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What causes canine sino-nasal aspergillosis? A molecular approach to species identification

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ABSTRACT

On the basis of phenotypic identification methods, *Aspergillus fumigatus* is reported as the most commonly identified aetiological agent of canine sino-nasal aspergillosis (SNA). However, definitive identification of *Aspergillus* spp. using phenotypic features alone is unreliable. The aim of this study was to determine the molecular identities of fungal species causing SNA in dogs. Genomic DNA was extracted from 91 fungal isolates from 90 dogs diagnosed with SNA in Australia, the USA and Belgium, and the ITS1-5.8S-ITS2 ribosomal DNA and partial β-tubulin regions were sequenced. Eighty-eight of 91 (96.7%) isolates were identified as *A. fumigatus* and 3/91 (3.3%) belonged to *Aspergillus* section *Nigri* spp. (*Aspergillus tubingensis*: 2/91; *Aspergillus uvarum*: 1/91). These findings confirm that *A. fumigatus* is the most common aetiological agent of canine SNA. This is the first report to document a pathogenic role for *A. tubingensis* and *A. uvarum* in dogs.

Introduction

Sino-nasal aspergillosis (SNA) is an important cause of chronic nasal disease in dogs. This fungal disease is most commonly seen in young to middle-aged dolichocephalic and mesaticephalic breeds, resulting in significant morbidity (Sharp et al., 1991; Sharman et al., 2010; Burrow et al., 2012). On the basis of phenotypic identification methods, *Aspergillus fumigatus* is the most commonly identified aetiological agent of canine SNA (Pomrantz et al., 2007; Billen et al., 2009). Other *Aspergillus* spp., including *A. flavus*, *A. niger* and *A. nidulans*, are implicated occasionally, although prevalence data and methodology of identification is lacking (Mathews and Sharp, 2006).

Reliable identification of *Aspergillus* spp. requires molecular analysis in addition to phenotypic methods (Balajee et al., 2005a, 2007; Sugui et al., 2010). It is difficult to differentiate *Aspergillus* spp. on the basis of their morphological appearance alone, as distinguishing sexual and asexual characteristics are difficult to identify, sporulation can be slow and, for species with intact sexual cycles, ascospores may be difficult to induce in vitro (Balajee et al., 2007; Alcazar-Fuoli et al., 2008). Furthermore, some *Aspergillus* spp. isolates can display atypical culture morphology compared to the type strain. Molecular methods of identification enable reliable differentiation of morphologically similar species, especially within *Aspergillus* section *Fumigati* (Alstruey-Izquierdo et al., 2012).

Cryptic *Aspergillus* spp. are becoming increasingly important in human and veterinary medicine due to their association with invasive disease that is refractory to treatment (Alcazar-Fuoli et al., 2008; Sugui et al., 2010; Barrs et al., 2012). Resistance to antifungal azole drugs has been reported amongst human beings with aspergillosis and different *Aspergillus* section *Fumigati* spp. isolates have different antifungal susceptibility profiles (Alcazar-Fuoli et al., 2008). The recently discovered *Aspergillus felis* is an important

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cryptic *Aspergillus* spp. known to cause invasive disease that is commonly refractory to therapy in cats (Barrs et al., 2013). Its discovery reflects the importance of accurate fungal species identification for determining clinical treatment and prognosis.

For molecular identification of species complex (section), the internal transcribed spacer regions (ITS1-5.8S-ITS2 regions) are ideal targets because they are present as multiple copies ($\geqslant$100 copies per fungal genome), universal fungal primers are available and they contain highly variable regions allowing differentiation of many species. Since variation within ITS1-5.8S-ITS2 regions is not sufficient to allow identification of all *Aspergillus* section *Fumigati* spp., additional sequences, including partial β-tubulin, rodlet A and/or calmodulin sequences are necessary for definitive species identification (White et al., 1990; Glass and Donaldson, 1995; Hong et al., 2005; Balajee et al., 2007; Samson and Varga, 2009; Sugui et al., 2010).

Garcia et al. (2012) recommended molecular identification of *Aspergillus* spp. that had previously been identified by morphology alone to determine both the validity of the phenotypic identification and the importance of *A. fumigatus*-like spp. in veterinary medicine. The aim of the present study was to determine the molecular identification of fungal species causing canine SNA using isolates obtained from different geographical regions. It was hypothesised that *A. fumigatus* is the most common cause of canine SNA.

Materials and methods

Isolates

Ninety-one *Aspergillus* spp. fungal isolates from 90 dogs with previously diagnosed SNA submitted to one of three mycology reference laboratories, or to one of four university veterinary teaching hospitals or diagnostic laboratories, from 1988 to 2012 were included in this study. There were 53 isolates from the USA, 31 from Australia and seven from Belgium (Table 1). Morphological identification was determined by microbiologists at participating institutions on the basis of gross colony morphology and microscopic morphology of fungal cultures grown on malt extract agar (MEA) (Samson et al., 2010), Sabouraud's dextrose agar containing chloramphenicol (0.5 g/L) and actidione (0.5 g/L), or potato flakes agar. Cultures were examined for growth rate and colony colour. Cultures were examined microscopically to determine length of the conidiophores, size and shape of the vesicle, whether the conidial head was radiate or columnar, uniseriate or biseriate, and the size, colour and shape of the conidia. Signalment of 71/90 dogs from which isolates were collected was also available for review.

Table 1
Country of origin and isolation date of fungal pathogens in 91 isolates of canine sinonasal aspergillosis.

Year	Number of isolates	Number of isolates		
		Australia[a]	USA[b]	Belgium[c]
1988	1	1	0	0
1992	1	1	0	0
1994	2	2	0	0
2000	1	1	0	0
2002	1	0	1	0
2004	2	0	2	0
2005	6	1	5	0
2006	3	1	2	0
2007	9	3	6	0
2008	10	4	6	0
2009	13	4	9	0
2010	15	6	9	0
2011	9	1	8	0
2012	18	6[A]	5	7
Total	91	31	53	7

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[A] Two isolates were sampled from the same clinical case.

DNA isolation and PCR

Samples were sub-cultured onto MEA and incubated in the dark at 28 °C for 7 days. Fungal genomic DNA was extracted using the Roche High Pure PCR Template Preparation Kit with an additional bead beating step, as per the European *Aspergillus* PCR initiative (White et al., 2010). A conventional panfungal PCR was performed, targeting the rDNA gene cluster, including the ITS1, 5.8S gene and ITS2 regions using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The partial β-tubulin gene was amplified using primers bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass and Donaldson, 1995). For isolates where the molecular identification did not match the phenotypic identification, an additional PCR was performed targeting the partial calmodulin gene using degenerate primers cmd5 (5′-CCGAGTACAAGGARGCCTTC-3′) and cmd6 (5′-CCGATRGAGGTCATRACGTGG-3′) (Hong et al., 2005).

PCR products were purified using the same primers and the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), and sequenced at the Australian Genome Research Facility, Westmead, Australia. Sequences were edited using BioEdit Sequence Alignment Editor version 7.1.3.0 (Hall, 1999). Species identification was determined using BLAST (NCBI, 2012) and Centraalbureau voor Schimmelcultures (CBS-KNAW, 2012) databases. Pairwise and multiple sequence alignments were performed using the ClustalW algorithm option in BioEdit. Phylogenetic trees were constructed with Mega 5.05 (Tamura et al., 2011) using the maximum likelihood discrete data method (tree searching method of 1000 replicate trees) and bootstrapping to determine phylogenetic tree accuracy (Felsenstein, 1983).

Results

Phenotypic identification

Eighty-seven fungal isolates from cases of canine SNA in Australia, the USA and Belgium were identified as *Aspergillus* section *Fumigati* spp., one from Australia as *Aspergillus* section *Restricti* and three from Australia (2) and the USA (1) as *Aspergillus* section *Nigri* spp., on the basis of gross colony and microscopic morphology of fungal cultures (Table 1). Of the 87 *Aspergillus* section *Fumigati* isolates, 82 had typical *A. fumigatus* morphology and five had atypical *A. fumigatus* morphology, including one isolate from the USA identified provisionally as *A. felis* and one from Australia as *A. viridinutans*. Two isolates were cultured from the same dog in one case of SNA from Australia, one with typical *A. fumigatus* morphology and the other with atypical *A. fumigatus* morphology; on molecular analysis, both isolates were identified as *A. fumigatus*.

Molecular identification

Eighty-eight of 91 (96.7%) isolates were identified as *A. fumigatus* and had 100% identity with the GenBank type strains for *A. fumigatus* for both ITS and partial β-tubulin sequences. Two isolates from the USA were identified as *A. tubingensis* (*Aspergillus* section *Nigri*) and one from Australia as *A. uvarum* (*Aspergillus* section *Nigri*) (Table 2). Two isolates identified as *A. fumigatus* demonstrated a single-nucleotide polymorphism (SNP) at base pair position 12 (G/A) in the non-coding region of the partial β-tubulin gene sequence. Three isolates identified as *A. fumigatus* displayed a double peak (C/C, T) at base pair position 62 of the ITS sequence.

Maximum likelihood and bootstrapping analysis of the partial β-tubulin sequences demonstrated that all sequences identified as *A. fumigatus* were clustered with the GenBank type strain for *A. fumigatus* in 99% of replicate trees (see Appendix A: Supplementary Fig. 1). Both isolates identified as *A. tubingensis* were clustered with the GenBank type strain for *A. tubingensis* in 83% of replicate trees (see Appendix A: Supplementary Fig. 2). The isolate identified as *A. uvarum* was a sibling, being in the same clade and having the

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Table 2
Phenotypic and molecular identity of fungal pathogens in 91 isolates of canine sino-nasal aspergillosis.

Number of isolates	Phenotypic identification	Dates of isolation	ITS[a]	*ben*A[a]	*cal*M[a]
87	*Aspergillus* section *Fumigati*	1988–2012	*A. fumigatus* (100%) (KF314727; KF314726[b])	*A. fumigatus* (100%) (KF314730; KF314729[c])	*A. fumigatus* (100%) (KF476656)
1	*Aspergillus* section *Restricti*	1994	*A. fumigatus* (100%) (KF314727)	*A. fumigatus* (100%) (KF314730)	*A. fumigatus* (100%) (KF476656
2	*Aspergillus* section *Nigri*	2007, 2010	*A. tubingensis* (100%) (KF314725)	*A. tubingensis* (100%) (KF314728)	
1	*Aspergillus* section *Nigri*	2012	*A. uvarum*/*A. japonicus*/*A. aculeatus*/*A. violaceofuscus* (100%) (KF476654)	*A. uvarum* (100%) (KF476655)	

[a] Species (% nucleotide identity relative to the type strain) (GenBank accession numbers).
[b] GenBank accession numbers for two *A. fumigatus* isolates that demonstrated a single-nucleotide polymorphism at base pair position 12 in the non-coding region of the partial β-tubulin gene sequence.
[c] GenBank accession numbers for three A. *fumigatus* isolates that displayed a double peak at base pair position 62.

closest relationship with the GenBank type strain for *A. uvarum* in 95% of replicate trees (see Appendix A: Supplementary Fig. 2).

The molecular identification of four isolates with atypical morphological characteristics was further confirmed by an additional PCR targeting the calmodulin gene. This included one isolate phenotypically identified as *Aspergillus* section *Restricti* and three atypical *Aspergillus* section *Fumigati* isolates, including the two isolates provisionally identified as *A. viridinutans* and *A. felis*. All had 100% identity to the *A. fumigatus* type strain for partial β-tubulin, ITS and calmodulin sequences. Phylogenetic analysis of the calmodulin sequences confirmed the molecular identity of all four isolates as *A. fumigatus* in 99% of replicate trees (see Appendix A: Supplementary Fig. 3).

Signalment

For the 71 cases in which signalment details were available for review, the median age at diagnosis was 6 years (range 1–14 years). There were 55 (77%) male dogs (6 entire, 49 neutered) and 16 (23%) females (2 entire, 14 neutered). Male dogs were over-represented amongst all countries (Australia 25/30; USA 24/34; Belgium 6/7). Breeds commonly affected were Golden retriever (10/71, 14%), Miniature schnauzer (5/71, 7%) and Labrador retriever (4/71, 6%).

Discussion

The results of this study confirmed the hypothesis that *A. fumigatus* is the most common aetiological agent of canine SNA, with 97% of all isolates being identified by PCR and sequencing of the ITS and partial β-tubulin genes as *A. fumigatus*. The molecular identity of fungal pathogens causing canine SNA has been investigated in two smaller studies (Peeters et al., 2008; Barrs et al., 2012). In evaluating the molecular identity of isolates that cause feline upper respiratory tract aspergillosis, seven canine SNA samples were included for comparison (Barrs et al., 2012). Similar to the findings of the current study, all were identified as *A. fumigatus* by PCR and sequencing of the ITS1-5.8S-ITS2 region.

Peeters et al. (2008) used real time PCR to determine the presence of fungi in mucosal biopsies from 14 dogs with clinically confirmed SNA, comparing these with dogs with other nasal cavity diseases and dogs with no nasal cavity disease (control group). A genus-specific assay detected *Penicillium* or *Aspergillus* spp. in all 14 SNA samples, but when species-specific assays were performed for *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*, only 7/14 were positive for *A. fumigatus*. The authors hypothesised that the negative results with the species-specific assays were either due to insufficient *A. fumigatus* DNA in the samples, or because *Penicillium* spp. or other *Aspergillus* spp. were the aetiological agents.

The only other *Aspergillus* spp. identified as aetiological agents of SNA in this study were from *Aspergillus* section *Nigri*. Also known as the black aspergilli, they are clearly distinguishable from *Aspergillus* section *Fumigati* on the basis of gross colony characteristics. Previous reports of *A. niger* as a cause of canine SNA were based on phenotypic identification alone (Mathews and Sharp, 2006). In a study involving molecular identification of *Aspergillus* section *Nigri* isolates from 41 human mycoses, there were similar frequencies of *A. niger* (20 isolates) and *A. tubingensis* (19 isolates) causing disease (Hendrickx et al., 2012). In the present study, we identified *A. tubingensis* and *A. uvarum* as aetiological agents of canine SNA for the first time.

Aspergillus tubingensis, a member of the *A. niger* clade, has been identified as an aetiological agent of mycotic rhinosinusitis, invasive aspergillosis, otomycosis and ocular infection in human beings (Balajee et al., 2009; Szigeti et al., 2011; Hendrickx et al., 2012). It is difficult to distinguish *A. tubingensis* from other members of the *A. niger* clade phenotypically. *Aspergillus uvarum*, a member of the *A. aculeatus* clade in section *Nigri* has been identified as a contaminant of grapes in the Mediterranean basin (Perrone et al., 2008) and has also been isolated from indoor air samples in the USA (Jurjević et al., 2012). Prior to the current study, it has not been reported as a cause of disease in human beings or animals; however, *A. uvarum* has only recently been discovered and a thorough description of its niches and pathogenic potential is still pending. The dog from which the isolate was grown also had a growth of *Penicillium* spp. *Aspergillus* section *Nigri* spp. have been reported to have varying minimum inhibitory concentrations for antifungal drugs, reflecting the importance of accurate identification (Samson et al., 2007; Balajee et al., 2009; Varga et al., 2011; Hendrickx et al., 2012).

The findings of this study are similar to those described for cats with SNA, where *A. fumigatus* and *Aspergillus* section *Nigri* spp. are the most common agents of disease (Whitney et al., 2005; Furrow and Groman, 2009; Barrs et al., 2013). Other species of *Aspergillus* reported to cause SNA in cats include *A. lentulus*, *A. felis* and *A. thermomutatus* (*Neosartorya pseudofischeri*), which are all members of *Aspergillus* section *Fumigati* (Barrs et al., 2013).

Cats are also susceptible to a more invasive form of upper respiratory tract aspergillosis known as sino-orbital aspergillosis (SOA). The most common agents of SOA in cats are the recently described novel species *A. felis* and *A. udagawae* (Kano et al., 2008, 2013; Barrs et al., 2013). SNA is the predominant form of upper respiratory tract aspergillosis in dogs, with only one case report of SOA being described in a dog with a mixed infection of *A. fumigatus* and *Pseudallescheria boydii* based on phenotypic identification (Willis et al., 1999).

Molecular identification matched phenotypic identification in the majority of samples in this study, with only 3/91 isolates

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(3%) identified to species level incorrectly, although over a third of isolates were not identified to species level using phenotypic methods. This low result is similar to finding reported by Garcia et al. (2012), where 1/80 (1%) isolates identified phenotypically as *A. fumigatus* was found to be *A. lentulus* on molecular identification.

In the present study, isolates identified as *A. felis* (sampled in 2012), an atypical *Aspergillus* section *Fumigati* sp. (sampled in 2010), *A. viridinutans* (sampled in 1992) and *A. restrictus* (sampled in 1994) by microscopic and macroscopic methods, were identified molecularly as *A. fumigatus*, demonstrating that some *A. fumigatus* isolates can phenotypically imitate other *Aspergillus* subgenus species. Isolates that are slow to sporulate can be misidentified based on phenotypic features alone. Prolonged incubation (21 days) of the isolate identified as *A. felis* resulted in fungal colony change from white to green; *Aspergillus fumigatus* typically sporulates within 7 days and the conidia are green (Latge, 1999). The slow sporulation phenotype is common amongst cryptic (*A. fumigatus*-mimetic) species, such as *A. udagawae* and *A. lentulus* (Balajee et al., 2006); however, as demonstrated here, this feature is not reliable for species identification. Mutant melanin-deficient strains have also been described and these may affect accurate phenotypic identification (Heinekamp et al., 2013). This could explain the misidentification of the remaining three isolates that remained white after prolonged incubation (>3 months). Additionally, all four isolates were incubated at 50 °C, since *A. fumigatus* has a unique tolerance for growth at high temperatures (Chang et al., 2004). All four isolates demonstrated growth at 50 °C, as did an additional six isolates of *A. fumigatus* that were routinely tested for growth at 50 °C at one of the participating mycology reference laboratories. Given that *A. fumigatus* is the most common cause of canine SNA, a practical approach for identification of *Aspergillus* isolates from affected dogs in veterinary clinical laboratories would be to test for growth at 50 °C and apply molecular identification techniques for isolates that fail this test. However, it should be noted that occasionally non-thermotolerant isolates of *A. fumigatus* can be encountered.

Previous studies have suggested a link between fungal gene sequence polymorphisms and fungal adaptation, including drug resistance and phylogenetic relationships. Such diversity has been associated with protein-encoding regions of genes, such as the partial β-tubulin and cyp51A genes (Mellado et al., 2001; Balajee et al., 2005b). As the double peaks seen in this study were in non-coding regions and the sequences had a close phylogenetic relationship with the *A. fumigatus* type strain, they are likely to be non-significant.

The signalment of dogs in this study is consistent with previous reports of canine SNA (Sharman et al., 2010). The breeds most commonly affected were mesaticephalic and dolichocephalic breeds and the age at diagnosis is consistent with reports in the literature of young to middle-aged dogs being most commonly affected (Sharp et al., 1991; Sharman et al., 2010). The male predisposition in this study supports previous reports where a male to female ratio of 1.9:1 was found in a total of 377 reported cases (Sharp et al., 1991; Zonderland et al., 2002; Johnson et al., 2006; Sharman et al., 2010).

Conclusions

A. fumigatus is the most common aetiological agent of canine SNA. This is the first report to document a pathogenic role for *A. tubingensis* and *A. uvarum* in dogs. Molecular identification should routinely be partnered with phenotypic identification methods for accurate diagnosis of aetiological agents of fungal disease in companion animals.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tvjl.2014.01.009.

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