



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-00532458>

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.

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

4
5 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.
6 Day^b, C. Clercx^a

7
8 ^a Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, University of Liège,
9 Boulevard de Colonster 20(B44), 4000 Liège, Belgium

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

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

14 ^d Department of Veterinary Animal Production, Biostatistics, Veterinary Faculty, University of Liège,
15 Boulevard de Colonster, 20/B43, 4000 Liège, Belgium

16

17 ***corresponding author: F.Billen**

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

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

21

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* (CAPurAspAg) 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 CAPurAspAg 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 CAPurAspAg, 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

45

46 Introduction

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

54 The value of several techniques detecting serum *Aspergillus*-specific antibodies in the
55 diagnosis of SNA has been evaluated. Agar gel double immunodiffusion (AGDD) is
56 inexpensive and easy to perform but this method does not to allow the quantification of serum
57 immunoglobulins. The first studies evaluating AGDD in the diagnosis of SNA reported up to
58 100% sensitivity and specificity (Lane and Warnock, 1977; Poli et al., 1981; Sharp et al.,
59 1984). Harvey and O'Brien (1983), however, reported 15% false positive results in dogs with
60 nasal tumours. In addition, AGDD result might be negative in early stages of SNA or where
61 *Penicillium*, rather than *Aspergillus*, is the cause of SNA (Harvey and O'Brien, 1983;
62 Mathews and Sharp, 2006). Enzyme-linked immunosorbent assay (ELISA) is a rapid
63 quantitative method that allows the detection of very low concentrations of antibodies. Khan
64 et al. (1984) reported a sensitivity of 78 to 100% and a specificity of 78 to 96% for ELISA in
65 the diagnosis of canine SNA. A major drawback of these early serological studies is the lack
66 of standardisation of the antigen preparations used. Although highly purified *Aspergillus*
67 antigens have become commercially available, the value of serology in the diagnosis of SNA
68 using these antigen preparations is only poorly documented since, to the authors' knowledge,
69 only a single study investigating the diagnostic value of AGDD in canine SNA has used such
70 antigens (Pomrantz et al., 2007).

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

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

83

84 **Materials and methods**

85 Animals

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

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

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

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

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

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

122 Sera from 33 dogs without nasal disease were also included in the present study.
123 Twenty dogs were client-owned (five Labrador retrievers, two Leonberger dogs, two German
124 Shepherd dogs and eleven dogs each from a single breed) suffering from various orthopaedic
125 diseases and thirteen were healthy beagle dogs. This group of control (CTRL) dogs included
126 20 males (16 entire) and 13 females (12 entire). Their mean age was 5.0 ± 3.7 years and their
127 mean body weight was 29.2 ± 15.9 kg. None of these dogs had history or clinical signs of
128 upper respiratory disease, immune-mediated disease or systemic immune suppression. The
129 study protocol for blood sampling in healthy beagle dogs was reviewed and approved by the
130 university institutional animal care and use committee of the University of Liège.

131

132 Aspergillus precipitins determination by AGDD

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

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

153

154 Anti-*Aspergillus* IgG determination by indirect ELISA

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

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

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

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

190

191 GM determination by sandwich ELISA

192 The commercially available sandwich ELISA (Platelia™ *Aspergillus*) was used.
193 Serum samples were processed according to manufacturer's instructions. A cut-off value of
194 0.5 was used as proposed by the manufacturer, as this value is now universally accepted in

195 Europe and the USA for human serum GM testing (Aquino et al., 2007; Maertens et al.,
196 2007). In consequence, sera with an OD index > 0.5 were considered positive.

197

198 Statistics

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

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

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

221

222 **Results**

223 Animals

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

228

229 AGDD

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

233

234 IgG ELISA

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

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

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

247

248 AGDD versus IgG ELISA

249 A positive IgG ELISA result was obtained in three out of the four dogs with SNA that
250 were negative in AGDD. A positive AGDD result was obtained in one dog with SNA that
251 was negative in IgG ELISA (Table 1). Only one dog with SNA was negative by both tests.

252 Sensitivity, SP, PPV and NPV of both serological methods were not statistically different.

253 While AGDD and IgG ELISA had a SP of 100 and 96.8%, respectively, their SE was lower
254 (i.e. 76.5 and 88.2 respectively). However, when results of the IgG ELISA were combined
255 with those of AGDD, the sensitivity increased to 94.1% (table 2) but the SE, SP, PPV and
256 NPV did not significantly differ relative to those values computed for the individual tests.

257

258 GM ELISA

259 Galactomannan was detected in the serum of 24% (4/17) of dogs with SNA (Table 1),
260 11% (2/18) of patients with NT, 9% (1/11) of dogs with LPR and 24% (8/33) of CTRL
261 animals. The SE, SP, PPV and NPV of GM quantification in the diagnosis of SNA are
262 reported in table 2. There was no significant difference between the 4 groups of dogs
263 regarding the GM index (figure 2). The median GM index was 0.35 (range 0.107-0.747), 0.21
264 (range 0.13-0.822), 0.27 (range 0.157-0.687) and 0.33 (range 0.122-1.5) in dogs with SNA,
265 dogs with NT, dogs with LPR and CTRL animals, respectively. Only three dogs had a GM
266 index above 1 (i.e. 1.29, 1.15 and 1.50) and these animals belonged all to the CTRL group. If
267 the cut-off of the GM index was increased to an optical density index of 1 (as in a large

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

270

271 GM ELISA versus AGDD versus IgG ELISA

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

279

280 **Discussion**

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

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

293 which up to 100% sensitivity was commonly reported (Lane and Warnock, 1977; Poli et al.,
294 1981; Sharp et al., 1984). The higher sensitivity reported in those earlier studies might be
295 related to the use of multiple *Aspergillus* antigenic preparations.

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

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

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

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

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

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

348 AGDD and IgG ELISA results were in agreement in 13 dogs with SNA (76.5%) (i.e.
349 12 dogs with both results being positive and 1 dog with both results being negative). This is
350 similar to what is observed in human patients with allergic aspergillosis in whom AGDD and
351 IgG ELISA results are generally in agreement (Richardson et al., 1983). The lack of complete
352 agreement between AGDD and IgG ELISA, however, is not completely understood. One
353 explanation might be that ELISA detects high-affinity antibodies while AGDD detects both
354 low- and high-affinity antibodies (Butler et al., 1978). It also remains possible that some
355 precipitating antigenic compounds are present in the extract in concentrations too low to cause
356 a detectable precipitate in AGDD, but are predominantly bound to the well-surface in the
357 ELISA (Hearn et al., 1985). Discrepancy may also be, in part, due to the fact that the IgG
358 ELISA measured only antibodies of the IgG classes while in the AGDD test, some observed
359 antigen-antibody reactions may represent the presence of precipitating antibodies of the IgM
360 classes (Hearn et al., 1985) or simply that serum precipitating antibodies may not necessarily
361 correlate to those detected in liquid phase by ELISA.

362 To the authors' knowledge, this study is the first report of serum GM quantification in
363 canine SNA. In humans, the sensitivity of the GM ELISA (Platelia™) varies considerably
364 with the immune status of the patient tested. In profoundly immune compromised patients,
365 sensitivity is generally higher than 90%, while in less immune compromised patients,
366 sensitivity may be as low as 30% (Husain et al., 2004; Kwak et al., 2004; Maertens et al.,
367 2002). Finally, in non-immune compromised patients with localized disease (e.g.

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

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

387 Accumulating evidence in humans with IA suggests that concomitant antifungal
388 therapy may delay the detection of fungal antigen in blood and may decrease the maximum
389 concentration of antigen reached in the serum due to reduced fungal growth (Marr et al.,
390 2005). In the present study, five out of the 17 dogs with SNA had received oral antifungal
391 therapy at the time of diagnosis (i.e. all five dogs were negative for serum GM detection); and
392 this may have interfered with GM testing.

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

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

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

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

417

418

419 **Acknowledgements**

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

422

423 **References**

424 Aquino, V.R., Goldani, L.Z., Pasqualotto, A.C., 2007. Update on the contribution of
425 galactomannan for the diagnosis of invasive aspergillosis. *Mycopathologia* 163, 191-
426 202.

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

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

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

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

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

- 443 Dye, C., Peters, I., Tasker, S., Caney, S.M., Dye, S., Gruffydd-Jones, T.J., Johnson, E., 2005.
444 Preliminary study using an indirect ELISA for the detection of serum antibodies to
445 *Alternaria* in domestic cats. *Vet. Rec.* 156, 633-635.
- 446 Garcia, M.E., Caballero, J., Cruzado, M., Andrino, M., Gonzalez-Cabo, J.F., Blanco, J.L.,
447 2001. The value of the determination of anti-*Aspergillus* IgG in the serodiagnosis of
448 canine aspergillosis: comparison with galactomannan detection. *J Vet Med B Infect*
449 *Dis Vet Public Health* 48, 743-750.
- 450 Gosepath, J., Mann, W.J., 2005. Role of fungus in eosinophilic sinusitis. *Curr Opin*
451 *Otolaryngol Head Neck Surg* 13, 9-13.
- 452 Guillot, J., Sarfati, J., de Barros, M., Cadore, J.L., Jensen, H.E., Chermette, R., 1999.
453 Comparative study of serological tests for the diagnosis of equine aspergillosis. *Vet.*
454 *Rec.* 145, 348-349.
- 455 Harvey, C.E., O'Brien, J.A., 1983. Nasal Aspergillosis-Penicillois, In: Bonagura, J.D. (Ed.)
456 *Kirk's Current Veterinary Therapy VIII*. W.B. Saunders Co, Philadelphia, pp. 236-
457 240.
- 458 Hearn, V.M., Donaldson, G.C., Healy, M.J., Trotman, D.M., 1985. A method to determine
459 significant levels of immunoglobulin G to *Aspergillus fumigatus* antigens in an
460 ELISA system and a comparison with counterimmunoelectrophoresis and double
461 diffusion techniques. *J. Immunoassay* 6, 165-187.
- 462 Herbrecht, R., Letscher-Bru, V., Oprea, C., Lioure, B., Waller, J., Campos, F., Villard, O.,
463 Liu, K.L., Natarajan-Ame, S., Lutz, P., Dufour, P., Bergerat, J.P., Candolfi, E., 2002.
464 *Aspergillus* galactomannan detection in the diagnosis of invasive aspergillosis in
465 cancer patients. *J. Clin. Oncol.* 20, 1898-1906.
- 466 Husain, S., Kwak, E.J., Obman, A., Wagener, M.M., Kusne, S., Stout, J.E., McCurry, K.R.,
467 Singh, N., 2004. Prospective assessment of *Platelia Aspergillus* galactomannan

- 468 antigen for the diagnosis of invasive aspergillosis in lung transplant recipients. *Am J*
469 *Transplant* 4, 796-802.
- 470 Jensen, H.E., Latge, J.P., 1995. An analysis of antibodies against *Aspergillus fumigatus* in
471 bovine serum by immunoblotting and enzyme-linked immunosorbent assays. *APMIS*
472 103, 124-130.
- 473 Johnson, L.R., Drazenovich, T.L., Herrera, M.A., Wisner, E.R., 2006. Results of rhinoscopy
474 alone or in conjunction with sinuscopy in dogs with aspergillosis: 46 cases (2001-
475 2004). *J. Am. Vet. Med. Assoc.* 228, 738-742.
- 476 Khan, Z.U., Richardson, M.D., Warnock, D.W., Lane, J.G., 1984. Evaluation of an enzyme-
477 linked immunosorbent assay (ELISA) for the diagnosis of *Aspergillus fumigatus*
478 intranasal infection of the dog. *Sabouraudia* 22, 251-254.
- 479 Kwak, E.J., Husain, S., Obman, A., Meinke, L., Stout, J., Kusne, S., Wagener, M.M., Singh,
480 N., 2004. Efficacy of galactomannan antigen in the Platelia *Aspergillus* enzyme
481 immunoassay for diagnosis of invasive aspergillosis in liver transplant recipients. *J.*
482 *Clin. Microbiol.* 42, 435-438.
- 483 Lane, J.G., Warnock, D.W., 1977. The diagnosis of *Aspergillus fumigatus* infection of the
484 nasal chambers of the dog with particular reference to the value of the double
485 diffusion test. *J. Small Anim. Pract.* 18, 169-177.
- 486 Maertens, J., Theunissen, K., Lodewyck, T., Lagrou, K., Eldere, J.v., 2007. Advances in the
487 serological diagnosis of invasive *Aspergillus* infections in patients with
488 haematological disorders. *Mycoses* 50, 2-17.
- 489 Maertens, J., Van Eldere, J., Verhaegen, J., Verbeken, E., Verschakelen, J., Boogaerts, M.,
490 2002. Use of circulating galactomannan screening for early diagnosis of invasive
491 aspergillosis in allogeneic stem cell transplant recipients. *J. Infect. Dis.* 186, 1297-
492 1306.

- 493 Marr, K.A., Laverdiere, M., Gugel, A., Leisenring, W., 2005. Antifungal therapy decreases
494 sensitivity of the *Aspergillus* galactomannan enzyme immunoassay. *Clin. Infect. Dis.*
495 40, 1762-1769.
- 496 Maschmeyer, G., Haas, A., Cornely, O.A., 2007. Invasive aspergillosis: epidemiology,
497 diagnosis and management in immunocompromised patients. *Drugs* 67, 1567-1601.
- 498 Mathews, K.G., Sharp, N.J.H., 2006. Canine Nasal Aspergillosis-Penicilliosis, In: Green,
499 C.E. (Ed.) *Infectious Diseases of the Dog and Cat*. Saunders Elsevier, St. Louis, pp.
500 613-620.
- 501 Ouchterlony, O., 1949. Antigen-antibody reactions in gels. *Acta Pathol. Microbiol. Scand.* 26,
502 507-515.
- 503 Peeters, D., Day, M.J., Clercx, C., 2005. An immunohistochemical study of canine nasal
504 aspergillosis. *J. Comp. Pathol.* 132, 283-288.
- 505 Peeters, D., Peters, I.R., Helps, C.R., Dehard, S., Day, M.J., Clercx, C., 2007. Whole blood
506 and tissue fungal DNA quantification in the diagnosis of canine sino-nasal
507 aspergillosis. *Vet. Microbiol.* 128, 194-203.
- 508 Poli, G., Ponti, W., Balsari, A., Addis, F., Mortellaro, C.M., 1981. *Aspergillus fumigatus* and
509 specific precipitins in dogs with turbinate changes. *Vet. Rec.* 108, 143-145.
- 510 Pomrantz, J.S., Johnson, L.R., Nelson, R.W., Wisner, E.R., 2007. Comparison of serologic
511 evaluation via agar gel immunodiffusion and fungal culture of tissue for diagnosis of
512 nasal aspergillosis in dogs. *J. Am. Vet. Med. Assoc.* 230, 1319-1323.
- 513 Richardson, M.D., Turner, A., Warnock, D.W., Llewellyn, P.A., 1983. Computer-assisted
514 rapid enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of
515 aspergillosis. *J. Immunol. Methods* 56, 201-207.
- 516 Saunders, J.H., Clercx, C., Snaps, F.R., Sullivan, M., Duchateau, L., van Bree, H.J.,
517 Dondelinger, R.E., 2004. Radiographic, magnetic resonance imaging, computed

- 518 tomographic, and rhinoscopic features of nasal aspergillosis in dogs. *J. Am. Vet. Med.*
519 *Assoc.* 225, 1703-1712.
- 520 Sharp, N.J., Burrell, M.H., Sullivan, M., Cervantes-Olivares, R.A., 1984. Canine nasal
521 aspergillosis: serology and treatment with ketoconazole. *J. Small Anim. Pract.* 25,
522 149-158.
- 523 Sharp, N.J.H., 1998. Aspergillosis and Penicilliosis, In: Green, C.E. (Ed.) *Infectious Diseases*
524 *of the Dog and Cat.* W.B. Saunders Co., Philadelphia, pp. 404-413.
- 525 Van Cutsem, J., Meulemans, L., Van Gerven, F., Stynen, D., 1990. Detection of circulating
526 galactomannan by Pastorex Aspergillus in experimental invasive aspergillosis.
527 *Mycoses* 33, 61-69.
- 528 Verweij, P.E., Weemaes, C.M., Curfs, J.H., Bretagne, S., Meis, J.F., 2000. Failure to detect
529 circulating Aspergillus markers in a patient with chronic granulomatous disease and
530 invasive aspergillosis. *J. Clin. Microbiol.* 38, 3900-3901.
- 531 Windsor, R.C., Johnson, L.R., Sykes, J.E., Drazenovich, T.L., Leutenegger, C.M., De Cock,
532 H.E., 2006. Molecular detection of microbes in nasal tissue of dogs with idiopathic
533 lymphoplasmacytic rhinitis. *J. Vet. Intern. Med.* 20, 250-256.
- 534 Young, R.C., Bennett, J.E., 1971. Invasive aspergillosis. Absence of detectable antibody
535 response. *Am. Rev. Respir. Dis.* 104, 710-716.
- 536
- 537

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.

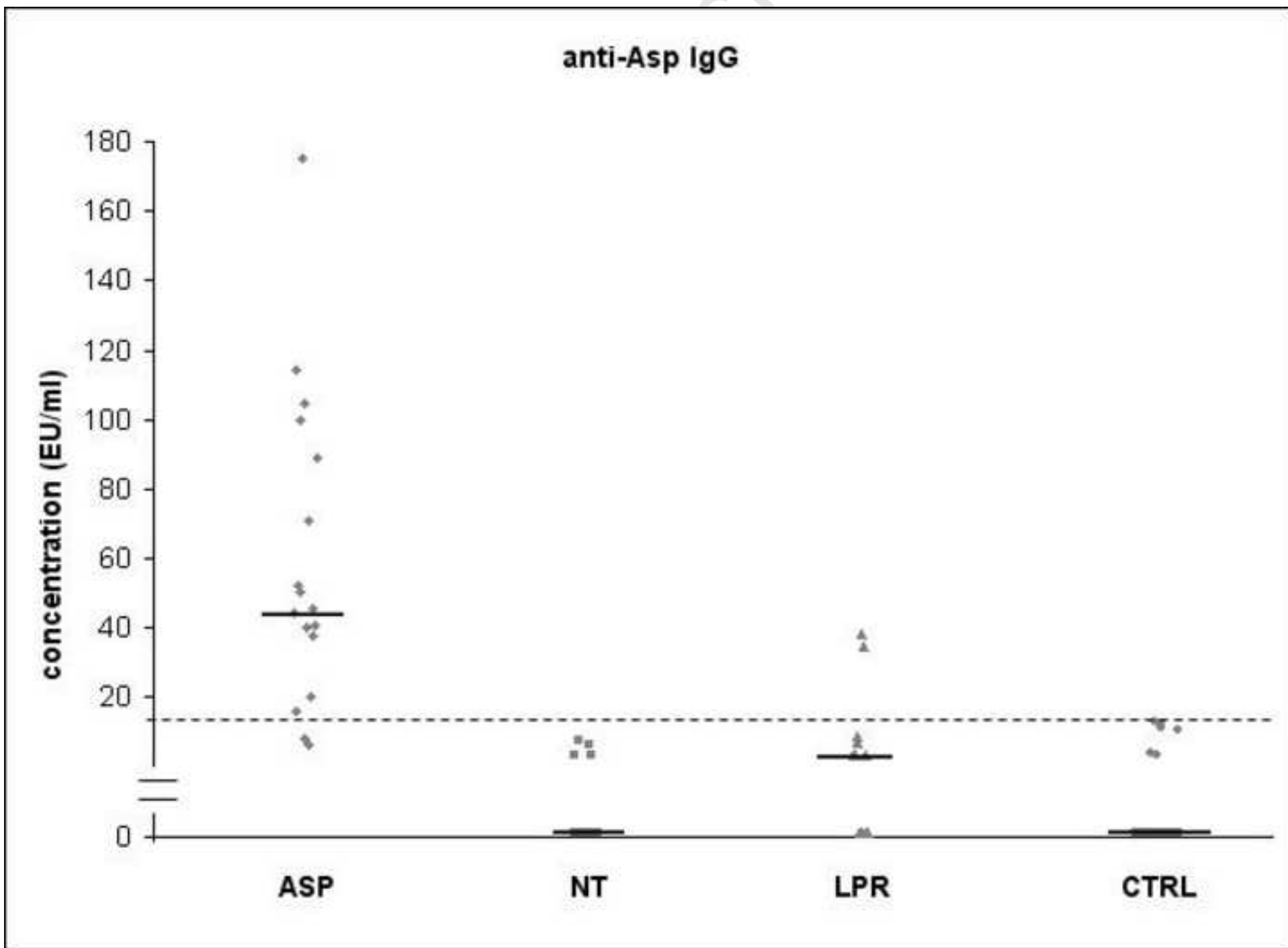
546

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

Figure 1



galactomannan

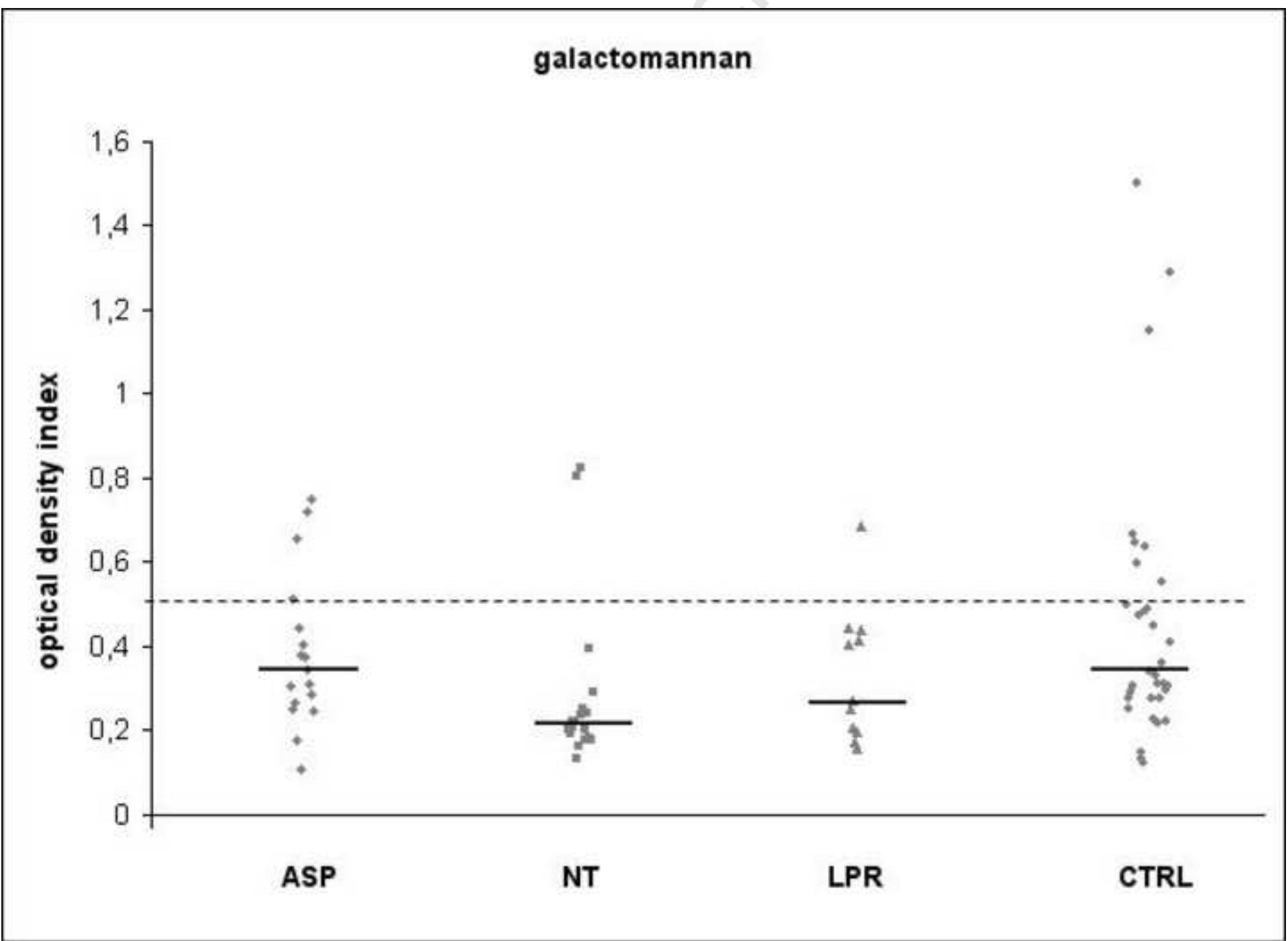


Table 1. Comparison of Diagnostic Methods in Dogs with SNA.

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 (PlateliaTM))

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))