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Canine distemper virus detection by different methods of One-Step RT-qPCR

Detecção do vírus da cinomose canina por diferentes métodos de One-Step RT-qPCR

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ABSTRACT

Three commercial kits of One-Step RT-qPCR were evaluated for the molecular diagnosis of Canine Distemper Virus. Using the kit that showed better performance, two systems of Real-time RT-PCR (RT-qPCR) assays were tested and compared for analytical sensitivity to Canine Distemper Virus RNA detection: a One-Step RT-qPCR (system A) and a One-Step RT-qPCR combined with NESTED-qPCR (system B). Limits of detection for both systems were determined using a serial dilution of Canine Distemper Virus synthetic RNA or a positive urine sample. In addition, the same urine sample was tested using samples with prior centrifugation or ultracentrifugation. Commercial kits of One-Step RT-qPCR assays detected canine distemper virus RNA in 10 (100%) urine samples from symptomatic animals tested. The One-Step RT-qPCR kit that showed better results was used to evaluate the analytical sensitivity of the A and B systems. Limit of detection using synthetic RNA for the system A was 11 RNA copies μL^{-1} and 110 RNA copies μl^{-1} for first round System B. The second round of the NESTED-qPCR for System B had a limit of detection of 11 copies μl^{-1}. Relationship between Ct values and RNA concentration was linear. The RNA extracted from the urine dilutions was detected in dilutions of 10^{-3} and10^{-2} by System A and B respectively. Urine centrifugation increased the analytical sensitivity of the test and proved to be useful for routine diagnostics. The One-Step RT-qPCR is a fast, sensitive and specific method for canine distemper routine diagnosis and research projects that require sensitive and quantitative methodology.

Key words: *canine distemper, molecular diagnosis, One-Step RT-qPCR, urine, centrifugation.*

RESUMO

Três kits comerciais de One-Step RT-qPCR foram avaliados para o diagnóstico molecular do Vírus da Cinomose Canina.Utilizando o kit que apresentou melhor desempenho, dois sistemas de RT-PCR em tempo real (RT-qPCR) foram comparados quanto à sensibilidade analítica na detecção do RNA do Vírus da Cinomose Canina:One-Step RT-qPCR (Sistema A) e One-Step RT-qPCR seguido da NESTED-qPCR (Sistema B).Os limites de detecção dos dois sistemas foram determinados utilizando diluição seriada de RNA sintético do Vírus da Cinomose Canina ou de uma amostra de urina positiva. Adicionalmente, uma amostra de urina foi avaliada com centrifugação ou ultracentrifugação prévia. Os kits comerciais de One-Step RT-qPCR amplificaram o RNA do vírus da cinomose canina em 10 (100%) amostras de urinas de animais sintomáticos. O kit de One-Step RT-qPCR que apresentou melhor resultado foi utilizado para avaliar a sensibilidade analítica dos sistemas A e B. Na reação da curva padrão com RNA sintético, o limite de detecção do sistema A foi de 11 cópias de RNA μL^{-1}. No sistema B foi de 110 cópias de RNA μL^{-1} na One-Step RT-qPCR e 11 cópias de RNA μL^{-1} na NESTED-qPCR. A relação entre os valores de Ct e concentração de RNA foi linear. O RNA extraído das diluições da urina foi detectado nas diluições de 10^{-3} e10^{-2} pelos sistemas A e B, respectivamente. A centrifugação prévia da urina aumentou a sensibilidade analítica da análise e mostrou ser importante para a rotina diagnóstica. A reação de One-Step RT-PCR é um método rápido, sensível, específico e aplicável na rotina de diagnóstico molecular da cinomose e em projeto de pesquisa que requer metodologia quantitativa e sensível.

Palavras-chave: *cinomose canina, diagnóstico molecular, One-Step RT-qPCR, urina, centrifugação.*

INTRODUCTION

Canine Distemper (CD) is a severe and often lethal infectious disease that affects dogs and a broad range of terrestrial and aquatic animals (CARVALHO et al., 2012). The disease is caused by

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Canine Distemper Virus (CDV), a ***Morbillivirus*** of the ***Paramyxoviridae*** family (ICTV, 2014). CDV is an enveloped and pleomorphic virion with a helical capsid that is associated with a single, non-segmented RNA genome of negative polarity. Direct contact with nasal, oral secretions or urine of infected animals is the major route of CDV transmission. Recently, outbreaks of distemper have been identified in dogs and an increasing number of host species, which has been the subject of many studies to elucidate the potential mutations and viral mechanisms that may be involved in host susceptibility (ZHAO et al., 2010; MEGID et al., 2013; DI SABATINO et al., 2014). Clinical signs of canine distemper consist of respiratory and/ or gastrointestinal symptoms that may or may not be associated with neurological symptoms (CARVALHO et al., 2012).

Diagnosis of canine distemper is often based on clinical suspicion. However, due to disease without typical findings of distemper, the similarity of clinical signs with other ailments and high spread of disease, the laboratory diagnosis is crucial. In the past decade, several gel-based molecular assays were developed for CDV detection (FRISK et al. 1999; HEADLEY et al., 2009; ROSA et al., 2012). However, to increase efficiency detection of CDV in samples with low viral load the NESTED RT-PCR has been shown to be the most sensitive method of CDV diagnoses (KIM et al., 2001; DI FRANCESCO et al., 2012).

RT-qPCR assays have been applied to detect and quantify canine distemper virus in clinical specimens of naturally infected dogs or to distinguish vaccine strains from wild strains, using two-step and real-time probes chemistry (ELIA et al., 2006; SCAGLIARINI et al., 2007; FISCHER et al., 2013; WILKES et al., 2014). Recently, surface plasmon resonance (SPR), electrochemical impedance spectroscopy (EIS) and conjugated gold nanoparticles methodologies were also developed for CDV detection (BASSO et al., 2013; 2015a; 2015b).

There is a wide range of commercials reagents and kits for RT-qPCR that use different chemistry amplification. The RT-qPCR yields depend on protocols standardisation, reagents, gene target, primers, template preparation and analytical method used. Various samples types, such as blood, conjunctival swabs and urine, have been used for CDV diagnosis. Urine is useful in the ante mortem diagnosis of distemper, easier to collect than other body fluids and shows great sensitivity in different clinical presentations of canine distemper (GEBARA et al., 2004; ELIA et al., 2006). The aim of this study was to evaluate three commercial kits of One-Step RT-qPCR and to compare the analytical sensitivity of One-Step RT-qPCR (System A) and One-Step RT-qPCR combined with NESTED-qPCR (System B) to detect CDV RNA in urine samples from dogs with clinical suspicion of CDV infection.

METHODS

Twenty urine samples from dogs were tested to compare the ability of CDV RNA detection of three commercial kits of One-Step RT-qPCR. Urine samples from dogs with clinical suspicion of CDV such as conjunctivitis, bronchitis, catarrhal pneumonia, gastroenteritis or neurological disturbances and 10 urines samples from healthy dogs without sign of CD were collected. RNA was harvested from one hundred microliters of urine using the Total RNA Purification Kit[a] according to the manufacturer's instructions and stored at -80°C until further use.

One-Step RT-qPCR was performed using three commercial kits designated as I, II and III, according to each manufacturer's instructions. Two microliters of RNA extracted was used in a final volume reaction of 20µL.The primer pair used in the three One-Step RT-qPCR kits was CDV-For (5'-AGC TAG TTT CAT CTT AAC TAT CAA ATT-3') and CDV-Rev (5'-TTA ACT CTC CAG AAA ACT CAT GC-3') which produced an amplicon of 83 bp (905–931 and 966–987 nucleotide nucleoprotein positions, respectively), designed by ELIA et al., (2006), in final concentration of 300nM. A negative control was performed using nuclease-free water.

The reactions prepared with Kit I contained 1XRT-PCR Mix, 0.2µL of RT Mix for One-Step RT-qPCR, primers and nuclease free water. Cycling conditions were: reverse transcription at 50°C for 10min, PCR initial activation step at 95°C for 5min, 40 cycling of denaturation at 95° for 30s and annealing/extension 60°C for 30s, followed by dissociation curve.

The reactions prepared with Kit II contained 1X RT-PCR Mix, 20U of RT Mix for One-Step RT-qPCR, 0.2µL of Reference Dye for Quantitative PCR, primers and ultrapure water.Cycling conditions were: reverse transcription at 44°C for 45min, PCR initial activation step at 94°C for 2min, 40 cycling of denaturation at 94° for 15s and annealing / extension 60°C for 1min, followed by dissociation curve.

The Kit III reactions were performed using 1X RT-PCR Mix, 1X RT Mix for One-Step RT-qPCR, 0.27µM of Reference Dye for Quantitative

PCR, primers and ultrapure water. Cycling conditions were: reverse transcription at 37°C for 15min, PCR initial denaturation at 95°C for 10min, 40 cycling of denaturation at 95° for 15s, annealing at 60°C for 30s and extensionat 72°C for 30s, followed by dissociation curve.

The One-Step RT-qPCR (System A) and One-Step RT-qPCR combined with NESTED-qPCR (System B) were compared using the RT-qPCR Kit I, which showed better dissociation curves, reaction time and CT averages than other tested kits. The One-Step RT-qPCR of System A was performed using the primer pair CDV-For and CDV-Rev with a primer final concentration of 300nM. The One-Step RT-qPCR of System B was performed using the primer pair P-1 sense (5'-ACAGGATTGCTGAGGACCTAT-3') and P-2 antisense (5'-CAAGATAACCATGTACGGTGC-3') (769-789 and 1055-1035 nucleotide nucleoprotein position of sense and antisense primers, respectively) described by FRISK et al. (1999), which produced an amplicon of 287bp. Negative reactions were applied to the NESTED-qPCR reaction carried out with the CDV-for and CDV- rev primer pair, stated in the System A, using the GoTaq® Green Master Mix[b] according to manufacturer's recommendations. Primer concentrations used were 300nM and 200nM in the One-Step RT-qPCR and NESTED-qPCR, respectively. Reactions were performed on a 7300 Real Time PCR System[c] in a final volume of 20µL. Negative controls were carried out with urine RNA extracted from a healthy dog without clinical of distemper and nuclease-free water.

The synthetic RNA was provided to access the efficiency and analytical sensitivity of the two RT-qPCR systems. One fragment of 287bp generated by RT-qPCR was cloned into the pGEM-T easy vector[b] and transformed into ***Escherichia coli*** DH-5α. The recombinant plasmid was quantified using a NanoDrop®ND-1000 Spectrophotometer. The CDV-plasmid was treated with restriction enzymes *NaeI* and *NdeI*[d] and separated via electrophoresis on a 1% agarose gel. Fragment of 692bp of CDV containing binding sites of the T7 polymerase was purified using Nucleospin® Gel and PCR Clean Up[e] according to the manufacturer's guidelines. The fragment was *in vitro* transcribed using MEGA shortscript™ T7[f] according to the manufacturer's instructions. Product of transcription was treated with DNAseI[g] to eliminate residual DNA and has been tested using RT-qPCR without the reverse transcriptase to assess RNA purity.

The transcribed RNA was quantified using Qubit® 2.0 Fluorometer[h] and the copies number was estimated. The quantified RNA was diluted in sample matrix in 10-fold serial dilutions and was subjected to System A and B in duplicate. Standard curve of both systems was constructed from serial 10-fold dilutions ($1.1x10^5$ to $1.1x10^0$ RNA copies per µl) of synthetic RNA using the Applied Biosystems 7000 System SDS software.

One RT-qPCR positive urine sample was diluted to 10^{-5} in RT-qPCR negative urine and, after RNA extraction, tested in duplicate using the RT-qPCR Systems A and B.

Additionally, to verify the amount of RNA in the urine sample under centrifugation, 1ml of sample was centrifuged at 8,000 x *g* for 8 minutes and 1ml was ultracentrifuged at 100,000 x *g* for 2 hours. One hundred microliters of the pelleted samples were used for RNA extraction and subjected to the two RT-qPCR systems.

RESULTS AND DISCUSSION

All ten RNA samples extracted from urine samples of dogs with clinical suspicion of CDV (100%) were positive with the three kits used. Ten RNA samples extracted from urines samples of healthy dogs and the negative control did not show amplification or peaks in the melting curve analysis. The RT-qPCR duration and average CT values were 2:00h/27.8, 3:20h/24.3 and 2:45h/29.3 for the Kit I, II and III, respectively. Melting temperature peaks varied from 73.8°C to 74.5°C, 74.6°C to 75.8°C and 74.2°C to 75.7°C for the Kit I, II and III, respectively. Kit II resulted in the lowest average CT values; however, in melting curve analysis of positive samples, there was a broad base, nonspecific peaks and melting temperature variation of 1.2ºC. Kit I and III analyses showed melting temperature variations of 0.7ºC and 1.5ºC, respectively, but non-specific peaks were not observed.

Three commercial kits were able to detect CDV RNA in urine samples. Over the past several years, real-time PCR has become the leading tool for the detection and quantification of nucleic acids. Increase of commercial master mix kits enabled diagnostic laboratories to implement real-time PCR. It has been demonstrated that the reverse transcription reaction mainly depends on the initial molecular step (the reverse transcription), choice of master mix reagents, quality of enzymes, concentration of reaction mix components, such as primers, probes and methodology used that can impact the sensitivity of results for real-time PCR assays (SERGERIE et al., 2007; BUZARD et al.,

2012; PICARD-MEYER, 2015). In this study, the Kit I was faster, with better melting curves showing better performance and was used to evaluate the analytical sensitivity of Systems A and B.

The standard curve of One-Step RT-qPCR Systems A and B was generated and showed linearity over the entire quantification range. System A showed a slope of -3.5, a coefficient of linear regression (R^2) of 0.99 and an efficiency of 93.0%. System B showed a slope of -3.65, a linear regression (R^2) 0.99 and an efficiency of 87.0%. System A was able to detect synthetic CDV RNA down to 1.1 x10 RNA copies μL^{-1}. System B amplified the synthetic RNA down to $1.1x10^2$ copies μL^{-1} in the RT-qPCR and 1.1 x10 RNA copies μL^{-1}in the NESTED-qPCR. System A detected CDV RNA in diluted urine down to 10^{-3} and System B detected CDV RNA in diluted urine down to10^{-2} (Table 1).

The NESTED reaction usually increases the analytical sensitivity of conventional PCR reactions for CDV detection and is often applied for diagnostic purposes. When using conventional reaction, the limit of detection of N gene of CDV was increased hundred fold using the One-Step RT-PCR combined with the NESTED PCR (KIM et al., 2001). The CDV was detected in 50.0% of urine samples from symptomatic dogs by One-Step RT-PCR and in 75.0% of urine samples when combined with NESTED-PCR (SHIN et al., 2004). Thus, in the present study, the System A showed superior analytical sensitivity compared to System B due to greater reaction efficiency. The qPCR is more sensitive, faster and reduces the risk of broad contamination. Composition of reagents, gene target and primers pair plays a role in the reaction yield. The reaction with the primer pair CDV-for and CDV-rev (System A) showed improved efficiency and sensitivity compared to the P-1 and P-2 primer pair (System B), probably due to the primer used. Particularly in studies of diagnostic methods that perform the NESTED-PCR, there is a high risk of broad laboratory contamination. Overall, qPCR is performed only from negative samples, previously detected in melting curve analyses, without opening positive tubes, reducing the risk of cross contamination and making this method a useful option for studies that require this tool.

The System A detected RNA down to 1.1 x 10 RNA copiesμL^{-1} and was more sensitive than two-step probe based chemistry using the same primer pair (ELIA et al., 2006). Benefits of SYBR Green I One-Step RT-qPCR assays over conventional RT-PCR and two-step qPCR detection methods, particularly for diagnosis, include their large dynamic range, ability to be scaled up for high through put applications and superior analytical sensitivity and specificity due to the curve dissociation (DUPOUEY et al., 2014). These assays are also faster, more practical in considering all of the required reagents in the RNA transcription reaction, optimise laboratory time and present a good cost to benefit ratio (SCHMITTGEN et al., 2000).

CDV is one of the most important infectious agents within canine population and has worldwide distribution and frequently affects unvaccinated young dogs (LAMB; KOLAKOFSKY, 2001). However, distemper has been known to occur in vaccinated dogs and outbreaks in various other hosts naturally infected, such as non-human primates have been investigated (BEINEKE et al., 2009; SAKAI et al., 2013). The definitive diagnosis of distemper often requires the laboratory tests to confirm disease without the typical symptoms and the similarity of clinical signs with other ailments. System A showed to be a sensitive and practical method that can be applied to detect CDV and can be used for target quantification in academic laboratory applications.

The urine centrifuged at 8,000 x *g* for 8 minutes showed increases in RNA copy number

Table 1 - Comparison of absolute viral quantification of urine dilution samples, centrifuged urine and ultracentrifuged urine in system A and B.

Sample	System A (copiesμL^{-1})	System B (copiesμL^{-1})
Urine 10^0	$2.8x10^5$	$7.1x10^4$
Urine 10^{-1}	$4.0x10^3$	$1.1x10^3$
Urine 10^{-2}	622.6	129.39^4
Urine 10^{-3}	29.3	-
Urine 10^{-4}	-	-
Urine 10^{-5}	-	-
Centrifuged Urine	$9.0x10^5$	$1.7x10^5$
Ultracentrifuged Urine	$9.7x10^5$	$2.4x10^5$

detection, which was approximately 3.2 and 2.3 times in System A and B, respectively. Nucleoprotein gene is considered the most conserved gene of the ***Morbillivirus*** family and the most expressed gene due to the transcriptional organization. As a result, this gene has been commonly used for CDV diagnosis. Urine is currently the best sample for CDV *ante-mortem* diagnosis and has been usefully applied. CDV causes cystitis and inclusion bodies, which can be found clearly in the cytoplasm or less frequently in the nucleus of urinary bladder cells by histology. Most likely, the RNA virus is detected in the urine due to infected urinary epithelial cell flaking and the presence of free virus in the urine. Detection of CDV RNA extracted from samples centrifuged at 8,000 x *g* for 8 minutes revealed a higher quantity of virus RNA (approximately three fold), most likely due to infected cell sedimentation containing the intracytoplasmic viral RNA. Ultracentrifugation results in concentrated viral particles, which increase sensitivity slightly when compared to centrifugation. Urine centrifugation at 8,000 x *g* for 8 minutes prior to RNA extraction is easy to perform, increases diagnostic sensitivity and is an important tool, particularly in samples with low viral load.

CONCLUSION

In conclusion, the three kits of One-Step RT-qPCR were able to detect CDV in urine samples. The Kit I was faster, with better melting curves indicating better performance. The paper describes a One-Step RT-qPCR and a One-Step RT-qPCR combined with NESTED-qPCR (System A and B, respectively) with the primer pair CDV-for and CDV-rev showing better analytical sensitivity. One-Step RT-qPCR is a sensitive and rapid method, which will support routine canine distemper laboratory surveillance and facilitate research projects that require sensitive and quantitative CDV detection. In addition, centrifugation of urine samples prior to RNA extraction appears to increase the test sensitivity.

SOURCES AND MANUFACTURES

a- Norgen Biotek Corporation,Thorold, ON, Canada.
b- Promega, Madison, WI, USA.
c- Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA.
d- Fermentas/Thermo Scientific, Burlington, Canada.
e- Macherey-Nagel, Düren, Germany.
f- Invitrogen, Life Technologies Corp., Carlsbad, CA, USA.
g- Sigma Sigma-Aldrich, St Louis, MO, USA.
h- Life Technologies Corporation, Carlsbad.

DECLARATION OF CONFLICTING INTERESTS

The authors of the manuscript disclose that there is no conflict of interest and that there were no commercial relationships with the manufacturers of any products used in this article.

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