ORIGINAL ARTICLE

# Should Trichrome Stain Be Used on All Post–Liver Transplant Biopsies with Hepatitis C Virus Infection To Estimate the Fibrosis Score?

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Recurrent hepatitis C is virtually universal after liver transplantation; however, an individual patient's clinical course and disease burden are highly variable and difficult to predict. The fibrosis score determined on posttransplant biopsies appears to be a sensitive and specific marker of disease progression and severity. Currently, the fibrosis score is determined from hematoxylin and eosin (H&E)–stained tissue sections supplemented by variable use of trichrome stain or other connective tissue–specific stains. In this study, we compare the fibrosis score on H&E stain with that obtained with trichrome stain in posttransplant liver biopsies of patients with hepatitis C. A total of 197 liver biopsies from 105 allograft patients with hepatitis C were reviewed. The mean fibrosis stage was 1.0 ± 1.25 with H&E stain versus 1.69 ± 1.42 with trichrome stain ($P < 0.00001$). The trichrome staging score was higher in 53.3%, lower in 3%, and the same in 43.7%. The fibrosis stage was raised by 2 or more points in 17.8% and elevated into a bridging category in 14.7%. No significant differences in clinical and laboratory levels were measured in patients with higher fibrosis scores. In conclusion, the hepatic fibrosis score is significantly underestimated by H&E stain in the posttransplant setting in patients with hepatitis C. The fibrosis stage may be an indicator of significant liver damage in these patients. Accuracy of its determination may be most easily facilitated by employment of a connective tissue stain. ***Liver Transpl 14:695-700, 2008.*** © **2008 AASLD.**

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Currently, chronic infection with hepatitis C virus (HCV) is the single most common indication for orthotopic liver transplant in adults, representing 40% to 50% of transplanted individuals and those waiting for transplant.[1] Approximately 6800 to 8500 chronically HCV-infected individuals are on liver transplant waiting lists throughout the United States, and approximately 2000 to 2500 transplants are performed on this patient population annually.[1,2] Allograft HCV infection occurs universally in patients undergoing liver transplantation for chronic HCV disease, and recurrence of HCV-associated liver lesions within the graft occurs in 60% to 80% of patients.[3-7] In one study, histologic recurrence of HCV disease occurred in up to 90% of patients within 5 years of transplant.[8] Overall, 20 to 40% of liver transplant recipients with recurrent HCV disease will progress to cirrhosis within 5 years.[5,7,9] This is in stark contrast to the less than 5% of individuals transplanted for non–HCV-related diseases who progress to cirrhosis during the same time period.[10] In transplanted patients, the natural history of HCV disease is accelerated, and patients have an excess risk of death or retransplantation for liver failure 10 to 15 years after transplantation.[3] As a result, various strat-

**Abbreviations:** Alk phos, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Creat, creatinine; GGT, glutamyl transpeptidase; H&E, hematoxylin and eosin; HCT, hematocrit; HCV, hepatitis C virus; SD, standard deviation; T Bili, total bilirubin; WBC, white blood cell count.

Studies were performed after institutional review board approval.

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egies to both prevent and treat recurrent HCV disease in allograft livers have been devised, and they include preemptive antiviral therapy before transplant, prophylactic antiviral therapy without evidence of recurrence, and initiation of antiviral therapy based on histologic evidence of recurrence.[11] The best strategy to use is controversial. Currently, the most common practice is to wait until there is histologic evidence of recurrent HCV disease, seen as increased allograft fibrosis or increased inflammation, before treatment with pegylated interferon and/or ribavirin is initiated.[11]

Emphasis has often been put on the fibrosis stage because it appears to be a significant indicator of advanced liver damage and impending liver failure.[12] In Knodell et al.'s original article,[13] the histology activity index, which included the fibrosis score, was based on hematoxylin and eosin (H&E)–stained and trichrome-stained tissue. The trichrome stain was used because it strongly and specifically stains collagen and provides better contrast with which to visualize the degree of fibrosis than H&E. In a modification to the original scoring system, the authors made no mention as to whether trichrome should be used when the fibrosis stage is being assessed.[14] The primary literature has been vague about the use of trichrome stains in this setting. Many publications fail to mention if trichrome was used and, if employed, whether it was used in all cases. Nevertheless, in practice, the great majority of pathologists employ a connective tissue stain when examining native liver biopsies; this is not necessarily true, however, when post–liver transplant cases are reported. Rapidity of diagnosis, with almost all major transplant centers offering same-day processing, has always been considered most important, primarily so that acute rejections can be treated quickly. A trichrome stain in most laboratories does not come out until the next day and would hold up the official signout of the case. Times have changed though, and with better immunosuppression, acute rejection diagnoses have fallen somewhat.[15-17] Perhaps most importantly, the transplant population has also changed, with many more hepatitis C patients now requiring monitoring as part of treatment protocols. Therefore, we undertook this study in order to compare the fibrosis stage on hematoxylin-stained tissue with the fibrosis stage on trichrome-stained tissue in allograft biopsies of HCV-positive recipients to determine if universal trichrome staging should be mandated when a fibrosis score is being reported.

## PATIENTS AND METHODS

### Patients

One hundred five patients with chronic HCV infection underwent liver transplantation at the University of Rochester (Rochester, NY) between January 2002 and January 2006. These patients included 82 men and 23 women, who had a combined total of 197 liver biopsies with a mean of 283 ± 44.9 days or median of 8.7 months post liver transplant. The HCV genotype distribution was as follows: 1 (not otherwise specified), 24 patients; 1a, 24 patients; 1b, 32 patients; 2, 4 patients; 3, 5 patients; and unknown, 16 patients. Each patient had 1 to 6 biopsies available for review, and the mean age of this population was 50.3 ± 7.7 years (Table 1). Only patients with biopsies greater than 1 month post transplant were included in order to exclude histology secondary to immediate postoperative complications. The patients' clinical findings, including genotype, HCV RNA viral load, biochemical abnormalities (blood urea nitrogen, creatinine, white blood cell count, hematocrit, platelet count, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyl transpeptidase), and immunosuppression type and level were also recorded from the time of biopsy.

**TABLE 1. Characteristics of the Patients in the Study**

| Characteristic | Value |
|---|---|
| Number of patients | 105 |
| Number of biopsies | 197 |
| Age (years, median) | 50.3 ± 7.7 |
| Gender (male/female) | 82/23 |
| Biopsy timing (months post transplant, median) | 8.7 |
| Hepatitis C genotype | |
| 1 | 24 |
| 1a | 24 |
| 1b | 32 |
| 2 | 4 |
| 3 | 5 |
| Not performed/unknown | 16 |

### Study Design

All needle biopsies included in the study met at least minimal criteria for adequacy (greater than 6 portal triads) and usually contained 12 to 22 portal triads. Formalin-fixed, paraffin-embedded sections had been cut at 4 μm and yielded a total of 3 H&E-stained microscope slides with 3 serial sections per slide. Gomori trichrome stain was performed on step-sectioned intervening slides also cut at 4 μm. They were required to be full or complete cuts of the parenchyma. If this was not the case, a trichrome stain was performed over one of the H&E-stained sections representative of a full cross section of tissue to ensure the most valid comparison of the slides. Twenty-eight of 197 biopsies were originally stained with trichrome. For those biopsies initially stained by H&E alone, a trichrome stain was obtained in the aforementioned manner, in many cases with a step section, and the case was restaged. In those cases that had originally been staged with the use of the trichrome stain, the pathologist was blinded to the original interpretation and was given the H&E slide alone and asked to render a fibrosis score. In all cases, H&E and trichrome stains were viewed and staged independently. A single experienced hepatic pathologist (C.K.R.), blinded to the patient identification and the

**TABLE 2. H&E Versus Trichrome Stain Fibrosis Scores in All 197 Biopsies**

| Fibrosis Score on H&E Stain | | Trichrome Stain Compared to H&E Stain | | |
|---|---|---|---|---|
| Score | n | Same [n (%)] | Higher [n (%)] | Lower [n (%)] |
| 0 | 94 | 46 (48) | 48 (52) | 0 (0) |
| 1 | 45 | 15 (33.3) | 29 (64.5) | 1 (2.2) |
| 2 | 38 | 17 (44.7) | 18 (47.3) | 3 (7.9) |
| 3 | 10 | 3 (30) | 5 (50) | 2 (20) |
| 4 | 5 | 2 (40) | 3 (60) | 0 (0) |
| 5 | 4 | 2 (50) | 2 (50) | 0 (0) |
| 6 | 1 | 1(100) | 0 (0) | 0 (0) |
| Overall | 197 | 86 (43.7) | 105 (53.3) | 6 (3.0) |

**Abbreviation:** H&E, hematoxylin and eosin.

**TABLE 3. H&E Versus Trichrome Stain Fibrosis Scores in Biopsies for Which Trichrome Was Initially Employed**

| Fibrosis Score on H&E Stain | | Trichrome Stain Compared to H&E Stain | | |
|---|---|---|---|---|
| Score | n | Same [n (%)] | Higher [n (%)] | Lower [n (%)] |
| 0 | 8 | 3 (37) | 5 (63) | 0 (0) |
| 1 | 7 | 2 (28) | 4 (58) | 1 (14) |
| 2 | 10 | 4 (40) | 6 (60) | 0 (0) |
| 3 | 2 | 0 (0) | 1 (50) | 1 (50) |
| 4 | 0 | 0 (0) | 0 (0) | 0 (0) |
| 5 | 1 | 1 (100) | 0 (0) | 0 (0) |
| 6 | 0 | 0 (0) | 0 (0) | 0 (0) |
| Overall | 28 | 10 (36) | 16 (57) | 2 (7) |

**Abbreviation:** H&E, hematoxylin and eosin.

original fibrosis stage, performed all of the grading in this study. A second pathologist (R.L.) was responsible for organizing cases, ordering trichrome stains, and presenting the slides to the grading pathologist. This was done as a further safeguard to ensure unbiased readings. The fibrosis stage was evaluated according to Ishak and colleagues,[14] with the score ranging from 0 to 6 (absent to cirrhosis). H&E-stained slides were prepared in a standard fashion. Gomori trichrome–stained slides were performed as outlined in Gomori's original publication.[18]

### Statistical Analysis

The differences in the fibrosis stage obtained between H&E and Gomori trichrome stains were compared with the *t* test (SPSS Windows, base version 14.0).

## RESULTS

The mean fibrosis stage for all 197 biopsies, as determined by H&E stain, was 1.0 ± 1.25 (median: 1.0, range: 0-6). The mean fibrosis stage as determined by Gomori trichrome stain was significantly higher at 1.69 ± 1.42 (range: 0-6; $P < 0.00001$). Overall, the trichrome stain staging score was higher in 53.3%, lower in 3%, and the same in 43.7% of biopsies. The distributions of the fibrosis score on H&E stain and its comparison with trichrome stain are shown in Table 2.

Of the 197 biopsies, trichrome stain was initially performed on 28, involving 23 patients. Three patients had trichrome performed on several biopsies. Overall, the trichrome fibrosis score was higher than H&E alone in 57%, the same in 36%, and lower in 7% (Table 3). The mean fibrosis score in this group on H&E stain was 1.36.

Of the 197 total biopsies, the fibrosis score was raised by 2 or more points following the use of trichrome stain in 35 (17.8%) and was elevated into a bridging fibrosis category (stage 3 or higher) from a nonbridging category (stages 0, 1, or 2) in 29 biopsies (14.7%). Among the 28 biopsies for which trichrome already had been performed, the connective tissue stain increased for 8 (28.6%) into a bridging fibrosis category.

No significant differences in the clinical and laboratory levels were measured in patients with fibrosis scores upgraded into a bridging category by trichrome stain (Table 4).

## DISCUSSION

In this study, we have demonstrated that H&E stain alone estimates a significantly lower fibrosis score than

**TABLE 4. Characteristics of Patients with Fibrosis Scores Significantly Upgraded by Trichrome Stain**

| | Fibrosis Score ≥ 3 | | Fibrosis Score < 3 | | *P* |
|---|---|---|---|---|---|
| Total biopsies | 30 | 164 | | | |
| Clinical variable | n | % | n | % | |
| Male/female | 25/30 | 83 | 125/164 | 76 | |
| Failed transplant | 0/30 | 0 | 9/164 | 5.5 | |
| Laboratory variable | Mean | SD | Mean | SD | |
| BUN (mg/dL) | 30.8 | 14.7 | 30.5 | 17.1 | 0.92 |
| Creatinine (mg/dL) | 2.1 | 2.7 | 1.6 | 1.0 | 0.33 |
| HCT (%) | 35.7 | 6.7 | 35.6 | 6.7 | 0.97 |
| WBC | 6.0 | 3.8 | 6.8 | 4.4 | 0.32 |
| Platelets (thousand/μL) | 146.7 | 91.2 | 167.3 | 85.7 | 0.26 |
| T. Bili (mg/dL) | 4.8 | 8.1 | 4.3 | 10.5 | 0.79 |
| AST (U/L) | 119.8 | 88.4 | 122.0 | 132.8 | 0.91 |
| ALT (U/L) | 138.6 | 123.9 | 168.1 | 236.8 | 0.32 |
| Alk phos (U/L) | 544.0 | 865.2 | 374.2 | 354.0 | 0.30 |
| GGT (U/L) | 792.5 | 1050.9 | 803.6 | 1317.5 | 0.97 |
| Tacrolimus level (ng/mL) | 8.2 | 6.3 | 7.9 | 4.9 | 0.84 |
| Genotype | n | % | n | % | |
| Hepatitis C 1a or 1b genotype | 11/11 | 100% | 69/78 | 88% | |

**Abbreviations:** Alk phos, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Creat, creatinine; GGT, glutamyl transpeptidase; HCT, hematocrit; SD, standard deviation; T Bili, total bilirubin; WBC, white blood cell count.

trichrome-stained tissue in hepatic allograft biopsies from liver transplant patients with recurrent HCV disease. Overall, our results show that when trichrome stain was used, the fibrosis stage was greater than predicted by H&E stain in 53.3% and rose by 2 or more points in 17.8% of biopsies. The group of patients for which trichrome stain increased their fibrosis stage into the bridging category (fibrosis score ≥ 3) showed no significant difference in biochemical parameters. Therefore, liver biopsy remains the gold standard test for determination of fibrosis.

The most clinically important finding of our study is that trichrome stain elevated a significant percentage of total biopsies (14.7%) and biopsies in which a trichrome was initially obtained (28.6%) from fibrosis stage 2 to fibrosis stage 3, which is the threshold proposed by many groups as the point at which HCV antiviral therapy should be initiated.

Recurrent HCV disease in post–orthotopic liver transplant patients is the most common cause of graft loss in liver transplant recipients with pretransplant HCV infection.[11] Although the diagnosis of recurrent HCV infection is easily accomplished by amplification of HCV RNA, the demonstration of HCV disease recurrence is more difficult and relies on allograft liver biopsy for determination of disease activity and progression.

Controversy exists as to when the initiation of antiviral therapy should begin because the rate of sustained viral response is less than 30% in the posttransplant population, and the drugs given for treatment are required to be taken for long periods of time and have several side effects. Most large North American liver transplant centers elect to treat only those patients with histologic disease of moderate to severe activity, progressive disease, or persistently elevated liver enzymes.[19] Most centers are now performing protocol liver biopsies at prescribed intervals in order to assess disease severity and progression by semiquantitatively estimating the degree of inflammation and fibrosis.[19] In particular, the fibrosis stage appears to be a significant indicator of disease progression and impending graft failure.[20] For this reason, most groups have adopted a fibrosis threshold at which they initiate HCV antiviral therapy. The optimal threshold level is not currently known, but many groups use early bridging to bridging fibrosis (Ishak fibrosis score ≥ 2-3 or equivalent) as their cutoff point.[10,12,21,22] In 2003, the International Liver Transplant Society Expert Panel Consensus Conference acknowledged that the optimal timing of treatment for recurrent HCV disease in the posttransplant setting has yet to be determined but suggested that patients with recurrent HCV disease with grade 2 fibrosis or higher be given a trial of antiviral therapy.[10] As such, accurate and reliable determination of the fibrosis score is imperative.

Traditionally, trichrome stains have been employed as a connective tissue stain in order to accentuate collagen and other cellular constituents in tissue sections. Although the word *trichrome* literally means 3 colors, the term *trichrome stain* is a general name for a number of techniques that use multiple (2 or more) acidic stains of contrasting colors to differentially stain tissue components.[23] In tissue, it has been used to enhance the contrast between collagen fibers, muscle fibers, fibrin, and erythrocytes.[23] The Gomori trichrome staining technique is a 1-step method that employs a combination of an acid triphenylmethane dye with a sulfonated azo dye in the presence of phosphotungstic or phosphomolybdic acid and dilute acetic acid.[18] At our institution, chromotrope 2R and aniline blue are used, and

under these conditions, collagen is stained blue and muscle fibers and erythrocytes are stained red. When it is applied to 4-μm tissue sections of liver, the areas of fibrosis (collagen) are stained blue, and the hepatocytes are magenta. The nuclei of all cells are black because of counterstaining with iron-hematoxylin.[18] H&E stains all stroma, including muscle, fibrin, cytoplasmic contours, and collagen, a similar color of red-pink. In most cases, an experienced pathologist can make a reasonably accurate determination of the actual tissue on the basis of the context and tissue type; however, when he or she does so, it is an educated guess at best. The advantage of using a trichrome stain is that it enhances the contrast between collagen and the background stroma, and this allows for a more accurate assessment of collagen quantity. We selected the Gomori trichrome stain over Masson's trichrome stain because of its simple, 1-step staining procedure, which uses commonly available, low-cost chemical dyes. The simplified nature of its protocol requires less histotechnologist labor and produces quick, reproducible results. At our institution, the Gomori trichrome stain is performed daily, and our lead histotechnologist regards it as a highly reliable stain that rarely is repeated for technical reasons (the rate of repetition has been estimated to be less than 0.5%). Photomicrographs of representative examples in which the fibrosis score was significantly increased when trichrome stain was employed are illustrated in Fig. 1.

In addition to accurately determining the stage of fibrosis, which relies on portal fibrous expansion, portal-to-portal bridging, and portal-to-central vein bridging, the trichrome stain allows for assessment of sinusoidal fibrosis, which is notoriously difficult to appreciate on H&E-stained tissue (Fig. 1E,F). This may be an important feature to recognize and report in the future, as many believe it is the cause of hepatic synthetic dysfunction and portal hypertension.[24]

The trichrome stain was initially performed on only 28 of 197 biopsies (14.2%). This rather low rate of trichrome utilization was in keeping with our program's policy of rapid signout of all liver transplant biopsies. In biopsies that had an initial trichrome stain performed, the reasons for its use were multiple and included cases in which the patient was doing poorly clinically, diagnostic problem cases, cases with larger amounts of steatosis, cases in which bridging was suspected on H&E stain, and cases in which the biopsy was from a patient who was chronologically further out from transplant. As a result of the findings of our study, we have modified our protocol for the handling of allograft biopsies, and we now automatically obtain a trichrome stain on all liver biopsies from orthotopic liver transplant patients infected with HCV who are greater than 1 month post transplant. We selected the 1-month cutoff because most patients rarely have significant fibrosis so soon after transplant; some centers may wish to extend this period to 2 or even 3 months, depending on the comfort level of the pathologist and clinicians.

A limitation of our study, which exists in all studies reporting a single observer's findings, is that the results

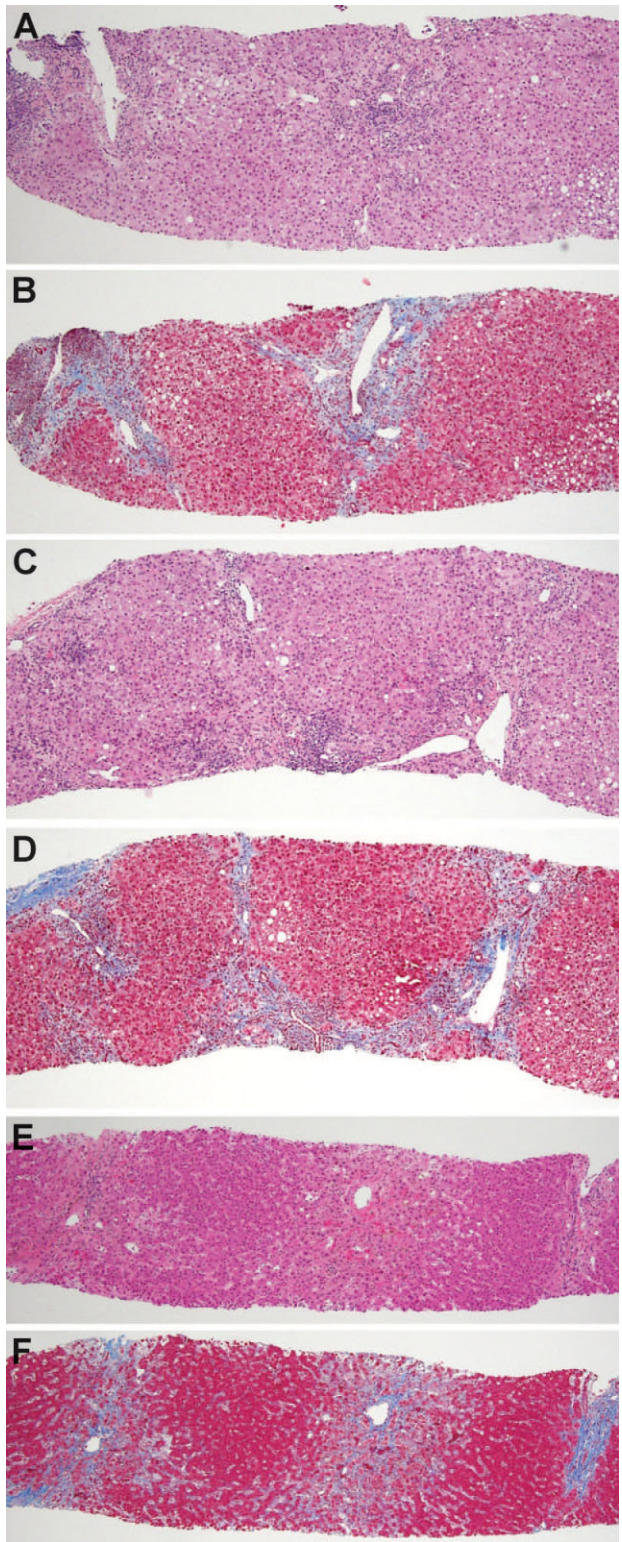


**Figure 1. Comparison of H&E and trichrome stains. (A,C,E) H&E sections from 3 different cases paired with (B,D,F) their respective trichrome stains demonstrate that a more accurate quantitation of collagen (blue color) is made possible with a contrasting connective tissue stain. (E,F) The third case shows a centrilobular and sinusoidal-type pattern of fibrosis rather than a predominantly portal pattern as seen in the first 2 cases (magnification, ×100). Abbreviation: H&E, hematoxylin and eosin.**

can be affected by variable interpretation of the biopsy and this lack of reproducible assessment can lead to false conclusions. We acknowledge that intrarater variability is a potential source of error within our study. Although we do not specifically address the subject, the results of others suggest that experienced liver pathologists have excellent reproducibility with respect to fibrosis scoring with the Ishak system. One report found intraobserver agreement to be between 90% and 94% when the same biopsy was scored multiple times.[25] In addition, we believe that the small amount of intrarater variability that is present in our study is offset by the large number of cases evaluated (n = 197).

In conclusion, although the Hepatic Activity Index score may be somewhat reflective of the biochemical parameters and suggest hepatic injury, these same parameters in many instances are less indicative of the fibrotic response.[14] It is also, of course, this laying down of connective tissue that has the most long-lasting consequences with respect to hepatic function and the future course of disease. It is therefore important that this fibrosis score be accurate, reproducible, and standardized, which we believe may best be achieved through the routine use of a trichrome stain. In summary, because of the increased numbers of hepatitis C patients undergoing liver transplantation, we suggest that pathology programs serving this population consider the use of a connective tissue stain, a practice that has been in almost universal employment in all native liver biopsies for many years.

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