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Original Article

Evaluation of a genus-specific ELISA and a commercial *Aspergillus* Western blot IgG[®] immunoblot kit for the diagnosis of aspergillosis in common bottlenose dolphins (*Tursiops truncatus*)

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Abstract

Aspergillosis is a fungal infection with high mortality and morbidity rates. As in humans, its definitive diagnosis is difficult in animals, and thus new laboratory tools are required to overcome the diagnostic limitations due to low specificity and lack of standardization. In this study of common bottlenose dolphins (*Tursiops truncatus*), we evaluated the diagnostic performance of a new commercial immunoblot kit that had been initially developed for the serologic diagnosis of chronic aspergillosis in humans. Using this in a quantitative approach, we first established its positive cutoff within an observation cohort of 32 serum samples from dolphins with “proven” or “probable” diagnosis of aspergillosis and 55 negative controls. A novel enzyme-linked immunosorbent assay (ELISA) test was also developed for detecting anti-*Aspergillus* antibodies, and results were compared between the two assays. Overall, the diagnostic performance of immunoblot and ELISA were strongly correlated ($P < .0001$). The former showed lower sensitivity (65.6% versus

90.6%), but higher specificity (92.7% vs. 69.1%), with no cross-reaction with other fungal infections caused by miscellaneous non-*Aspergillus* genera. When assessing their use in a validation cohort, the immunoblot kit and the ELISA enabled positive diagnosis before mycological cultures in 42.9% and 33.3% subjects addressed for suspicion of aspergillosis, respectively. There was also significant impact of antifungal treatment on the results of the two tests ($P < .05$). In all, these new serological methods show promise in aiding in the diagnosis of aspergillosis in dolphins, and illustrate the opportunity to adapt commercial reagents directed for human diagnostics to detect similar changes in other animals.

Key words: marine mammals, blood test, cetaceans, immunoblot, serology, galactomannan.

Introduction

Aspergillosis is a fungal airborne infection caused by ubiquitous molds belonging to the *Aspergillus* genus. In humans, aspergillosis is responsible for a wide array of distinct clinical conditions categorized as invasive form, chronic disease, for example, aspergilloma, and/or allergic affection, for example, allergic bronchopulmonary aspergillosis.^{1,2} Mortality rates of invasive aspergillosis in humans are estimated at 30–100% and depend on both the underlying medical condition and the delay in achieving a definitive diagnosis.^{2,3} In spite of recent development of new laboratory tools like quantitative polymerase chain reaction (qPCR) or measurement of galactomannan (GM) antigen, the routine diagnosis of invasive aspergillosis remains difficult:⁴ it is indubitably achieved when suggestive histopathological findings are reported from biopsy samples. Such procedures, however, are invasive and often not readily achievable.⁴ Evaluating new noninvasive methodologies are thus essential in aiding the diagnosis of aspergillosis.

Definitive diagnosis of aspergillosis in animals is also challenging,⁵ especially in avian species.^{6–8} In marine mammals, *Aspergillus*-related disease is assumed to be rare, but has been reported with increasing frequency: 66.7% of the 18 reported cases have been published after the year 2000.^{9–12} The major species responsible for infection in cetaceans belong to the *Fumigati* section,¹² *Nigri* section and *Terrei* section in which *A. fumigatus stricto sensu* (*ss*), *A. niger ss* and *A. terreus ss* are the most frequently isolated,¹³ respectively. In marine mammals, development of aspergillosis is thought to be based on a chronic invasive process which is generally indicative of another disease and/or (sub-)acute physiologic stress,¹⁴ but it is rarely associated with severe immunosuppression and profound neutropenia like in humans.¹⁵ Underlying pulmonary disease may affect host defense mechanisms, leading to colonization and potential invasion of bronchial tissue by *Aspergillus* spp.¹⁶ However, other organs, including the brain, may also be infected following systemic dissemination.^{9,11,17} In marine mammals, and especially in

dolphins, laboratory tools are largely less developed than for humans.^{11,18,19} For instance, qPCR has not been implemented in veterinary laboratories, and advanced medical imaging is not as easily accessible. Also, as reported in humans, sensitivity of blood cultures in bottles placed in automate is very low for *Aspergillus* spp.²⁰ Positive culture from respiratory specimens does not definitively diagnose a true infection, but may reflect a simple colonization of the upper airways or represent an environmental contaminant.⁴ Furthermore, although it was not specifically reported in cetaceans, GM detection was demonstrated to be not totally reliable in some other animal species.²¹ Therefore, alternative non-invasive methods derived from human medicine need to be developed in order to increase the accuracy of the diagnosis in animals, and to make it readily available at lower costs. In such a context of chronic aspergillosis with no associated immunocompromised status, serological assays to detect antibodies directed against *Aspergillus* would be relatively easy and relevant to perform in animals, like dolphins.^{22,23} In this study, we assessed the diagnostic performance of a new commercial immunoblot assay for detection of anti-*Aspergillus* antibodies, the *Aspergillus* Western blot IgG[®] kit (LDBio Diagnostics, Lyon, France), in comparison with a novel genus-specific enzyme-linked immunosorbent assay (ELISA).

Methods

Study samples

Case definitions

Classification of fungal disease in dolphins was derived from the definition utilized in human medicine,^{1,15,24–26} which included cases subdivided into “proven” and “probable” infections. The definitive diagnosis of “proven” aspergillosis was based upon histopathological evidence of fungal disease, using Periodic Acid-Schiff or Gomori-Grocott’s Methenamine silver staining (i.e., for *Aspergillus*-diseased cases: presence of invasive, 45°-branched, septate filaments within tissues).⁴ The diagnosis

of “probable” fungal infection was based upon positive mycological culture on selective fungal media (Sabouraud dextrose agar) at 30°C and/or 37°C for 7 days, in conjunction with examination findings, clinical signs (e.g., wheezing, changes in vocalizations, weight loss, elevated respiration pace, anorexia, ...), imaging investigations (granuloma on celioscopy/endoscopy, infiltrates and peri-bronchial lesions on computed tomography and X-ray radiography), response to antifungal treatment, and any medical records that corroborated the diagnosis. GM antigen detection was performed according to the manufacturer’s recommendations (Bio-Rad Clinical Diagnostics, Hercules, CA, USA),^{6,8,21,27} but it was not considered to be a pivotal criterion for the diagnosis of “probable” aspergillosis in dolphins in this study, since it has not been validated so far in such a context.⁴ Dolphins that fell into the category of “possible” aspergillosis (i.e., dolphins with positive mycological cultures or clinical signs of invasive fungal infection, but not both simultaneously^{1,24,25}) were excluded. Clinically normal dolphins served as the healthy controls in this study.

Observation cohort

Samples from this group were retrospectively assessed to set the diagnostic parameters/cutoffs of the serological assays. They had been opportunistically collected during routine or clinical health assessments from common bottlenose dolphins (*Tursiops truncatus*) under human care in the United States of America. Tubes had been totally fulfilled with blood drawn from the tail fluke veins (with no usage of alcohol for preliminary cutaneous disinfection) through an 18–20 gauge, 1–1.5-inch needle (or butterfly set). Samples were thereafter archived in the Department of Pathology and Laboratory Medicine, Division of Comparative Pathology (University of Miami, Miller School of Medicine, Miami FL, USA) and in the facility of the National Marine Mammal Foundation (San Diego CA, U.S.A.) until testing. There were no non-inclusion criteria based on sex/age or underlying disease.

Overall, 32 samples had been obtained from *Aspergillus*-diseased cases with “proven” or “probable” diagnosis. Most were attributable to species of the *Fumigati* section, except eight characterized by species of the *Flavi*, *Nigri*, and *Terrei* sections. Nine samples were drawn from dolphins affected by other invasive fungal infections, sometimes concurrent in certain individuals, including candidiasis ($n = 6$), cryptococcosis ($n = 2$), histoplasmosis ($n = 2$), *Coccidioides immitis* infection ($n = 2$), mucormycosis ($n = 1$, in association with “proven” aspergillosis), and served as non-*Aspergillus* controls (except for the dolphin with mixed infection which was included in the case group). Forty-seven samples were obtained from clinically normal

dolphins, with no biological evidence of invasive fungal infection.

Validation cohort

Ninety-nine distinct serum samples were subsequently acquired from 21 animals (17.8 years \pm 9.8; 30.6% males) with suspected aspergillosis or undergoing post-diagnostic follow-up. They were sent to the investigator center (Division of Comparative Pathology, University of Miami, Miller School of Medicine, Miami, FL, USA) and prospectively tested for serological methods. Concomitantly, these dolphins underwent other routine and specialized diagnostic procedures to establish a definitive diagnosis during their clinical care. For example, all were sampled for mycological cultures.

Another subset of serum samples was obtained from 32 free-ranging dolphins (estimated mean age = 14.0 years \pm 3.0; 36.4% males) living in Sarasota Bay (FL, USA), and archived in the collection of the National Marine Fisheries Service Scientific Research, Mote Marine Laboratory before diagnostic testing.²⁸

Serological tests

Pre-processing step

Upon arrival to the investigator center (Division of Comparative Pathology, University of Miami, Miller School of Medicine, Miami, FL, USA), all samples were stored at -80°C with no intervention until they were thawed, and centrifuged for 10 minutes (min) at 3000 g just before being utilized for testing.

ELISA

Briefly, each serum sample was systematically tested in eight serial dilutions performed in phosphate-buffered saline (PBS) buffer, ranging from 1:64 to 1:8192 dilutions. Fifty microliters of each dilution was placed into one well of a 96-well microtiter plate that had been manually coated with bulk *Aspergillus* ID antigens made of pooled mycelial-phase culture filtrates of *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* (IMMY, Norman, OK, USA). Samples were incubated at 37°C for 30 min. After a washing step with Tween-saline solution, 50 μl of horseradish peroxidase (HRP-) conjugated rabbit anti-bottlenose dolphin IgG_{h+1} (Bethyl, Montgomery, TX, USA), diluted 1: 10,000 in PBS, was added to each well, and incubated at 37°C for 30 min. Finally, 50 μl of chromogen ABTS[®] solution (KPL, Gaithersburg, MA, USA) was added to each well after an additional washing step. The optical density (OD), related to reaction intensity after 0, 5, 10, and 15 min, was read at 405 nm, in a SPECTRAMax[®] microplate spectrophotometer (Molecular Devices, Downingtown, PA, USA), following a

5-second-long auto-shaking sequence. An OD of 0.2 or higher was considered positive. For each run, a highly positive serum, tested previously with positive titer over 1: 1024, was used as a control.

Immunoblot testing

Each serum sample was tested using the *Aspergillus* Western Blot IgG[®] kit (LDBio Diagnostics, Lyon, France) according to the manufacturer's recommendations. For immunodetection, 1.2 ml of alkaline phosphatase-(AP-) conjugated rabbit anti-bottlenose dolphin IgG_{h+l} (diluted 1: 1000, Bethyl, Montgomery, TX, USA) was distributed into each channel. The immunoblot patterns were compared to a positive control supplied by the manufacturer that was revealed by anti-IgG conjugate (LDBio Diagnostics, Lyon, France). Each sample was examined in triplicate and independently read by two experts. According to previously published works,²⁹ a specific focus was made on four immunoblot bands, located at 30, 22, 18–20, and 16 kDa, that have been shown to be specific for *Aspergillus* sensitization in humans. By adding the intensity results of each abovementioned band (which was individually scored from 0 to 4, according to comparison with the score of the positive control), a global Western blot (WB) score was ranked on 16 points for each sample.

Data analysis

Statistical analyses were performed using XLStat Premium v.18.07[®] software (Addinsoft, Paris, France). Methods validation was achieved according to prior recommendations.³⁰ Receiver operating characteristic (ROC) curves and areas under the curve (AUC) were generated in order to establish positive cutoff with the WB score of the commercial kit. Lower and upper cutoff values were calculated using Youden's J statistic³¹ and ROC curves.³⁰ A true positive was defined as a positive result that was in agreement with the definitive diagnosis of "proven" or "probable" aspergillosis in the observation cohort. Diagnostic measures were then calculated to assess performance: sensitivity, specificity, positive and negative predictive values, and likelihood ratios. The comparison of diagnostic performance was made using the Wilcoxon signed-rank test. The Pearson's rank correlation coefficient was used to assess the correlation between results of the different serology assays. Statistical association was tested by regression models, expressed in odds ratio (OR) with 95% confidence interval (95% confidence interval [CI]). Missing data, for example, when the total volume of sample was insufficient to complete all the analyses, were managed by the method of mean imputation. The α -risk was adjusted at 0.05.

Ethics

No animal was anesthetized, euthanized, or sacrificed for the purposes of this study. The serum samples utilized were prospectively banked during veterinary assessments in normal and clinical dolphins.

This study was conducted using archived samples collected from Six Flags Aquarium, Georgia Aquarium, and US Navy marine mammals as part of their routine care. The US Navy Marine Mammal Program (MMP) houses and cares for a population of dolphins in San Diego Bay (CA, USA). The MMP is Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited and adheres to the national standards of the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Animal Welfare Act. Samples obtained from free ranging dolphins in Sarasota Bay (FL, USA) were collected under National Marine Fisheries Service Scientific Research Permit No. 15543 and Mote Marine Laboratory IACUC approval, with support from Dolphin Quest, Inc., the Chicago Zoological Society, and the Office of Naval Research. Georgia Aquarium is a USDA/APHIS approved and inspected facility, and a member of the Association of Zoos and Aquariums and the Alliance of Marine Mammal Parks and Aquariums. Georgia Aquarium has been awarded the Humane Conservation certification by the American Humane Association. Biologic samples for this study were taken as part of a preventive medicine program or for medical monitoring purposes.

Results

Assessment of the serological assays within the observation cohort

Results are displayed in Table 1. Notably, no difference for GM results were observed between the *Aspergillus*-diseased cases and the controls (index 0.2 ± 0.1 vs. 0.3 ± 0.1 , $P > .05$).

ELISA

The ROC curve showed AUC at 0.844, sensitivity and specificity at 96.9%, [82.6–100.0%] and 36.4%, [24.9–49.6%] by the lower cutoff value set at 1: 256 titer, and at 90.6%, [74.8–97.4%] and 69.1%, [55.9–79.7%] by the upper cutoff established at 1: 512 titer (Fig. 1A, upper left panel). The upper positive threshold allowed correct separation of *Aspergillus*-diseased dolphins and controls (Fig. 1A, upper middle panel), but cross-reaction was noticed to be frequent, especially for cetaceans infected with *C. immitis* and *Cryptococcus* sp. (Table 2). In these dolphins, the respective titers ranged from 1: 1024 to 1: 8192. For this reason, the positive predictive value was found moderate, at 63.0%. There was no significant impact of the

Table 1. Demographics and characteristics of the observation cohort of common bottlenose dolphins. The group assignment was made according to the case definition for diagnosis of aspergillosis (for details, see the Methods section).

Study population of the observation cohort (N = 87)	Mean (\pm SD) or Number (%), [95% confidence interval]	
	<i>Aspergillus</i> -diseased cases ^Y (N ₁ = 32)	Controls ^{A‡} (N ₂ = 55)
Age (years):	22.5 (\pm 10.8), [18.4 – 26.5]	16.0 (\pm 10.3), [13.0 – 19.0]
Sex (male):	16 (50.0%), [32.7 – 67.3%]	20 (36.4%), [23.7 – 49.1%]
Diagnosis of fungal infection:		
- proven	19 (59.4%), [42.4 – 76.4%]	8 (14.6%), [5.2 – 23.9%] ^ψ
- probable	13 (40.6%), [23.6 – 57.6%]	/
Clinical course:		
- respiratory ¹	20 (62.5%), [45.7 – 79.3%]	1 (1.8%), [0.0 – 5.3%]
- disseminated ²	8 (25.0%), [10.0 – 40.0%]	11 (20.0%), [9.4 – 30.6%]
Routine biological findings:		
- fungal culture isolating <i>Aspergillus</i> section <i>Fumigati</i> (positive)	24 (75.0%) [60.0 – 90.0%]	/
- fungal culture isolating <i>Aspergillus</i> section non- <i>Fumigati</i> (positive)	8 (25.0%) [10.0 – 40.0%]	/
- fungal culture isolating other genera than <i>Aspergillus</i> (positive)	1 (3.1%) [0.0 – 9.2%]*	8 (14.6%), [5.2 – 23.9%]
Results of anti- <i>Aspergillus</i> serological assays:		
- galactomannan antigen measurement (index)	0.2 (\pm 0.1), [0.2 – 0.3]	0.3 (\pm 0.1), [0.2 – 0.4]
- immunoblot testing (Western blot score/16)	8.7 (\pm 4.2), [7.1 – 10.2]	3.1 (\pm 2.4), [2.5 – 3.8]
- ELISA (1: dilution)	1,024 (1,536), [128 – 16,384] [⋄]	256 (384), [0 – 8192] [⋄]

ELISA, enzyme-linked immunosorbent assay; N, number; SD, standard deviation; /, 0 (0.0%), [0.0 – 0.0%].

^Y Eight dolphins were independently tested twice or more when repeated blood work had been performed at separate times

^A Included samples from clinically normal control dolphins ($n = 47$) and from control dolphins undergoing non-*Aspergillus* invasive fungal infection ($n = 8$).

[‡] Seven dolphins were independently tested twice or more when repeated blood work had been performed at separate times.

^ψ Included cases of candidiasis (two were due to *Candida glabrata* and two to *C. tropicalis*), cryptococcosis, histoplasmosis, and coccidioidosis, sometimes concomitant in unique dolphins.

¹ That is, coughing, abnormal vocalizations, hard chuffing, radiological changes and all signs related to tracheitis, bronchitis, pneumonia, pleurisy . . .

² That is, at least to distinct organs were shown to be involved.

* For one case, necropsy revealed concomitant aspergillosis and mucormycosis of the trachea.

[⋄] Median (interquartile range), [min. – max. value].

Aspergillus species responsible for the infection on ELISA results ($P = .8$).

Immunoblot testing

Overall, immunoblot analysis confirmed that antibodies against *Aspergillus* proteins of 30, 22, 18–20, and 16 kDa were consistently observed in dolphins with “proven” or “probable” aspergillosis (Fig. 2). Presence of the 22 kDa band showed the highest sensitivity, but the lowest specificity (100.0% and 10.3%, respectively). The band at 16 kDa was rarely present during “proven” or “probable” aspergillosis and had sensitivity estimated at 53.10%, but was the most specific (100.0%). Sensitivity was correct for band at 30 kDa (94.4%), and specificity was high for the band at 18–20 kDa (97.1%). When evaluating the four abovementioned immunoblot bands together by adding their respective intensities, the lower positive cutoff values for the WB score was determined to be 5 out of a total of 16, while the upper value was set at 6 out of 16, respectively (Fig. 1B, lower left and middle panels). ROC curve showed AUC at 0.855, sensitivity and speci-

ficity at 78.1% [60.9–89.1%] and 78.2%, [65.4–87.1%] by the lower cutoff value, and at 65.6% [48.2–79.6%] and 92.7% [82.1–97.5%] by the upper threshold, respectively (Table 2). Overall, positive results were strongly associated with diagnosis of aspergillosis (odds ratio [OR] = 21.3, 95% CI [6.1–73.7], $P < .0001$). There was no significant influence upon the WB score from the *Aspergillus* species which was found to be involved in the infectious process ($P = 1.0$). The invasive fungal infections due to non-*Aspergillus* genera, for example, *Cryptococcus*, *Candida*, *Mucor*, *Rhizopus*. . . , provided WB scores below the positive cutoff. Notably, the dolphin with aspergillosis secondary to mucormycosis, had a WB score value just below the upper positive threshold (5/16).

Diagnostic performance of the immunoblot testing was compared with that of the ELISA in Table 2. The two methods were statistically correlated (Pearson’s coefficient $R^2 = 0.577$, $P < .0001$). Of all the dolphins with “proven” or “probable” diagnosis of aspergillosis, none were positive in immunoblot testing, while concurrently negative with the ELISA method.

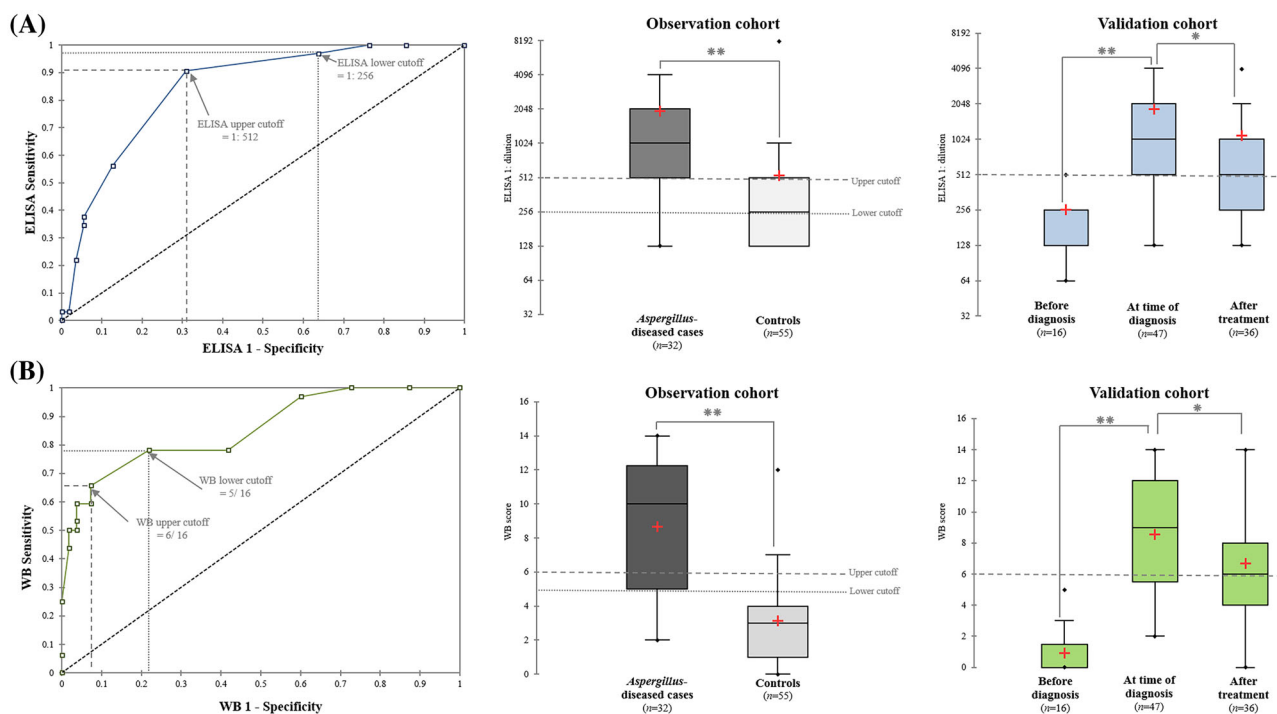


Figure 1. Diagnostic performance of the novel *Aspergillus* genus-specific ELISA (A) and the immunoblot testing (*Aspergillus* Western blot IgG[®] kit, LDBio Diagnostics, Lyon, France) (B) in common bottlenose dolphin blood samples. A, – upper left panel, ROC curve for the ELISA in the observation cohort; upper middle panel, box plot showing distribution of the ELISA 1: dilution titers, in the observation cohort. One extreme value, positive at a 1: 16,384 dilution, is not shown because out of the graph; upper right panel, box plot showing distribution of the ELISA 1: dilution titer in the validation cohort. B, lower left panel, ROC curve for the immunoblot testing; lower middle panel, box plot showing distribution of the Western blot (WB) scores for immunoblot testing; lower right panel, box plot showing distribution of the global WB scores of immunoblot testing in the validation cohort. * $P < .05$; ** $P < .0001$; AUC, area under curve; ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic; WB, Western blot. This Figure is reproduced in color in the online version of *Medical Mycology*.

Table 2. Diagnostic performance of the novel enzyme-linked immunosorbent assay (ELISA) and the immunoblot testing (*Aspergillus* Western blot IgG[®] kit, LDBio Diagnostics, Lyon, France) for detection of anti-*Aspergillus* antibodies in common bottlenose dolphin serum samples. - Positive cutoff values that were used for calculation are indicated directly inside the columns. The Wilcoxon signed-rank tests were used for statistical analysis. In brackets: 95% confidence intervals. ELISA, enzyme-linked immunosorbent assay.

	ELISA Positive cutoff \geq 1: 512 dilution	Commercial <i>Aspergillus</i> Western blot IgG [®] kit Positive cutoff \geq 6 /16	P-value
Sensitivity	90.6% [74.8 – 97.4]	65.6% [48.2 – 79.6]	0.016
Specificity	69.1% [55.9 – 79.7]	92.7% [82.1 – 97.5]	0.002
Positive predictive value	63.0% [49.1 – 77.0]	84.0% [69.6 – 98.4]	0.065
Negative predictive value	92.7% [84.7 – 100.0]	82.3% [72.7 – 91.8]	0.131
Positive likelihood ratio	2.9 [1.9 – 4.4]	9.0 [3.4 – 24.0]	
Negative likelihood ratio	0.1 [0.0 – 0.4]	0.4 [0.2 – 0.6]	

Assessment of the serological assays within the validation cohort

Dolphins under human care

The ELISA was sporadically falsely-positive for three of them in which the diagnosis of aspergillosis was not concomitantly/subsequently confirmed. In contrast in six cases, ELISA was positive earlier than mycological cultures (in average 88 ± 55 days earlier). Besides, it resulted in the

diagnosis in another case, while concomitant cultures constantly remained sterile. The antifungal treatment had a significant impact upon decreasing the ELISA titers from 1: 1024 to 1: 512 ($P = .039$) (Fig. 1A, upper right panel).

The immunoblot testing confirmed the suspected diagnosis of aspergillosis in 16 out of the 21 bottlenose dolphins. Importantly, it provided a positive diagnosis in three cases in which the mycological culture remained sterile

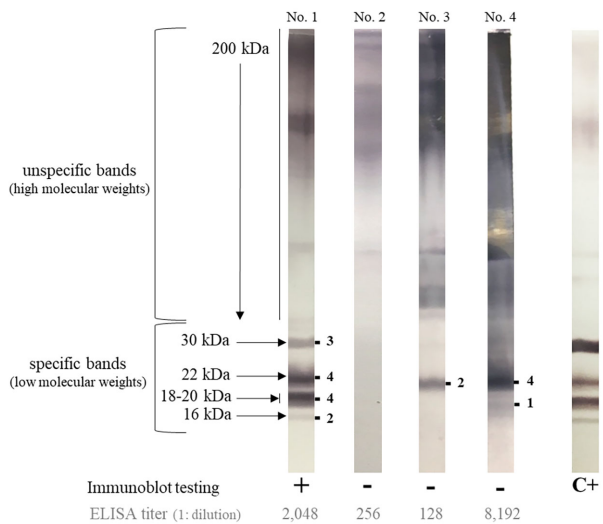
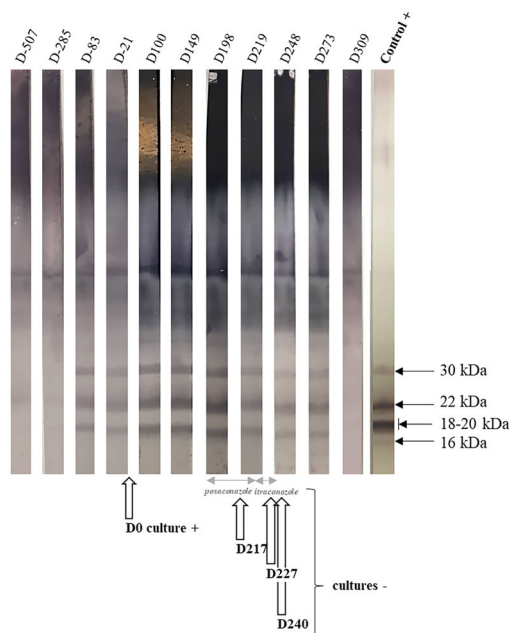


Figure 2. Examples of anti-*Aspergillus* immunoblot testing (*Aspergillus* Western blot IgG[®] kit, LDBio Diagnostics, Lyon, France) in common bottlenose dolphin blood samples. *Left panel*, examples of one positive (+) and three negative (–) patterns. The global Western blot score for specimen No. 1 is 13/16, as calculated by the sum of the respective band intensities;²⁹ *right panel*, pattern obtained with the human positive control (C+). AP, alkaline phosphatase; C+, positive control; ELISA, enzyme-linked immunosorbent assay; kDa, Kilo Dalton. This Figure is reproduced in color in the online version of *Medical Mycology*.

throughout the study period. In addition, the immunoblot test indicated diagnosis prior to the *Aspergillus* culture in six dolphins, in average 103 ± 66 days earlier. It also reflected positive response to antifungal treatment ($P = .002$), with a decrease in the mean WB score from 8.9 to 6.7/16 (Fig. 1B, *lower right panel*). Conversely, one animal showed no response on immunoblot testing to antifungal therapy over a 3.5-year period following diagnosis, that is, WB scores were stable at 13–14/16, whereas ELISA titers were fluctuating from 1: 512 to 1: 16,384. While this animal had substantial clinical improvement on antifungal therapy, necropsy revealed rare fungal hyphae characterized by regular septa, parallel walls, and acute angle branching present only within devitalized airway cartilage. The animal died of causes unrelated to pulmonary aspergillosis. An example of serological follow up of another animal is shown in Figure 3.

Free-ranging dolphins

All but six were negative with ELISA method: one was positive at a 1: 2,048 titer and showed multiple papilloma lesions on the rectal mucosa, the vulva, and the urinary papilla. This dolphin also had multiple pinpoint depressed dark brown skin lesions on the right side and mild



Sampling date *	Immunoblot (sum/ 16)	ELISA (1: dilution)	GM antigen (index)
D-507	1	256	0.1
D-285	0	128	0.1
D-83	9	512	0.2
D-21	9	2,048	0.2
D100	12	2,040	0.1
D149	12	512	0.1
D198	12	512	0.1
D219	9	256	0.4
D248	6	1,024	0.2
D273	6	512	0.3
D309	0	256	NA

Figure 3. Example of anti-*Aspergillus* serological follow up in a 15 year-old-male common bottlenose dolphin (D0 being the reference day when mycological culture became positive). ELISA and immunoblot testing (*Aspergillus* Western blot IgG[®] kit, LDBio Diagnostics, Lyon, France) were both positive 12 weeks before the mycological culture (D-83). Worsening in clinical condition, especially appearance of abnormal vocalizations, marked the start for antifungal therapy (D166). Serological status returned negative 2 months after withdrawing ITRACONAZOLZ (D309), although nonspecific increase of antibody rates was transiently noticed with ELISA testing (D248). Notably, as shown in the right column, measurements of GM antigen were never consistent with the actual medical condition at time of sampling. D, day (D0 being the first day when mycological culture became positive); ELISA, enzyme-linked immunosorbent assay; GM, galactomannan antigen; kDa, Kilo Dalton; NA, not available. This Figure is reproduced in color in the online version of *Medical Mycology*.

peritoneal effusion in the left abdomen. Another dolphin, exhibiting a 1: 1024 titer in ELISA, had skin lesions of unknown cause above the right pectoral flipper.

Due to low volume samples, only 13 free-ranging dolphins were tested using the immunoblot. All but one were negative. For this one positive animal, the WB score was estimated at 7/16, and ELISA results were below 1: 64 titer, while no clinical signs were reported.

Discussion

Although ELISA has been widely used to detect anti-*Aspergillus* antibodies in serum,³² precipitin detection by immunodiffusion remains the reference standard for serological diagnosis of chronic or subacute aspergillosis in immunocompetent or slightly immunocompromised human patients.^{33–35} However, as they are less labor-intensive and allow detailed study of the immune response, immunoelectrophoresis and immunoblotting techniques have been progressively developed, often replacing immunodiffusion for diagnostic confirmation testing.^{29,32,35} Increasing availability of commercial standardized kits approved for diagnostic purposes has reduced the usual limitations that were noticed with in-house immunoelectrophoresis and immunoblotting, such as poor intra- and inter-laboratory reproducibility and extended turnaround times.^{22,33} For example, ready-to-use immunoblot kits have already been utilized in human medicine for the diagnosis of HIV infection,³⁶ Lyme borreliosis,³⁷ and toxoplasmosis.³⁸ Nonetheless, such assays have been rarely applied in veterinary medicine, and not evaluated so far,³⁹ with the exception of a few in-lab techniques like that for the diagnosis of *Encephalitozoon cuniculi* infection in rabbits.³⁹ As aspergillosis can be a serious medical condition in dolphins, and its diagnosis can also be challenging, it seems vital to evaluate novel diagnostic tools, such as commercial immunoblot kit.

From this study, the *Aspergillus* Western blot IgG[®] kit (LDBio Diagnostics, Lyon, France), as well as the genus-specific ELISA method with which it was compared, appeared convenient to process, and easy to interpret, for the diagnosis of aspergillosis in common bottlenose dolphins. It is noteworthy that the multi-well plate reading for the ELISA testing is objective with spectrophotometer devices, but results of the immunoblot testing were more detailed for studying immunoreactivity. Addressing the lower molecular weight bands at 30, 22, 18–20, and 16 kDa through a quantitative approach,²⁹ we found similar patterns as those reported for human samples, for example, the 16 kDa band was often very slightly visible and the least sensitive of all, but very specific. Likewise, there were no obvious alternate patterns—in either banding or intensity of bands—in the dolphin samples in comparison with the human ones. Al-

together, our findings highlighted a high specificity of the immunoblotting, that is, 92.7% versus 94.0% in previous study carried out in humans,²⁹ especially because the positive cutoff was herein voluntarily set at a high value. Furthermore in this reagent kit, the manufacturer prioritized obtaining standardized purified antigens, although the nature of these antigens has not been disclosed thus far. Importantly, the results from the immunoblot testing showed no cross-reaction in blood samples from dolphins infected with fungi belonging to non-*Aspergillus* genera like *Coccidioides* sp. In contrast, and as previously reported,⁴⁰ several cross-reactions to *Coccidioides* and *Cryptococcus* spp. occurred when using the bulk antigens in the ELISA. Conversely to the purified proteins in the immunoblot kit, these bulk antigens are compound of mixture of unpurified proteins and other moieties like carbohydrates that are common among many fungal species. One should also note that both the ELISA and the immunoblot testing were reliable for the diagnosis of fungal infection due to most of the *Aspergillus* species, including those that do not belong to the *Fumigati* section, like *A. niger* and *A. flavus*. In this study, although the respective sensitivities were lower than those observed in some groups of human patients (100.0% and 41.7% in cystic fibrosis and non-cystic fibrosis patients for the immunoblot testing),²⁹ the two tests appeared to rival mycological cultures, at least when considering serology as a preliminary screening step among the panel of tools dedicated to diagnosis. The current study showed a high positive predictive value of the immunoblot testing for detecting *Aspergillus* infection in a screening approach. It also demonstrated the diagnostic benefits of repeated blood measures over the course of disease.

Results of this work need to be carefully interpreted, because animals are more likely to be colonized and/or chronically infected than humans, with variable clinical symptoms depending on the species. In animal-based studies involving natural infection, there may be a poor reliability due to the lack of consensus regarding the disease classification. It is particularly hard to classify correctly aspergillosis in cetaceans, because the disease features are somewhat different from invasive aspergillosis in neutropenic humans. In dolphins, aspergillosis is only moderately invasive, but its evolution is more chronic than in immunocompromised patients. In light of these considerations, pathology and immune response of aspergillosis in cetaceans should be rather compared to those of subacute/chronic human aspergillosis, like the form observed in patients with morbidity factors, for example, tuberculosis, sarcoidosis, autoimmune diseases, chronic obstructive pulmonary disease, and so forth.¹⁴ Nonetheless, the concept based on categorization of fungal infection used for humans (who represent a single mammal species) should not be correctly

applicable for all the other animals. However, to reduce misclassification of our study animals, we were forced to use a composite criterion based on clinical data and histopathology/mycological culture, as it is consensually approved for use in human infections.⁴ Notably, unlike in clinical trials recently reported in human patients with aspergillosis,^{41,42} there were more “proven” cases than “probable” ones in our observation cohort. One possible explanation relies on *post-mortem* evaluation which allows reliable diagnosis of “proven” aspergillosis through gross and histopathology observations.^{4,43} Conversely, the negative controls in the observation cohort could appear as a possible limitation due to underestimation: this group of dolphins was considered apparently healthy based on normal bloodwork and the lack of clinical signs consistent with aspergillosis, but only a few had been tested via mycological cultures before their inclusion. For these reasons, subclinical fungal infection or colonization cannot be definitely ruled out in these animals. In addition, it has been shown in humans that persistent colonization in clinically asymptomatic humans is a pathway to infection, and can induce sustainable antibody response.^{22,29} Since *Aspergillus* spp. is ubiquitous in the terrestrial and aquatic environment, exposure may be common in diverse populations of cetaceans in the wild and under human care.⁴⁴ It is, debatable, however, if seroconversion is a sufficient argument to start antifungal therapy or if instead, this should reinforce the need for more frequent monitoring or additional diagnostics.⁴⁵ In this study, antibodies appeared sustainable over time, including after long antifungal treatment, although their rates statistically decreased. Additional studies are now required with more cases that will undergo repeated measures after treatment to better assess antibody longevity.

In conclusion, studies focusing on specific diagnostic approaches, like this one, help to improve basic knowledge about fungal disease in wildlife and to overcome the lack of standardization, together with the inability to invest in expensive diagnostic tools. Herein, the novel ELISA and the immunoblot testing appeared valuable to detect anti-*Aspergillus* antibodies in dolphins. To our knowledge, this is the first time that a commercial immunoblot test initially intended for humans has been successfully used in animals. The uniformity of the reagents, as well as the availability of free software allowing scanning and easy reading of the blot patterns like ImageJ[®] software (<https://imagej.nih.gov/ij/>) or Image Studio Lite[®] software (<https://www.licor.com/>),⁴⁶ would allow reproducibility, specificity to fungal genus, and reliable inter-laboratory results.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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