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Short communication

Detection of *Neospora caninum* in dog organs using real time PCR systems

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Abstract

Neospora caninum is a parasite responsible for paresis in dogs. The dog can harbour enkysted parasites in several organs. The detection of *N. caninum* was performed using 3 different real time PCR systems all amplifying the NC5 DNA region. One system was based on Sybr® green, one on Plexor™ technology and the last on Taqman® probe. Comparison of the three methods indicated that the detection limit was 1 equivalent genome on pure DNA but that this detection limit increased in the presence of foreign DNA using the Sybrgreen and Plexor systems. Therefore, the Taqman system was chosen to detect *N. caninum* in liver and spleen of naturally infected dogs. The overall prevalence was 32.2%. Comparison between PCR results and serological results using IFAT showed that among the 28 PCR positive dogs only 9 were seropositive and that 8 seropositive dogs were PCR negative. Therefore serology can underestimate the real carriage in dogs. However, PCR methods must be improved in terms of sensitivity and inhibition problems.

Keywords: *Neospora caninum*; Dog; Real time PCR; Organs

1. Introduction

Neospora caninum is a heteroxenous cyst-forming apicomplexan parasite responsible for diseases in animals. The major clinical manifestations are hind limb paralysis in dog and abortion in cattle worldwide (Dubey et al., 2006). The dog is the definitive host and the cattle are the intermediate host (Georgieva et al., 2006). Dogs are infected by ingestion of contaminated food of bovine origin. In dogs, the parasite can reach organs such as the brain, spinal cord, retina, muscles, thymus, heart, liver, kidney, stomach, adrenal gland, and skin (Peters et al., 2000) where it can form cysts and persist for a long time leading to chronic disease (Georgieva et al., 2006). Reactivation of the parasite can occur when the immune system of the host is depressed (Georgieva et al., 2006). Serology is the principal method of *N. caninum* diagnosis. It includes IFAT considered as the Gold standard (Dubey and Schares, 2006), ELISA including a lot of variations and commercially available kits (Dubey and Schares, 2006), DAT (Packham et al., 1998) and Western blot as a confirmation test. Nevertheless, serology is an

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indirect diagnosis test. Moreover, in case of early or chronic infection with cysts formation, the serology could be negative even if the parasite is present in the host. Therefore, direct diagnosis methods can be useful. These methods include immunohistochemistry, animal infection or PCR (Dubey and Schares, 2006). PCR has been demonstrated to be more sensitive to immunohistochemistry (Dubey and Schares, 2006). Classical PCR where the amplification and the detection are separated was the first technique described in *N. caninum* amplification (Payne and Ellis, 1996; Yamage et al., 1996). Semi-nested or nested PCR was also developed to increase the sensitivity (Buxton et al., 1998; Baszler et al., 1999). The major targets were the locus Nc5, a repetitive sequence present in the *N. caninum* genome and the ITS1 locus present in ribosomal DNA operons (Gondim et al., 2004). Real time PCR systems using a specific Taqman probe (Collantes-Fernandez et al., 2002) or hybridisation probes (Müller et al., 2002) were also described. In this study, the Nc5 sequence was selected since the ITS1 is potentially more variable (Gondim et al., 2004). A sensitive detection method of *N. caninum* in dog organs by real time PCR using either specific Taqman® probe, Plexor™ or Sybr® green systems was described.

2. Materials and methods

2.1. Reference material

N. caninum strain NC-1 (Dubey et al., 1988) was used to produce reference DNA. Briefly, NC-1 tachyzoites (ATCC50843; Dubey et al., 1988) were used to infect vero cells (ATCC CRL-1587). The growing tachyzoites led to the vero cells lyses and the tachyzoites were withdrawn. The total content of the culture flask was centrifuged (1500 × *g* for 20 min) and the pellet were resuspended in phosphate buffered saline (PBS, pH 7.4) and flushed through a 25 gauge needle in order to lyse to vero cells. The tachyzoites were harvested by centrifugation (1500 × *g* for 20 min). The pellets were resuspended in PBS (pH 7.4) and the number of tachyzoites in the suspension was determined using a haemocytometer.

2.2. Indirect fluorescence antibody test

The IFAT was performed as previously described (Dubey et al., 1988). Briefly, 10^4 tachyzoites per well were used to coat a 10-wells tefloned slide. The slides were washed and 20 μl of diluted sera (1/50–1/800) were added to the wells for 25 min at 37 °C. After washing, a FITC-conjugated anti-dog IgG (Sigma–Aldrich, St Louis, USA) was added and incubated as above. After washing, the slides were mounted and positive tachyzoites were looked for under epifluorescence microscope (400×).

2.3. Organs

From November 2004 to June 2005, 87 dogs from a dog pound in Algiers were euthanised by pentobarbital overdose and the liver, the spleen and the mesenteric lymph nodes were removed by surgery. The samples were conserved transported to the lab on ice in a cooling bag. In the lab, the organs were stored at −20 °C till DNA extraction. Moreover, none of the studied dogs showed neosporosis symptoms.

2.4. DNA extraction

The DNA was extracted from 50 mg of each organ or from NC-1 tachyzoitzes (4×10^6 ml^{-1}) using a phenol chloroform based method (Sambrook et al., 1989). The organs were incubated in a lysis buffer (Tris 10 mM, EDTA 100 mM, pH 8, SDS 0.5%) containing proteinase K (0.1 mg ml^{-1}) at 56 °C for 18 h. The lysate was extracted twice with a phenol/chloroform/isoamylic alcohol (25/24/1, v/v/v) solution. The DNA was precipitated with ethanol in the presence of 3 M sodium acetate. After centrifugation (13,000 × *g* for 30 min), the pellets were washed with 70% ethanol. The final DNA pellets were resuspended in 200 μl Tris EDTA buffer. The DNA concentration was spectrophotometrically estimated (Nanodrop 100, Isogen, The Netherlands). Finally the DNA was stored at −20 °C until use. For NC-1 DNA extraction, the DNA was extracted using the same protocol as above on 1 ml of pelleted tachyzoites but the lysis time was reduced to 3 h.

2.5. PCR systems

The primers were selected on base of the NC5 sequence (Genbank accession number X84238). For the different real time PCR systems, the amplifications were performed on ABI7000 (Applied Biosystems, Foster City, CA, USA). The PCR efficiency has been calculated by using the slope of the standard curve $E = (1 - 10^{-1/\text{slope}}) \times 100$.

2.6. Sybrgreen system

For the Sybrgreen real time system the primers were modified from NP7-NP4 system (Baszler et al., 1999) using the Oligo6 software (Medprobe, Oslo, Norway). Upper primer: 561U20: GGGAGTTGGTAGCGGT-