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Development of a Peptide Nucleic Acid Probe to *Trichosporon* Species and Identification of Trichosporonosis by Use of *In Situ* Hybridization in Formalin-Fixed and Paraffin-Embedded (FFPE) Sections

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In order to identify *Trichosporon* species in formalin-fixed and paraffin-embedded sections from which visual discrimination of non-*glabrata Candida* species is mostly ineffective but critical for the choice of antifungals, we tested the usefulness of a newly designed peptide nucleic acid probe (PNA) for *in situ* hybridization (ISH). Results confirmed the usefulness of ISH with our PNA probe in identifying *Trichosporon* species from *Candida albicans.*

Trichosporon species have been reported as the second or third most common agents of yeast fungemia (1–3), and the prevalence has increased, particularly in patients with hematologic malignancies (4–6). Since *Trichosporon* spp. exhibit low susceptibility to candins (7), histopathological examination is important as one of the useful diagnostic procedures, even though it is regarded as poor for the identification of *Trichosporon* species from other dimorphic yeasts, namely, non-*glabrata Candida*, species, because of their overall similarities (8–10). Therefore, the establishment of an auxiliary diagnostic method for use in routine pathological laboratories is required for diagnosis of disseminated trichosporonosis. In the present study, we report attempts to develop a specific peptide nucleic acid (PNA) probe to *Trichosporon* spp. and evaluate this method for identification of the fungus in formalin-fixed and paraffin-embedded (FFPE) tissue sections by using *in situ* hybridization (ISH).

We employed FFPE tissues both from experimentally infected mice and autopsies with a proven diagnosis. Specific-pathogen-free male, 8-week-old Institute of Cancer research mice were injected intravenously with 3×10^7 yeast cells of *Trichosporon asahii* (strain 015), *T. asahii* (strain 336), or *Candida albicans* (J2-15), and their kidneys were obtained 3 days after infection and processed by a conventional method. Lungs from autopsies with disseminated candidiasis and trichosporonosis were also used. Trichosporonosis was diagnosed by DNA sequence analysis. Candidiasis was culture proven (EC Toho approved; 20047).

The antisense PNA probe targeting the 26S rRNA of *Trichosporon* spp. (N terminus-CGG ACA ATC GAA GAC) was hypothetically designed based on a comparison of the sequences of 26S rRNA genes of *Trichosporon* spp. and other pathogenic fungi available in the GenBank database. To identify *C. albicans*, we also used an antisense PNA probe targeting the 26S rRNA of *C. albicans* (N terminus-TAC TTG TGC GCT ATC GGT) (11). Furthermore, to estimate retention and hybridizability of the target RNA in samples, we used a panfungal antisense PNA probe (N terminus-TAC TTG TGC GCT ATC GGT) (12). The oligonucleotide probes used in this study were made by Fasmac Co., Ltd. (Kanagawa, Japan), and the N terminus of the PNA probes was conjugated to fluorescein isothiocyanate (FITC). The process of obtaining FFPE tissues and the ISH procedure were performed as described previously (12, 13).

ISH showed strong positive signals against *Trichosporon* spp. 26S rRNA within yeast-like elements present in renal tissues from mice infected with *T. asahii* (Fig. 1A), whereas these signals were not observed in specimens derived from mice infected with *C. albicans* (Fig. 1B). On the other hand, the panfungal PNA probe reacted with *T. asahii* and *C. albicans* (Fig. 1C and D) and confirmed the retention and hybridizability of rRNA. In an additional evaluation using autopsy samples, ISH preparation showed that the PNA probe against *Trichosporon* spp. was strongly reactive with yeast-like elements of *Trichosporon* spp. (Fig. 2C), whereas the PNA probe against *C. albicans* was not reactive with any *Trichosporon* spp. (Fig. 2D). Conversely, the PNA probe against *Trichosporon* spp. was not reactive with organisms from subjects with candidiasis (Fig. 3C), but its organisms showed strong positive signals when the PNA probe targeting *C. albicans* was applied (Fig. 3D). Whereas the *Trichosporon* species-specific probe we designed in the study showed acceptably strong signals for *T. asahii* in tissue sections from both experimental infections and autopsy samples, it should be confirmed whether the probe actually reacted for non-*asahii Trichosporon* species in FFPE tissues.

Trichosporon spp. present certain morphological features in pathological specimens (14). However, their morphological similarities to other fungi, especially non-*glabrata Candida* species, lead to difficulties in the identification of trichosporonosis. Hence, the establishment of an auxiliary diagnostic method for use in routine pathological laboratories would be useful for a diagnosis of disseminated trichosporonosis with histopathological differentiation from candidiasis. Although a few studies have at-

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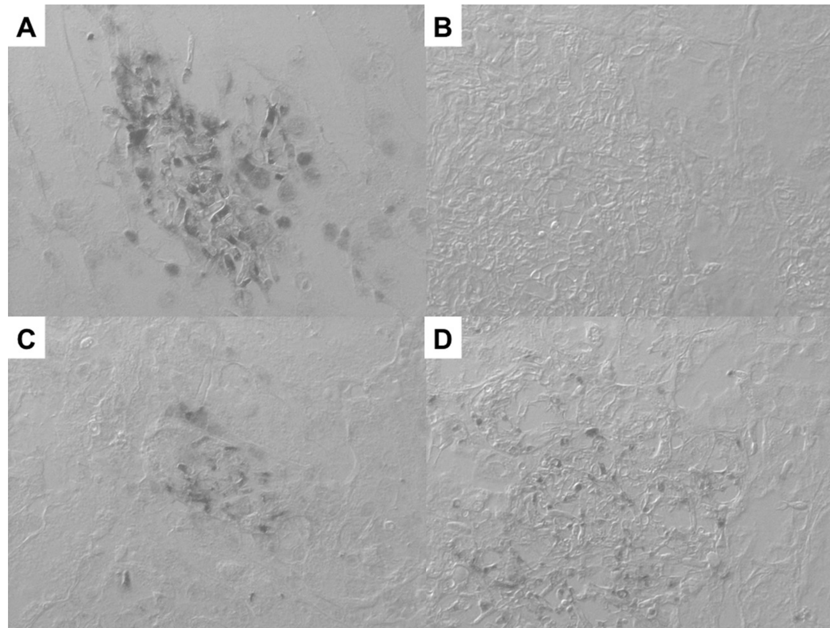

FIG 1 Specificity verification of the *Trichosporon* spp. PNA probe and assessments of rRNA retention and its hybridizability in experimentally infected mice. (A) ISH using the *Trichosporon* spp. PNA probe in renal tissue from mice infected with *T. asahii*. Strong positive signals against 28S rRNA of *Trichosporon* spp. were observed in the specimen. (B) ISH using the *Trichosporon* spp. PNA probe in renal tissue from mice infected with *C. albicans*. Positive signals were not observed in the specimen. (C) ISH result with the panfungal PNA probe in renal tissue from mice infected with *T. asahii*. Strong positive signals were observed in the specimen. (D) ISH result with the panfungal PNA probe in renal tissue from mice infected with *C. albicans*. Strong positive signals were observed in the specimen. Magnification, ×400.

tempted to identify other fungi in histopathological specimens by using ISH (15–17), no investigations have utilized ISH for the diagnosis of trichosporonosis.

The diagnosis of trichosporonosis by immunohistochemistry using a self-made antibody to *Trichosporon* has been reported (18, 19); however, these antibodies are not available for commercial use, there are limitations for their use, and their specificity could not be confirmed. Therefore, we developed

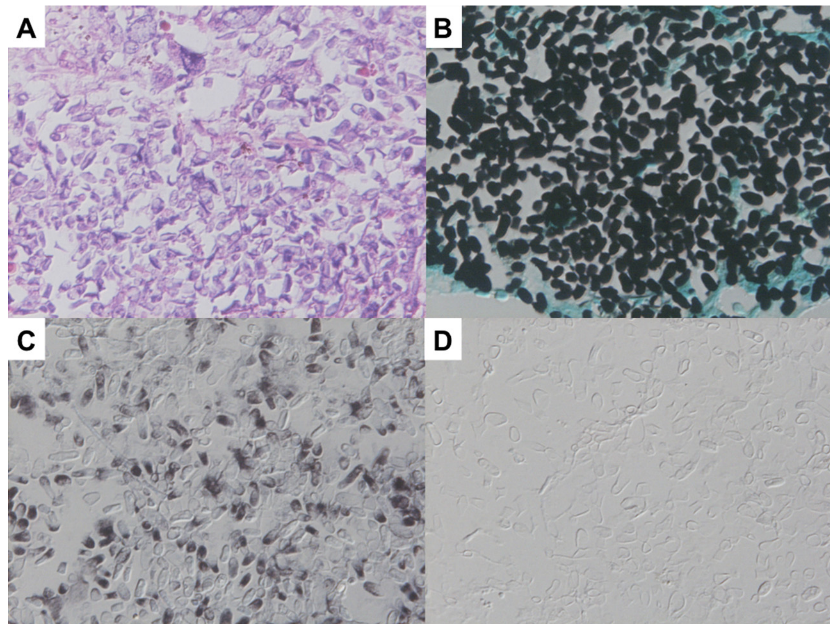

FIG 2 Results of ISH with a pulmonary lesion of disseminated trichosporonosis confirmed by DNA sequence analysis. (A) Pathological findings with hematoxylin and eosin stain. Histological examination revealed foci consisting of yeast formations of organisms. (B) Findings with Grocott's stain. Grocott's stain showed oval or square yeast-like elements within foci of infection. (C) Result of ISH with the *Trichosporon* spp. PNA probe. The PNA probe against *Trichosporon* spp. was strongly reactive with the yeast-like elements of *Trichosporon* spp. (D) ISH result with the *C. albicans* PNA probe. The PNA probe against *C. albicans* was not reactive with any *Trichosporon* spp. organisms.

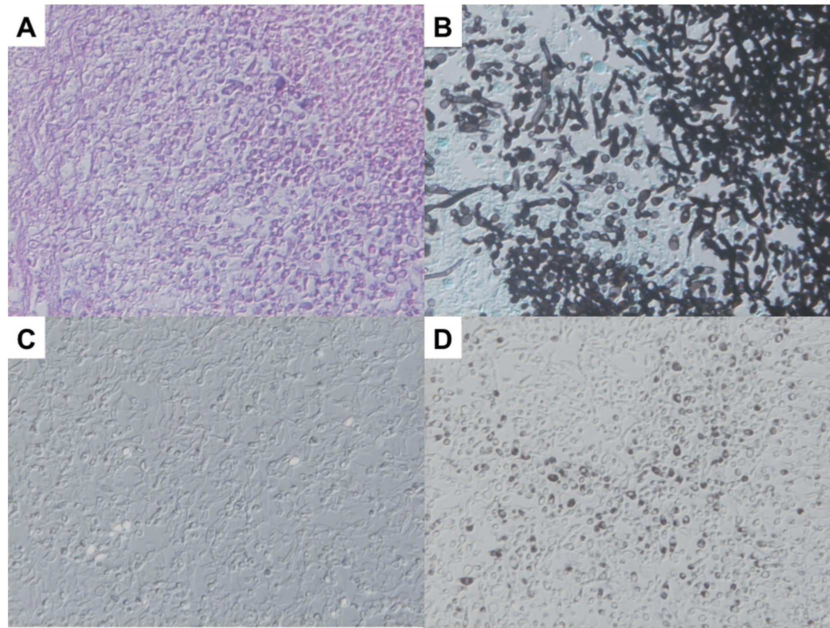

FIG 3 Results of ISH for pulmonary lesions in the case of culture-proven *C. albicans*. (A) Pathological findings with hematoxylin and eosin stain. Histological examination revealed foci consisting of pseudohyphal and yeast formations of organisms. (B) Results with Grocott's stain. Grocott's stain showed oval yeast-like and pseudohyphal elements within the foci of infection. (C) ISH result with the *Trichosporon* spp. PNA probe. The PNA probe against *Trichosporon* spp. was not reactive with any organisms of *C. albicans*. (D) ISH result with the *C. albicans* PNA probe. The PNA probe against *C. albicans* was strongly reactive with pseudohyphal and yeast-like elements of *C. albicans*.

the auxiliary utility of ISH for the pathological diagnosis of trichosporonosis from FFPE tissues. Although PCR has been regarded as a sensitive and useful assay for the detection of *Trichosporon* species (20, 21), the application of this technique to pathological specimens has the disadvantage of being highly susceptible to contamination and formalin fixation, potentially leading to diagnostic mistakes. In addition, PCR-based molecular techniques have the difficulty of DNA release in DNA extraction due to the rigid fungal cell wall (22). Accordingly, we now have to regard that FFPE tissue also limits the use of PCR because of DNA degradation and low yield on extraction protocols. On the other hand, ISH has little contamination risk and does not require nucleic acid extraction. From the viewpoint of the above-mentioned properties, ISH may overcome the disadvantage of PCR-based molecular techniques that use FFPE sections. Recently, ISH techniques employing PNA probes for rRNA have been developed as useful techniques for the differentiation of medically important *Candida* spp. (11, 23). These novel properties enabled PNA probes to hybridize to complementary nucleic acid targets with high specificity and rapid binding kinetics (24, 25).

In conclusion, we wish to emphasize that ISH with our probe can be valuable in distinguishing *Trichosporon* spp. from non-*glabrata Candida* species in FFPE tissues, since we demonstrated that our newly designed PNA probes targeting the 26S rRNA showed a specific signal intensity for *Trichosporon* spp. in various kinds of tissue sections.

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M.S. and Y.O. wrote the manuscript as major and equal contributors. D.S., H.N., and T.I. sampled publications. S.Y.M. advised the first author on ISH. N.T., M.W., and T.N. carried out the histopathological evaluation. K.S. integrated the data and gave final approval to the manuscript as a corresponding author. All authors contributed towards the conceptualization, writing, reading, and approval of the final manuscript.

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