minutes) or 75°C (for 30 minutes), can reduce

infection. Ultraviolet light has not been shown to have a significant effect on reducing viral load [9].

Good hygiene regarding the feeding, housing, and care of dogs is also crucial for preventing CCoV. It is important to keep food and water bowls, as well as the animals' bedding, clean through periodic disinfection with virucidal solutions. Likewise, treating conditions such as coprophagia is vital, given that the fecal-oral route is the primary mode of infection [9]. Vaccines for CCoV prevention exist and contain inactivated and attenuated viruses. However, although they are safe, they do not provide complete protection, as they reduce but do not eliminate viral replication in the intestinal tract. Additionally, since these vaccines are based on classic CCoV IIa viruses, they do not protect against CCoV-I strains, and protection against variant type II strains is uncertain [3]. In Chile, there are various commercial brands authorized by the SAG that aim to protect against CCoV, including Vanguard Plus CPV/CV, Quantum CV, and Recombitek C6/CV (all inactivated), from the laboratories Pfizer Inc., Intervet Inc., and Sanofi Pasteur S.A., respectively [13]. Given the background information and the difficulty of diagnosing this disease based solely on clinical presentation, this thesis project proposes a conventional RT-PCR molecular protocol using *in silico* -designed primers targeting the M gene-a conserved coronavirus gene-to provide an alternative diagnostic option. Thus, here were propose a reverse transcription-polymerase chain reaction (RT-PCR) protocol for the detection of CCoV, with design for optimal primers *in silico* for the amplification of the canine coronavirus M gene and to present a molecular proposal for the detection of canine coronavirus.

Materials and Methods

This project can be carried out in any laboratory in the Third World, thanks to Kary Mullis's brilliant idea [14] and the availability of various bio-tools that are still free on the Internet.

To Design Primers *in Silico* for the Amplification of the Canine Coronavirus M Gene.

The GenBank® database will be used to access published and known sequences of the canine coronavirus M gene. These sequences will be aligned using the free online program Clustal Omega to obtain a consensus sequence containing at least two candidate regions for subsequent primer design. The free-access online tool OligoPerfect™ Designer will be used to generate a ranking of optimal primers based on the following criteria:

a. Primer length: 18-25 nucleotides

b. GC content (%GC): approximately 50%

c. Melting temperature (Tm): 50-60°C

d. Absence of secondary structures: primers must not form dimers or hairpin structures.

The amplicon length will be determined based on the length of the consensus sequence and the location of the candidate regions (e.g., 500 bp).

To Provide a Molecular Protocol for the Detection of Canine Coronavirus.

Following RNA extraction (Annex 1), the proposed conditions for molecular detection will utilize the previously designed primers, alongside the general parameters for a conventional RT-PCR reaction, as detailed below:

a) RT-PCR Reaction

Reaction Mixture

To perform the RT-PCR procedure, the Invitrogen® "SuperScript™ III One-Step RT-PCR System with Platinum™ Taq" kit will be used in accordance with the manufacturer's procedural guidelines: 25µL of "2X Reaction Mix" (buffer containing 0.4 mM of each Deoxynucleotide Triphosphate (dNTP) and 3.2 mM MgSO4), 10µL of RNA template (positive control, negative control, or nuclease-free water), 2µL of each primer (as defined above), 2µL of "SuperScript™ III RT/Platinum™ Taq Mix," and nuclease-free water to achieve a total volume of 50µL.

b) Amplification of Genetic Material

An Apollo thermal cycler (CLP, USA; or another) with 96 wells (0.2 mL each) will be used to perform the reaction. The amplification program will begin with reverse transcription at 48°C for 30 minutes, followed by initial denaturation and activation of Platinum Taq™ at 95°C for 2 minutes. Subsequently, a sequence of 40 cycles will be performed (denaturation at 95°C for 30 seconds; annealing at 55°C* [determined using a temperature gradient thermal cycler] for 30 seconds; elongation at 72°C for 30 seconds), followed by a final elongation step at 72°C for 7 minutes.

c) Visualization of the Amplified Product

The RT-PCR product can be visualized via agarose gel electrophoresis in TAE (Tris-acetate-EDTA) buffer, run at 90 V for 40 minutes, using an appropriate molecular size marker. Afterward, the gel will be incubated in GelRed for 30 minutes at room temperature and observed under a UV transilluminator, with the image recorded photographically. A DNA fragment of the expected size (e.g., 500 bp) compared to the marker will be considered positive. DNA fragments of the expected size may be sent to specialized companies, such as Genytec Ltda., for sequencing. The obtained nucleotide sequences will be aligned using Clustal Omega to generate a consensus sequence for each fragment. These consensus sequences can then be entered into BLAST to confirm their identity and determine the percentage of nucleotide identity relative to sequences available in GenBank®.

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Conflict of Interest

None.

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