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A PCR-based assay for screening substrates for *Aspergillus fumigatus* for application in kiwi hatcheries

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Abstract: Captive facilities across New Zealand strive to mimic natural conditions for captive animals as closely as possible. In the case of the kiwi (*Apteryx* spp.), captive habitats are augmented with natural stimuli such as soils, leaf litter, bark, plants, logs, and mosses. Interaction with these introduced stimuli has been shown to encourage normal foraging behaviour and is speculated to aid in inoculating young animals with healthy microbial communities. However, introducing non-sterile natural stimuli into the captive environment also carries the risk of exposing kiwi to diseases such as aspergillosis, coccidiosis, and candidiasis. Aspergillosis is of particular concern to rearing facilities – the disease is most commonly attributed to exposure to *Aspergillus fumigatus*, an opportunistic fungal pathogen. Here we present a PCR-based screen to qualitatively detect the presence and/or absence of *A. fumigatus* in soils. Soil samples collected from nesting sites of rowi (Ōkārito brown kiwi, *Apteryx rowi*) in the Ōkārito region of the West Coast were screened for *A. fumigatus* using a species-specific primer set coupled with a basic DNA extraction. Willowbank Wildlife Reserve soil and substrate samples were also screened as a baseline comparison representing captive rearing facilities. Results from the assays showed that the extraction technique was effective at isolating *A. fumigatus* DNA at detectable levels from a variety of soils, and that Ōkārito soils did not harbour a higher abundance of *A. fumigatus* than those found at Willowbank. This preliminary screening method could be used by facilities in New Zealand to quickly and cheaply screen soils and substrates for *A. fumigatus* before introducing them to captive enclosures.

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Key words: *Aspergillus fumigatus*, aspergillosis, kiwi, hatcheries, Ōkārito, soils

INTRODUCTION

Captive rearing of endangered species is the cornerstone of conservation programs in New Zealand. For the rarest of the rare, such as rowi (Ōkārito brown kiwi, *Apteryx rowi*) and kākāpō (*Strigops habroptilus*), captive rearing efforts have helped to dramatically improve survivability and stabilise populations (Colbourne *et al.* 2005; Holzapfel *et al.* 2008). However, the sensitivity of these species to captive conditions remains a challenge. In the case of kiwi (Family Apterygidae),

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a nocturnal, territorial ground-dwelling forager, these challenges include restricted foraging spaces, disruptions to their chronobiology, unnatural diets, and exposure to foreign microbes and antimicrobials (Taborsky & Taborsky 1992, 1995; Dickens *et al*. 2006; Becker *et al*. 2014; Pan & Yu 2014; Waite *et al*. 2014). The extent to which these factors affect the long-term survivability of captive-bred kiwi in the wild is not yet fully known. While some variables, such as territory size, are inherently unavoidable, efforts have been made by rearing facilities to carefully recreate a "wild" environment for kiwi in captivity. Captive diets are often supplemented with live invertebrates to teach young kiwi how to forage and enclosures are filled with a wide variety of stimuli such as deep soils, rocks, plants, logs, mosses, leaf litter, and hutches to encourage exploratory behaviour in birds that would be roaming several hectares in search of food in the wild (Fraser *et al.* 2009). Studies have shown that including a variety of natural stimuli for captive animals to interact with not only helps with behavioural development but also with the establishment of a properly attuned microbiome – the collection of bacteria, fungi, protists, and other microorganisms that form a symbiotic community with kiwi as their host (Colston & Jackson 2016; Berg *et al*. 2020). Louden *et al*. (2014) and Becker *et al.* (2014) showed that including an "environmental reservoir" of relevant symbiotic organisms in the form of introduced soils or substrates in captive environments established a more favourable microbiome in amphibians (Becker *et al.* 2014; Loudon *et al.* 2014). Further, San Juan *et al.* (2021) showed that soil bacteria comprised a vast proportion of the gut microbiome in wild kiwi, stressing the importance of these soil organisms. However, care must be taken when adding these stimuli – not all microorganisms present are symbiotic, and introducing diseases into habitats remains a major risk. One such disease that is carefully guarded against in captive facilities, hatcheries, and wildlife sanctuaries across New Zealand is aspergillosis (Fraser *et al*. 2009; Glare *et al*. 2014; Tell *et al*. 2019; Hauck *et al.* 2020). This disease is caused by the inhalation of conidia (spores) of species in the fungal genus *Aspergillus* and their subsequent proliferation in the lungs (Bossche *et al.* 1988; Fischer *et al*. 2018; Arné *et al.* 2021). The majority of worldwide aspergillosis cases are attributed to one species in particular, *Aspergillus fumigatus* (Bossche *et al.* 1988; Fischer *et al.* 2018; Arné *et al*. 2021). Recent studies have highlighted that *A. fumigatus* is omnipresent in most soil types in many kiwi sanctuaries, either in hyphal or conidial form (Glare *et al*. 2014). The disease is often fatal for young or immunocompromised birds and can cause long-term damage to animals that do survive infection (Ainsworth & Rewell 1949). Captive facilities employ multiple strategies to reduce the likelihood of aspergillosis cases in the animals in their care. These include the regular replacement of soil, leaf litter, and substrates in enclosures, spore counts by external laboratories, daily cleaning and aeration of bedding materials and proper storage of these materials in dry, well-ventilated spaces (Fraser *et al*. 2009). Aspergillosis was responsible for 24 kiwi deaths since 2003 and a recent outbreak in captive kākāpō (*Strigops habroptilus*) at a sanctuary on Codfish Island (Whenua Hou) resulted in 21 cases of infection and a total of 9 deaths (Gartrell 2021). These statistics demonstrate the importance of preventing aspergillosis in captive habitats, especially those of rare and endangered species. Given the tenuous stability of kiwi populations, it is critical that any modifications to their habitat, including soils in enclosures and as probiotic food additives as well as substrates for bedding are screened for the presence of *A. fumigatus* to minimise the risk of exposure for captive birds. However, regular screening is currently not undertaken due to limitations of available methods. Current methods of *Aspergillus* detection include culturing the pathogen from soils or samples of infected tissue and performing colony and spore counts for quantification, as well as Polymerase Chain Reaction (PCR) and sequencing to confirm taxonomic identification (Glare *et al*. 2014). This is a time-consuming process and requires taxonomic expertise. Commercial testing is also available to captive facilities in the form of multiplex quantitative PCR (qPCR) testing of soils and tissues (D. Tisnall, *pers. comm.* 2022). These quantitative assays are thorough and provide accurate results of both the numbers of *Aspergillus* colony forming units (CFU) per gram of sample as well as genotyping to determine the species of *Aspergillus* present. However, due to the cost per sample these tests are deployed only when disease-onset occurs and can have long turnaround times. We provide a test-case for a simple species-specific PCR based assay that could be deployed to routinely screen substrates from kiwi habitats to proactively minimise exposure to *Aspergillus fumigatus*. This study describes the optimisation and testing of a simple qualitative PCR assay to specifically detect *A. fumigatus* in soil samples. Soil and other substrates such as peat moss from Willowbank Wildlife Reserve, and soil from the Ōkārito Reserve, a natural habitat of rowi, were tested and compared in this study. A primer set developed by Serrano *et al.* (2011) was used to selectively amplify *A. fumigatus* DNA found in samples. The two sets of soil samples, from the captive rearing facility and a native reserve were tested to understand the differences in the baseline levels of *Aspergillus fumigatus* in the two habitats.

METHODS

Sample collection and DNA extraction

All field sampling from the Ōkārito Reserve and Willowbank Wildlife Reserve (WWR) was carried out using sterilised equipment (autoclaved, 121°C, 15 psi, 60 minutes) and with permission from the relevant authorities of each site. The Ōkārito Reserve was chosen as a suitable native habitat that best represents the types of soils and flora that wild kiwi might be exposed to – the region comprises around 90km^2 of beech (Fuscospora), rimu (*Dacrydium cupressinum*) and mānuka (*Leptospermum scoparium*) forest as well as extensive wetlands and is home to the only wild population of rowi. Willowbank is a major kiwi captive rearing facility in New Zealand, receiving a large proportion of rowi chicks each year for rearing via Operation Nest Egg (Colbourne *et al*. 2005). The facility provided an accurate representation of the typical conditions of captive kiwi habitats, maintained to the National Kiwi Husbandry Standard (Fraser *et al*. 2009). From the Ōkārito Reserve, soils were collected by field teams from the field teams from the Department of Conservation at five different egg-collection sites throughout the Reserve, for a total of 5 kg. Samples were stored at 4°C and transported to Manaaki Whenua Landcare Research (MWLR) in Lincoln for processing. Five-gram subsamples (n = 36) were collected from the total and stored at -18°C. From Willowbank, multiple samples of soil (n = 17, 5 g each) and bedding materials such as peat moss (n = 17, 5 g each) and straw (n = 17, 1 g each) were collected from rowi enclosures. To extract and suspend environmental DNA, 50 mL of sterile water (Milli-Q) was added to each sample in an autoclaved flask. Flasks were shaken on a Ratek Orbital Mixer (Ratek Instruments, Boronia, Australia) at 160 RPM for 30 minutes. One mL was then extracted into a clean microcentrifuge tube, heated at 95°C to lyse microbial cells, centrifuged at 11,000 RPM in a 5145-D benchtop centrifuge (Sigma-Aldrich, Darmstadt, Germany) for four minutes and the supernatant transferred into new 1.7 mL microcentrifuge tubes.

Assessment of DNA extraction efficiency using PCR with broad fungal ITS primers

To test the efficacy of the rapid DNA extraction technique, ITS1-F_KYO1 forward (5′-CTHGGTCATTTAGAGGAASTAA-3′) and ITS2_KYO1 reverse (5′-CTRYGTTCTTCATCGDT-3′) primers developed by Toju *et al*. (2012) were used to broadly amplify fungal DNA in a random selection of extracted soil and substrate samples (n = 16). DNA from an isolate of *A. fumigatus* (conc. 2 ng/μL) was extracted at the MWLR laboratory in Auckland (ICMP accession number 23465) to be used as a positive control. The 15 μL PCR mix consisted of 7.50 μL of 2× KAPA Plant PCR Buffer (KAPABiosystems, Wilmington, MA), 0.60 μL of each primer (10 μM), 0.12 μL of 3G KAPA DNA polymerase (KAPABiosystems), 5.18 μL of PCR-grade water (Milli-Q) and 1.00 μL of sample DNA. The PCR protocol was as follows – denaturation at 95°C for 2 minutes, then 34× cycles of 95°C for 20 seconds, 50°C for 20 seconds, 72°C for 30 seconds and a final extension of 1 minute. Gel electrophoresis with a standard 2% agarose gel and 5 μL of PCR product per lane was used to visualise PCR products and confirm expected fragment sizes of 300–350 bp.

Optimisation of species-specific *A. fumigatus* RodA primers

Annealing temperature

The rodlet A region of *A. fumigatus* DNA was targeted using RodA forward (5′-ACATTGACGAGGGCATCCTT-3′ and reverse (5′-ATGAGGGAACCGCTCTGATG-3′) primers (Integrated DNA Technologies, Auckland, New Zealand) as described by Serrano *et al*. (2011). RodA primers were optimised for specificity to *A. fumigatus* using a gradient PCR with annealing temperatures ranging from 50–65.8°C. Pure *A. fumigatus* DNA samples were used (n = 16) in two dilution series, 1:10 and 1:100, to assess differences in signal strength. The 15 μL PCR mix used for this protocol was the same as above, but with the ITS1-F_KYO1 and ITS2_KYO1 primers replaced with RodA forward and reverse primers. PCR products were visualised using the same gel electrophoresis method as above.

Efficiency & species-specificity

RodA primers were tested for specificity to *A. fumigatus* compared to DNA from a closely related species, *Aspergillus niger*. DNA from a culture of *A. niger* (conc. 2 ng/μL) (ICMP accession number 2523) was obtained to act as a negative control for this test. Additionally, primers were assessed for efficiency in the presence of soil-based inhibitors. Random soil and substrate samples were chosen (n = 4) and spiked with equal amounts of either *A. fumigatus* or *A. niger* DNA. The same PCR and gel electrophoresis method as above was used, but with the optimised annealing temperature.

Detection of *A. fumigatus* in Ōkārito and WWR soils and substrates using optimised RodA primers

The optimised assay was employed to screen for the presence of *A. fumigatus* in samples collected from soils, and other substrates within the captive

rearing facility, as well as soil samples collected in the natural habitat of rowi, i.e the Ōkārito Reserve (n = 85). Assay results were assessed via gel electrophoresis, with bands detected at ~320 bp considered positive for *A. fumigatus*. Data were recorded in an Excel spreadsheet and analysed as below.

Statistical analysis

To determine whether there was a significant difference in *A. fumigatus* presence between the two sample groups (Ōkārito vs WWR), a general linear model (GLM) was used. Samples positive for *A. fumigatus* were labelled with a 1, and negatives with a 0, to create a presence absence matrix which was exported to RStudio (version 1.4) for analysis. RStudio packages Hmisc (Harrell Jr & Harrell Jr 2019) (version 4.6-0) and lme4 (Bates *et al.* 2007) (version 1.1-27.1) were used to run a GLM for binomial data. Ōkārito soil samples, as baseline in the natural habitat, were compared against all other soil types, with soil groups retained as a random effect in the model and using the formula, *Aspergillus.presence ~ samples + (1/sample.type).*

RESULTS

Optimal PCR mix and protocol of the assay for screening

15 µL PCR mix – 7.50 µL of 2× KAPA Plant PCR Buffer (KAPABiosystems), 0.60 µL of each primer (10 µM), 0.12 µL of 3G KAPA DNA polymerase (KAPABiosystems), 5.18 µL of PCR-grade water (Milli-Q) and 1.00 µL of sample DNA.

Protocol – denaturation at 95°C for 2 minutes, then 34× cycles of 95°C for 20 seconds, 65.8° for 20 seconds, 72°C for 30 seconds, final extension 1 minute.

Detection of *A. fumigatus* DNA in soil and substrates and statistical analysis of Ōkārito vs WWR groups

RodA primers were used to amplify *A. fumigatus* DNA from environmental DNA extracted from Ōkārito soil and WWR soil and substrate samples. A faint band of ~320 bp indicated the presence of *A. fumigatus* DNA and therefore a positive sample. It was expected that any amount of extracted and amplified fungal DNA in samples would be very low, due to the resistance of fungal conidia to lysis. Therefore, samples positive for *A. fumigatus* may have only shown a faint band that could be mistaken for a negative. To mitigate this, all samples displaying even a faint band would be counted as positive. Figure 1 shows the gel electrophoresis output with positive samples highlighted, and Table 1 shows a summary of positive and negative results. Overall, Ōkārito soils had a positive rate of 2.9%. WWR run soils had a rate of 5.8%, and WWR peat moss and straw a rate of 17.6% and 5.8% respectively. After being split into two groups (Ōkārito vs WWR) a GLM (lme4) with a fit of maximum likelihood found no significant difference between the positive rates of the two groups (Ōkārito vs WWR, p = 0.254). Table 2 shows a summary of the GLM results.

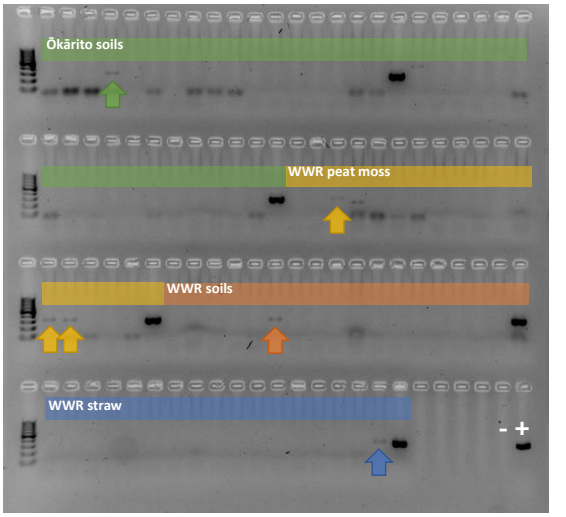

Figure 1. Gel electrophoresis image of Ōkārito natal soil samples compared against Willowbank Wildlife Reserve peat moss, soils and straw, coloured as green, yellow, orange, and blue from left to right. Arrows highlight samples positive for *Aspergillus fumigatus* for each substrate type. Dark bands represent positive controls, faint bands positive samples.

Assessment of DNA extraction efficiency

PCR amplification of samples post-extraction using broad fungal ITS primers showed positive results across all samples. Gel electrophoresis visually confirmed the presence of amplified DNA from a variety of fungal species. These results confirmed the ability of the extraction method to adequately isolate and suspend fungal DNA from soil and substrates.

Optimal annealing temperature for RodA primer set

A gradient PCR confirmed specificity of the primers at higher temperatures as stated by Serrano *et al.* (2011). Clear bands of ~300 bp (the target amplicon size) in the gel electrophoresis output showed the highest specifity to target *A. fumigatus* DNA at an annealing temperature of 65.8°C.

Table 1. Total counts for each soil/substrate type positive for detectable *Aspergillus fumigatus* content and their percentage (WWR is Willowbank Wildlife Reserve).

Soil/substrate	Positive counts	Total counts	Percentage positive
Ōkārito soil	1	34	2.9
WWR soil	1	17	5.8
WWR peat moss	3	17	17.6
WWR straw	1	17	5.8

Table 2. Generalised linear mixed model output comparing both soil groups against each other. Soil type (natal, run, peat moss, straw) was included as a factor.

	Standard Error	z-value	p-value
Intercept	0.4709	-4.713	2.44e-06
Natal vs WWR	1.1189	-1.142	0.254 ^{ns}

Fainter bands were visible in different size ranges as the temperature decreased. 65.8°C was used as the annealing temperature for all further PCR amplification protocols that used RodA primers in this study.

Primer cross-specificity to closely related species and efficiency in the presence of potential PCR inhibitors

The RodA primer set showed no cross-specificity to close relatives of *A. fumigatus* such as *A. niger*. Purified *A. fumigatus* DNA amplified strongly, whereas no amplification was reported for purified DNA of *A. niger* at comparable concentrations. It was also clear that soil samples spiked with *A. fumigatus* had an equal level of amplification to pure DNA samples, indicating that any PCR inhibitors present in the soil and substrate extracts did not sufficiently inhibit amplification.

DISCUSSION

We provide an optimised PCR-based rapid screening method for *A. fumigatus* and test its application at a captive-rearing facility, Willowbank Wildlife Reserve (WWR), that houses rowi. We find that background levels of *A. fumigatus* in soils from the participating captive-rearing facility are comparable to those in the natural habitat of the rowi. Below we discuss the applicability of this method, especially proactive use in kiwi captive-rearing facilities, and its limitations.

Detection of *A. fumigatus* in soils and substrates

Aspergillus fumigatus is a common soil-borne fungus that is well-known to hatcheries and captive-rearing facilities as the largest contributor to cases of aspergillosis. As such, all soils and substrates used in rowi enclosures at Willowbank were screened to provide an accurate overview of the presence of *A. fumigatus*. While we found no significant difference between numbers of positive samples of natal soils compared to WWR soil, peat moss and straw, peat moss from the brooder boxes of young kiwi exhibited the highest proportion of positive samples.

Peat Moss

Peat moss is an ideal substrate for fungal growth, with a high humidity and nutrient content and a supportive matrix structure (Gorham & Rochefort 2003). WWR peat moss is stored in dry environments to minimise fungal growth.

Straw

The straw used as bedding material in hutches was suspected to be the highest risk substrate for *A. fumigatus* by WWR keepers (B. Brett, *pers. comm.* 2020). As such, straw is regularly inspected and replaced by staff to minimise the risk of fungal growth. However, straw is often obtained as whole bales from local agricultural providers, with little record of its storage conditions before arriving at WWR – it has been found that an important determinant of *Aspergillus* levels in substrates is age of the substrate and storage condition (Glare *et al.* 2014). However, our screen only detected a contamination rate of 5.8%, much lower than that of peat moss and comparable with general WWR soils. This may have been due to the fact that we only collected ~1g of straw per sample due to its bulk.

Soils

Soils at WWR that are used in outdoor habitats are sourced from multiple local areas. *A. fumigatus* is known to have a ubiquitous presence in soils, and so we suspected a high rate of contamination from WWR soils. However, WWR soil had the same number of positive samples as straw, with a rate of 5.8%. This may have been due to a lack of nutrients or humidity in these high-turnover agricultural soils, as well as efforts by WWR keepers to regularly replace soil to ensure it does not stagnate.

Ōkārito soil

Ōkārito soil samples were collected from five different egg collection sites, 1 kg from each. These

samples were then subsampled and statistically pooled to form the "Natal soil" group. Ōkārito soils had the lowest level of *A. fumigatus*, at 2.9%, despite samples including a large proportion of dead plant material such as roots and a high moisture content. Heterogenous soils from old growth forests are typically rich in fungal diversity (Jansson & Hofmockel 2020). As a result, the high species diversity observed in such soils may reduce the proliferation of a few dominant species and thereby reduce the load of *A. fumigatus*.

Advantages and applications of the assay

This assay was developed to quickly and accurately screen substrates for *A. fumigatus* in an effort to ensure their introduction to rowi enclosures did not exascerbate the risk of aspergillosis for birds in captivity. While commercial testing options through veterinary clinics are often available for this purpose, we sought to develop a faster protocol that could provide a simple positive or negative result without the need for extensive laboratory testing. This assay does not require a typical DNA extraction kit, can be used with a wide variety of substrates, and DNA sequencing is not necessary due to the specifity of the primers used. Large numbers of samples can be screened in a matter of hours using minimal equipment at any moderately equipped PC2 laboratory. Furthermore, this assay does not rely on a clinical case of aspergillosis to arise before being implemented – it can be used as a preventative method to reduce the risk of aspergillosis in captive environments. Regular application of the assay, especially when new captive habitats are established, could help monitor contamination levels over time and determine when soils and substrates should be replaced.

Limitations of the assay

While we developed an assay that could be used proactively to limit exposure of *A. fumigatus* containing substrates to kiwi in captivity, confirmation of highly contaminated samples via sequencing is recommended. Our rapid DNA preparation method may allow 'leaking' of PCR inhibitors which may vary across soils and substrate types (Schrader *et al.* 2012). In our tests, while this was not an issue, this may be problematic in other sample types such as high humic acid containing soils. Our method could be further improved by employing a DNA extraction kit specifically optimised for the sample type. These kits efficiently remove PCR inhibitors present in a sample (Whitehouse & Hottel 2007), but can be expensive and time consuming for large numbers of samples. Further, our DNA preparation method may not efficiently lyse conidial cells – the predominant disease causing agent in aspergillosis (Fischer *et al.* 2018). Fischer *et al.* (2018) demonstrated that *A. fumigatus* conidia can suvive temperatures of up to 60°C (Vallejo-Cardona *et al.* 2017). As such, we chose a temperature of 95°C to ensure complete degradation of spores. Even so, no chemical additions were made to the extraction solution that could have helped to degrade conidial cells. Again, these agents are found in commercial extraction kits, which would further optimise this step in future. Aspergillosis can also be caused by other species of *Aspergilli*, such as *A. niger* or *A. flavus* (Serrano *et al.* 2011). However, discussions with the Department of Conservation and WWR keepers determined that a focus on *A. fumigatus* specifically was imperative for this study since this species is responsible for the majority of aspergillosis cases in New Zealand (K. McInnes, *pers. comm.* 2021). Moving forward, further primers targeting these alternative species could also be applied to this screen in a multiplexed fashion (Xu *et al.* 2000; San Juan *et al.* 2021).

CONCLUSIONS

Aspergillosis is a significant contributor to the mortality rates of captive avian species in New Zealand wildlife sanctuaries. Efficient and thorough testing of captive environments for *A. fumigatus* remains an important component of captive rearing. The purpose of this study was to demonstrate a simple yet effective PCR-based method for qualitative testing of substrates for *A. fumigatus* in captive habitats. A primer set was optimised for specificity to *A. fumigatus* and efficiency in the presence of soil-based contaminants. Findings indicated that this screen is useful in the context of qualitatively detecting the presence or absence of *A. fumigatus* in various soil and substrate types, and could be applied as a cost-effective routine screen for wildlife sanctuaries concerned about aspergillosis.

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