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Surveillance of *Vittaforma corneae* in hot springs by a small-volume procedure

Jung-Sheng Chen [a], Tsui-Kang Hsu [c, 1], Bing-Mu Hsu [b, **], Tung-Yi Huang [b], Yu-Li Huang [d], Men-Fang Shaio [e], Dar-Der Ji [e, *]

[a] *Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan, ROC*
[b] *Department of Earth and Environmental Sciences, National Chung Cheng University, 168 University Road, Minhsiung Township, Chiayi County, 62102, Taiwan, ROC*
[c] *Department of Ophthalmology, Cheng Hsin General Hospital, Taipei, Taiwan, ROC*
[d] *Department of Safety Health and Environmental Engineering, National Kaohsiung First University of Science and Technology, Kaohsiung, Taiwan, ROC*
[e] *Department of Tropical Medicine, National Yang-Ming University, No.155, Sec.2, Li-Nong Street, Taipei, 112, Taiwan, ROC*

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ABSTRACT

Vittaforma corneae is an obligate intracellular fungus and can cause human ocular microsporidiosis. Although accumulating reports of *V. corneae* causing keratoconjunctivitis in both healthy and immunocompromised persons have been published, little is known about the organism's occurrence in aquatic environments. Limitations in detection sensitivity have meant a large sampling volume is required to detect the pathogen up to now, which is problematic. A recent study in Taiwan has shown that some individuals suffering from microsporidial keratitis (MK) were infected after exposure to the pathogen at a hot spring. As a consequence of this, a survey and analysis of environmental *V. corneae* present in hot springs became an urgent need. In this study, sixty water samples from six hot spring recreation areas around Taiwan were analyzed. One liter of water from each sample site was filtered to harvest the fungi. The positive samples were detected using a modified nested PCR approach followed by sequencing using specific SSU rRNA gene primer pairs for *V. corneae*. In total fifteen *V. corneae*-like isolates were identified (25.0% of sites). Among them, six isolates, which were collected from recreational areas B, C and D, were highly similar to known *V. corneae* keratitis strains from Taiwan and other countries. Furthermore, five isolates, which were collected from recreation areas A, C, E and F, were very similar to *Vittaforma*-like diarrhea strains isolated in Portugal. Cold spring water tubs and public foot bath pools had the highest detection rate (50%), suggesting that hot springs might be contaminated via untreated water sources. Comparing the detection rate across different regions of Taiwan, Taitung, which is in the east of the island, gave the highest positive rate (37.5%). Statistical analysis showed that outdoor/soil exposure and a high heterotrophic plate count (HPC) were risk factors for the occurrence of *V. corneae*. Our findings provide empirical evidence supporting the need for proper control and regulations at hot spring recreational waters in order to avoid health risks from this pathogen. Finally, we have developed a small volume procedure for detecting *V. corneae* in water samples and this has proved to be very useful.

1. Introduction

The Microsporidia are a diverse group of obligate intracellular fungi that infect insects, animals and humans. Currently there are over one hundred microsporidial genera known and almost 1500 species; many are found at high frequency in aquatic environments (Stentiford et al., 2013). The first recognized human microsporidial infection was described by Nageli 1857 and involved silkworms (Nageli, 1857). From 1985 onwards, various studies have reported opportunistic microsporidial infections in AIDS patients, with the symptoms including diarrhea and weight loss. Improvements in diagnostic methods have led to an increased awareness of the Microsporidia and more recent studies have shown that

* Corresponding author.
** Corresponding author.
E-mail addresses: bmhsu@ccu.edu.tw (B.-M. Hsu), darder.ji@gmail.com (D.-D. Ji).
[1] Equal contribution to the first author.

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Microsporidia also are able to cause diseases in immunocompetent individuals (Bryan et al., 1991; Desportes et al., 1985; Didier et al., 2004; Weber et al., 2000). To date, seven genera of Microsporidia have been associated with human diseases (*Enterocytozoon* spp., *Encephalitozoon* spp., *Trachipleistophora* spp., *Tubulinosema acridophagus*, *Anncaliia algerae*, *Pleistophora* sp., and *Vittaforma corneae*), and clinical manifestations of human pathogenic microsporidia have been described in detail by US Center of Disease Control (Didier, 2005).

A case of corneal microsporidiosis was reported in 1993 that affected an immunocompetent patient and the pathogen was named *Nosema corneum* (Silveira et al., 1993). *N. corneum* was later renamed *Vittaforma corneae* because of the morphological characteristics of the fungi's spores in the infected cornea of mice. Various studies have confirmed that *V. corneae* is able to cause ocular microsporidiosis in both immunocompetent and immunocompromised patients (Deplazes et al., 1998; Shadduck et al., 1990; Sharma et al., 2011). Evidence from these studies suggests that *V. corneae* may present a significant risk of ocular microsporidiosis. In India, thirty cases of ocular microsporidiosis were reported between 2006 and 2008, and the number of infections was found to be increasing year by year (Reddy et al., 2011b). In Singapore, there were 124 confirmed cases of microsporidial keratitis reported between 2004 and 2009, and many of these reported cases were HIV-negative patients (Loh et al., 2009). Both studies showed that the risk of microsporidial keratitis (MK) was associated with seasonal rainfall. In the Singaporean study, 62 (50%) of the 124 patients had soil/mud exposure prior to infection. The first confirmed outbreak of microsporidial keratoconjunctivitis was reported in 2012 in Singapore. The outbreak was caused by *V. corneae*, and most of the patients had had prolonged soil or muddy water exposure during rugby games (Kwok et al., 2013; Lam et al., 2013; Tan et al., 2013). The above studies suggest that the disease occurs as a result of environmental exposure. In Taiwan, 23 microsporidial keratitis cases were reported between 2006 and 2011, and 14 (60.9%) had had prior exposure to hot springs (Fan et al., 2012). The same study also found that in four patients, the lapse time between hot spring exposure and symptom onset was less than 3 days. Microsporidia spores are heat resistant and therefore it is likely that spore exposure may be the major route of transmission when there is human infection with *V. corneae*.

Studies of Microsporidia in the aquatic environmental have mostly focused on *Enterocytozoon bieneusi* (Hu et al., 2014; Ma et al., 2015), and only two studies have reported evidence indicating the presence of *V. corneae* in aquatic environments (Dowd et al., 1998; Fournier et al., 2000). The amount of *V. corneae* in surface water is usually low, and this will often affects detection accuracy. The two studies targeting *V. corneae* were carried out by filtering large volumes of water (more than 10 L) and detection was via single-step PCR. Collection of large volumes of water as a sample is both time-consuming and costly, and the approach differs dramatically regarding the sample volume normally used for detecting waterborne pathogens, which is typically less than 1 L. In addition, while a single step PCR method is commonly used for pan-microsporidial detection, if the ambient concentration of *V. corneae* is low, which is expected to be the case in most water bodies, this method will be a relatively unsuitable approach to detecting *V. cornea* in an aquatic environment. In this context, a clinical study has shown that the use of diagnostic PCR can improve the diagnostic rate for *V. corneae* infections by 25% compared with a standard microscopic examination (Bharathi et al., 2013).

Although a pan-microsporidial one-step PCR yielded a 1148bp fragment of the 18S rRNA gene had been developed, which could be used for the detecting *V. corneae*, a more specific one-step PCR amplified a 472bp fragment within the above region was established to improve the detection of *V. corneae* by Bharathi et al. (Bharathi et al., 2013; Raynaud et al., 1998). In a number of studies, nested PCR, which involves a combination of different PCR tests, has been successfully used for the detection of protozoan/fungal pathogens in water samples (Badiee et al., 2015; Coupe et al., 2005; Miller and Sterling, 2007). Nevertheless, up to the present, the nested PCR method has not been used for detecting *V. corneae*.

Little is known about the potential for exposure to *V. corneae* in hot springs in Taiwan or elsewhere. We hypothesize that the use of nested PCR for the detection of *V. corneae* will enhance the detection limit for this organism and result in a requirement for less than 1 L of water sample when carrying out surveillance for *V. corneae* in hot spring water systems.

2. Materials and methods

2.1. Detection methods for V. corneae

Three methods were used in this study to detect *V. corneae*, namely two one-step PCR approaches and a modified nested PCR approach. The first method (the “one-step universal PCR”) used the universal primers described by Raynaud et al. (1998) and is primarily a method used for pan-microsporidia detection. The second method (the “one-step specific PCR”), as described by Bharathi et al. (2013)), consists of a primer set specific for *V. corneae*. For the third method (nested PCR), the primer sets used in the two one-step PCR methods were combined with some modifications. The outer primer set was V1/V1r, which targets the SSU-rRNA gene of *Microsporidia* spp. and amplifies from position 1 to position 1148 of the *V. corneae* SSU-rRNA gene based on the sequence from the NCBI nucleotide database (GenBank access no. U11046.1). The inner primer set, VC/VC-R, is able to amplify a 472 bp fragment from within the *V. corneae* SSU-rRNA gene in a species-specific manner. The region amplified is from position 192 to position 663 of the *V. corneae* SSU-rRNA gene as defined above. The primers used for each method are summarized in Table 1.

The thermal cycling conditions for the one-step universal PCR analysis consisted of 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 1.5 min, with a final extension of 72 °C for 5 min. The amplicons from this PCR were purified using a PCR cleanup kit (Qiagen, Taiwan). The thermal cycling conditions of the one-step specific PCR method consisted of 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s, with a final extension of 72 °C for 10 min. For the nested PCR analysis, the initial thermal cycling conditions used were the same as for the outer primers, and this was followed by a second diagnostic PCR amplification using the same conditions as above for the inner primers.

2.2. Evaluation of detection limit

To determine the limit of detection (LOD) for each method, a 13-order serial dilution of plasmid DNA was prepared. This created 5×10^{-2} to 5×10^{10} plasmid copies per reaction. A *V. corneae* strain that was isolated from a microsporidial keratitis patient in Taiwan was obtained from the Taiwan CDC and this was used as a positive control (MK patient). The isolated chromosomal DNA of this strain was then amplified using the one-step universal PCR approach. Subsequently, the PCR product was cloned into the T&A Cloning Vector (Real Biotech Co., Taiwan) and then transformed into *Escherichia coli* DH5α cells. After selection and confirmation of the insert, the plasmid DNA was extracted using a Plasmid DNA Extraction kit. This was the DNA used to prepare the 13-order serial dilutions. After PCR, samples were separated by electrophoresis on a 2% agarose gel and the DNA product visualized using a UV