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Development and Validation of Ultra-Rapid Periodic Acid–Schiff Stain for Use in Identifying Fungus on Frozen Section

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- **Context.**—There is no standardized process for utilization of periodic acid–Schiff during intraoperative frozen sections to identify fungal organisms.

- **Objective.**—To develop a rapid staining process for fresh tissue with periodic acid–Schiff during intraoperative consultation and develop an appropriate control block.

- **Design.**—Muscle tissue was inoculated with 2 species of fungus (Aspergillus fumigatus and Paecilomyces spp) and grown at 3 different temperatures for 72 hours. Inoculated tissue was embedded in optimal cutting temperature compound, cut, and stained using a modified periodic acid–Schiff stain. The optimal control was determined for future use as the standard control. Multiple control slides were cut and stained, using successively shorter time intervals for each step. The staining process that provided accurate results in the shortest amount of time was deemed ultra-rapid periodic acid–Schiff. This method was validated by carryover studies and clinical specimens.

- **Results.**—Paecilomyces spp incubated at 30°C for 72 hours was the most optimal positive control with numerous yeast and hyphal forms. The fastest staining process involved 2 minutes of periodic acid and Schiff reagent and 10 dips of light green solution. Tap water was as effective as distilled water. Validation was successfully achieved. Clinical cases all stained identical to formalin-fixed, paraffin-embedded tissue stained with hematoxylin-eosin and periodic acid–Schiff.

- **Conclusions.**—Ultra-rapid periodic acid–Schiff provides fast and reliable identification of fungal organisms on fresh tissue. Development of a concurrent positive control allows for quality control and validation.

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Over the past century, the intraoperative frozen section has played an integral role in the diagnosis and management of surgical patients. Intraoperative consultations provide real-time diagnoses from histologic examination of fresh tissue, which can aid and guide appropriate surgical management. One of the earliest and most famous approaches to frozen section was developed in 1905 by Louis B. Wilson, MD, chief of Pathology at Mayo Clinic, Rochester, Minnesota. After standardizing his approach using methylene blue, Dr Wilson was able to provide the surgeon histopathologic information within 2 to 5 minutes. Today, most medical centers use hematoxylin-eosin (H&E) stains for the interpretation of frozen sections. Modern intraoperative consultation can provide pivotal information regarding benign versus malignant diagnoses, and the greatest advantage is histologically accurate rapid results. Frozen sections decrease the turnaround time of specimens from 16 to 24 hours for formalin-fixed, paraffin-embedded tissue (FFPE), to less than 30 minutes. Rapid turnaround time can guide intraoperative management based on diagnosis. While intraoperative frozen sections have been instrumental in cancer diagnosis, staging, and margin status, little work has been done to expand the utility of frozen sections.

Currently, identification of fungus on H&E-stained frozen section is limited, as organisms are frequently rare, or translucent in appearance and difficult to identify. Sensitivity and specificity vary based on clinical suspicion and quantity of fungal organisms present. The vast majority of current studies examine the utility of identification in the diagnosis of acute invasive fungal rhinosinusitis. Review of published case series of acute invasive fungal sinusitis demonstrated a sensitivity range of 72.7% to 87.5% and a specificity range of 72.7% to 100%. One study examined the utility of margin status on frozen section for soft tissue wound debridement, demonstrating 68.4% sensitivity and 100% specificity for assessing clear margins.

Our institution is a military level 1 trauma center with an active burn unit where we care for hundreds of civilian and military burn patients each year. Fungal infection in these patients is frequent and is a time-sensitive diagnosis as it is related to increased morbidity and mortality. Using tradi-

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tional methods and routine processing, specimens require up to 48-hours turnaround time when standard fungal stains are ordered. Rush processing can typically reduce turnaround time to approximately 24 hours. Recent publications have demonstrated that periodic acid–Schiff (PAS) can be modified to provide early diagnosis of fungal infection on fresh tissue during intraoperative consultation (deemed periodic acid–Schiff fungus–frozen section [PASF-fs]). These studies demonstrated excellent success in identification of fungal organisms in the diagnosis of fungal rhinosinusitis compared with H&E frozen section alone. Herein, we aimed to develop an ultra-rapid PAS stain and determine the fastest turnaround time that allows for identification of fungal organisms on frozen section. We also aimed to develop a quality control tissue block to be stained simultaneously with the submitted tissue, to provide a validated and reproducible method for intraoperative diagnosis of fungal organisms on frozen sections.

**MATERIALS AND METHODS**

**Phase 1: Development of an Adequate Control Block**

It is recommended practice for histology labs to provide a quality control slide per batch or per slide for special stains and immunohistochemistry. To provide the highest quality product, an adequate control slide should be created to be stained in parallel with the submitted intraoperative tissue. Because we need to interpret PAS on frozen sections, a control block of frozen tissue would be adequate. The ideal control would show both yeast and hyphal elements, with positive staining confined to fungal organisms and minimal background staining. In addition, it would be made from a material that allows for easy cutting (minimal adipose or calcium creating artifact). Lean muscle was selected as our control tissue. Fresh refrigerated muscle was taken from meat (low fat chicken, steak, etc) could be substituted. Muscle tissue was cut into 0.5 × 0.5 × 0.5-cm fragments. In each of 2 test tubes, 10 drops of 10% yeast extract solution was added to 10 mL of sterile water. Yeast organisms of Aspergillus fumigatus and Paecilomyces spp were added to respective test tubes. The solution was vortexed to mix thoroughly. Two wire loops of the solution were added to each cut fragment of muscle tissue in individual sterile containers (6 in total). The tissue was then incubated at 25°C, 30°C, and 37°C for 72 hours. At 48 to 72 hours, visible fungal growth was identified in each container. Three days after inoculation, tissue was embedded in optimal cutting temperature compound, frozen at −20°C, and cut into 8-μm sections on a Thermo Scientific Microm HM 550 cryostat. All specimens were stained via a modified PASF-fs (Table 1) to demonstrate that the previously reported PASF-fs could stain the fungal organisms at the study institution, and to compare which incubation grew the most optimal control. At our institution, we used periodic acid 0.5% aqueous, Schiff reagent, and Fast Green Substitute for Light Green Working Solution from Poly Scientific R&D Corp. Three pathologists then reviewed the potential control slides and compared the quality and quantity of fungal organisms present. Potential controls were evaluated based on several factors, including if both hyphae and yeast organisms were present, if tissue was intact enough to allow adequate frozen section and if fungal invasion was present. An optimal control was selected by expert consensus.

**Phase 2: Development of Ultra-Rapid PAS (URPAS) Staining Process**

After phase 1 was completed, the best control was recreated following identical steps to 1.1 above. Specimen was embedded in optimal cutting temperature, frozen, and cut into 8-μm sections. Sections were stained using the same reagents as 1.1; however, the total duration of each step was decreased stepwise until the specimen spent less total time than a typical H&E stain line (Table 2). Slides were compared on the quality of staining of fungal organisms. The fastest time was then tested using tap water instead of distilled water (Round 8) to determine whether the distilled water was a necessary component of the process. The real-world time to perform the entire process from embedding the tissue to cover-slipping was documented and compared with previously reported PASF-fs.

**Phase 3: Validation of URPAS**

To ensure multiple frozen sections and controls can be stained in the same reagents without carryover of fungal organisms, a validation study was performed. Alternating sections of fungal positive control tissue (pulled from step 1.2) and fungal-negative fresh tissue was cut on the same cryostat. Slides were stained, in series, in the same reagent containers following the optimal URPAS method defined in 1.2. Four pathologists reviewed the validation slides. Validation was considered successful when all 10 fungal positive control slides had identifiable fungal organisms and all 10 fungal negative slides had no fungal organisms present.

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**Table 1. Steps for Modified Periodic Acid–Schiff Fungus–Frozen Section (PASF-fs) (Performed Using Poly Scientific R&D Corp Reagents)**

| Reagents | 0.5% periodic acid fast, Schiff’s reagent, working light green solution, distilled water |
| --- | --- |
| Procedure |  |
| Step 1 | Quick fix slide with 1 dip in 95% ethanol |
| Step 2 | Wash slide in distilled water |
| Step 3 | Submerge slide in 0.5% periodic acid for 5 min |
| Step 4 | Wash slide in distilled water |
| Step 5 | Submerge slide in Schiff reagent for 5 min |
| Step 6 | Wash slide in distilled water for 1 min |
| Step 7 | Submerge slide in working light green solution for 30 s |
| Step 8 | Wash slide in distilled water |
| Step 9 | Dehydrate slide in 95% ethanol, then 100% ethanol |
| Step 10 | Submerge slide in 2 changes of xylene for 10 dips |
| Step 11 | Coverslip with synthetic resin (Permount) |

**Table 2. Stepwise Decrease in Staining Duration of Modified Periodic Acid–Schiff Fungus–Frozen Section (PASF-fs)**

| Round No. | Steps Performed for Each Round of Staining |
| --- | --- |
| Round 1 | Periodic acid–Schiff for 5 min. Step 6 for 1 min, step 7 for 30 s |
| Round 2 | Periodic acid–Schiff for 4 min. Step 6 for 45 s, step 7 for 20 s |
| Round 3 | Periodic acid–Schiff for 3 min. Step 6 for 30 s, step 7 for 15 s |
| Round 4 | Periodic acid–Schiff for 2 min. Step 6 for 10 dips, step 7 for 10 dips |
| Round 5 | Periodic acid–Schiff for 1 min. Step 6 for 10 dips, step 7 for 10 dips |
| Round 6 | Periodic acid–Schiff for 30 s. Step 6 for 10 dips, step 7 for 10 dips |
| Round 7 | Periodic acid–Schiff for 10 s. Step 6 for 10 dips, step 7 for 10 dips |
| Round 8 | Fastest round with clearly visualized fungus replacing distilled water with tap water |

*a Refer to Table 1 for entire steps.  
b While tap water worked in the studied lab, the pH and water chemistry may affect results. It is recommended if distilled water is readily available to exclusively use distilled water.
After validation with control tissue, clinical cases over a 3-month period that were submitted as a rush order fresh specimen with clinical indication of “rule out fungus” had URPAS performed on the tissue. Clinical validation was considered successful if at least 10 positive and 10 negative blocks were collected and had identical results to FFPE blocks with H&E and PAS staining. Results were compared with microbiology cultures if preformed simultaneously.

RESULTS

Development of an Adequate Control Block

Muscle tissue was inoculated in the fungal lab with A. fumigatus and Paecilomyces spp and incubated at 25°C, 30°C, and 37°C for 72 hours. After 72 hours all samples had a visible fungal cap adherent to the muscle tissue. All frozen sections demonstrated easily identifiable fungal elements on PAS-fs. Paecilomyces spp had an even mixture of yeast and hyphal forms at 25°C and 30°C. At 37°C, Paecilomyces demonstrated predominately hyphal forms and a decreased quantity of fungal organisms. A. fumigatus grew predominantly yeast forms at 25°C and 30°C with rare single hyphal forms identified. At 37°C, A. fumigatus grew abundant hyphal forms. Both 25°C and 30°C demonstrated relatively intact muscle tissue that cut relatively easily on the cryostat. At 37°C, the muscle began to break down substantially, increasing the difficulty of cutting a quality slide without artifact. Tissue invasion by fungal elements was evident on all cut sections. Figure 1, A through L, shows fungal organisms in H&E and PAS-fs at each incubation temperature. The optimal specimen was Paecilomyces spp incubated at 30°C. Both hyphal and yeast forms were present, and the tissue remained intact for frozen sectioning. Perpendicular sectioning to the fungal cap allowed for the highest number of fungal organisms to be present for identification.

Development of URPAS

Tissue sections were stained with modified PAS with decreasing time in each reagent until only 10 dips of each reagent was performed according to Table 2. Staining results are demonstrated in Figure 2, A through H. Fungal hyphae are clearly visualized within rounds 1 through 4 with decreasing visibility beginning on round 5. Round 4 was optimal as the fastest stain time while still effectively staining every fungal element. During round 8, round 4 was retested using tap water and found to be as effective as distilled water and was significantly easier to acquire. The steps performed in round 4 consisted of 2 minutes each for periodic acid and Schiff reagent, produced the most optimal fungal identification, and was deemed URPAS for frozen section. The steps to perform URPAS is displayed in Table 3. The real-world time to preform PAS-fs in the studied lab with a single person preforming the process from embedding to coverslip was 15 minutes 40 seconds on a single block of tissue and 16 minutes 43 seconds on 2 blocks of tissue. For comparison, and H&E-stained frozen took 5 minutes 9 seconds for 1 block and 6 minutes 11 seconds for 2 blocks. URPAS took 6 minutes and 50 seconds for 2 blocks (URPAS will always be a minimum of 2 blocks if the control block is used).

Validation of URPAS

Positive control tissue was alternated with negative control tissue (fresh raw chicken) until 10 of each control was sectioned and stained according to the steps in Table 3. Each pair of positive and negative control tissue were simultaneously stained. Between pairs of slides, the tap water rinse was replaced. Staining results are illustrated in Figure 3, A through T. All positive slides had clearly visible fungal organisms and all negative slides had no identifiable fungal organisms present.

In a 3-month timespan, there were 6 clinical cases submitted fresh with a clinical indication of “rule out fungus.” Five of the cases were from burn patients while 1 was a nasal mucosa ulcer. These 6 cases resulted in 33 blocks of tissue that were tested with URPAS. There were 20 blocks positive for fungal elements and 13 blocks that were negative. All 33 blocks matched the FFPE diagnosis. Quantities of fungal elements ranged from rare to abundant.

Types of tissue tested were soft tissue (adipose and skeletal muscle)—23 blocks; skin—4 blocks; nasal mucosa—3 blocks; and prostate—3 blocks. All tissue types had both positive and negative blocks. Only 1 case was completely

Figure 1. Image comparison at ×400 magnification of fungal organisms stained with modified periodic acid–Schiff (PAS) frozen section staining. A through F, Paecilomyces spp. A, Hematoxylin-eosin (H&E) of 25°C; B, PAS of 25°C; C, H&E of 30°C; D, PAS of 30°C; E, H&E of 37°C; and F, PAS of 37°C. G through L, Aspergillus fumigatus. G, H&E of 25°C; H, PAS of 25°C; I, H&E of 30°C; J, PAS of 30°C; K, H&E of 37°C; and L, PAS of 37°C.
negative for fungus on both URPAS and FFPE, a 2-part skin punch biopsy specimen. Figure 4 demonstrates a positive URPAS stained clinical case. Only 4 of 6 cases had corresponding microbiology fungal cultures performed simultaneously. The only case negative on URPAS and FFPE, a skin punch biopsy specimen, was additionally negative on fungal culture. One case was positive on URPAS and FFPE in 8 of 10 blocks but was negative on fungal culture. Two cases were positive on URPAS, FFPE, and fungal culture; 1 was positive for Aspergillus species, and the other was positive for Fusarium species.

DISCUSSION

The development of an easily accessible and reusable control slide was crucial in the development of the URPAS to verify the accuracy of fungal identification. In this study, Paecilomyces spp incubated at 30°C was considered the most optimal. However, Paecilomyces spp could easily be grown at 25°C (room temperature) if access to an incubator is difficult to obtain. Having a positive control, ready to stain alongside the intraoperative specimen, improves validity and reliability of this method. As this new process is implemented at the study institution, it is predicted that a large amount of control tissue will be needed. Appropriate caution should always be taken when handling fungal organisms to include adequate personal protective equipment and use of fume hoods. Personnel involved in preparing the positive control tissue should be educated in the proper handling of infectious organisms.

To have a prefrozen, readily available control block, the optimal control tissue frozen in optimal cutting temperature was cut off the chuck with a scalp blade and stored in a −20°C freezer until needed. On receiving an intraoperative frozen section for identification of fungal elements, the premade control tissue is applied to a new chuck and the prefrozen block can be reattached to the chuck, allowing a positive control frozen section to be cut immediately. Alternatively, to increase speed and utility, the tissue block may remain frozen on the chuck in the freezer.

The standard PAS staining procedure involves multiple time-consuming steps, making it unwieldy for intraoperative consultation. While the modified PAS is faster, the shortest staining intervals for optimal staining had not been previously evaluated. Using URPAS, 2 minutes in periodic acid and 2 minutes in Schiff reagent is as fast as a standard manual H&E-stain line. Performing manual H&E and URPAS simultaneously could allow for optimal fungal identification with no delay in intraoperative results compared with H&E alone. In real-world testing, URPAS preformed almost 10 minutes faster than the previously described PAS-Fs, taking approximately the same amount of time as an H&E-stained slide while additionally providing a control block for pathologists to verify the stain worked appropriately. It should be noted that exact time to perform these steps depends greatly on general frozen-section experience, laboratory set up, and familiarity with this new process.

During validation, it is recommended to use a commercially available meat product that is not already contaminated by fungal organisms (fresh raw chicken was used for this study). If fungal elements are detected in the meat product, but do not resemble the species of fungus inoculated in the

Table 3. Steps to Perform Ultra-Rapid Periodic Acid–Schiff (URPAS), Determined to be the Most Optimal Staining Procedure

| Step No. | Steps to Perform Stain |
|---------|------------------------|
| Step 1  | Quick fix slide with one dip in 95% ethanol |
| Step 2  | Wash slide in tap water |
| Step 3  | Submerge slide in 0.5% periodic acid for 2 min |
| Step 4  | Wash slide in tap water |
| Step 5  | Submerge slide in Schiff reagent for 2 min |
| Step 6  | Wash slide in tap water for at least 10 dips |
| Step 7  | Submerge slide in working light green solution for 10 dips |
| Step 8  | Wash slide in tap water |
| Step 9  | Dehydrate slide in 95% ethanol, then 100% ethanol |
| Step 10 | Submerge slide in 2 changes of xylene for 10 dips |
| Step 11 | Coverslip with synthetic resin (Permount) |

a While tap water worked in the studied lab, the pH and water chemistry may affect results. It is recommended if distilled water is readily available to exclusively use distilled water.

Figure 2. Image comparison at x400 magnification illustrating how fast a modified periodic acid–Schiff (PAS) stain can be performed, termed ultra-rapid PAS (URPAS). Refer to Tables 1 and 2 for times and details. A, Round 1; B, round 2; C, round 3; D, round 4; E, round 5; F, round 6; G, round 7; and H, round 8 (tap water).
control, it is recommended to repeat with a different fresh tissue instead of determining the method invalid. If there is fungal carry over during the staining process, reevaluate technique to see where improvement can be made. While the validation was performed at the study institution, the reagents were removed from the refrigerator immediately before use. The first 3 positive controls that were stained were lighter than the last 7 (Figure 3, A through T). It is hypothesized this is related to the temperature of the reagents during the first 5 to 10 minutes outside of the refrigerator. To reduce this temperature effect, periodic acid can be stored and used at room temperature. Schiff reagent should be refrigerated during storage, but removed before performing URPAS, and allowed to come to room temperature as frozen-section blocks are made and cut into slides. All reagents should be stored and discarded according to the specific manufacturer’s recommendations.

CONCLUSIONS

URPAS is an institutionally validated method of staining that can rapidly highlight fungal organisms during intraoperative consultation by frozen section and guide clinical management. This is the first PAS method that can be performed within the same time frame as a standard manual H&E, preventing delay in frozen section diagnosis. In addition, it is the first frozen PAS method that uses a control block to ensure the stain performed as intended. When fungus is detected, more information can be provided to the clinical team regarding tissue invasion and angioinvasion compared with culture. Also, this process will frequently leave additional tissue to be paraffin embedded in the histology lab, allowing for routine histologic confirmation by PAS. The systemic and stepwise approach used in this study provides a confident framework for other labs to implement a similar technique. This process can dramatically improve turnaround time over traditional FFPE or culture by providing information to the clinical team in a confident manner.

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