



Original Article

Histopathological study on the prevalence of trichosporonosis in formalin-fixed and paraffin-embedded tissue autopsy sections by *in situ* hybridization with peptide nucleic acid probe

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Received 18 March 2019; Revised 4 August 2019; Accepted 6 September 2019; Editorial Decision 27 August 2019

Abstract

Trichosporon species are some of the most common pathogenic yeasts in Asia, and many are resistant to echinocandin antifungal drugs. Effective treatment of fungal infections requires the selection of appropriate antifungals and the accurate identification of the causal organism. However, in histopathological specimens *Trichosporon* spp. are often misidentified as *Candida* species due to morphological similarities. *In situ* hybridization (ISH) is a useful technique for identifying fungal species in formalin-fixed and paraffin-embedded (FFPE) tissue sections. Although many novel probes for ISH are available, the practical use of ISH for identification of fungi remains limited, in part due to the lack of adequate verifications. We conducted a two-center retrospective observational study in which the ISH technique was used to differentiate *Trichosporon* spp. and *C. albicans* in FFPE tissue from autopsy specimens. The study included 88 cases with blood stream yeast infection without *Cryptococci* extracted from 459 autopsy files of cases with proven invasive fungal infection (IFI). Positive signals for the *Trichosporon* spp. protein nucleic acid (PNA) probe and *C. albicans* PNA probe were seen for 7 and 35 cases, respectively, whereas the remaining 46 were negative for both. For the *Trichosporon* spp.- positive specimens, 5/7 were reported as candidiasis in autopsy records. Our results suggested that accurate histological identification of fungal infections remains challenging, but ISH may be a suitable approach to support histological findings. In addition, this retrospective study suggested that trichosporonosis may have high prevalence among cases of bloodstream yeast infections in Japan.

Key words: invasive fungal infection, *in situ* hybridization, *Trichosporon*.

Introduction

Invasive fungal infection (IFI) is a serious and refractory disease that has high mortality among immunocompromised patients. Rapid identification of causative fungi is important for selecting

appropriate antifungals that may directly contribute to favorable patient outcome and reducing health care costs.¹ Although identification by culture is generally accepted as the gold standard for identifying causative fungi in clinical specimens obtained from

infection sites, low assay sensitivity, potential for contamination, and time required are significant issues for correct diagnosis of fungal infection relative to that for diagnosis of bacterial or other microbial infections. As such, molecular procedures that supplement histological identification of causative fungi are being introduced.² Histopathological diagnosis is accepted as one of the best diagnostic procedures for IFI. Moreover, the superiority of histopathological approaches is widely accepted based on the speed, low cost, and ease of evaluation of the invasiveness of causative fungi offered by these methods. However, specimens cannot always be obtained for histological examination and some fungal species are difficult to identify by light microscopy examination of tissue sections alone.^{2,3}

Research into molecular diagnostic techniques involving polymerase chain reaction (PCR) or *in situ* hybridization (ISH) for identification of causative agents of invasive fungal infection is ongoing. These techniques enable identification of causative species more rapidly than culture techniques. In addition, newly available commercial peptide nucleic acid fluorescent *in situ* hybridization (PNA FISH) assays in conjunction with blood culture bottles have become popular for clinical use.⁴ Although several novel primers and/or probes for fungi have been introduced, there are limited studies concerning the direct applications of these probes for clinical specimens and the use of ISH for formalin-fixed and paraffin-embedded (FFPE) tissue sections requires adequate verification.^{2,5}

Candida albicans is the most common cause of invasive infections related to yeast and accounts for about half of all *Candida* spp. infections. However, over the past few decades, reports of the incidence of non-*albicans* *Candida* spp. infections worldwide have been increasing, as is the frequency of isolation of these fungi.^{6,7} After the launch of echinocandin, this class of antifungal drugs has seen broad use worldwide and in the latest guidelines is recommended as the first-line treatment for invasive candidiasis.⁸ The safety of echinocandin is generally accepted and its effectiveness in targeting some non-*albicans* *Candida* spp., including those that are azole-resistant, is confirmed.⁸ On the other hand, *Trichosporon* species, which are mostly resistant to echinocandins, are emerging as common sources of yeast infections in many Asian countries, including Japan.⁹ Indeed, some cases of echinocandin-breakthrough trichosporonosis have been reported in recent decades.^{7,10,11} In such cases, the patients often had an initial diagnosis of generalized candidiasis.¹² Thus, although the importance of identification of *Trichosporon* spp. and *Candida* spp. using histopathological examination is accepted, the morphological similarity of these two yeasts, which both produce yeast and hyphae forms in human tissues, complicates accurate identification using only routine histological examination approaches.³ For selection of optimal antifungal drugs, supplemental molecular procedures are now needed to complement histopathological diagnosis of these fungal infections. We previously reported the development of an ISH procedure using PNA probes that are specifically designed

to detect *Trichosporon* spp.¹³ However, this molecular technique has not yet been adequately tested on FFPE tissue sections from clinical subjects.

The present study aimed to evaluate the usefulness of the ISH procedure involving a *Trichosporon* spp.-specific PNA probe applied to FFPE tissue sections from autopsy subjects, most of whom had been diagnosed as having generalized candidiasis. The results of this study may also contribute to the elucidation of the past prevalence of trichosporonosis in Japan.

Methods

Study setting

This study considered 8,054 autopsies performed on cases from all major clinical departments at two major regional hospitals in the Tokyo metropolitan area: Toho University Medical Center Omori Hospital (cases between January 1975 and December 2012) and the Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital (cases between January 1987 and December 2012). All study samples were from cases having a diagnosis of IFI that was histologically verified by confirmation of the presence of fungal elements in infection foci.¹⁴ We found 459 cases of IFI during the observation period. Among these, we extracted cases that were recorded as having bloodstream yeast infection defined as affecting two or more deep, sterile solid organs comprising the heart, lung, kidney, liver, spleen, adrenal glands, thyroid, and brain. Two organs were considered since colonization of the respiratory tract by candida is common and defining cases as having invasive infection is difficult when *Candida* infection is limited to the lung.^{8,15} Therefore, we excluded candida pneumonia accompanied by superficial infections such as esophageal candidiasis and candida cystitis. Cryptococcal infections were also excluded because histological identification of this type of yeast is generally accurate, and it rarely exhibits hyphal growth.

A total of 105 cases meeting these inclusion criteria were extracted from the autopsy records; most cases were diagnosed as having invasive candidial infection, although several were documented only as having yeast-related diseases. Specimens from these 105 autopsies were prepared for histological confirmation of the diagnosis of bloodstream yeast infections.

The study protocol was approved by the Ethics Committee of Toho Medical University (A19023_A18055_27070).

Materials

Sections taken from blocks of FFPE tissue from the 105 autopsies were mounted on slide glasses. The samples were deparaffinized and stained with hematoxylin and eosin as well as periodic acid-Schiff (PAS) reaction. Another routine special stain, such as Grocott's methenamine silver (GMS) or Alcian blue staining, was used as required. Three expert pathologists participated in histopathological diagnosis using light microscopy

to confirm the previous histological diagnosis of invasive yeast infection. Five cases infected with encapsulated yeast diagnosed as cryptococcosis and 12 cases of mould infection confirmed during review of our study were excluded from the cohort. We identified the morphological features of fungi based on Guarner et al.³

In situ hybridization using peptide nucleic acid probe

A total of 88 FFPE tissues from autopsies confirmed as blood stream yeast infections without cryptococcosis were included in this study. PNA probes that were previously confirmed to show specific targeting of *Trichosporon* spp. 26 s rRNA (N terminus-CGG ACA ATC GAA GAC) and *Candida albicans* 26 s rRNA (N terminus-ACA GCA GAA GCC GTG) were used.¹³ The oligonucleotide probes were made by Fasmac Co., Ltd. (Kanagawa, Japan), and the N-terminus of the PNA probes was conjugated to fluorescein isothiocyanate (FITC).

The ISH technique was carried out according to the method previously described by Shinozaki et al.¹⁶, with minor modifications. Briefly, 4 μm -thick sections of FFPE tissue containing foci having the highest concentration of yeast for each case were prepared for ISH. Kidney tissue sections were the most common samples. After deparaffinization and rehydration of the sample using standard protocols, DNA alignments in the sections were exposed by heating the section in 1 mM EDTA buffer (pH 8.0) for 30 minutes in a 98°C water bath (Thermo Fisher Scientific K.K., Yokohama, Kanagawa). After cooling at room temperature for 20 minutes, the sections were treated with 10 $\mu\text{g}/\text{ml}$ proteinase K (Nippon Gene Co., Ltd., Tokyo, Japan) for 10 minutes at 37°C. Then, 1 $\mu\text{g}/\text{ml}$ PNA probe dissolved in hybridization buffer (Nippon Gene Co., Ltd., Tokyo, Japan) was applied and hybridized at 56°C for 90 minutes followed by incubation at 94°C for 5 minutes to denature the rRNA. After repeated washings with 2 \times standard saline citrate (SSC) at 56°C, anti-FITC antibody (Roche Diagnostics K.K., Tokyo, Japan) and horseradish peroxidase-labeled polymer solution (Nichirei Biosciences, Inc., Tokyo, Japan) were used to detect hybridization signals. Finally, the peroxidase reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dojindo Laboratories, Kumamoto, Japan) in 0.1 M phosphate buffer (pH 7.3) containing 3% H_2O_2 , 1% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 1% $(\text{NH}_4)_2\text{Ni}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$. Dark brown staining seen in the tissue indicated a positive result.

All ISH were performed for both autopsy samples and control samples taken from mice experimentally infected with either *Trichosporon asabii* (strain 015) or *Candida albicans* (strain J2-15), which were prepared for a previous study.¹³ Furthermore, we confirmed the specificity of both probes used in this study with FFPE of cultures from several non-*albicans* *Candida* spp. (*C. glabrata* [strain TIMM10634], *C. tropicalis* [strain TIMM 0313], and *C. parapsilos* [strain TIMM0292]) species.

Histological evaluation of inflammation using a tissue response grading system

Since IFIs are generally opportunistic infections, a wide histological spectrum of infected foci were recognized because both the tissue response of the host and virulence of causative fungi varied from case to case. Therefore, to evaluate whether tissue response, especially infiltration of neutrophils, affected ISH signals, we histologically evaluated the extent of inflammation in the foci of yeast infections using a tissue response grading system (grade 1–3) to determine the numbers of fungi and host inflammation cells in the specimens.¹⁷ This grading was used to show the relationship between host immune response and ISH results. To focus on the host immune response, the modified grading scheme was: Grade 1: No response or infiltration of inflammatory cells; grade 2: Mild to moderate infiltration of inflammatory cells; and grade 3: Strong infiltration of inflammatory cells. Three expert pathologists scored the samples after analysis by light microscopy. For foci having more than one grade, the dominant grade was used. Index sample images for each tissue grade are shown in Figure 1.

Polymerase chain reaction (PCR) and sequencing

To confirm that samples showing no ISH signals indeed lacked DNA specific to either yeast, PCR was carried out for all negative samples. The Internal Transcribed Spacer (ITS) region of fungal ribosomal DNA was amplified to determine the quality of fungal DNA in samples showing no signal for either ISH PNA probe (*C. albicans* and *Trichosporon* spp.) Three fungus-specific PCR assays for ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') forward/ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') reverse, ITS3 (5'-GCATCGATGAAGAACGCAGC-3') forward/ITS4 (5'-TCCTCCGCTTATTGATATGC-3') reverse and ITS1 forward/ITS4 reverse) were used according to a previously described method.¹⁸ DNA was extracted using a commercially available kit (QIAamp DNA FFPE Tissue Kit, Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions. DNA was extracted from three 10 μm -thick FFPE sections taken from the same block as that used for ISH. Each PCR mixture included 5 μl extracted DNA, 1 μl 1.25 U/ μl MightyAmp DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.5 μl of 100 μM primer, 25 μl 2 \times Mighty Amp Buffer Ver.2 (Takara Bio Inc., Shiga, Japan) and dH_2O . PCR was performed using a T100 thermal cycler (Bio-Rad Laboratories, CA, USA) with initial denaturation at 98°C for 2 minutes, followed by 40 cycles of denaturation at 98°C for 10 seconds, annealing for 15 seconds at 55°C, and extension for 30 seconds at 68°C. A 3 minute extension step at 68°C was included at the end of amplification. The PCR products were electrophoresed on a 1.5% agarose gel and visualized under UV light. All PCR-positive samples were sequenced, and the results were compared with sequences in the MycoBank database.

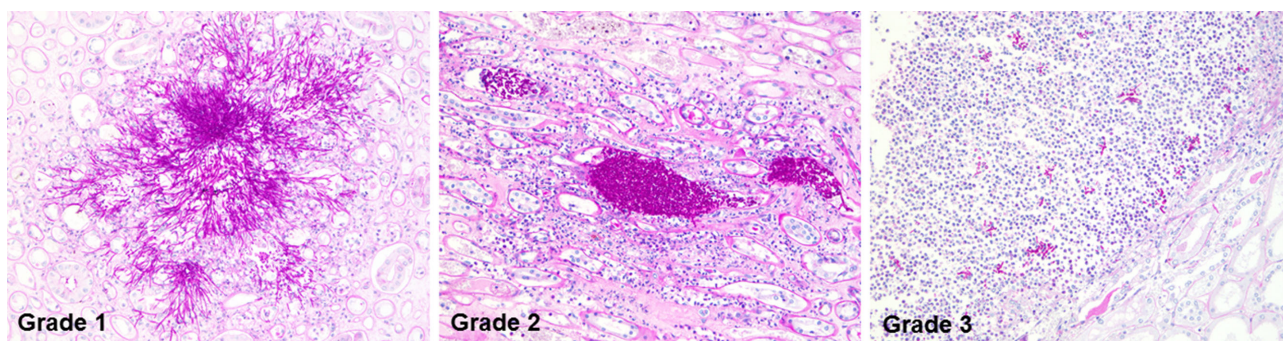


Figure 1. Tissue response grading system. The inflammatory cell infiltrates largely comprising neutrophils against invading fungi were classified into three grades to describe the strength of the host immune defense. Index cases for each grade are shown: Grade 1: Absence of inflammatory infiltrate with extensive fungal growth presenting as both yeast and hyphal forms; grade 2: Mild to moderate inflammatory infiltrate with intermediate fungal growth; grade 3: Marked inflammatory infiltrate with a few yeast cells present (200x total magnification, periodic acid-Schiff (PAS) reaction).

A similarity >99% for amplicons was required to identify the presence of fungi.

Statistical analysis

The Cochran-Armitage test for trend was used to evaluate the relationship between tissue grade scores and ISH results. *P* values < .05 were considered statistically significant. All statistical analyses were performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). EZR is a modified version of R Commander designed to add statistical functions that are frequently used in biostatistics.¹⁹

Results

Demographic information for study subjects

The median age of the autopsied patients was 54.9 years old. The study included several neonatal cases, and more than half of the total cases (60.2%) were male. Underlying diseases present in our study subjects are shown in Table 1.

In situ hybridization results

Among the 88 samples analyzed, seven and 35 samples showed positive signals for *Trichosporon* spp.- and *C. albicans*-specific probes, respectively. A representative case (case 30) that had a positive signal for the *Trichosporon* spp.-specific probe is shown in Figure 2 and a representative case (case 22) that had a positive signal with the *C. albicans*-specific probe is shown in Figure 3. A total of 46 samples gave no signals for either probe and no sample was simultaneously positive for both probes (Fig. 4). Additional details are shown in Supplementary Figures S1 and S2a, b, c. One of the seven samples that had positive ISH signals for the *Trichosporon* spp. PNA probe was from a specimen taken from an autopsy filed as trichosporonosis

Table 1. Demographic information for 88 autopsy samples.

Characteristics	no.(%) or median [range]
Age [yr]	54.9 [0-88]
Gender	
Male	53 (60.2)
Female	35 (39.8)
Underling disease ^a	
Hematological disorders	29 (33.0)
Leukemia ^b	19 (21.6)
Malignant lymphoma	4 (4.5)
Aplastic anemia	3 (3.4)
Other hematologic disorders	6 (8.0)
Solid organ tumors	33 (37.5)
Gastrointestinal carcinoma	13 (14.8)
Lung carcinoma	5 (5.7)
Gall bladder and bile duct carcinoma	4 (4.5)
Hepatocarcinoma	3 (3.4)
Urinary bladder carcinoma	3 (3.4)
Other carcinoma	5 (5.7)
Other underling disease ^c	31 (35.2)
Hepatitis	4 (4.5)
Kidney failure	4 (4.5)
Cardiovascular disease	4 (4.5)
Systemic lupus erythematosus	3 (3.4)
Acquired immunodeficiency syndrome	1 (1.1)
Kidney transplantation	1 (1.1)
Others	14 (15.9)

^aAutopsy samples may be from patients that had more than one underlying condition.

^b“Leukemia” includes both acute and chronic leukemia.

^cDiseases having 3 or more cases and those that cause severe immunosuppressive conditions are listed.

for which the causative fungus was confirmed by culture as *Trichosporon cutaneum*. On the other hand, five of the samples that gave positive signals for *Trichosporon* spp. were from autopsies recorded as candidiasis, whereas one was diagnosed

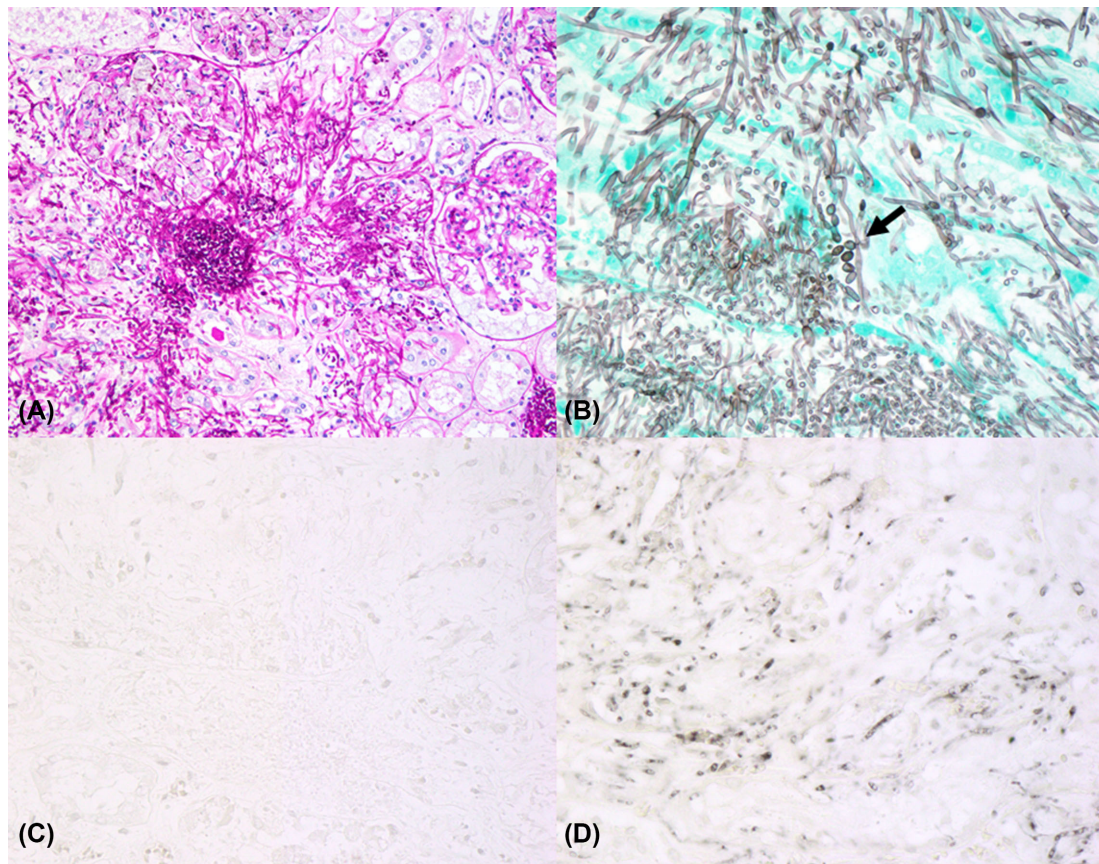


Figure 2. Representative case positive for PNA probe targeting *Trichosporon* spp. Images are from a representative case (case 30) that showed positive staining for *Trichosporon* spp. (A) Invasive yeast with hyphae forms in kidney tissue with no inflammatory cell response (Periodic acid-Schiff (PAS) reaction, 200 \times). (B) Arthroconidia (arrow) with hyphae forms (Grocott's methenamine silver (GMS) stain, 400 \times). (C) Negative reaction for protein nucleic acid (PNA) probe targeting *C. albicans* (400 \times). (D) Positive reaction for PNA probe targeting *Trichosporon* spp. The positive result is indicated by dark brown staining of the tissue (400 \times).

only as having an unspecified fungal infection. Of the seven *Trichosporon* spp. positive cases, four were from patients that had a hematological malignancy (three leukemia and one multiple myeloma).

Neither probe showed positive signals for control FFPE sections prepared from cultures of several of non-*albicans* *Candida* spp. species (*C. glabrata*, *C. tropicalis*, and *C. parapsilos*).

Polymerase chain reaction (PCR) and sequencing results

No PCR products were obtained for the target region containing ITS1/ITS2 (0/46 samples), which amplified the shortest fragment out of the three assays. However, in the region targeted by ITS3/ITS4 and ITS1/ITS4, which amplify longer fragments, nearly all samples (46/46 and 45/46, respectively) had positive results upon PCR amplification.

Although PCR products from 41 samples in the ITS3/ITS4 assay and 19 samples in the ITS1/ITS4 assay indicated the presence of fungal DNA-PCR amplicons by sequencing analysis, no DNA sequences corresponded to yeasts.

Tissue response grading

Histological characteristics of each lesion reflecting tissue response to the invading yeast were evaluated using our tissue response grading system and summarized in Figure 5 in accordance with differences in causative fungi that were confirmed in this study by ISH. An increased likelihood of a positive signal for ISH with either probe was associated with lower tissue grading scores ($P = .02$ for *C. albicans* PNA probe and $P = .01$ for *Trichosporon* spp. PNA probe by Cochran-Armitage test for trend).

Discussion

The present study aimed to elucidate the prevalence of trichosporonosis in autopsy samples taken from individuals in Japan, most of whom might be diagnosed as having candidiasis. Differentiation between *Trichosporon* spp. and *Candida* spp. using routine histopathological examination alone can be difficult since both yeasts have similar dual-shaped histological features that imply the presence of both yeast and hyphal forms

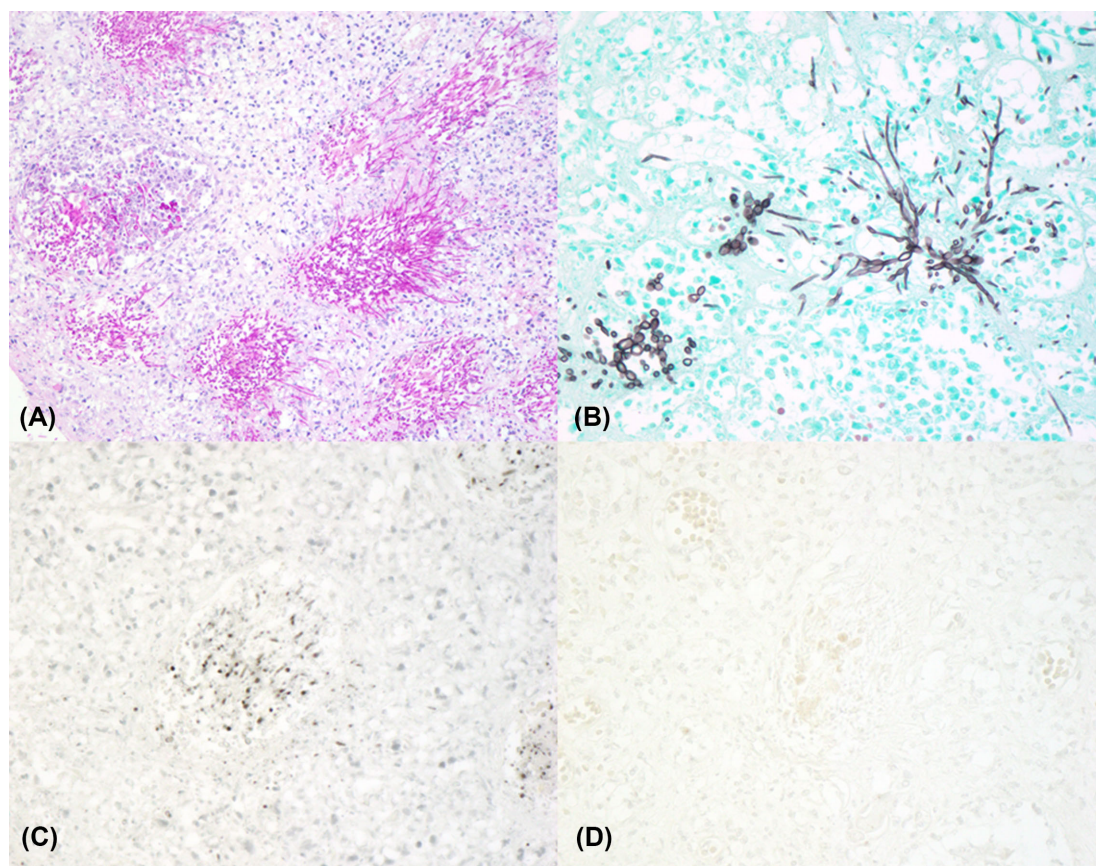


Figure 3. Representative case positive for PNA probe targeting *C. albicans*. Images are from a representative case (case 22) that showed positive staining for *C. albicans*. (A) Invasive yeast with hyphae forms in kidney tissue with moderate numbers of inflammation-related cells, mainly neutrophils (Periodic acid-Schiff [PAS] reaction, 200 \times). (B) Pseudohyphae and budding are visible (Grocott's methenamine silver [GMS], 400 \times). (C) Positive reaction of PNA probe targeting *C. albicans*. The positive result is indicated by dark brown staining of the tissue (400 \times). (D) Negative reaction of PNA probe targeting *Trichosporon* spp. (400 \times).

in human tissues. Therefore, we used ISH to help differentiate these species in FFPE sections taken at autopsy from cases confirmed as having blood stream yeast infections without cryptococcosis.

Among the 88 autopsy samples tested, 42 samples showed positive signals by ISH using either *Trichosporon* spp.- or *C. albicans*-specific probes. None of the samples showed positive signals for both probes.

There are several possible reasons for the relatively low ratio of positive samples. First, DNA alignment in FFPE samples could be impaired. Indeed, several studies reported that false negatives for PCR of samples from FFPE specimens can be caused by nicks and gaps in DNA that develop during formalin fixation and could result in the predominance of shorter products than that expected for the targeting primers.^{20,21} The overall low prevalence of positive signals for our ISH tests could be due to a similar mechanism, particularly since ISH does not include a procedure for DNA amplification, and thus this method may be more vulnerable to disruptions in the structure of the targeting alignment that form after hydrolysis of nucleic acids in the sample during formalin fixation. The fixation of most of the FFPE

tissue samples in this study for one week or more is one limitation of our study, although such samples did provide a feasible means for obtaining large numbers of samples.

Second, the lower positive ratio for ISH results could also be due to the extensive inflammation response to fungi. In this study we found that cases having a weaker tissue inflammation response (e.g., grade 1 and 2) had a higher prevalence of positive signals for both probes in FFPE sections. Thus, there was an inverse relationship between the positive rate of ISH and strength of inflammation. Specifically, the gene targeted by probes could be damaged by superoxides and proteolytic enzymes produced by certain inflammatory cells, mainly neutrophils and macrophages.²² Together, the extended formalin fixation and DNA damage induced by inflammatory cells may account for the low sensitivity of ISH.

A third possibility is that cases infected with non-*albicans* *Candida* might have generalized infection, which neither of the probes we used can detect. This possibility is supported in part by our finding that three cases having invading yeasts that were morphologically identified as *C. glabrata* (case 18, 38, and 44) did not show positive signals for either probe.

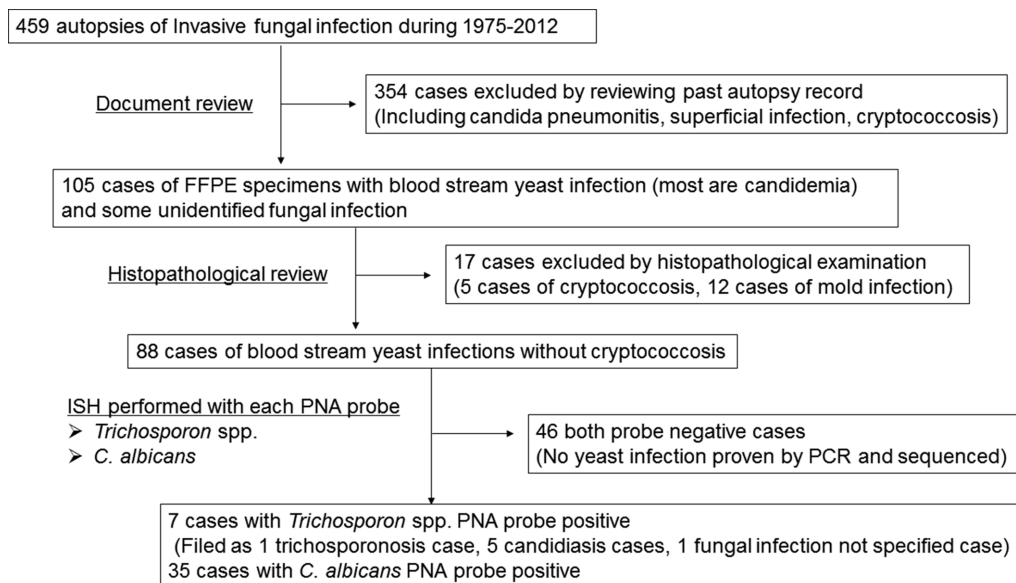


Figure 4. Flowchart indicating steps from subject enrollment to results. Overview of how 88 cases of blood stream yeast infections without cryptococcosis were extracted from review of past autopsy records and histopathological results, followed by results of *in situ* hybridization (ISH) using PNA probes targeting *C. albicans* and *Trichosporon* spp.

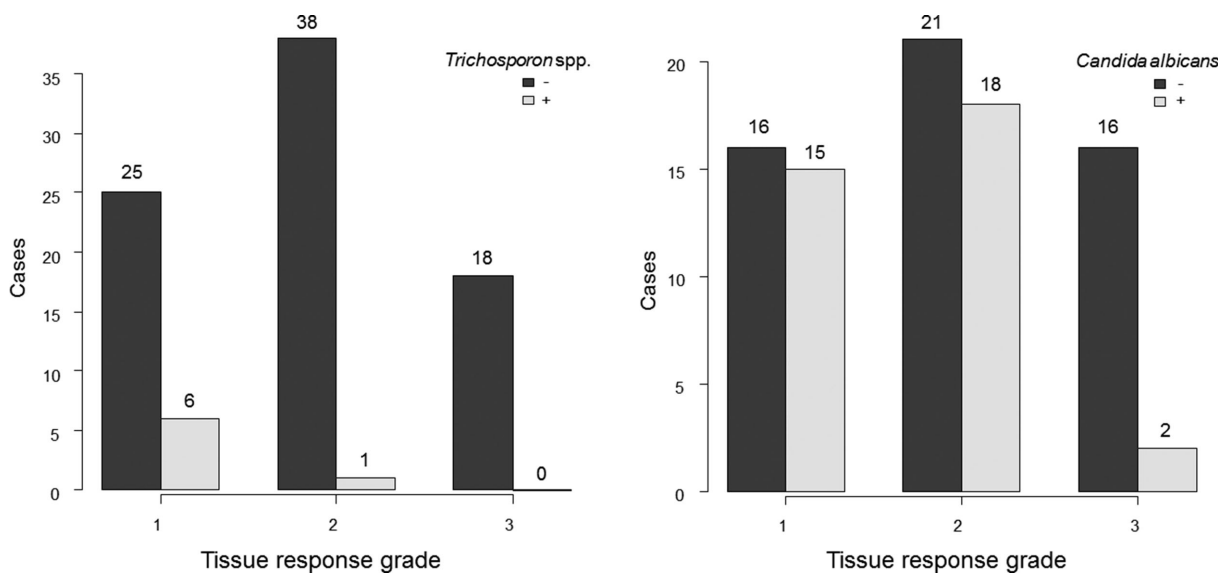


Figure 5. Relationship between rate of positive ISH and strength of inflammatory response. No cases that were scored as grade 3 had positive signals for the *Trichosporon* spp. PNA probe. In cases with trichosporonosis, most had positive signal consistent with grade 1. Similarly, there were a small number of cases scored as grade 3 that were positive for the *C. albicans* PNA probe ($P = .02$ and $P = .01$ for *C. albicans* and *Trichosporon* spp. PNA probe, respectively, by Cochran-Armitage test for trend).

Therefore, to determine whether fungal DNA was indeed damaged in FFPE samples that were negative for both probes with ISH, PCR assays were carried out on these samples using panfungal primers. Although the PCR assay indicated that the FFPE samples that yielded double negative ISH results may have contained damaged DNA, the cause of such damage and when it occurred (e.g., formalin fixation, inflammation, during processing) was unclear. This is one limitation of this study. However, the lack of simultaneous positive signals for both probes

supports the ability of ISH to provide reliable information for identification of causative dual-shaped yeast in samples that do have positive signals with this procedure.

The seven cases with probable trichosporonosis based on positive signals for the *Trichosporon* spp. probe suggest that the prevalence of generalized trichosporonosis among patients diagnosed with IFI at the two institutions is around 1.5% (7/459). Although there are few reports of the prevalence of trichosporonosis in Japan, the prevalence that emerged from our

study results was higher than the 0.1%–0.2% rate that was previously reported in a review article summarizing nationwide IFI autopsy records.²³ Part of this discrepancy between this previously reported rate and our findings may be due to the lack of supplementary molecular techniques for histological diagnosis. Most of the pathology departments in Japan conduct histopathological examination using special stains (e.g., GMS and PAS) alone; however, molecular techniques are not routinely used in Japan for the diagnosis of IFI. Notably, five out of the seven cases indicating a positive signal for the *Trichosporon* spp. PNA probe were recorded as having candidiasis infection. These results suggest that trichosporonosis could have been misidentified as generalized candidiasis in these cases because of the similarity in the shape of these two yeasts in tissue sections.

Some reports suggested that *Trichosporon* spp. is the second most common yeast isolated from individuals having malignant hematological disease.^{7,24} Only two reports published in English have provided high-quality data regarding the prevalence of trichosporonosis in Japan that was confirmed by the analysis of blood culture and immunohistochemistry examination using FFPE tissues from autopsy subjects.^{25,26} Both of the reports described trichosporonosis as the second most common invasive yeast infection following candidiasis in a group of patients with hematological malignancy. In these patients, the prevalence of trichosporonosis ranged from 4.4 to 10% of the total IFI population and 9.8 to 14.3% of patients with invasive yeast infections. Unfortunately, the reproducibility of the immunohistochemistry techniques, especially the specificity of the antibody used to diagnose *Trichosporon* spp. in these reports, may not have been appropriately verified. Our results showed that the ratio of trichosporonosis to candidiasis was much higher than previous reports, around 1:5.²³ If the higher prevalence mirrors the actual status, the reliance on histological identification of dual-shaped yeasts that have both forms, yeast and hypha, may be associated with the previously reported lower rate of *Trichosporon* spp. infection relative to that of *Candida* spp.

In addition to the possibility that fixation times of 1 week or more could have affected DNA alignment that in turn produced the lower than expected rate of positive ISH results, we did not confirm the ISH test results by culture, because diagnosis of IFI in autopsy subjects was generally made only by histopathological examination without any mycological analysis. Therefore, a future study should use culture results to verify ISH findings. Another limitation is that our study population may not reflect nationwide rates of disease, particularly since the number of autopsies performed in Japan is decreasing, and most autopsies are performed for individuals that had serious debilitating diseases. Whereas histopathological identification is accepted as a standard diagnostic procedure for fungal infection, unacceptable repeatability of this procedure has been reported.²⁷ Molecular techniques, mainly those involving PCR and ISH, have recently been applied as supplemental procedures for histological

identification of infectious disease agents. These methods cannot replace histopathological examination because in their current form they cannot be routinely used to evaluate all specimens. Therefore, analyses that combine histopathological examination and molecular techniques could provide the best approach for distinguishing causative yeast strains that have similar forms, such as *Trichosporon* spp. and *C. albicans*.

Applying ISH to complement histological examination of FFPE samples to diagnose IFI is potentially useful to accurately distinguish *Trichosporon* spp. from *Candida* spp. The results of this study support the usefulness of ISH as a supplemental procedure to histological examination to diagnose trichosporonosis. ISH can also be used as a supplemental method to retrospectively analyze autopsy specimens to elucidate the actual prevalence of trichosporonosis. Further investigation of the accuracy and reproducibility of ISH is required for standardization and validation for its use as a supplemental diagnostic method.

Supplementary material

Supplementary data are available at MMYCOL online.

Acknowledgments

This study was supported by the Japan Agency for Medical Research and Development (AMED) under grant number JP19fk0108045, and JP19fk0108094, and the Japan Society for the Promotion of Science (JSPS) KAKENHI grant number JP26860250 and project grants from Toho University (# 29-47).

The funders had no role in the study design, data collection and interpretation, or publication.

Declaration of interest

Conflicts of interest: K.S. received research grants from Pfizer Inc., Dainippon-Sumitomo Pharma and Astellas Pharma Inc.; he also received payments for lectures from Dainippon-Sumitomo Pharma, and for consulting from Miraca Holdings Inc. All other authors declare that they have no competing interests.

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