Short Report

Immunohistopathology of *Prototheca wickerhamii* in Cutaneous Lesions of Protothecosis

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ABSTRACT

Protothecosis is a rare infection caused by pathogenic algae of the genus *Prototheca*. *Prototheca wickerhamii* causes cutaneous/subcutaneous opportunistic infections in humans and small animals. The diagnosis of protothecosis is based on histopathological examination of this organism, which can be confused with other fungi and inflammatory cells in infected tissues. In this study, immunohistopathological investigation was made of infected cutaneous human and animal tissues exhibiting protothecosis using rabbit antiserum against *P. wickerhamii*. Serum detected *P. wickerhamii* in human and feline protothecosis tissues, and did not react with *Candida albicans* in the human kidney tissues showing candidiasis. This antiserum can therefore differentiate *P. wickerhamii* cells from the yeast-like cells of *C. albicans* and *Prototheca zopfii* in target tissues.

Key words: antiserum, immunohistopathology, protothecosis, *Prototheca wickerhamii*

Introduction

Protothecosis is a rare infection caused by the genus *Prototheca* (Trebouxiophyceae, Chlorophyta), which is classified as an achnorophyllous alga closely related to the green algae of the genus *Chlorella*. This genus is widely distributed throughout the natural world in sewage, soil, lakes and marshes. Although protothecosis is recognized with sufficient frequency that individual reports of disease are not routinely published, the relative prevalence of disease is still considered low: by the year 2000, only 108 human infections had been reported in the medical literature; by 2006, only 44 canine infections had been reported; and by 2009, only 5 feline infections had been reported. The most pathogenic algae in humans and small animals is *Prototheca wickerhamii*, which can cause opportunistic infections cutaneously and subcutaneously. The diagnosis of protothecosis depends on histopathological examination of skin biopsies. However, endospores of this organism may be confused with other fungi and inflammatory cells in infected tissues. Therefore, a specific antiserum against the alga should be useful for definitive diagnosis of this infection. In this study, immunohistopathological investigation was performed on infected tissues of cutaneous human and animal protothecosis using rabbit antiserum against *P. wickerhamii*.

Materials and methods

Strains

Type strains of *P. wickerhamii* (ATCC16529) were used in this study. Isolates were grown in liquid *Prototheca* isolation medium (PIM) at 37°C for 3 days and were then collected by centrifugation.
tion (700 × g, 10 min). Cells were washed twice in saline (0.15 M sodium chloride) and then fixed in saline containing 0.05% formalin, followed by storage in formalinized saline for 60 min at room temperature. Finally, cells were washed three times with saline and standardized based on hemocytometer counts.

**Immunization of rabbits**

Immunization was carried out at Japan Bio Serum Co., Ltd. (Hiroshima, Japan). Briefly, 4 adult male New Zealand white rabbits (Japan Bio Serum Co., Ltd.) were immunized with 14 daily intravenous injections of 1 × 10^7 cells four times. Animals were euthanized by pentobarbital, and serum was collected 7 days after the conclusion of the immunization schedule. Serum was purified by affinity chromatography with a protein A binding column.

**Antibody absorption test with Candida albicans**

In order to prevent cross-reactivity with other yeasts, *C. albicans* TIMM5030 was grown in liquid Sabouraud’s dextrose medium (1% of peptone and 4% of dextrose) at 37°C for 2 days, and was then collected by centrifugation (700 × g, 10 min). Yeast cells were washed twice in saline and fixed in saline containing 0.05% formalin, followed by storage in formalinized saline for 60 min at room temperature. Finally, cells were washed three times with saline, and collected by centrifugation. Purified serum and a 1/2 volume of yeast cells were incubated at 37°C for 48 h with shaking, followed by centrifugation. This serum was then collected and used for immunohistopathological examination.

**Immunohistopathological examination on human and feline tissues**

Human and feline, cutaneous protothecosis tissues were immunohistochemically analyzed in this study. Both cases were diagnosed based on to the results of histopathological examination and isolation of *P. wickerhamii*. As a negative control, bovine mammary gland with *P. zopfii* infected tissue and human candidiasis kidney tissue were used in this study.

Tissues from these cases were fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and embedded in paraffin. These were then sectioned at 5μm and placed on slides overnight at 37°C. Formalin-fixed paraffin sections of human kidney candidiasis tissue were used as negative controls for serum cross-reactivity. Sections were deparaffinized in xylene for 1.5 h, washed twice in 100% ethanol and once in 95% ethanol, and then with distilled water. Antigen retrieval was performed by incubating the mounted sections in an autoclave for 10 min in boiling 9 mM sodium citrate (pH 6.0). Sections were then washed twice with PBS and blocked for 1 h at 25°C in blocking buffer (phosphate buffered solution; PBS with 10% bovine serum albumin; BSA). Without washing, sections were incubated with the serum diluted 1:800 with blocking buffer overnight at 4°C. After sections were washed three times in PBS, staining was performed using anti-rabbit Nichirei simple stain Lat MAX-PO MULTI (Nichirei, Tokyo, Japan) with 3,3′-diaminobenzidine (DAB; Dako, Tokyo) as the chromogen. After DAB treatment, counter-staining was performed with Mayer’s hematoxylin (Sigma-Aldrich Co. LLC, Tokyo) and eosin solution (Wako Pure Chemical Industries, Ltd.).

**Results**

**Detection of *P. wickerhamii* in human and feline tissues by immunohistochemistry**

The serum detected *P. wickerhamii* in human and feline protothecosis tissues, and did not react with bovine mammary gland with *P. zopfii* infected tissue but *C. albicans* in human renal candidiasis tissue (Fig. 1).

**Discussion**

In previous reports, *P. wickerhamii* was found to be a common agent in cutaneous/subcutaneous opportunistic infections in human and small animals. Therefore, it is very important to identify this alga in tissues and to successfully treat human protothecosis caused by *P. wickerhamii*, as success rates of treatment are low (59%–76%) with limited susceptible drugs. The main consideration in the differential diagnosis is a deep, cutaneous mycosis, and common fungal infections including cryptococcosis, histoplasmosis, sporotrichosis, chromomycosis and other yeast-like pathogens. The general morphologic features of histopathological appearance in cutaneous protothecosis are spherical, nonbudding, sporangia with a thick, double-layer wall, morula-like appearance and filled with multiple endospores.

The rabbit antiserum against *P. wickerhamii* used in this study showed no cross-reactions with...
C. albicans or P. zopfii in bovine mastitis tissues. Therefore, this antiserum is able to differentiate P. wickerhamii cells from yeast-like cells of C. albicans and P. zopfii in the target tissues. Moreover, we investigated for cross-reactions with Cryptococcus neoformans cells and Sporothrix schenckii cells that were fixed in saline containing 0.05% formalin. The antiserum used in this study also showed no cross-reactions with these cells (data were not shown here).

However, further investigations into the immunohistopathological examination of protothecosis cases remain necessary.

Postscript

All procedures were carried out in accordance with the “Act on Welfare and Management of Animals and administration of animal welfare and management and Proper-handling guideline for Raising and Keeping of Laboratory Animals” of the Nature Conservation Bureau Ministry of the Environment of Japan (http://www.env.go.jp/nature/dobutsu/aigo/2_data/nt_h180428_88.html).

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References


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