HAL
open science

Comparison of the value of measurement of serum galactomannan and -specific antibodies in the diagnosis of canine sino-nasal aspergillosis

F. Billen, D. Peeters, I.R. Peters, C.R. Helps, P. Huynen, P. de Mol, L. Massart, M.J. Day, C. Clercx

To cite this version:

F. Billen, D. Peeters, I.R. Peters, C.R. Helps, P. Huynen, et al.. Comparison of the value of measurement of serum galactomannan and -specific antibodies in the diagnosis of canine sino-nasal aspergillosis. Veterinary Microbiology, 2009, 133 (4), pp.358. 10.1016/j.vetmic.2008.07.018 . hal-00532458

HAL Id: hal-00532458

https://hal.science/hal-00532458v1

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Comparison of the value of measurement of serum galactomannan and *Aspergillus*-specific antibodies in the diagnosis of canine sino-nasal aspergillosis

Authors: F. Billen, D. Peeters, I.R. Peters, C.R. Helps, P. Huynen, P. De Mol, L. Massart, M.J. Day, C. Clercx

PII: S0378-1135(08)00292-7
DOI: doi:10.1016/j.vetmic.2008.07.018
Reference: VETMIC 4109

To appear in: *VETMIC*

Received date: 20-5-2008
Revised date: 18-7-2008
Accepted date: 24-7-2008

Please cite this article as: Billen, F., Peeters, D., Peters, I.R., Helps, C.R., Huynen, P., De Mol, P., Massart, L., Day, M.J., Clercx, C., Comparison of the value of measurement of serum galactomannan and *Aspergillus*-specific antibodies in the diagnosis of canine sino-nasal aspergillosis, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.07.018

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Manuscript ACCEPTED MANUSCRIPT

Comparison of the value of measurement of serum galactomannan and *Aspergillus*-specific antibodies in the diagnosis of canine sino-nasal aspergillosis.

F. Billen[a*], D. Peeters[a], I.R. Peters[b], C.R. Helps[b], P. Huynen[c], P. De Mol[c], L. Massart[d], M.J. Day[b], C. Clercx[a]

[a] Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20(B44), 4000 Liège, Belgium

[b] Division of Veterinary Pathology, Infection and Immunity, School of Clinical Veterinary Science, University of Bristol, Langford BS40 5DU, United Kingdom.

[c] Department of Biomedical Sciences, Medical microbiology, Medicine Faculty, University of Liège, CHU, Avenue de l'Hopital B23, 4000 Liège, Belgium

[d] Department of Veterinary Animal Production, Biostatistics, Veterinary Faculty, University of Liège, Boulevard de Colonster, 20/B43, 4000 Liège, Belgium

[*]corresponding author: F.Billen

Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, University of Liège, Boulevard de Colonster 20(B44), 4000 Liège, Belgium

Tel: +32 4 366 42 00 Fax: +32 4 366 42 41 E-mail: fbillen@ulg.ac.be

Accepted Manuscript

ACCEPTED MANUSCRIPT

Abstract

Serology is currently used for the diagnosis of canine sino-nasal aspergillosis (SNA). However, the accuracy of serological testing using commercially available, standardized purified antigen preparations of *Aspergillus* (CAPur*Asp*Ag) has only be poorly documented.

The aim of the present study was to assess the diagnostic value of an agar-gel double immunodiffusion (AGDD) test and an anti-*Aspergillus* IgG ELISA, using CAPur*Asp*Ag and the commercially available Platelia™ test for the detection of serum galactomannan.

Sera from 17 dogs with SNA, 18 dogs with a nasal tumour (NT), 11 dogs with lymphoplasmacytic rhinitis (LPR) and 33 control dogs were tested with the 3 methods.

AGDD result was positive in 76.5% of dogs with SNA, whereas all sera from dogs with non-fungal nasal disease and control dogs were negative. A positive IgG ELISA result was obtained in 88% of dogs with SNA and in 18% of dogs with LPR. All patients with NT and control dogs had a negative IgG ELISA result. The Platelia™ test was positive in 24% of dogs with SNA, 11% of dogs with NT, 9% of dogs with LPR and 24% of control dogs.

The results of this study suggest that (1) the detection of serum *Aspergillus*-specific antibodies with AGDD or ELISA, using CAPur*Asp*Ag, provides excellent specificity and good sensitivity, (2) the specificity is higher for AGDD (100%) than for ELISA (96.8%) while sensitivity is higher for ELISA (88.2%) than for AGDD (76.5%) and (3) serum galactomannan quantification with the Platelia™ test is unreliable for the diagnosis of canine SNA.

Keywords

Dog; sino-nasal aspergillosis; serology; immunodiffusion; ELISA; galactomannan

Accepted Manuscript

ACCEPTED MANUSCRIPT

Introduction

Canine sino-nasal aspergillosis (SNA) is most commonly caused by *Aspergillus fumigatus* (Sharp, 1998). While SNA may be suspected based on history and clinical signs, confirmation of the diagnosis may remain challenging. Several diagnostic methods have been evaluated in SNA (De Lorenzi et al., 2006; Johnson et al., 2006; Pomrantz et al., 2007; Saunders et al., 2004). Nevertheless, false positive and false negative results can occur with most methods (De Lorenzi et al., 2006) and a accurate simple non-invasive diagnostic test is still lacking.

The value of several techniques detecting serum *Aspergillus*-specific antibodies in the diagnosis of SNA has been evaluated. Agar gel double immunodiffusion (AGDD) is inexpensive and easy to perform but this method does not to allow the quantification of serum immunoglobulins. The first studies evaluating AGDD in the diagnosis of SNA reported up to 100% sensitivity and specificity (Lane and Warnock, 1977; Poli et al., 1981; Sharp et al., 1984). Harvey and O'Brien (1983), however, reported 15% false positive results in dogs with nasal tumours. In addition, AGDD result might be negative in early stages of SNA or where *Penicillium,* rather than *Aspergillus*, is the cause of SNA (Harvey and O'Brien, 1983; Mathews and Sharp, 2006). Enzyme-linked immunosorbent assay (ELISA) is a rapid quantitative method that allows the detection of very low concentrations of antibodies. Khan et al. (1984) reported a sensitivity of 78 to 100% and a specificity of 78 to 96% for ELISA in the diagnosis of canine SNA. A major drawback of these early serological studies is the lack of standardisation of the antigen preparations used. Although highly purified *Aspergillus* antigens have become commercially available, the value of serology in the diagnosis of SNA using these antigen preparations is only poorly documented since, to the authors' knowledge, only a single study investigating the diagnostic value of AGDD in canine SNA has used such antigens (Pomrantz et al., 2007).

ACCEPTED MANUSCRIPT

In human medicine, standard techniques based on *Aspergillus* antibody detection are not specific and sensitive enough to be used for the diagnosis of invasive aspergillosis (IA) because most patients fail to generate serum antibody (Young and Bennett, 1971). Therefore, diagnostic techniques have focused on the detection of circulating *Aspergillus* antigens, such as galactomannan (GM) (Aquino et al., 2007). In dogs, evaluation of serum GM detection has been limited to one study in which only dogs with IA were investigated (Garcia et al., 2001). Results were inconsistent when compared with the detection of *Aspergillus*-specific antibodies by ELISA.

The aim of this study was to assess the diagnostic value of a range of serological tests in the diagnosis of canine SNA. Commercially available standardized purified *Aspergillus* antigen preparation was used to develop an AGDD test and ELISA for detection of serum antibody. The sandwich ELISA test (Platelia™) was applied for the detection of serum GM.

Materials and methods

Animals

Sera from 46 dogs with nasal disease were retrospectively available for this study. In all dogs, diagnostic rhinoscopy was performed with a flexible paediatric bronchoscope (Fujinon BRO-YP2, Onys SA, Brussels, Belgium) and/or with a rigid endoscope (cystoscope K Storz SL 30°, Karl-Storz-Endoscopy Belgium SA, Strombeek-Bever, Belgium). When fungal plaque-like lesions were present, fungal cultures were performed from these lesions. In all other cases, fungal culture was performed from nasal swabs. All cultures were performed on Sabouraud-chloramphenicol-actidione medium at 37°C. Biopsies were taken in all patients for histopathological examination with biopsy forceps under endoscopic guidance. In dogs with fungal plaque-like lesions, the mucosa of the distal part of the nasal cavity was sampled, while in dogs with nasal tumour, the mass was sampled. In the other dogs with nasal disease,

ACCEPTED MANUSCRIPT

the most abnormal/inflamed region of the mucosa was biopsied. All sera were stored at -20°C until analysed.

Seventeen of these dogs had SNA. Affected animals included three Rottweilers, three mixed-breed dogs, two Beauceron sheepdogs and nine dogs each from a single breed. There were eleven males (seven entire) and six females (three entire) with a mean age of 6.0 ± 3.9 years and a mean body weight of 30.5 ± 10.4 kg. SNA was suspected based on history and clinical signs. The diagnosis was confirmed when fungal plaques were perendoscopically observed in the frontal sinus and/or nasal cavity and a positive fungal culture was obtained from fungal plaque-like lesions. *A. fumigatus* was the only fungus isolated in the present study.

Eighteen dogs suffered from nasal tumours (NT). Represented breeds included three golden retrievers, three mixed-breed dogs, two Rottweilers and ten dogs each from a single breed. There were seven males (six entire) and 11 females (four entire). The mean age of these dogs was 9.7 ± 2.7 years and their mean body weight was 28.4 ± 14 kg. Diagnosis of NT was based in all dogs on history, clinical signs, perendoscopic observation of a nasal mass and histopathological confirmation of neoplasia.

Eleven dogs were diagnosed with idiopathic lymphoplasmacytic rhinitis (LPR). Represented breeds included three mixed dogs and eight dogs each from a single breed. This group included five males (three entire) and six females (three entire). The mean age of these dogs was 7.8 ± 2.7 years and their mean body weight was 23.0 ± 15.8 kg. In all dogs, idiopathic LPR was diagnosed based on history, clinical signs, endoscopic findings (unilateral or bilateral mucopurulent nasal secretions, mucosal oedema/congestion with mild turbinate destruction, and the absence of fungal plaque, nasal mass, foreign body, dental abscess or oro-nasal fistula), negative fungal culture and histopathological findings compatible with LPR.

ACCEPTED MANUSCRIPT

Initial diagnosis of LPR was also confirmed by the absence of progression of the nasal disease during follow-up of the patient.

Sera from 33 dogs without nasal disease were also included in the present study. Twenty dogs were client-owned (five Labrador retrievers, two Leonberger dogs, two German Shepherd dogs and eleven dogs each from a single breed) suffering from various orthopaedic diseases and thirteen were healthy beagle dogs. This group of control (CTRL) dogs included 20 males (16 entire) and 13 females (12 entire). Their mean age was 5.0 ± 3.7 years and their mean body weight was 29.2 ± 15.9 kg. None of these dogs had history or clinical signs of upper respiratory disease, immune-mediated disease or systemic immune suppression. The study protocol for blood sampling in healthy beagle dogs was reviewed and approved by the university institutional animal care and use committee of the University of Liège.

Aspergillus precipitins determination by AGDD

The AGDD test was performed based on the technique of double immunodiffusion described by Ouchterlony (Ouchterlony, 1949). A 1% preparation of agarose (Sigma, St Louis, Missouri) in phosphate-buffered saline (PBS) (pH 7.4, 0.01 M) was poured into plastic petri dishes to a depth of 3 mm. A template was used to cut a pattern of six 5 mm diameter wells arranged 4 mm apart (edge to edge) around a central well also of 5 mm diameter. Twenty microliters of the antigen solution (1/2 dilution in PBS), composed of a standardized purified antigen preparation (Aspergillus Immunodiffusion Antigen (ref. 100501), Meridian Bioscience Europe s.a./n.v., Nivelles, Belgium) (1.486 mg/ml protein content) derived from the mycelial phase of cultures of *A. fumigatus*, *A. niger* and *A. flavus* was loaded into the central well. Twenty microliters of positive control serum (Goat Anti-Aspergillus Immunodiffusion Control Serum (ref. 100901), Meridian Bioscience Europe s.a./n.v., Nivelles, Belgium) (1/2 dilution in PBS), containing antibodies specific to *A. fumigatus*, *A.*

ACCEPTED MANUSCRIPT

flavus and *A. niger*, and twenty microliters of each test dog sera (1/2 dilution in PBS) were loaded to each of the peripheral wells in such a way that each dog serum was adjacent to a well containing the positive control serum allowing detection of precipitin bands of identity. The plates were incubated in a moist chamber at room temperature for a minimum of 24 hours and then observed for the development of precipitin lines. Detection of the precipitin bands was facilitated by use of a beam of high-intensity light with the plate held over a dark background with the light projected from below at approximately 45° angle to the plate surface.

Anti-*Aspergillus* IgG determination by indirect ELISA

Polyvinylchloride microtitre plates (Titerteck Immunoassay Plate, Helsinki, Finland) were coated with 75µl of the same antigen preparation used for the AGDD diluted in PBS to a final concentration of 20 µg/ml and incubated overnight at 4°C. Plates were subsequently blocked with 75 µl of 1% (wt/vol) polyvinylpyrrolidone (Sigma, St Louis, Missouri) in PBS for 1 hour at room temperature. Doubling dilutions of each serum sample (50 µl) in 5% non-fat milk in PBS plus 0.05 % Tween 20 (PBST) were made on each plate from a starting dilution of 1:50 and incubated for 2 hours at 37°C. Fifty µl of a rabbit anti-dog IgG alkaline phosphatase conjugated antibody solution (Sigma, St Louis, Missouri) (dilution of 1:10000 in PBST) was added and incubated for 1 hour at 37°C. On each plate, all samples were fully titrated (from 50 to 6400) and a positive control reference serum from a dog with SNA confirmed by fungal culture of a nasal plaque and positive AGDD test was titrated on each plate. In order to confirm the specificity of the ELISA, negative controls omitting each reagent were performed on each plate. All incubations were carried out in a humidified chamber, and plates were washed three times with 150 µl of PBS between incubations. Checkerboard titrations were used to determine optimum dilutions of reagents and serum

ACCEPTED MANUSCRIPT

samples. The plates were developed with Sigma Fast p-nitrophenyl phosphate (pNPP) alkaline phosphatase substrate (Sigma, St Louis, Missouri) in the dark for 1 hour at room temperature before being read at 405 and 492 nm with a Multiscan Biochromic Plate reader (Labsystems, Helsinki, Finland).

Data were analysed in Excel. By plotting $\log_{10}$ optical density (OD) values against $\log_{10}$ serum dilutions, curves for standard and test serum samples were generated. Sample and standard curves were compared for parallelism and where this was not evident; dilution points on the sample curve were omitted. A minimum of three dilution results were deemed necessary to obtain a dilution curve. IgG concentrations in the serum samples we expressed as ELISA Units (EU) per ml with the serum standard having a concentration of 100 EU/ml. Serum samples whose concentration was below the detection limit of the ELISA and thus not sufficient to produce a three point dilution curve or whose dilution curve was not parallel to the standard were assigned a value of 0 EU/ml.

Inter- and intra-plate coefficients of variation were determined by running 16 repeats of the standard serum sample on 4 separate plates with 4 repeats on each plate. The cut-off value was established using either the mean + 3 standard deviations (SD) of the IgG concentration of the 33 healthy control dog sera (Jensen and Latge, 1995) or receiver-operating characteristic (ROC) analysis. Both methods resulted in a cut-off value of 15 EU/ml. Sera with an anti-*Aspergillus* IgG concentration > 15 EU/ml were consequently considered as positive.

<u>GM determination by sandwich ELISA</u>

The commercially available sandwich ELISA (Platelia™ *Aspergillus*) was used. Serum samples were processed according to manufacturer's instructions. A cut-off value of 0.5 was used as proposed by the manufacturer, as this value is now universally accepted in

ACCEPTED MANUSCRIPT

Europe and the USA for human serum GM testing (Aquino et al., 2007; Maertens et al., 2007). In consequence, sera with an OD index > 0.5 were considered positive.

Statistics

The SAS computer software package was used for all statistical analyses. Age and body weight of dogs were assessed for normality by use of the Kolmogorov-Smirnov test, and were compared among groups of dogs (i.e. SNA, NT, LPR, and CTRL dogs) by use of a one-way ANOVA. Sex status was compared among groups of dogs by the use of a contingency table (4x4) with a Fisher's Exact Test. Results of the IgG ELISA and GM ELISA were assessed for normality before and after logarithmic transformation by using the Kolmogorov-Smirnov test. As the IgG ELISA data were normally distributed following logarithmic transformation, 1EU was added to all results to allow transformation of samples assigned an anti-Aspergillus IgG concentration of 0 EU/ml. In dogs with SNA, duration of symptoms (in months) and *Aspergillus*-specific IgG concentration were compared between dogs that showed a positive AGDD result and patients with a negative AGDD result, using a T-Test. Comparisons between groups (i.e. SNA, NT, RLP, and CTRL dogs) were made using Generalized Linear Model (GLM) procedures. For each diagnostic method used, a contingency table (2x4) with a Fisher's Exact Test was used to compare the number of positive results between the groups of dogs. Positive and negative results of GM ELISA and IgG ELISA were compared by using a contingency table (2x2) with a Fisher's Exact Test. Statistical differences were considered significant when $P<0.05$.

For each diagnostic method, sensitivity (SE), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV) were calculated with 95% confidence intervals, following a binomial distribution. Perendoscopic visualisation of fungal plaques was used as

Accepted Manuscript

ACCEPTED MANUSCRIPT

the gold standard for the diagnosis of SNA. Intra- and inter-plate coefficients of variation of the IgG ELISA were calculated with a spreadsheet program.

Results

Animals

There was no significant difference between the four groups of dogs with respect to sex and body weight. However, dogs with NT were significantly older than dogs with SNA and CTRL dogs ($P<0.05$) and dogs with LPR being significantly older than CTRL animals ($P<0.05$).

AGDD

Thirteen sera (76%) from dogs with SNA were positive by AGDD (Table 1). Sera from all dogs with non-fungal nasal disease and CTRL animals were negative. The SE, SP, PPV and NPV of AGDD for the diagnosis of SNA are given in table 2.

IgG ELISA

The intra- and inter-plate coefficients of variation of this ELISA were 9 and 5% respectively. The sera from 14 dogs with NT, 4 dogs with LPR and 27 CTRL dogs could not generate a dilution curve including at least 3 dilution points within the range of the standard serum; therefore these samples were assigned an *Aspergillus*-specific IgG concentration of 0 EU/ml.

Using the calculated cut-off value of 15 EU/ml, a positive IgG ELISA result was obtained for sera from 15 (88%) dogs with SNA (Table 1) and from 2 (18%) dogs with LPR. Sera from dogs diagnosed with NT and CTRL dogs were all negative. The SE, SP, PPV and NPV of the IgG ELISA in the diagnosis of SNA are reported in table 2. Dogs with SNA had significantly

ACCEPTED MANUSCRIPT

higher (P<0.0001) *Aspergillus*-specific IgG concentrations (median: 45.3 EU/ml) than dogs with NT (median: 0 EU/ml), dogs with LPR (median: 3.34 EU/ml) and CTRL animals (median: 0 EU/ml) (figure 1).

AGDD versus IgG ELISA

A positive IgG ELISA result was obtained in three out of the four dogs with SNA that were negative in AGDD. A positive AGDD result was obtained in one dog with SNA that was negative in IgG ELISA (Table 1). Only one dog with SNA was negative by both tests. Sensitivity, SP, PPV and NPV of both serological methods were not statistically different. While AGDD and IgG ELISA had a SP of 100 and 96.8%, respectively, their SE was lower (i.e. 76.5 and 88.2 respectively). However, when results of the IgG ELISA were combined with those of AGDD, the sensitivity increased to 94.1% (table 2) but the SE, SP, PPV and NPV did not significantly differ relative to those values computed for the individual tests.

GM ELISA

Galactomannan was detected in the serum of 24% (4/17) of dogs with SNA (Table 1), 11% (2/18) of patients with NT, 9% (1/11) of dogs with LPR and 24% (8/33) of CTRL animals. The SE, SP, PPV and NPV of GM quantification in the diagnosis of SNA are reported in table 2. There was no significant difference between the 4 groups of dogs regarding the GM index (figure 2). The median GM index was 0.35 (range 0.107-0.747), 0.21 (range 0.13-0.822), 0.27 (range 0.157-0.687) and 0.33 (range 0.122-1.5) in dogs with SNA, dogs with NT, dogs with LPR and CTRL animals, respectively. Only three dogs had a GM index above 1 (i.e. 1.29, 1.15 and 1.50) and these animals belonged all to the CTRL group. If the cut-off of the GM index was increased to an optical density index of 1 (as in a large

ACCEPTED MANUSCRIPT

number of earlier studies in human IA), only 3 CTRL dogs were considered positive for GM antigenemia.

GM ELISA versus AGDD versus IgG ELISA

No relationship could be found between positive and negative results of GM ELISA and *Aspergillus*-specific antibodies (AGDD and IgG ELISA) detection. The four dogs with SNA that had serum GM detected were also positive in AGDD and IgG ELISA but the two dogs with LPR and the 8 CTRL animals that were positive for serum GM detection were negative in AGDD and IgG ELISA. Moreover, the three dogs showing the highest antigenemia (i.e. optical density index > 1) were CTRL dogs with negative AGDD and IgG ELISA results.

Discussion

The present study has investigated the value of GM antigenemia measurement and two serological tests (i.e. AGDD and IgG ELISA) in the diagnosis of canine SNA. Both serological tests were performed with a commercially available standardized purified *Aspergillus* antigen preparation extracted from cultures of *A. fumigatus*, *A. niger*, and *A. flavus*.

The specificity of AGDD in the diagnosis of canine SNA was 100% as no false positive results were obtained. This is similar to the findings of most previous studies (Lane and Warnock, 1977; Poli et al., 1981; Pomrantz et al., 2007; Sharp et al., 1984). One study reported positive AGDD in some dogs with nasal tumour but the method used in that study was not given in detail (Harvey and O'Brien, 1983). The sensitivity of AGDD in the present study was 76% which is similar to a recent report in which the same standardized purified antigen preparation was employed (Pomrantz et al., 2007) but lower than in other studies in

ACCEPTED MANUSCRIPT

which up to 100% sensitivity was commonly reported (Lane and Warnock, 1977; Poli et al., 1981; Sharp et al., 1984). The higher sensitivity reported in those earlier studies might be related to the use of multiple *Aspergillus* antigenic preparations.

According to Mathews and Sharp (2006), AGDD can be negative in early cases of canine SNA. In the present study, there was no significant difference between the mean duration of clinical signs in dogs with SNA with a negative AGDD result (7.3 ± 9.3 months) and affected dogs with a positive AGDD result (3.0 ± 1.6 months). In consequence, the negative AGDD results obtained in some dogs with SNA in the present study are unlikely to reflect the stage of disease. According to Mathews and Sharp (2006), nasal *Penicillium* infection can also result in a negative AGDD test result in a dog with SNA when only *Aspergillus* antigens are used in the test. This is unlikely to be the reason for the negative AGDD results in some dogs with SNA as only *A. fumigatus* was cultured from nasal samples from dogs in the present study.

Since there is no purified anti-*Aspergillus* canine antibody commercially available, serum from a dog with SNA confirmed by rhinoscopy, fungal culture and AGDD was used as a positive control for the IgG ELISA. Since the test serum samples may have contained other antibody sub-classes or non-specific cross-reactive antibodies resulting in false positive anti-*Aspergillus* antibody titres, sample dilution curves were assessed for parallelism and compared with the positive control serum to ensure that only antibodies with similar affinities to the antigen preparation used were measured (Dye et al., 2005).

In this study, low concentrations of *Aspergillus*-specific IgG antibodies were found in a large number of dogs without SNA. This was expected as dogs are frequently exposed to ubiquitous *Aspergillus* antigens and may mount an immune response to this fungus without contracting the clinical disease (Day and Penhale, 1988). Similarly, serum *Aspergillus*-specific antibodies have been detected in healthy horses and cows (Guillot et al., 1999; Jensen

ACCEPTED MANUSCRIPT

and Latge, 1995). When positive reactions occur in serological assays using complex preparations of fungal antigens, the assay may be detecting normal baseline antibody as opposed to an induced humoral immune response to *Aspergillus* spp.. This seems to be the case in horses where no differentiation could be made between healthy and *Aspergillus*-infected horses based on *Aspergillus*-specific IgG concentrations (Guillot et al., 1999). As the majority of dogs with SNA had higher concentrations of *Aspergillus*-specific IgG antibodies than dogs with non-fungal nasal disease and CTRL dogs, *Aspergillus*-infected dogs could be differentiated readily from dogs that may have been exposed to the fungus from environmental sources.

Two (18%) dogs with idiopathic LPR had a positive IgG ELISA result. One explanation could be that both dogs were incorrectly classified within the LPR group. As other diagnostic tests, such as diagnostic imaging or sinus trephination, were not realized in these dogs, one could assume that these dogs could have suffer from SNA localized to the frontal sinus without nasal involvement (Johnson et al., 2006). However, these dogs did not received antifungal therapy and none developed SNA during a follow-up period of at least 8 months. The role of *Aspergillus* spp. in the pathogenesis of LPR could be another explanation for an increased *Aspergillus*-IgG antibody concentration in some dogs with LPR. In people, it has been postulated that fungal organisms might represent the immunologic target initiating and maintaining the disease process in patients with chronic rhino-sinusitis (Gosepath and Mann, 2005). A recent human study reported the presence of *Aspergillus*-specific antibodies in 11% of patients with chronic rhino-sinusitis. However, antibodies were detected with AGDD and not with ELISA (Cameli-Rojas et al., 2004). In dogs, the relationship between fungus and LPR has only recently been investigated in two studies comparing quantitatively fungal DNA in nasal mucosa of dogs with SNA, LPR, NT and healthy control dogs. Yet, results were divergent. Windsor et al. (2006), using a broad pan-fungal assay, detected more

ACCEPTED MANUSCRIPT

fungal DNA in the nasal mucosa of dogs with LPR than in dogs with NT or healthy control dogs while Peeters et al. (2007), using a *Penicillium* and *Aspergillus* assay, did not find different amounts of fungal DNA between dogs with LPR and healthy control dogs. Other studies investigating the immunological response to fungus in LPR are needed to clarify the role of fungus in LPR in dogs.

AGDD and IgG ELISA results were in agreement in 13 dogs with SNA (76.5%) (i.e. 12 dogs with both results being positive and 1 dog with both results being negative). This is similar to what is observed in human patients with allergic aspergillosis in whom AGDD and IgG ELISA results are generally in agreement (Richardson et al., 1983). The lack of complete agreement between AGDD and IgG ELISA, however, is not completely understood. One explanation might be that ELISA detects high-affinity antibodies while AGDD detects both low- and high-affinity antibodies (Butler et al., 1978). It also remains possible that some precipitating antigenic compounds are present in the extract in concentrations to low to cause a detectable precipitate in AGDD, but are predominantly bound to the well-surface in the ELISA (Hearn et al., 1985). Discrepancy may also be, in part, due to the fact that the IgG ELISA measured only antibodies of the IgG classes while in the AGDD test, some observed antigen-antibody reactions may represent the presence of precipitating antibodies of the IgM classes (Hearn et al., 1985) or simply that serum precipitating antibodies may not necessary correlate to those detected in liquid phase by ELISA.

To the authors' knowledge, this study is the first report of serum GM quantification in canine SNA. In humans, the sensitivity of the GM ELISA (Platelia™) varies considerably with the immune status of the patient tested. In profoundly immune compromised patients, sensitivity is generally higher than 90%, while in less immune compromised patients, sensitivity may be as low as 30% (Husain et al., 2004; Kwak et al., 2004; Maertens et al., 2002). Finally, in non-immune compromised patients with localized disease (e.g.

ACCEPTED MANUSCRIPT

aspergilloma) circulating GMs are usually not detected (Verweij et al., 2000). In the present study, the GM ELISA with a cut-off set at 0.5 had a SE of 23.5% in the diagnosis of canine SNA. Using a cut-off of 1, no serum from dogs with SNA was positive. Several reasons can be proposed to explain the poor SE of the GM test. The most logical hypothesis would be that GM antigens were not detected in most serum samples from dogs with SNA because no GM is released into the bloodstream due to the fact that this disease seems non-invasive (Peeters et al., 2005). Negative antigenemia in most dogs with SNA is in accordance with the negative findings in horses with guttural pouch aspergillosis which is also considered to be a non-invasive infection (Guillot et al., 1999).

The second explanation relates to the presence of serum antibodies that may form complexes with GM antigens and consequently interfere with the performance of the GM ELISA. One study has shown that the sensitivity of the GM ELISA was lower in human patients with serum *Aspergillus*-specific antibodies than in those without serum *Aspergillus*-specific antibodies (Herbrecht et al., 2002). The IgG ELISA test designed in the present study detected antibodies against a large number of epitopes present in crude extracts of *Aspergillus*, including probably GM epitopes. Thus, the presence of anti-GM antibodies in the serum of dogs with SNA could have impaired the detection of serum GMs in these dogs. Nevertheless, the lack of correlation between GM antigenemia and the *Aspergillus*-specific IgG concentration in these dogs does not favour this hypothesis.

Accumulating evidence in humans with IA suggests that concomitant antifungal therapy may delay the detection of fungal antigen in blood and may decrease the maximum concentration of antigen reached in the serum due to reduced fungal growth (Marr et al., 2005). In the present study, five out of the 17 dogs with SNA had received oral antifungal therapy at the time of diagnosis (i.e. all five dogs were negative for serum GM detection); and this may have interfered with GM testing.

ACCEPTED MANUSCRIPT

Finally, it has been shown in human patients with IA that the concentration of circulating GM may fluctuate widely over time (Van Cutsem et al., 1990). The existence of these fluctuations in dogs with SNA is at present unknown. Due to the retrospective nature of this study, serial sampling was not performed.

The specificity of GM antigenemia is generally greater than 90% in humans with IA (Maschmeyer et al., 2007). In the present study, about 18% of non-aspergillic dogs had a positive GM ELISA. Several causes of false positive results have been discovered in human patients. Transient antigenemia or cross-reactivity with other serum components have been proposed to account for the false positive results in patients with IA (Husain et al., 2004). Hence, false-positive GM ELISA results for patients treated with β-lactam antimicrobials (amoxicillin/clavulanic acid) has also been described (Aubry et al., 2006). Although fourteen out the 79 dogs of the present study had received amoxicillin/clavulanic acid, only two of them (one dog with SNA and one CTRL dog) had a positive GM ELISA result, suggesting a negligible potential influence of the antibiotic on the results.

One perceived drawback of this study might be the small number of diseased dogs sampled and in consequence, the small number of serum in each category. Larger studies should be conducted in order to verify and confirm the utility of these serological tests in canine SNA.

In conclusion, results of this study suggest that serum GM quantification with GM ELISA (Platelia™) is unreliable for the diagnosis of canine SNA and that the detection of serum anti-*Aspergillus* antibodies by AGDD or IgG ELISA, using a standardized purified commercially available *Aspergillus* antigen preparation, has excellent specificity and good sensitivity. Specificity is higher for AGDD than for IgG ELISA while sensitivity is higher for IgG ELISA than for AGDD.

ACCEPTED MANUSCRIPT

Acknowledgements

This research was supported by a grant from the Huynen Foundation from the University of Liège, Belgium.

References

Aquino, V.R., Goldani, L.Z., Pasqualotto, A.C., 2007. Update on the contribution of galactomannan for the diagnosis of invasive aspergillosis. Mycopathologia 163, 191-202.

Aubry, A., Porcher, R., Bottero, J., Touratier, S., Leblanc, T., Brethon, B., Rousselot, P., Raffoux, E., Menotti, J., Derouin, F., Ribaud, P., Sulahian, A., 2006. Occurrence and kinetics of false-positive Aspergillus galactomannan test results following treatment with beta-lactam antibiotics in patients with hematological disorders. J. Clin. Microbiol. 44, 389-394.

Butler, J.E., Feldbush, T.L., McGivern, P.L., Stewart, N., 1978. The enzyme-linked immunosorbent assay (ELISA): a measure of antibody concentration or affinity. Immunochemistry 15, 131-136.

Cameli-Rojas, V., Mata-Essayag, S., de Capriles, C.H., Magaldi, S., de Perez, E.G., Garrido, L., Balderrama-Caballero, D., 2004. Aspergillus species in patients with chronic rhinosinusitis. Mycoses 47, 47-49.

Day, M.J., Penhale, W.J., 1988. Humoral immunity in disseminated Aspergillus terreus infection in the dog. Vet. Microbiol. 16, 283-294.

De Lorenzi, D., Bonfanti, U., Masserdotti, C., Caldin, M., Furlanello, T., 2006. Diagnosis of canine nasal aspergillosis by cytological examination: a comparison of four different collection techniques. J. Small Anim. Pract. 47, 316-319.

ACCEPTED MANUSCRIPT

Dye, C., Peters, I., Tasker, S., Caney, S.M., Dye, S., Gruffydd-Jones, T.J., Johnson, E., 2005. Preliminary study using an indirect ELISA for the detection of serum antibodies to Alternaria in domestic cats. Vet. Rec. 156, 633-635.

Garcia, M.E., Caballero, J., Cruzado, M., Andrino, M., Gonzalez-Cabo, J.F., Blanco, J.L., 2001. The value of the determination of anti-Aspergillus IgG in the serodiagnosis of canine aspergillosis: comparison with galactomannan detection. J Vet Med B Infect Dis Vet Public Health 48, 743-750.

Gosepath, J., Mann, W.J., 2005. Role of fungus in eosinophilic sinusitis. Curr Opin Otolaryngol Head Neck Surg 13, 9-13.

Guillot, J., Sarfati, J., de Barros, M., Cadore, J.L., Jensen, H.E., Chermette, R., 1999. Comparative study of serological tests for the diagnosis of equine aspergillosis. Vet. Rec. 145, 348-349.

Harvey, C.E., O'Brien, J.A., 1983. Nasal Aspergillosis-Penicillosis, In: Bonagura, J.D. (Ed.) Kirk's Current Veterinary Therapy VIII. W.B. Saunders Co, Philadelphia, pp. 236-240.

Hearn, V.M., Donaldson, G.C., Healy, M.J., Trotman, D.M., 1985. A method to determine significant levels of immunoglobulin G to Aspergillus fumigatus antigens in an ELISA system and a comparison with counterimmunoelectrophoresis and double diffusion techniques. J. Immunoassay 6, 165-187.

Herbrecht, R., Letscher-Bru, V., Oprea, C., Lioure, B., Waller, J., Campos, F., Villard, O., Liu, K.L., Natarajan-Ame, S., Lutz, P., Dufour, P., Bergerat, J.P., Candolfi, E., 2002. Aspergillus galactomannan detection in the diagnosis of invasive aspergillosis in cancer patients. J. Clin. Oncol. 20, 1898-1906.

Husain, S., Kwak, E.J., Obman, A., Wagener, M.M., Kusne, S., Stout, J.E., McCurry, K.R., Singh, N., 2004. Prospective assessment of Platelia Aspergillus galactomannan

Accepted Manuscript

ACCEPTED MANUSCRIPT

antigen for the diagnosis of invasive aspergillosis in lung transplant recipients. Am J Transplant 4, 796-802.

Jensen, H.E., Latge, J.P., 1995. An analysis of antibodies against Aspergillus fumigatus in bovine serum by immunoblotting and enzyme-linked immunosorbent assays. APMIS 103, 124-130.

Johnson, L.R., Drazenovich, T.L., Herrera, M.A., Wisner, E.R., 2006. Results of rhinoscopy alone or in conjunction with sinuscopy in dogs with aspergillosis: 46 cases (2001-2004). J. Am. Vet. Med. Assoc. 228, 738-742.

Khan, Z.U., Richardson, M.D., Warnock, D.W., Lane, J.G., 1984. Evaluation of an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of Aspergillus fumigatus intranasal infection of the dog. Sabouraudia 22, 251-254.

Kwak, E.J., Husain, S., Obman, A., Meinke, L., Stout, J., Kusne, S., Wagener, M.M., Singh, N., 2004. Efficacy of galactomannan antigen in the Platelia Aspergillus enzyme immunoassay for diagnosis of invasive aspergillosis in liver transplant recipients. J. Clin. Microbiol. 42, 435-438.

Lane, J.G., Warnock, D.W., 1977. The diagnosis of Aspergillus fumigatus infection of the nasal chambers of the dog with particular reference to the value of the double diffusion test. J. Small Anim. Pract. 18, 169-177.

Maertens, J., Theunissen, K., Lodewyck, T., Lagrou, K., Eldere, J.v., 2007. Advances in the serological diagnosis of invasive Aspergillus infections in patients with haematological disorders. Mycoses 50, 2-17.

Maertens, J., Van Eldere, J., Verhaegen, J., Verbeken, E., Verschakelen, J., Boogaerts, M., 2002. Use of circulating galactomannan screening for early diagnosis of invasive aspergillosis in allogeneic stem cell transplant recipients. J. Infect. Dis. 186, 1297-1306.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Marr, K.A., Laverdiere, M., Gugel, A., Leisenring, W., 2005. Antifungal therapy decreases sensitivity of the Aspergillus galactomannan enzyme immunoassay. Clin. Infect. Dis. 40, 1762-1769.

Maschmeyer, G., Haas, A., Cornely, O.A., 2007. Invasive aspergillosis: epidemiology, diagnosis and management in immunocompromised patients. Drugs 67, 1567-1601.

Mathews, K.G., Sharp, N.J.H., 2006. Canine Nasal Aspergillosis-Penicilliosis, In: Green, C.E. (Ed.) Infectious Diseases of the Dog and Cat. Saunders Elsevier, St. Louis, pp. 613-620.

Ouchterlony, O., 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. 26, 507-515.

Peeters, D., Day, M.J., Clercx, C., 2005. An immunohistochemical study of canine nasal aspergillosis. J. Comp. Pathol. 132, 283-288.

Peeters, D., Peters, I.R., Helps, C.R., Dehard, S., Day, M.J., Clercx, C., 2007. Whole blood and tissue fungal DNA quantification in the diagnosis of canine sino-nasal aspergillosis. Vet. Microbiol. 128, 194-203.

Poli, G., Ponti, W., Balsari, A., Addis, F., Mortellaro, C.M., 1981. Aspergillus fumigatus and specific precipitins in dogs with turbinate changes. Vet. Rec. 108, 143-145.

Pomrantz, J.S., Johnson, L.R., Nelson, R.W., Wisner, E.R., 2007. Comparison of serologic evaluation via agar gel immunodiffusion and fungal culture of tissue for diagnosis of nasal aspergillosis in dogs. J. Am. Vet. Med. Assoc. 230, 1319-1323.

Richardson, M.D., Turner, A., Warnock, D.W., Llewellyn, P.A., 1983. Computer-assisted rapid enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of aspergillosis. J. Immunol. Methods 56, 201-207.

Saunders, J.H., Clercx, C., Snaps, F.R., Sullivan, M., Duchateau, L., van Bree, H.J., Dondelinger, R.E., 2004. Radiographic, magnetic resonance imaging, computed

ACCEPTED MANUSCRIPT

tomographic, and rhinoscopic features of nasal aspergillosis in dogs. J. Am. Vet. Med. Assoc. 225, 1703-1712.

Sharp, N.J., Burrell, M.H., Sullivan, M., Cervantes-Olivares, R.A., 1984. Canine nasal aspergillosis: serology and treatment with ketoconazole. J. Small Anim. Pract. 25, 149-158.

Sharp, N.J.H., 1998. Aspergillosis and Penicilliosis, In: Green, C.E. (Ed.) Infectious Diseases of the Dog and Cat. W.B. Saunders Co., Philadelphia, pp. 404-413.

Van Cutsem, J., Meulemans, L., Van Gerven, F., Stynen, D., 1990. Detection of circulating galactomannan by Pastorex Aspergillus in experimental invasive aspergillosis. Mycoses 33, 61-69.

Verweij, P.E., Weemaes, C.M., Curfs, J.H., Bretagne, S., Meis, J.F., 2000. Failure to detect circulating Aspergillus markers in a patient with chronic granulomatous disease and invasive aspergillosis. J. Clin. Microbiol. 38, 3900-3901.

Windsor, R.C., Johnson, L.R., Sykes, J.E., Drazenovich, T.L., Leutenegger, C.M., De Cock, H.E., 2006. Molecular detection of microbes in nasal tissue of dogs with idiopathic lymphoplasmacytic rhinitis. J. Vet. Intern. Med. 20, 250-256.

Young, R.C., Bennett, J.E., 1971. Invasive aspergillosis. Absence of detectable antibody response. Am. Rev. Respir. Dis. 104, 710-716.

Accepted Manuscript

ACCEPTED MANUSCRIPT

538 **Figure legend**

539 **Figure 1.** Results of the *Aspergillus*-specific IgG ELISA in 17 dogs with sino-nasal
540 aspergillosis (SNA), 18 dogs with nasal tumour (NT), 11 dogs with idiopathic
541 lymphoplasmacytic rhinitis (LPR) and 33 control dogs (CTRL). The cut-off value (dashed
542 line) corresponds to an *Aspergillus*-specific IgG concentration of 15 EU/ml. The *Aspergillus*-
543 specific IgG concentration of the serum from 14 dogs with NT, 4 dogs with LPR and 27
544 CTRL dogs was below the detection limit of the ELISA. These sera were assigned a
545 concentration of 0 EU/ml. Bar = median.

546

547 **Figure 2**. Results of serum GM determination by the sandwich ELISA (Platelia™) in 17 dogs
548 with sino-nasal aspergillosis (SNA), 18 dogs with nasal tumour (NT), 11 dogs with idiopathic
549 lymphoplasmacytic rhinitis (LPR) and in 33 control dogs (CTRL). The cut-off value (dashed
550 line) corresponds to an optical density index of 0.5. Bar = median.

551

552

Accepted Manuscript

Figure 1

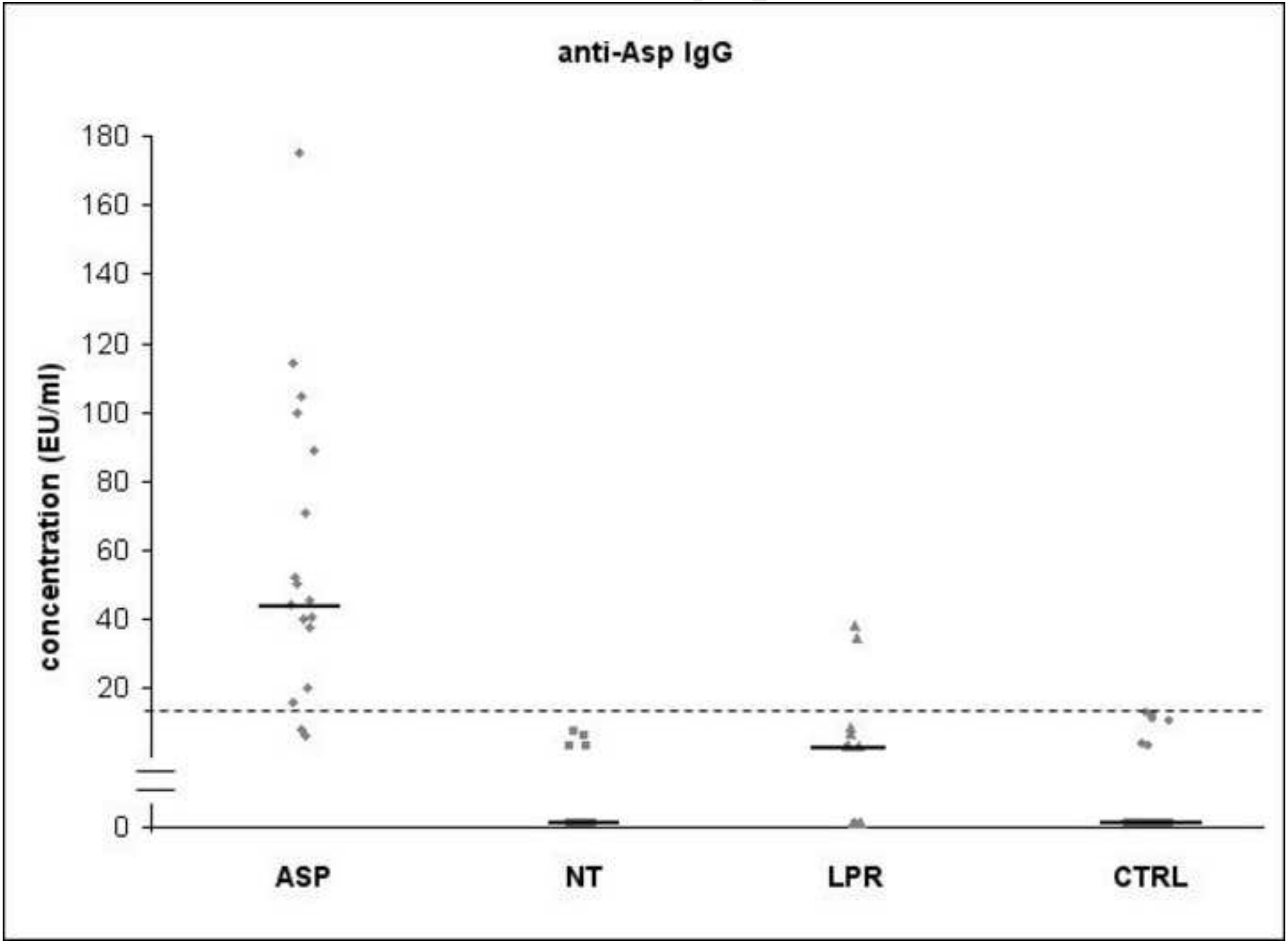
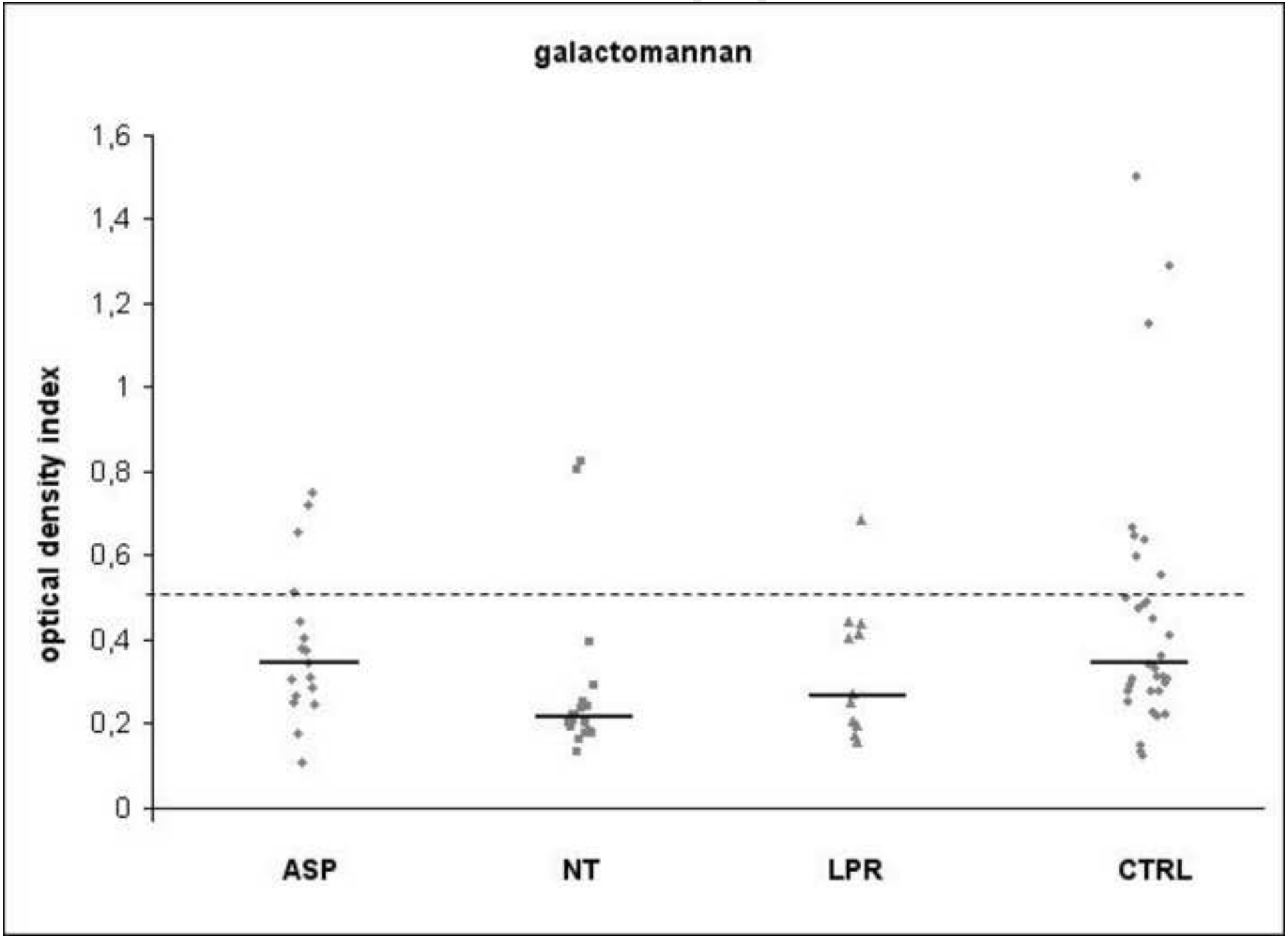

anti-Asp IgG
concentration (EU/ml)
180
160
140
120
100
80
60
40
20
0
ASP
NT
LPR
CTRL

Figure 2


galactomannan
optical density index
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
ASP
NT
LPR
CTRL

ACCEPTED MANUSCRIPT

Table 1. Comparison of Diagnostic Methods in Dogs with SNA.

Dog with SNA (number)	AGDD (+/-)	IgG ELISA (EU/ml)	GM ELISA (ODIndex)
1	-	**88.94**	0.243
2	+	**40.51**	**0.747**
3	+	**45.30**	0.285
4	+	**37.69**	0.308
5	+	**20.49**	0.345
6	+	**70.94**	**0.721**
7	+	6.49	0.376
8	-	**40.45**	0.402
9	-	7.97	0.379
10	+	**104.90**	0.107
11	+	**175.40**	0.441
12	+	**50.54**	**0.657**
13	+	**100.00**	0.174
14	-	**52.23**	0.266
15	+	**16.04**	**0.512**
16	+	**114.58**	0.248
17	+	**44.30**	0.304

Positive results are in bold, according to the respective cut-off values (i.e. >15 EU/ml for the IgG ELISA and > 0.5 Optical Density Index for the GM ELISA (Platelia™))

Accepted Manuscript

ACCEPTED MANUSCRIPT

Table 2. Analysis of Diagnostic Methods in Dogs with SNA.

	AGDD		IgG ELISA		AGDD + IgG ELISA		GM ELISA	
	(%)	**(95%CI)**	**(%)**	**(95%CI)**	**(%)**	**(95%CI)**	**(%)**	**(95%CI)**
SE	**76.5**	50.1-93.1	**88.2**	63.5-98.5	**94.1**	71.2-99.8	**23.5**	6.8-49.8
SP	**100**	94.2-100	**96.8**	88.8-99.6	**96.8**	88.8-99.6	**82.3**	70.4-90.8
PPV	**100**	75.2-100	**88.2**	63.5-98.5	**88.9**	65.2-98.6	**26.7**	7.7-55.1
NPV	**93.9**	85.1-98.3	**96.8**	88.8-99.6	**98.4**	91.1-99.9	**79.7**	67.7-88.7

Comparison of sensitivity (SE), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV) of serum precipitin detection with agar-gel double immunodiffusion (AGDD), *Aspergillus*-specific IgG concentration with indirect ELISA and serum GM detection by sandwich ELISA (Platelia™) (with 95 % confidence intervals(CI))

Accepted Manuscript