Original Article

Comparison between the Effectiveness of Polymerase Chain Reaction and In Situ Hybridization in Detecting the Presence of Pathogenic Fungi by Using the Preserved DNA in Formalin-Fixed and Paraffin-Embedded Tissues

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SUMMARY: In situ hybridization (ISH) has been recognized as an important technique for identifying the causative fungi in the foci of infection observed in histopathological specimens which was processed from formalin-fixed and paraffin-embedded (FFPE) tissues. However, few basic studies have conducted an evaluation of the DNA preservation for use in ISH in comparison to polymerase chain reaction (PCR). The latter is a DNA amplification-based modality. In the present study, we analyzed 65 FFPE lung tissue specimens collected from autopsy cases for comparing the usefulness of ISH and PCR analysis. As a result, the positive identification rates for PCR were strikingly low; a majority of these results can be assumed to be false negative because the presence of fungi had been confirmed by histopathological analysis. In contrast, panfungal ISH targeting of the 28S rRNA showed a higher sensitivity than the 230-bp panfungal PCR primers did (80.0% versus 4.6%, respectively). Furthermore, over 60% of the samples we examined showed a favorable intensity of the ISH signal. Therefore, in conventional postmortem FFPE tissues, the state of DNA preservation may be more favorable for ISH than PCR analysis.

INTRODUCTION

Whereas the identification of the causative agents of invasive fungal infections (IFIs) is important for clinical management of the disease (1), conventional diagnostic modalities sometimes provide negative or unreliable results. Consequently, alternative molecular detection modalities have been used increasingly in recent decades to identify invasive fungal elements present in formalin-fixed and paraffin-embedded (FFPE) tissues (2). Polymerase chain reaction (PCR) is the most commonly used analytical approach for detecting such invasive fungal elements, but recent studies have shown that in situ hybridization (ISH) can also be used for identifying the fungal elements present in FFPE tissues (3–6). However, variability in the sources of FFPE materials makes it difficult to perform an exact comparison of the effectiveness of the two diagnostic modalities. We therefore conducted a comparative investigation of PCR

analysis and ISH to examine the DNA preservation using FFPE tissues collected from autopsies of patients with confirmed IFIs. Such basic studies may provide beneficial insights into the usefulness of molecular diagnostic pathology in the study of IFIs.

MATERIALS AND METHODS

Materials: We used lung tissues from 65 autopsies performed from 1971 to 2006 in the Toho University Medical Center, Omori Hospital for the study. The presence of fungal infections was confirmed through histopathological analysis of all samples by 3 expert pathologists who reexamined the samples using hematoxylin and eosin staining and Grocott's methenamine silver (GMS) staining. Since it has been previously reported that the lung was the most common organ involved irrespective of major fungal species, the lung was adopted as the representative organ of IFI (7). Tissues obtained from the autopsies were fixed in 15% unbuffered formalin before being paraffin embedded and stored at room temperature. Postmortem interval (PMI) was documented, but the duration of fixation during tissue sampling was unknown.

The 65 autopsy cases included those of 39 men and 26 women. Malignancy was the most common underlying

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disease in 37 cases, 26 of which showed hematological malignancy. The demographics and clinical characteristics are summarized in Table 1. The protocols we used were approved by the Ethics Committee of Toho University Faculty of Medicine (#20047).

PCR: (i) DNA extraction: Three serial sections (5- μ m thickness) were cut with a microtome from each of the lung FFPE blocks and placed in sterile 1.5-ml microcentrifuge tubes. The microtome was cleaned thoroughly with 100% ethanol before each block was cut. Tissues in the microcentrifuge tubes were deparaffinized 3 times with fresh xylene for 15 min, followed by rehydrating 3 times in 100% ethanol for 15 min, and then air-dried for 45–60 min. Next, we added 200 μ l of Tris-EDTA (TE) buffer (pH 8.0) containing 1% SDS to the air-dried deparaffinized tissues, mixed and then heated for 10 min at 98°C. The tissues were spun down and 10 μ l of protease K (Nippon Gene Co., Tokyo, Japan) adjusted to a concentration of 20 mg/ml was added to the mixture, which was incubated overnight at 48°C. The DNA was purified twice with phenol and then precipitated with 8 μ l of 5 M NaCl and 500 μ l of 100% refrigerated ethanol for 2 h at –20°C. The frozen mixture was centrifuged for 20 min at 4°C and at 12,000 rpm, the supernatant was discarded, and the pellet was washed with 1 ml of 75% refrigerated ethanol to remove the unwanted salt content. Finally, the pellet was resuspended in 30 μ l of TE buffer.

(ii) PCR analysis: Primers used in this study are listed in Table 2 (synthesized by FASMAC Co., Kanagawa, Japan). Human β -globin primers and panfungal primers were used to assess the quality of DNA from the human and fungal specimens, respectively, which were preserved in the FFPE blocks. Each PCR mixture contained 2.5 μ l of 10 $\times$ PCR buffer, 0.75 μ l of MgCl₂,

0.125 μ l of Taq polymerase (iTaQ™ DNA polymerase; Bio-Rad Laboratories, Richmond, Calif., USA), 0.5 μ l of dNTP (dNTP Mix; Bio-Rad Laboratories), 0.5 μ l of each primer (0.4 μ M), and 0.5 μ l of DNA template solution. Ultrapure water was added to increase the volume of the mixture to 25 μ l. The thermal cycler (MyCycler™ Thermal Cycler; Bio-Rad Laboratories) for PCR was programmed in the following manner: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 1 min; and extension at 72°C for 1 min followed by a final extension phase at 72°C for 1 min. Positive (cultured fungal) controls were used for every run. The products amplified by PCR were detected using 3% agarose gel electrophoresis and were visualized under UV irradiation.

ISH: (i) Nucleic acid probes: To determine the efficacy of ISH using the FFPE autopsy tissues, a panfungal peptide nucleic acid (PNA) probe targeting the 28S rRNA was used to assess the rRNA preserved in the specimens (6). The PNA probe was labeled with fluorescein isothiocyanate (FITC), which was synthesized by FASMAC Co., for commercial use.

(ii) ISH procedure: ISH was performed using modified previously described methods (6). Briefly, 4- μ m-thick sections were cut using a microtome and mounted on PLATINUM coated glass slide (Matsunami Glass Ind., Ltd., Osaka, Japan). The sections were routinely deparaffinized 3 times in xylene, each time for 5 min, followed by 3 immersions in 100% ethanol, each for 5 min, and then washed in sterile distilled water. The pretreatments are as follows: first, the tissue sections were heated in 1 mM of EDTA buffer (pH 8.0) in a water bath at 98°C for 30 min; second, the sections were cooled at room temperature for 30 min; and lastly, they were digested with 10 μ g/ml of protease K for 10 min at 37°C. Then, the sections were carefully washed 3 times in sterile distilled water for 5 min, dehydrated 3 times in 100% ethanol for 1 min, and then air-dried. We then applied 20 μ l of the hybridization solution (formamide:hybridization buffer = 1:1) with a concentration of 1 μ g/ml to each slide. The rRNA in the tissues was denatured in a deep block for glass slides (AL-96MP; TAITEC Co., Saitama, Japan) in a block thermostatic oven (DTU-2B; TAITEC Co.) for 5 min at 94°C, followed by immediate cooling on ice for 5 min. Hybridization was incubated for 90 min at 56°C in a humidity chamber containing 2 $\times$ standard saline citrate (SSC) solution. After the hybridization, excess or unbound probes were removed by washing the slides 3 times with 0.2 $\times$ SSC at 56°C for 10 min. The signals were then visualized by routine immunostaining with an

Table 1. Demographic and clinical characteristics of 65 patients

Characteristic	No. of patients
Age	53.26 (SD, 2.75; Median, 60)
Gender (female:male)	26:39
Period	1971–2006
Underlying diseases	
Leukemia	26 (D, 7; OT, 4)
Malignant tumor of solid organs	11 (D, 1; OT, 3)
Autoimmune diseases	6 (D, 1; OT, 1)
Aplastic anemia	4 (D, 2)
Hepatic cirrhosis	4 (D, 1; OT, 1)
Fulminant hepatitis	3 (D, 1)
Others	11 (D, 7; OT, 1)

D, disseminated infection; OT, old tuberculosis.

Table 2. Primers used for PCR analysis

Primer pair	Amplicon	Sequence	Specificity (Ref.)
PC03/PC04	110 bp	5'ACACAACCTGTGTTCACTAGC3' 3'CAACTTCATCCACGTTCAACC5'	Human β -globin gene (8)
PC03/GH21	250 bp	5'ACACAACCTGTGTTCACTAGC3' 3'TGACAAAGCCCCATACGCT5'	Human β -globin gene (8)
U1/U2	230 bp	5'GTGAAATTGTTGAAAGGGAA3' 3'GACTCCTTGGTCCGTGTT5'	Panfungal 28S rRNA gene (9)
18SF1/5.8SR1	300 bp	5'AGGTTTCCGTAGGTGAACCT3' 3'TTCGCTGCGTTCTTCATCGA5'	Panfungal ITS1 rRNA gene (10)

Table 3. PCR and ISH results data set

Case no.	Age ¹⁾	Year ²⁾	ST ³⁾ (y)	PMI ⁴⁾ (hr)	Pangungal ISH			β -globin PCR		Panfungal PCR	
					Allred score			PC03 PC04	PC03 GH21	U1/U2 28S	18SF1/5.8SR1 ITS1
					IS ⁵⁾	PS ⁶⁾	TS ⁷⁾	110 bp	250 bp	230 bp	300 bp
1	60	1971	40	10	3	5	8	—	—	—	—
2	19	1972	39	unknown	2	3	5	—	—	—	—
3	55	1972	39	3.000	1	3	4	+	—	—	—
4	51	1972	39	1.670	1	1	2	+	—	—	—
5	41	1973	38	unknown	2	5	7	+	—	—	—
6	16	1973	38	3.000	2	3	5	+	—	—	—
7	50	1974	37	1.780	0	0	0	+	—	—	—
8	60	1975	36	7.080	1	2	3	+	—	—	—
9	45	1977	34	15	3	3	6	—	—	—	—
10	70	1978	33	12.58	0	0	0	—	—	—	—
11	56	1978	33	13.38	2	4	6	—	—	—	—
12	65	1978	33	10	2	3	5	—	—	—	—
13	31	1980	31	unknown	2	2	4	—	—	—	—
14	10	1980	31	6.620	1	1	2	—	—	—	—
15	12	1980	31	1.250	2	4	6	—	—	—	—
16	30	1981	30	1.830	3	5	8	—	—	—	—
17	54	1981	30	2.000	3	5	8	—	—	—	—
18	78	1981	30	11	0	0	0	—	—	—	—
19	48	1981	30	1.150	3	3	6	—	—	—	—
20	24	1982	29	1.200	3	4	7	—	—	—	—
21	59	1983	28	2.500	3	5	8	—	—	—	—
22	82	1984	27	1.22	3	2	5	—	—	—	—
23	34	1984	27	5.500	2	5	7	—	—	—	—
24	19	1985	26	4.45	2	4	6	—	—	—	—
25	28	1985	26	15.42	1	1	2	—	—	—	—
26	84	1985	26	1.000	2	3	5	—	—	—	—
27	65	1986	25	14.25	0	0	0	—	—	—	—
28	60	1986	25	20	2	2	4	—	—	—	—
29	32	1988	23	3.000	2	1	3	—	—	—	—
30	66	1988	23	3.500	2	1	3	—	—	—	—
31	39	1988	23	4.670	3	2	5	—	—	—	—
32	64	1989	22	7.270	2	4	6	—	—	—	—
33	65	1990	21	11.4	1	2	3	—	—	—	—
34	38	1991	20	8.000	3	2	5	—	—	—	—
35	35	1992	19	1.250	2	3	5	—	—	—	—
36	73	1992	19	2.670	1	2	3	—	—	—	—
37	71	1992	19	9.650	2	3	5	+	—	—	—
38	62	1992	19	15	1	2	3	—	—	—	—
39	39	1992	19	1.780	3	5	8	—	—	—	—
40	75	1993	18	15.3	0	0	0	—	—	—	—
41	83	1993	18	8.680	0	0	0	+	—	+	—
42	73	1994	17	7.130	0	0	0	—	—	—	—
43	49	1994	17	12	3	5	8	+	—	—	—
44	75	1994	17	4.330	3	4	7	—	—	—	—
45	54	1995	16	1.000	3	1	4	—	—	—	—
46	60	1995	16	9.370	3	4	7	—	—	—	—
47	21	1995	16	1.620	3	4	7	—	—	—	—
48	83	1997	14	6.000	0	0	0	—	—	—	—
49	72	1998	13	9.480	3	5	8	—	—	—	—
50	70	1998	13	12.4	3	2	5	—	—	—	—
51	78	1998	13	13	3	4	7	+	—	—	—
52	66	1998	13	15.18	2	1	3	+	—	+	—
53	75	1998	13	15.43	3	3	6	+	—	—	—
54	6	1999	12	2.130	3	4	7	—	—	—	—
55	77	2000	11	3.830	0	0	0	+	—	—	—
56	57	2000	11	2.200	3	5	8	—	—	—	—
57	68	2000	11	17	3	4	7	—	—	—	—
58	85	2000	11	1.500	2	4	6	—	—	—	—
59	67	2002	9	6.000	3	4	7	—	—	—	—
60	73	2004	7	2.000	0	0	0	+	—	—	—
61	67	2004	7	20.42	1	1	2	—	—	—	—
62	57	2004	7	17	0	0	0	—	—	—	—
63	65	2006	5	22	0	0	0	—	—	—	—
64	15	2006	5	2.550	2	2	4	+	+	+	—
65	1	2006	5	9.280	0	0	0	—	—	—	—

¹⁾: Age of autopsy subject.²⁾: Year of autopsy performed.³⁾: Storage term of paraffin block.⁴⁾: Postmortem interval.⁵⁾: Intensity score.⁶⁾: Proportion score.⁷⁾: Total score.

anti-FITC primary antibody (Roche Diagnostics K. K., Tokyo, Japan) diluted 1:400 with blocking buffer (0.01 M 100 ml PBS at pH 7.2, 1 g albumin, and 0.1 g NaN₃) and a horseradish peroxidase-labeled secondary antibody (Nichirei Biosciences, Inc., Tokyo, Japan). Before treating with the primary antibody, the slides were immersed in 1% H₂IO₆ for 5 min to block the endogenous peroxidase. Finally, the peroxidase sites were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dojindo Laboratories, Kumamoto, Japan) in 0.1 M phosphate buffer (pH 7.3) containing 3% H₂O₂, 1% CoCl₂·6H₂O, and 1% (NH₄)₂Ni(SO₄)₂·6H₂O (11).

(iii) Evaluation of ISH with the Allred score: The slides were examined under a light microscope. A positive signal was noted as the presence of dark blue staining of the fungal forms. To assess the signal precisely, the Allred score was employed to evaluate the ISH result. Specifically, the intensity score (IS) was based on a 4-point system: a score of 0, 1, 2, and 3 for none, light, medium, and dark staining, respectively (see Fig. 2). The numerical value for the percentage of staining (proportion score [PS]) was determined using a geometric rather than a linear division: no cells stained = 0; 0–1/100 cells stained = 1; 0–1/10 cells stained = 2; 1/10–1/3 cells stained = 3; 1/3–2/3 cells stained = 4; and 2/3–1 cells stained = 5. Addition of the IS values and PS values showed the total score (12,13). The total number of fungal elements was determined using sections stained with the GMS stain.

Statistics: The variables were compared using a Mann-Whitney *U*-test or independent samples *t* test. A two-tailed *P* value of <0.05 was considered statistically significant. All statistical calculations were performed using SPSS version 11.5 for Windows.

RESULTS

Evaluation of nucleic acid preservation in the FFPE autopsy tissues: Since most of the autopsy studies do not perform isolation and identification of the microorganisms from the foci of infection, a supplemental modality to identify the causative fungi becomes important. In this study, we compared the usefulness of PCR by using human β -globin and panfungal primers and that of ISH by employing panfungal probes. We found that PCR analysis with 110-bp β -globin primer gave a positive identification rate of 23.1% (15/65), which was the highest for our investigation. Positive rates for PCR using the 250-bp β -globin, 230-bp panfungal, and 300-bp panfungal primers were 1.5%, 4.6%, and 0%, respectively (Table 3). In contrast, ISH using the panfungal probes showed a positive signal of 80.0% (52/65).

Effect of PMI and storage duration on the PCR analysis: Compared to biopsy or surgical specimens, FFPE autopsy specimens possess unique properties, which may lead to a special DNA preservation status. Although the duration of fixation of the autopsy tissues in formalin was unknown to us, data on PMI and storage time were able to be collected from the autopsy records (Table 3), and analyzed statistically. As shown in Table 4, PCR analysis results revealed no significant relationship to the storage duration and PMI.

Table 4. Results of univariate analysis

		ST	PMI
PCR β -globin (110 bp)	+	22.87 $\pm$ 13.17	7.06 $\pm$ 5.20
	–	21.80 $\pm$ 8.95	7.77 $\pm$ 6.11
sig. (two-tailed)		<i>P</i> = 0.901	<i>P</i> = 0.694

ST, storage term; PMI, postmortem interval.

Evaluation of ISH with the Allred score: For ISH, it is routine to morphologically identify and evaluate the true-positive signal. To evaluate the results more precisely, an Allred score (12,13) was used, which assigns a numerical value to both the overall stain intensity and stain pattern. Such a score might provide us with a clear and objective understating of the ISH results. We found that 66.2% of the cases possessed an intensity score $\geq$ 2, and 63.1% of the cases got a total score of more than 4 (Table 3, Fig. 1, Fig. 2).

DISCUSSION

In recent years, there has been an increasing interest in the use of ISH with the microtome sections obtained from FFPE tissues in order to identify the causative fungi in involved tissue (6). However, few studies have documented the reliability of ISH as compared to that of conventional PCR amplification-based diagnostic modalities. We therefore performed a comparative investigation using ISH and PCR analysis to detect fungus-specific nucleic acids preserved in the FFPE tissues collected from autopsy cases in which the presence of filamentous fungi had been confirmed by prior histopathological examination.

We would like to discuss briefly about the essential procedure for processing FFPE tissues to be used in a supplemental molecular modality. To retrieve the nucleic acids from the FFPE tissues (14,15), the samples were heated in alkaline solutions followed by digestion with protease K (6,16), prior to performing PCR and ISH. However, the positive identification rates of PCR analysis were unacceptably low, the highest of which was only 23.1% with a 110-bp β -globin primer. These results can be assumed as false negative because the presence of fungi had been histologically confirmed in all the samples we used. Since it has been previously reported that formalin fixation occasionally introduces nicks, gaps, and consequent fragmentation in DNA alignments (17,18), PCR might produce inadequate amplicons during the early phase of amplification, most of which might be shorter than that expected. If these inadequate amplicons would be predominant, the final PCR products would contain little amount of adequate amplicons. The results of our study confirmed the findings of a previous report showing that the average length of the DNA fragments was 300–400 bases in biopsy tissues, but it was much shorter in postmortem FFPE tissues (19). The superiority of the biopsy tissues in preserving the DNA may be attributable to a short-term exposure to formalin along with the use of 10% neutral buffered formalin, which creates a relatively stable environment for DNA preservation (20–22). On the other hand, there was no significant relationship in positive detection rates using the 110-bp β -globin primer be-

Fig. 1. Results of the panfungal ISH ($\times 400$). The pictures are numbered in the order of the age of the blocks, increasing from older to younger. For details of the Allred score, refer to Table 3.

tween the targeted DNA preservation and the storage duration or PMI of the samples. This fact suggests that a favorable detection using PCR analysis might be possible if an optimal formalin fixation is performed, but

its reliability should be verified by future studies. In addition, it would be necessary to test the effectiveness of the recently introduced polymerases (23) in order to obtain a higher PCR efficiency.

Fig. 2. Variations in the ISH signal intensity. The histogram comprising the intensity score 0, 1, 2, and 3 of the Allred scoring system indicates the representative signal intensity of none, light, medium, and dark staining, respectively. Twenty-four (36.9%) cases showed an intensity score of 3, while 43 (66.2%) cases displayed an intensity score ≥ 2 .

As opposed to PCR, ISH has been considered a type of molecular diagnostic modality for which it is not necessary to extract and amplify DNA. Our study showed that the panfungal ISH probes targeting the 28S rRNA exhibited a higher sensitivity than PCR analysis using the panfungal primers (80.0% versus 4.6%, respectively). To assess the ISH signal more precisely in the present study, we employed the Allred score, which was originally introduced to evaluate the signals indicated by immunohistochemistry with the use of an antibody against the hormone receptors of breast cancer cells in FFPE tissues (12,13). Our results showed that over 60% of samples possessed an intensity score of more than 2 and a total score of more than 4. Accordingly, ISH may overcome the DNA size limitation of PCR, and provide a direct, rapid, and localizable result.

Although immunohistochemistry using specific antibodies designed against the fungi has been accepted as one of the supplemental modalities for FFPE tissues, the cross-reactivity of a polyclonal antibody or the low sensitivity of a monoclonal antibody are the difficulties that yet need to be addressed (24,25). Therefore, we amplified the ISH signal in the study with the use of an anti-fluorescein antibody (26), followed by visualization with DAB to enhance very weak but specific signals, which were present in some samples. In addition, the procedure might enable not only visualization under a light microscope, but also allow permanent preservation of the visualized signals in the tissue sections. Moreover, the convenience of species-specific target-gene sequences available from GenBank would make the commercial synthesis of the ISH probes easier. Some studies on ISH have shown favorable results with specific probes (3–6). With the development of an automated and standardized method, this approach is expected to become more widely available and cost-effective. However, we also found some ISH samples that showed

no signal at fungi in the tissues. This fact could be induced by tremendously severe fragmentation of the target sequences and/or the intractable RNA-protein cross-linkage in the paraffin-embedded tissues, which may lead to greatly reduced accessibility of the hybridization targets.

In conclusion, whereas the DNA preserved in conventional postmortem FFPE tissues might be severely degraded for use in the detection of pathogenic fungi using PCR analysis, ISH can be considered a superior detection method. This finding may in part be attributable to the fact that ISH has no amplification steps that may potentially lead to false negative results. Therefore, in the future, standardizing formalin fixation methods and modifying the conventional preservation procedures are necessary steps for optimizing DNA detection, along with the development of a supplemental molecular assay for histopathological materials.

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