

Utility of fungal polymerase chain reaction on nasal swab samples in the diagnosis and monitoring of sinonasal aspergillosis in dogs

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Abstract

Background: In dogs with sinonasal aspergillosis (SNA) the utility of PCR in the diagnosis and monitoring of the disease after treatment has not been assessed.

Objectives: To evaluate the presence of fungal DNA using quantitative PCR targeting *Aspergillus fumigatus* (*Aspfum*) and *Aspergillus* spp. (Pan*Asp*), and PCR targeting multiple fungal species (Pan*Fun*), in samples obtained from nasal cavities of dogs with SNA, other nasal diseases and healthy dogs.

Animals: Sixty-two dogs including 20 with SNA, 12 with cured SNA (of which 10 are from the SNA group), 20 dogs with Non-SNA nasal disease, and 20 healthy dogs.

Methods: Prospective cross-sectional study. *Aspfum*, Pan*Asp*, and Pan*Fun* were performed on blindly collected nasal swabs obtained in anesthetized dogs.

Results: In SNA dogs, *Aspfum* and Pan*Asp* were positive in 13/20 and 14/20 dogs. In all dogs in the 3 other groups, *A. fumigatus* DNA was not detected using *Aspfum*. Pan*Asp* was positive in 3 non-SNA dogs: 1 with cured SNA and 2 with Non-SNA nasal disease. A Ct cut-off value of 33.3 for *Aspfum* demonstrated 65% sensitivity and 100% specificity. A Ct cut-off value of 34.5 for Pan*Asp* demonstrated 70% sensitivity and 96.2% specificity. Pan*Fun* was positive in 16/20, 12/12, 19/20, and 7/20 dogs in the SNA, cured SNA, Non-SNA, and healthy groups, respectively.

Conclusion and Clinical Importance: *Aspfum* and Pan*Asp* on blindly collected nasal swabs can be useful for the detection of SNA at diagnosis and at cure, especially when more invasive methods are not available.

KEYWORDS

aspergillosis, diagnosis, dogs, nasal swabs, PCR

Abbreviations: AGGD, agar-gel double immunodiffusion; *Aspfum*, qPCR targeting *A. fumigatus*; AUC, area under the curve; DNA, deoxyribonucleic acid; LPR, lymphoplasmacytic rhinitis; Pan*Asp*, qPCR targeting *Aspergillus* spp.; Pan*Fun*, PCR targeting all fungi; qPCR, quantitative polymerase chain reaction; ROC, receiver operating characteristics; SNA, sinonasal aspergillosis.

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1 | INTRODUCTION

Sinonasal aspergillosis (SNA) accounts for 7% to 11% of dogs with chronic nasal disease.¹⁻³ It is frequently diagnosed in young to middle-aged dolichocephalic or mesocephalic breeds.¹⁻³ *Aspergillus fumigatus* is recognized as the most common etiological agent of SNA in dogs, although a pathogenic role has been documented for *Aspergillus tubingensis*, *Aspergillus uvarum*, and *Penicillium* spp.^{4,5} Dolichocephalic and mesocephalic breeds are suggested to be healthy carriers of the *Aspergillus* fungus.⁴

Idiopathic lymphoplasmacytic rhinitis (LPR) is a major differential for early SNA as it is also frequently diagnosed in middle-aged dolichocephalic breeds. LPR is characterized by histologic abnormalities such as lymphoplasmacytic to mixed inflammation in the nasal mucosa without any identifiable cause.⁶ The potential role of fungi in LPR has been investigated.⁷⁻⁹ High levels of fungal deoxyribonucleic acid (DNA), not including *Aspergillus* spp., are detected in nasal biopsies of dogs with LPR, suggesting that fungal organisms can be causally associated with the inflammation.⁷⁻⁹ Two fungi, *Alternaria* spp. and *Cladosporium* spp., have been identified in the nasal flora of dogs with LPR, though they have also been identified in healthy dogs and dogs with nasal neoplasia.⁷⁻⁹ The role of fungi in the etiopathogenesis of LPR has not been determined.

Early detection of SNA can be challenging and clinical signs are often vague, such as a decreased appetite.⁴ Suggestive clinical signs include mucopurulent nasal discharge, epistaxis, sneezing, nasal planum pain, ulceration or depigmentation.⁴ Nasal airflow could be normal or higher on the ipsilateral side. Costly and invasive methods are usually necessary to obtain a diagnosis.¹⁰ Culture of fungal material, cytology, histopathology and serology are specific diagnostic methods but demonstrate poor to moderate sensitivity.^{4,11-13} Definitive diagnosis requires imaging studies and rhinoscopy.⁴ Furthermore, confirmation of cure after treatment is difficult to assess: follow-up rhinoscopy to confirm absence of fungal material in the nasal cavities/sinuses is strongly advised even when clinical signs are improved or resolved.^{14,15}

Real-time quantitative polymerase chain reaction (qPCR) assays have been designed for the detection of *Aspergillus fumigatus* species (*Aspfum*) and *Aspergillus* genera (*PanAsp*), as well as PCR targeting all fungi (*PanFun*).^{16,17} The sensitivity and specificity of these tests in the diagnosis and monitoring of dogs with SNA has not been reported.

We aimed to determine the diagnostic value of *Aspfum*, *PanAsp* and *PanFun*, using blindly collected deep swabs, in the diagnosis and monitoring of SNA. We also aimed to investigate the presence of fungal DNA in dogs with other chronic nasal diseases and in healthy dolichocephalic dogs. Our hypotheses were that, using blindly collected deep swabs, (1) positive qPCR and PCR results would be obtained in dogs with confirmed, active SNA, and (2) negative qPCR and PCR results would be obtained in dogs after confirmed cure from SNA. We further hypothesized that (3) positive fungal qPCR and PCR results can occur in dogs with other chronic nasal diseases such as LPR, and (4) in healthy dogs of breeds predisposed to SNA.

2 | MATERIAL AND METHODS

2.1 | Study sample

The study was performed at the veterinary clinic of companion animals of the University of Liège (Belgium), between 2017 and 2019. This study was approved by the ethical committee of the University of Liège (approval number: 1854) and all samples were obtained with informed and written owner consent. Four groups of dogs were defined: dogs with SNA at diagnosis (SNA group), dogs that had had SNA with confirmed cure (cured SNA group), dogs with other chronic nasal diseases, such as LPR and nasal neoplasia (Non-SNA nasal disease group), and healthy dolichocephalic dogs (Healthy control group).

Dogs were included in the SNA group after confirmation of the diagnosis based on the following criteria: clinical signs of chronic upper respiratory disease (sneezing, unilateral or bilateral mucopurulent nasal discharge, reverse sneezing, epistaxis), positive rhinoscopic findings with direct visualization of fungal plaques associated with turbinate destruction, and positive fungal culture of plaques. When available, the CT scan was used to show typical features of nasal aspergillosis including cavitory destruction of the turbinates with abnormal soft tissue within the nasal cavity, presence of a rim of soft tissue along the frontal and sinus bones, and thick reactive bone, as well as to assess possible cribriform plate lysis. Administration of oral antifungal treatment at the time of referral was recorded. Endoscopic debridement was performed under general anesthesia, in sternal recumbency, using a pediatric bronchoscope (Pediatric bronchoscope Fujinon EB-4105; Onys SA). A 2% enilconazole (Imaverol; Janssen-Cilag SA) solution was prepared and infused in each sinonasal cavity, as previously described.¹⁵ Briefly, under general anesthesia, the dog was placed in dorsal recumbency with the nasopharyngeal area blocked, nose pointed upward, and the solution was infused until it reached the nostrils in order to immerse both sinonasal cavities, for 15 minutes. Dogs with CT-scan evidence of cribriform plate damage had rhinotomy with surgical debridement and direct topical enilconazole administration.¹⁸ No oral antifungal treatment was prescribed; treatment was discontinued in dogs that received antifungal agents before diagnosis.

The SNA cured group included dogs that had recovered from SNA after perendoscopic debridement or surgical management and topical therapy. Short-term cure was determined 3 to 4-weeks after treatment and was based on resolution of clinical signs and absence of fungal plaques at recheck rhinoscopy combined with negative fungal culture. Additionally, long-term cure was based on resolution of clinical signs at least 1 year after the last treatment, assessed by phone. The Non-SNA nasal disease group was composed of dogs with either LPR or neoplasia. The diagnosis of LPR was based on the presence of chronic, bilateral nasal discharge, the absence of lethargy or decreased appetite, the absence of sinus pain, compatible rhinoscopic findings (mucous or mucopurulent nasal secretions, mucosal edema/congestion, possible mild turbinate remodeling and absence of necrotic or fungal material), and appropriate histopathological changes in biopsy samples of the nasal mucosa (lymphoplasmacytic inflammation of the lamina propria). When available, diagnosis was also based on CT scan findings (presence of soft tissue opacification, fluid accumulation, mild turbinate destruction only and

TABLE 1 Nucleic acid sequences of probes and primers

Test	PCR type	Target species	Primers/probes	Sequence	Reference
Aspfum	qPCR	<i>Aspergillus fumigatus</i>	Reverse primer	ATATGCTTAAGTTCAGCGGGT	Salehi ¹⁶
			Aspfum probe	6FAM-CAGCCGACACCCAACCTTTATTTTT-BBQ	
PanAsp	qPCR	<i>Aspergillus</i> spp.	Reverse primer	ATATGCTTAAGTTCAGCGGGT	Salehi ¹⁶
			Forward primer	GCGTCATTGCTGCCCTCAAGC	
PanFun	End-point PCR	<i>Pan Fungal</i>	ITS1	TCCGTAGGTGAACCTG CGG	Maniam ¹⁷
			ITS4	TCCTCCGCTTATTGATATGC	

Abbreviations: Aspfum, qPCR targeting *A. fumigatus*; PanAsp, qPCR targeting *Aspergillus* spp.; PanFun, PCR targeting all fungi; PCR, polymerase chain reaction; qPCR, quantitative PCR.

absence of other diseases, such as SNA or tumoral process).⁶ Cases with acute clinical signs, with moderate to significant endodontic disease or when rhinoscopy showed an intranasal foreign body, or moderate to severe destructive rhinitis were excluded.

Nasal neoplasia was diagnosed based on history, clinical signs, CT-scan when available, rhinoscopic findings (presence of obstructive abnormal tissue in the nasal cavity, nasopharynx or in both of these localizations), and confirmation with histopathology.

Finally, the Healthy control group included client-owned, healthy, dolichocephalic dogs, age-matched with SNA dogs. Healthy status was based on the absence of reported clinical signs, a normal physical examination and normal blood test results (hematology and biochemistry).

2.2 | Nasal swabs sample collection

Nasal swabs were taken under general anesthesia in all dogs, and before performing rhinoscopy in dogs with nasal disease. In the cured SNA group, nasal swabs were performed 30 seconds before to the recheck rhinoscopy (which was 3-4 weeks after endoscopic debridement or surgical management). All dogs were premedicated with butorphanol (0.2 mg/kg; Butomidor, Richter Pharma) IV in combination with medetomidine (5 µg/kg; Medetor, CP-Pharma). Propofol (2-4 mg/kg; Propofol, Zoetis) was used for induction. Anesthesia was maintained with isoflurane (Iso-Vet; Eurovet).

A nasal smear was taken either from the left (in healthy dogs) or the most affected nasal cavity based on history and clinical exam. Samples were taken under general anesthesia in intubated dogs. For this purpose, the nare was maintained open using a sterile speculum and a sterile swab (Copan, FLOQSwabsTM, 553C, Brescia, Italy) was introduced blindly through the speculum up to approximately the caudal third of the nasal cavity. Three gentle complete circular movements were used to brush the mucosa before withdrawal of the swab through the speculum. The swab tip was then cut and stored in a sterile cryotube and banked at -80°C until batched analyses to extract DNA. In SNA and Non-SNA nasal disease groups, the procedure was before rhinoscopy.

2.3 | Sample analysis

All samples were transported to the pathology department of University of Liege. Genomic DNA was isolated from the swab using a

DNeasy Blood & Tissue Kits as previously described (QIAGEN Benelux BV; Antwerp, Belgium).¹⁹ Deoxyribonucleic acid samples were stored at -80°C until further molecular analysis. Primers and probes selected for qPCR and PCR testing are described in Table 1.^{16,17} Adequate measures were used to avoid PCR assay contamination, as recommended in the literature.²⁰ Duplicate qPCRs for Aspfum and PanAsp were performed using Luna Universal qPCR Master Mix (New England BioLabs), as previously published.¹⁶ Cycles of PCR were as follows: an initial step of 5 minutes at 95°C, followed by 45 cycles at 95°C for 30 seconds, then 55°C for 30 seconds, and then “elongation” at 72°C for 1 minute; the final cycle was followed by extension at 72°C for 3 minutes. All qPCR samples were analyzed in duplicates. PanAsp was designed to detect the species *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus nidulans/Aspergillus versicolor*. Amplicons from Aspfum and PanAsp were not sequenced. Quantitative PCR results were interpreted as very high load (cycle threshold [Ct] < 20), high load (20.1-24.0), moderate load (24.1-28.0), low load (28.1-32.0), very low load (32.1-39.9). Results were considered negative if the Ct value was greater than 40. PanFun PCR was performed using end-point PCR with the Taq PCR Kit (New England BioLabs). Primers ITS1 and ITS4 were used.¹⁷ PCR cycles were as follows: an initial step of 5 minutes at 95°C, followed by 45 cycles at 95°C for 30 seconds, then 50°C for 30 seconds, and then “elongation” at 72°C for 1 minute; the final cycle was followed by extension at 72°C for 3 minutes. After gel electrophoresis, sequences of amplicons of expected size were obtained by Sanger sequencing (Eurofins Genomics). β-Actin was used as a housekeeping gene to test for PCR inhibitors.²¹ The positive control used was total DNA from a fungal plaque confirmed as positive for *A. fumigatus* by culture; the negative control used was water. The nucleotide sequences were subjected to NCBI (National Center for Biotechnological Information) Nucleotide BLAST (Basic Local Alignment Search Tool) analysis for species identification. In some cases, despite the positive amplification of the amplicon on gel, the sequencing could not identify any agent due to mixed infections, results were considered not readable.

2.4 | Statistical analysis

All statistical analyses were performed with XLSTAT software (version 2019.4.2) for Windows. Normality was checked with Shapiro-Wilk tests in all groups. Accordingly, Kruskal-Wallis tests with Dunn post

hoc tests and Bonferroni correction were used to compare Ct value of *Aspfum* and PanAsp results between SNA, SNA cured, Non-SNA nasal disease and Healthy control groups. A Ct value of 40 was arbitrarily assigned to dogs with a negative qPCR result. Mann Whitney U test was used to compare Ct the values of *Aspfum* and PanAsp between dogs in the SNA group that had received oral administration of antifungal therapy or not. Nonparametric data were expressed as median, minimum and maximum range and interquartile range (IQR). Receiver operating characteristics (ROC) curves were created to assess the ability of *Aspfum* and PanAsp to diagnose *Aspergillus* spp. The area under the curve (AUC) with the 95% confidence interval (CI 95%) and the Ct value that resulted in the greatest combination of sensitivity and 1-specificity was identified using easyROC software (v. 1.3.1). The correlation between Ct values obtained either by *Aspfum* or by PanAsp was analyzed with the Pearson's correlation coefficient. Regarding PanFun, Monte Carlo method with Marascuilo procedure as post hoc analysis was used to compare proportions of *Aspergillus* spp. in SNA, SNA cured, Non-SNA nasal disease and Healthy control groups. For all tests, a P-value less than .05 was considered significant.

3 | RESULTS

3.1 | Study sample

Sixty-two dogs were included in the study. Twenty dogs met the inclusion criteria for definitive diagnosis of SNA, all had a positive culture of *A. fumigatus*. A CT-Scan was performed in 11 dogs in the SNA group. The location of the fungal plaques was exclusively nasal in 4/20, in sinus in 1/20 (confirmed with CT) and sinonasal in 13/20 dogs. In 2 dogs, the location was not definitely solely nasal, as the sinuses were not accessible during rhinoscopy and no CT scan was available to exclude sinus involvement. A CT-Scan was performed in 11 dogs in the SNA group; in 2 of them, cribriform plate damage was obvious and they were treated surgically. Six SNA group dogs were receiving antifungal therapy (itraconazole: Sporanox; Janssen-Cilag SA, 5 mg/kg PO q 12 hours) with median time before admission of 14 days, ranging from 14 to 60 days. Twelve dogs were included in the SNA cured group, of which 10 came from the SNA group at diagnosis and 2 independent dogs were added. Twenty dogs were included in the Non-SNA nasal disease group: 15 dogs had LPR; 5 dogs had a nasal tumor, with 4 having nasal carcinoma and 1 palatine osteosarcoma. Twenty dogs were included in the Healthy control group.

3.2 | *Aspergillus fumigatus* qPCR results (*Aspfum*)

Aspfum was positive in 13/20 SNA group dogs (65%) with a median Ct value of 31.05 (range min/max, 23.2-40; IQR: 27.23-40) and negative in all dogs in the 3 other groups. Dogs in the SNA group had a mean Ct value significantly lower than in the 3 other groups ($P < .01$; Figure 1; Table 2). A Ct cut-off value of 33.3 showed a sensitivity of 65% and a specificity of 100% for the diagnosis of aspergillosis

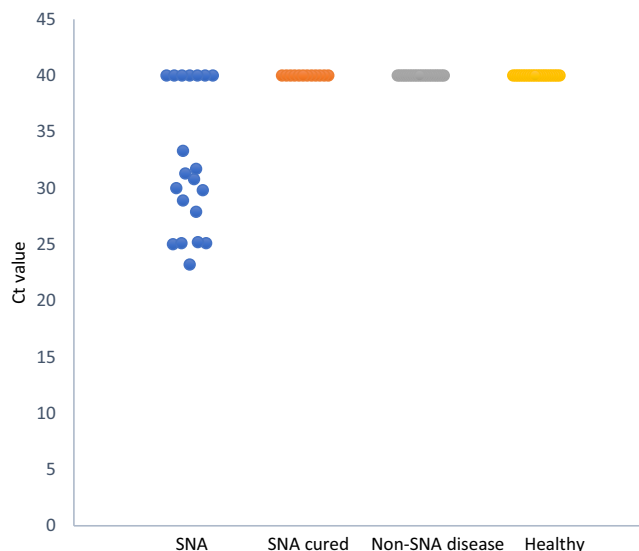


FIGURE 1 scattergram of qPCR *Aspfum* (*Aspergillus fumigatus*) test results, expressed as Ct value, in the 4 groups of dogs: SNA (sinonasal aspergillosis), cured SNA, Non-SNA nasal disease and healthy. Dogs with SNA had a mean Ct significantly lower than in the 3 other groups ($P < .01$). A Ct value of 40 is considered as a negative result. Ct, cycle threshold; qPCR, quantitative polymerase chain reaction

(AUC = 82.5% [CI 95%: 70.5-94.5]; $P < .01$; Figure 2). Among the 6 dogs treated with antifungal therapy at admission, 5 dogs (83.3%) were negative. Administration of antifungal therapy at the time of admission was associated with a higher Ct value ($P = .01$). When excluding dogs being treated with itraconazole at the time of admission, the sensitivity and specificity of *Aspfum* were 86% and 100%, respectively. The dog with focal sinus SNA had a positive result Ct value of 30.

3.3 | *Aspergillus* spp. qPCR results (PanAsp)

PanAsp was positive in 14 dogs (70%) in the SNA group, with a median Ct value of 31.95 (range min/max, 24.3-40; IQR: 28.6-40). PanAsp was also positive in 1 dog in the SNA cured group (8.33%) at a very low load (Ct value of 34.1; Table 2). In the Non-SNA nasal disease group, 1 dog with LPR and 1 dog with nasal neoplasia (nasal carcinoma) were tested positive at a very low load with Ct value of 33.1 and 37.2, respectively. PanAsp was negative in all healthy control dogs. The median Ct value of dogs in the SNA group was significantly lower ($P < .01$); however, an overlap of Ct values between groups was present (not all individual Ct values were lower for SNA group dogs compared with dogs in the other groups; Figure 3). A Ct cut-off value of 34.5 had a sensitivity of 70% and a specificity of 96.2% (AUC = 83.8% [CI 95%: 72.2-95.4]; $P < .01$) for sinonasal aspergillosis (Figure 4). Among the 6 dogs being treated with itraconazole at the time of admission, 5 specimens (83.3%) were PanAsp negative. Administration of antifungal therapy at the time of diagnosis was associated

TABLE 2 Results of *Aspfum*, Pan*Asp*, and PanFun in study population

Group	N	Positive <i>Aspfum</i>	Positive Pan <i>Asp</i>	Positive PanFun	Fungal species (PanFun)
SNA	20	13/20 (65%)	14/20 (70%)	16/20 (80%)	<i>Aspergillus fumigatus</i> (9/16), <i>Cladosporium</i> spp. (2/16), <i>Pithomyces chartarum</i> (1/16), not readable ^a (3/16), <i>Legionella</i> spp. ^b (1/16), <i>Vorticella</i> spp. ^b (1/16)
SNA cured	12	0	1/12 (8.33%)	12/12 (100%)	<i>Cladosporium</i> spp. (4/12), <i>Blumeria graminis</i> (2/12), <i>Caloplaca</i> spp. (1/12), <i>Dissoconium</i> spp. (1/12), not readable ^a (3/12), <i>Quercus</i> spp. ^b (1/12)
Non-SNA nasal disease	20	0	2/20 (10%) LPR: 1/15 Tumor: 1/5	19/20 (95%) LPR: 14/15 Tumor: 5/5	LPR: <i>Cladosporium</i> spp. (4/14), <i>Aspergillus</i> spp. (1/14), <i>Mycocacia uda</i> (1/14), <i>Malassezia</i> spp. (1/14), <i>Penicillium brevicompactum</i> (1/14), <i>Vishniacozyma victoriana</i> (1/14), <i>Zymoseptoria tritici</i> (1/12), <i>Stictis radiata</i> (1/14), <i>Plicaturopsis crispa</i> (1/14), <i>Urtica dioica</i> ^b (1/14), <i>Corynespora</i> spp. ^b (1/14) Tumor: <i>Aspergillus niger</i> (1/5), <i>Cladosporium</i> spp. (1/5), <i>Peniophora</i> spp. (1/5), not readable ^a (2/5)
Healthy	20	0	0	7/20 ^c (35%)	<i>Penicillium</i> spp. (1/20), <i>Legionella</i> spp. ^b (3/20), not readable ^a (3/20)

Abbreviations: *Aspfum*, qPCR targeting *A. fumigatus*; Healthy, healthy dolichocephalic dogs; LPR, lymphoplasmacytic rhinitis; Pan*Asp*, qPCR targeting *Aspergillus* spp.; PanFun, PCR targeting all fungi; PCR, polymerase chain reaction; qPCR, quantitative PCR; SNA cured, dogs that recovered from SNA; SNA, dogs with sinonasal aspergillosis (group).

^aNot readable: positive results with sequencing unusable secondary to the presence of several fungal in the sample.

^bNonfungal agent.

^cPercentage of positive dogs in healthy dolichocephalic dogs was significantly lower than other three groups.

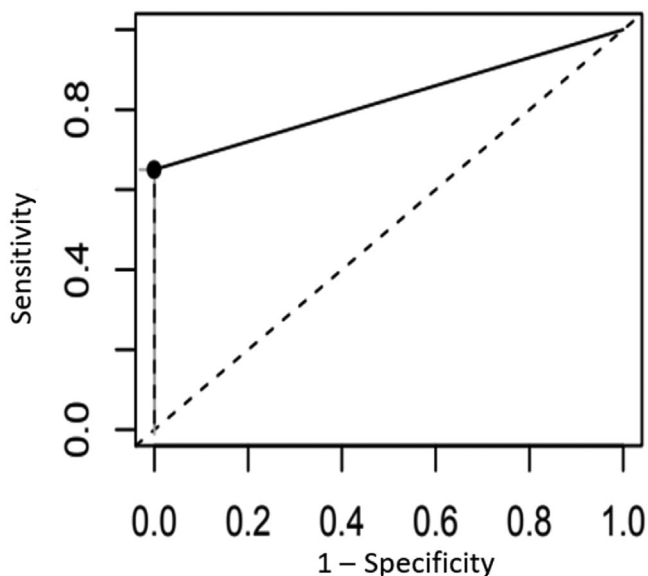


FIGURE 2 Receiver operating characteristics curves of qPCR *Aspergillus fumigatus*. Ct cut-off value of 33.3 can be used to diagnose aspergillosis with a specificity of 100% and a sensitivity of 65% (area under the curve = 82.5% [95% confidence interval: 70.5-94.5]; $P < .01$)

with higher Ct value ($P = .03$). When excluding dogs treated with itraconazole before admission, the sensitivity and specificity were 93% and 94%. The Ct values for *Aspfum* qPCR was slightly lower and strongly correlated with the Ct values for Pan*Asp* ($r = 0.8$, $P < .01$). The dog with focal SNA had a positive result with a Ct value of 31.5.

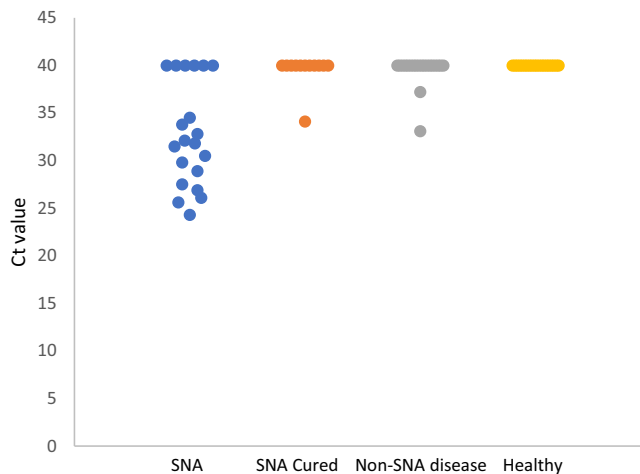


FIGURE 3 scattergram of qPCR tests results for Pan*Asp* (*Aspergillus* spp.), expressed as Ct value, comparing the 4 groups SNA (sinonasal aspergillosis), cured SNA, Non-SNA nasal disease and healthy. Dogs with SNA had a mean Ct significantly lower than in the 3 other groups ($P < .01$). A Ct value of 40 is considered as a negative result. Ct, cycle threshold; qPCR, quantitative polymerase chain reaction

3.4 | Pan-fungus PCR results (PanFun)

PanFun PCR results are shown in Table 2. The PCR PanFun was positive in (16/20) 80%, (12/12) 100%, (19/20) 95%, and (7/20) 35%, in the SNA group, the SNA cured group, the Non-SNA nasal disease and the Healthy control groups, respectively. PanFun PCR in Healthy control dogs was more often negative than in the 3 other groups

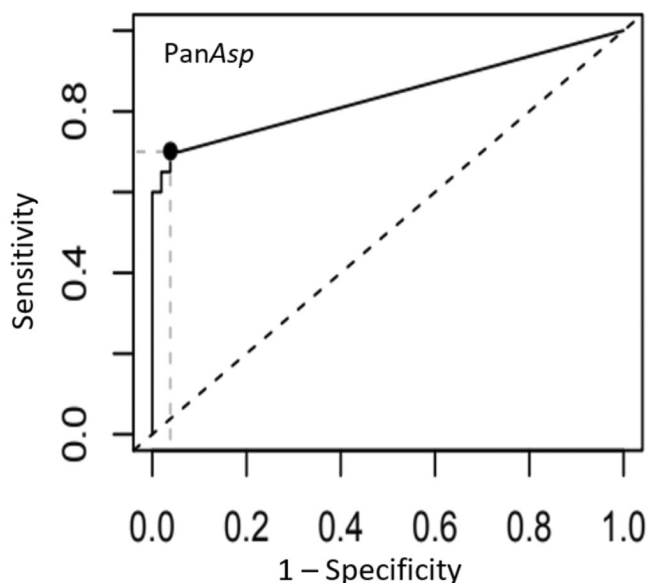


FIGURE 4 Receiver operating characteristics curves of qPCR *Aspergillus* spp. Ct cut-off value of 34.5 can be used to diagnose aspergillosis with a specificity of 96.2% and a sensitivity of 70% (area under the curve = 83.8% [95% confidence interval: 72.2-95.4]; $P < .01$)

($P = .02$). In the SNA group, *Aspergillus* spp. were detected in 9 dogs, all of which were positive for *Aspergillus fumigatus* using *Aspfum*. In non-SNA nasal disease, 2 dogs were positive for *Aspergillus* spp.: 1 with LPR and 1 with nasal neoplasia. In the SNA cured group and Healthy control group, PanFun did not detect *Aspergillus* spp. Regarding the 6 SNA dogs being treated with itraconazole at the time of admission, 2 had positive results either for *A. fumigatus* or *Cladosporium* spp., 3 had negative results and the last 1 had a positive result for a nonfungal agent. Sequencing after PanFun PCR could not identify a single agent in 13.8% (10/72) of dogs: 2 in SNA group, 3 in cured SNA group, 2 in Non-SNA nasal disease group and 3 in the Healthy control group. The PanFun PCR identified nonfungal agents in 11.1% (8/72).

4 | DISCUSSION

This study assessed the presence of fungal DNA using qPCR targeting *Aspfum*, PanAsp and PCR PanFun. Samples for PCR were obtained from blindly collected deep swabs of the nasal cavities in 4 groups of dogs: dogs with SNA at diagnosis and dogs after cure from SNA, dogs with other chronic nasal diseases, and healthy dolichocephalic dogs. Using a Ct cut-off value of 33.3, the sensitivity of *Aspfum* was 65% with a specificity of 100%. Using a Ct cut-off value of 34.5, PanAsp had a sensitivity of 70% with a specificity of 96.2%. When excluding dogs being treated with itraconazole at the time of admission, the sensitivities of *Aspfum* and PanAsp increased up to 86% and 93%, respectively.

The deep, blind sampling method used in this study was quick, easy and minimally invasive to perform. This technique was selected rather than direct sampling of a fungal plaque under rhinoscopic guidance in order to mimic what can be performed in practice, when more sophisticated and invasive techniques, such as rhinoscopy and CT-scan, are unavailable. Care was taken to avoid contamination of the sample from the muco-cutaneous junction of the nares by inserting the swab into the nose through a sterilized speculum.

Quantitative PCR for *Aspfum* and PanAsp using our blind nasal swab collection method provided a moderate sensitivity and excellent specificity. Compared to other available tests, sensitivities of *Aspfum* and PanAsp were low. Indeed, the detection of serum aspergillus specific antibodies with agar-gel double immunodiffusion (AGDD) or ELISA provide good sensitivity and excellent specificity, with a higher sensitivity for ELISA (88.2%) compared to AGDD (76.5%) and higher specificity for AGDD (100%) compared to ELISA (96.8%).¹¹ Culture of fungal plaques at 37°C has good sensitivity (88%) and excellent (100%) specificity.¹² Other diagnostic tests such as cytology and histology on blinded collection have poor sensitivity to diagnose SNA with fungal hyphae detected in 20% to 41% of cases.^{13,22,23}

Our study suggested that administration of itraconazole at the time of sampling can decrease sensitivity of the qPCR testing. When those dogs were removed from the statistical analysis, the sensitivity of both *Aspfum* and PanAsp increased. We hypothesize that per os antifungal treatment could decrease the fungal load in the nasal secretions without preventing growth of fungal colonies inside the plaques. By using a deep but blinded sampling method, we might have not reached the fungal plaques, hence did not detect fungal DNA in these positive cases.

A strong correlation was present between Ct values of both qPCR tests with for *Aspfum* and PanAsp. While *Aspfum* testing appears appropriate, PanAsp testing is an interesting tool to detect the small percentage of SNA cases related to other *Aspergillus* species. Indeed, occasional cases are caused by *A. flavus* or *A. niger*.⁴ In comparison with Ct values obtained in other studies, the Ct values obtained in the present study correspond to rather low DNA load, while we would have expected higher DNA loads.^{19,24,25} This could be explained by the blind sampling method used in this study, since we did not sample fungal plaques directly. One dog with focal sinus plaque location had a positive Ct value of 30 and 31.5 using PanFun and PanAsp, respectively, indicating that fungal DNA can be present in the plaques as well as in nasal secretions (assuming no plaque material came into direct contact with the nasal swab). In our study, 2 dogs with SNA had a very low load Ct values for *Aspfum* and PanAsp. The significance of a high absolute copy number is still questionable: positive results with high Ct values (such as above 33) must be interpreted with caution and along with other parameters, including clinical data. In any case, independently from molecular testing, advanced and invasive procedures, such as CT-scan and rhinoscopy remain the gold standard for final diagnosis. These represent not-to-be-missed procedures before initiation of sinonasal infusion protocols, which are considered the preferred treatment for SNA in dogs.^{4,15,26}

In cured SNA dogs, there was a good correlation between negative PCR results and clinical cure, including rhinoscopic findings and follow-up. Only 1 dog was positive via PanAsp qPCR at very low load Ct value at follow-up. Despite this positive result, this dog did not have any expected clinical sign of SNA. It is worth noting that PCRs detect the presence of fungal DNA, but do not assess whether the fungus is alive or dead.

We suggest that Aspfun and PanAsp qPCR could be used at check-up when rhinoscopic findings make the clinician doubt about complete cure (persistence of atypical secretions) or when rhinoscopy is not available. Positive qPCR at recheck would indicate a stronger need to repeat rhinoscopy. In Non-SNA nasal disease group, PanAsp, but not Aspfun, was also positive in 2 dogs at very low load Ct value (above 32.1): 1 dog with LPR and 1 with nasal neoplasia. Based on the absence of positive results in dogs from the healthy control group, we might consider that the positive dog in the cured SNA and the 2 dogs in Non-SNA nasal disease group are true positives, indicating the presence of a very low load of fungal DNA, and compatible with absence of SNA disease. This is not surprising since *Aspergillus* spp. is a saprophytic fungus and up to 1000 conidia of this fungus are thought to be inhaled on a daily basis in humans: finding traces of fungus anywhere, including inside a nasal cavity is plausible.^{4,18,26}

In cured SNA and Non-SNA nasal disease groups, PanFun was positive in 100% and 93% of cases respectively, while in the SNA group, it was positive in 80% (16/20) only, indicating that PanFun test using blind nasal collection is not appropriate for the diagnosis of SNA. In cured SNA and Non-SNA nasal disease groups, *Cladosporium* spp. was the most frequently identified fungus. This fungus is part of the LPR mycobiota in dogs.⁹ The same study also reported other fungi for the first time, such as *Aspergillus* spp., *Mycoacia uda*, *Malassezia* spp., *Vishniacozyma victoriae*, *Zymoseptoria tritici*, *Stictis radiata*, *Plicaturopsis crispa*, *Peniophora* spp. in the nasal flora of dogs with LPR and nasal neoplasia. These results suggest that *Aspergillus* spp. is not the major component of the mycobiota of dogs with cured SNA and Non-SNA nasal disease. The role of fungi as components of the nasal flora, pathogens or contaminants remains to be clarified. Fungal involvement in the pathogenesis of LPR is difficult to assess, as they could be opportunistic agents in dogs with chronic nasal disease. Given that the nasal filtration capacity is decreased due to decreased muco-ciliary clearance and turbinate destruction, it would not be surprising to find the presence of small amounts of fungi in the respiratory tract, as has been observed in human medicine.^{18,27} However, pathogens found in the nasal cavity (fungi, bacteria, viruses or their associated products) might play a role in initiating or perpetuating mucosal inflammation.^{28,29} In our Healthy control group, *Penicillium* spp. was found in 1 individual. This fungus is a saprophytic agent and was reported as a cause of fungal rhinitis; however, its presence in a healthy dolichocephalic dog could also indicate its physiologic presence in healthy animals.⁵ These results show that *Aspergillus* spp. does not seem to be the major component of the mycobiota of dogs in healthy predisposed breeds neither. However, quantitative studies would be required to compare the presence of fungi in dolichocephalic versus nondolichocephalic dogs using comparative

housekeeping genes. In healthy dogs, PanFun identified the bacteria *Legionella* spp. in 3 dogs. Further studies are needed to determine whether dogs can be subclinical carriers, or whether this result is due to a contamination of the PCR assays.

There were several limitations to this study. Storage methods could have negatively affected quality of amplicons and therefore part of our results. Both freezing and cryopreservation time can negatively decrease sensitivity.³⁰ Normalization of both qPCR methods, *Aspfun* and *PanAsp*, using comparative housekeeping genes was not performed. Although the *PanAsp* and *Aspfun* assays used were based on previously described primers, specificity of both qPCR has not been completely explored. Cross reactions with closely related species might have accounted for some of the positive results.¹⁶ The PanFun PCR did not permit identification of different fungi present in a single sample since it only identifies the predominant fungus. Therefore, a positive test with the assay might represent the presence of *Aspergillus* species. Moreover, in some instances DNA from flora and fauna was amplified. In a previous study, PanFun PCR has rare cross-reactivity between closely related *Aspergillus* spp. (sub) species.¹⁶ Such contamination and cross-reactivity can affect results and might mask the presence of fungi. All these pitfalls can negatively affect the accuracy of PanFun to diagnose SNA on nasal swab.

In conclusion, the present study showed that positive *Aspfun* and *PanAsp* qPCR testing using blindly collected deep nasal swabs is strongly suggestive of aspergillosis in suspected cases, while negative testing does not exclude the disease. This simple, inexpensive and fast alternative diagnostic method can best be performed before administration of antifungal therapy. These tests might also serve in the assessment of a cure at posttreatment check-up when complete cure is questionable, especially when more invasive methods are not available.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the ethical committee of the University of Liege (approval number: 1854) and all samples were obtained with informed and written owner consent.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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REFERENCES

1. Tasker S, Knottenbelt CM, Munro EA, et al. Aetiology and diagnosis of persistent nasal disease in the dog: a retrospective study of 42 cases. *J Small Anim Pract.* 1999;40(10):473-478.
2. Meler E, Dunn M, Lecuyer M. A retrospective study of canine persistent nasal disease: 80 cases (1998-2003). *Can Vet J.* 2008;49(1):71-76.
3. Lobetti RG. A retrospective study of chronic nasal disease in 75 dogs. *J S Afr Vet Assoc.* 2009;80(4):224-228.
4. Peeters D, Clercx C. Update on canine sinonasal aspergillosis. *Vet Clin North Am Small Anim Pract.* 2007;37(5):901-916, vi, 916.
5. Talbot JJ, Johnson LR, Martin P, et al. What causes canine sino-nasal aspergillosis? A molecular approach to species identification. *Vet J.* 2014;200(1):17-21.
6. Windsor RC, Johnson LR, Herrgesell EJ, de Cock HEV. Idiopathic lymphoplasmacytic rhinitis in dogs: 37 cases (1997-2002). *J Am Vet Med Assoc.* 2004;224(12):1952-1957.
7. Windsor RC, Johnson LR, Sykes JE, et al. Molecular detection of microbes in nasal tissue of dogs with idiopathic lymphoplasmacytic rhinitis. *J Vet Intern Med.* 2006;20:250-256.
8. Peeters D, Peters IR, Helps CR, Dehard S, Day MJ, Clercx C. Whole blood and tissue fungal DNA quantification in the diagnosis of canine sino-nasal aspergillosis. *Vet Microbiol.* 2008;128(1-2):194-203.
9. Mercier E, Peters IR, Billen F, et al. Potential role of *Alternaria* and *Cladosporium* species in canine lymphoplasmacytic rhinitis. *J Small Anim Pract.* 2013;54(4):179-183.
10. Sissener TR, Bacon NJ, Friend E, Anderson DM, White RAS. Combined clotrimazole irrigation and depot therapy for canine nasal aspergillosis. *J Small Anim Pract.* 2006;47:312-315.
11. Billen F, Peeters D, Peters IR, et al. Comparison of the value of measurement of serum galactomannan and *Aspergillus*-specific antibodies in the diagnosis of canine sino-nasal aspergillosis. *Vet Microbiol.* 2009;133(4):358-365.
12. Billen F, Clercx C, Le Garèrès A, et al. Effect of sampling method and incubation temperature on fungal culture in canine sinonasal aspergillosis. *J Small Anim Pract.* 2009;50(2):67-72.
13. Pomrantz JS, Johnson LR, Nelson RW, et al. 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.* 2007;230(9):1319-1323.
14. Schuller S, Clercx C. Long-term outcomes in dogs with sinonasal aspergillosis treated with intranasal infusions of enilconazole. *J Am Anim Hosp Assoc.* 2007;43(1):33-38.
15. Vangrinsven E, Girod M, Goossens D, Desquilbet L, Clercx C, Billen F. Comparison of two minimally invasive enilconazole perendoscopic infusion protocols for the treatment of canine sinonasal aspergillosis. *J Small Anim Pract.* 2018;59(12):777-782.
16. Salehi E, Hedayati MT, Zoll J, et al. Discrimination of *Aspergillosis*, *Mucormycosis*, *Fusariosis*, and *Scedosporiosis* in formalin-fixed paraffin-embedded tissue specimens by use of multiple real-time quantitative PCR assays. *J Clin Microbiol.* 2016;54(11):2798-2803.
17. Maniam R, Selvarajah GT, Mazlan M, Lung Than LT. Pulmonary papillary adenocarcinoma with *Aspergillus versicolor* infection in a dog. *Med Mycol Case Rep.* 2017;19:25-29.
18. Claeys S, Lefebvre JB, Schuller S, et al. Surgical treatment of canine nasal aspergillosis by rhinotomy combined with enilconazole infusion and oral itraconazole. *J Small Anim Pract.* 2006;47(6):320-324.
19. Fastrès A, Canonne MA, Taminiau B, et al. Analysis of the lung microbiota in dogs with *Bordetella bronchiseptica* infection and correlation with culture and quantitative polymerase chain reaction. *Vet Res.* 2020;51(1):46.
20. Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature.* 1989;339(6221):237-238. doi:10.1038/339237a0. Erratum in: *Nature* 1989 Jun 8;339(6224):490.
21. Piorkowski G, Baronti C, de Lamballerie X, et al. Development of generic Taqman PCR and RT-PCR assays for the detection of DNA and mRNA of β -Actin-encoding sequences in a wide range of animal species. *J Virol Methods.* 2014;202:101-105. doi:10.1016/j.jviromet.2014.02.026
22. Johnson LR, Drazenovich TL, Herrera MA, Wisner ER. Results of rhinoscopy alone or in conjunction with sinuscopy in dogs with aspergillosis: 46 cases (2001-2004). *J Am Vet Med Assoc.* 2006;228(5):738-742.
23. De Lorenzi D, Bonfanti U, Masserdotti C, et al. Diagnosis of canine nasal aspergillosis by cytological examination: a comparison of 4 different collection techniques. *J Small Anim Pract.* 2006;47(6):316-319.
24. Danesi P, Ravagnan S, Johnson LR, et al. Molecular diagnosis of *Pneumocystis pneumonia* in dogs. *Med Mycol.* 2017;55(8):828-842.
25. Wang J, Wang J, Li R, Shi R, Liu L, Yuan W. Evaluation of an incubation instrument-free reverse transcription recombinase polymerase amplification assay for rapid and point-of-need detection of canine distemper virus. *J Virol Methods.* 2018;260:56-61.
26. Hazuchova K, Neiger R, Stengel C. Topical treatment of mycotic rhinitis-rhinosinusitis in dogs with meticulous debridement and 1% clotrimazole cream: 64 cases (2007-2014). *J Am Vet Med Assoc.* 2017;250:309-315.
27. Van de Veerdonk FL, Gresnigt MS, Romani L, et al. *Aspergillus fumigatus* morphology and dynamic host interactions. *Nat Rev Microbiol.* 2017;15(11):661-674.
28. Ragab A, Clement P, Vincken W, Nolard N, Simones F. Fungal cultures of different parts of the upper and lower airways in chronic rhinosinusitis. *Rhinology.* 2006;44(1):19-25.
29. Gosepath J, Brieger J, Vlachtsis K, Mann WJ. Fungal DNA is present in tissue specimens of patients with chronic rhinosinusitis. *Am J Rhinol.* 2004;18(1):9-13.
30. Chatterjee A, Saha D, Niemann H, Gryshkov O, Glasmacher B, Hofmann N. Effects of cryopreservation on the epigenetic profile of cells. *Cryobiology.* 2017;74:1-7.

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