Detection of antibodies against domestic cat hepadnavirus using baculovirus-expressed core protein

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Abstract

A novel orthohepadnavirus (domestic cat hepadnavirus [DCH]) similar to human hepatitis B virus has been recently detected in serum and liver samples from domestic cats with chronic hepatitis and hepatocellular carcinoma. Molecular investigations by independent research groups around the world have revealed positivity rates ranging from 6.5% to 12.5% in blood samples and up to 14.0% in liver tissue. In this study, we screened an age-stratified collection of feline sera (*n* = 256) by using an antibody detection enzyme-linked immunosorbent assay based on the recombinant core antigen of DCH (DCHc). Specific antibodies (DCHc Abs) were detected with a prevalence of 25.0%. The DNA of DCH was detected in 35.9% (23/64) of seropositive cats and only in 1.0% (2/192) of seronegative animals. Based on the serological (IgG and IgM anti-DCHc) and virological status, the possible stages of DCH infection were predicted.

1 INTRODUCTION

The family Hepadnaviridae comprises small hepatotropic enveloped viruses, with a partially double-stranded DNA genome of approximately 3.0−3.4 kb in length, containing four overlapping open reading frames (ORFs) encoding for the polymerase (L), surface (S), core (C), and X proteins (Seeger & Mason, 2015). Based on their divergent genomic sequences and narrow host range of infection, hepadnaviruses are classified into five genera: Parahepadnavirus, Metahepadnavirus, Herpetohepadnavirus, Avihepadnavirus, and Orthohepadnavirus (Magnius et al., 2020). The genus Orthohepadnavirus includes at least 12 species whose members infect mammals, with hepatitis B virus (HBV), a major human pathogen, as the type species of the genus (Magnius et al., 2020; Seeger et al., 2013). In the last few years, a variety of distantly HBV-related orthohepadnaviruses has been identified in diverse mammalian species, including cats (Aghazadeh et al., 2018). The first feline orthohepadnavirus infection was documented in 2018 in Australia, from a 7-year-old male neutered domestic shorthair cat diagnosed with multicentric large B-cell lymphoma and concomitant infection with feline immunodeficiency virus (FIV). Using specific polymerase chain reaction (PCR), the novel virus, named domestic cat hepadnavirus (DCH), was detected in 10% of FIV-infected cats and fully sequenced (Aghazadeh et al., 2018). Subsequently, viruses genetically close to the Australian DCH strain have been detected in cat whole blood and serum samples with rates of 10.8% in Italy (Lanave et al., 2019), 12.4% in Thailand (Piewbang et al., 2020), and 12.3% in Malaysia (Anpuanandam et al., 2021). The potential clinical impact of DCH on feline health and its possible role in the development of liver disease is currently under investigation (Capozza, Decaro, et al., 2021). A positive correlation between an increased level of markers indicative of structural or functional liver damage and high serum viral loads (>104 genome copies per millilitre) has been observed (Lanave et al., 2019; Capozza, Lanave, et al., 2021). In addition, analysis by PCR and in situ hybridization (ISH) of formalin-fixed, paraffin-embedded biopsies of diseased and normal feline liver revealed the presence of DCH in 43.0% of chronic hepatitis and 28.0% of hepatocellular carcinoma (HCC) es, whereas the virus was not found in cholangitis es, biliary carcinomas, and normal liver samples (Pesavento et al., 2019). Histological lesions associated with inflammation and neoplasia mirroring the features observed in HBV-associated hepatopathies (Desmet et al., 1994) have been identified in cats with DCH-associated chronic hepatitis or HCC (Pesavento et al., 2019). The epidemiology and pathobiology of DCH in cats is still uncertain, but the possible parallelisms with HBV might help understand the biology of DCH. The natural history of HBV infection and the spectrum of the disease are diverse and variable in severity, evolution, and prognosis, ranging from acute asymptomatic and self-limiting forms to symptomatic disease and to progressive chronic hepatitis B (Fattovich, 2003; Liaw & Chu, 2009). A substantial proportion of patients develops cirrhosis and hepatocellular carcinoma, whereas others have lifelong quiescent viral activity not requiring antiviral therapy (Seto et al., 2018). For detection of HBV and predicting the stage of infection in human patients, antigens, antibodies, and viral genome are profiled/quantified and this information is coupled with haematological tests (Kao, 2008; Huang et al., 2020; Lee & Kim, 2021). Among the HBV serological markers, the presence of antibodies raised to the core antigen (anti-HBc) is compatible with acute, resolved, chronic, and occult HBV infection (Kao, 2008). Several enzyme-linked immunosorbent assay (ELISA) tests based on recombinant HBc are commercially available and routinely employed for detection of anti-HBc in blood and organ donors. For DCH, detection and quantification of viral genome represents the only diagnostic tool (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Lanave et al., 2019; Piewbang et al., 2020).

Herein, we report the development of an antibodies detection ELISA based on the recombinant DCH core protein (DCHc). The ELISA test was employed to investigate the antibody response to DCHc in an age-stratified sera collection from household cats. All sera were also analyzed by quantitative PCR to evaluate the possible correlation between the serological response against DCHc and the viraemic status.

2 MATERIALS AND METHODS

2.1 Sampling

A total of 256 household cat serum samples, submitted to the laboratory of the veterinary hospital of the Faculty of Veterinary Medicine of Teramo (Italy) for presurgical evaluation, were randomly selected. Data on the clinical status of animals were not available. The cats were grouped according to the age: <1 year, 1–3 years, 3-year age groups from 4 to 12, and >12 years of age. Informed consent was obtained from all animal owners.

2.2 Expression of the core protein (DCHc) in the baculovirus system

The full-length core encoding gene (573-nt in length) of the DCH strain ITA/2018/165-83 (GenBank accession no. MK117078) (Lanave et al., 2019), containing recognition sites for BamHI, respectively, before the start and after the stop codon was synthesized and cloned into the plasmid pUC57 (GeneScript). To generate the recombinant baculovirus transfer vector, the core gene was excised from pUC57 vector and inserted into BamHI site of the pAcYM1 plasmid (Matsuura et al., 1987) under the control of the polyhedrin promoter. The presence and the correct orientation of the insert were evaluated by PCR, digestion enzyme, and sequence analysis. Recombinant vector was then purified and co-transfected into Spodoptera frugiperda (Sf21) cells with linearized baculovirus DNA (BacPAK6, Bsu36 I digest; Clontech) using Cellfectin II Reagent (Invitrogen, Milan, Italy). The recombinant baculovirus was selected using X-Gal blue/white screening and plaque purified on Sf21 cells (King & Possee, 1992). For large-scale production of the core protein, 100 ml Sf9 cells (1 × 106 cell/ml) suspension culture (King & Possee, 1992) was infected with the recombinant baculovirus at a multiplicity of infection of 3 plaque forming units/cell. The culture medium after separation from the cell debris at 4 days postinfection (PI) was concentrated by ultracentrifugation in SW28 rotor (Beckman) at 27,000 rpm for 3 h through a 17% sucrose cushion in TEN-buffer (100 mM NaCl; 50 mM Tris-HCl, pH 7.5; 1 mM EDTA). The final pellet was resuspended in 1 ml of TEN-buffer, analyzed by SDS-15% polyacrylamide gel electrophoresis (PAGE), and visualized by Coomassie brilliant blue staining. Protein concentration was determined by measuring the optical density at 280 nm (OD280) and visually by running on SDS-15% PAGE aliquots containing bovine serum albumin standards (Promega Corporation, Milan, Italy).

The immunogenicity of the recombinant protein was assessed by western blot (WB) using sera collected from two cats that repeatedly resulted positive for DCH DNA during a longitudinal observational study (Capozza, Lanave, et al., 2021; Lanave et al., 2019). Antigenic cross-reactivities between the DCH core and other recombinant viral proteins of feline origin was ruled out in WB using rabbit hyperimmune sera raised, respectively, against feline calicivirus (Di Martino et al., 2007) and feline GIV.2 norovirus virus-like particles (VLPs) (Di Martino et al., 2010) (data not shown).

Electron microscopy (EM) analysis was performed by adsorbing of 10-µl drop of recombinant protein onto carbon-coated copper 400-mesh EM grids for 15 min and then stained for 1 min with 2% phosphotungstic acid (pH 7.2).

2.3 Antibody detection ELISA

For the ELISA development, mock-infected Sf9 insect cells lysate and DCHc recombinant protein, both at final concentrations of 1 µg/ml, were coated onto 96-well EIA plates (Costar, Italy) at 100 µl per well in carbonate–bicarbonate buffer (0.05 M, pH 9.6) and incubated at 4°C overnight (Di Martino et al., 2014). The wells were washed five times with 0.1% Tween-Phosphate-Buffered Saline (PBS-T) and then blocked with 200 µl of SuperBlock™ Blocking Buffer (Invitrogen, Paisley, UK) at room temperature for 1 h. To determine the optimal working dilutions, twofold dilutions of sera positive for IgG or IgM in WB were prepared starting with a dilution of 1:25 until 1:400. Each serum dilution was added to wells coated with either the DCHc antigen or mock-infected Sf9 cells. The sample dilution was considered optimal when for each serum tested was obtained a positive/negative ratio (OD405 of DCHc antigen/OD405 of mock infected cells) ≥2.0 and OD405 value close to 0.5 for IgM and 1.0 for IgG. Based on the results, serum samples (100 µl) were diluted, respectively, to 1:50 for IgM or to 1:100 for IgG in SuperBlock™ Blocking Buffer and added to the antigen-coated wells. After incubation at 37°C for 1 h, plates were washed five times with 0.1% PBS-T and then incubated with horseradish peroxidase-conjugated goat anti-cat IgM (Bio-Rad, Italy) or goat anti-cat IgG (Sigma-Aldrich, Milan, Italy), respectively, at dilution of 1:40,000 and 1:5000 for 30 min at 37°C. The reaction was developed with the addition of 100 µl per well of 2,20-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Invitrogen) substrate and incubation at room temperature for 12 min. Absorbance was measured at 405 nm using a multiskan automatic plate reader (ThermoLabsystems, Finland). The cut-off point of the ELISA (OD405 ≥ 0.3 for IgM; OD405 ≥ 0.8 for IgG) was established as the mean of the OD405 readings of 25 cat serum samples negative in WB for IgM or IgG anti-DCHc plus 2 standard deviations. For each sample tested, a positive/negative ratio (OD405 of DCHc/OD405 of mock-infected cells) ≥2.0 was used to evaluate the background binding. To evaluate the repeatability of the ELISA, 10 serum samples (five positive sera and five negative sera) were selected randomly. Inter-assay variability was assessed testing samples in five replicates on plates in different experiments, whilst intra-assay variability was determined testing each sample in five replicates within the same experimental session. The intra-assay coefficients of variation (CV) ranged from 4.3% to 7.1%, and the inter-assay CV value ranged from 5.8% to 8.7%. For each serum sample, ELISA assay was repeated twice. Also, all sera resulting positive in ELISA was further assessed in WB.

2.4 Molecular screening for DCH

Total DNA was extracted from 200 µl of each serum sample by using the QIAamp Cador Pathogen Mini Kit (Qiagen S.p.A., Milan, Italy), following the manufacturer's instructions and stored at −80°C until use. The presence of DCH DNA was assessed by quantitative PCR (qPCR), targeting a 105 nucleotide (nt) fragment of the polymerase region, as previously described (Lanave et al., 2019). Quantification was performed using TaqMan Fast Advanced Master Mix (Invitrogen) in a 25-µl volume comprising 5 µl of extracted DNA and 20 µl of master mix. Primers and TaqMan probe were used at concentrations of 200 and 100 nM, respectively. Tenfold serial dilutions (from 100 to 109 copies) of a

plasmid standard TOPO XL PCR containing a 1.4 kb long fragment of the Australian reference strain AUS/2016/Sydney (GenBank accession no. MH307930) (Aghazadeh et al., 2018) were used in each PCR run.

2.5 Statistical analysis

Statistical analysis was performed using Graphpad Prism Software (https://www.graphpad.com/scientific-software/prism/). Fisher exact test and Student's t-test were used to determine difference in prevalence rates among the age groups and difference between serologic and molecular results. The significance level of the tests was set at $p < 0.05$.

3 RESULTS

3.1 Expression of the recombinant core antigen

The core protein gene of the DCH strain ITA/2018/165-83 (Lanave et al., 2019) was successfully cloned into a baculovirus transfer vector (Figure 1a,b) to generate a recombinant baculovirus. By SDS-15% PAGE, a band with a molecular weight of ∼21.7 kDa corresponding in size to the DCH core protein was observed in the supernatant of Sf9 insect cells from 96 h PI (Figure 1c). The antigenicity of the recombinant core protein, DCHc, was confirmed by WB (Figure 1d), using field sera collected from cats resulted molecularly positive for DCH (Capozza, Lanave, et al., 2021; Lanave et al., 2019). Furthermore, EM analysis showed that the DCHc recombinant protein was able to self-assembly into VLPs with an estimated diameter of approximately 27 nm (Figure 2).

3.2 Serological screening to detect antibodies to DCHcAg

The DCHc protein was employed to develop an antibody detection ELISA to investigate the presence of specific IgM and IgG antibodies (DCHcAbs) in an age-stratified feline population. Out of 256 feline serum samples tested, a total of 64 (25.0%) reacted with DCHc. Of these, 55 sera (21.5%, 55/256) were positive only for IgG with an OD405 ranging from 0.8 to 2.2 (mean OD405 of 1.2), five samples (2.0%, 5/256) reacted only for IgM (mean OD405 of 0.5) and an additional four sera (1.6%, 4/256) possessed either IgM or IgG (mean OD405 of 0.4 and 0.9, respectively). All the positive sera obtained in ELISA, either for IgM or IgG DCHcAbs, were confirmed in WB assay. By examining the age-related patterns of anti-DCHc seropositivity, the prevalence was 20.0% (5/25) in cats <1 year of age and 32.6% (15/46) in the 1- to 3-year age group. The seropositivity rates declined to 17.8% (8/45) in cats of 4–6 years of age and to 19.1% (8/42) in the 7- to 9-year age group, increasing markedly in the 10–12 years age group (29.2%, 14/48) and in animals older than 12 years (28.0%, 14/50). There were no statistically significant differences comparing by Fisher's exact test the seroprevalences among the various age groups ($p > 0.05$).

3.3 Molecular investigation

All the feline sera were screened molecularly. Viral DNA was found in a total of 25/256 cats, with an overall prevalence of 9.8%. The mean and median values of DCH in the feline sera were 1.1 × 103 and 2.1 × 102. By examining the distribution of the molecular positivity across various age groups, the highest rates were found in cats

<1 year of age (16.0%, 4/25), followed by the 10- to 12-year age group (14.3%, 6/48) and older than 12 years (11.1%, 5/50), although without statistically significant difference compared to other age groups by Fisher's exact test ($p > 0.05$). The detection rate of DCH DNA was 35.9% (23/64) in seropositive cats and 1.0% (2/192) in seronegative (Table 1). This difference was strongly supported statistically ($p < 0.0001$). Furthermore, a significant difference in the means of the OD405 values between molecularly positive and negative cats ($p = 0.0294$) was observed by unpaired t-test. The median OD405 value was 1.35 for viraemic cats and 1.1 for cats resulted negative in qPCR. Out of 23 viraemic cats, 19 (82.6%) possessed only DCHc IgG, three (13.0%) were positive both for IgM and IgG and one (4.3%) serum had only IgM (Table 2).

TABLE 1. Results of the serological and molecular screening for domestic cat hepadnavirus (DCH) in the feline sera

Anti-DCHc AbsELISA	Sera tested	Positive qPCR	Negative qPCR
Positive anti-DCHcAg	64 (25.0%)	23 (35.9%)	41 (64.1%)
Negative anti-DCHcAg	192 (75.0%)	2 (1.0%)	190 (99.0%)
Total	256	25 (9.8%)	231 (90.2%)

TABLE 2. Distribution of the seropositivities for IgM and IgG anti-DCHc in the sera collection analyzed

IgM anti-DCHc	IgM and IgG anti-DCHc	IgG anti-DCHc	Total
Anti-DCHc Abs	5 (7.8%)	4 (6.2%)	55 (85.9%)	64
qPCR	1 (4.3%)	3 (13.0%)	19 (82.6%)	23

4 DISCUSSION

Serological response against HBc is considered a marker of exposure to HBV infection (Kao, 2008). The recombinant HBV core protein has been successfully expressed using a variety of cell systems including bacteria (Lanford et al., 1987; Pasek et al., 1979), yeast (Miyanohara et al., 1986), mammalian cells (Roossinck et al., 1986), and insect cells (Lanford & Notvall, 1990). HBV core protein possesses the property to self-assemble in VLPs eliciting a strong immune response when inoculated in mice (Lanford & Notvall, 1990).

In this study, recombinant DCH core particles were generated in insect cells using baculovirus expression system and employed to develop an antibody detection ELISA to study DCH seroprevalence in a population of household cats randomly selected among animals hospitalized for surgery. Anti-DCHc Abs were detected with an overall prevalence of 25.0% (64/256), remarking the results of direct epidemiologic investigations (Aghazadeh et al., 2018; Anpuanandam et al., 2021; Lanave et al., 2019; Piewbang et al., 2020). On screening of all sera by qPCR, DCH DNA was identified in 9.8% (25/256) of cats. This rate was higher than those found in adult pet cats in Australia (6.5%) (Aghazadeh et al., 2018) and like the rates previously reported in an Italian feline population (10.8%) (Lanave et al., 2019), Thailand (12.4%) (Piewbang et al., 2020) and Malaysia (12.3%) (Anpuanandam et al., 2021). Interestingly, 92% (23/25) of molecular positive cats possessed also DCHc Abs, whilst only 8% (2/25) of seronegative cats resulted

positive in qPCR. In addition, a significant correlation between the OD405 values and the detection rate of DCH DNA was found by comparing viraemic cats (OD405 ≥ 1.35) with non-viraemic animals (OD405 ≤ 1.1). Overall, these findings indicate that anti-DCHc Abs might be used as a serologic marker with diagnostic value in the feline clinical practice for detection of DCH infection.

Analyzing the age-related trend, the highest seroprevalence was revealed in cats aged 1–3 years (32.6%, 15/46), followed by the 10–12 years age group (29.2%, 14/48) and animals older than 12 years (28.0%, 14/50). A different pattern was observed in the molecular detection rates, as the highest positivity for DCH DNA was found in cats <1 year of age (16.0%, 4/25), confirming previous findings that many infections may occur in young animals (Lanave et al., 2019). However, in our analysis high molecular rates were also found in cats older than 10 years of age. Combining the molecular and serological results, the U-shaped DCH age-related profile emerged in this study could be consistent with the aptitude of DCH to give persisting infection, as occurs in about 95% of human patients infected with HBV perinatally or in early childhood (Lavanchy, 2004; Liaw & Chu, 2009). Our hypothesis on DCH disease patterns is also supported by the evidence that long-term DCH infection may establish in some animals (Capozza, Lanave, et al., 2021).

During acute HBV infection the first serum marker is the surface antigen (HBsAg), followed by anti-HBc IgM (1−2 weeks after HBsAg), both which persist for up to 6 months. Although anti-HBc IgG are detectable later, they show a lifelong persistence (Liaw & Chu, 2009). In our analysis, a high proportion of seropositive animals possessed only anti-DCHc IgG (55/64, 85.9%), whilst a serological status compatible with an early stage of infection was observed in nine sera (9/64, 14.1%) that were positive only for IgM (5/64, 7.8%) or both for IgM and IgG (4/64, 6.25%). Viral DNA was detected in one out of five cats with IgM and in three out of four cats with both IgM and IgG, confirming an active replication stage. Furthermore, a serological pattern consistent with a very early stage of infection, before DCHc seroconversion, was revealed in two samples that were negative serologically, but positive for DCH DNA. The viral loads detected in the viraemic sera analyzed in this investigation were generally lower (mean value 1.1 × 103) than those reported in a previous study (Lanave et al., 2019) that focused on cats with a clinical suspect of infectious disease or with a haematochemical profile suggestive of liver injury. A significant correlation between high DCH DNA load (≥104 viral genome copies per millilitre) and liver damage was observed (Lanave et al., 2019). Similarly, high HBV DNA loads in blood are detectable in acute or chronic stages of disease (Seeger & Mason, 2015). In the present survey, the feline population assessed was apparently healthy at the time of sampling, even if detailed individual hematological data were not available. Accordingly, we hypothesized that DCH infections with low viral load may represent a status of inactive carrier, as observed in overtly healthy human individuals with occult HBV infection, in which viral DNA is always detectable in the liver and close to or under the limits of detection in the blood. Individuals with occult infection are negative for both HBsAg and anti-HBs, but positive for anti-HBc. Also, when detectable, serum HBV DNA is present at level <103 viral genome copies (equivalents to about <200 IU/ml) (Raimondo et al., 2008). The risk of HBV reactivation is frequently observed in patients under immunosuppressive diseases or immunosuppressive treatments (Wang et al., 2017). Likewise, a correlation between DCH infection and simultaneous infection by immunosuppressive feline retroviruses has been observed in cats (Lanave et al., 2019). Non-viraemic cats with anti-DCHc Abs (64.0%; 41/64) could be either animals entered into a stage of occult infection or animal

recovered from acute DCH infection. Seroconversions to anti-HBs and anti-HBc are observed in the blood of human patients who recover from HBV infection. However, the presence of anti-HBc alone has also been associated to false positive reaction, due to the lack of a serological standardized diagnostic procedure (Wang et al., 2017). In this study, false positive reactions were ruled out re-testing all positive sera by WB for IgM and IgG, confirming the results obtained in ELISA. Therefore, evaluating the stage of DCH infection using only anti-DCHc Abs as marker is not sufficient and the development of novel serological test based on the S antigen of DCH would be required.

In conclusion, baculovirus-expressed DCHc protein was used to develop an ELISA and to assess the prevalence of anti-DCHc Abs in the sera of cats. By coupling serological and molecular information for DCH, the vast majority of viraemic cats were found to possess anti-DCHc Abs that could be used as a marker of DCH infection. The significance of anti-DCHc Abs in non-viraemic cats requires further investigations. Making parallelisms with HBV infection, the pattern of anti-HBs response could provide additional diagnostic information. Implementing the diagnostics of DCH in cats with other serological markers, such as the S antigen (DCHs) and S-specific antibodies (anti-DCHs), will be necessary to decipher the stage of DCH infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STATEMENT

Ethical statement is not applicable (Decreto Legislativo 4 March 2014, No. 26) since investigations was performed on cat serum samples submitted to the laboratory of the veterinary hospital of the Faculty of Veterinary Medicine of Teramo (Italy) for presurgical evaluation.

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Figure legend

FIGURE 1 (a) Polymerase chain reaction (PCR) amplification of the domestic cat hepadnavirus (DCH) core gene after cloning in the pAcYM1 baculovirus transfer vector. Line M: BenchTop 1 kb DNA ladder; line 1: DCH gene. (b) BamHI digestion of the pAcYM1 baculovirus transfer vector. Line M: BenchTop 1 kb DNA ladder; line 1: the recombinant vector digested with BamHI. (c) SDS-15% PAGE of the recombinant DCHc protein of the strain ITA/2018/165-83 (Lanave et al., 2019) and visualization by Coomassie brilliant blue staining. Line 1: Precision Plus protein Standards (Bio-Rad, Italy); lines 2 and 3: bovine serum albumin standards (Promega Corporation, Milan, Italy) at concentration, respectively, of 1 µg/µl and 100 ng/µl; line 4: DCHc protein concentrated from the supernatants of Sf9 insect cells at 96 h postinfection (PI). The arrow indicates the band of ~21.7 kDa corresponding in size to the putative molecular weight of DCH core protein. (d) Western blot (WB) analysis of the DCHc protein using a feline serum previously resulted positive for DCH DNA (Lanave et al., 2019). The work serum dilution was 1:100, followed by the adding of horseradish peroxidase-conjugated goat anti-cat IgG (Sigma-Aldrich, Milan, Italy) at dilution of 1:5000. Line 1: Precision Plus protein Standards; line 2: mock-infected Sf9 insect cells; line 3: recombinant DCHc protein. The arrow indicates the band of positivity

FIGURE 2

Electron microscopy analysis of domestic cat hepadnavirus (DCH) core particles. Bar, 50 nm. The arrows indicate DCHc recombinant protein self-assembled into virus-like particles (VLPs) with an estimated diameter of approximately 27 nm

Table legend

TABLE 1. Results of the serological and molecular screening for domestic cat hepadnavirus (DCH) in the feline sera

TABLE 2. Distribution of the seropositivities for IgM and IgG anti-DCHc in the sera collection analyzed