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Investigation of dogs as a reservoir of Penicillium marneffei in northern Thailand

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SUMMARY

Background: Penicillium marneffei is a dimorphic pathogenic fungus endemic in Southeast Asia that usually causes disseminated disease, mainly in immunocompromised individuals, especially those with HIV infection. Untreated cases are usually fatal. The only known natural reservoir exists in bamboo rats and there is no firm evidence that these animals are involved in direct transmission to humans. The risk of infection is not restricted to those living in endemic areas; HIV-infected individuals who travel to Southeast Asia have also become infected by *P. marneffei*. Hence, there must exist sources to which even tourists are exposed on a short-term basis.

Design and methods: Penicillium is known to infect dogs and this animal is common in the streets and temple areas of Chiang Mai, where there is one of the highest incidences of *P. marneffei* infection in the world. Dogs have not been well studied as a possible reservoir. To investigate this possibility, we took nasal swabs from 83 outdoor dogs and performed culture and nested polymerase chain reaction (PCR) to detect *P. marneffei*.

Results: We found that approximately 13% of nasal swabs from dogs in Chiang Mai, Thailand were positive when tested by two different PCR methods, but culture results were negative. Sequencing the products from both PCR reactions showed 100% identity with *P. marneffei*, whereas no other known fungi shared both sequences.

Conclusions: Our results suggest that dogs might be an animal reservoir for *P. marneffei* in northern Thailand. This observation should be confirmed by additional studies.

1. Introduction

Penicillium marneffei infection is an opportunistic mycotic disease that is endemic in Southeast Asia and Southern China.[1–4] Penicilliosis has emerged as one of the most common infections in HIV-infected patients residing in endemic areas,[3–10] with over 8300 cases in Thailand by 2009 (http://epid.moph.go.th). Most cases of penicilliosis are disseminated infections,[2] clinically resembling histoplasmosis, cryptococcosis, and tuberculosis.[11,12] The common clinical features include fever, weight loss, anemia, generalized lymphadenopathy, hepatosplenomegaly, pulmonary involvement, mucosal ulcers, and skin lesions.[4–8,12–14] Infected tissues can show a mixture of reactions, including necrosis, suppuration, and granuloma formation.[7,15,16]

P. marneffei is a dimorphic fungus exhibiting a mycelial form at 25 °C and a yeast form at 37 °C.[4,14,17] The yeast-like organisms vary from 3 to 8 microns in length,[8,15] with a characteristic intracytoplasmic transverse septum that is clear on routine stains such as Diff-Quik and Papanicolaou.[8,18] More recently, polymerase chain reaction (PCR)-based tests have been developed that can specifically identify *P. marneffei*.[4,19–22] The reactions use oligonucleotide primers based either on the 18S rRNA gene sequence or the *ITS1-5.8S-ITS2* rDNA region of *P. marneffei*. This technique is more rapid than culture (10 h compared to up to 7 days) and much more sensitive, detecting as little DNA template as 1.8 fg.

The infection responds to antifungal treatment, but untreated cases, especially in immunocompromised patients, are usually fatal.[8] Hence identification of possible sources of the infection is clinically important. The risk of infection is not restricted to those living in endemic areas. HIV-infected individuals who travel to Southeast Asia have also become infected by *P. marneffei*.[12,23,24] This implies two things: (1) short-term exposure is all that is

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necessary for infection in susceptible individuals, and (2) at least one source of infection must exist in an environment contacted by tourists even on a short-term basis.

Animal reservoirs in northern Thailand have been identified to include the bamboo rat (*Rhizomys sinensis*), the hoary bamboo rat (*Rhizomys pruinosus*), the large bamboo rat (*Rhizomys sumatrensis*), and the small bay bamboo rat (*Cannomys badius*).[18,25–27] However, these animals are generally not in contact with humans and there is no firm evidence of direct transmission from rat to man.[1] In Thailand, there are numerous other outdoor animals including dogs, cats, rats, and elephants to which both local people and tourists might be exposed, either directly (contact with the animal) or indirectly (contact with aerosolized secretions/excretions). *Penicillium* species are known to cause nasal infection in North American dogs.[28,29] The literature on this infection is extremely scanty and the species of Penicillium have not yet been determined. Symptomatic animals have nasal discharge and ulceration of external nares and epistaxis.[29] However, up to 40% of seemingly normal dogs will have a nasal swab positive for Aspergillus or Penicillium on culture.[29] No comparable studies currently exist on dogs in Thailand, but these animals are common at temples and market areas where tourists might visit.

We postulated that dogs in northern Thailand might be a reservoir of *P. marneffei* and performed a study on nasal swabs obtained from outdoor dogs for the presence of *P. marneffei*. To increase detection, both culture and PCR were employed. A positive result would not only increase our understanding of *P. marneffei* infection but would also have public health implications. A negative result would eliminate this animal as a source of concern for transmission of this disease to local Thais and tourists.

2. Materials and methods

2.1. Specimen collection

We hypothesized that outdoor dogs in Chiang Mai might be carriers of *P. marneffei* since they are exposed to other animals and to soil in various locations. Hence, nasal swabs were obtained from 83 outdoor dogs in Chiang Mai, Thailand, with the cooperation of temple directors at selected temples in Chiang Mai for dogs living within temple camps. All swabs were taken under the supervision of the Faculty of Veterinary Medicine, Chiang Mai University.

2.2. PCR reactions

DNA was extracted from the nostril specimens using the QIAamp Blood Minikit (Qiagen GmbH, Germany) according to the manufacturer's instructions. Samples were initially screened by one or the other of two PCR tests, as previously described.[19,21,22] To maximize specificity, only specimens positive by both PCR reactions would be considered as positive. Accordingly, any specimen that was positive by one PCR test was then tested by the other test.

The first PCR method was a nested procedure performed as previously described.[21,22] The primers used for the first PCR reaction were: RRF1, 5′-ATCTAAATCCCTTAACGAGGAACA-3′; RRH1, 5′-CCGTCAATTTCTTTAAGTTTCAGCCTT-3′. For the second PCR reaction they were: Pm1, 5′-ATGGGCCTTTCTTTCTGGG-3′; Pm2, 5′-GCGGGTCATCATAGAAACC-3′. The first set of primers amplifies a portion of the 18S rRNA gene that is fungus-specific and the second set amplifies a portion of the 18S rRNA gene of *P. marneffei*. For the first PCR reaction, the parameters were: 95 °C for 5 min, then 35 cycles at 95 °C for 30 s; 55 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 7 min. For the second PCR reaction, the parameters were: 95 °C for 5 min, then 15 cycles at 95 °C for 30 s; 68 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 7 min. The positive control was DNA from *P. marneffei* at 8 ng/μl and the negative control was a 'no template' control. PCR products were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. This method can detect *P. marneffei* DNA in amounts as low as 1.8 fg. Since this value was obtained from laboratory culture specimens, it was decided to determine the sensitivity of detection for the experimental conditions of this study. For this purpose, DNA extracted from *P. marneffei* was added to a suspension prepared from dog nostril specimens to create serial DNA dilutions of 800 pg/μl, 80 pg/μl, 8 pg/μl, 800 fg/μl, 80 fg/μl, 8 fg/μl, and 0.8 fg/μl. Nested PCR was performed and the sensitivity of this method was determined to be 80 pg/μl.

The second PCR method was performed as previously published.[19] The sequences of these primers were: PM2, 5′-GATGGACTGTCTGAGTACC-3′; PM4, 5′-ATGGTGGTGACCAACCCCCGCA-3′. These primers amplify a portion of the *ITS1-5.8S-ITS2* rDNA region of *P. marneffei* that was reported as specific for this organism.[19] In our study, this PCR test was used to confirm *P. marneffei* detected by the first method. The PCR parameters were: 95 °C for 5 min, then 35 cycles at 95 °C for 30 s; 65 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 7 min.

2.3. Sequence analysis

Automated DNA sequencing of the PCR products was performed using the Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, USA) and the ABI BigDye Terminator v3.1 cycle sequencing kit according to the manufacturer's instructions. The NCBI BLAST program (http://www.ncbi.nlm.nih.gov) was used to search for nucleotide sequence identity.

2.4. Fungal culture

All samples were also set up for culture of *P. marneffei*. Nasal swabs were cultured using Sabouraud's dextrose agar plates, and incubated at room temperature for 5–7 days. Microscopic examination was performed by mounting the fungi in lactophenol cotton blue. The details of hyphae and spore arrangement were examined to identify the fungal type.

3. Results

3.1. PCR results

Of the 83 samples, 51 were initially tested by the PCR reaction that amplifies 18S rRNA. Twenty-six of these samples gave a positive result. Positive cases were then further studied by the second PCR method that amplifies the *ITS1-5.8S-ITS2* rDNA region. Of those 26 samples, seven remained positive. The other 32 samples were initially tested by the PCR reaction that amplifies the *ITS1-5.8S-ITS2* rDNA region and 17 of these gave positive results. We then performed the PCR test using primers for 18S rRNA and four cases remained positive. Thus, 11/83 (13%) samples gave positive PCR results with both primer pairs.

Three of the samples positive with both sets of primers were sequenced. For the 390-base pair product obtained from the primers for the 18S rRNA gene of *P. marneffei*, all three samples showed identical results with 100% identity to *P. marneffei*. However, a BLAST search also showed 100% identity to other species of *Penicillium* (*purpurogenum, verruculosum, diversum*) and the fungi *Acremonium cellulolyticus* and *Talaromyces flavus*. For the primers used to amplify the *ITS1-5.8S-ITS2* rDNA region of *P. marneffei*, those sequences are found in *P. marneffei* and *P. verruculosum*, but not in *P. purpurogenum*, *P. diversum*, *A. cellulolyticus* and *T. flavus*. Hence, even if the first PCR reaction was amplifying one of these fungi instead of *P. marneffei*, it would

not be detected by the second PCR reaction. The primer sequences are shared by *P. marneffei* and *P. verruculosum*, but the product sequence is not identical. Sequencing the product obtained for the *ITS1-5.8S-ITS2* rDNA region showed 100% identity to *P. marneffei*.

3.2. Culture results

Of the 83 samples, the culture results were as follows: *Cladosporium sp* was grown in 31 samples, *Fusarium sp* in three, *Aspergillus sp* in five, *Aspergillus fumigatus* in one, *Penicillium sp* (not *marneffei*) in two, yeast in 29, unidentified fungus in 35, and there was no growth in 15 samples. More than one organism was recovered from 28 samples. For the 11 samples positive by both PCR methods, *Cladosporium sp* was grown in three samples, yeast in three, unidentified fungus in five, and there was no growth in three samples. More than one organism was recovered from two samples: one of these grew yeast and an unidentified fungus, and the second did as well, together with *Cladosporium sp*.

4. Discussion

Animal reservoirs in northern Thailand have been identified to include the bamboo rat (*R. sinensis*), the hoary bamboo rat (*R. pruinosus*), the large bamboo rat (*R. sumatrensis*), and the small bay bamboo rat (*C. badius*).[18,25–27] Infected rats appear healthy[1] and therefore a carrier may not appear ill. While bamboo rats are a natural reservoir, these animals are generally not in contact with humans and there is no firm evidence of direct transmission from rat to man.[1] However, bamboo rats and HIV-positive patients have been found to share genetically similar strains of *P. marneffei*, suggesting rat-to-human transmission might be possible or co-infection from a common but still unidentified source.[30] One scenario is that death of the bamboo rat results in the production of large quantities of spores, thereby serving as an amplifying host for human *P. marneffei* infections.[30] Alternatively, transmission could be indirect, with some other animal serving as an intermediate between the bamboo rats and humans.

We postulated that outdoor dogs in northern Thailand might be a possible reservoir of this pathogen given that nasal Penicillium infection has been found in dogs in North America. Using a PCR-based approach, we found that using primers for either 18S rRNA or the *ITS1-5.8S-ITS2* rDNA region, about 50% of specimens gave a positive result. However, when those positive samples were checked using the other set of PCR primers, the number of positives was reduced by a further 50–75%, such that overall 11/83 samples were positive with both sets of primers. Thus, although each PCR method was supposedly specific for *P. marneffei*, there were apparently false-positives. This may reflect that the sequences for the PCR primers were based on identifying *P. marneffei* in human samples, and distinguishing it from other human pathogenic fungi. However, the range of organisms in dogs is much less well studied, and there may be overlap in primer sequences with other fungal organisms that involve dogs but not humans. In particular, the nasal region of dogs is much more in contact with fungi in nature and soil compared to humans. Sequencing results lend support to this concept. The PCR product from primers designed to amplify the 18S rRNA gene of *P. marneffei* showed 100% identity to *P. marneffei* but also with *P. purpurogenum*, *P. verruculosum*, and *P. diversum*, and the fungi *A. cellulolyticus* and *T. flavus*. *Talaromyces stipitatus* has been shown to co-exist with *P. marneffei* in soil,[31] although this has not been reported specifically for *T. flavus*.

For this reason, we felt it was important to confirm a positive result by one PCR method using the other PCR method. Although the primers for this second reaction would not amplify most of the above organisms, they would give a positive result for both *P. marneffei* rather than *P. verruculosum*. We found that the sequence obtained for the PCR product from primers designed to amplify the *ITS1-5.8S-ITS2* rDNA region showed 100% identity to *P. marneffei* rather than *P. verruculosum*, confirming that we were amplifying *P. marneffei*. With this in mind, samples that are positive by both methods are much more likely to be true-positives. This would mean that a significant proportion (~13%) of dogs in Chiang Mai are carriers of *P. marneffei*.

It is curious that none of the specimens in the study grew *P. marneffei* by routine culture. The reasons for this are not clear; however PCR-based methods are more sensitive than culture and are able to detect even small numbers of organisms in samples that might be negative by culture. This situation has been reported specifically for *P. marneffei*, for which soil samples positive by PCR yielded negative cultures.[31] Moreover, samples negative by routine culture have sometimes required animal inoculation to yield a positive result.[27] These observations imply culture of *P. marneffei* from environmental samples is not always straightforward, although why this should be the case is not known. Lastly, it is also possible that some other organism is being detected by PCR based on identical nucleotide sequences between *P. marneffei* and some yet unidentified non-pathogenic fungus occurring in nature, particularly soil. Until other fungi from soil are studied in greater detail, it is unknown if any will have identical sequences to *P. marneffei*.

Even if dogs are carriers of *P. marneffei*, the manner in which humans acquire *P. marneffei* infection is unclear. An experimental model in mice reproduced systemic infection by intratracheal instillation of the organism.[32] The primary site of involvement was the lung in this model. Inhalation may be the point of entry for humans;[33] however in those with clinical disease, lung involvement is not common. In AIDS patients, the incidence of infection is higher in Chiang Mai (20%) than in Bangkok (2%). There is an increased incidence of infection during the rainy season in Chiang Mai, Thailand,[34] but the reason for this is unknown. One hypothesis is that soil has higher fungal contamination during the rainy season. Previous studies found that *P. marneffei* was present in occasional bamboo rat burrows.[2,27] However, this finding has not been confirmed in subsequent studies.[30,35,36] *P. marneffei* has been detected in soil samples in Thailand, including elephant camps and temple grounds.[31] The organism can survive in sterile soil for several weeks, but can only survive a few days in non-sterile soil.[30,31] Thus, dogs might acquire the organism through nasal contact with soil. This could be equally true for elephants, another animal frequently contacted by tourists. A study of the carrier status of domesticated elephants would be of value, although beyond the scope of this present study. Similarly, knowing the movement patterns of tourists within endemic areas might provide additional clues to settings where *P. marneffei* might be found and has not yet been searched for.

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Ethical approval: All animal work was carried out under the supervision of, and in accordance with the institutional guidelines of the Faculty of Veterinary Medicine, Chiang Mai University.

Conflict of interest: No conflict of interest.

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