Rapid Approaches for Diagnosis of Canine Distemper Virus in Live and Dead Dogs in Egypt

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BACKGROUND: Canine Distemper Virus infection (CDV) is a highly contagious disease of high morbidity and mortality rates in dogs. The causative virus is CDV which is a Morbillivirus, CDV is a pantropic virus characterized by multisystemic infection and high case fatality, with worldwide distribution, so rapid diagnosis and quarantine of the infected dogs with starting suitable treatment was required. The aim: this study aimed to achieve rapid diagnosis of canine distemper virus infection on ante mortem and post mortem aspects. Material and Methods: One healthy control disabled dog, 53 infected dogs with suggestive clinical signs for CDV infection were checked by (a) clinical examination; (b) Rapid immunochromatography (IC) on conjunctival swabs (c) qRT-PCR on blood and tracheal exudates to confirm presence or absence of CDV, by expression analysis of CDV-F gene. Then all 53 examined dogs isolated and received supportive treatment but all died and disabled controlled one exposed to soft death. qRT-PCR were conducted on tissue samples from all 54 dogs for detection CDV-F gene expression values in different tissues. Results: Clinical signs suggestive for CDV infection recorded in all 53 examined dogs, 24 of 54 dogs were positive for IC. Gene expression analysis test detected high values for CDV-F gene expression in tracheal exudates and blood samples of 36 live dogs, while the expression values were also high in tissue samples from different organs of 36 dead dogs. Statistical comparison of IC to qRT-PCR showed that values were 72%, 100%, 81.4%, 100% and 64.2 for sensitivity, specificity, accuracy, PPV, negative PV, respectively. No effect of sex, age, and breed on results using Chi-square test. Prevalence of CDV infection was 66% among population of this study. Conclusion: this study concluded that detection of clinical signs suggestive for CDV with application of IC and qRT-PCR together should be applied as rapid diagnosis on ante mortem level, while qRT-PCR could be used for rapid post mortem diagnosis of CDV infection.

Keywords: Canine Distemper Virus, CDV, qRT-PCR, Immune-chromatography, Dogs, Egypt.
Infected dogs develop a biphasic fever [6,8], in the first viraemic stage generalized infection of all lymphoid tissues characterized by transient fever with lymphoid depletion, lymphopenia, leukocytosis, icterus, and fever. The second viraemic stage is characterized by generalized fever with lymphopenia, lymphadenopathy, and lymphosarcoma [6,9,10]. In the second viraemic stage secondary viraemia associated with fever and mass infection of all parenchymal tissues, e.g. respiratory tract, GIT, CNS, skin. During the second stage various clinical signs may be found as nasal discharge, anorexia, conjunctivitis, gastrointestinal tract signs, respiratory signs and neurological disturbance [6,11,12]. Intestinal pneumonitis and rhinitis is sequel of respiratory tract infection, vomiting, diarrhoea and dehydration caused by GIT tract infection, hyperkeratosis of skin.

Neurological signs in the form of hyperexcitability, cerebellar rigidity, seizures, paraparesis, sensory ataxia and cerebellar signs which developed as result of distribution of CDV in CNS tissue [13-15].

Diagnosis of CDV infection depends mainly on isolation of the virus in tissue culture (canine cells) but this is a time consuming, taking several days to week [16]. In spite of vaccination against canine distemper many decades, CDV infection still remain fatal disease of dogs [17].

Because of CDV causes a contagious disease of high mortality rates. The need for the use of rapid techniques is urgent, in order to isolation and quarantine of infected dogs with starting early treatment [18].

No published data regarding diagnosis of CDV in Egypt, in addition to importance of establishing definitive diagnostic system for the control of this disease is urgent. So this study aimed to set a definitive scheme for accurate, rapid diagnosis including clinical signs, the use of immune-chromatography (IC) and quantitative Real Time-PCR (qRT-PCR) as rapid test for detection of CDV even in low levels of viraemia in live and dead dogs.

Material and Methods

Duration of the study

This study has been conducted during the period between January 1, 2015 and October 30, 2018.

Ethical approval and informed consent

We informed and received the permission of the owners of dogs included in this study for taking samples used in this work. Samples were collected as per standard sample collection procedure without any harm to animals. We received agreement of owner of disabled control puppy for applying soft death, also we received agreement of all dog owners included in this study for using isolated diseased dogs for scarification taking different tissue samples.

Chemicals

For molecular analysis, Trizol was bought from Invitrogen (Carlsbad, CA, USA). The reverse transcription and polymerase chain reaction (PCR) kits were obtained from Fermentas (Glen Burnie, MD, USA). For IC analysis, direct IC kits were purchased from Bionote Inc., Korea.

Examined animals

(a) 53 diseased dogs were examined. Each dog was examined clinically to detect the clinical manifestations of the disease. (b) One congenitally disabled healthy German Shepherd puppy of 90 day age old suffered from congenital disability in his hind limb and according to private request of the owner to make soft death of his disabled puppy [19, 20].

Clinical examination

Fifty three infected dogs and a healthy control disabled puppy were received at a Clinic at 6 October district located in Giza Governorate, Egypt. History of the examined dogs including breed, sex, age, past medical data history, and registered vaccination were recorded. Dogs subjected to general and specific clinical examination according to Gaskell et al. [1]. The severity of the clinical signs observed in this study was recorded as severe, moderate, mild or acute or sub-acute forms.

Sampling

Conjunctival swabs were collected from all clinically infected cases dogs and a healthy control disabled puppy was checked by rapid IC test (rapid CDV Ag test kit, Veterinary Department, Technical University of Berlin, Germany) for qualitative detection of Canine Distemper virus antigen in Conjunctival swabs. Conjunctival swabs were carried out on 53 infected dogs and a healthy control disabled puppy for detecting clinical signs of CDV viral infection [21].

Molecular study

Extraction of total RNA and cDNA synthesis

Tracheal exudates and blood from live dogs (n=54), in addition to liver, spleen, myocardium, lung, brain tissues and lymph nodes (medialastinal and retropharyngeal) samples were collected from all infected dogs after death and control disabled dog after soft death (n=54) according to Ramas and Wetzlel [19] and Reilly [20] and used for RNA extraction using TRIzol® Reagent (Invitrogen, Germany). Kit. The isolation method was carried out according to the manufacturer’s instructions of the above Kit. Approximately 50 mg of the samples were homogenized in 1 ml of TRIzol® Reagent in Eppendorf tubes. Afterwards, total RNA was dissolved and preserved in diethylpyrocarbonate (DEPC)-treated water up to use [22, 23].

To assess the RNA yield and purity of the total RNA, RNAse-free DNase I (Invitrogen, Germany) was used to digest DNA contamination. A small drop of isolated RNA was examined by spectrophotometer at the 260/280 nm ratio. To avoid RNA damaging, aliquots of RNA were prepared after isolation for either reverse transcription reaction or otherwise for storing at −80°C up to use.

To synthesize the complementary DNA (cDNA) isolated RNA from all samples were reverse transcribed into cDNA. The reaction volume was carried out in 20 µl. The reaction volume was prepared according to the instructions of the RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). The reverse transcription (RT) reaction was performed for 10 min at 25°C. Afterwards, the tubes of the reaction were put in thermo-cycler machine for 60 min at 42°C, and then the reaction was terminated for 5 min at 90°C. The PCR products containing the cDNA were kept at 4°C up to use for DNA amplification [22, 23].

Quantitative Real Time-PCR (qRT-PCR) Primer design

Specific primers used in this study were designed for different regions of F gene which codes virus fusion protein using primer3 program as illustrated in Table 1.

A StepOne Real-Time PCR System (Applied Biosystem, USA) was used to assess the copy of the cDNA of tracheal exudates, liver, spleen, myocardium, lung, brain tissues and lymph nodes (medialastinal and retropharyngeal) to detect the expression values of the Canine Distemper virus F-gene. A volume of 25 µl of reaction mixtures was prepared containing 12.5 µl of SYBR® green (TakRa, Biotech. Co. Ltd.), 0.5 µl of forward and reverse primers, 6.5 µl DNA-RNA free water, and 2.5 µl of the synthesized cDNA. A melting curve of the reaction was performed for each qRT-PCR termination at 95°C to assess the quality of the primers. To verify that the reaction of the qRT-PCR does not have any contamination PCR tubes containing non template control were used. The sequences of specific primers of the Canine Distemper virus F-gene were used Table 1. The relative quantification of the target gene to the reference (GAPDH, [24]) was determined by using the 2−ΔΔCT method [22, 23].

Table 1. CDV primers used in this study.

<table>
<thead>
<tr>
<th></th>
<th>Gene Name</th>
<th>Primer name</th>
<th>Accession number</th>
<th>Estimated size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CDV-F</td>
<td>F: CTTGGCAAAATGTTGCTT</td>
<td>M21849.1</td>
<td>1579 - 1821</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH</td>
<td>F: GAGAAAAGCTGGGAAATG</td>
<td>Tarlinton [24]</td>
<td>193</td>
</tr>
</tbody>
</table>

Based on available CDV genome sequences, CDV/Canine Distemper virus

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form that was fatal usually in puppies.

Fifty three naturally infected puppies, 1
disabled healthy control male German Shepherd
of age 2 to 4 months of history with no
vaccination against Distemper virus were kept
under close observation and taking all sanitary
and epidemiological measures for prevention
of infection spread according to recommended
protocol for Distemper virus control.

The observed clinical signs were:
- Non neurological signs: fever 40 – 41.7 ºC
serous – mucopurulent nasal and conjunctival
discharge, coughing, dyspnea, vomiting and
diarrhea as shown in Table 2 and Fig. 1.

- Neurological signs: includes showing gums,
seizures, circling, continues fits of high voices,
later end by limb ataxia and weakness, head
tremors, head tilt, depression, behavior changes
end by death as shown in Table 2.

Immune-chromatography (IC)
All hospitalized infected puppies that showed
clinical signs were checked by rapid Immune-
chromatography (IC) for qualitative detection
of canine distemper viral antigen in conjunctival
discharge, the 26 mentioned puppies were positive
when checked by rapid IC test as mentioned in
Fig. 2 and Table 3, while control disabled puppy
were negative.

Molecular study
Expression levels of CDV-F gene in dog tissues

** Fig. 2. Immune chromatography test kit showing positive results for CDV infection

Table 3. Positive and negative male and females dogs examined for CDV using Immune-chromatography.

<table>
<thead>
<tr>
<th>Breed</th>
<th>No. of cases</th>
<th>Breed size</th>
<th>Age (month)</th>
<th>Sex</th>
<th>Fever (ºC)</th>
<th>Clinical Signs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small</td>
<td>Large</td>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>German Shepherd</td>
<td>16</td>
<td>-</td>
<td>16</td>
<td>2-3</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>15</td>
<td>-</td>
<td>15</td>
<td>2-3</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Yorkshire</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>2-3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Labrador Retriever</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>2-3</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Griffon</td>
<td>5</td>
<td>-</td>
<td>5</td>
<td>2-3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Boxer</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>3-4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>8</td>
<td>46</td>
<td></td>
<td>28</td>
<td>26</td>
</tr>
</tbody>
</table>

* Fever 40 – 41.7 ºC serous – mucopurulent nasal and conjunctival discharge, coughing, dyspnea, vomiting and diarrhea
** Shewing gums, seizures, circling, continues fits of high voices, later end by limb ataxia and weakness, head tremors, head tilt, depression. The severity of clinical signs evaluated as mild, moderate and severe.
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TABLE 4. Comparison between immune-chromatography (IC) according qRT-PCR findings of examined cases of CDV in live dogs.

<table>
<thead>
<tr>
<th>Method of identification</th>
<th>IC Positive</th>
<th>IC Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>qRT-PCR</td>
<td>26(T+) 18(F-)</td>
<td>18(T-)</td>
<td>54</td>
</tr>
</tbody>
</table>

(T+): True positive, (T-): True negative, (F+): False positive, (F-): False negative
Sensitivity= 72%   Positive pre-detective values (PPV)= 100%
Specificity= 100%   Negative pre-detective values (NPV)= 64.2%
Accuracy= 81.4%

& hospitals. It proved to be specific, easily in performance for detection of CDV antigen in conjunctival swabs of examined dogs as reported by An et al. [21].

IC succeeded in detection of 26 infected cases among 54 examined dogs, due to lack in its sensitivity for detection the infected cases harboring low concentration of viremia below 105.5 TCID50/ml [27].

In this work Gene expression analysis test (qRT-PCR) tried as highly sensitive, specific test with high accuracy in detection of CDV-F gene expression using specific primer on blood and tracheal exudate of examined dogs as recommended by Elia et al. [18] and Budaszewski & von Messling [28].

qRT-PCR in this study succeeded in detection of 36 infected cases among 54 suspected cases due to its high sensitivity in detection of CDV-F gene expression even in very low virus concentration which may reach to 102 as confirmed by Elia et al. [18].

These results proved that gene expression analysis test was an accurate, conclusive, confirmatory, qualitative test in diagnosis of CDV infection in live dogs as confirmed by many authors [Elia et al. [18] and Shaw & Ihle [26]. Moreover that qRT-PCR was a sensitive anti mortem diagnostic technique especially reliable in sub acute and chronic stages of CDV infection [17, 18, 28-30].

Results of qRT-PCR when applied in liver, spleen, myocardium , lung , retropharyngeal & mediastinal lymph nodes and brain tissue of dead dogs, showed that CDV-F gene expression values were high in lung & lymph nodes than that values of liver, spleen, myocardium and brain significantly (P<0.05), while there is no significant difference between expression values of liver, spleen, myocardium, and brain tissues agreed with Elia et al. [18].

The above mentioned results recommend the use of qRT-PCR as quantitative assay for measuring CDV load & distribution in different infected organs by estimating CDV-F gene expression quantitively, with high accuracy because it can detect CDV even in level 106 to 108. So, qRT-PCR served as reproducible assay for studying pathogenetic mechanisms induced by CDV [17, 18, 30]. Moreover, the gene expression values in blood and tracheal exudate were high as that recorded in lung tissue, these findings nominate qRT-PCR as rapid molecular assay for studying CDV spread and shedding [18, 28, 30-35].

Conclusion
This study concluded that detection of clinical signs suggestive for CDV, application of rapid IC and molecular assaying by qRT-PCR could serve as rapid, accurate, confirmatory methods of CDV diagnosis on ante-mortem level without need for CDV isolation, while qRT-PCR was considered as accurate assay for rapid diagnosis on post-mortem level.

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Conflict of Interest Statement

The author whose name is listed immediately below certify that he has no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Author contribution

The author performed the study plan and design. Romane Adieb Awad collected the samples from the clinic, and had carried out the clinical examination and IC laboratory work. Romane Adieb Awad carried out the work of clinical evaluation of the diseased dogs, treatment, clinical follow-up and assessment. He also has carried out the molecular work included in this study. Romane Adieb Awad carried out the work of clinical evaluation from the clinic, and had carried out the clinical examination and IC laboratory work. Romane Adieb Awad collected the samples for Prof. Dr. Wagdy K. B. Khalil for his great help in achieving molecular work and finalizing of this manuscript. The present study received no financial support.

Ethical consideration

The owners of dogs were informed and permission was received from them. The dogs included in this study for taking samples used in this work. Samples were collected as per standard sample collection procedure without any harm to animals. Individual permission received from the owner of the disabled control puppy and on his request to do soft death of his disabled control puppy. The proposal of this study had approval from National Research Center committee no: 10/01/2015.

References


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**تقنيات سريعة لتشخيص فيروس الدستمبر في الكلاب الحية والميتة في مصر**

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تعتبر عدوى الاصابة بفيروس الدستمبر فى الكلاب من الامراض الوبائية ذات معدل الاصابة والنفوق العالي في الكلاب. مسبب المرض هو فيروس الدستمبر وهو عبارة عن فيروس موربيلى وهو فيروس يصيب اجهزة مختلفة في جسم الكلاب ويتسبب في مئات الى الألاف من وفيات الكلاب في العالم. التشخيص السريع وعزل الكلاب المريضة واتباع الإجراءات الوقائية تساعد في انقاذ الكلاب. تكمن أهمية الدراسة في تتبع توزيع فيروس الدستمبر في الكلاب على مستوى البلاد وتحديد الدلتا المرضية. وقد تم استخدام تقنيات DNA-Sequencing و PCR لتشخيص فيروس الدستمبر في كلاب نقلت إلى مصر من عرب بوروندي وعلماء الفيروسات في الجامعة الورطوطية، وعلماء الوبائيات في الوطنية للبحوث البيطري. وقد تم استخدام الامينو كروماتوجرافى في كلاب لمعرفة تفاعل البلمرة المتسلسل العكسى الكمي للكشف عن فيروس الدستمبر. دراسة عينت من كلب بمعدل 24 كلب واظهر اختبار الامينو كروماتوجرافى ارتفاع في تفاعل البلمرة المتسلسل العكسى الكمي في الدم. كان معدل الامينو كروماتوجرافى 91.6% مع معدل الامينو كروماتوجرافى في الدم 94.5%. وخلال فحص الامينو كروماتوجرافى استخدمت مادة تمثل في نوع الإدمان والدردش الأكلي في نتائج الفحص. وقابلة الدقة (بدقة) في الفحص 91.6% مع معدل الامينو كروماتوجرافى في الدم 94.5%.

**توصيات**

تعتبر الدراسة ناجحة في استخدام الامينو كروماتوجرافى في التشخيص السريع. وعند استخدام الاختبار الامينو كروماتوجرافى في الكلاب الميتة، يمكن استخدام الاختبار بالامينو كروماتوجرافى للتشخيص السريع. وعند استخدام الاختبار الامينو كروماتوجرافى في الكلاب الحية، يمكن استخدام الاختبار بالامينو كروماتوجرافى للتشخيص السريع.