Amyloidosis is a heterogeneous group of disorders caused by extracellular deposition of amyloid, chemically identified as low-molecular-weight subunits of different misfolded proteins.1 Currently at least 35 amyloidogenic proteins have been documented.2 The clinical manifestation of amyloidosis is remarkably protean, depending on the type of amyloidogenic protein, affected organs, and amount of deposition. These manifestations may include nephrotic-range proteinuria, restrictive cardiomyopathy, cardiac conduction abnormalities, unexplained hepatomegaly, enlarged muscles (most notably the tongue), easy bruising, impaired coagulation, and autonomic and peripheral neuropathy, among others. In many cases, the clinical manifestation and imaging studies may be suggestive of amyloidosis, but the diagnosis of amyloidosis can be confirmed only by the presence of amyloid in tissue biopsy. Yet in other cases, tissue amyloidosis with significant clinical implication can be incidentally identified in asymptomatic patients. These considerations necessitate the implementation of a highly sensitive and specific approach to examining tissue specimens for amyloid deposition.

The diagnosis of amyloidosis involves 2 steps: identification of tissue amyloid deposition followed by identification of the amyloidogenic proteins. The second step classically involved immunostaining of the tissue sections with a battery of antibodies against different amylogenic proteins. More recently, this technique has been superseded by mass spectrometry of laser-microdissected amyloid protein from tissues.3 It is obvious that both approaches are possible only after tissue amyloid deposition is accurately identified.

Regardless of the chemical nature, all types of amyloidosis share a common feature: a β-sheet configuration of the amyloidogenic proteins, leading to distinctive histochemical features detectable by special stains. These stains include thioflavin T or thioflavin S fluorescence, Sirius red, p-dimethylaminobenzaldehyde–nitrite reaction, and Congo red.4 Most of these stains are limited by variable sensitivity and low specificity; thus, only Congo red remains as the most reliable stain in the clinical setting.5,6 Traditionally, tissue amyloid deposit is suspected in hematoxylin-eosin (H&E)–stained tissue sections as acellular, amorphous, glassy eosinophilic material, which is then confirmed by a
salmon pink color in the Congo red stain. However, false-positive and false-negative congophilia are well documented, which impairs the diagnostic sensitivity and specificity of Congo red stain. This problem is indeed not infrequent.

In a previous study focusing on examination of Congo red stain using Texas Red–filtered fluorescence microscopy (TRFM), the well-recognized increased diagnostic sensitivity of this technique was confirmed, but the study also made an anecdotal observation that this technique helps improve the specificity of the Congo red stain, as also previously alluded to by Linke. This potentially important diagnostic utility is, however, not well recognized. The current study aims to comprehensively evaluate this possible improved specificity of Congo red stain by TRFM.

**MATERIALS AND METHODS**

Ninety-two specimens were studied in both a retrospective and a prospective manner. The cases were categorized into 3 groups. Group I included 15 cases with a definitive diagnosis of amyloidosis, including 8 kidney biopsies, 2 bone marrow biopsies, 2 fat pad aspiration biopsies, a heart biopsy, a colon biopsy, and a maxillary sinus content. This group served as a positive control to standardize the histochemical procedure and the optical instrument. Group II consisted of 63 cases in which amorphous eosinophilic structures reminiscent of amyloid protein were seen on H&E-stained tissue sections, some of which are well known for false-positive congophilia. These structures included elastic tissue, hyalinized fibrous tissue, hyalinized vascular wall tissue, glomerular mesangial matrix, fibrillar glomerulonephritis, desmoplastic stroma associated with invasive carcinoma, smooth muscle bundles with cautery artifact, thyroid colloid, intravascular and extravascular pooled red blood cells, bone spicules, necrotic adipose tissue, ovarian corpora albicantia, and uromodulin or hyaline renal tubular casts. The true nature of these structures was readily recognizable on light microscopic (LM) examination of H&E-stained sections. Verheoff-van Gieson stain for elastic tissue, Masson trichrome stain for fibrous tissue and fibrin, periodic acid–Schiff stain for uromodulin, or electron microscopy was used in some cases to confirm the nature of the amyloid-like tissue. This group served as the main module to evaluate the specificity of TRFM-enhanced congophilia. Group III consisted of 14 cases in which amyloid and amyloid look-alikes were seen in the same tissue section, often side by side, and both displaying congophilia, with equal or slightly differential staining intensity. These cases included thyroid amyloid and colloid, vascular amyloid in bowel with lymphoid aggregates, red blood cells, myocardiocytes, bone marrow amyloid and bone spicules, renal amyloid and tubular cell cytoplasm/tubular casts/sclerotic vascular wall tissue, and synovial amyloid and hyalinized fibrous tissue. This group served to test the premise that TRFM would provide a definitive diagnosis of amyloidosis by differentially enhancing congophilic structures.

A definitive diagnosis (“amyloidosis” or “no amyloidosis”), which is the foundation of this study, was achieved with rigorous criteria in each case. These include not only the morphology of the studied tissue samples, but also findings in tissue samples from other organs, laboratory data, clinical findings, and follow-up for each patient included in this study.

Specimens from each group were processed in the same laboratory setting and under the same technical conditions, including processing, staining, and optical examination. Tissue sections 4 to 10 μm thick were stained for Congo red. The protocol proposed by Puchter et al, consisting of Congo red stain in alkaline 80% ethyl alcohol solution saturated with NaCl, was used. This protocol was selected because it is documented to best prevent nonspecific tissue binding of Congo red dye molecules. Consecutive tissue sections were stained with H&E. A microscope with both LM and immunofluorescence capacity with a Texas Red filter (excitation peak 596 nm/emission maximum of 620 nm) was used (Olympus microscope, Olympus America, Center Valley, Pennsylvania). This microscopic setting allowed for examination of exactly the same area by routine LM and TRFM by switching the filters. The H&E and Congo red-stained sections of each case were examined by routine LM, with and without a polarizer, to identify amyloid and amyloid–look-alike areas. Birefringence, including that of apple-green appearance, was recorded for each case, but its presence was not required for a definitive diagnosis of amyloidosis. Exactly the same areas identified in Congo red stain by LM were then examined under fluorescence with Texas Red filter. The Congo red-stained sections were scored as either positive or negative for congophilia under LM. It was noted that if there was amyloid, the enhancement was uniformly strong for all areas with amyloid deposition, with an appearance well above the background. If there was no amyloid, the congophilic area was not different from the background in terms of enhancement. Therefore, enhancement by TRFM was scored as positive or negative. The results from using each method were compared and statistical analysis was performed to provide diagnostic specificity using Microsoft Excel.

**RESULTS**

In group I cases, the amyloid deposits typically appeared glassy and amorphous on H&E stain. This material displayed variable congophilia, but was strongly enhanced by TRFM in each case (Figures 1, A and B, and 2, A and B). Of note, in 2 cases (an endomyocardial biopsy and an abdominal fat pad aspiration), the congophilia of amyloid deposits was so faint that it was missed by initial LM examination; however, the congophilia was strongly enhanced by TRFM, leading to an unequivocal diagnosis of amyloidosis. Furthermore, reexamination of the same deposits by LM now revealed convincing, albeit weak, congophilia.

In group II cases, the amorphous eosinophilic structures reminiscent of amyloid were seen by LM, raising the suspicion of amyloidosis. When sections from these cases stained with Congo red were examined with LM and TRFM, 3 patterns of congophilia and enhancement were observed (Table). In 10 cases, neither congophilia nor enhancement was detected, accurately negating the diagnosis of amyloidosis. In 39 cases, congophilia was present, raising the possibility of amyloidosis; however, the congophilia was not enhanced by TRFM, thus supporting the diagnosis of no amyloid deposition (Figures 3, A and B, 4, A through C, 5, A through D, 6, A through D, 7, A through C, and 8, A through C). In the remaining 11 cases, both congophilia and enhancement of variable intensity were seen (Figure 9, A and B), representing a failure of TRFM to resolve an ambiguous diagnostic possibility of amyloidosis raised by congophilia observed on LM.

In group III cases, both amyloid and amyloid-mimicking structures in the same tissue section showed congophilia, often of slightly different intensity but with many appearing equal. In each of these cases, the amyloid-specific congophilia was strongly enhanced, in sharp contrast to poor or no enhancement of the adjacent nonamyloid congophilia, allowing for a specific diagnosis of amyloidosis against a background of equivocal congophilia by LM (Figures 10, A and B, 11, A and B, 12, A and B, 13, A and B, and 14, A and B).

The Table summarizes the findings in cases from groups I and II. The calculated specificity to the detection of amyloid by LM was 0.21 (95% CI, 0.11–0.33). When TRFM was used, the specificity increased to 0.83 (95% CI, 0.71–0.91).
Figure 1. Renal amyloidosis: diffuse glomerular congophilia seen on light microscopic examination (A), strongly enhanced by Texas Red-filtered fluorescence microscopy (B) (original magnification ×200 [A and B]).

Figure 2. Abdominal fat pad biopsy in a patient with amyloidosis. There is rather weak congophilia seen on light microscopic examination (A), which is strongly enhanced by Texas Red-filtered fluorescence microscopy (B), confirming the presence of amyloid (original magnification ×200 [A and B]).

Figure 3. Diabetic nephropathy. Congophilia is noted in mesangial matrix, arterial wall, tubular cast, and tubular cells on light microscopic examination (A). None of these areas are enhanced by Texas Red-filtered fluorescence microscopy (B) (original magnification ×400 [A and B]).
DISCUSSION

The current diagnosis and management of amyloidosis call for identification of tissue amyloid deposition followed by determining its chemical type. Chemical typing currently achieved by either panel immunohistochemistry or mass spectrometry is tissue based and thus hinges on an accurate tissue diagnosis of amyloidosis. The Congo red stain is essential for this goal, yet its limited sensitivity and specificity are well recognized.

The Congo red stain may be weak or faint, thus failing to detect the amyloid, especially when amyloid deposition is scant. This limited sensitivity has called for remedies including using thicker tissue sections, procedural modifications to enhance the staining intensity, examination under a stronger light source, and, perhaps most effectively, examination under Texas Red fluorescence, as shown by several studies.\textsuperscript{9,11,12}

A source of even more vexation, indeed a rather frequent problem, is the limited specificity of the Congo red stain for amyloid. A variety of structures/tissues, unrelated to amyloid, can appear salmon pink by the Congo red stain. Corrective measures have been reported ever since the utility of the Congo red stain for the detection of amyloid deposits was described by Bennhold\textsuperscript{13} in 1922. Several procedural modifications have been developed to prevent nonspecific binding of Congo red dye molecules to tissue, with limited success. First reported in 1927, apple-green birefringence under polarized LM was described as a
distinguishing feature of amyloid fibrils from other fibrillar proteins like collagen and elastin. Apple-green birefringence ever since has been ingrained in the literature as a prerequisite for the diagnosis of amyloidosis. This dictum is, however, not only inaccurate but also misleading. The detection of birefringence may require the most optimal light sources and thus the birefringence may not be visible under routine optical instrumentation in otherwise typical amyloid deposition. It was shown as early as 1969, by Klatskin, that under intense illumination and specific orientation of tissue sections stained with Congo red and examined under polarized microscopy, focal green birefringence can be seen in the absence of amyloidosis. Most recently, international consensus indicates that the birefringence of amyloid in fact often appears as shades of yellow and blue, with or without shades of green. Against this controversial background literature, we are of the opinion that there is a need to best use all available methods for an accurate diagnosis of amyloid. On balance, birefringence, in spite of its limitations, remains an important diagnostic adjunct. Other, more recent modifications to enhance the diagnostic yield of the Congo red stain, including Congo red and immunohistochemistry using monoclonal antibodies specific to AA amyloid, have encountered limited success and, at the same time, still face the daunting task of panel immunohistochemistry. Methods are still needed to improve the diagnostic specificity of the Congo red stain.

Examination of Congo red–stained tissue sections under fluorescent light was first described by Cohen et al in 1959 on specimens obtained from animal models. Later, Puchtler and Sweat in 1965 showed that examination of Congo red–stained sections under fluorescence microscopy was a sensitive tool to detect small amounts of amyloid deposits. More recently, the sensitivity of this method was further substantiated by other authors. However, despite the growing body of evidence that supports the use of Congo red fluorescence microscopy to enhance the sensitivity of amyloid detection in histology sections, limited data are available regarding the specificity of this technique. Our study aimed at evaluating the ability of Congo red fluorescence microscopy using the Texas Red filter to mitigate the false positivity seen with LM and thus enhance the specificity of Congo red stain in detecting amyloid deposits in histology sections from a variety of tissue types.

In the current study, TRFM confirmed the diagnosis of amyloidosis in each of the 15 cases in group I by specifically and strongly enhancing the congophilic areas. The congophilia was rather obvious in 9 cases but was so weak/faint that it was not recognized on initial LM examination in 2 cases (heart and fat pad biopsies). LM reexamination after TRFM, however, demonstrated convincingly observable congophilia. These observations certainly corroborate previous observations on TFRM-mediated increased diagnostic sensitivity. The main purpose for including this group in the

Figure 6. Endometriosis-associated urinary bladder elastosis. Elastosis on hematoxylin-eosin stain (A) confirmed by Verhoeff-van Gieson elastic stain (B). Congophilia is observed on Congo red stain by light microscopic examination (C) but is not enhanced by Texas Red–filtered fluorescence microscopy (D) (original magnification ×200 [A through D]).

Figure 7. Shoulder joint ligament from a patient with degenerative joint disease. Amyloid-like tissue on hematoxylin-eosin stain (A) is strongly congophilic by light microscopic examination (B), but is not enhanced by Texas Red–filtered fluorescence microscopy (C) (original magnification ×200 [A through C]).
current study, however, is to ascertain that the techniques confirmed to be valid for the sensitivity evaluation were applied to the specificity study groups.

In 50 of the 63 cases in group II, congophilia was focally noted in areas with at least some LM features of amyloid. The congophilia displayed variable intensity, but was convincingly strong in many cases. Importantly, the congophilic areas showed apple-green birefringence when polarized in many of these cases. In some of these cases, the nature of the congophilic amyloid-like structures was readily identifiable, such as in renal tubular uromodulin casts, glomerular hyalinosis, lamina propria fibrosis of colon, and endometriosis-elastosis of urinary bladder (Figure 6). In other cases, the true nature of the congophilic deposited material was undetermined. An example was an endomyocardial biopsy with intramyocyte dense eosinophilic material that showed intense congophilia on light microscopic examination (Figure 8). In either situation, amyloidosis was effectively ruled out on additional clinicopathologic correlation. Under TRFM, 39 of these 50 cases showed no enhancement of the congophilic area, providing support for the diagnosis of no amyloidosis. However, some level of enhancement was noted in the remaining 11 cases, including thyroid colloid (Figure 9), red blood cells, and dense fibrous tissue. Thus, false positivity was not entirely mitigated by using TRFM, calling for a judicious consideration of all pertinent observations in the differential diagnosis of amyloidosis.

Perhaps of more diagnostic conundrum than cases with either rather obvious amyloidosis or cases with amyloid look-alike are those in which these 2 types of tissue lesion are seen together in the same tissue sections and both display congophilia; group III included these cases. They not only exemplify a not infrequent problem, but also allow for simultaneous testing of both sensitivity and specificity of the Congo red stain. Although the number of tested cases was rather small, we found that the TRFM achieved its intended goal quite well. It clearly enhanced the amyloid and, at the same time, failed to enhance the amyloid-like material in spite of the congophilia in both.

We think that every practicing pathologist would encounter cases in which Congo red stain, in spite of repetition and proper technical consideration, yields an equivocal result (from too much red everywhere to patchy faint red). TRFM can be very helpful in this context. Thus, there are cases in which Congo red is quite faint, but if this faint Congo red is enhanced, the diagnosis of amyloidosis is confirmed (pertinently illustrated in Figure 2). If there is adequate congophilia, but this is not enhanced, this is probably not amyloidosis (pertinently illustrated in Figure 3).
The mechanism for Texas Red fluorescence–mediated enhancement of both diagnostic sensitivity and specificity of the Congo red stain has not been elucidated. This mechanism perhaps involves a chemically specific bonding of amyloid molecules and Congo red dye molecules during staining and the fluorochrome nature of the Congo red dye. X-ray diffraction studies have shown that formation of amyloid of any chemical type starts with monomeric polypeptides specific for each amyloid chemical type assembling in a cross–β-sheet configuration in which these peptide molecules are arranged in horizontal parallel rows. These sheets proceed to form amyloid fibrils by vertical end-to-end stacking.\textsuperscript{21,22} A truly positive Congo red stain represents a chemical reaction in which specific chemical bonds are created between Congo red dye molecules and amyloid molecules, thus placing the dye molecules within the amyloid molecules in a specific spatial orientation, which accounts for the LM appearance and perhaps also some distinctive optical features of a truly positive Congo red stain such as birefringence or dichroism.\textsuperscript{23} Congo red is a fluorochrome dye; that is, it can be seen by fluorescence microscopy. However, this visibility requires a specific spatial orientation and a suitable filter for the fluorescence scope. These considerations may account for the amyloid-specific enhancement by TRFM. On the other hand, Congo red dye molecules may bind nonamyloid structures by electrostatic forces or nonspecific bonding, accounting for a false-positive Congo red stain that would not be enhanced by fluorescence examination.\textsuperscript{24}

In conclusion, examination of Congo red–stained tissue sections under fluorescence microscopy with Texas Red filter increases the diagnostic yield and the specificity for detection of amyloid in formalin-fixed, paraffin-embedded tissue.
Figure 12. Intestinal amyloidosis. On light microscopy, congophilia is noted in the vessel wall and in the intravascular pooled red blood cells (A). Texas Red–filtered fluorescence microscopy strongly enhances the vascular amyloid-specific congophilia, but not the nonspecific congophilia of the pooled red blood cells (B) (original magnification ×200 [A and B]).

Figure 13. Renal amyloidosis. On light microscopy, congophilia is noted in a glomerulus and an arterial wall (A). Texas Red–filtered fluorescence microscopy enhances the amyloid-specific glomerular congophilia, but not the nonspecific vascular congophilia (B) (original magnification ×400 [A and B]).

Figure 14. Pulmonary fibrosis. Congophilia is noted in corpora amylacea and in the interstitium on light microscopy (A). Texas Red–filtered fluorescence microscopy strongly enhances the corpora amylacea–associated congophilia, confirming that amyloid is a component of the corpora amylacea, a well-known observation. In contrast, the nonspecific interstitial congophilia is not enhanced (B) (original magnification ×200 [A and B]).
References


