Accepted Manuscript

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PII: S1090-0233(14)00516-4
DOI: http://dx.doi.org/doi: 10.1016/j.tvjl.2014.12.020
Reference: YTVJL 4368

To appear in: *The Veterinary Journal*

Accepted date: 21-12-2014

Please cite this article as: V.R. Barrs, B. Ujvari, N.K. Dhand, I.R. Peters, J. Talbot, L.R. Johnson, F. Billen, P. Martin, J.A. Beatty, K. Belov, Detection of *Aspergillus*-specific antibodies by agar gel double immunodiffusion and IgG ELISA in feline upper respiratory tract aspergillosis, *The Veterinary Journal* (2014), http://dx.doi.org/doi: 10.1016/j.tvjl.2014.12.020.

Detection of *Aspergillus*-specific antibodies by agar gel double immunodiffusion and IgG ELISA in feline upper respiratory tract aspergillosis

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Highlights

- Feline antibodies against cryptic *Aspergillus* spp. cross react with an aspergillin containing *A. fumigatus* antigens.
- Brachycephalic cats are prone to upper respiratory tract aspergillosis (URTA).
- The agar gel immunodiffusion (AGID) assay has low sensitivity for diagnosis of URTA.
- IgG ELISA has high sensitivity and specificity for diagnosis of URTA.
- This study provides evidence that cats with URTA are systemically immunocompetent.

Abstract

Feline upper respiratory tract aspergillosis (URTA) is an emerging infectious disease. The aims of this study were: (1) to assess the diagnostic value of detection of *Aspergillus*-specific antibodies using an agar gel double immunodiffusion (AGID) assay and an indirect immunoglobulin G (IgG) ELISA; and (2) to determine if an aspergillin derived from mycelia of *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus flavus* can be used to detect serum antibodies against 'cryptic' *Aspergillus* spp. in *Aspergillus* section *Fumigati*. Sera from cats with URTA (group 1: n = 21) and two control groups (group 2: cats with other upper respiratory tract diseases, n = 25; group 3: healthy cats and cats with non-respiratory, non-fungal illness, n = 84) were tested. Isolates from cats with URTA comprised *A. fumigatus* (n = 5), *A. flavus* (n = 1) and four cryptic species: *Aspergillus felis* (n = 12), *Aspergillus thermomutatus* (*Neosartorya pseudofischeri*, n = 1), *Aspergillus lentulus* (n = 1) and *Aspergillus udagawae* (n = 1).

Brachycephalic purebred cats were significantly more likely to develop URTA than other breeds (P <0.013). The sensitivity (Se) of the AGID was 43% and the specificity (Sp) was 100%. At a cut-off value of 6 ELISA units/mL, the Se of the IgG ELISA was 95.2% and the Sp was 92% and 92.9% for groups 2 and 3 cats, respectively. *Aspergillus*-specific antibodies against all four cryptic species were detected in one or both assays. Assay Se was not associated with species identity. Detection of *Aspergillus*-specific antibodies by IgG ELISA has high Se and Sp for diagnosis of feline URTA.

Keywords: Aspergillosis; *Aspergillus* spp.; Sino-nasal; Sino-orbital; Feline

Introduction

Feline upper respiratory tract aspergillosis (URTA) is increasingly being recognised (Barrs and Talbot, 2014). There are two anatomical forms of disease, sino-nasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA) (Barrs et al., 2012, 2014). A strong association has been identified between the infecting fungal species and the anatomical form of disease; SNA is most commonly caused by *Aspergillus fumigatus*, while *Aspergillus felis*, a recently discovered 'cryptic' species in *Aspergillus* section *Fumigati* (*Aspergillus viridinutans* complex), is the most common cause of SOA (Barrs et al., 2013, 2014; Barrs and Talbot, 2014). So-called cryptic species are indistinguishable on morphological features from *A. fumigatus sensu stricto*.

Similar to SNA in dogs, feline SNA is usually non-invasive, such that fungal hyphae do not penetrate the respiratory mucosa (Whitney et al., 2005); in contrast, in SOA fungal hyphae invade sino-nasal and paranasal tissues. Invasive mycoses typically occur in immunocompromised hosts. However, systemic immunodeficiency has not been detected in most cats with URTA (Barrs et al., 2012), one exception being a cat with feline leukaemia virus (FeLV) infection (Goodall et al., 1984).

The sensitivity (Se) of serological tests for detection of fungal antigens or *Aspergillus*-specific antibodies in aspergillosis depends on the systemic immunocompetence of the host as reflected by the ability to clear fungal antigen from the circulation and to mount an antibody response. An ELISA to detect a fungal cell wall antigen, galactomannan (GM), in serum (Platelia *Aspergillus* EIA, Bio-Rad) has a Se of up to 90% in immunocompromised patients, including neutropenic human patients with pulmonary aspergillosis and dogs with disseminated invasive aspergillosis (DIA) (Maertens et al., 1999; Garcia et al., 2012).

However, the Se of this test is <30% in non-neutropenic human patients with aspergillosis, in immunocompetent dogs with SNA and in cats with URTA (Billen et al., 2009; Kitasato et al., 2009; Whitney et al., 2013).

Conversely, detection of serum *Aspergillus*-specific antibodies by agar gel double immunodiffusion (AGID) or by immunoglobulin G (IgG) ELISA has a high test Se in immunocompetent patients, including dogs with SNA (67-88%) and humans with chronic pulmonary aspergillosis (74-94%) (Pomrantz et al., 2007; Billen et al., 2009; Guitard et al., 2012; Ohba et al., 2012). A detectable antibody response is mounted in <30% of neutropenic humans with aspergillosis and dogs with DIA (Day et al., 1985; Hope et al., 2005; Schultz et al., 2008).

We hypothesised that *Aspergillus*-specific antibodies would be detectable in the majority of cats with URTA, since most cats with URTA are not, as far as it is possible to currently evaluate, systemically immunocompromised. The aims of this study were: (1) to assess the diagnostic value of detection of *Aspergillus*-specific antibodies using an AGID assay and an indirect IgG ELISA; and (2) determine if a commercial aspergillin derived from mycelia of *A. fumigatus, Aspergillus niger* and *Aspergillus flavus* can be used to detect serum antibodies against cryptic *Aspergillus* spp. in *Aspergillus* section *Fumigati*.

Materials and methods

Signalment data and serum (1-2 mL per cat) were collected prospectively from cats diagnosed with URTA (group 1), cats with upper respiratory tract (URT) signs not attributable to aspergillosis (group 2) and from cats without respiratory or fungal disease (group 3). Samples were collected with informed consent according to the guidelines of the

Animal Ethics Committee of the University of Sydney (approval number N00/9-2012/5774, date of approval 22 June 2012). Serum samples were collected at the time of diagnosis and were stored at -80 °C for batch testing.

Animals

Group 1: Cats with upper respiratory tract aspergillosis (n = 21) - Inclusion criteria for cats with URTA were a complete medical history, consistent clinical signs, identification of fungal hyphae on cytology and/or histopathology of tissue from the sino-nasal cavity or orbit, and a positive fungal culture (Barrs et al., 2012). Cases with mixed fungal infections were excluded. Isolates were identified using phenotypic features and comparative sequence analyses of the internal transcribed spacer (ITS) regions (ITS1-5.8S-ITS2), partial β-tubulin and/or partial calmodulin genes (Barrs et al., 2013), except for *A. fumigatus* identification, where consistent phenotypic features and demonstration of growth at 50 °C was an acceptable alternative to molecular identification (Barrs and Talbot, 2014). Isolates comprised *A. fumigatus* (*n* = 5), *A. flavus* (*n* = 1) and four cryptic species in *Aspergillus* section *Fumigati*, i.e. *A. felis* (*n* = 12), *Aspergillus thermomutatus* (syn. *Neosartorya pseudofischeri*, *n* = 1), *Aspergillus lentulus* (*n* = 1) and *Aspergillus udagawae* (*n* = 1) (Table 1).

Cats were classified as having SOA (*n* = 12) or SNA (*n* = 9) based on the presence (SOA) or absence (SNA) of a retrobulbar mass on computed tomography (CT) or magnetic resonance imaging (MRI) at diagnosis. Sera were tested for antibodies against feline immunodeficiency virus (FIV) and FeLV antigen (IDEXX SNAP Combo, IDEXX Laboratories). Medical histories were analysed for the presence of co-morbidities. All cats were neutered, comprising 11 male neutered (MN) and 10 female neutered (FN) cats, and the median age was 5 years (range 2-14 years). Breeds comprised domestic crossbred (*n* = 8),

Persian (*n* = 4), Ragdoll (*n* = 3), Himalayan (*n* = 2), British shorthair (*n* = 1), Scottish shorthair (*n* = 1), Cornish Rex (*n* = 1) and Abyssinian (*n* = 1).

Group 2: Control cats with other URT disease (n = 25) - Inclusion criteria were: (1) consistent clinical signs, e.g. sneezing, nasal discharge; (2) absence of fungal hyphae on cytology or histology of tissue collected from the sino-nasal cavity; and/or (3) serological, histopathological or endoscopic diagnosis of another URT disease. Standard diagnostic investigations included latex antigen cryptococcal serology (CALAS, Meridian Bioscience), upper airway endoscopy, CT examination of the sino-nasal cavity, fungal culture and biopsy. This group included cats with chronic rhinosinusitis (*n* = 9), nasal neoplasia (*n* = 10) (lymphoma, *n* = 4; adenocarcinoma, *n* = 3; squamous cell carcinoma, *n* = 2; osteosarcoma, *n* = 1), upper respiratory cryptococcosis (*n* = 5) and nasopharyngeal stenosis (*n* = 1). All cats were neutered (13 MN, 12 FN). The median age was 11 years (range 4-16 years). Breeds comprised domestic crossbred (*n* = 14), Persian (*n* = 2), Siamese/Oriental (*n* = 2), Russian blue (*n* = 2), Cornish Rex (*n* = 2), British shorthair (*n* = 1), Burmilla (*n* = 1) and Tonkinese (*n* = 1).

Group 3 (i and ii): Control cats without respiratory or fungal disease (n = 84) - Inclusion criteria for group 3 (i) were healthy cats presenting to the Valentine Charlton Cat Centre (VCCC) for neutering, vaccination or wellness examination and for group 3 (ii) were sick cats presenting to the VCCC for non-fungal, non-respiratory illness. Exclusion criteria for groups 3 (i) and 3 (ii) were any clinical signs within the last 4 weeks or findings at physical examination suggestive of respiratory disease. This group comprised (i) 36 healthy cats, including five male entire (M), 11 MN, six female entire (F) and 14 FN, and (ii) 48 cats presented for non-fungal, non-respiratory illness (one M, 23 MN, 24 FN). Diagnoses in cats

with non-respiratory disease included hyperthyroidism or post radio-iodine treatment of hyperthyroidism recheck (*n* = 12), enteropathy e.g. enteritis, intestinal foreign body (*n* = 11), chronic kidney disease (*n* = 8), allergic skin disease (*n* = 4), central nervous system disease (*n* = 3), diabetes mellitus (*n* = 2), pancreatitis (*n* = 2), cholelithiasis (*n* = 1), chyloabdomen (*n* = 1), anaemia (*n* = 1), dog bite wound (*n* = 1), portosystemic shunt (*n* = 1) and idiopathic hypocalcaemia (*n* = 1).

Overall, there were 39 males (six M, 33 MN) and 45 females (six F, 39 FN) in group 3; the median age was 8 years (range 0.7-19.5 years). The median age of group 3 (i) cats was 3 years (range 0.7-12 years) and the median age of group 3 (ii) cats was 12 years (range 1-19.5 years). Breeds comprised domestic crossbred (*n* = 67), Burmese (*n* = 3), Ragdoll (*n* = 3), Devon Rex (*n* = 2) and one each of Cornish Rex, Abyssinian, Siamese, Birman, Bengal, Persian, Russian blue, British shorthair and Singapura.

Agar gel double immunodiffusion

Detection of precipitating anti-*Aspergillus* antibodies by AGID (Ouchterlony method) was performed using a commercially available test-kit (Fungal Immunodiffusion Kit, Meridian Bioscience) comprising agar immunodiffusion plates, an aspergillin derived from the mycelial phase of cultures of *A. fumigatus, A. niger* and *A. flavus* with a protein content of 1486 µg/mL (Aspergillus Immunodiffusion Antigen reference number 100501, Meridian Bioscience) and goat anti-*Aspergillus* immunodiffusion control serum (reference number 100901, Meridian Bioscience). Testing was performed in accordance with the manufacturer's instructions using 20 µL each of control sera, test serum and aspergillin. All samples were tested in duplicate. Gels were examined for the presence of precipitin bands of identity or partial identity after 24 h and again after an additional 48 h incubation (final reading) in a

humidified chamber at room temperature. Visualisation of precipitin bands was facilitated by directing a high intensity light beam at a 45° angle below the plate, with the latter held against a black background.

Aspergillus-specific IgG quantification by indirect ELISA

An indirect ELISA for detection and quantification of *Aspergillus*-specific IgG antibodies in canine sera using the same aspergillin as for the AGID was modified for use in cats (Billen et al., 2009). Binding activity using polyvinylchloride or polystyrene 96 well plates was assessed as similar. Two commercially available secondary antibodies, rabbit anti-cat IgG (H&L), ALP conjugated, were evaluated (SAB 37008-1, Sigma; AS10 1479, Agrisera).

The assay was optimised by performing checkerboard titrations to determine the optimal dilutions of antigen, cat serum and secondary antibody. Inter- and intra-plate coefficients of variation were calculated by running 40 repeats of the positive control sample (pooled positive control sera from seven cats with confirmed aspergillosis and a positive AGID result) on four separate plates with 10 repeats on each plate. Test samples were run in duplicate and each plate contained a duplicate positive control, a negative control (pooled negative control sera from 15 healthy controls with a negative AGID result), and a blank (phosphate buffered saline, PBS, plus 0.05% Tween 20, Sigma; PBS-T). Sera were titrated in doubling dilutions from 1:800 to 1:102,400.

Ninety-six well enzyme immunoassay (EIA)/radioimmunoassay (RIA) polystyrene plates (Costar 3590, Corning) were coated with 75 µL aspergillin (2.5 µg protein/mL) and incubated at 4 °C overnight. Plates were blocked with 75 µL 1% w/v polyvinylpyrrolidone

(Sigma) in PBS for 1 h at room temperature. Fifty microlitres of patient sera was diluted in 5% non-fat milk in PBS-T, titrated on plates in doubling dilutions from 1:800 to 1:102,400 and incubated for 2 h at 37 °C. Fifty microlitres of 1:8000 rabbit anti-cat IgG (H&L), ALP conjugated, antibody solution (SAB 37008-1, Sigma) diluted in PBS-T was added to each well and incubated for 1 h at 37 °C. Next, 200 µL Sigma*FAST* p-nitrophenyl alkaline phosphate substrate (Sigma) was added to each well, incubated in the dark for 45 min then stopped with 50 µL of 3 M NaOH. Optical density (OD) was determined using a plate reader with a 405 nm and 492 nm wavelength filter (Benchmark Plus microplate spectrophotometer, Bio-Rad Laboratories). All incubations were performed in a humidified chamber and wells were washed three times between incubations with 150 µL PBS.

Statistical analysis

The mean age of cats was compared between groups using a general linear model. Sex proportions were compared using a χ^2 test. For the purpose of statistical analyses, breeds were grouped into brachycephalic (Persian/Persian-cross, Himalayan, Ragdoll, Birman, Burmilla, British/Scottish shorthair) and non-brachycephalic (Domestic short/longhair, Cornish/Devon Rex, Bengal, Russian blue, Oriental, Siamese, Tonkinese, Singapura). Proportions of cats in brachycephalic and non-brachycephalic groups, and proportions of positive test results for cats infected with *A. fumigatus* versus cryptic species were compared using Fisher's exact test.

Values for median ELISA units (EU) in group 1 were compared between AGID positive and AGID negative cats, and between cats with *A. fumigatus* infections and those infected with cryptic species, using non-parametric Mann and Whitney *U* tests. For analysis of ELISA data, the geometric mean optical OD for each set of duplicate serum samples was

calculated and $\log_{10}$ OD values were plotted against $\log_{10}$ serum dilutions for positive control and test sera in Microsoft Excel. The curves generated were compared for parallelism and IgG concentrations were expressed as EU/mL, with the positive control serum standard having a concentration of 100 EU/mL (Billen et al., 2009). Serum samples with fewer than three dilution points within the linear range of the standard, and thus considered to have antibody concentrations below the detectable limit of the ELISA (<2.5 EU/mL), were assigned a value of 0 EU/mL. An association between age and IgG quantification in EU/mL in controls was investigated using simple linear regression.

Cut-off values were established by determination of the mean plus three SD of the IgG concentration of the controls and by receiver operating characteristic (ROC) analysis. ROC analysis was conducted by fitting a logistic regression model of log EU values on the binary outcome (1 or 0) created by specifying the URTA group as 1 and the control group as 0 (Dohoo et al., 2009). ROC analyses were conducted for group 2, group 3 and both groups combined. The optimal cut-off value for each analysis was determined using Youden's J index. Se and specificity (Sp) at the determined cut-off values were reported as described by de Silva et al. (2013). Analyses were conducted using SAS 2002-2003 (SAS Institute/IBM). A 5% level of significance was used for all statistical tests.

Results

Cats

In group 1, one cat (cat 5) was determined to be FIV-infected on the basis of a positive FIV antibody response and no history of FIV vaccination; the other 20 cats in group 1 tested negative for FIV and FeLV (Table 1). The mean age of cats in group 1 (6.3 years) was significantly different from that of cats in group 2 (9.8 years; P <0.01) and group 3 (i) (4.0

252 years, P <0.01), but not from the combined group 3 (i and ii: 8.2 years, P = 0.1) or a
253 combined control group (groups 2 + 3: 8.7 years; P = 0.06). There were no significant
254 differences in sex between groups (P = 0.9). The proportions of brachycephalic breeds were
255 significantly different between groups 1 and 2, and between groups 1 and 3, but not between
256 groups 2 and 3 (P = 0.2); 11/21 (52%) group 1 cats were brachycephalic compared to 4/25
257 (16%) group 2 cats (P <0.05) and 4/84 (5%) group 3 cats (P < 0.01).
258
259 *Agar gel double immunodiffusion*
260 Nine of 21 sera (43%) from cats with URTA (group 1) were positive in the AGID
261 (Table 1). Sera from all 25 cats in group 2 and 84 cats in group 3 tested negative. The Se, Sp,
262 positive predictive value (PPV) and negative predictive value (NPV) of AGID for the
263 diagnosis of URTA are given in Table 2. Of the nine cats with positive AGID results, one was
264 infected with *A. fumigatus* and eight were infected with cryptic species, including *A. felis* (n =
265 7) and *A. udagawae* (n = 1) (Table 1). There was no association between test result (positive
266 or negative) and infecting species (*A. fumigatus* versus cryptic species; P = 0.3).
267
268 *IgG ELISA*
269 The intra- and inter-plate coefficients of variation of the ELISA were 7.4% and 9.3%,
270 respectively. Sera from cats that did not generate a dilution curve with a minimum of three
271 dilution points within the range of the standard serum were assigned an *Aspergillus*-specific
272 IgG concentration of 0 EU/mL (20/25 group 2 cats and 74/84 group 3 cats). Cut-off values
273 calculated using the mean plus three SD of the IgG concentration and using ROC analysis
274 were similar, yielding results of 5.6 and 6.0 EU/mL, respectively, regardless of the control
275 group used. Se and Sp were optimal at a cut-off value of 6 EU/mL (Table 3); at this cut-off

value, the Se was 95.2%, the Se was 92.0%, the PPV was 90.9% (95% confidence interval, CI, 70.8-98.6%) and the NPV was 95.5% (95% CI 78.8-99.3%).

Using the calculated cut-off value of 6.0 EU/mL, a positive IgG ELISA result was obtained for sera from 20/21 (95.2%) cats with URTA (range 6.3-797.9 EU/mL) (Table 1), from 2/25 (8.0%) cats, both with cryptococcal rhinitis, in group 2 (8.7 and 80.7 EU/mL) and 6/84 (7.1%) cats in group 3 (7.3-8.9 EU/mL) (Fig. 1). The median *Aspergillus*-specific IgG concentration in cats with URTA (group 1) was 55.7 EU/mL. Among cats with URTA, there was no significant difference in *Aspergillus*-specific IgG concentrations in cases with *A. fumigatus* infection (median 67 EU/mL; $n = 5$) and cases with infection by cryptic species (other members of the *A. fumigatus* complex; median 56.6 EU/mL; $n = 15$; $P = 0.1$). There was no association between test result (positive or negative) and infecting species (*A. fumigatus* versus cryptic species; $P = 0.3$). The median *Aspergillus*-specific IgG concentrations of cats with positive AGID results was 78.3 EU/mL, compared to 31.95 EU/mL for cats with negative AGID results ($P = 0.2$). There was no significant effect of age on EU values of combined groups 2 and 3 ($P = 0.05$) or group 3 alone ($P = 0.2$).

Discussion

In this study, we demonstrated that antibodies against four cryptic species of *Aspergillus* (*A. felis, A. udagawae, A. lentulus* and *A. thermomutatus*) can be detected in feline serum with assays utilising a commercial aspergillin derived from *A. fumigatus, A. niger* and *A. flavus*. Although this result was not unexpected given the close phylogenetic relationship of these cryptic species to *A. fumigatus* (Barrs et al., 2013; Novakova et al., 2014), it is important to demonstrate this cross reactivity, given the high frequency of infections with

such cryptic species in cats. *A. felis* and *A. udagawae* are the two most commonly reported species of *Aspergillus* to cause SOA in cats (Kano et al., 2008, 2013; Barrs et al., 2013, 2014).

There was a marked difference in the Se of the AGID and the IgG ELISA for detection of *Aspergillus*-specific antibodies, while the Sp for both assays was high. In contrast to the IgG ELISA, which detects one class of antibody, immunodiffusion assays detect precipitins (Crowle, 1973). In AGID assays, optimal diffusion depends on many factors including sufficiently large antigen (Ag) and antibody (Ab) reservoirs to maintain infinite pools of reactants (Kunkel, 1988). Since a commercial test kit was utilised in the present study, optimisation of the assay was not performed. The same commercial AGID has been evaluated for diagnosis of canine SNA, which is caused by *A. fumigatus* in >95% of cases, with reported Se of 57-67% (Pomrantz et al., 2007; Pomrantz and Johnson, 2010; Barrs and Talbot, 2014). Another commercial AGID (Immuno-Mycologics) had a Se of 31% for diagnosis of canine SNA (Peeters and Clercx, 2007). The highest reported Se of 76.5% using an AGID for diagnosis of canine SNA utilised a customised Ouchterlony method (Billen et al., 2009).

The IgG ELISA had high Se and Sp overall, indicating that the production of *Aspergillus*-specific IgG is a reliable indicator of URTA. Depending on the cut-off value and control group used, the Se of the assay was 91-100% and the Sp was 92-100%. Cases with URT diseases other than aspergillosis (group 2) represent the most relevant control group in a clinical situation. Of interest, both cats with false positive IgG results in group 2 had cryptococcosis. The high antibody titre detected in one cat with sino-orbital cryptococcosis (80.7 EU/mL) was repeatable. The cat had a latex cryptococcal antigen titre of 1024 (Meridian, CALAS 2010) and *Cryptococcus gattii* was cultured from the nasal cavity. On CT

there was a retrobulbar mass arising from the nasal cavity, but yeasts were not seen on cytology of fine-needle aspirate biopsies. The cat was treated with itraconazole for one year until the LCAT decreased to zero and clinical signs resolved. Possible explanations for the high antibody titre are a false positive disease result or a true result due to co-infection with *Aspergillus* spp., which is possible, but unlikely. Concurrent pulmonary cryptococcosis and aspergillosis has been documented rarely in humans (Lin et al., 2006; Enoki et al., 2012).

The high frequency of *Aspergillus*-specific IgG and the low frequency of retroviral infection by serology in group 1 cats (0% for FeLV; 4.7% for FIV) provides further evidence that cats with URTA are not systemically immunocompromised (Whitney et al., 2013). Other causes of immunosuppression documented in cats with DIA, such as feline panleukopenia, feline infectious peritonitis or prolonged corticosteroid therapy (Ossent, 1987), were not evident amongst cats with URTA tested in the present study. However, local disease that may have predisposed to sino-nasal cavity fungal colonisation was identified in two cats; one cat with *A. fumigatus* infection had concurrent nasal adenocarcinoma (cat 17) and one cat with *A. flavus* infection had plant material removed from the nasal cavity during endoscopy (cat 20) (Table 1). To further our understanding of the immunopathogenesis of this disease, additional studies of the humoral response to URTA, including quantification of IgM and IgA in affected cats, are warranted.

Our finding that pure bred cats of brachycephalic conformation were significantly more likely to develop URTA confirms a predisposition which, until now, has only been suspected (Tomsa et al., 2003; Whitney et al., 2005; Barrs et al., 2012). Impaired sinus aeration and drainage associated with brachycephalic skull conformation that favours fungal colonisation has been proposed as a mechanism for this breed association (Tomsa et al.,

2003). A heritable disorder of innate immunity has also been proposed (Barrs and Talbot, 2014). Chronic invasive granulomatous fungal rhinosinusitis of humans, similar to feline SOA, occurs in immunocompetent people in the Indian subcontinent, especially those working in agriculture and construction (Thompson and Patterson, 2012). In contrast to feline SOA, the aetiological agent is usually *A. flavus*. *A. flavus* is an uncommon cause of URTA in cats and only a single case has been identified previously (Malik et al., 2004).

Using a cut-off value of 5 EU/mL to optimise the IgG ELISA for Se makes this assay an ideal screening test for URTA in cats with consistent clinical signs; positive results should be corroborated with additional tests, such as fungal culture. Assay Sp was not 100% even at the cut-off value optimised for Sp (9 EU/mL) in group 2 cats, the most clinically relevant control group. Therefore, serology should not be relied upon as the sole diagnostic test for URTA.

Conclusions

Detection of *Aspergillus*-specific IgG by AGID and ELISA was highly specific for the diagnosis of aspergillosis in cats. The Se of IgG detection by ELISA was high, whereas the Se of detection using AGID was low. Depending on the cut-off value used, the ELISA has good discriminatory power to distinguish between presumed environmental exposure, which increases with age, and that induced by colonisation and infection. This study provides further evidence that feline URTA affects systemically immunocompetent individuals.

Conflict of interest statement

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This study was funded by an Australian Companion Animal Health Foundation grant (015/2013). The sponsors were not involved in any aspect of the study or in the decision to publish this manuscript. The authors thank many colleagues for contributions of clinical samples for this study.

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515 **Figure legend**

516

517 Fig. 1. ELISA units/mL for 21 group 1 sera (cats with aspergillosis; black circles), 25 group 2
518 sera (control cats with other upper respiratory tract disease; black stars) and 84 group 3 sera
519 (control cats, either healthy or sick with non-respiratory disease; grey circles). Lines represent
520 means ± standard deviations.

521

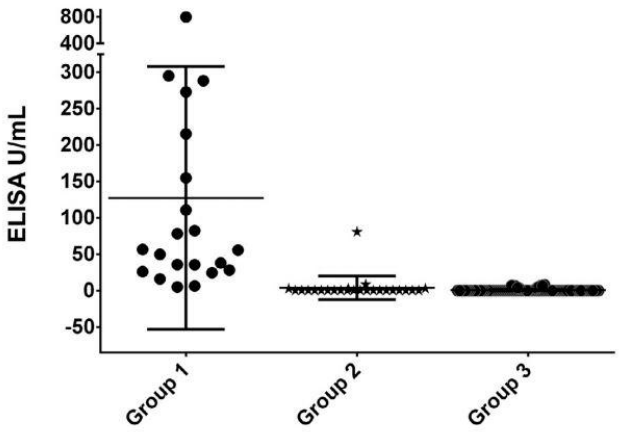

Table 1

Fungal species and serology results for group 1 cats with upper respiratory tract aspergillosis.

Cat [a]	Age (years)	Sex	Breed	Form	Fungal species [b]	AGID	ELISA (units/mL)
1	2	MN	Ragdoll	SNA	*A. thermomutatus* *(N. pseudofischeri)*	-	35.7
2	2	FN	DSH	SOA	*A. felis*	+	273.1
3	5	FN	Cornish Rex	SOA	*A. felis*	-	797.9
4	13	MN	DSH	SNA	*A. felis*	-	5
5	14	FN	Persian cross	SNA	*A. lentulus*	-	38
6	3	MN	DSH	SOA	*A. felis*	-	26
7	8	FN	Persian	SOA	*A. felis*	+	215.5
8	2	MN	British shorthair	SOA	*A. felis*	+	110.7
9	7	MN	Persian	SNA	*A. fumigatus*	-	28.2
10	2	MN	Himalayan	SOA	*A. felis*	+	35.8
11	8	MN	DLH	SOA	*A. udagawae*	+	55.7
12	8	FN	Scottish shorthair	SNA	*A. fumigatus*	+	56.6
13	5	FN	DSH	SOA	*A. felis*	+	154.9
14	4	MN	Ragdoll	SOA	*A. felis*	+	49.7
15	3	FN	Himalayan	SOA	*A. felis*	+	78.3
16	2	FN	DSH	SOA	*A. felis*	-	295.1
17	14	FN	Abyssinian	SNA	*A. fumigatus*	-	16.1
18	3	MN	Ragdoll	SOA	*A. felis*	-	288.42
19	14	FN	Persian	SNA	*A. fumigatus*	-	24.6
20	4	MN	DSH	SNA	*A. flavus*	-	6.3
21	7	MN	DSH	SNA	*A. fumigatus*	-	82.4

AGID, agar gel immunodiffusion; DSH, domestic shorthair; DLH, domestic longhair; FN, female neutered; MN, male neutered; SNA, sino-nasal aspergillosis; SOA, sino-orbital aspergillosis.

[a] *Aspergillus* spp.; *A. thermomutatus* syn. *Neosartorya pseudofischeri*; country of origin was Australia except cats 17 (USA), 19 (UK) and 21 (Belgium).

[b] Signalment of cat and molecular identity of isolates for cats 1-15 has been reported elsewhere (Barrs et al., 2013, 2014).

Table 2

Diagnostic accuracy of agar-gel double immunodiffusion in 21 cats with sino-nasal and sino-orbital aspergillosis.

	Control group 2 [a] (*n* = 25)		Control group 3 [b] (*n* = 84)		Control groups 2 and 3 (*n* = 109)	
	%	95% CI	%	95% CI	%	95% CI
Se	42.9	21.9-66.0	42.9	21.9-66.0	42.9	21.9-66.0
Sp	100.0	86.2-100.0	100.0	95.7-100.0	100.0	96.6-100.0
PPV	100.0	66.2-100.0	100.0	66.2-100.0	100.0	66.2-100.0
NPV	87.5	50.2-100.0	97.7	79.2-93.4	90.1	83.3-94.8

CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

[a] Cats with other upper respiratory tract diseases (excluding aspergillosis).

[b] Healthy controls (*n* = 36) and sick cats (*n* = 48) with non-fungal, non-respiratory illness.

Table 3

Performance of IgG ELISA for detection of *Aspergillus*-specific antibodies at different cut-off values.

			Cut-off value (EU/mL)					
			5		6		9	
Controls	AUC	95% CI for AUC	Se	Sp	Se	Sp	Se	Sp
Group 2	0.97	0.92-1.00	100.0% (21/21)	92.0% (23/25)	95.2% (20/21)	92.0% (23/25)	90.5% (19/21)	96.0% (24/25)
Group 3	0.97	0.98-1.00	100.0% (21/21)	91.7% (77/84)	95.2% (20/21)	92.9% (78/84)	90.5% (19/21)	100.0% (84/84)
Group 2 and 3	0.99	0.97-1.00	100.0% (21/21)	91.7% (100/109)	95.2% (20/21)	92.7% (101/109)	90.5% (19/21)	99.1% (108/109)

CI, confidence interval; AUC, area under curve; SE, sensitivity; SP, specificity.

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