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Detection of *Candida albicans* mRNA from Formalin-Fixed, Paraffin-Embedded Mouse Tissues by Nested Reverse Transcription-PCR

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Histopathology archives represent a vast source of infectious disease specimens that can be used to elucidate important disease processes. In this report, we describe a method to detect *Candida albicans* gene expression from infected, formalin-fixed, paraffin-embedded mouse tissue. By use of glass beads to break open the fungal cells and proteinase K treatment, RNA was extracted routinely from tissue sections that had been fixed for up to 72 h. Upon reverse transcription of the RNA and nested PCR, the procedure detected *C. albicans* "housekeeping" and putative virulence genes.

Candida albicans is a commensal of the oral cavity, gastrointestinal tract, and female genital tract, where it persists in equilibrium with the microbial flora and the host's immune system; however, alterations in the physiological or immunological status of the host can lead to infections ranging from superficial mucosal lesions to life-threatening systemic diseases in immunocompromised patients (16). *Candida* species are the most common fungal pathogens of humans and rank as the fourth most frequent cause of nosocomial bloodstream infections in the United States (21).

The extraction of nucleic acid from fixed tissue is particularly important since it allows the use of archival material for retrospective studies. Molecular analysis of archived, fixed, pathological specimens can facilitate disease classification and can help to clarify important aspects of the disease process. This methodology is especially important for defined biopsies, for which extensive clinical data are available. Analyzing quantitative changes in gene expression either by the host or the invading pathogen may help identify therapeutic targets and virulence genes. However, the deleterious effects of chemical fixatives on nucleic acids (e.g., degradation, chemical modification of the bases, and protein cross-linking) are well documented (2, 10, 14). Moreover, prolonged tissue fixation and the chemical nature of the fixative can have a drastic effect on the ability to extract nucleic acid from embedded tissues (5, 17). Nevertheless, extraction of DNA from these tissues has been accomplished, and archived tissue specimens are now being used to study changes in gene expression. However, the ability to extract RNA from fixed tissues adds a second layer of complexity due to the fragile nature of RNA and the necessity of producing cDNA. In addition, a high degree of sensitivity and specificity is required when the target is the infectious agent rather than host tissues. In situ hybridization has been used to detect *Candida* species in fresh tissue sections using probes that target rRNA (11); however, no reports to date have utilized this methodology to study gene expression in fixed tissues. This is presumably due to inadequate sensitivity, and the fungal cell wall may also preclude in situ reverse transcription (RT)-PCR. To circumvent this problem, nested PCR has recently been used as a diagnostic tool for detecting DNA isolated from infected, formalin-fixed, paraffin-embedded tissue (3, 7, 13); however, no studies to date have explored the feasibility of analyzing fungal gene expression from formalin-fixed tissues. In this report we utilized nested RT-PCR to detect *C. albicans* gene expression from formalin-fixed, paraffin-embedded mouse tissues.

Tissues, RNA extraction, and RT-PCR. Germfree immunodeficient Tgε26 mice were colonized (alimentary tract) by oral inoculation with a pure culture of *C. albicans* SC5314. Oroesophageal candidiasis was lethal for Tgε26 mice at 4 to 5 weeks after colonization (1) with infections occurring in the stomach, palate, tongue, and esophagus. Tissues from uninfected germfree mice were used as controls. Tissues were fixed in phosphate-buffered saline containing 10% formaldehyde for 1.5 or 72 h, processed in graded alcohol, and embedded in paraffin. Histopathology and *C. albicans* colony counts from the tissues were performed to verify the presence or absence of fungal infection (data not shown), and reports of them have been published previously (1).

Twenty-micrometer sections from formalin-fixed, paraffin-embedded composites of stomach, esophagus, tongue, and palate tissue were cut with a microtome and processed immediately. In order to prevent carryover with contaminating RNA, a fresh blade was used for each sample. Cut sections were placed in a 1.5-ml tube (two sections per tube) and were deparaffinized by mixing with 1 ml of xylene for 20 min at room temperature with agitation. To remove the xylene, samples were collected by centrifugation at 20,000 × *g* for 5 min and were washed three times with 1 ml of absolute ethanol. After removal of the last ethanol wash, pellets were air dried for 10 min.

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One of the reasons that RNA retrieval from formalin-fixed tissues is problematic is that the RNA is resistant to both extraction and enzymatic manipulation due to cross-linking with proteins (10, 18). Proteinase K readily solubilizes formalin-fixed tissue and releases RNA from the cross-linked matrix, allowing it to be purified following phenol chloroform extraction. However, *C. albicans* possesses a rigid cell wall that consists mainly of polysaccharide and only a small amount of protein (4). Therefore, RNA was extracted using the paraffin block RNA isolation kit (Ambion) according to the manufacturer's instructions with the following modifications: after resuspension of the samples in 200 μl of 1-mg/ml proteinase K, the samples were vortexed continuously for 30 min in the presence of acid-washed glass beads (425 to 600 μm) to break open the fungal cells. The samples were incubated at 45°C for 24 h, followed by three extractions with acid phenol chloroform, which was essential to eliminate trace amounts of DNA. The RNA was precipitated overnight at −20°C with an equal volume of isopropanol and was washed two times with 75% ethanol.

Formalin also chemically modifies RNA (14). Monomethylol groups are added to all four bases at various rates, which inhibits cDNA synthesis. However, the majority of the methylol groups can be removed from bases by heating at 70°C in Tris-EDTA buffer (14). Consequently, the resulting pellet was resuspended in 10 μl of Tris-EDTA buffer and incubated at 70°C for 30 min. The samples were then treated with 4 U of DNase I for 25 min at 37°C, extracted with acid phenol chloroform, and precipitated overnight by the addition of isopropanol. The final pellet was resuspended in 10 μl of diethyl pyrocarbonate-treated water.

It is likely that, in its degraded state, the mRNA will have lost some or all of its 3′ poly(A) tails. Further, among the bases of RNA, adenine appeared the most susceptible to electrophilic attack due to fixation (14). Consequently, random primers (as opposed to oligo[dT] primers) were mixed with 5 μl of the RNA and were reverse transcribed into cDNA by using the retroscript kit (Ambion). Routinely, 2 to 4 μl of the resulting mix was then subjected to PCR amplification by using mouse or *C. albicans*-specific primers, respectively.

RNA extracted from formalin-fixed tissues is significantly degraded and fragmented (9, 10). The degree of degradation correlates with the length of time that the tissues were fixed. Consequently, long fragments generally cannot be PCR amplified. Therefore, primers were designed to amplify fragments less than 140 bp in length. The forward and reverse primers used for PCR amplification were as follows: 5′-TTCCTCAAG TTCACCTACC and 5′-CGAAGACCACGGTTCAG (mouse *S15* gene); 5′-CACAAACCAATACATAATG and 5′-GTAG ACAGTGACATCAGC and the nested primers 5′-TCAGAT TTCTCTAAAGTCG and 5′-TGACATCAGCTTGAGTGG (*C. albicans EFB1*); and 5′-TTCTGGTGGTAGTGGTGG and 5′-ATGGCACTGGTATCATCAGC-3′ and the nested primers 5′-TACCTGTAGATCCTATGG and 5′-TGGTATCATC AGCGTATTG (*C. albicans SAP9*).

For amplification of the mouse *S15* gene, after an initial denaturation at 95°C for 2 min, the samples were subjected to 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. Identical conditions were employed for *EFB1* and *SAP9* amplification, except the annealing temperatures were 58 and 56°C, respectively, and the samples were subjected to 30 cycles of amplification by using the outer primer set, followed by an additional 30 cycles of amplification by using the nested primer set. An aliquot (1 μl) of the first PCR product was used as template for nested PCR. Previous reports using nested PCR of DNA isolated from infected and fixed tissues have detected the DNA equivalent of 10 cells per sample. However, if required, a higher level of sensitivity may be achieved by increasing the amount of template or the number of PCR cycles or by using more product after the first amplification step.

For each PCR amplification run, multiple controls were included. Positive controls included 10 pg of genomic DNA/RNA prepared from fresh cultures of *C. albicans* or RNA prepared from fresh tissue (Ambion). Negative control PCRs were identical to the test PCRs without the addition of template. To detect the presence of contaminating DNA in the RNA samples, identical RT reactions lacking reverse transcriptase were PCR amplified. RNA extracted from uninfected mouse tissue was used to demonstrate that the products were *C. albicans* specific. In addition, the identity of *C. albicans EFB1* and *SAP9* PCR products was confirmed by DNA sequence analysis. Product carryover was avoided by physically separating tubes containing infected and noninfected samples. In addition, gloves were changed after handling template samples, aerosol barrier pipette tips were always used, and reaction master-mixes lacking template were prepared before aliquoting to the individual PCR tubes. Transcripts were detected routinely from at least three sections that were analyzed separately from the same formalin-fixed, paraffin-embedded block. This procedure was repeated using four independently fixed blocks from four different mice in addition to germfree controls.

Detection of mouse transcripts from formalin-fixed, paraffin-embedded tissues. It is well documented that increasing the fixation time decreases the ability to isolate intact nucleic acid (17). Therefore, tissues were fixed for 1.5 or 72 h in neutral-buffered formalin; the ages of the paraffin-embedded tissues were 6 and 18 months old, respectively. To confirm that the samples contained cDNA that was capable of being amplified by PCR, the samples were also tested for the presence of the mouse *S15* gene (8). PCR amplification resulted in a product of the predicted size of 115 bp (Fig. 1). This product was of a size similar to that obtained after PCR amplification by using template prepared from fresh tissue (Fig. 1, lane 3). PCR products were detected irrespective of the length of time that the tissue had been fixed, although the strength of the signal was weaker from the 72-h fixed sample.

Detection of *C. albicans* transcripts from infected, formalin-fixed, paraffin-embedded mouse tissues. Since the RNA preparation contained mainly mouse and a small amount of *C. albicans* RNA, individual PCR primers were BLAST searched against the EMBL database to ensure *C. albicans*-specific amplification. Primers were generated to target the *C. albicans* "housekeeping" *EFB1* gene (6, 20). Primers for *EFB1* amplification were designed to span an intron of 365 bp in size (12). Consequently, a 97-bp product was expected after nested amplification by using cDNA as a template, while a 462-bp product was expected when genomic DNA was present. The effi-

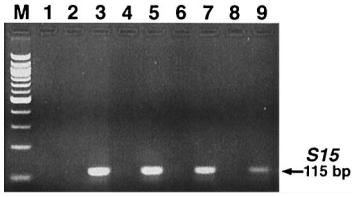

FIG. 1. Detection of mouse *S15* expression from formalin-fixed, paraffin-embedded mouse tissue. PCR amplification was performed by using cDNA prepared from the following: fresh tissue (lane 3), germ-free tissue that was fixed for 1.5 h (lane 5), and *C. albicans*-infected tissue that was fixed for 1.5 h (lane 7) or 72 h (lane 9). To test for the presence of contaminating DNA, PCR amplification was also performed by using RNA extracted from the following: fresh tissue (lane 2), uninfected tissue that was fixed for 1.5 h (lane 4), and *C. albicans*-infected tissue that was fixed for 1.5 h (lane 6) or 72 h (lane 8). The negative control PCR lacked template (lane 1). Lane M, 100-bp DNA ladder.

ciency of the *EFB1* primers was initially tested by using 10-fold serial dilutions of *C. albicans* genomic DNA (Fig. 2). By use of conventional PCR, an *EFB1* PCR-amplified product was produced with 1 ng of genomic DNA. Conversely, nested PCR produced an *EFB1* PCR-amplified product with only 1 pg of DNA, indicating that, under these conditions, nested PCR was approximately 1,000-fold more sensitive than conventional PCR.

The nested RT-PCR strategy was performed on RNA prepared from the formalin-fixed, paraffin-embedded tissues (Fig. 3A). A 97-bp *EFB1* product was detected routinely from cDNA prepared from tissues fixed for 1.5 or 72 h. Moreover, the product size was clearly different from that obtained by using genomic DNA as template, indicating that the starting template was cDNA and not DNA (Fig. 3A, compare lanes 2 and 6). Conventional PCR did not result in visually detectable amplification products, indicating that nested PCR was required to generate the desired sensitivity and specificity (data not shown). RT-PCR products were also not obtained from uninfected tissues, indicating that the products were *C. albicans* specific. The lack of PCR-generated products from these samples was not due to the presence of PCR inhibitors, as the mouse *S15* gene could be readily amplified using these templates (Fig. 1).

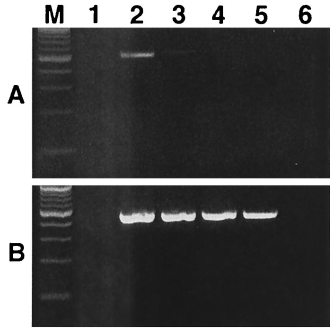

FIG. 2. Sensitivity of conventional (A) and nested (B) PCR. Conventional PCR that uses *EFB1* primers was performed on *C. albicans* genomic DNA by using 1 ng (lane 2), 100 pg (lane 3), 10 pg (lane 4), 1 pg (lane 5), and 0.1 pg (lane 6) of DNA. Nested PCR was then performed by using 1 μl of the conventional PCR product as template. PCR amplification was also carried out in the absence of template (lane 1). Lane M, 100-bp DNA ladder.

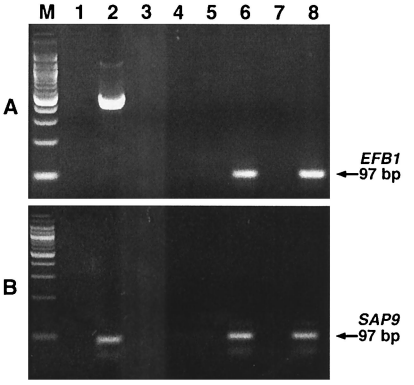

FIG. 3. Detection of *C. albicans* gene expression from formalin-fixed, paraffin-embedded mouse tissue. Nested PCR amplification was performed with *EFB1* (A) or *SAP9* (B) primers. PCR amplification was performed by using cDNA prepared from the following: uninfected tissue that was fixed for 1.5 h (lane 4) and *C. albicans*-infected tissue that was fixed for 1.5 h (lane 6) or 72 h (lane 8). To test for the presence of contaminating DNA, PCR amplification was also performed by using RNA extracted from uninfected tissue (lane 3) and *C. albicans*-infected tissue that was fixed for 1.5 h (lane 5) or 72 h (lane 7). The negative control PCR lacked template (lane 1), while the positive control PCR contained 10 pg of *C. albicans* genomic DNA (lane 2). Lane M, 100-bp DNA ladder.

EFB1 is one of the few *C. albicans* genes that contain an intron (12). To demonstrate that the procedure could also distinguish gene expression from an intronless gene and to detect *C. albicans* transcripts other than that generated by a housekeeping gene, nested primers were designed against the secretory aspartyl proteinase 9 (*SAP9*, predicted size of 97 bp) gene, a putative virulence factor. This gene was chosen because, while low and sporadic levels of *SAP9* transcripts have been detected under specific conditions in vitro (15), sustained expression has been detected during oropharyngeal candidiasis (19). Nested RT-PCR detected *SAP9* gene expression from fixed tissue irrespective of the length of fixation (Fig. 3B).

RT-PCR is an invaluable tool to study gene expression when the amount of sample is limited and to achieve the desired specificity when the sample is "contaminated" with a significant amount of heterologous RNA. Other techniques for assessing gene expression such as in situ hybridization are limited by the level of sensitivity and the lack of quantitative expression data. The data presented demonstrate the feasibility of studying *C. albicans* gene expression from infected, formalin-fixed, paraffin-embedded mouse tissues and should allow subsequent experiments that use tissues present in many pathology departments. If combined with real-time PCR (9), it may enable not only qualitative but also quantitative analysis of gene expression during the disease process. Since proteinase K is routinely used for nucleic acid extraction of human tissues (3, 13) and glass beads are commonly used to disrupt fungal cell walls, the procedure should be applicable to most fungus-infected human tissues.

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