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NUCLEIC ACID AMPLIFICATION TESTING OF URINE SAMPLES FROM DOGS WITH DISTEMPER-LIKE DISEASE

TESTAREA PRIN AMPLIFICAREA ACIDULUI NUCLEIC A PROBELOR DE URINĂ DE LA CÂINI CU SUSPICIUNE CLINICĂ DE BOALA CARRÉ

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ABSTRACT | REZUMAT

Canine Distemper (CD) is a viral disease that affects a great variety of mammal species, both pets and wild animals (e.g., dogs, coyotes, foxes, ferrets and raccoons) but which shows a special interest for the infectious pathology of dog. Canine distemper virus (CDV) or *Canine morbillivirus* is a single-stranded RNA virus of the genus *Morbillivirus* (family *Paramyxoviridae*). Phylogenetic analysis revealed that CDV is close related with Phocine distemper virus (PDV), Measles virus (MeV), and Rinderpest virus (RPV). There are eighteen recognized lineages, seven out of which are considered the main ones. They are as follows: Asia-1, Asia-2, America-1, America-2, Arctic-like, European wildlife, and Europe. Each of these lineages has been discovered through sequence analysis of the "H" gene. In this study 20 urine samples of patients with distemper-like disease were analysed using RT-PCR technique. All the patients presented seizures and myoclonia; non-specific symptomatology that made it necessary to use the RT-PCR technique for a diagnosis with a high confidence. Only four samples provided positive results after being tested using the RT-PCR.

Keywords: Real-Time PCR, Canine Distemper Virus, Canine morbillivirus, Dog pathology

Boala Carré este o boală virală care afectează o varietate mare de specii de mamifere, atât domestice cât și sălbatice (ex. câini, coioți, vulpi, dihori și ratoni), dar care prezintă un interes aparte pentru patologia infecțioasă a câinelui. *Canine distemper virus* (CDV) sau *Canine morbillivirus* este un virus ARN monocatenar din genul *Morbillivirus* (familia *Paramyxoviridae*). Analiza filogenetică a arătat că CDV este strâns înrudit cu Phocine distemper virus (PDV), Measles virus (MeV) și Rinderpest virus (RPV). Există optsprezece tulpini CDV recunoscute, dintre care șapte sunt considerate principalele. Acestea sunt Asia-1, Asia-2, America-1, America-2, Arctic-like, European wildlife și Europe. Fiecare dintre aceste linii a fost descoperită prin analiza secvenței genei „H". În acest studiu s-au analizat 20 de probe de urină recoltate de la câini cu simptome de boala posibil asociate bolii Carré, folosind tehnica RT-PCR. Toți pacienții prezentau convulsii și mioclonie; simptomatologie nespecifică care a făcut necesară utilizarea tehnicii RT-PCR pentru un diagnostic cu grad de încredere ridicat. Doar patru probe au furnizat rezultate pozitive după ce au fost testate prin RT-PCR.

Cuvinte cheie: Real-Time PCR, Distemper virus, Canine morbillivirus, patologie canină

Canine Distemper (CD), also known as Carré's disease, is a viral disease that affects a great variety of mammal species. Numerous species of the order *Carnivora* proved their susceptibility to Canine distemper virus (CDV), and mortality varies significantly from one species to another. Susceptibility to infection has been reported in *Ailuridae* (red panda family), *Canidae* (dog, coyote, wolf, and fox), *Hyaenidae* (hyena), *Mustelidae* (e.g., weasel, mink, badger, and marten), *Procyonidae* (raccoon),*Ursidae* (bear), *Viverridae* (civets) and more recently *Felidae* (cats in experimental infections) (8). It is believed that the disease began in the 18th century, though it is believed to have started in South America, rather than Europe, as a result of a mutation of the Measles virus (1-4). Even though Edward Jenner was the first scientist to describe CD from a clinical point of view in 1809, it was not until the early 1900s when Henri Carré managed to isolate the CDV and thus demonstrate its aetiology (3).

CDV is a member of the genus *Morbillivirus*, in the family *Paramyxoviridae*. It is known as one of the most fatal viral diseases in dogs, second only to Rabies. It is a relatively large virus, measuring around 150-250 nm. It is a single-stranded RNA virus with a helical lipoprotein envelope. Its viral genome is made up of 15690 nucleotides, including the 6 genes that code the synthesis of its structural proteins: Nucleocapsid, Phosphoprotein,Matrix Protein, Fusion Protein, Hemagglutinin, and Viral polymerase (10, 11, 13, 17, 34).

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There are eighteen recognized lineages, seven out of which are considered the main ones. They are Asia-1, Asia-2, America-1, America-2, Arctic-like, European wildlife, and Europe. Each of these lineages has been discovered through sequence analysis of the "H" gene (24, 30, 36, 45). The discovery of several related viruses of the same genus, such as *Phocine morbillivirus* and *Cetacean morbillivirus* and CDV's similarity to the *Measles morbillivirus* make it intriguing to scientists, and also suggest that the virus may be able to mutate, thus becoming a zoonotic agent. There is evidence for the possible involvement of CDV in human multiple sclerosis (5-8, 31, 27). Even though CDV has first appeared to be a virus that could only infect certain species, it is now clear that it has mutated, therefore being able to successfully infect and replicate in large members of the *Felidae* family. Multiple casualties amongst lions (*Panthera Leo*) have been observed in the African national park Serengeti since 1994, due to hyenas (*Crocuta Crocuta*), which acted as vectors for CDV (12, 29, 33, 44). CD evolve at a multi-systemic viral disease with wide variety of clinical manifestations and lesions that can be associated with several other protozoal, bacterial and viral infections. Therefore, the confirmatory exam by using virological (e.g., CDV-Ag detection and nucleic acid amplification testing) and / or immunological (e.g., ELISA IgG and ELISA IgM) methods must be considered (9).

The aim of study was the RT-PCR analysis of urine samples of patients with distemper-like disease (e.g., seizures and myoclonia).

MATERIALS AND METHODS

Animals and biological samples

Dogs with age between 1 and 8 years (Fig. 1), from several different breeds (Fig. 2), and with distemper-like disease were sampled (20 urine samples) for RT-PCR analysis. All the patients presented seizures and myoclonia. Urine was sampled on sterile tubes, with no additives in order to preserve the properties. Urine was collected free catch or by catheterization. Disposable gloves are used for each animal. Urine samples were refrigerated until primary processing (16, 25).

RNA isolation from urine samples

Method was described previously by Gurau et al. (2021) (22). In this step, all categories of RNA in the biological sample were isolated. The RNA extraction was based on the ability to selectively bind silica membranes / adsorption of nucleic acids on the surface of paramagnetic particles (46). The advantages of this approach are mainly the speed of obtaining nucleic acids with a satisfactory degree of purity for most subsequent applications, ease of use and standardization of workflow and last but not least the possibility of automation of extraction (46). Pure Link Genomic Digestion Buffer and proteinase K were added to the pre-

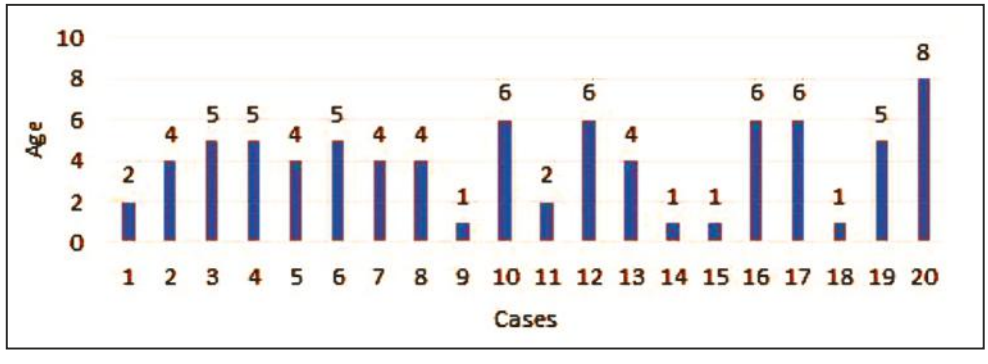

Fig. 1. The age of tested animals

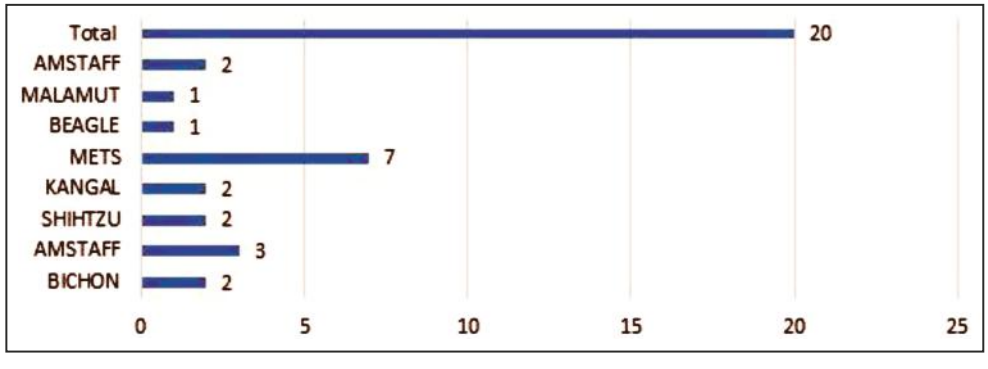

Fig. 2. The breeds of tested animals

viously well-homogenized biological sample and incubated at 55 °C with vortexing for 1 hour. After incubation, tubes were centrifuged for 3 minutes at maximum speed and then the supernatant was transferred to a new Eppendorf tube.

Then RNase A was added to obtain a purified RNA extract. The next step was to bind the RNA. PureLink® Genomic RNA kits bind RNA using a silica-based membrane in the presence of salts. The lysate ~ 640 µl was transferred to the colony and centrifuged at 10,000 x g 1 min after the addition of PureLink Genomic Lysis / Binding Buffer and ethanol 96°-100° of molecular biology purity. The washing step was performed with 2 wash buffer solutions. RNA elution was performed in 50 µl PureLink Genomic Elution Buffer (10 mM Tris-HCl, pH 9.0, 0.1 mM EDTA) (46).

RNA genomic quantification from urine

RNA quantification was performed in order to observe the results on the RNA concentrations in the biological samples. Total RNA quantification was done using the Qubit dsRNA HS test kit (Invitrogen, Thermo Fisher Scientific, Applied Biosystems, US) in conjunction with the Qubit 4 fluorometer (47).

The quantification protocol begins with the preparation of the working solution by diluting the Qubit ds RNA HS 1: 200 reagents in Qubit dsRNA HS Buffer. The final volume for each sample must be 200 µL. 190 µL Qubit working solution is used for the standard and 198 µL Qubit working solution for the sample. Qubit reagent is used in an amount of 10 µL for each standard and 2 µL for samples with a final volume of 200 µL (Table 1). Then vortex for 2-3 seconds, incubate at room temperature for 2 minutes and read the amount of RNA on the Qubit 4 fluorometer. The standards are noted first, following the results. The results are in ng / µL. The results can then be exported in formal excel using a USB device. The results are stored in the fluorometer internal memory. Samples thus quantified are stored in the freezer at -80°C until PCR amplification for sequencing in the next step of the research (47).

RNA Amplification

Amplification consisted of a series of 40 cycles, each containing 3 sequences: denaturation for 5 seconds at 96°C, alignment (primers in the RNA strand) with a duration of 5 seconds at temperature of 60°C, and the extension consisting in the formation of new RNA strands by Taq-polymerase at the temperature of 68°C, for 3 seconds. The final stage of Real-time PCR consisted of a final 1-minute elongation cycle at 72°C.

Real-time PCR is a quantitative method for determining the electronic amount of DNA present in a sample and displaying it in real time. The outcome is portrayed as a graph, with the abscissa representing the number of cycles done and the ordinate representing the fluorescence level emitted. The PCR cycle in which the amplification curve exceeds the immunofluorescence threshold is known as the cycle threshold (ct). Negative and/or positive controls are used in conjunction with the test sample in order to validate the PCR test's efficiency. A control with a conflicting result (positive for a negative control or negative for a positive control) negates the result of all tests analysed with that control (32). The primers were nucleotide sequences that connect precisely to the investigated DNA and can be used to amplify a section of the genetic information that is deemed unique to a certain type of organism and were provided in a commercial kit for CDV diagnostic (Omnigen, Omnivet, Romania).

RESULTS AND DISCUSSIONS

Out of 20 cases examined by using the PCR technique, only 4 turned out to be positive, that represents 20% of the patients tested.

The positive cases are listed below.

Case no. 1

- Sample no 2 (Fig. 3): Masha;
- General information: Kangal, 4 months old, Male;
- Clinical signs: neurological signs, dyspnoea, and coughing;
- The dog had been vaccinated with DHPPI;
- Real-time PCR: An upward curve was recorded at the level of the 28.75 cycle, revealing an average quantity of CDV RNA in the sample.

Case no. 2

- Sample no 5 (Fig. 4): Bella;
- General information: half-breed, 2 months old, female;

Table 1

Standards and Samples RNA Quantification Protocol

<table>
<tr><td colspan="2" rowspan="2">Reagent used volume</td><td>Standard 1</td><td>10 µL</td><td>Sample 1</td><td>2 µL</td></tr>
<tr><td>Standard 2</td><td>10 µL</td><td>Sample 2</td><td>2 µL</td></tr>
<tr><td>Qubit dsRNA HS Buffer</td><td>199 µL</td><td rowspan="2">Reagent used</td><td rowspan="2">190 µL</td><td rowspan="2">Reagent used</td><td rowspan="2">198 µL</td></tr>
<tr><td>Qubit dsRNA HS reagent</td><td>1 µL</td></tr>
<tr><td>Total</td><td>200 µL</td><td colspan="4">Final volume needed for reading results 200 µL</td></tr>
</table>

- Clinical signs: myoclonus, and nasal secretions observed;
- Real-time PCR results: An upward curve was recorded at the level of the 29.62 cycle, revealing an average quantity of CDV DNA in the sample.

Case no. 3

- Sample no 7 (Fig. 5): Max;
- General information: half-breed, 6-year-old, male;
- Clinical signs: Generalized myoclonus, fever, hypertension, dyspnoea, and hard pad disease;
- Real-time PCR results: An upward curve was recorded at the level of the 27.42 cycle, revealing an average quantity of CDV RNA in the sample.

Case no. 4

- Sample no 16 (Fig. 6): Lord;
- General information: half-breed, 8-year-old, male;
- Clinical signs: icterus, hypertension, and fever;
- Real-time PCR results: An upward curve was recorded at the level of the 25.87 cycle, revealing an average quantity of CDV RNA in the sample.

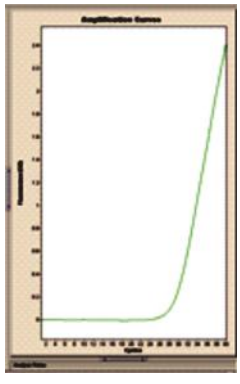

Fig. 3. Case no. 1. An upward curve was recorded at the level of the 28.75 cycle, revealing an average quantity of CDV RNA in the sample

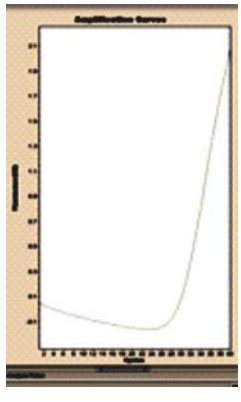

Fig. 4. Case no. 2. An upward curve was recorded at the level of the 29.62 cycle, revealing an average quantity of CDV DNA in the sample

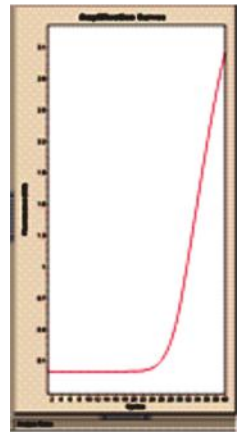

Fig. 5. Case no. 3. An upward curve was recorded at the level of the 27.42 cycle, revealing an average quantity of CDV RNA in the sample

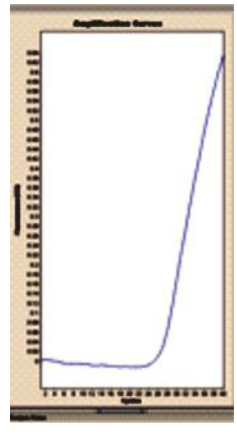

Fig. 6. Case no. 4. An upward curve was recorded at the level of the 25.87 cycle, revealing an average quantity of CDV RNA in the sample

Two out of the positive cases were aged younger than 6 months, which proves that even though maternal antibodies could still be present, the immune status was not strong enough to repel and avoid an infection or the maternal antibodies fade gradually if vaccination it is not done at the right time (19, 18, 26, 28). However, this does not exclude the possibility of an immunization that did not occur when the vaccination was administered, which is, though, a very rare event.

The two other cases were above the age of 6 years of age, which shows that vaccination is the only way the disease could be avoided to a very high degree (14, 15) or CDV can take place at any age category, if immunization scheme is not followed. This could also show a lack of consistency in vaccination (35, 37, 39, 43).

Three out of positive cases were male patients and a similar number were half-breed dogs. These results are similar to other previous research that suggests a higher susceptibility for the unvaccinated male population to contract the virus and develop disease (19,

20, 26) and different from others where notable differences in the number of antibodies that vary from male to female were not recorded (38,39,40).

When it comes to clinical manifestations shown by patients suffering from CDV, all of them showed neurological and respiratory signs. Fever was also encountered in all of the cases. All of the patients showed dyspnoea and shortness of breath. Diarrhoea has to be taken into consideration as it leads to anorexia (41, 42). As studies have shown, epithelial damage could be one of the clinical manifestations found in patients suffering from CDV (21, 23). Nevertheless, hard pad disease was only diagnosed in one of the patients, aged 6, male.

CONCLUSIONS

After performing RT-PCR tests on 20 patients that were susceptible to CDV and observing the clinical signs of the positive patients, only four cases were positive after being tested using the RT-PCR. Half of the dogs with positive results were young, under the age of 1 year, with clinical respiratory signs. Also, all of the RT-PCR-positive patients showed neurological signs, such as myoclonus, epileptic seizures, and partial paresis, which are unspecific signs. In this study the nucleic acid amplification testing is proved to be very specific and sensible.

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