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Confirmed cases of *Trichosporon asahii* in South Africa causing mortality: a case series

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Abstract

Trichosporon is an emerging, medically important anamorphic basidiomycetous fungal genus that includes the causative agents of deep-seated and superficial infections in humans and other mammals. Systemic Trichosporonosis has been reported twice previously in birds and was previously considered to be clinically insignificant and rarely associated with disease in birds. This paper describes two cases of confirmed *Trichosporon asahii var. asahii* in different bird species with no currently identified common epidemiological factors. Clinical signs included respiratory distress; neurological signs and progression to death. After serial testing of various tissues *Trichosporon asahii var. asahii* was isolated, cultured and confirmed as the causative agent via PCR in both cases.

Introduction

Trichosporon spp. is an emerging, medically important fungal genus that includes the causative agents of deep-seated and superficial infections in humans and other mammals. *Trichosporon spp* have been isolated in both diseased and healthy birds. *Trichosporon cutaneum* was isolated from 7.8% of cloacae in migratory birds (Cafarchia et al., 2006). In a study to evaluate and assess organisms for their possible use in probiotics, *Trichosporon spp* were excluded due to their association of disease in both humans and mammals (Garcia-Hernandez et al., 2012). In 1988, a deceased Green-winged Macaw (*Ara chloropterus*) was diagnosed with systemic trichosporonosis; in this particular case, causes for immunosuppression were suspected but not investigated (Taylor, 1988). A Cockatiel (*Nymphicus hollandicus*) was diagnosed with dermatitis caused by *Trichosporon* spp. and following positive biopsy and culture results, was successfully treated with ketoconazole (Gartrell et al., 2005).

In early February 2016, unwell birds began presenting to a primary and referral avian practice located in Klapmuts, Western Cape, South Africa. The patients presented with a variety of clinical signs including respiratory signs, gastrointestinal upset, skin lesions and neurological signs consisting of ataxia, torticolis and hind limb paresis. The disease was not confined to a genus, species or age group. Presenting species included but was not limited to African Grey Parrots *(Psittacus erithacus)*, Blue and Gold Macaws *(Ara arauana)*, Chickens (*Gallus gallus domesticus*), Eclectus parrots *(Eclectus roratus)*, Lovebirds *(Agapornis spp.)*, Falcons (*Falciniformes spp*), Red-tailed Black Cockatoos *(Calyptorhynchus banksia)* and Umbrella Cockatoos *(Cacatua alba)*. The youngest patient in this case series was a two-week-old Blue and Gold Macaw chick and the eldest, a 10-year-old African Grey Parrot. Subclinical cases were also detected during routine surgical sexing and incidentally on radiographic studies. Samples were collected both ante- and post-mortem from clinically affected patients. *Trichosporon asahii var asahii* was confirmed in two different cases and a mixture of other organisms were isolated, included *Streptococcus* spp., *Nocardia* spp., and *Pasteurella* spp., but viral isolation and electron microscopy were negative for viruses. The appropriate authorities were contacted during the initial stages of the outbreak and it was established that an overall increase in the number of fungal cases in South Africa was occurring. There appeared to be no geographical predilection and as the outbreak progressed, clinical signs became more varied, aggressive and systemic. Over the ensuing 12 months the number of cases has continued to increase. And to date, 14 have been treated at this facility with advanced fungal disease. This case series outlines two cases with contrasting husbandry, environmental factors and age.

Clinical Reports

Case One

In October 2016, a two-year-old aviary bred African Grey cock presented for respiratory distress. The patient displayed signs of oropharyngeal and possible respiratory infections. Radiographs and coelomic endoscopy were performed which revealed severe airsacculitis. The patient received subcutaneous fluid therapy, assisted feeding and air sac cannulation. The patient died after two days of hospitalisation. Post mortem examination was performed which revealed tracheal granulomatous lesions, grey exudate on the serosal surface of the air sacs and pulmonary congestion (Figure 1). Tissue samples were not submitted for histopathology.

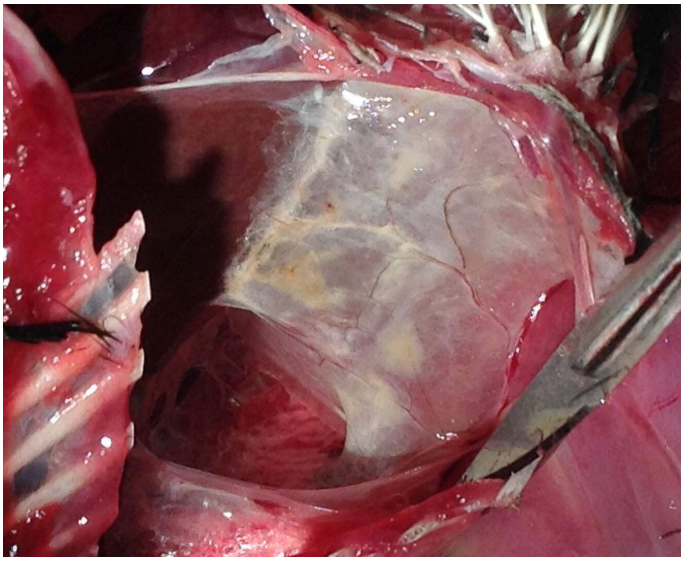

Figure 1. African Grey with grey exudate on the serosal surface of the air sacs

Case Two

In November 2016, a two-month-old Red-tailed Black Cockatoo chick presented for weight loss and difficulty breathing. The patient was anorexic and in very poor body condition so was treated with immediate supportive care including subcutaneous fluid therapy, assisted feeding, active heating and oxygen therapy. Radiographs revealed soft tissue opacity lesion in the syringeal region. A presumptive diagnosis of aspergillosis was made and the patient was treated with Ketoconazole (30mg/kg PO BID; VetsBrands, Gauteng, South Africa). The patient died within 24 hours. An in-house post mortem examination was performed which revealed proventricular dilatation with thick grey material in the lumen, severe white-yellow granulomatous nodules in the trachea extending to the level of the syrinx (Figure 2), severe airsacculitis and pink to purple small, round coloured nodular lung lesions. The thoracic and abdominal air sacs were most severely affected. Samples were collected for culture and submitted to a veterinary diagnostic laboratory.

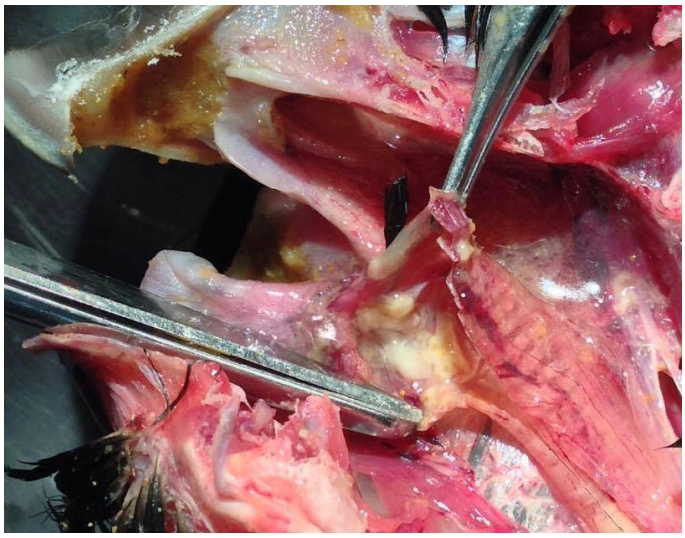

Figure 2. Red Tailed Black Cockatoo with severe tracheal granulomatous lesions

Culture and Sensitivity and PCR

Case One

A pure culture of an unidentified, budding yeast was obtained from the necrotic lung and air sacs; additionally, morphologically similar yeast was obtained in high numbers from the proventricular sample and microscopy revealed budding cells and hyphal forms in the proventricular tissue. Physiological tests conducted on the yeast resulted in a presumptive *Paracoccidioides* spp. diagnosis. In addition, the disease signs included lethargy, lack of appetite, weight loss and necrotic lesions in the nasopharyngeal and oropharyngeal areas and trachea and lungs of birds (ultimately resulting in death) matched those of paracoccidioidomycosis. Nevertheless, it was recommended that molecular methods be used to confirm the identity of the yeast. Thus, a representative yeast isolate was submitted to be identified using large-subunit rDNA D1/D2 domain sequence analysis (Fell et al. 2000). The isolate was found to be a representative of *Trichosporon asahii*.

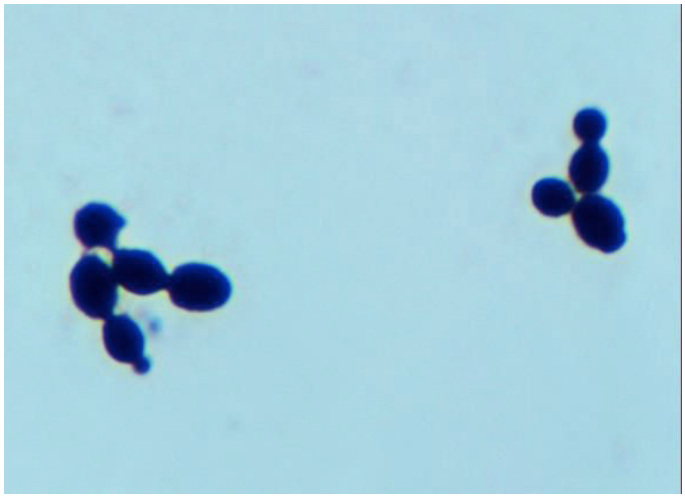

Figure 3. A micrograph of Gram-positive, yeast cells showing multipolar budding. The yeast originate from the necrotic lung and air sac samples. Micrograph 1000x magnification.

Case Two

A pure culture of an unidentified, budding yeast (Figure 3) was obtained from the necrotic lung and air sac samples; additionally, a morphologically similar yeast occurred in high numbers in proventricular necropsy samples, while microscopy revealed budding cells and hyphal forms in the proventricular tissue (Figure 4). Similar to Case One, the yeast was tentatively identified as a *Paracoccidioides* species. A representative yeast isolate was subsequently submitted to be identified using large-subunit rDNA D1/D2 domain sequence analysis (Fell et al. 2000). Similar to Case One, the isolate was found to represent *T. asahii*.

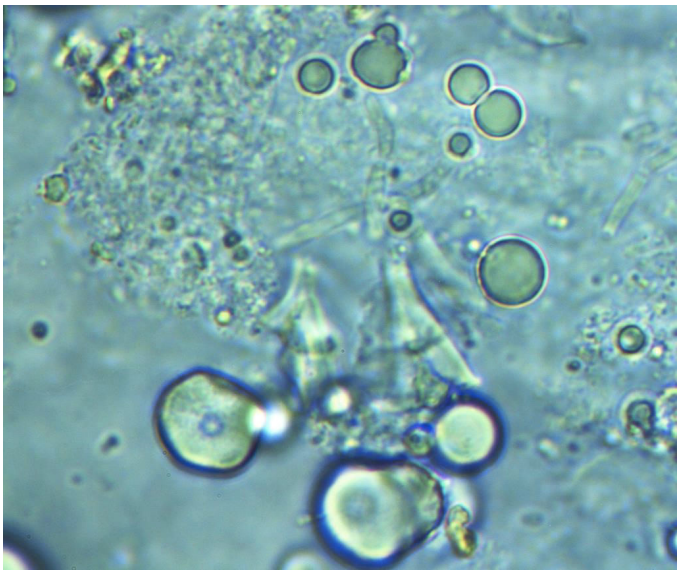

Figure 4 . Hyphae suspended in the proventriculus tissue at 1000x magnification.

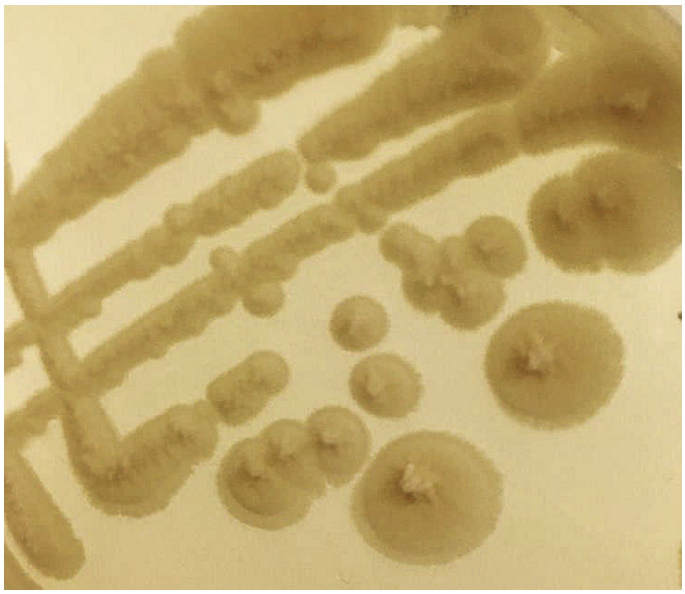

Figure 5. Creamy colonies, typical of *Trichosporon asahii,* that are widely fissured near the bottom with a zonate and fimbriate margin. The strain originated from the necrotic lung and air sacs.

Viral investigation

Electron microscopy and viral isolation were performed on samples with negative results on all samples submitted. PCR for Circovirus, Polyomavirus and Herpesvirus were negative.

Isolation of *Trichosporon asahii* from avian clinical samples and environmental samples

Trichosporon asahii was first identified as a multi-polar budding yeast cells cultured on a *Streptococcus* spp. selective blood agar (Blood Agar Base with Streptococcus Selectatab - Mast Group, Derby Road, Bootle, Merseyside, UK) from a throat swab of an African Grey parrot (case one) that displayed signs of inappetence, weight loss, and lethargy in October 2016. The colonial morphology of the yeast at 36°C was best described as tiny, grey colonies surrounded by α-haemolysis. Unfortunately, mainly due to slow growth of the colonies, the 'dimorphic fungus' was lost to bacterial overgrowth. However, and similarly, a multi-polar budding yeast agent with hyphal structures was the only microorganism detected microscopically in the proventricular tissue of case two. Following microscopic analysis of the necropsy sample, no dimorphic yeast isolates displaying the characteristic multi-polar budding cells under microscopy were isolated on culture media. The culture was dominated by a rapid-growing *Candida albicans* isolate and many bacterial species, including members of the family Enterobacteriaceae, a *Nocardia* spp. and a *Streptococcus* spp. Viridans Group .

In December 2016, a slow-growing, dimorphic yeast-like organism obtained from the proventricular tissue of case one was detected in moderate numbers on *Streptococcus* spp. selective blood agar, as well as Sabouraud dextrose agar (Mast Group, Bootle, UK). Microscopic analysis of the yeast revealed Gram-positive, multi-polar budding cells using the Gram-stain technique. The organism formed tiny, grey colonies with α-haemolysis on *Streptococcus* spp. selective blood agar at 36°C as previously described; while on Sabouraud dextrose agar (Mast Group, Bootle, UK), a slow-growing, white, powdery colonial morphology was observed at 26°C. The slow-growing yeast colonies were difficult to identify due to bacterial overgrowth, a problem experienced previously with this organism. Microscopic analysis revealed Gram-positive, multipolar budding yeast cells and hyphal structures also as previously observed. The yeast was identified to the species level using a 28S rDNA PCR technique; sequencing and a Blast search of the amplicon revealed 100% 28S rRNA gene sequence identity to *Trichosporon ashaii*.

Recommended Treatment protocols

Many cases with similar clinical signs presented over ensuing months. Following the positive identification of the organism from the birds in this case series, a broad-spectrum treatment protocol was developed for suspected cases, the protocols were adjusted as more information was obtained from cases submitted for pathology. Due to the severity of the cases in this report and the undesir-

able outcome of death if left un-treated, suspected cases that were radiographed and showed similar lesions were started on the following protocol.

The patients initially received supportive care and were treated with a combination of Ketoconazole (20-30mg/kg PO BID) and Amoxicillin Clavulanic Acid (125mg/kg PO/IM BID). The dose and duration was determined by the severity of the clinical signs. Critical patients were placed into ICU, severely dyspnoeic patients with tracheal obstruction benefited from coelomic air sac cannulation. Nebulisation therapy did not prove effective and was found to cause patients to deteriorate rapidly and was removed from the treatment protocol. Of these patients that were considered to have moderate respiratory disease, which included oculo-nasal discharge, choanal lesions, and respiratory distress with tail bobbing, seven out of nine patients survived when treated with the above protocol.

The success rate with patients is good, however the neurological patients required modification of the treatment; they were commenced on Voriconazole (15mg/kg PO BID), Amoxicillin Clavulanic Acid (125mg/kg PO/IM BID) and Enrofloxacin (20mg/kg PO BID). This was because Voriconazole can cross the blood brain barrier and penetrate the cerebrospinal fluid. Antimicrobial therapy was started based on culture results, as *Trichosporon* spp. was frequently associated in various samples from these birds. An African Grey Parrot that presented with acute onset of neurological signs (impaired vision, disorientation and hindlimb paresis) was commenced on the adjusted protocol and was able to walk after five days of therapy.

Discussion

Trichosporon asahii is an asexual basidiomycetous yeast characterised by globose or ovoid cells occurring singly or in pairs while growing in liquid culture (Sugita, 2011). However, septate hyphae with barrel-shaped arthroconidia are also formed. Colonies on Sabouraud's glucose agar are typically white to creamy, semi-shiny, smooth, and widely fissured near the bottom, with a zonate and fimbriate margin (Figure 5) (Karashima et al., 2002; Ichikawa et al., 2016). All the avian clinical isolates described in the present study displayed the characteristic white, powdery colonial morphology associated with clinical isolates.

It has been found that while representatives of *T. asahii* are unable to ferment carbohydrates they are able to aerobically assimilate a wide range of monomeric carbohydrates, including hexoses and pentoses, as well as polyols and organic acids (Karashima et al., 2002; Ichikawa et al., 2016). Furthermore, it was demonstrated that strains belonging to *T. asahii* can readily assimilate the amino acid L-Lysine as well as the polyamine cadaverine, and they are all able to hydrolyse urea and some may even grow at temperatures as high as 40°C. The ability of fungi to grow at these elevated temperatures potentially allows them to be pathogens of warm-blooded animals, including man (Robert et al. 2015).

It is well known that *T. asahii* is the most common causative agent of trichosporonosis in humans, a potentially life-threatening infection caused by members of the genus *Trichosporon* (Colombo et al., 2011). This yeast was isolated from different clinical samples including infected blood, lungs, nails, skin and urine (Sugita, 2011). It is known to cause localised systemic as well as disseminated infections in patients with acute leukaemia (de Hoog et al., 2000).

Interestingly, despite being well-known opportunistic pathogens for decades, relatively little was known about the virulence factors of *Trichosporon spp* (De Hoog et al. , 2000; Sugita. 2011). Nevertheless, it was found that the more pathogenic strains of *T. asahii* do have a higher haemolytic and biofilm formation ability than those that are less pathogenic (Sun et al.. 2012). The ability to produce biofilms highlights the potential to colonise medical devices, while haemolytic ability reflects the activity of the enzymes involved in host cell degradation. Such enzymes include proteases, phospholipases, lipases and DNases; all of which were found to be produced by representatives of *T. asahii* (Bentubo et al., 2014).

All the avian clinical isolates identified during the present study produced a notable zone of α-haemolysis on blood agar, which gradually progressed to large zones of β-haemolysis as the colonies enlarged during incubation. Such a response on blood agar is known to be indicative of pathogenicity (Sun et al., 2012) and may be an important virulence factor contributing to the ability of *T. asahii* to invade tissue and cause systemic disease in birds.

Another potential virulence factor of *T. asahii* may be the ability to secrete the enzyme β-N-acetylhexosaminidase, which is thought to degrade N-acetyl-β-D-glucosamine on the surface of the host's macrophages, thereby preventing the macrophages from recognising the invading fungal cells (Ichikawa et al., 2004). A well-known potential virulence factor among fungi, which *T. asahii* also shows, is the ability to undergo phenotypic switching as observed on agar media. It was previously contended that such phenotypic switching is an attribute of fungal virulence, facilitating invasion and escape from host defences (Odds, 1997). Furthermore, it was suggested that phenotypic switching by *T. asahii* would affect various characteristics and genes that may play a role in fungal invasion of the host (Ichikawa et al., 2004).

Since *T. asahii* commonly occurs in air samples, exposure to this yeast is generally via inhalation of airborne spores (Sugita et al., 2004; Cordeiro et al., 2010; Duarte-Oliveira et al., 2017). It was also found that under certain climat-

ic conditions airborne *T. asahii* propagules are known to cause summer-type hypersensitivity pneumonitis in humans (Sugita et al., 2004). During the present study, we were unable to isolate *T. asahii* from seed and commercial bird feed; however, we did isolate the yeast from a contaminated kelp product exposed to beach sand, municipal water and air prior to packaging and spoilage. Since fungal counts in the municipal water in the Western Cape are usually very low, and the region experienced unusually hot dry weather accompanied by strong dusty South Easterly winds for three years prior to the current trichosporonosis outbreak (Baudoin et al., 2017), it is likely that the birds were infected with airborne spores of *T. asahii*.

Previously this genus was considered a commensal, but considering our findings, it is possible that isolates may vary in pathogenicity. Pathogenicity may be evaluated by studies of the different species of the genus and further genetic characterisation. The potential exists that an unknown cause of severe immunosuppression led to the fulminant overgrowth and death caused by opportunistic Trichosporonosis, or that this strain is particularly virulent and is acting as a primary pathogen. Investigation is ongoing to determine the aetiopathogenesis and the clinical disease course of this pathogen and the virulence factors associated with the current outbreak.

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