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REVIEW ARTICLE

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The current status of avian aspergillosis diagnoses: Veterinary practice to novel research avenues

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Abstract

Aspergillus fungal infections continue to be a significant cause of morbidity and mortality in birds that can, in part, be attributed to the lack of a diagnostic "gold standard" for *Aspergillus* infection, and which delays the diagnosis, treatment, and outcome of avian patients. At present, none of the available methods in veterinary care can detect aspergillosis early enough and with the accuracy, precision, and specificity required of an ideal diagnostic tool. Therefore, researching methods of *Aspergillus* detection is still an active area of inquiry, and novel techniques continue to emerge. This review will provide a brief overview of current clinical methods, with an emphasis on avian care, in addition to a series of techniques in development that could offer distinct advantages over existing methods.

KEYWORDS
Aspergillus, avian, diagnostic, fungal infection

1 | INTRODUCTION

Aspergillus is a ubiquitous conidial fungus whose spores are universally inhaled and ingested.[1] In healthy humans, the innate and cellular immune systems prevent penetration of germinating spores through the alveolar epithelium, and block infection.[2] However, in immunocompromised patients, *Aspergillus*, most commonly *A fumigatus*,[1] can cause a spectrum of acute and chronic respiratory conditions, and in its most aggressive form, invasive aspergillosis (IA) can lead to widespread infection and death.[2] Similarly, although mammals are normally resistant to aspergillosis, reports in dogs, cats, horses, cows, marine mammals, and nonhuman primates exist.[3-5] Aspergillosis is pervasive in both the domestic and wild avian communities, where it is a common cause of respiratory distress and morbidity.[6,7]

In addition to adverse health effects, *Aspergillus* infections in medical practice incur a substantial socioeconomic burden. Mortality in immune compromised human patient populations, such as those with hematologic malignancies, organ transplants, and HIV infections, are particularly high and seen in as many as 90% of patients.[8] Therefore, IA confers a grim prognosis, often complicating existing medical conditions in immunosuppressed patients. In addition to personal and societal loss, the monetary cost of treating a patient that has complications from IA along with that of preexisting medical conditions, can exceed $69 000 (US dollars).[8] The situation is paralleled in veterinary patients and domesticated and wild avian species, where an aspergillosis diagnosis and treatment can incur additional resources, time, and money for zoos, livestock, and pet owners.

In vulnerable human patient populations, IA treatments can be prophylactic,[9] empirical, or preemptive,[10,11] depending on a patient's medical condition, the degree and duration of possible neutropenia, and the diagnostic test results for aspergillosis.[8] This cautious line of treatment is justified due to the high mortality rate associated with IA. However, significant shortcomings to this line of treatment exists. First, antifungal drugs are potentially toxic, so using them prophylactically could expose patients to drug side effects and possible toxicities.[12] Second, prophylactic medical treatments and the prophylactic use of these drugs in agricultural settings can lead to increases in resistance to the current arsenal of therapies,[13,14] which lowers antifungal efficacy and necessitates the discovery of new agents.

A resolution to resistance problems would be the accurate, sensitive, and early diagnosis of aspergillosis. However, despite the large number of clinical techniques available, none have proven specific, sensitive, and rapid enough for efficient and reliable aspergillosis diagnosis (Table 1). This lack of a "gold standard" for detection in humans, birds, and other mammals poses a problem for timely and accurate aspergillosis treatments. Therefore, many researchers are actively seeking ways to overcome this hurdle. This review will briefly cover routinely used clinical methods in birds and mammals

TABLE 1 Aspergillosis diagnostics: advantages and disadvantages

Methods	Advantages	Disadvantages
Radiography	• Rapid • Noninvasive • Cost-effective • Equipment widely available in many veterinary settings	• Only indicates infection and is not pathognomonic • Imaging of certain areas may be obscured by muscle and bone • Absence of radiological signs in the early stages of infection • Radiation exposure • Limited to diagnosis in airway passages • Requires sedation
Computed tomography (CT)	• Rapid • Noninvasive • More specific and sensitive than radiography • 3D imaging capability may capture obscured features	• Only indicates infection and is not pathognomonic • Requires highly specialized imaging equipment • Radiation exposure • Deep sedation/anesthesia required
Magnetic resonance imaging (MRI)	• Rapid • Noninvasive • More specific and sensitive than X-rays • 3D imaging capability may capture features obscured in X-rays	• Only indicates infection and is not pathognomonic • Requires highly specialized imaging equipment • Costlier than CT • Deep sedation/anesthesia required
Endoscopy	• Rapid • Simultaneous biopsy and swab collection possible • Enables visualization and extent of lesions and therapy • Relatively cost-effective compared with CT and MRI	• Invasive • Only indicates infection and is not pathognomonic • Limited to diagnosis in the airway passage • Requires sedation
Histology	• Relatively rapid • Sensitive • Cost-effective	• Cannot definitively identify genus due to similarities with other filamentous fungi • Can be invasive, depending on the tissue biopsy needed • Biopsy may be hazardous in ill patients
Cytology	• Relatively rapid • Cost-effective	• Can be invasive, depending on location of sample collection • Only indicates infection and is not pathognomonic
Fungal culture	• Can identify genus and species, therefore pathognomonic • Culture grown from a normally sterile cavity is generally definitive for diagnosis • Can be used to simultaneously test for susceptibility to antifungal agents • Cost-effective	• Can be invasive, depending on location of sample collection • Time consuming, delays diagnosis and possibly treatment • Relatively insensitive since culture may fail to grow; a negative test does not necessarily rule out *Aspergillus* infection • Contamination by exogenous spores may cause false positives • Requires human judgment to identify species
Serologic *Aspergillus* antibody detection	• Pathognomonic and specific to *Aspergillus* • Rapid • Noninvasive • Cost-effective • Amenable to high-throughput	• Low sensitivity • Susceptible to false positives since antibody presence could arise from prior exposure to *Aspergillus*
Galactomannan GM assay (ELISA-based)	• Pathognomonic and relatively specific for *Aspergillus* • Rapid • Noninvasive if testing serum • High-throughput	• Relatively lower sensitivity in serum compared with BAL • Cross-reactivity with other fungal species possible • Effectiveness dependent on underlying medical condition • Interference possible from certain drug treatments

but will also delve into the novel avenues being pursued for the detection of *Aspergillus* species.

2 | PREDISPOSING FACTORS AND SPECIES SUSCEPTIBILITY

In birds, veterinarians can use environmental signs to provide clues of possible acute or chronic aspergillus infections.[6,7] Environmental factors include cage conditions, humidity, temperature, hygiene,[15] contaminated food or bedding,[16,17] trauma, or recent stressors such as transportation.[18] Immune suppression is also a contributing element to the development of an *Aspergillus* infection.[19] A predisposition based on species has been implicated by clinical observation[20-22] and experimental studies,[23,24] but was dismissed by another study.[25] Some species might be more predisposed to infection, but in some avian species (ie, penguins), infections could be due to their poor housing environments.[7,26,27]

There are fewer reports of aspergillosis in mammals compared with that of the avian species. However, cases have been described in dogs, cats, horses, cows, camels, marine mammals, and nonhuman primates.[3-5,28] Environmental factors and contamination of bedding or feed contribute to the sources of *Aspergillus* spores.[3]

3 | CLINICAL EXAMINATION

Symptoms in birds are variable and depend on whether the infection is acute or chronic and on the location of infection; however, none of these observations are pathognomonic.[6,7] Symptoms include, to name a few, an altered breathing depth and rate (dyspnea, tachypnea), open-beak breathing, rhinitis, change in vocalization or aphonia, reduction of stamina and athletic performance, beak deformation, ocular discharge, blepharitis or swelling, dermatitis and folliculitis, lethargy, inappetence, anorexia, diarrhea, vomiting, unilateral wing droop in flight or inability to fly or stand, central nervous signs, and peripheral nerve paresis.[15,22,29-35]

Aspergillosis in dogs is usually limited to the sinonasal area, as reflected by the most common symptoms: sneezing, epistaxis, ulceration of the nasal planum, facial pain, and mucopurulent discharge.[3,36,37] Similar to dogs, infection in cats is most frequently seen in the sinonasal and sino-orbital regions.[4] In horses, aspergillosis is localized to the head causing intermittent epistaxis, mucopurulent nasal discharge, dysphagia, laryngeal paralysis, and Horner's syndrome.[3,38] In camels, aspergillosis causes scrotal granulomas.[28]

All symptoms of aspergillosis found through physical examination and patient history are nonspecific and do not represent stand-alone diagnostics. However, they do serve as the first line of inquiry to direct further clinical investigations or to bolster findings from more specific diagnostic tests.

4 | CONVENTIONAL AND DIGITAL RADIOGRAPHY, ENDOSCOPY, AND ADVANCED DIAGNOSTIC IMAGING

A routine and widely available technique for aspergillosis diagnosis is a radiographic examination, which is rapid and inexpensive and can indicate whether there are signs of pulmonary infection.[6,7] Avian patients need to be manually restrained and either anesthetized or sedated to limit movement and calm breathing. Even though radiological examination is noninvasive, the requirement for sedation makes this test a risky option for critically ill patients.[29,39]

Orthogonal (ie, lateral and ventrodorsal) views depict the lower respiratory tract clearly, but the cranial lung fields and trachea can remain obscured by surrounding muscle and bone.[29,40] The range of locations where aspergillosis can occur, and the nature of radiographic signs that are seen with this infection, vary across avian species. However, radiographically, aspergillosis may manifest itself as granulomas in the oropharynx, periorbital sinuses, trachea, syrinx, lungs, and air sacs, as loss of definition in the cardiac silhouette, as increased line-shaped radiodensities in the caudal lung borders, as hyperinflation or asymmetry in the air sacs, or thickening of the air sac membranes.[41,42] Digital radiography and specialized software have improved the quality and resolution of radiographs, enabling a more detailed delineation of visible structures and therefore has completely replaced conventional radiography.

Radiographic abnormalities in avian cases of aspergillosis are nonspecific, and do not confer a definitive diagnosis, but may be useful for ruling out other illnesses. However, if radiographic evidence is visible, it indicates that birds are already in the advanced stages of disease.[43] Therefore, lack of radiologic signs does not preclude the presence of aspergillosis since radiographic evidence is not always seen in early infections, and veterinarians might need to use other diagnostic methods to rule out aspergillosis.

Computed tomography (CT)[44] and magnetic resonance imaging (MRI)[45] are more advanced imaging modalities with greater resolution and three-dimensional capabilities compared with conventional radiography. CT and MRI can show suspicious areas of infection that are not seen with radiography and endoscopy (ie, lower bronchi, lung parenchyma, internal organs, spinal cord, brain). Furthermore, CT and MRI can highlight the invasive nature of aspergillosis; however, these modalities require deep sedation or anesthesia, which precludes their use in severely ill patients.[46,47] In a recent study, although CT was recommended for aspergillosis diagnosis in cranes, false negative CT scan results were produced in seven out of 10 cases, which included cases of mild air sacculitis, membrane opacification, and small plaques.[48] Like radiography, neither CT nor MRI can conclusively diagnose aspergillosis, so the increased cost of using these imaging modalities compared with radiography may not be justified.[6]

Endoscopy can also be used to diagnose aspergillosis, and although invasive, it can provide a more concrete diagnosis since lesions can be visually inspected.[6,7] It can also be used to obtain biopsy and swab samples for further histologic and cytologic analysis (see Section 5 below) or therapy (granuloma removal, air sac irrigation, endosurgical laser debridement).[22,49-51] Endoscopic signs depend on the stage of the disease. In avian air sacs, neovascularization, fluid accumulation, thickening, and cloudy patches are seen earlier in the disease process and plaque-like lesions that can be pigmented by a greenish-gray fungal "grass-like" growth in the later stages. *Aspergillus fumigatus* is the most common culprit in birds.[52] Lesions can be localized or disseminated in IA. Although endoscopy is invasive, it has the benefit of being able to collect biopsies for histopathology and swabs for fungal culture. Risks associated with endoscopy can occur in advanced cases where granulomas might cause changes to the normal air sac/thoracic anatomy that can result in bleeding and death by insertion of the endoscope.

Although aspergillosis is much less common in mammals compared with birds, similar diagnostic imaging modalities have been employed in canine patients for the diagnosis of aspergillosis, including rhinoscopy,[3,36,37] radiography,[3,36,37,53] CT,[3,36,37,54] and MRI.[36,37,53,55] MRI and CT use in feline patients has also been documented,[4,56] and endoscopic examination of the guttural pouch and radiography have been reported in horses.[3,38] As for clinical

observations, definitive diagnoses by imaging is not possible, but serves as an indication that an infection is present and further analysis by more conclusive methods is required, such as identification of the microorganism using fungal cultures (Table 1).

5 | CYTOLOGY, FUNGAL CULTURE, AND HISTOPATHOLOGY

In avian species, endoscopy (see Section 4 above) is more useful compared with other imaging methods because of its ability to collect specimens for cytology, fungal culture, and histology providing a more definitive diagnosis. Therefore, endoscopy, in combination with cytology and culture, is presently considered the "gold standard" for an aspergillosis diagnosis. Samples collected can be tissue biopsies, swabs, or fluid.

Biopsy tissue sections can be histologically analyzed using H&E, Grocott's, and periodic acid-Schiff (PAS) staining methods.[57,58] PAS is helpful because it provides a counterstain that reveals host tissue.[58] Fungal granulomas appear as necrotic cores interspersed with fungal hyphae and surrounded by heterophil granulocytes, lymphocytes, macrophages, and plasma cells. Granulomas of avian aspergillosis appear in two modes, either as: (i) deep nodules typical of organs with nonaerated parenchyma, or (ii) as nonencapsulated superficial, diffuse forms common to serosal and lung tissues.[59] *Aspergillus* hyphae are 5-10 μm thick, straight, parallel, and septate, with angular dichotomous branching.[58]

Although genus classification is possible using classical staining, misidentification can occur due to the similarities with other filamentous fungal species. Therefore, newer more specific methods use immunohistochemical staining.[58,60,61] Alternatively, microdissection of histologic sections can be used to separate fungal matter from avian tissue for more definitive molecular characterizations (see PCR section below).[62] Calcium oxalate crystals have been detected in histologic sections of respiratory tract tissue infected with *Aspergillus* in humans, horse, dog, oxen, dolphins, and birds.[63] Therefore, the authors recommend routine polarization of slides to check for calcium oxalate crystals, especially for respiratory tract fungal infections.

Collected fluid samples can be assessed cytologically for signs of inflammation by analysis of heterophils, macrophages, and lymphocytes (Figure 1).[64] Definitive identification of *Aspergillus* as the invading pathogen requires culturing on specific substrates (ie, Sabouraud agar, potato agar) and observation of colony morphology and color, followed by microscopic characterization of conidia, conidiophores, and ontogeny.[58,65] Culture analysis requires collection from otherwise sterile locations to avoid contamination by exogenous spores, as could be the case for tracheal, nasal, or pharyngeal swabs, which can lead to false-positive results. On the other hand, a negative result does not necessarily rule out aspergillosis since a culture could simply fail to grow.[66]

Fungal culture, cytology, and histopathology have also been used to diagnose aspergillosis in dogs[36,67] and horses.[38] Fungal cultures followed by phenotypic characterization is affordable and usually provides a definitive diagnosis for aspergillosis (Table 1). However, fungal cultures are labor intensive, are susceptible to misidentification due to similarities with other fungal species, and require time, which causes delays in treatment and could worsen prognoses. Despite its advantages, newer methods have been introduced, such as molecular and immunologic techniques, reviewed below.

6 | HEMATOLOGY, SERUM BIOCHEMISTRY, AND PROTEIN ELECTROPHORESIS

Aspergillus infection in birds elicits an immune response which might be reflected in the patient's WBC and differential cell count.[6,7,64] Total WBC counts can increase and a heterophilic leukocytosis, monocytosis, or lymphopenia can be present.[68,69] In cases with depressed heterophil counts or functions, the total WBC count can be normal or decreased. Heterophils can show toxic changes that

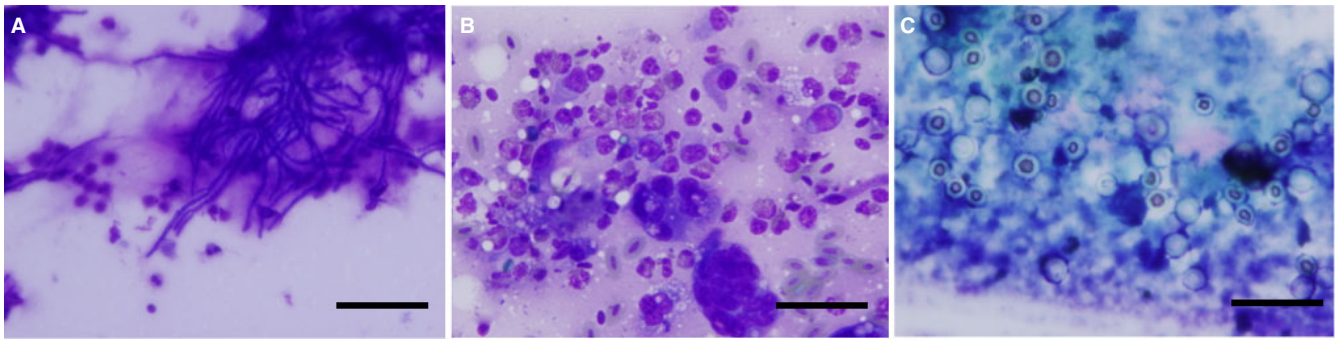

FIGURE 1 Photomicrographs of cytological analyses from airsac fluid aspirates at three different stages of aspergillosis, magnification ×100, scale bar 20 μM (photo courtesy: DFH/C. Silvanose). A, *Aspergillus fumigatus* hyphae stained with modified Giemsa, indicating active aspergillosis, which was confirmed with an endoscopically-obtained air sac biopsy from a gyrfalcon. B, Giant cells and fungal spores stained with modified Giemsa, indicating chronic aspergillosis, which was confirmed with an endoscopically-obtained air sac biopsy from a gyrfalcon. C, Formation of Hülle cells after a voriconazole antifungal treatment stained with a modified Giemsa stain, indicating regression of aspergillosis, which was confirmed with an endoscopically-derived air sac biopsy from a gyrfalcon

are associated with *Aspergillus* infection, but this change is nonspecific and can be associated with other diseases.[7]

Serum or plasma biochemistry can be performed with other more specific diagnostic tests for aspergillosis.[6,7] Changes in plasma variables (ie, proteins, metabolites, electrolytes) could determine which organs are affected by *Aspergillus* and its toxins, and the severity of the infection. Blood biochemistry may also be used to monitor recovery as levels can normalize with treatment. Commonly measured variables include: (i) aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alanine transaminase (ALT), and glutamate dehydrogenase (GLDH) to indicate liver damage; (ii) gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP) for biliary tract damage; (iii) creatinine kinase (CK) which reflects prolonged muscle breakdown from emaciation; and (iv) electrolytes and mineral composition that can indicate renal disease or failure.[6,7]

A relatively large class of plasma proteins, called acute-phase proteins (APPs), are biomarkers of inflammation and infection. Some APPs investigated in birds with aspergillosis are haptoglobin (HP) and serum amyloid A (SAA),[70,71] although others have also been tested.[72] The combined results from these studies in various bird species indicate that HP and SAA levels can increase or decrease with *Aspergillus* infection based on species, age, and prognosis. Therefore, the status of using APPs for aspergillosis diagnosis is at present ambiguous and requires further clarification. There is also a precedent for the use of APPs in the diagnosis of *Aspergillus* infection in the sinuses of dogs.[73]

To the best of our knowledge, studies on the utility of plasma or serum protein electrophoresis (SPE) for aspergillosis diagnosis are limited to birds. Rather than just measuring total serum/plasma proteins, SPE identifies the various components of each protein fraction, providing a more detailed picture of the spectrum of proteins present.[74] Since total protein levels do not change in some patients with aspergillosis, SPE can identify changes in the various fractions. Research into the use of SPE in penguins, psittacines, and raptors is well documented.[70,75-78] Most SPE studies on avian blood have employed agarose gel systems. Pilot studies with the more advanced capillary systems have been performed, but are, at present, impractical for birds, due to the large volume of required blood.[79] SPE is most informative when appropriate references from normal, healthy birds are available; and therefore, is limited to well-documented avian species.[6]

All tests presented in this section are nonspecific and cannot be used as stand-alone test for aspergillosis diagnoses, and are usually employed as adjuvants to bolster findings from more specific tests, to indicate sites of infection, or monitor response to therapy. Also, negative results or values within the normal range do not rule out an *Aspergillus* infection.

7 | SEROLOGY

Serologic assays for the presence of anti-*Aspergillus* antibodies have been tested as possible pathognomonic diagnostic tests of aspergillosis (Table 1).[6,7] Antibody quantification by indirect hemagglutination assays (IHA), agar gel immunodiffusion (AGID) assays, or enzyme-linked immunosorbent assays (ELISA) have been used in chickens,[80] turkeys,[80,81] psittacines,[77,82] pigeons,[83] ducks,[84] penguins,[82,85,86] and raptors.[25,70,82,87]

Anti-*Aspergillus* antibodies increased in pigeons inoculated with *Aspergillus*, suggesting a positive correlation between the presence of antibodies and exposure to *Aspergillus*, although the response diminished after the second week postexposure.[83] This also supported the potential usefulness of anti-*Aspergillus* antibodies as biomarkers of aspergillosis. However, larger studies in falcons failed to support this notion, since antibody titers determined by IHA did not correlate with clinical signs, fungal culture, endoscopy, or necropsy.[87] ELISA also did not discern significant differences in anti-*Aspergillus* antibody concentrations in penguins with or without clinical symptoms.[82]

Furthermore, although seroconversion was observed in falcons that developed aspergillosis, the high incidence of conversion in raptors, used as controls, precluded the use of anti-*Aspergillus* antibodies as a useful diagnostic test in falcons.[70] Control raptors could have been exposed to exogenous environmental *Aspergillus* that did not progress to infection. ELISA results in other birds were similarly inconsistent, with an occasional inability to detect antibodies in psittacines with confirmed aspergillosis.[77,82] In cases where antibodies were present, no correlation with prognosis was found.

Despite the potential for an *Aspergillus*-specific diagnosis, it seems that anti-*Aspergillus* antibody quantification may not be useful due to the high background in healthy individuals, which could be caused by the ubiquitous dispersal of *Aspergillus* in the environment, and hence repeated exposure of birds. Anti-*Aspergillus* antibody detection in dogs,[36,37] cats,[4] horses,[88] and cattle is also difficult,[89] where healthy controls show positivity, although titers might be higher in infected animals. Overall, the environmental presence of *Aspergillus* makes antibody detection tests unreliable and must be interpreted with care and in the context of other diagnostic results.

8 | *ASPERGILLUS* BIOMARKERS

Diagnostic methods of aspergillosis have been developed based on the detection of *Aspergillus* antigens or nonimmunogenic *Aspergillus* components. The two most well-known biomarkers are galactomannan (GM)[90] and (1-3)-β-D-glucan (BG),[91] both components of the *Aspergillus* cell wall. GM is relatively specific to *Aspergillus*, and positive results are considered pathognomonic for aspergillosis. A commercial FDA-cleared ELISA kit, Platelia™ (BioRad Laboratories, Marnes-La-Coquette, France) exists for GM detection. (1-3)-β-D-glucan is present in the walls of several fungal genera, so positive results are not specific for *Aspergillus* and should be considered more of a panfungal test. Since BG is nonimmunogenic, BG detection is based on enzymatic horseshoe crab coagulation[91] and has been marketed as the FDA-cleared Fungitell (Associates of Cape Cod, Beacon Diagnostics, Falmouth, MA, USA). Both GM and BG have been widely studied in human patients, but less extensively in avian and other veterinary patients, although a few studies have tested the

reliability of these tests in veterinary practice. In addition to GM and BG, we will discuss emerging methods still in the research stages of development.

8.1 | Antigens

The specificity and sensitivity of the GM assay in human patients are widely documented (Table 1).[92,93] Although generally reliable and applicable to both bronchoalveolar lavage (BAL) and noninvasive serum samples, other factors such as concurrent patient treatment can confound the results.[92,94] The full spectrum of interferences associated with the GM assay is still uncertain; however, antifungal administration could limit fungal growth and thus inhibits GM release, and antibiotics may contain trace amounts of GM or contaminants that cross-react with the GM antibody.[92,94] In addition, due to fluctuations in the specificity and sensitivity of the GM assay, a definitive diagnosis for aspergillosis requires combination with other diagnostic methods (eg, radiological, culture, and molecular).[95] The effectiveness of the GM assay in being able to detect aspergillosis depends on the underlying medical condition and performs relatively poorly in solid organ transplant patients compared with hematological malignancies.[96] Although initially hailed as a noninvasive serum method, later studies demonstrated that GM was more sensitive in samples collected by invasive BAL.[97]

Although lagging behind human trials, GM has been tested on avian aspergillosis diagnoses with mixed results and suggests that the GM assay might only be suitable for an aspergillosis diagnosis in certain avian species. Trials in falcons showed a low sensitivity and poor correlation between the GM index and aspergillosis status, which was possibly due to the limited dissemination of *Aspergillus* in those test cases.[70,98] Although several reasons were suggested,[70] one modification that can improve experimental outcomes might be to perform assays on air sac lavages (ASL) rather than serum, since *Aspergillus* detection in BAL fluid was more sensitive compared with that of serum in human patients.[93,97] Additionally, the kinetics of GM release are not completely understood, even in humans. Therefore, the time frame for maximal GM levels and its relation to stage of infection needs to be determined for optimal detection. In psittacines, GM fared better with a greater association of positive GM samples and confirmed aspergillosis cases.[75,76] GM testing in canine and feline patients failed to consistently detect elevated GM levels in patients with confirmed aspergillosis.[4,36] GM detection in serum and urine on both experimentally infected and spontaneous cases of aspergillosis in cattle showed that serum GM positivity correlated with aspergillosis status and might be a useful diagnostic marker in cattle.[99]

Due to deficiencies in the GM assay (see below), research seeks to find a superior *Aspergillus* antigen for earlier, more reliable detection with lower cross-reactivity and less interference from drugs and underlying medical conditions. Recently, three immunologic assays have been developed to detect *Aspergillus*. In the first assay, a JF5 mouse MAb antibody integrated into a lateral flow device binds to an *Aspergillus* extracellular glycoprotein antigen.[100] This system is similar to a pregnancy test, has excellent potential for point-of-care aspergillosis testing, is relatively inexpensive and could benefit veterinarians at zoos, farms, and in the wild, since serum or ASL samples can be directly applied to the device to produce rapid results within minutes. In human trials, some successes and some setbacks have been found. Initial studies demonstrated superior sensitivity to GM detection,[101] but later work did not support these findings.[102] Nevertheless, the convenience of the JF5 lateral flow device might be useful if the sensitivity issue can be addressed, or lateral flow technology could be adopted into other platforms.[103]

Another lateral flow device was developed to detect aspergillosis in urine from IA animal models and human patients.[104] The study employed a murine MAb476 antibody that recognizes GM-like *Aspergillus* antigens with relatively low cross-reactivity to other fungal pathogens. However, neat urine contained an inhibitory substance that interfered with the assay, so the urine samples had to be processed before they could be used. Nevertheless, the test could detect GM-like antigens in the urine of experimentally infected guinea pigs and human patients diagnosed with possible or probable IA, but the sample size was too small to assess device performance characteristics.

An ELISA-based method was developed using antibodies against *A fumigatus* thioredoxin reductase GLiT (TR).[105] Tests in animal models and patient serum samples demonstrated that these antibodies could detect *Aspergillus*, but not better than the GM test. Active areas of research include finding *Aspergillus*-specific antigens that induce stronger and more specific immune responses.[106] Neither the lateral flow JF5 device nor the anti-TR assay has been used in veterinary research or practice.

8.2 | (1-3)-β-D-glucan

(1-3)-β-D-glucan is a nonimmunogenic component of fungal cell walls; and therefore, the BG assay relies on an enzymatic horseshoe crab coagulation cascade reaction, which produces a colorimetric output (Table 1).[91] Unfortunately, BG is a cell wall component in multiple fungal genera, and thus, the assay is not specific for aspergillosis. The reliability of BG has been tested extensively in human patients, and the key findings can be summarized with a few central points. Like the GM assay, the BG assay performance varies across patient groups. Meta-analyses place the sensitivity and specificity from blood samples in the range of 56.8%-77.1% and 81.3%-97.0%, respectively.[93] False-positive results due to cross-reactivity with cellulose have also occurred,[107] and the test, being panfungal, is incapable of determining the invading fungal species. Regardless of its deficiencies, duplicate positive tests are considered strongly indicative of an invasive fungal disease, and when used in conjunction with more specific tests for *Aspergillus*, it may provide a diagnosis.[108]

The BG assay appeared to be more suitable than the GM assay for avian aspergillosis diagnoses with an *average BG cut-off* that covered all avian species tested and that could differentiate positive and negative birds, in most instances (approximately 83%).[109] However, as with the GM assay, the results were species dependent. For

positive aspergillosis cases, the greatest *average BG levels* were seen in infected sea birds, while the lowest were observed in raptors and experimentally infected quails. Like the GM assay, the BG assay was less reliable in raptors but could distinguish sick and healthy raptors better than the GM assay.

Currently, GM and BG study results demonstrate that these methods might be useful for avian aspergillosis diagnoses, particularly in psittacine and sea birds, which warrants further studies.[98,109] However, the more extensive human studies indicate that diagnostic tests suffer from drawbacks that limit sensitivity and specificity, even under optimal conditions (Table 1). Therefore, these methods will likely never be an ideal choice to diagnose aspergillosis in birds and other veterinary species, and might still require a diagnosis using an adjuvant method. Newer point-of-care formats in lateral flow devices could bring portability, low cost, and ease of use;[100,103,104] however, the sensitivities of these devices are lower than that of ELISA-based GM assays.[102]

9 | MOLECULAR

9.1 | Polymerase chain reaction

The advent of PCR brought a sensitive and cost-effective diagnostic platform for numerous diseases including aspergillosis (Table 2).[58] Although PCR is not part of routine clinical practice for IA diagnoses, it was recently recommended for inclusion in the guidelines to diagnose at-risk patients.[93] PCR can also differentiate between *Aspergillus* species, generally based on the 18S rRNA gene, furthering its diagnostic power.[58,110] Additionally, genes conferring antifungal resistance can be targeted, in parallel, to guide treatment options.[111] PCR is rapid and amenable to massive throughput, and equipment and reagents are routine in most laboratories due to continually decreasing costs.

Despite the many advantages of PCR, this method has not been integrated into clinical diagnostic guidelines, primarily due to a lack of standardization, although evidence suggests it should be integrated.[93,110] Obstacles to routine implementation come from exogenous spore colonization, and workup steps and reagents that can introduce contaminants and cause false-positive results. Clinical samples that inhibit PCR reagents and small PCR reagent working volumes can cause false-negative results.[94,112] Nevertheless, PCR is widely used in aspergillosis diagnostics and too numerous to mention in this review.

Active PCR research examining this method as a diagnostic tool to detect aspergillosis is ongoing, and several variations have emerged, including nested PCR, quantitative PCR (qPCR), and ELISA-PCR.[110] PCR is more sensitive than GM for detection in serum but has decreased sensitivity in BAL samples.[93] Like GM or BG, combined diagnostics are needed with PCR and two consecutive tests required to make a definitive diagnosis.[95,110] Nevertheless, commercial kits are available, such as the MycAssay™ (Myconostica, Manchester, UK) based on real-time qPCR (RT-qPCR).

In contrast to the extensive reliability, specificity, and sensitivity tests performed on PCR for aspergillosis diagnoses in humans, the method is in its infancy in veterinary medicine. Nevertheless, PCR has been tested on cultured isolates from psittacines,[113] falcons,[113] ostriches,[114] penguins,[115] pigeons,[116] chickens,[117,118] and white storks;[62,119] however, these tests are still mostly in the research stages. PCR analysis and identification of micro-isolated fungal hyphae from avian lung biopsies has been tested.[62] PCR has also been tested on isolates from bovine udders, but it was not used as a diagnostic tool.[120] One crucial distinction between PCR in humans vs birds is in the sampling protocol. DNA is extracted directly from the clinical samples for PCR testing of human blood, plasma, and serum. In contrast, in birds, most PCR tests are on samples from cultured isolates,[113-119] which introduces additional steps and increases the amount of time needed for analyses. Therefore, future PCR testing in birds and mammals should adopt similar protocols to those used in human samples (ie, extracting fungal DNA directly from biological fluids).[112]

The polymerase chain reaction assay is increasingly available in many laboratories due to decreasing costs of PCR instruments and a wider availability of reagents. The advantages of using PCR to diagnose aspergillosis are numerous. Shorter turnaround times when DNA is extracted directly from samples will provide a significant benefit for making decisions with regard to treatment. In addition, the PCR platform is amenable to high-throughput with simultaneous analysis of samples, including analysis of genes that confer antifungal resistance.[111] The RT-qPCR format allows for more quantitative methods and could be used to determine fungal burden and the likelihood of IA.[58] PCR can identify pathogens at the species or strain level, which cannot be accomplished with the GM or BG assays. In addition, PCR protocols are compatible with many sample types, including blood, serum, plasma, BAL fluid, tissue biopsies, and paraffin-embedded tissues. Recently, mobile insulated isothermal PCR (iiPCR) units have become available to detect pathogens in the field. iiPCR has been successfully used on site to detect various human, avian, porcine, and equine diseases.[121] Although not field tested for *Aspergillus* detection, it may be possible to apply such technologies to detect aspergillosis in animals in the field.

PCR does have some drawbacks.[58,94,112] False positives can occur because of the ubiquitous presence of *Aspergillus* spores that could contaminate clinical samples or PCR reagents. Cross-reactivity with unknown fungal species or mis-priming could also occur. False negatives can be caused by small reagent volumes used in setting up PCR reactions, or by biological molecules in clinical samples that might inhibit PCR reagents or suboptimal DNA extraction. However, its greatest disadvantage at present is the lack of standardization among different labs that perform PCR,[93] in contrast to the GM assay, which has an internal control supplied by the manufacturer.

Lack of standardization is the main reason why PCR was not adopted into the EORTC/MSG guidelines for the clinical diagnosis of aspergillosis (European Organisation for the Research and Treatment of Cancer/Mycoses Study Group). Recently, however, a meta-analysis of PCR studies on human samples suggested that PCR sensitivity (blood: 84%-88%, BAL: 76.8%-79.6%) was superior to that of the

TABLE 2 *Aspergillus* detection methods in research stages: advantages and disadvantages

Methods	Advantages	Disadvantages
Polymerase chain reaction (PCR)	• Noninvasive • Rapid • Can identify down to species level but targeted • Can test for antifungal susceptibility by PCR for resistance genes • Sensitive • High-throughput • qPCR can be quantitative • Versatile samples: blood, serum, plasma, BAL, tissue, paraffin-embedded tissue • Cost-effective • Possible field use	• Lack of standardization • Potential cross-reactivity of PCR primers • Exogenous spores can lead to false positives • Requires cell lysis and DNA purification steps which increase chances of contamination • Low sample and reagent volumes can lead to false negatives • Potential PCR reagent inhibitors in clinical samples can lead to false negatives • Certain types of PCR require specialized probes • Cannot locate site of infection
Nucleic acid sequence-based amplification (NASBA)	• Noninvasive • Rapid • Can identify down to species level but targeted • Sensitive and quantitative • High-throughput • Fewer restrictions on primer design compared with qPCR • Reduced likelihood of carryover contamination compared with PCR • Isothermal, does not require temperature cycling • Cost-effective	• Lack of extensive studies to fully characterize capabilities • Potential cross-reactivity of NASBA primers • Exogenous spores can lead to false positives • Requires cell lysis and RNA purification steps which increase chances of contamination • RNA is less stable than DNA • Low sample and reagent volumes can lead to false negatives • Certain types of NASBA require specialized probes • Cannot locate site of infection
Loop-mediated isothermal amplification (LAMP) and rolling-circle amplification (RCA)	• Noninvasive • Rapid • Can identify down to species level but targeted • Sensitive and quantitative • High-throughput • Fewer restrictions on primer design compared with qPCR • Reduced likelihood of carryover contamination compared with PCR • Isothermal, does not require temperature cycling • LAMP is highly specific due to the need of 4 simultaneous primers for amplification • Cost-effective	• Lack of extensive studies to fully characterize method's capabilities • Potential cross-reactivity of primers • Exogenous spores can lead to false positives • Requires cell lysis and DNA/RNA purification steps which increase chances of contamination • Low sample and reagent volumes can lead to false negatives • Certain methods require specialized probes • Cannot locate site of infection
MAb JF5 lateral flow device for serum and bronchoalveolar lavage (BAL) testing	• Specific for *Aspergillus* but targeted • Rapid • Noninvasive if testing serum • Point-of-care and easy to perform • Cost-effective	• Sensitivity compared with GM and BG assays is uncertain • Potential cross-reactivity with other species • Cannot locate site of infection • Performance characteristics still unknown
MAb476 lateral flow device for urine testing	• Specific for *Aspergillus* but targeted • Noninvasive • Rapid • Point-of-care and easy to perform • Cost-effective	• Urine samples require processing and cannot be used with neat urine • Sensitivity compared with GM and BG assays is unknown • Potential cross-reactivity with other species • Cannot locate site of infection • Performance characteristics still unknown
Thioredoxin reductase GLiT (ELISA-based)	• Specific for *Aspergillus* but targeted • Noninvasive • Rapid • High-throughput • Cost-effective	• Sensitivity compared with GM and BG assays is unknown • Potential cross-reactivity with other species • Cannot locate site of infection • Performance characteristics still unknown

(Continues)

TABLE 2 (Continued)

Methods	Advantages	Disadvantages
Proteomic	• Potential to identify down to strain level • Untargeted so pathogen can be identified if it is not *Aspergillus* and reference spectrum is in the database • Mass spectrum collection is amenable to automation • Quality control methods established, standards developed • Ability to test for antifungal susceptibility during the culturing step • Potential to develop detection directly from biosamples • Low sample cost	• Time consuming at present due to culturing requirement • Contamination possible throughout the culturing procedure and sample preparation steps • Requires construction and careful curation of a database of reference spectra • Cannot identify the pathogen if a reference spectrum is not available in the database • Culturing conditions need to be carefully maintained as for database • Procurement of MALDI-TOF equipment is expensive • Cannot locate site of infection
Gliotoxin and methylated gliotoxin detection	• Noninvasive • Rapid • Sensitive and quantifiable • Standards and precise reagents known • Gliotoxin release is associated with hyphal growth, detection could coincide with early stage of infection • Amenable to automation • Low sample cost	• Not specific for *Aspergillus* since gliotoxin is released by other fungal pathogens • Relation of fungal metabolite level to time course, severity of infection, and EORTC/MSG IA status needs to be established • Sample preparation and metabolite extraction increases labor and potential contamination • Calibration required to test HPLC-MS/MS performance • Procurement of HPLC-MS/MS equipment is expensive • Cannot locate site of infection
Triacetylfusarinine C (TAFC) detection	• Noninvasive • Rapid • Sensitive and quantifiable • Standards and precise reagents known • TAFC release is associated with nutrient procurement for hyphal growth, detection could coincide with early stage of infection • Amenable to automation • Low sample cost	• Not specific for *Aspergillus* since TAFC is released by a few other fungal pathogens • Relation of fungal metabolite level to time course, severity of infection, and EORTC/MSG IA status needs to be established • Sample preparation and metabolite extraction increases labor and potential contamination • Calibration required to test UPLC-MS/MS performance • Procurement of UPLC-MS/MS equipment is expensive • Cannot locate site of infection
^{18}F-fluoro-2-deoxy-D-glucose (^{18}F-FDG) positron emission tomography/computed tomography (PET/CT)	• Noninvasive • Rapid • Functional and metabolic imaging modality offers more information over purely imaging methods • Imaging based method that can locate the site of infection and the extent of dissemination • Availability, use, and safety of ^{18}F-FDG is widely documented • PET equipment already available in clinics	• Exposes patient to low level of ionizing radiation • Sensitivity appears promising, but only a limited number of studies have been completed • Specificity unknown but is likely to be relatively low • PET is highly specialized and costly imaging equipment • PET scan is relatively expensive compared with X-ray or conventional CT

(Continues)

GM (blood: 79.3% BAL: 83.6%-85.7%) and BG (blood: IA: 56.8-77.1) assays on blood samples.[93] On the other hand, PCR specificity (blood: 75%-76%, BAL: 93.7%-94.5%) was greater than that of the GM assay (blood: 80.5%-86.3%, BAL: 89.0%-89.4%) for BALs, but was lower in specificity than that of the GM assay for blood. Accordingly, PCR has now been recommended to be included in the EORTC/MSG guidelines for the clinical diagnosis of aspergillosis.[93] Results also suggest that PCR could be a valuable tool for aspergillosis diagnosis in veterinary medicine.

9.2 | Isothermal amplification methods

Real-time nucleic acid sequence-based amplification (NASBA) is an isothermal RNA amplification method that can detect

TABLE 2 (Continued)

Methods	Advantages	Disadvantages
^{68}Ga-TAFC (gallium- triacetylfusarinine C) positron emission tomography (PET)	• Noninvasive • Rapid • Relatively specific for *Aspergillus* but targeted • Imaging based method which can locate the site of infection and the extent of dissemination • TAFC release is associated with nutrient procurement for hyphal growth, detection could coincide with early stage of infection • Differentiates between active Aspergillus growth and colonization *versus* the presence of inert spores • PET equipment already available in clinics	• Exposes patient to low level of ionizing radiation • Safety of ^{68}8Ga-TAFC administration still needs to be tested • TAFC is not unique to *Aspergillus*, therefore cross-reactivity is possible with other fungal genera • ^{68}Ga production requires very specialized radio facilities • PET is highly specialized and costly imaging equipment • PET scan is relatively expensive compared with X-ray or conventional CT • Limited studies, so sensitivity is uncertain at present
eNose detection of volatile organic compounds (VOCs)	• Noninvasive • Rapid • Possibility of point-of-care • Potential to be specific for *Aspergillus* but performance characteristics are still being determined • Untargeted, so pathogen could potentially be identified that is not *Aspergillus* • Specifically detects disease state, not just the presence of *Aspergillus* • Low sample cost • Relatively low equipment cost	• May be limited to lung infections • Cannot locate site of infection • Exogenous substances from the air/environment might contaminate samples • Confounding parameters still unknown • Environment can influence eNose performance • Calibration required to test instrument performance • Requires construction and validation of prediction models
Gas chromatography mass spectrometry (GC-MS) detection of volatile organic compounds (VOCs)	• Noninvasive • Rapid • Potential to be specific for *Aspergillus* but performance characteristics still being determined • Could potentially be performed in an untargeted format so pathogens could possibly be identified that is not *Aspergillus* • Standards and precise reagents known • Amenable to automation • Low sample cost	• May be limited to lung infections • Cannot locate site of infection • Exogenous substances from the air/environment might contaminate samples • Breath samples may require preconcentration • Confounding parameters still unknown • Calibration required to test instrument performance • A panel of metabolites unique to *Aspergillus* (or other IFD) need to be discovered in vitro first • Procurement of GC-MS equipment is expensive
Mass spectrometry (MS) metabolomics	• Noninvasive • Rapid • Potential to be specific for *Aspergillus* but performance characteristics still being determined • Untargeted, so pathogen could potentially be identified that is not *Aspergillus* • Reveals additional metabolic data which could offer insight into biological processes eg, host-guest interaction • Standards and precise reagents known • Relatively high sensitivity • Amenable to automation • Low sample cost	• Requires sample manipulation/component separation, increases chances of introducing contaminants or altering the native state of metabolites • Confounding parameters still unknown • Specificity for *Aspergillus* still uncertain • Requires construction and validation of prediction models • Procurement of GC or HPLC/UPLC or MALDI-MS equipment is expensive • Cannot locate site of infection

(Continues)

Aspergillus.[94,122-124] This technique is as sensitive as PCR with fewer primer restrictions compared with qPCR, but its ability to diagnose IA has not been evaluated in clinical medicine. *Aspergillus* detection has also been tested using isothermal DNA and RNA amplification methods, such as loop-mediated isothermal amplification (LAMP) and the rolling-circle amplification (RCA).[125] These methods are simpler and faster than PCR because they do not require temperature cycling and can also identify pathogens at the species level, but the sensitivity and specificity compared with PCR has not yet been determined.

TABLE 2 (Continued)

Methods	Advantages	Disadvantages
Nuclear magnetic resonance (NMR) metabolomics	• Noninvasive • Rapid • Semiquantifiable • Analysis performed on unadulterated, native biosamples • Analysis does not require identification of new or unique *Aspergillus* metabolites • Specifically detects disease state, not just the presence of *Aspergillus* • Reveals additional metabolic data which could offer insight into biological processes eg, host-pathogen interaction • Standards and precise reagents known • Amenable to automation • Low sample cost	• Relatively low sensitivity • Confounding parameters still unknown • Specificity for *Aspergillus* still uncertain • Requires construction and validation of prediction models • Procurement of NMR equipment is expensive • NMR equipment needs expensive routine maintenance • Cannot locate site of infection

10 | PROTEOMICS BY MASS SPECTROMETRY

The -omics era has ushered numerous novel techniques,[126,127] among them proteomics, which can simultaneously analyze all proteins and protein fragments in biological samples. Usually, the resultant spectra from proteomic inquiries are unique "fingerprints" of a particular sample that can be used for identification purposes when compared with a reference database (Table 2). Mass spectrometry (MS), principally MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry), is the most commonly used method. The mass spectrum fingerprint is so unique that pathogens can be identified down to the species and even the strain level (Figure 2), which has been verified in *Aspergillus* and other fungal pathogen cultures, and in isolates cultured from patient samples.

The first MALDI-TOF MS to characterize *Aspergillus* were performed on spores.[128] The resultant spectra were simple with few peaks, but each peak was distinct for each of the four *Aspergillus* species tested. Later methods used preextraction steps to enrich the resultant spectra, which enabled the characterization of additional species. A study involving twelve different *Aspergillus* species demonstrated a 100% and 95%-100% species and strain prediction, respectively, reaffirming the potential of MALDI-TOF MS as a diagnostic tool.[129] A modification of MALDI-TOF MS, surface-enhanced laser desorption ionization (SELDI), which includes additional sample adsorption and washing steps, could discriminate between *A fumigatus* and *A lentulus*, two very closely related species, which are difficult to resolve by current clinical methods.[130] Despite similarities, *A lentulus* is naturally resistant to several antifungals; therefore, knowing which strain is involved in an infection can guide appropriate treatment.

To establish the utility of MALDI-TOF MS in clinical scenarios, a series of studies were conducted on clinical isolates from patients.[131-140] In these studies, known fungal species including genera other than *Aspergillus* were used to construct databases, and clinical isolates were compared against these databases for classification. To unambiguously assign and verify species, designations were generally accomplished by performing DNA sequencing on a combination of: (i) β-tubulin, (ii) calmodulin, (iii) actin, and (iv) the internal transcribed spacers, ITS1 and ITS2, that flank the 5.8S ribosomal DNA (ITS1–5.8S–ITS2). MALDI-TOF MS could consistently predict the fungal species from patient-derived isolates, if a reference spectrum was available.[131-140]

MALDI could identify common *Aspergillus* species more frequently than the rarer ones. However, when reference spectra from rarer species was included, MALDI-TOF MS was better able to characterize species.[134,136-138] In the past few years, numerous multihospital studies in several countries have shown the clinical application of this technology and the potential for standardization. MALDI-TOF MS could correctly determine fungal species and even strains, when a reference spectrum was available.

MALDI-TOF MS is very sensitive and only requires minute sample quantities.[94] Spectra collections are rapid and amenable to high-throughput and automation. As long as a reference spectra is available, MALDI-TOF MS can not only determine whether or not an infection is caused by *Aspergillus* but it can also identify other pathogens that could be causing invasive fungal disease (IFD). Despite numerous advantages of MADI-TOF MS, the need to culture clinical isolates lengthens the overall process prior to analyses and also increases the possibility of introducing contaminants. MALDI-TOF MS pathogen detection directly from blood cultures has been moderately successful for microorganisms other than *Aspergillus*, so there is potential to move past the need for a culturing step if methods can be adequately developed.[141-143]

In addition, clinical isolates cannot be identified unless comparative reference spectra exist; therefore, database collections are required, which include strain and geographical variants.[94] Because of increased sensitivity, minute changes in culturing and solvent extraction procedures will impact the spectra. So after a database is constructed, care must be exercised to culture clinical isolates and prepare samples in exactly the same manner as the references. Accurate identification also requires careful instrument calibration and selection of appropriate standards. Many of these challenges are technical and can be overcome with careful standardization protocols. Finally, although high costs are associated with MALDI-TOF MS

purchase and maintenance, this technology shows great promise in aspergillosis diagnosis, which could be applicable to human, avian, and other veterinary patients.

11 | METABOLIC

Recently, a host of methods have emerged based on metabolites of aspergillosis (**Table** 2).[144] These techniques are made possible by the primary and, in some instances, unique and rich secondary *Aspergillus* metabolisms.[145] *Aspergillus* infection can leave traces in the host by the release of *Aspergillus*-specific metabolites or mixtures of metabolites.[145] Alternatively, infections can initiate changes to the host's metabolome, which can be detected.[127,146] In both instances, cutting-edge analytical methods have been developed to detect the "signature" of pathogen infection. At present, these methods are not used in clinical medicine, but research on animal models and patient samples have shown promise for use in veterinary settings.[144]

11.1 | Secondary metabolite detection

Germinating spores of *Aspergillus* and a few other fungi secrete mycotoxins. Gliotoxin is a mycotoxin secondary metabolite and a potent immune suppressor.[147-150] Therefore, gliotoxin detection could coincide with the early stages of infection and serve as a diagnostic tool for aspergillosis. Earlier methods of gliotoxin detection were semiquantitative biological assays,[149] but more accurate analytical methods were introduced for accurate and precise quantitative evaluations.[147,148,150]

One study performed high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to quantify gliotoxin with accuracy and sensitivity according to analytical standards for setting detection limits.[147] HPLC-MS/MS was used to detect gliotoxin in human serum and was compared with the GM assay results performed in tandem. The authors discovered that most serum samples negative on GM assay were also negative for gliotoxin.[147] However, serum samples positive for GM were also often negative for gliotoxin, which could have been caused by false-positive GM results or diminishing levels of gliotoxin in the later stages of infection, when fungal burden and hence GM levels were greater. Since the study did not score patient serum samples according to the EORTC/MSG criteria for IA diagnoses, it was not possible to ascertain whether false-positive GM results were at play or if patients were in the later stages of IA.

Another plausible explanation was that the instability of gliotoxin in vivo was due to its chemical structure, which contains a reactive disulfide bond. Another study sought to address this possibility by measuring the levels of bis(methylthio)gliotoxin (bmGT), a gliotoxin derivative of greater stability due to methylation of the disulfide bond, by high-performance thin layer chromatography (HPTLC).[148] The study showed that bmGT levels were better at determining IA

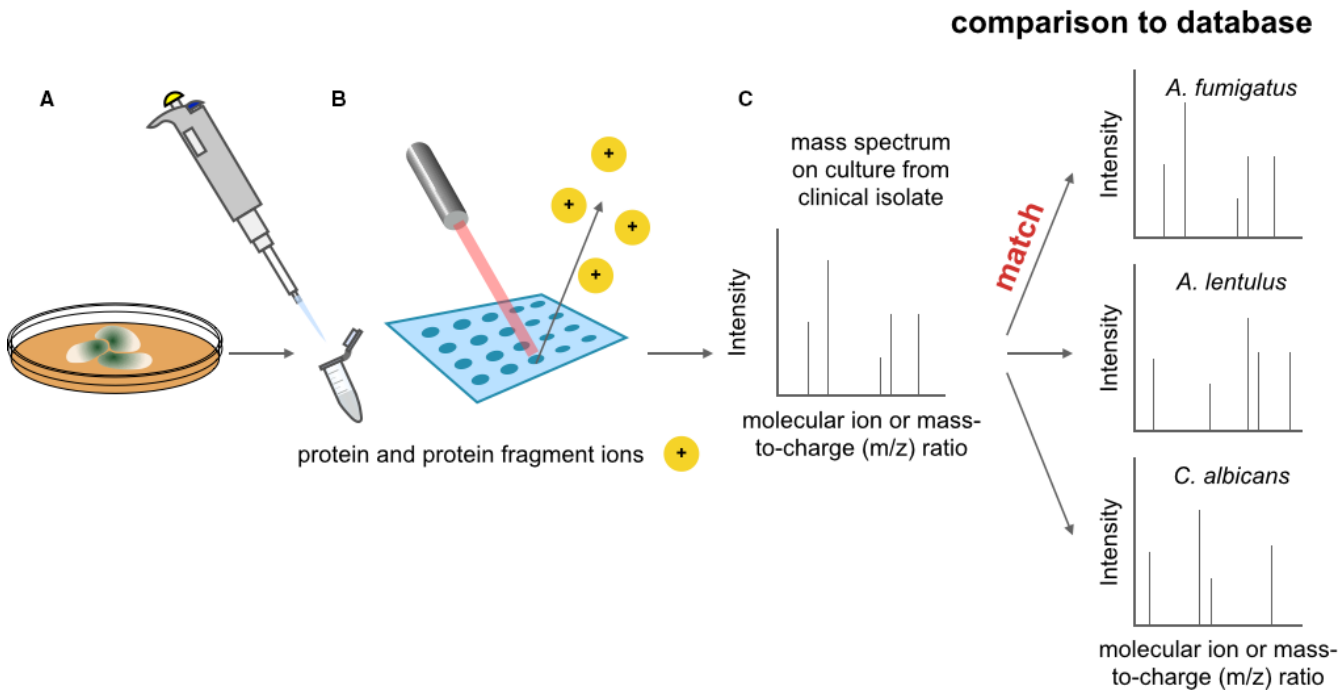

FIGURE 2 Illustration of the work-flow for proteomic analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. A, Pathogenic fungi are cultured from clinical isolates. B, A mixture of spores, conidia, and/or mycelia is collected from the colony surface and mixed in a small volume of solvent. The sample is applied to a target-plate where the MALDI laser will ionize the sample, and embedded in a matrix, a material to aid ionization. Sample ions travel through the mass spectrum machine where they are separated by size to produce a mass spectrum. C, The mass spectrum collected for the clinical isolate is matched to a spectrum from a database of known fungal pathogens. Identification is not generally possible if a reference spectrum is not available, and care must be taken to culture clinical isolates in a similar manner to strains used to construct the database

status than GM levels measured with the GM assay; however, the two diagnostic tests used together had a positive predictive value of 100%, while almost completely avoiding false negative results.

Gliotoxin was also examined as a biomarker of aspergillosis in several veterinary medical studies on birds and other veterinary species, with detection in cattle udder tissues,[151] avian lung and air sac tissues,[152] and turkey poult, and chicken lung tissue samples;[117,153] however, these studies did not include an in-depth analysis of gliotoxin levels as a diagnostic tool. Gliotoxin testing of *Aspergillus* cultured from clinical isolates from chickens,[117] turkeys,[154] and cattle[120] has also been performed, but similarly, the gliotoxin levels were not measured.

Triacetylfusarinine C (TAFC) is another secondary metabolite that has been used as a biomarker of *Aspergillus* infection. It is a siderophore that scavenges iron for fungal growth during infection.[155] TAFC is relatively unique to *Aspergillus* and has, therefore, been proposed as a method to predict aspergillosis.[155] Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) has quantified TAFC in human serum, and levels were found to positively correlate with GM levels (≥0.5 index threshold).[155] Interestingly, TAFC was present more often in suspected IA cases than in probable/proven IA cases, which the authors suggested could have arisen because probable/proven cases received antifungal treatments. In addition, some serum from patients with suspected IA were negative for GM but positive for TAFC, indicating that either false negative GM results were to blame, or that siderophore secretion might be an early event and occur before the onset of significant fungal burden.

Ergoline alkaloid fumigaclavine A (FuA), a major mycotoxin produced by *A fumigatus*, has been detected in falcon blood and respiratory tissue from a broad variety of avian birds using an enzyme immunoassay (EIA).[156] Fumigaclavine alkaloids are generated in vivo by *A fumigatus* during clinical avian aspergillosis. However, the function of these secondary metabolites in disease pathogenesis remains unknown.

At present, most serum metabolite quantification studies have been limited to human studies or research studies using animal models.[147-150,155] Studies in animals and birds are limited to biopsy or necropsy tissue samples, or from isolate cultures that have much less diagnostic value.[117,120,151-154] Nevertheless, the precise relation that gliotoxin, bmGT, and TAFC concentrations have to the detection of aspergillosis and its infection stages remains to be resolved and will be necessary for future clinical diagnostics in human and veterinary medicine.

11.2 | Metabolite uptake

Radiolabeled metabolites combined with positron emission tomography (PET) have been investigated as potential diagnostic tools for an aspergillosis diagnosis. A series of studies using ^{18}F-fluoro-2-deoxy-D-glucose (^{18}F-FDG) demonstrated possible diagnostic utility,[157-159] but the full benefits and pitfalls remain to be elucidated. ^{18}F-FDG is taken up by pathogens and immune cells that accumulate at sites of infection, and constitute the basis for identifying lesions.[160] Compared with purely anatomical CT, functional and metabolic imaging by ^{18}F-FDG/PET could identify all fungal lesions detected by CT, in addition to smaller nodules not revealed by CT.[157] A combination of CT and ^{18}F-FDG/PET could differentiate between invasive and noninvasive pulmonary aspergillosis, depending on the pattern of ^{18}F-FDG uptake in lesions.[158] In addition, ^{18}F-FDG/PET can be adopted to monitor recovery following treatment.[159]

Despite several advantages over CT and MRI, such as potentially greater sensitivity and detection of smaller nodules, the specificity of ^{18}F-FDG/PET for aspergillosis diagnosis is still uncertain, and it may be more useful in conjunction with more targeted tests such as the GM assay or PCR. ^{18}F-FDG uptake is nonspecific; and therefore, false positives are possible. In addition, ^{18}F-FDG uptake can cause misdiagnosis, as in a case of aspergillosis that was initially diagnosed as lung cancer.[161] To address the specificity issue, TAFC radiolabeling was tested as a potential PET tracer for aspergillosis diagnoses, because TAFC is a unique metabolite of *Aspergillus*.[162] Since TAFC is an iron scavenger, and gallium (Ga) and iron have similar chemical properties, complexes of ^{68}Ga-TAFC could be prepared. After ^{68}Ga-TAFC was administered, it was rapidly eliminated from healthy animals, but was retained in the lungs of rats with IA in a manner dependent on the severity of infection. Rats that were challenged with *Aspergillus* spores but did not develop IA, did not absorb ^{68}Ga-TAFC, suggesting that ^{68}Ga-TAFC intake was specific to active *Aspergillus* growth and division in the early stages of infection. Specificity studies demonstrated that *A fumigatus* absorbed ^{68}Ga-TAFC and took up another siderophore, ^{68}Ga-FOXE (ferrioxamine E), better than most other fungi and microorganisms, except for *S aureus*.[163] While ^{68}Ga-TAFC had higher specificity, ^{68}Ga-FOXE demonstrated greater sensitivity. Although preliminary, ^{68}Ga-TAFC studies are promising, but more are required. Moreover, potential toxic side effects of ^{68}Ga-TAFC or ^{68}Ga-FOXE administration will need to be determined, even though the use of gallium-68 in nuclear medicine is well established.[164]

As a diagnostic tool, PET technology with/without CT can identify the spread of infection to sites other than the lungs to determine the extent of *Aspergillus* dissemination. However, patients are exposed to radiation, which is a significant drawback, and animals and birds would also require sedation or anesthesia. Until now, ^{18}F-FDG/PET for aspergillosis diagnosis has only seen limited tests in humans, while ^{68}Ga-TAFC or ^{68}Ga-FOXE administration have only been examined in research animals with no medical or veterinary applications. However, since PET can be performed on sedated animals, this method may eventually be used for aspergillosis diagnosis of birds and other veterinary species.

11.3 | Volatile organic compounds

Several *Aspergillus* metabolites are volatile organic compounds (VOCs) that can be detected in the breath of patients with pulmonary aspergillosis.[165] Breath collection is noninvasive and is an ideal sample collection method for critically sick patients. Earlier works focused on VOCs, such as 2-pentylfuran, which were

relatively unique in *Aspergillus* compared with other fungal species, and which were also not part of the normal human metabolism. 2-pentylfuran was found to be elevated in patients suffering from aspergillosis and was suggested as a potential test.[166] However, this metabolite is also found in certain foods, and consumption could confound the test results.[167] Newer methods focus on VOC combinations that can be more predictive of or unique to aspergillosis.

Volatile organic compound detection can be achieved using eNose technologies, which are relatively inexpensive, portable electronic devices.[168-170] An eNose contains a series of sensors that respond physically, eg, registers a change in electrical resistance, upon the adsorption of volatile metabolites from VOC mixtures. Each volatile metabolite produces a response in the sensor array, and the mixture of VOCs produces a cumulative signal that is unique to a particular VOC mixture (Figure 3).[168,169] eNoses have been tested on breath collected from patients with prolonged chemotherapy-induced neutropenia (PCIN)[171] and cystic fibrosis (CF)[172] for whom aspergillosis was independently assessed by other methods (EORTC criteria and sputum culture, respectively). Breath collected from both uninfected controls and patients with probable or proven IA was analyzed using a Cyranose 320 eNose device (Sensigent, Baldwin Park, CA, USA). The resultant "breathprints" were statistically analyzed and used to create prediction models. The eNose device could predict aspergillosis status with a cross-validation of 89%, which demonstrated that the eNose device could predict IA in the presence of two different underlying diseases, PCIN[171] and CF.[172] Early detection is also possible if a prediction model can be built to detect the inflammatory response mounted against inhaled and germinating *Aspergillus* spores. In addition, the eNose device was trained to specifically recognize aspergillosis over other IFDs in an

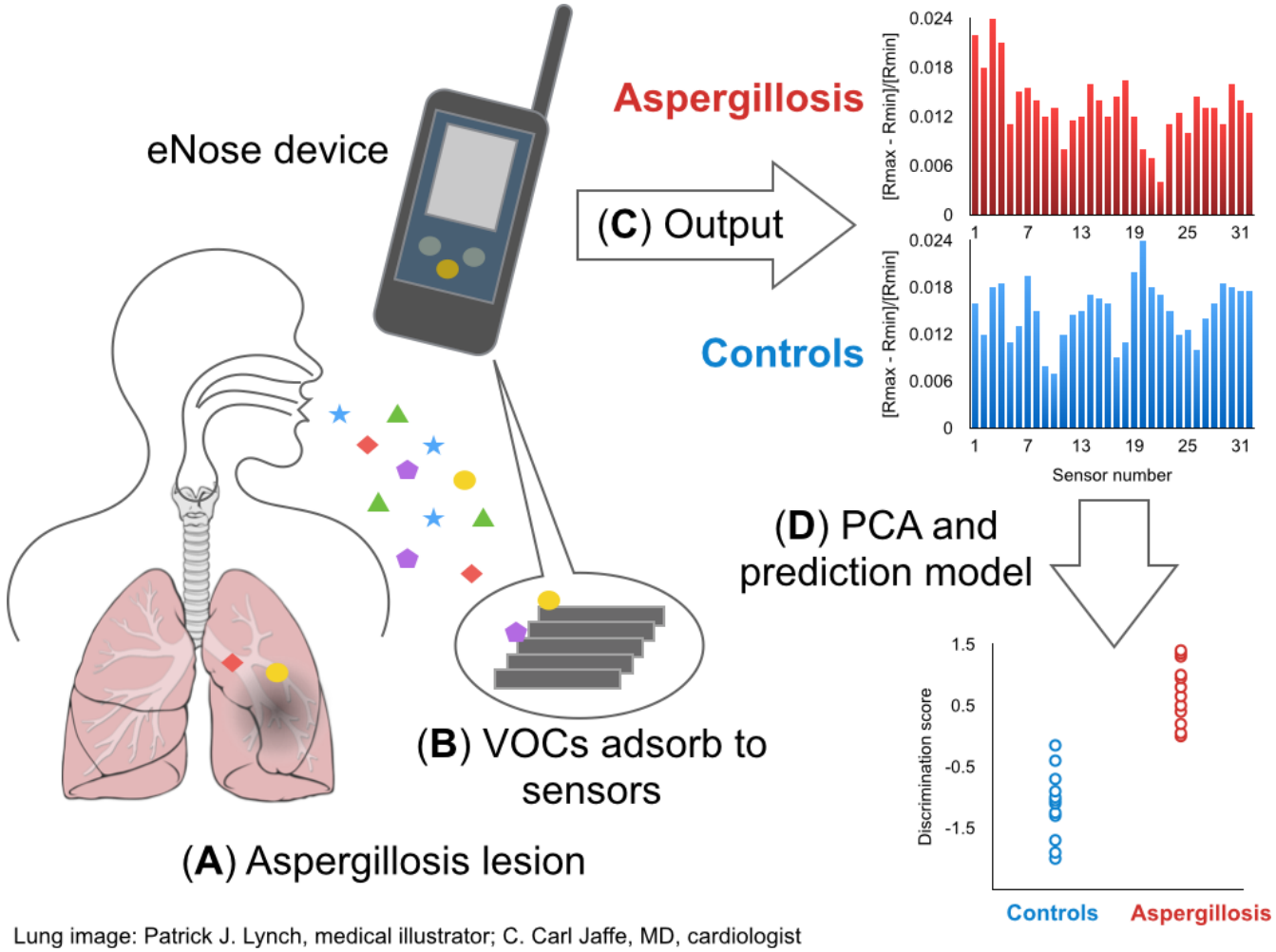

FIGURE 3 Graphic representation of the eNose operation. A, Aspergillosis lesion results in volatile organic compounds (VOCs) production, either due to metabolites from *Aspergillus* or from the host inflammatory response. B, Exhaled breath from patients containing VOCs is applied to the eNose. Each sensor within the eNose sensor array will have a physical response upon adsorption of components from the VOC mixtures, eg change in electrical resistance. The sensor array response is the cumulative effect of all sensors within the array and will vary depending on the unique combination of VOCs in the sample, which will differ between controls and patients with aspergillosis. C, Sensor outputs are recorded for all controls versus all IA cases for each sensor within the array as a function of change in sensor response. D, All collected data is analyzed by principal component analysis (PCA) and used to build a prediction model which is cross-validated. Blue: control patients, red: *Aspergillus*-infected patients

in vitro proof-of-concept study,[173] and the same principles could be applied to detect other IFDs.

Although the eNose technology for aspergillosis diagnoses has not reached clinical medicine, it has great potential for both medical and veterinary practices.[174-179] Also, since some eNose devices are handheld, point-of-care diagnostics are possible and ideal for use in field, zoo, and farm locations. Nevertheless, methods need to be standardized to apply eNose technology to human and veterinary medicine.

Volatile organic compounds can also be identified and quantified using gas chromatography mass spectrometry (GC-MS).[180] One study identified an *A fumigatus* VOC signature in vitro that was defined by a unique combination of terpenes compared with that of other *Aspergillus spp*. Then, an in vivo study identified two additional terpenes unique to aspergillosis, which resulted in a distinctive panel of eight terpenes.[180] In this same study, 64 patients were recruited, and their IA status was independently determined according to EORTC/MSG guidelines. Breaths were evaluated using GC-MS, and the eight terpenes characteristic of *Aspergillus* were identified and represented by a heat map. The data were statistically analyzed to predict IA status as compared with IA statuses according to the EORTC/MSG guidelines. GC-MS analysis correctly predicted 60 of the cases with 94% sensitivity and 93% specificity.[180]

Like eNose technology, GC-MS is also noninvasive; however, more specialized and expensive equipment is needed. Additionally, preliminary in vitro studies are required to identify unique metabolite mixtures, which might not translate to in vivo analyses. Although not used clinically, this preliminary study suggests that GC-MS could potentially be used to diagnose IA in human medicine. It could also be adapted to veterinary medicine if this eight terpene *Aspergillus* "signature" panel is present, or if another panel could be determined.

11.4 | Metabolomics

Like proteomics, metabolomics is another -omics technology that detects all metabolites in biological samples,[181] and can identify metabolites or mixtures unique to infecting pathogens or detect changes in infected host metabolic profiles that can be adapted to infectious disease diagnostics.[146,182] The most common metabolomic detection methods are nuclear magnetic resonance (NMR) and MS.[181] NMR is advantageous because it can be directly applied to biological samples in their native states, and is usually performed on liquid samples such as blood and serum,[183] although the technology for solid tissue sample analysis is forthcoming.[184] Organic solvents are needed to extract metabolites from biological samples for MS. The metabolites also need to be separated with either gas chromatography (GC) or high- or ultra-high-performance liquid chromatography (HPLC or UPLC).[185-187] Although MS has greater sensitivity, NMR has the advantage of detecting signals from all metabolites simultaneously in complex biological mixtures without requiring sample manipulation, which may alter the native state or introduce contaminants (Figure 4).[181]

Metabolomics experimentation requires data collection, digitization, and subsequent analyses using multivariate statistics of (i) the integral values of the NMR spectral peaks (ie, area under the peaks), and (ii), the intensities of the ion chromatogram peaks for the MS experiments. In this manner, systematically varying metabolites between healthy and disease biosamples can be identified. Since the pioneering metabolomics work of Jeremy K. Nicholson and his team at the Imperial College London, several studies on human metabolic phenotyping have been performed for disease diagnosis and prognosis.[188] However, this technology has not been as widely explored in IA diagnostics, although a pilot NMR study did investigate falcon aspergillosis in the Middle East.[189] In this study, NMR metabolomics were applied to falcon blood samples (gyrfalcons and gyr-x peregrine hybrids) and classified as either clinically healthy or confirmed to have aspergillosis.[189] Statistical analysis of resultant spectra clustered healthy and sick falcons into separate groups, indicating that each group possessed a distinct metabolic profile. The NMR peaks that contributed the most to the differences in the profiles were assigned to 3-hydroxybutyrate, which was shown to be greatly increased in raptors suffering from aspergillosis. Although this exploratory study is still in the early stages, the technique shows promise as a tool for aspergillosis diagnoses in human and veterinary medicine.[144,189]

12 | OUTLOOK AND FUTURE PERSPECTIVE

Despite the numerous diagnostic methods for *Aspergillus* detection and various guidelines available, the accurate and timely diagnosis of aspergillosis in humans, birds, and other veterinary species remains challenging. Most methods require additional analyses or the application of two or more different methods to improve the accuracy and sensitivity of a diagnosis. Therefore, none of the existing clinical techniques are ideal diagnostic methods, which should be: (i) capable of early detection, (ii) accurate and sensitive, (iii) minimally invasive, (iv) cheap, (v) robust, (vi), rapid, and (vii) ideally in a point-of-care format. A method that embodies all of these features would allow timely and accurate diagnoses so that appropriate treatments could be selected, thus improving patient prognoses. Conversely, more accurate diagnostics would result in fewer patients receiving unneeded fungal treatments, thus reducing the potential toxic side effects of antifungal drugs and decreasing the spread of resistance and cost of inpatient care in human and veterinary medicine.

At present, several promising methods used to diagnose aspergillosis are in the research stages of development. As mentioned, proteomic profiling of *Aspergillus* cultured from clinical isolates has exhibited high accuracy and sensitivity when using appropriate protocols and when reference spectra are available. Other methods are broad, ranging from precise, quantifiable techniques (GC-MS, HPLC-MS/MS, UPLC-MS/MS and NMR) to less expensive handheld portable eNose devices to more conventional medical diagnostic imaging by PET. Each offers advantages over existing methods which could help in making aspergillosis diagnoses in human and veterinary medicine. In particular, noninvasive methods, such as NMR metabolomics, VOCs, and serum metabolite

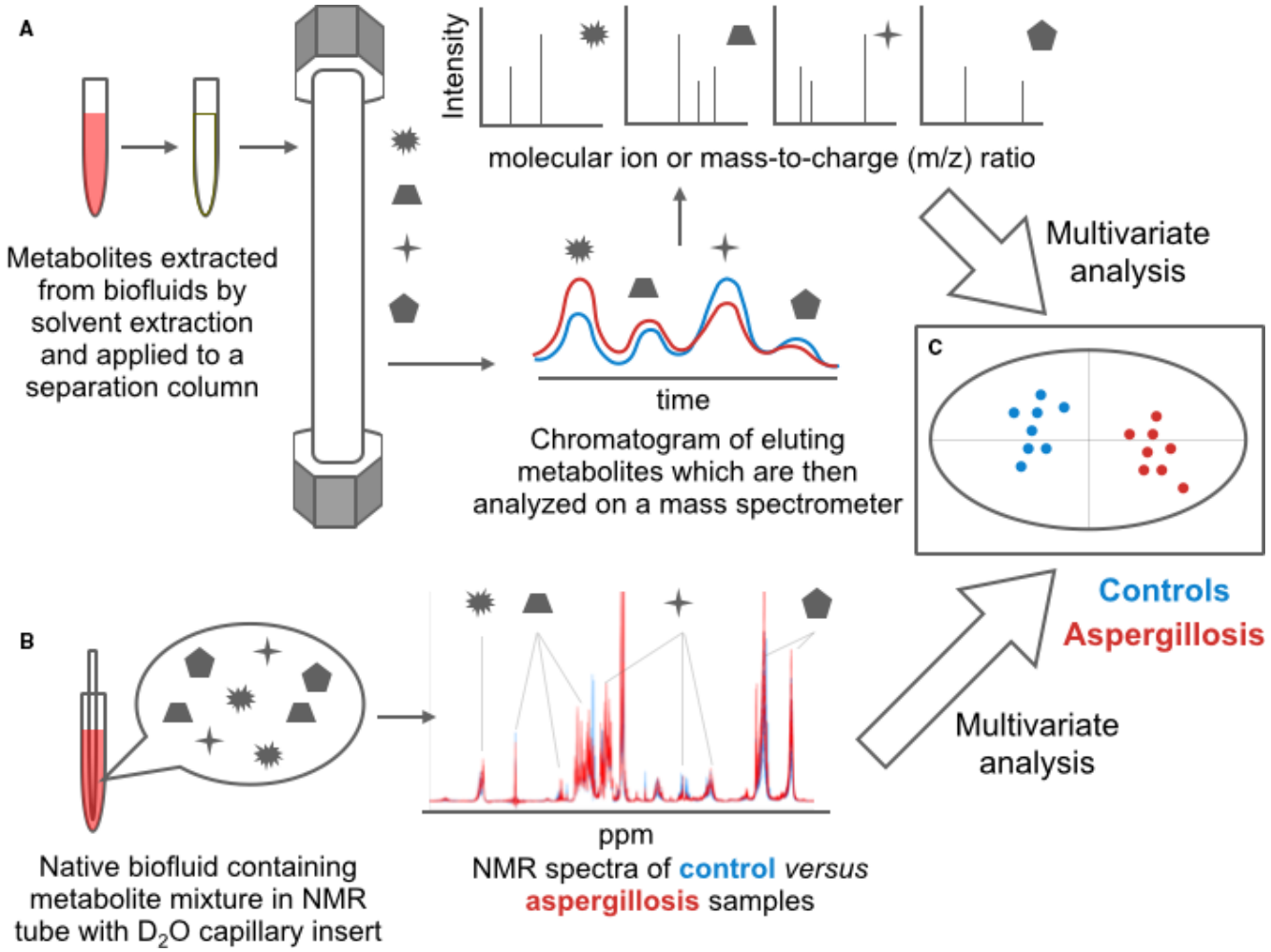

FIGURE 4 Schematic of metabolomics analysis by mass spectrometry (MS) and nuclear magnetic resonance (NMR). A, Biological samples are collected from patients. Depending on the MS technique employed, metabolites are preconcentrated or solvent extracted before separation by GC (gas chromatography) or HPLC (high-performance liquid chromatography). Each metabolite is then serially analyzed on a mass spectrometer, producing a spectrum where peaks correspond to metabolite molecular masses or mass-to-charge ratios. The combination of peak position during GC or HPLC separation with mass or mass-to-charge ratio is compared with known library compounds to identify sample metabolites. Relative, semiquantification can be obtained from areas under the extracted ion chromatogram peaks. B, Biological fluids from patients are directly loaded into NMR tubes without any treatment, and a D_2O (deuterated water) capillary is inserted. An NMR instrument collects a 1D ^{1}H-NMR spectrum of the biofluid that simultaneously records signals (peaks) of all the metabolites present while suppressing the ^{1}H signal from the water. The NMR spectrum contains peaks whose positions (ppm: parts per million) and splitting patterns correspond to metabolite structures that are matched to known libraries to identify metabolites present in the biofluid. Relative, semiquantitative information can be extracted from areas under the NMR spectrum peaks. C, All collected data are subjected to multivariate statistical analysis (principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), or orthogonal PLS-DA (O-PLS-DA), etc.) to reveal the distinct metabolic profiles from spectra collected on control versus patients with aspergillosis. Blue: control patients, red: *Aspergillus*-infected patients

analyses, and PET imaging would benefit patients too ill to undergo more invasive procedures, such as biopsy or ASL.

Further research is anticipated to bring about early, accurate, sensitive, rapid, and robust aspergillosis diagnoses in humans, avian, and other veterinary species.

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