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Inflammatory marker profiles in an avian experimental model of aspergillosis

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Aspergillosis is a common infection in avian species, but can be a challenge to diagnose. Inflammatory markers have been successfully used in mammals for diagnostic and monitoring purposes of various diseases. The aim of this study was to identify inflammatory markers that could aid in the diagnosis of aspergillosis in an avian species. Five-week-old Japanese quail were infected experimentally with *Aspergillus fumigatus*, and inflammatory markers were measured in plasma. In addition, lung tissues were cultured to quantify the fungal burden. Infected quail had higher plasma levels of ceruloplasmin, unsaturated iron-binding capacity (UIBC), iron, and total iron-binding capacity (TIBC), and lower levels of haptoglobin, compared with uninfected controls. There were positive linear relationships between *A. fumigatus* colony-forming units cultured from the lungs of infected quail, and levels of ceruloplasmin, UIBC, and TIBC. Quail that died prior to the end of the experiment (day 10 post-infection) had higher ceruloplasmin, UIBC, and TIBC, and lower haptoglobin levels than infected quail that survived. The inflammatory marker profile in quail infected with aspergillosis in this study differs from that seen in mammals, and from the pattern of inflammatory markers seen in birds with bacterial infections. Inflammatory markers could prove useful for diagnosing aspergillosis in birds, and for monitoring disease progression in infected avian species.

Keywords Aspergillosis, inflammatory markers, acute phase proteins, avian, experimental infection

Introduction

Aspergillosis is a common respiratory infection in companion birds and wild birds in rehabilitation centers [1,2] that is difficult to diagnose owing to its non-specific presentation. A definitive diagnosis of aspergillosis infection in avian species requires a combination of positive fungal culture and histopathology or cytology [3]. Less invasive diagnostic tests would be preferable, particularly for very ill birds that cannot withstand stressful diagnostic procedures.

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Detection of specific markers (e.g., antigen, antibody, nucleic acids) have been studied in mammalian species [4] and could augment the diagnostic capabilities in avian aspergillosis, but studies in birds (i.e., *Aspergillus* specific antibody titers, galactomannan antigenemia, and beta-D-glucan) indicate that these tests are currently not sensitive and/or specific enough to be considered diagnostic without confirmation by other testing methods [3,5,6].

Inflammatory markers, such as acute phase proteins (APPs), are increasingly used in veterinary diagnostics [7]. APPs are produced primarily by the liver and are up- or down-regulated in response to infection, injury, or stress. In mammals, several individual APPs are involved in host defense against *Aspergillus* [8–12]. Although there is less information available for avian species, there is evidence from protein electrophoretic studies that alterations in APP

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concentrations might occur in response to aspergillosis [13,14]. However, changes in protein fractions separated by protein electrophoresis are non-specific indicators of disease or injury, whereas a panel of several individual APPs could provide a greater degree of diagnostic specificity, as shown by several studies examining the diagnostic usefulness of APPs in mammals with aspergillosis, as well as a number of other diseases or disorders [12,15,16].

In the current study, five-week-old Japanese quail (*Coturnix japonica*) were infected experimentally with *Aspergillus fumigatus* conidia and six inflammatory markers were measured to assess their value for identifying and evaluating aspergillosis infections in birds.

Materials and methods

Bird care and housing conditions

Japanese quail were hatched and raised to the age of five weeks at the University of California, Davis Avian Sciences facility, CA, USA. At five weeks of age, quail were transferred to indoor animal rooms and housed individually in $8 \times 8 \times 14$-inch battery cages. Temperature was held constant and food (Layena, Purina Mills, St Louis, MO, USA) and water were available *ad libitum*. All procedures and conditions were in accordance with the University of California Institutional Animal Care and Use Committee guidelines.

Experimental infections

Two sets of experimental infections were conducted. *Aspergillus fumigatus* 10AF conidia were harvested from growth on potato dextrose agar in 0.05% Tween 80-saline as described previously [9,17]. In the first experiment, five-week-old Japanese quail ($n = 40$) were inoculated intratracheally (IT) with *A. fumigatus* conidia using a 27 gauge intravenous catheter with the stylet removed as described previously [18]. Inoculum doses administered via the catheter were 5×10^6, 8.2×10^6, 4.7×10^7, or 8×10^7 conidia per bird ($n = 10$ birds at each inoculum). In addition, eight quail were infected with 4.7×10^7 conidia using a stainless steel 19 gauge atomizer (Microsprayer model 1A-1B, Penn-Century, Wyndmoor, PA, USA). Only one inoculum dose was given with the atomizer in the first experiment, as an initial test of this inoculation method. Inocula were suspended in 100 µl Tween 80-saline. Twelve birds were not infected and served as controls.

In the second experiment, all inoculations were given IT via the atomizer. Two inoculum doses were included: 5×10^6 or 1×10^7 conidia per bird in 100 µl saline ($n = 21$ each). Also included were four uninfected control birds.

Birds were monitored three times daily and mortalities or signs of illness were recorded. Birds found in severe respiratory distress were humanely euthanized with an overdose of pentobarbital given IM. At 10 days post infection, all remaining birds were euthanized and necropsied. All birds were submitted for necropsy and histopathology.

Sample collection

Blood samples (approximately 1 ml per bird) were taken by jugular venipuncture prior to euthanasia. Blood was transferred to heparinized tubes and centrifuged. Plasma was separated and stored frozen at −80°C until analyses were performed.

Following euthanasia, the respiratory tract (from the proximal trachea to the pulmonary parenchyma, excluding the air sac membranes) was collected aseptically. No portions of the air sac membranes were collected owing to difficulties in uniformly collecting the same amount of membrane for culture.

The majority of the lung was collected for culture of *A. fumigatus*. Lung tissue was weighed and placed into 10 ml sterile saline containing 100 units of penicillin and 100 µg of streptomycin per ml. Small sections of the right and left lung lobes were collected for histology. Tissues collected for histology were fixed in 10% neutral buffered formalin and paraffin embedded. Four micrometer thick sections were cut, stained with hematoxylin and eosin, and evaluated for lesions consistent with aspergillosis.

Fungal culture

Fungal cultures were performed on the lung tissues of all quail euthanized on day 10 post-infection. Fungal burden in the lung tissues of infected quail was quantified by determining colony forming units (CFU) [9,17–19]. In brief, lung tissues were mechanically homogenized using a tissue homogenizer (Tissumizer, Tekmar, Vernon, BC, Canada) and serially diluted 10-fold. Aliquots were plated in duplicate on Sabouraud dextrose agar containing 50 mg of chloramphenicol per L, and incubated at 37°C for 24–48 h. Colonies were counted and the number of CFU per gram of lung tissue determined. The lower limit of the assay is approximately 10 CFU per tissue.

Inflammatory marker assays

Five inflammatory markers, haptoglobin, unsaturated iron-binding capacity (UIBC), total plasma iron, ceruloplasmin, and mannan-binding lectin (MBL)-dependent complement activity (hereafter ‘MBL/complement’) were measured in blood plasma using functional assays. Total iron-binding capacity (TIBC), which is the sum of

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plasma iron and UIBC, was also included as a sixth measure. Briefly, haptoglobin was measured using a commercially available kit (Haptoglobin PHASE colorimetric assay Tridelta Development Ltd, Maynooth, County Kildare, Ireland) according to the manufacturer's instructions, with one alteration: reactions were carried out in Eppendorf tubes and centrifuged to remove precipitates prior to transfer to a 96-well plate. UIBC and plasma iron were measured using a commercial kit (Teco Diagnostics, Anaheim, CA, USA), scaled down for use in 96-well plates. Plasma ceruloplasmin was measured following the method of Kim and Combs [20]. MBL/complement was measured following the hemolytic method of Kuipers et al. [21], with the exception that sheep RBC were used rather than chicken RBC, and MBL-deficient serum was not added as a source of complement components. Therefore, the assay assessed the complete MBL-complement pathway function of the components present in the sample plasma.

All samples were run in duplicate. Inter-assay and intra-assay coefficients of variation were as follows: plasma iron, 13.1%, 6.4%; UIBC, 14.7%, 16.8%; MBL, 13.2%, 1.3%; haptoglobin, 5.8%, 6.0%; ceruloplasmin 16.8%, 10.1%, respectively.

Statistical analyses

Statistical analyses were carried out in the R Statistical Programming Language [22], with the exception of diagnostic sensitivity and specificity analyses, which were carried out using MedCalc for Windows, version 12.2.1.0 (MedCalc Software, Mariakerke, Belgium). When the distribution of dependent variables did not approximate a normal distribution, transformations were used that produced the closest approximation to normal. Inflammatory markers were compared between control and infected groups, and between infected birds that survived and those that died, using two-tailed *t*-tests. The relationship between inflammatory markers and CFU was estimated using linear regression models. A result was considered statistically significant if $P < 0.05$.

Results

Mortality rate in experimentally infected quail

In experiment 1, the mortality rate by day 10 post-infection differed according to inoculation method and dose. The highest mortality rate (7/8 or 88%) was seen in the group infected with 4.7×10^7 conidia via the atomizer. The two highest inoculum doses given via catheter (8×10^7 and 4.7×10^7) resulted in mortality rates of 40% and 30%, respectively; there was no mortality in birds infected via catheter with the two lowest inoculum doses (5×10^6 and 8.2×10^6).

In experiment 2, mortality was 57% at the 5×10^6 inoculum dose given via the atomizer, and 71% at the higher 1×10^7 dose, also given via the atomizer.

Confirmation of aspergillosis by culture and histopathology

Lungs from 45 experimentally infected quail that were still alive on day 10 post-infection, and four uninfected control birds, were cultured to test for the presence of *A. fumigatus*. All control birds were culture-negative. Of the 45 experimentally inoculated quail, 40 had positive fungal cultures and five had no detectable infection. There were no obvious commonalities among the five inoculated birds that did not become infected, as they came from both experiments, and both inoculation methods and doses ranging from 5×10^6 to 8×10^7 were represented in this group. Four of these five culture-negative birds had inflammatory markers similar to those of control birds, while the 5th bird had some elevated inflammatory markers (data not shown). One of these birds was submitted for histology and had no lung or air sac lesions. All five of these birds were excluded from subsequent analyses.

A subset of infected birds ($n = 28$) were submitted for histology. One experimentally infected but culture-negative bird was included in this subset; lesions consistent with aspergillosis (granulomatous, multifocal tracheitis) were observed, but due to the lack of *Aspergillus* upon culture, this bird was excluded from the analysis. Histological changes consistent with aspergillosis were seen in 26 of 27 culture-positive birds examined. Histological changes observed included multifocal to coalescing foci of granulomatous and pleocellular inflammation with multifocal necrosis, fibrin, edema and intralesional fungi in the lungs and in some cases, accompanying granulomatous tracheitis (Fig. 1A–D). The histological changes in lungs of birds infected via catheter were similar to those reported previously [18]. The lungs from birds inoculated with the atomizer were similar to those inoculated with the catheter, but some of the birds inoculated with the atomizer had focal transmural inflammation and fibrosis at the focus of inoculation that sometimes served as a nidus for the *Aspergillus* infections and fungal tracheitis (Fig. 1A). Fungal nodules were infrequently present in thoracic and/or abdominal air sacs but were not taken for histologic examination. The respiratory tracts of control birds examined were unremarkable, with the exception of two birds that had a small number of very small foci of lymphocytic infiltrates, which is a nonspecific lesion and not suggestive of fungal tracheitis.

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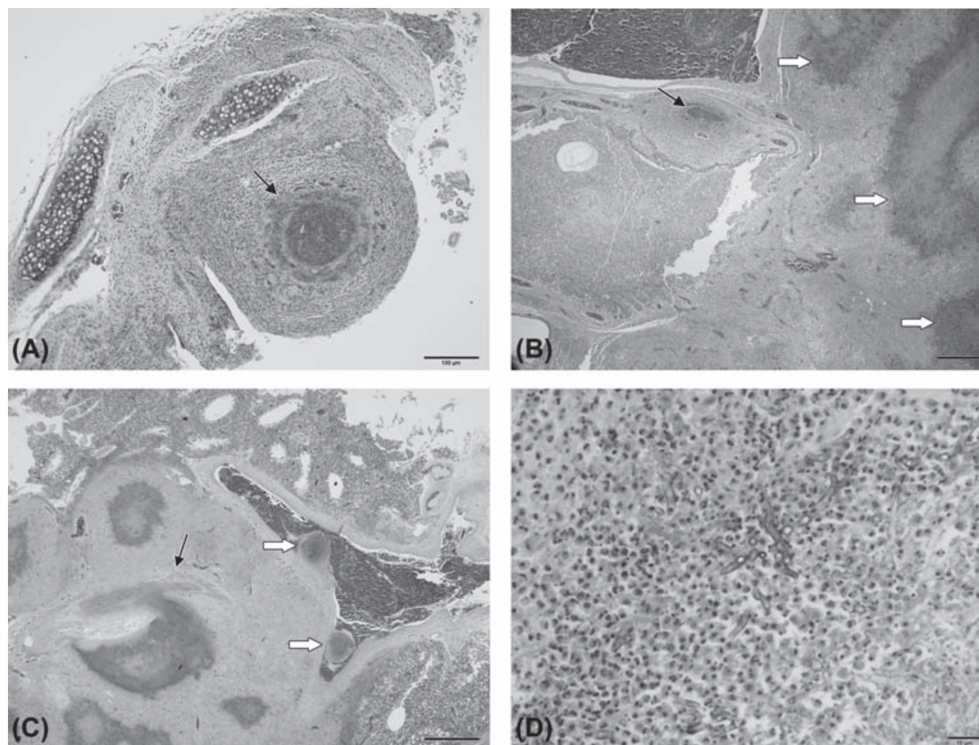

Fig. 1 (A) Trachea from a quail inoculated with 4.7×10^7 *Aspergillus fumigatus* conidia using a stainless steel 19 gauge atomizer. Note ulceration and granuloma in the mucosa of the trachea with central necrosis (arrow) surrounded by granulomatous inflammation. H&E stain. (B) Lungs from quail inoculated with *A. fumigatus* via catheter. Note bronchus on the left with plug of necrotic cell debris, granuloma in the wall of the branchus with associated cartilage necrosis (arrow) and dense cellular infiltrate in the lungs on the right with multifocal necrosis (open arrows). H&E stain. (C) Lungs from a quail inoculated with 4.7×10^7 *A. fumigatus* conidia using a stainless steel 19 gauge atomizer. Note granulomatous inflammation obliterating the bronchus (arrow) and multiple granulomas in the wall of the blood vessel (open arrows). H&E stain. (D) Lungs from a quail inoculated with 4.7×10^7 *A. fumigatus* conidia using a stainless steel 19 gauge atomizer. High magnification of necrotic loose debris in airway (lower right) and necrotic ulcerated bronchus with heterophilic inflammation (upper left) with fragments of *A. fumigatus*. H&E stain.

Inflammatory marker assays

Blood samples were obtained from 86 of the 92 quail experimentally infected with *A. fumigatus* in the two experiments. The majority of blood samples were collected on day 10 post-infection (p.i.). However, some samples ($n = 21$) were obtained at earlier time points (ranging from day 2–day 7 p.i.) from birds that were euthanized prior to day 10 due to severe respiratory distress. There were significant differences between uninfected and infected quail in all but one inflammatory marker: ceruloplasmin, ($P < 0.001$), UIBC ($P < 0.001$), iron ($P < 0.001$) and TIBC ($P < 0.001$) were all higher in infected birds; haptoglobin was lower in infected birds ($P = 0.037$) (Fig. 2). MBL was marginally higher in infected quail compared with controls ($P = 0.055$, Fig. 2).

There were also differences in some inflammatory marker levels between birds that died of experimental aspergillosis infection and those that were infected but survived. Ceruloplasmin, UIBC, and TIBC (all $P < 0.001$) were significantly higher, and haptoglobin significantly lower ($P = 0.007$), in quail that died of aspergillosis infection compared with those that survived (Fig. 3). Thus, infected birds that died showed the greatest change in inflammatory markers, whereas infected birds that survived showed the same pattern of inflammatory marker change, but to a lesser extent.

There were significant linear relationships between some inflammatory markers and the CFU of *A. fumigatus* cultured from the lungs of infected quail. Ceruloplasmin ($P < 0.001$), UIBC ($P = 0.008$), and TIBC ($P < 0.001$) were positively and significantly correlated with CFU in the lung tissues of infected birds (Fig. 4). Although inoculum dose was significantly correlated with CFU in the subset of quail infected via catheter ($P < 0.001$) (there were not enough survivors in the atomizer group for a similar test), when infectious dose was included in the linear models, it was not an independently significant predictor of inflammatory marker levels (all $P > 0.05$).

Specificity and sensitivity analyses were used to compare the diagnostic power of individual and combinations

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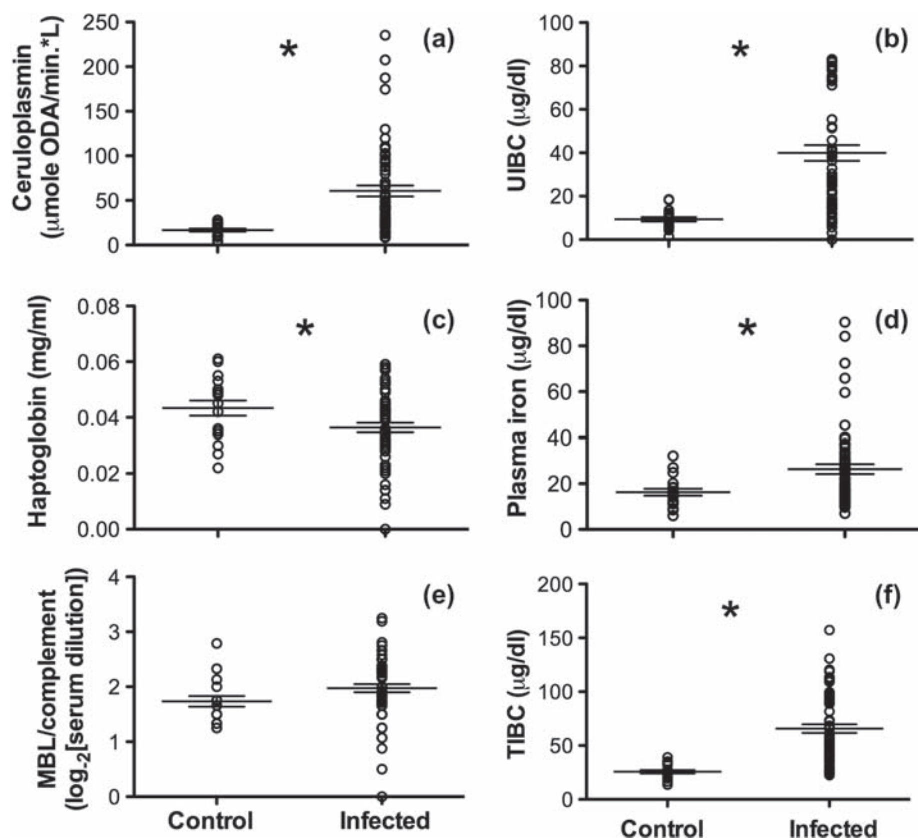

Fig. 2 Plasma levels of ceruloplasmin (a), unsaturated iron-binding capacity (UIBC) (b), haptoglobin (c), iron (d), mannan-binding lectin (MBL)/complement activity (e), and total iron-binding capacity (TIBC) (f) in healthy uninfected Japanese quail (*Coturnix japonica*), and quail experimentally infected with *Aspergillus fumigatus*. Bars indicate the group mean ± 1 standard error. The majority (65/86) of blood samples were collected on day 10 post-infection (p.i.); 21 samples were collected at earlier time points from birds that did not survive to day 10 p.i. *Indicates statistically significant differences between groups.

of inflammatory markers in this experimental infection setting. Results are given in Table 1. The single inflammatory marker with the highest test sensitivity was UIBC. Sensitivity was improved when two inflammatory markers were taken together; TIBC and UIBC, or TIBC and ceruloplasmin, provided the highest test sensitivity (Table 1); TIBC and ceruloplasmin provided the highest specificity and sensitivity. Adding a third inflammatory marker to achieve a small increase in sensitivity resulted in a drop in specificity, and vice-versa, indicating that a third marker did not add useful information.

Discussion

We found that ceruloplasmin, UIBC, plasma iron, and TIBC were increased in quail with aspergillosis. MBL-dependent complement activity was marginally higher in infected quail as well, while haptoglobin was significantly lower. Previous studies of inflammatory markers in birds with aspergillosis have reported increased circulating beta- and gamma-globulins and decreased albumin:globulin ratios [14,23], but to our knowledge, individual inflammatory markers have not been measured previously. A small number of studies have measured individual APPs in mammals infected with aspergillosis, and increased haptoglobin and C-reactive protein have been reported [12,15].

Ceruloplasmin, transferrin (UIBC), and MBL are positive acute phase proteins in birds [24–28] and increased concentrations of these proteins were expected in response to infection. In mammals, haptoglobin is also a positive APP, but in birds the direction of haptoglobin change might depend on the type of infection. Haptoglobin increases in birds with bacterial or protozoal infections [24,29,30], but either no change or a decrease in haptoglobin has been reported in birds responding to viral or fungal pathogens [25,31,32]. This study provides additional evidence that haptoglobin might be a negative acute phase protein in the context of fungal infections in birds.

Plasma iron is generally considered a negative acute phase reactant in both mammals and birds [33], and it is surprising that birds infected with aspergillosis in this study exhibited increased plasma iron levels. *Aspergillus*

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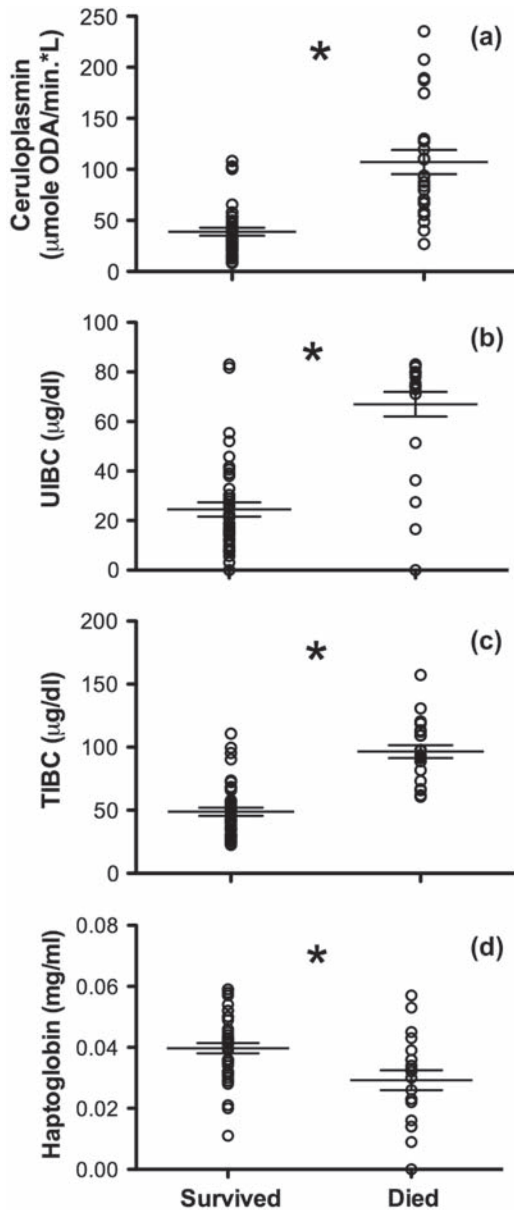

Fig. 3 Plasma levels of ceruloplasmin (a), unsaturated iron-binding capacity (UIBC) (b), total iron-binding capacity (TIBC) (c), and haptoglobin (d) in Japanese quail (*Coturnix japonica*) experimentally infected with *Aspergillus fumigatus* that survived to day 10 post-infection (p.i.) ($n = 65$), or died prior to day 10 p.i. ($n = 21$). Bars indicate the group mean ± 1 standard error. *Indicates statistically significant differences between groups.

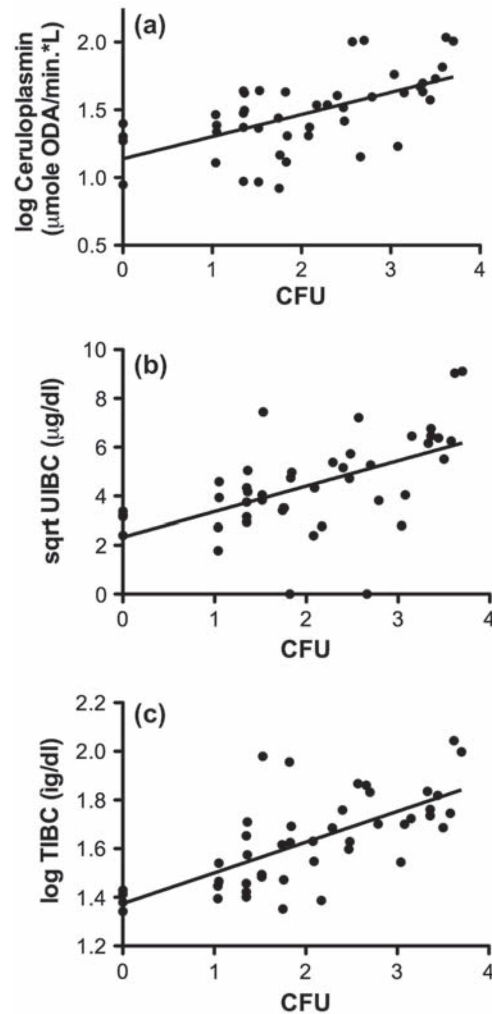

Fig. 4 Relationship between *Aspergillus fumigatus* colony-forming units (CFU) cultured from the lungs of infected Japanese quail, and ceruloplasmin (a) (log ceruloplasmin = 1.05 × CFU + 1.38), UIBC (b) (square root UIBC = 1.88 × CFU + 1.19), and TIBC (c) (log TIBC = 0.123 × CFU + 1.38) as shown by linear regression analysis.

fumigatus can impair iron uptake by host cells [10], which could lead to an increase in iron in the circulation. This increase in iron availability could also be linked to birds' susceptibility to aspergillosis.

We also found that there were relationships between some APPs and apparent disease severity. Ceruloplasmin, UIBC, and TIBC levels were all positively correlated with increasing numbers of CFU of *Aspergillus* cultured from quail lungs at the end of the experiment. In addition, ceruloplasmin, UIBC, and TIBC were significantly higher, and haptoglobin significantly lower, in quail that died of aspergillosis compared to those that were infected but survived through 10 days of infection. APPs have been found to be useful in evaluating the degree of injury or infection in mammalian species [34,35] and our study provides evidence that APPs may also be useful for the evaluation of infection in birds.

For diagnostic purposes, a panel of two inflammatory markers (TIBC and UIBC, or TIBC and ceruloplasmin), provided the most sensitive test for aspergillosis in this experimental setting. However, specificity would most likely be lower in a clinical setting with a less homogenous patient population, as other types of infections could result

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Table 1 Diagnostic sensitivity and specificity of inflammatory markers for experimental aspergillosis in Japanese quail (*Coturnix japonica*).

Marker	Positive test value	Sensitivity	Specificity	Negative predictive value	Positive predictive value
UIBC	> 13.5	79.7%	89.5%	56.7%	96.2%
Fe	> 16.4	71.4%	63.2%	40.0%	86.5%
TIBC	> 39.0	76.2%	100%	55.9%	100%
Ceruloplasmin	> 28.0	71.9%	100%	51.4%	100%
Haptoglobin	< = 0.044	74.6%	55.6%	40.0%	84.6%
MBL	> 1.75	65.6%	73.7%	38.9%	89.4%
TIBC + Ceruloplasmin	TIBC > 39.0 Or Cer > 28.0	88.9%	100%	73.2%	100%
TIBC + UIBC	TIBC > 39.0 Or UIBC > 13.5	88.9%	89.5%	71.0%	96.6%
UIBC + Ceruloplasmin	UIBC > 13.5 Or Cer > 28.0	85.7%	89.5%	65.1%	96.5%

UIBC, Unsaturated iron-binding capacity; Fe, plasma iron; TIBC, total iron-binding capacity; MBL, mannan-binding lectin + complement activity.

in elevated inflammatory markers. When distinguishing between birds with different types of infections, a panel of more than two inflammatory markers might yield better specificity. Haptoglobin in particular might be useful because haptoglobin levels decreased in quail with aspergillosis infections, but has been reported to increase in birds with bacterial or protozoal infections [24,29,30,36].

In conclusion, the APP pattern induced in an experimental avian model of *A. fumigatus* infection appears qualitatively different from APP patterns found in mammalian models of aspergillosis [12,15]. This difference is not surprising, as the inflammatory response in general is known to differ between mammals and birds [33]. The reasons for the differences between mammalian and avian inflammatory responses are unknown, but there are many possible causes, including differences in toll-like receptors (TLRs) that initiate the inflammatory response [37]. Regardless of the underlying mechanism, the unique combination of increased and decreased acute phase reactants in birds experimentally infected with aspergillosis could prove useful in the diagnosis of this disease.

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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