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1 Whole blood and tissue fungal DNA quantification in the diagnosis of
2 canine sino-nasal aspergillosis

3

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19

20 **Abstract**

21 Various combinations of tests are used to confirm the diagnosis of canine sino-nasal
22 aspergillosis (SNA) because false-positive and false-negative results can occur with
23 each test. Therefore, the aim of this study was to evaluate whether detection of fungal
24 DNA in blood and nasal tissue samples was of value in the clinical diagnosis of this
25 disease.

26 Four groups were included in the study (dogs with SNA, lymphoplasmacytic rhinitis or
27 nasal neoplasia, and control animals). Real-time PCR assays detecting DNA from all
28 *Penicillium* and *Aspergillus* species (PenAsp assay) or species-specific DNA from *A.*
29 *fumigatus*, *A. terreus*, *A. flavus* and *A. niger* were applied to whole blood and nasal
30 tissue samples. Results obtained by PCR were compared between the groups.
31 Sensitivity, specificity, positive and negative predictive values (PPV and NPV) for
32 fungal DNA detection were compared with those for alternative diagnostic procedures
33 including histopathology, serology and fungal culture.

34 Significantly more fungal DNA was detected by the PenAsp assay in tissue biopsies
35 from dogs with SNA than in the three other groups. Sensitivity, specificity, PPV and
36 NPV for this method were 1.00, 0.06, 0.32 and 1.00. *Aspergillus fumigatus* DNA was
37 detected in seven tissue biopsies from dogs with SNA and in one biopsy from a dog
38 with a nasal tumour. Sensitivity, specificity, PPV and NPV for this diagnostic test were
39 0.50, 0.97, 0.87 and 0.82. No significant difference was found between the groups with
40 respect to the amount of DNA detected in blood by the PenAsp assay. Sensitivity,
41 specificity, PPV and NPV for this method were 0.71, 0.24, 0.31 and 0.64. *Aspergillus*
42 *fumigatus* DNA was detected in the blood of three dogs with SNA and sixteen dogs
43 without SNA. Sensitivity, specificity, PPV and NPV for this diagnostic tool were 0.21,

44 0.45, 0.15 and 0.54. Detection of *A. fumigatus* DNA in nasal tissue had the highest
45 specificity, PPV and NPV but sensitivity of this method was low. Detection of fungal
46 DNA in whole blood was of no value in the diagnosis of SNA.

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47 **Keywords**

48 Dog; canine; aspergillosis; rhinitis; real-time PCR; DNA detection

49

50 **Abbreviations**

51 Afumi assay: *Aspergillus fumigatus* assay

52 Ct: threshold cycle

53 G3PDH: glyceraldehyde-3-phosphate dehydrogenase

54 gDNA: genomic DNA

55 LPR: lymphoplasmacytic rhinitis

56 NPV: negative predictive value

57 PCR: polymerase chain reaction

58 PenAsp assay: *Penicillium/Aspergillus* assay

59 PPV: positive predictive value

60 SNA: sino-nasal aspergillosis

61 **Introduction**

62 Sino-nasal aspergillosis (SNA) is a common cause of nasal discharge in the dog
63 and is most often caused by *Aspergillus fumigatus* (Sharp et al., 1991). Various
64 combinations of diagnostic tests, such as radiography, computed tomography,
65 rhinoscopy, histological examination, cytology, fungal culture, and serology are used to
66 confirm the diagnosis of canine SNA (De Lorenzi et al., 2006; Saunders and van Bree,
67 2003; Sharp et al., 1991). Currently, no single test can be used to make the diagnosis
68 because false-positive and false-negative results can occur with each (De Lorenzi et al.,
69 2006). Therefore, the gold standard for diagnosing this disease is considered to be the
70 direct visualization of fungal plaques by rhinoscopy or the microscopical observation of
71 fungal elements in samples taken for cytological or histopathological examination (De
72 Lorenzi et al., 2006; Johnson et al., 2006; Zonderland et al., 2002).

73 As the fungus does not invade the mucosa or disseminate throughout the body in
74 canine SNA (Peeters et al., 2005), this disease resembles a form of human fungal
75 sinusitis that occurs in immune competent patients. Although quantification of fungal
76 DNA by real-time polymerase chain reaction (PCR) is widely used in human medicine
77 for the diagnosis and monitoring of therapy of invasive aspergillosis (Ferns, 2006), we
78 are not aware of any report of the use of fungal DNA detection in the diagnosis of
79 human non-invasive fungal sinusitis.

80 Idiopathic lymphoplasmacytic rhinitis (LPR) is another common cause of nasal
81 discharge in the dog, but in this disease the clinical signs tend to be less severe than in
82 SNA (Windsor et al., 2004). The etiology of this condition in dogs is unknown. In
83 human medicine, airborne fungi have been shown to play a key role in the pathogenesis
84 of disease in some patients with chronic rhinosinusitis (Ponikau et al., 2006; Rao et al.,

85 2006). In a recent study, molecular analysis has shown higher loads of fungal DNA in
86 nasal biopsies from dogs with idiopathic LPR than in nasal biopsies from healthy dogs,
87 suggesting a possible role for fungal organisms in idiopathic LPR of dogs (Windsor et
88 al., 2006).

89 Therefore, the aims of the present study were (1) to evaluate the potential value
90 of detection of fungal DNA in samples of whole blood and nasal tissue in the diagnosis
91 of canine SNA, and (2) to investigate whether *Aspergillus* may have a role in the
92 pathogenesis of canine LPR.

93 **Materials and Methods**

94 **Animals**

95 Fourteen dogs with a diagnosis of aspergillosis involving the nasal cavities and
96 frontal sinus were included in the study. Ages ranged from 1 to 10 years (median 4.5).
97 Diagnosis of SNA was based on results of physical examination and observation of
98 typical intranasal or intrasinusal fungal plaques associated with turbinate destruction on
99 rhinoscopy/sinuscopy.

100 Seven dogs with a diagnosis of LPR were included in the study. Ages ranged
101 from 0.5 to 13 years (median 6). Diagnosis of LPR was made on the basis of clinical
102 signs, rhinoscopic (unilateral or bilateral mild to moderate turbinate lysis with
103 congestion and oedema of the mucosa, mucoid to mucopurulent secretions, and failure
104 to demonstrate a foreign body, neoplasia or fungal plaques in the nasal cavities) and
105 histopathological findings.

106 Thirteen dogs suffering from neoplasia of the nasal cavity were entered into the
107 study. Ages ranged from 6 to 18 years (median 10). Diagnosis was based on
108 visualisation of a mass during rhinoscopy and histopathological findings.

109 Eight client-owned dogs, euthanased for various independent reasons (non-
110 metastatic gastric carcinoma in two dogs, brain tumour in two dogs, and uncontrollable
111 aggression, congestive heart failure, pulmonary carcinoma and non metastatic
112 osteosarcoma in one dog each), and four healthy beagle dogs were sampled as control
113 animals. None of these dogs had clinical history or signs of upper respiratory disease,
114 immune-mediated disease or systemic immunosuppression. Ages of these dogs ranged
115 from 4 to 12 years (median 7.5).

116 Seven dogs with SNA had received oral antifungal therapy (ketoconazole in four
117 dogs and itraconazole in three dogs) for 8 to 15 days prior to rhinoscopy. In all other
118 animals, no systemic antifungal therapy had been administered for at least 2 months
119 preceding rhinoscopy.

120

121 **Sample collection**

122 Two ml of whole blood were collected from all dogs (except three control dogs)
123 into EDTA. The samples were stored at -70°C until DNA extraction. Two ml of clotted
124 blood were also collected and the serum harvested from these samples was stored at -
125 18°C until *Aspergillus* serology was performed.

126 All of the rhinoscopic and sinusoscopic examinations were performed by the same
127 investigator (CC). In diseased dogs, the nasal cavities were explored with a rigid
128 endoscope (Cytoscope K Storz SL 30°, Karl-Storz-Endoscopy Belgium SA, Strombeek-
129 Bever, Belgium) and, where possible, the frontal sinuses were investigated with a
130 flexible bronchoscope (Fujinon BRO-YP2, Onys, SA, Brussels, Belgium). In each dog
131 (including control animals), nasal secretions were blindly sampled with a dry swab for
132 fungal culture.

133 In dogs with SNA, perendoscopic mucosal biopsy samples were taken from the
134 distal part of the nasal cavity, near the junction with the frontal sinus. In dogs with LPR,
135 perendoscopic biopsies were taken where the mucosa appeared to be the most inflamed.
136 In dogs with nasal tumour, biopsies were taken from the tissue suspected to be
137 neoplastic. In control client-owned dogs, perendoscopic mucosal biopsy samples were
138 taken with owners' consent within 10 minutes of euthanasia from the posterior third of
139 the right nasal cavity, but the frontal sinuses could not be explored. In control beagle

140 dogs, perendoscopic biopsies were taken from the distal part of the nasal cavity.
141 Samples were placed in a 2.0-ml cryotube (CM.LAB, Vordingborg, Denmark), snap
142 frozen in liquid nitrogen and stored at -70°C.

143

144 **Histopathology**

145 Additional mucosal biopsy samples from each dog were placed in 10% neutral
146 buffered formalin for histopathological examination. Sections were stained with
147 haematoxylin & eosin and Grocott (for fungal organism) by standard histochemical
148 procedures (Bancroft and Cook, 1984). All biopsy sections were reviewed by a board-
149 certified veterinary histopathologist (MJD), without knowledge of their origin.

150

151 **Fungal DNA quantification**

152 **DNA Isolation**

153 Freeze-dried ampoules of *A. fumigatus* (IMI number: 360452), *A. flavus*
154 (367606), *A. niger* (358433) and *A. terreus* (348431) were obtained from CABI
155 Bioscience (Egham, Surrey, UK).

156 Genomic DNA (gDNA) was isolated from these *Aspergillus* samples using the
157 Macherey-Nagel NucleoSpin Blood Isolation Kit (ABgene, Epsom, UK) following
158 reconstitution of the freeze-dried samples with nuclease-free water. Three methods of
159 DNA extraction, which were suitable for application to the tissue biopsies and blood,
160 were tested against 200µl of the *Aspergillus* suspensions. These methods involved using
161 the lysis buffer and proteinase K from the extraction kit with or without additional
162 mechanical disruption. Method 1 involved incubation of the *Aspergillus* suspension
163 with lysis buffer and proteinase K in a 2ml Safe-Lock microcentrifuge tube (Eppendorf,

164 Cambridge, UK) at 55°C overnight in a shaking incubator (Vortemp 56EVC, Labnet,
165 Rutland, UK). Method 2 was the same as method 1 except that a 5mm stainless steel
166 ball was added to the microcentrifuge tube during the overnight incubation. Method 3
167 involved mechanical disruption of the *Aspergillus* using a TissueLyser (Qiagen Ltd,
168 Crawley, UK). The *Aspergillus* was pelleted by centrifugation at 10,000g for 5 minutes
169 and the supernatant removed. A 5mm stainless steel ball was added to the tube and the
170 pellet was snap-frozen with liquid nitrogen before shaking in the TissueLyser at 20
171 cycles per second for 2 minutes. This procedure was repeated three times before
172 addition of the lysis buffer and proteinase K and overnight incubation in the shaking
173 incubator. Following the overnight incubation, the extraction procedure was completed
174 as per the manufacturer's instructions.

175 The three extraction methods were tested with each of the *Aspergillus* species in
176 duplicate before testing with the *Penicillium* spp./*Aspergillus* spp. (PenAsp) real-time
177 PCR assay (Table 1). Method 1 was the least effective at extracting DNA. Method 2
178 was the most effective method which resulted in the lowest threshold cycle (Ct) value
179 with all of the *Aspergillus* species tested (Table 1). There was no advantage to use of
180 freeze-thaw cycles and the TissueLyser (method 3). This experiment was repeated with
181 *Aspergillus* suspensions mixed with EDTA blood from a dog which had been
182 previously determined to be negative for *Aspergillus*. The results of this experiment
183 were similar to those obtained with the *Aspergillus* suspensions, with method 2 being
184 the most effective (data not shown). Method 2 was therefore used for the extraction of
185 gDNA from the nasal biopsies and blood samples in the main experiment.

186 Genomic DNA was isolated from the endoscopic biopsies and blood samples
187 (200µl) using the Macherey-Nagel NucleoSpin Blood Isolation Kit (ABgene). The

188 tissue and blood were added to a 2ml Safe-Lock microcentrifuge tube (Eppendorf)
189 containing the lysis buffer and proteinase K (from the isolation kit) and a 5mm stainless
190 steel ball. Biopsies, but not blood samples, were disrupted using a TissueLyser (Qiagen
191 Ltd) by shaking the tubes at 20 cycles per second for 2 minutes. The microcentrifuge
192 tubes containing both sample types including the stainless steel balls were then
193 incubated at 56°C overnight in a shaking incubator (Vortemp 56EVC, Labnet) set at
194 1000rpm before completion of the extraction protocol as per the manufacturer's
195 instructions. The DNA was eluted with 100µl of elution buffer (from the extraction kit).

196

197 **Primer and Probe Design**

198 The assays specific for the ribosomal RNA gene sequences of *A. fumigatus*
199 (*Afumi*), *A. flavus*, *A. niger*, *A. terreus* and PenAsp assays were the same as used by
200 Haugland *et al.* (Haugland *et al.*, 2004) (Table 2). The only exception was the probe
201 sequence for the PenAsP assay which was altered by the use of three locked nucleic
202 acid bases rather than a 3' minor groove binder in order to raise the predicted annealing
203 temperature from that of the unmodified probe (underlined bases in Table 2). The
204 glyceraldehyde-3-phosphate dehydrogenase (G3PDH) specific assay was the same as
205 that used previously (Peters *et al.*, 2003). Primers and probes were synthesised by
206 Eurogentec Ltd. (Southampton, U.K.) and were reconstituted in EB buffer (10mM Tris
207 HCl pH 8.4, Qiagen Ltd.) before use.

208

209 **Real-Time PCR**

210 Real-time PCR was performed using HotStarTaq Master Mix (Qiagen Ltd.).
211 Gene-specific amplification was performed using 0.2 μ M of each primer, 0.1 μ M of
212 probe or SYBR Green 1 (1:100,000) (Sigma-Aldrich Ltd., Poole, Dorset) and 2.5 μ l of
213 gDNA in a final volume of 25 μ l. Magnesium chloride concentrations were adjusted to
214 4.5mM in the final reaction by addition of 25mM MgCl₂.

215 Sample incubations were performed in an iCycler IQ (Bio-Rad Laboratories,
216 Hemel Hempstead, U.K.) at 95°C for 15 minutes and then 45 cycles of 95°C for 10
217 seconds and 60°C (58°C for Afumi assay) for 15 seconds during which the fluorescence
218 data were collected. Threshold cycle values were calculated as the cycle when the
219 fluorescence of the sample exceeded a threshold level corresponding to 10 standard
220 deviations from the mean of the baseline fluorescence. The thermocycling protocol was
221 extended when SYBR Green I was used by heating samples from 75°C to 95°C in 0.5°C
222 increments with a dwell time at each temperature of 10 seconds during which time the
223 fluorescence data were collected in order to create a melt curve.

224 Nuclease-free water passed through the DNA isolation was analysed in a similar
225 manner to all other samples to control for sample contamination. A negative control of
226 nuclease-free water and a positive control sample with a known Ct value were included
227 with all sample runs to control for run-to-run Ct variation and all reactions were run in
228 duplicate.

229 Relative expression data for each of the gene targets were calculated using the
230 gBase applet for Microsoft Excel (<http://medgen.ugent.be/qbase/>). This applet calculates
231 a relative copy number for each sample, normalised against the measured housekeeper
232 gene (G3PDH), using the methods described by Vandesompele *et al.* (Vandesompele et

233 al., 2002). The sample with the fewest gene copies (latest Ct value) is given a relative
234 copy number of 1 and all other samples are given values relative to this sample.
235 Samples with no measured Ct value were assigned a relative copy number of 0. These
236 relative copy number results were used for the statistical testing and presentation in
237 graphical form.

238

239 **Reaction Efficiency**

240 A dilution curve (1:10 dilution) of gDNA from each of the *Aspergillus* species
241 was tested in both the species-specific and PenAsp assay in triplicate. A master mix was
242 made up and aliquotted into the PCR plate prior to addition of the template into each
243 reaction tube individually. A graph of Ct versus \log_{10} copy number of the sample from
244 the dilution series was produced. The slope of this graph was used to determine the
245 reaction efficiency.

$$246 \quad \text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

247

248 **Other diagnostic tools for diagnosing *Aspergillus* infection**

249 **Histopathology**

250 Diagnosis of SNA was made by histopathology only if branching septate hyphae
251 were observed on Grocott-stained sections.

252

253 **Serology**

254 Serological testing was performed by double agarose gel immunodiffusion
255 (Immuno-Mycologics, OK, USA) in order to determine the presence of serum antibody
256 specific for *Aspergillus*.

257

258 **Mycology**

259 Nasal secretions were inoculated on Sabouraud-dextrose-agar medium with
260 chloramphenicol within 3 hours of collection. The media were incubated at room
261 temperature (between 20 and 22°C) in ambient air. Positive results were more precisely
262 characterized by microscopic examination. A positive fungal culture within 10 days of
263 inoculation was considered compatible with fungal infection.

264

265 **Statistics**

266 The non-parametric (Kruskal-Wallis) analysis of variance was used to compare
267 the expression of each gene target among the four clinical groups examined in the study.
268 Significant results were further characterised using the Mann-Whitney Test. The level
269 of significance was $p < 0.05$ for all analyses.

270 For each diagnostic method (blood and tissue fungal DNA quantification,
271 histopathology, *Aspergillus* antibody titre and fungal culture), sensitivity, specificity,
272 positive predictive value (PPV) and negative predictive value (NPV) were calculated.
273 The gold standard for diagnosing SNA was the observation of typical fungal plaques
274 during rhinoscopy/sinuscopy.

275 **Results**

276 **Real-time assay validation**

277 All real-time PCR assays produced a single melt curve peak when run with
278 SYBR Green I and single band products of appropriate size were obtained with agarose
279 gel electrophoresis (data not shown). Reaction efficiencies were assessed using a ten-
280 fold serial dilution of gDNA using a minimum of six dilutions. All reactions had
281 efficiencies between 94 and 100% (Table 2) both with SYBR Green I and the Taqman
282 probes and these efficiencies are similar to those reported previously for these assays
283 (Haugland et al., 2004). These efficiencies were used in the calculation of the relative
284 copy number values. The species-specific assays showed no cross-reactivity with the
285 non-target species, as was reported previously (Haugland et al., 2004), and all species
286 were detected by the PenAsp assay.

287

288 **Fungal DNA quantification**

289 Fungal DNA was detected by the PenAsp assay in all nasal biopsy samples
290 except in the samples from two dogs with nasal neoplasia (Figure 1). Although the
291 relative copy numbers overlapped between the four groups, there was significantly more
292 fungal DNA in biopsies from dogs with SNA than in tissue from dogs of the three other
293 groups. There was significantly less fungal DNA in nasal biopsies from dogs with nasal
294 tumour than in control animals and dogs with LPR. Using the species-specific assays,
295 there was no *A. terreus*, *A. niger* or *A. flavus* DNA in any of the nasal biopsies.
296 However, *A. fumigatus* DNA was detected in seven biopsies from dogs with SNA and
297 in one biopsy from a dog with a nasal tumour (Figure 2).

298 Fungal DNA was detected by the PenAsp assay in the blood of 9/9 control dogs,
299 3/7 dogs with LPR, 10/13 dogs with nasal tumour and 10/14 dogs with SNA (Figure 1).
300 No significant difference could be found between the groups. *Aspergillus fumigatus*
301 DNA was detected in the blood of three dogs with SNA, nine dogs with nasal tumour,
302 three dogs with LPR and four control animals (Figure 2). There was no *A. terreus*, *A.*
303 *niger* or *A. flavus* DNA detected in the blood of any dog. Sensitivity, specificity, PPV
304 and NPV of tissue and blood fungal DNA detection by the PenAsp and the Afumi
305 assays in the diagnosis of SNA are given in Table 3.

306

307 **Other diagnostic tools**

308 **Histopathology**

309 The samples from control dogs all had normal histological structure. In sections
310 from dogs with LPR, the architecture of the mucosa was relatively well preserved with
311 focal oedema. The lamina propria was mildly to moderately infiltrated by lymphocytes
312 and plasma cells although, in some cases, neutrophils were predominant. In sections
313 from dogs with SNA, the lesions were more severe. An intense, ulcerative, mixed
314 inflammatory infiltration with neutrophils and mononuclear cells, associated with
315 necrosis and fibrin exudation was observed in most cases. In the group of dogs with
316 nasal neoplasia, eight dogs were diagnosed with carcinoma (adenocarcinoma in seven
317 dogs and squamous cell carcinoma in one patient), four dogs with sarcoma
318 (myxosarcoma, osteochondrosarcoma in one dog each, and undifferentiated sarcoma in
319 two dogs) and one dog with mast cell tumour.

320 Using Grocott-stained sections, septate branching hyphae were observed at the
321 surface of the mucosa in biopsy samples from only 6/14 dogs with SNA.

322 **Serology**

323 *Aspergillus* serology yielded a positive result in four dogs with SNA and in one
324 dog with LPR.

325 **Fungal cultures**

326 Fungal culture was positive in six dogs with SNA (all *A. fumigatus*). *Aspergillus*
327 *fumigatus* DNA was detected in the nasal mucosa of 5 of these 6 dogs. Fungal culture
328 was positive in one dog with nasal tumour (*A. terreus*). Sensitivity, specificity, PPV and
329 NPV of these tests in the diagnosis of canine SNA are given in Table 3.

330 **Discussion**

331 In the present study, we have evaluated the diagnostic value of detection of
332 fungal DNA in tissue and whole blood samples from dogs with SNA. Using a broad-
333 spectrum real-time PCR assay detecting ribosomal DNA from all *Penicillium* and
334 *Aspergillus* species, sensitivity and NPV of tissue fungal DNA detection were both
335 100%, but specificity and PPV of this diagnostic method were very low. This was in
336 part explained by the fact that fungal DNA was detected in the nasal mucosa of all
337 control dogs. This constitutes the first evidence that fungi are part of the normal flora of
338 the nasal cavity in the dog. Similarly, fungi, including species from the genera
339 *Penicillium* and *Aspergillus*, have been shown to be part of the background flora of the
340 nasal cavity in humans (Catten et al., 2001; Murr et al., 2006; Scheuller et al., 2004). In
341 the present study, there was significantly more fungal DNA in the nasal mucosa of dogs
342 with SNA than in that of dogs from the three other groups. However, the relative copy
343 numbers overlapped between the four groups, and no clear cut-off could clearly identify
344 dogs with SNA. The diagnostic value of this assay would likely have been increased by
345 deliberately collecting biopsy samples from fungal plaques visualized endoscopically
346 within the nasal cavity or sinus. However, in this study we instead elected to sample a
347 standard mucosal site in order to permit comparison between mucosal samples collected
348 from dogs in the different disease groups.

349 The results of the present study support the fact that canine SNA is mostly
350 caused by *A. fumigatus*. Using the species-specific assays, no DNA derived from *A.*
351 *terreus*, *A. niger* or *A. flavus* was found in any of the nasal biopsies. However,
352 sensitivity, specificity, PPV and NPV of mucosal *A. fumigatus* DNA detection in the
353 diagnosis of SNA were 50, 97, 87 and 82%, respectively. Although sensitivity was

354 moderately low, it was still greater than that of the routine diagnostic tests that were run
355 for comparison in the present study. The negative results obtained with the four
356 *Aspergillus* species-specific assays in nasal biopsies from seven dogs with SNA could
357 mean that, in these cases, the disease was caused by a *Penicillium* or an *Aspergillus*
358 species other than *A. fumigatus*, *A. terreus*, *A. niger* or *A. flavus*. However, there may
359 have been sufficient DNA from a mixture of fungal species present to give a positive
360 result with the general PenAsp assay but insufficient DNA of any single *Aspergillus*
361 species tested to give a positive result with the specific assay. Therefore, the samples
362 which were negative in the Afumi assay may still have had *A. fumigatus* present but in
363 insufficient quantity to be positive with the specific assay.

364 In the present study, fungal DNA detection in whole blood by the PenAsp assay
365 was of no value for diagnosing SNA, largely due to low specificity and PPV. This was
366 mostly related to the high number of positive results obtained from control dogs and
367 dogs with nasal tumour. A high number of false-positive results has been reported in
368 many studies examining the performance of blood DNA detection in the diagnosis of
369 invasive aspergillosis in humans (Halliday et al., 2006; Kami et al., 2001; Quindos,
370 2006). Although false-positive results could be due to contamination by airborne spores
371 during the PCR procedures (Kami et al., 2001; Loeffler et al., 1999), the likelihood of
372 such contamination has decreased significantly due to technical improvements in PCR
373 methodology (Quindos, 2006). Environmental contamination is not likely to have
374 occurred in the present study as strict precautions were taken, including the use of
375 dedicated HEPA filtered PCR cabinets for setting-up the PCR assays. Carryover of PCR
376 products (Kami et al., 2001) is unlikely due to the strict separation of each phase of the
377 work in separate laboratories with dedicated equipment (DNA extraction, PCR setup,

378 PCR running and amplicon analysis). An alternative explanation for this high number of
379 positive results is the true detection of fungal DNA in the blood of these patients.
380 However, none of the dogs had neutropenia or signs of systemic immune suppression
381 that could have prevented clearance of the fungal elements by neutrophils.

382 As the amount of fungal DNA was similar in the nasal mucosa of dogs with LPR
383 and control dogs, the present study does not clearly implicate *Penicillium* or *Aspergillus*
384 fungi in the pathogenesis of canine LPR. In a recent study using a broader pan-fungal
385 assay, there was more fungal DNA in the nasal mucosa of dogs with LPR than in that of
386 dogs with nasal tumour or control animals (Windsor et al., 2006). However, in that
387 study, the difference in fungal DNA load between dogs with LPR and control dogs was
388 due to the absence of DNA detected in the nasal mucosa of control animals (Windsor et
389 al., 2006). The discrepancy in the results of these studies can be related to differences in
390 PCR techniques, assays, samples and DNA extraction methods used (Ferns, 2006).
391 Alternatively, other fungi, from which DNA was not detected by the PenAsp assay,
392 might be involved in the pathogenesis of canine LPR. Indeed, in humans with chronic
393 rhinosinusitis, *Alternaria* is particularly suspected to trigger the local inflammatory
394 reaction (Shin et al., 2004).

395 One of the major obstacles to using PCR to quantify *Aspergillus* DNA is a
396 reliable and simple method for DNA extraction. The presence of a complex, sturdy cell
397 wall that is resistant to lysis impairs extraction of the DNA by conventional methods
398 employed for bacteria (Griffiths et al., 2006). Most fungal DNA extraction protocols are
399 time-consuming and are not suitable for use as a routine diagnostic technique (Velegaki
400 et al., 1999; Williamson et al., 2000). More recent extraction methods include freezing
401 and thawing, freezing and boiling, bead-beating and enzyme extraction (Griffiths et al.,

402 2006; Kabir et al., 2003). The best and quickest fungal DNA extraction methods involve
403 bead lysis with or without enzyme digestion (Griffiths et al., 2006). In the present study,
404 we have tested three extraction methods and selected the one that yielded the greatest
405 amount of fungal DNA and which was the quickest to perform. This method involved
406 enzyme digestion and bead lysis.

407 In conclusion, the detection of *A. fumigatus* DNA in nasal biopsy samples shows
408 the highest specificity, PPV and NPV for diagnosing canine SNA, but sensitivity of this
409 method is low. The results of the present study do not clearly implicate *Penicillium* or
410 *Aspergillus* fungi in the pathogenesis of canine LPR. However, other fungi could be
411 involved in the pathogenesis of this disease.

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- 496

497 **Table 1**

		<i>A. flavus</i>	<i>A. fumigatus</i>	<i>A. niger</i>	<i>A. terreus</i>
Method 1	Mean Ct	27.6	28.0	30.1	28.6
	SD	0.72	0.26	0.70	0.68
Method 2	Mean Ct	23.2	24.0	24.4	24.2
	SD	0.17	0.05	0.24	0.14
Method 3	Mean Ct	25.2	25.6	26.1	26.2
	SD	0.10	0.29	0.24	0.12

498

499 Three methods of DNA extraction were assessed by application to suspensions of the
500 four target *Aspergillus* species. Method 1 consisted of lysis buffer and proteinase K
501 added to the samples with overnight incubation at 56°C in a shaking incubator. Method
502 2 included the addition of a 5mm ball to the tubes during the overnight incubation.
503 Method 3 involved the use of freeze-thawing and mechanical disruption prior to lysis
504 buffer, proteinase K and the overnight incubation. The mean Ct and standard deviation
505 is shown for the three repeats of each extraction method.

506 **Table 2**

Assay	Product Size	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe Sequence (5' FAM - 3' BHQ-1)	Efficiency
PenAsp	~80	CGGAAGGATCATTACTGAGTG	GCCCCGCCGAAGCAAC	<u>CCA</u> ACCTCC <u>CC</u> ACCCG <u>TG</u>	94.0 to 100.2%
Afumi	136	GCCCCGCCGTTTCGAC	CCGTTGTTGAAAGTTTTAACTGATTAC	CCCGCCGAAGACCCCAACATG	99.7%
Aflav	89	CGAGTGTAGGGTTCCTAGCGA	CCGGCGGCCATGAAT	TCCCACCCGTGTTTACTGTACCTTAGTTGCT	100.7%
Anigr	79	GCCGGAGACCCCAACAC	TGTTGAAAGTTTTAACTGATIGCATT	AATCAACTCAGACTGCACGCTTTCAGACAG	95.9%
Aterr	72	ATCATTACCGAGTGCGTGCTTTA	CCCGCCGAAGCAACAAG	CCCAACCTCCCACCCGTGACTATTG	98.8%
G3PDH	90	TCAACGGATTTGGCCGTATTGG	TGAAGGGGTCATTGATGGCG	CAGGGCTGCTTTTAACTCTGGCAAAGTGGA	99.9%

507 Sequences of forward and reverse primers as well as the probe used in the real-time PCR reactions (Haugland et al., 2004). The probe
508 sequence used for the PenAsp assay was altered by addition of locked nucleic-acid (LNA) bases to increase the predicted annealing
509 temperature of the unmodified sequence. LNA bases are underlined in the table. All primers were desalted when purified and the probes
510 were HPLC purified.

511 **Table 3.**

	Sensitivity	Specificity	PPV	NPV
Fungal culture	0.43	0.97	0.86	0.78
Serology	0.31	0.97	0.80	0.76
Histopathology	0.14	1.00	1.00	0.71
Tissue fungal DNA detection (PenAsp assay)	1.00	0.06	0.32	1.00
Tissue fungal DNA detection (<i>A. fumigatus</i> specific assay)	0.50	0.97	0.87	0.82
Blood fungal DNA detection (PenAsp assay)	0.71	0.24	0.31	0.64
Blood fungal DNA detection (<i>A. fumigatus</i> specific assay)	0.21	0.45	0.15	0.54

512

513 Sensitivity, specificity, positive and negative predictive values (PPV and NPV) of

514 different diagnostic tests in the diagnosis of canine sino-nasal aspergillosis.

515 **Legend for figures**

516 **Figure 1.** Plots of the relative copy number of *Penicillium / Aspergillus* DNA in nasal
517 and blood samples assessed by real-time PCR in control dogs (HEA), dogs with nasal
518 tumour (TUM), dogs with idiopathic lymphoplasmacytic rhinitis (LPR) and dogs with
519 sino-nasal aspergillosis (ASP). The horizontal line corresponds to the median value of
520 that group. The samples with a relative copy number of 0 had no detectable template but
521 measurable amounts of G3PDH DNA. Significant differences between groups are
522 shown with the corresponding *P* values shown.

523

524 **Figure 2.** Plots of the relative copy number of *A. fumigatus* DNA in nasal and blood
525 samples assessed by real-time PCR in control dogs (HEA), dogs with nasal tumour
526 (TUM), dogs with idiopathic lymphoplasmacytic rhinitis (LPR) and dogs with sino-
527 nasal aspergillosis (ASP). The horizontal line corresponds to the median value of that
528 group. The samples with a relative copy number of 0 had no detectable template but
529 measurable amounts of G3PDH DNA.

Figure 1: Relative copy number for *Penicillium* spp. and *Aspergillus* spp. in nasal biopsies and blood from the four clinical groups

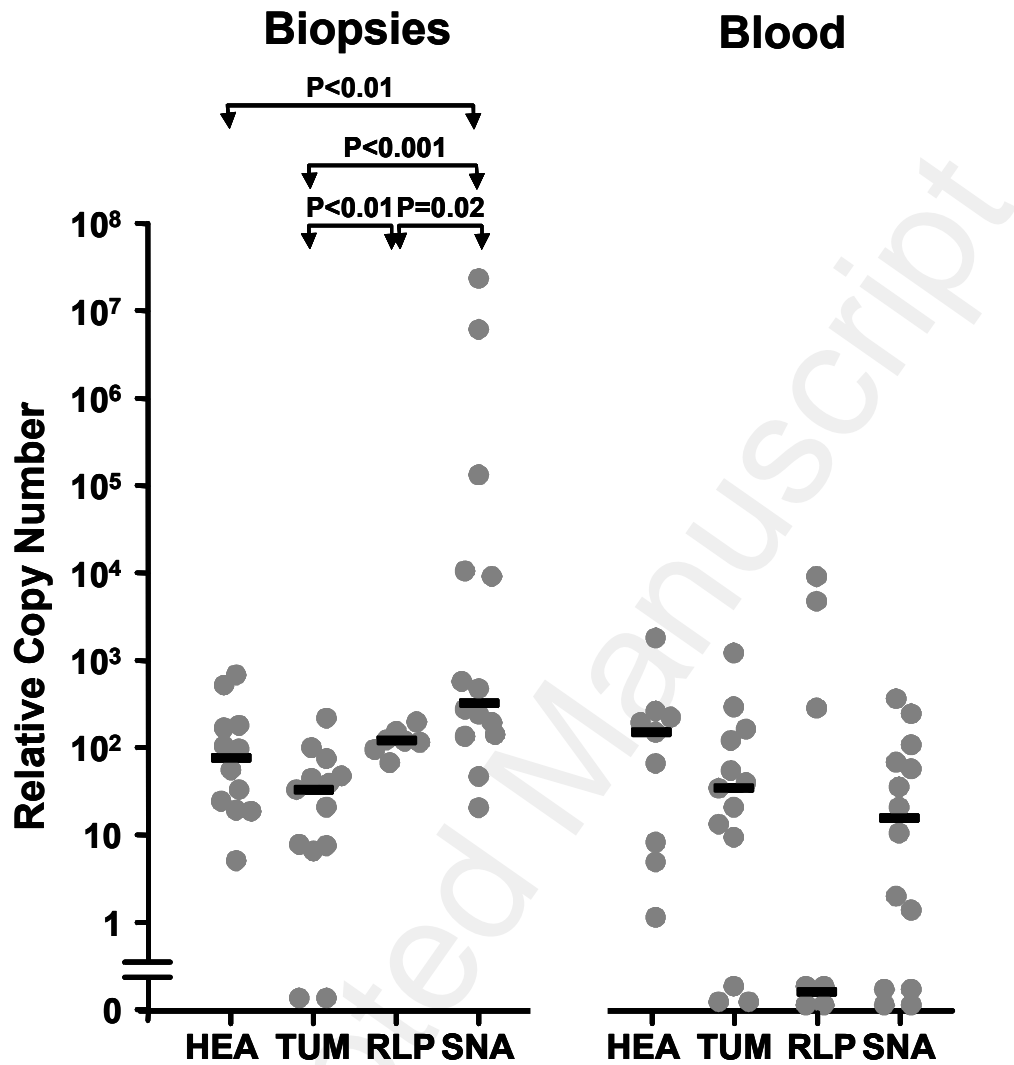


Figure 2: Relative copy number for *Aspergillus fumigatus* in nasal biopsies and blood from the four clinical groups

