



Original Article

Classification of amyloid deposits in diagnostic cardiac specimens by immunofluorescence[☆]

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Received 24 January 2008; received in revised form 25 April 2008; accepted 29 May 2008

Abstract

Background: At least 12 distinct forms of amyloidosis are known to involve the heart or great vessels. Patient treatment regimens require proper subtyping of amyloid deposits in small diagnostic cardiac specimens. A growing lack of confidence in immunohistochemical staining for subtyping amyloid has arisen primarily as a result of studies utilizing immunoperoxidase staining of formalin-fixed paraffin-embedded tissue. Immunofluorescence staining on fresh frozen tissue is generally considered superior to immunoperoxidase staining for subtyping amyloid; however, this technique has not previously been reported in a series of cardiac specimens. **Methods:** Amyloid deposits were subtyped in 17 cardiac specimens and 23 renal specimens using an immunofluorescence panel. **Results:** Amyloid deposits were successfully subtyped as AL, AH, or AA amyloid by immunofluorescence in 82% of cardiac specimens and 87% of renal specimens. In all cases, the amyloid classification was in good agreement with available clinical and laboratory assessments. A cross-study analysis of 163 cases of AL amyloidosis reveals probable systemic misdiagnosis of cardiac AL amyloidosis by the immunoperoxidase technique, but not by the immunofluorescence technique. **Conclusions:** Amyloid deposits can be reliably subtyped in small diagnostic cardiac specimens using immunofluorescence. The practical aspects of implementing an immunofluorescence approach are compared with those of other approaches for subtyping amyloid in the clinical setting. © 2008 Published by Elsevier Inc.

Keywords: Amyloid; Amyloidosis; AH Amyloid; AL Amyloid; AA Amyloid; Immunofluorescence; Misdiagnosis; Cardiac; Heart

1. Introduction*1.1. Types of amyloid involving the heart*

The amyloidoses comprise a family of diseases characterized by the formation of a specific type of protein deposit in tissues [1]. In these amyloid deposits, there is typically a single culprit protein that adopts an abnormal extended β -sheet conformation, which facilitates the formation of large insoluble fibrils. Amyloid deposits are classified based on the specific protein forming these amyloid fibrils. Currently, there are at least 27 distinct forms of amyloid, which differ in

their distribution (systemic vs. localized), mode of acquisition (hereditary vs. acquired), and clinical relevance [2]. It is the extended β -sheet structure of amyloid fibrils that allows Congo red dye to bind the deposits in an orderly fashion so as to display green birefringence upon the application of plane-polarized light. In addition to the specific culprit protein, most if not all amyloid deposits contain nonspecific proteins including serum amyloid P, apolipoprotein E, and heparan sulfate proteoglycans [1–4]. The proteoglycans in amyloid deposits allow these deposits to stain with sulfated alcian blue. Serum amyloid P is often exploited as a general immunohistochemical marker for all amyloid deposits in tissue and as a radio-labeled nuclear medicine probe to assess for whole-body amyloid involvement [5].

Of the 27 currently known types of amyloid, 12 types have been reported to involve the heart or great vessels (Table 1). In the setting of a plasma cell dyscrasia or other immunoglobulin-producing lymphoproliferative disorders,

[☆] This work was supported by the Department of Pathology, Massachusetts General Hospital.

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t1.1 Table 1
t1.2 Types of amyloid that involve the heart and great vessels

t1.3	Amyloid protein	Precursor protein	Systemic vs. localized	Associations or tissue localization	Reference
t1.4	AL	Immunoglobulin light chain	Systemic	Plasma cell dyscrasia	[6–8]
t1.5	AH	Immunoglobulin heavy chain	Systemic	Plasma cell dyscrasia	[9]
t1.6	ATTR	Transthyretin	Systemic	Senile systemic hereditary	[14,15]
t1.7					[16–18]
t1.8	AA	Serum amyloid A	Systemic	Chronic inflammation	[21]
t1.9	A β ₂ M	β ₂ -Microglobulin	Systemic	Hemodialysis	[22]
t1.10	AApoAI	Apolipoprotein A-I	Systemic	Hereditary	[23]
t1.11			Localized	Atherosclerotic plaques	[33]
t1.12	AApoAII	Apolipoprotein A-II	Systemic	Hereditary	[24]
t1.13	AApoAIV	Apolipoprotein A-IV	Systemic	Senile systemic	[19]
t1.14	AGel	Gelsolin	Systemic	Hereditary	[25]
t1.15	Abri	ABriPP	Systemic	Hereditary	[26]
t1.16	AANF	Atrial natriuretic factor	Localized	Atria	[27–30]
t1.17	AMed	Lactadherin	Localized	Arterial media	[31,32]
t1.18	–	Unknown	Localized	Cardiac valves	[34]
t1.19				Mural thrombi	[35]

cardiac amyloid may form as a result of the overproduction of a monoclonal amyloidogenic immunoglobulin [6–8]. Immunoglobulin-related amyloid is most often due to the deposition of the immunoglobulin light chain, AL amyloid, but occasionally results from the deposition of the immunoglobulin heavy chain, AH amyloid [9–12]. Transthyretin may form amyloid deposits in the heart either in patients with a normal wild-type gene sequence as a manifestation of senile systemic amyloidosis [13–15], or as part of a hereditary disease in patients carrying an amyloidogenic mutation in one of their transthyretin genes [16,17]. Over 80 such transthyretin mutations are currently known, some of which cause a late-onset clinical presentation, with patients presenting with cardiac amyloidosis in the seventh decade of life or later [18]. Senile systemic amyloidosis involving the heart may also result from the deposition of apolipoprotein A-IV [19]. Systemic amyloidosis due to serum amyloid A occurs in the setting of chronic inflammatory conditions. Such AA amyloidosis typically manifests with more involvement of the kidneys, liver, and spleen than the heart, but the heart may be involved in severe cases [20,21]. Chronic hemodialysis is associated with the systemic deposition of β 2-microglobulin containing amyloid, which may involve the heart [22]. In addition to transthyretin, mutations of a growing list of other genes are now known to result in systemic familial amyloidosis that involves the heart, specifically apolipoprotein A-I, apolipoprotein A-II, gelsolin, and Abri precursor protein [23–26].

In addition to these systemic amyloidoses, several localized forms of amyloid are encountered in the heart and great vessels. Atrial amyloid is a form of amyloid due to atrial natriuretic peptide, which is present in a majority of older adults, but its distribution is limited to the cardiac atria [27,28]. It has been suggested that the deposition of atrial amyloid may be related to the presence and/or severity of atrial fibrillation, but this association is not certain [29,30]. Small amyloid deposits derived from a fragment of lactadherin are frequently observed in the media of the aorta and

other large- and medium-sized arteries [31,32]. This arterial medial amyloid is associated with aging, but otherwise its clinical significance is unclear. Amyloid due to deposition of wild-type apolipoprotein A-I has been observed in atherosclerotic plaques [33]. Similar “dystrophic” amyloid has been observed in a high proportion of cardiac valves resected for calcific stenosis or rheumatic disease as well as in mural thrombi [34,35]. In these latter two cases, the nature of the amyloid is unclear; however, awareness of these localized forms of cardiovascular amyloid is necessary to avoid inappropriately attributing such amyloid to a clinically significant systemic process.

1.2. Necessity for the classification of amyloid in cardiac surgical specimens

Each case of cardiac amyloidosis is in effect any one of at least 12 distinct diseases, each with its distinct prognosis and clinical management. In the clinical management and treatment of patients with cardiac amyloidosis, a critical step is to identify the type of amyloid present in the tissue [36–39]. Thus, successful and proper classification of the amyloid deposits in diagnostic cardiovascular specimens has an important impact on patient care. Much of the recent outcry for proper classification of amyloid deposits is derived from the current treatment for AL amyloidosis [40–43]. Not uncommonly, this disorder is now treated with melphalan-based chemotherapy with autologous peripheral stem cell rescue. This treatment may be instituted in the setting of a normal bone marrow biopsy based primarily on the classification of the amyloid in the cardiac biopsy. The treatment-related mortality for this approach may reach as high as 10–25% [37]; thus misclassification of senile amyloid or hereditary amyloid as AL amyloid must be avoided. In addition, in some medical centers, chemotherapy and stem cell rescue for AL amyloidosis are preceded by cardiac transplantation, again emphasizing the necessity for accurate subtyping of the amyloid [44–46].

Certainly, an important step toward working up a patient with cardiac amyloidosis is to determine whether there is a circulating monoclonal paraprotein. However, with traditional approaches, relying on the presence of a paraprotein has led to the misdiagnosis of AL amyloidosis in patients who actually have hereditary amyloidoses [47,48]. In addition, some patients with AL amyloidosis lack a detectable circulating paraprotein. Based on traditional serum protein electrophoresis with immunofixation (SPEP-IF), about 10% of patients with systemic amyloidosis and a detectable paraprotein actually have a hereditary amyloidosis, and, conversely, about 15% of patients with AL amyloidosis have no detectable paraprotein [47]. The new serum-free light chain (SFLC) analyses appear more sensitive than the older SPEP-IF technique; however, both false negatives and false positives are likely to continue to be a problem [49]. Similarly, assessing for amyloidogenic mutations in the DNA is of value in suspected cases of hereditary amyloidosis. However, a patient with such a mutation could still develop AL amyloidosis, and relying only on the DNA sequence could also lead to a misdiagnosis. These concerns highlight the necessity to routinely and accurately subtype amyloid deposits in diagnostic cardiovascular specimens.

1.3. Approaches to the classification of amyloid

Certainly, the predominant techniques available to surgical pathologists to identify proteins in diagnostic specimens are immunohistochemical techniques. In the amyloid subtyping literature, there is at times apparent confusion concerning the fact that there are distinct types of immunohistochemical techniques, specifically immunoperoxidase staining used with formalin-fixed paraffin-embedded tissue and immunofluorescence staining, which in diagnostic

settings is typically only performed on fresh frozen tissue due to the high background of formalin-fixed tissue. There are many reports of utilizing immunoperoxidase staining to subtype amyloid deposits in formalin-fixed paraffin-embedded tissue [13,14,21,50–58]. However, at many institutions, this approach is felt to be not reliable, and reports of low success rates and even outright misdiagnoses have been linked to the use of immunoperoxidase staining [47,48,59–62]. The unreliability of immunoperoxidase staining for subtyping amyloid derives primarily from the high background staining associated with the antibodies that are often used, and the fact that most amyloid deposits in the heart and kidney will stain to some degree with most of the relevant antibodies. This latter effect is overcome by using a panel of antibodies and classifying the amyloid based on the antibody that yields the most intense staining [62,63]. Subtyping amyloid deposits by staining with only one antibody is unreliable. In surgical pathology subspecialties with ready access to frozen tissue, particularly renal pathology, the high background staining encountered with the immunoperoxidase technique has been circumvented by the use of immunofluorescence staining on fresh frozen tissue [64–67]. There have been no reports of using immunofluorescence to systematically subtype cardiac amyloidosis.

2. Methods

2.1. Patients

The patients included in this study are detailed in Tables 2 and 3. Patient 1 has been reported previously [46]. All cases were evaluated in the Department of Pathology at Massachusetts General Hospital. The cardiac amyloid cases consisted of four autopsies and 12 surgical specimens received from

t2.1 Table 2
t2.2 Patients in this study with cardiac amyloid that was analyzed by immunofluorescence

t2.3 Patient no.	Specimen	Age/gender	Amyloid Type	SFLC	SPEP-IF ^a	BM-ISH ^b
t2.4 1	LV Biopsy	46 M	Lambda	Lambda	Negative	–
t2.5 2	RV Biopsy	47 F	Lambda	Lambda	VI Lambda BJ	–
t2.6 3	RV Biopsy	62 M	Lambda	Lambda	VI Lambda BJ	–
t2.7 4	RV Biopsy	37 F	Lambda	Lambda	IgG Lambda	Lambda
t2.8 5	RV Biopsy	62 M	Lambda	Lambda	IgG Lambda	–
t2.9 6	LV Biopsy	67 M	Indeterminate	Kappa	IgA Kappa	–
t2.10 7	Apical Core	58 M	Lambda	Lambda	Lambda BJ	Lambda
t2.11 8	RV Biopsy	75 M	Lambda	–	–	–
t2.12 9	RV Biopsy	56 M	Lambda	Lambda	IgG Lambda	–
t2.13 10	RV Biopsy	67 M	Indeterminate	Negative	Negative	–
t2.14 11	RV Biopsy	77 M	Indeterminate	–	–	–
t2.15 12	RV Biopsy	48 F	Lambda	Negative	Negative	Lambda
t2.16 13	RV Biopsy	55 M	Kappa	–	Kappa BJ	–
t2.17 14	Autopsy	65 M	Lambda	–	IgG Lambda	–
t2.18 15	Autopsy	44 M	Lambda	Lambda	Negative	Lambda
t2.19 16	Autopsy	64 F	IgG	Kappa	–	–
t2.20 17	Autopsy	72 F	Lambda	Lambda	–	Lambda

t2.21 For all, “–” indicates test not performed or results not available.

t2.22 ^a VI indicates very low concentration band 12.5–25 mg/dl; BJ, Bence Jones protein.

t2.23 ^b Clonality of bone marrow plasma cell clone determined by in situ hybridization of bone marrow plasma cell mRNA for kappa and lambda light chain.

t3.1 Table 3
t3.2 Patients in this study with renal amyloid that was analyzed by immunofluorescence

t3.3	Patient no.	Specimen	Age/gender	Amyloid type	SFLC	SPEP-IF ^a	Associated disease
t3.4	18	Biopsy	46 F	Lambda	–	–	
t3.5	19	Biopsy	71 M	Indeterminate	–	IgG Kappa	
t3.6	20	Biopsy	61 M	Lambda	–	Lambda BJ	
t3.7	21	Biopsy	74 F	Lambda	–	IgM Lambda	
t3.8	22	Biopsy	64 F	Lambda	–	–	
t3.9	23	Biopsy	57 M	Lambda	–	Negative	
t3.10	24	Biopsy	49 M	Indeterminate	–	–	
t3.11	25	Biopsy	77 F	Lambda	–	IgG Lambda	
t3.12	26	Biopsy	78 M	Lambda	–	IgA Lambda	
t3.13	27	Biopsy	70 F	Lambda	–	IgG Lambda	
t3.14	28	Biopsy	65 F	Lambda	–	–	
t3.15	29	Biopsy	47 M	Kappa	–	–	
t3.16	30	Biopsy	49 F	Kappa	Kappa	Vl IgG Kappa	
t3.17	31	Biopsy	63 M	AA	–	Vl IgG Lambda	Rheumatoid arthritis
t3.18	32	Biopsy	74 M	Lambda	–	–	
t3.19	33	Biopsy	75 F	AA	–	–	Myeloproliferative disorder
t3.20	34	Biopsy	50 M	Lambda	–	–	
t3.21	35	Biopsy	84 M	Lambda	–	–	
t3.22	36	Biopsy	38 F	Lambda	–	IgA Lambda	
t3.23	37	Biopsy	61 M	Kappa	–	IgG Kappa	
t3.24	38	Nephrectomy	64 M	AA	–	Vl IgG Kappa	Crohn's disease
t3.25	39	Nephrectomy	49 M	AA	–	–	Familial mediterranean fever
t3.26	40	Nephrectomy	45 F	Indeterminate	–	Negative	

t3.27 For all, “–” indicates test not performed or results not available.

t3.28 ^a Vl indicates very low concentration band 12.5–25 mg/dl; BJ, Bence Jones protein.

189 2003 to 2007, and one archived surgical specimen from 1996.
190 The cardiac surgical specimens consisted of 10 right
191 ventricular endomyocardial biopsies, two left ventricular
192 endomyocardial biopsies, and one left ventricular apical core
193 segment removed for ventricular assist device placement. The
194 kidney amyloid cases consisted of 20 core needle biopsies
195 and three surgical nephrectomies obtained from 2000 to 2007.
196 The presence of amyloid was determined based on the
197 presence of extracellular material in formalin-fixed paraffin-
198 embedded tissue that stained with Congo red, and with Congo
199 red stain displayed green birefringence upon the application
200 of plane-polarized light. All activities were approved by the
201 hospital's Human Subjects Institutional Review Board.

202 2.2. Immunofluorescence

203 The amyloid deposits were subtyped in fresh frozen tissue
204 using immunofluorescence. The primary antibodies em-
205 ployed are listed in Table 4. For direct immunofluorescence,
206 fluorescein isothiocyanate (FITC)-conjugated primary anti-
207 bodies were utilized. For indirect immunofluorescence, pig
208 anti-rabbit and rabbit anti-mouse FITC-conjugated secondary
209 antibodies were obtained from Dako and used at dilutions of
210 1:50 and 1:40, respectively. For subtyping cardiac amyloid,
211 2- μ m-thick frozen sections were stained with a panel of
212 antibodies consisting of lambda, kappa, serum amyloid A,
213 transthyretin, and apolipoprotein A-I. Stained sections were
214 examined using an Olympus BX60 fluorescence microscope
215 equipped with a Spot digital camera. The amyloid subtype
216 was assigned based on the antibody that yielded the most

intense staining. To assign a subtype to the amyloid, it was
required that staining for the antigen be substantially greater
than that of all other antigens in the panel. If the cardiac
amyloid was not assigned based on the initial panel, then a
secondary panel was employed which consisted of IgG, IgA,
IgM, β 2-microglobulin, and fibrinogen. The kidney amyloid
deposits were subtyped using a panel consisting of lambda,
kappa, serum amyloid A, fibrinogen, IgG, IgA, and IgM. The
optimal staining conditions were determined for each of the
primary antibodies, including their specificity in their ability
to detect antigens, by staining of previously archived frozen
renal tissue with amyloid (λ -AL, κ -AL, and AA) and
nonamyloid (IgG, IgA, IgM, and fibrinogen) deposits,
paraffin-embedded sections of myocardium with senile
systemic amyloidosis (transthyretin) and atherosclerotic
plaques with amyloid (apolipoprotein A-I) from autopsies,

Table 4
Primary antibodies used for immunofluorescence

Antigen	Source	Type	Method	Dilution
Lambda	Dako	Polyclonal	Direct	1:20
Kappa	Dako	Polyclonal	Direct	1:20
Serum amyloid A	Dako	Monoclonal	Indirect	1:20
Transthyretin	Dako	Polyclonal	Indirect	1:70
Apolipoprotein A-I	Biogenesis	Monoclonal	Indirect	1:100
IgG	Dako	Polyclonal	Direct	1:30
IgA	Dako	Polyclonal	Direct	1:20
IgM	Dako	Polyclonal	Direct	1:20
Fibrinogen	Dako	Polyclonal	Direct	1:20
β 2-Microglobulin	Dako	Monoclonal	Indirect	1:50

233 and frozen myocardium with endothelial-specific staining
 234 (β 2-microglobulin). For these control tissues, frozen tissue
 235 was stained by immunofluorescence as detailed above, and
 236 paraffin-embedded tissue was stained by immunoperoxidase
 237 using standard techniques.

238 2.3. Statistics

239 Comparison of observed and expected frequencies was
 240 performed using the exact binomial test. *P* values less than
 241 .05 were considered significant.

242 3. Results

243 3.1. Classification of amyloid by immunofluorescence

244 Of the 17 cardiac specimens (Table 2), the amyloid was
 245 successfully subtyped by immunofluorescence in 14 cases

(82%). These 14 cases included 12 cases of λ -AL amyloid 246
 (Fig. 1) and one case each of κ -AL amyloid (Fig. 2) and IgG- 247
 AH amyloid (Fig. 3). The IgG-AH amyloid case was an 248
 autopsy in which the cardiac amyloid had a prominent 249
 vascular pattern and was also present in arteries in the kidney, 250
 liver, spleen, pancreas, and parathyroid glands. In one of the 251
 three cases not subtyped successfully, there was clinical 252
 evidence of a monoclonal gammopathy. The disproportionately 253
 high number of AL cases compared with senile 254
 amyloid cases observed in our series is likely related to the 255
 availability of frozen tissue, as patients with suspected AL 256
 amyloid are biopsied in our institution for amyloid subtyping. 257

Of the 23 renal specimens (Table 3), the amyloid was 258
 subtyped in 20 cases (87%). These 20 cases entailed 13 cases 259
 of λ -AL amyloid, three cases of κ -AL amyloid, and four 260
 cases of AA amyloid (Fig. 4). All of the cases of AA amyloid 261
 occurred in patients with chronic inflammatory conditions. 262
 As with the cardiac specimens, in one of the three cases not 263

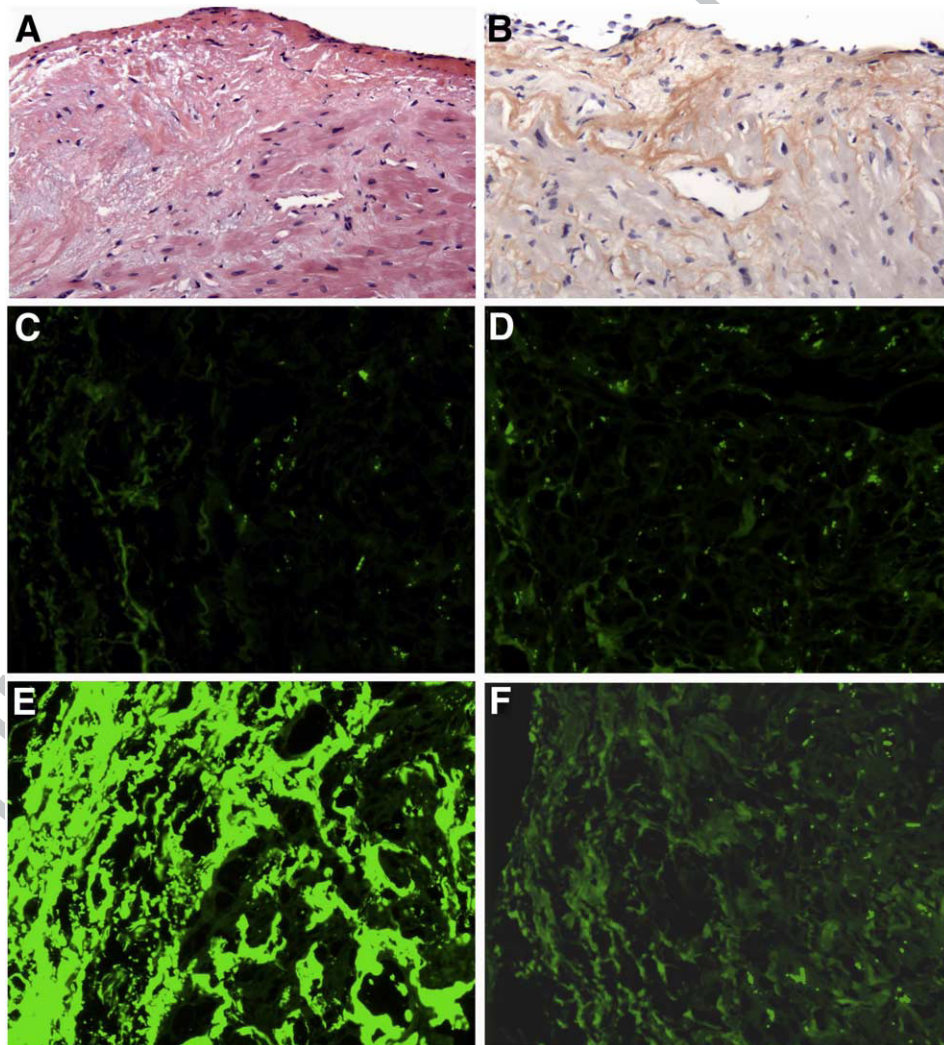


Fig. 1. λ -AL Amyloid in an endomyocardial biopsy. Hematoxylin and eosin-stained section (A) and Congo red-stained section (B) showing amyloid with a predominant interstitial pattern. Immunofluorescence staining of frozen myocardium showing staining for (C) apolipoprotein A-I, (D) κ light chain, (E) λ light chain, and (F) transthyretin, with more intense staining of the amyloid for λ light chain compared with the other antigens. Original magnification 400 \times .

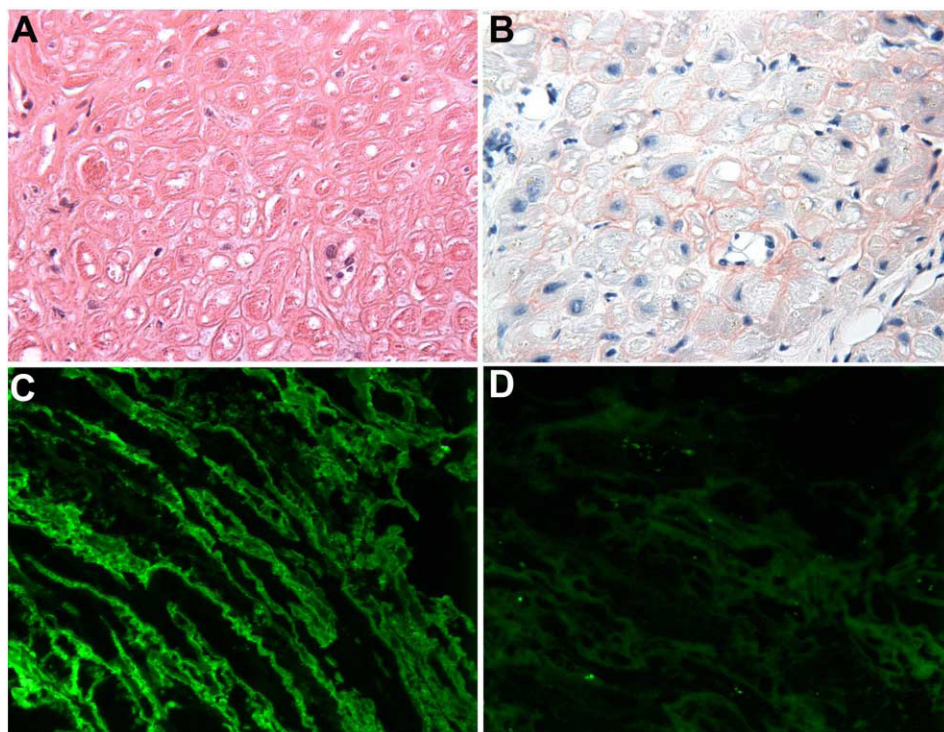


Fig. 2. κ -AL Amyloid in an endomyocardial biopsy. Hematoxylin and eosin-stained section (A) and Congo red-stained section (B) showing amyloid with a predominant interstitial pattern. Immunofluorescence staining of frozen myocardium showing staining for (C) κ light chain and (D) λ light chain, with more intense staining of the amyloid for κ light chain. Original magnification 400 \times .

264 subtyped successfully, there was clinical evidence of a
 265 monoclonal gammopathy. It is not certain whether these two
 266 cases represent false negatives by the immunofluorescence
 267 panel or in fact represent inherited amyloidoses presenting in
 268 the setting of a monoclonal gammopathy.

269 3.2. Validating the assignment of AL amyloidosis

270 For 12 of the 13 cardiac specimens and for eight of the 16
 271 kidney specimens classified as AL amyloidosis, there was
 272 independent assessment of the light chain clone by SPEP-IF,
 273 SFLC analysis, and/or in situ hybridization of the plasma
 274 cells in the bone marrow (BM-ISH). In all 20 of these cases,
 275 the immunofluorescence determination of the light chain
 276 restriction (κ vs. λ) was confirmed by the independent
 277 assessment. There were 10 cases in which there was an
 278 assignment of the light chain restriction by SFLC analysis
 279 and in which SPEP-IF was also performed. In five of these
 280 10 cases, the SPEP-IF was either negative or only identified a
 281 nonspecific very low concentration band, demonstrating the
 282 enhanced sensitivity of SFLC analysis. There were two cases
 283 of cardiac amyloid subtyped as λ -AL by immunofluorescence
 284 in which both the SFLC analysis and SPEP-IF were
 285 negative. In one of these cases, the plasma cells in the bone
 286 marrow showed λ restriction by in situ hybridization,
 287 indicating that false negatives will continue to occur when
 288 assessing for circulating paraproteins even with the more
 289 sensitive SFLC analysis.

290 As a second method of validation, for all of the cases
 291 classified as AL amyloidosis, the number of cases classified
 292 as κ divided by the number of cases classified as λ (κ/λ ratio)
 293 was determined. Even though κ light chain restriction is more
 294 common than λ light chain restriction in plasma cell
 295 neoplasms, λ is more common than κ in patients with AL
 296 amyloid by 3:1, based on large clinical databases [68,69].
 297 Thus, in autopsy series, the κ/λ ratio is expected to be ~ 0.33 .
 298 Assessment of the κ/λ ratio from several reported autopsy
 299 series employing immunoperoxidase staining confirms this
 300 ratio (Table 5). These autopsy studies utilized primarily
 301 proprietary antibodies and in one case commercial antibody
 302 preparations available prior to 1990. In addition to being
 303 more amyloidogenic than κ light chains, λ light chains that
 304 are amyloidogenic also have been reported to have greater
 305 tropism for the heart and kidneys than do amyloidogenic κ
 306 light chains [70]. Thus when assessing a series of surgical
 307 specimens from these two organs, the κ/λ ratio is expected to
 308 be ≤ 0.33 . Previous immunofluorescence studies on amyloid
 309 deposits in kidney surgical specimens using commercially
 310 available antibodies in fact have demonstrated the κ/λ ratio
 311 to be 0.24 (Table 5). In our kidney surgical specimens, the κ/λ
 312 ratio was 0.23, in good agreement with the previous studies.
 313 Importantly for the cardiac surgical specimens in our series,
 314 the κ/λ ratio obtained by immunofluorescence was 0.11 and
 315 thus was also as expected, ≤ 0.33 . In contrast, assessment of
 316 three previous studies employing commercially available
 317 antibodies to subtype amyloid in cardiac surgical specimens

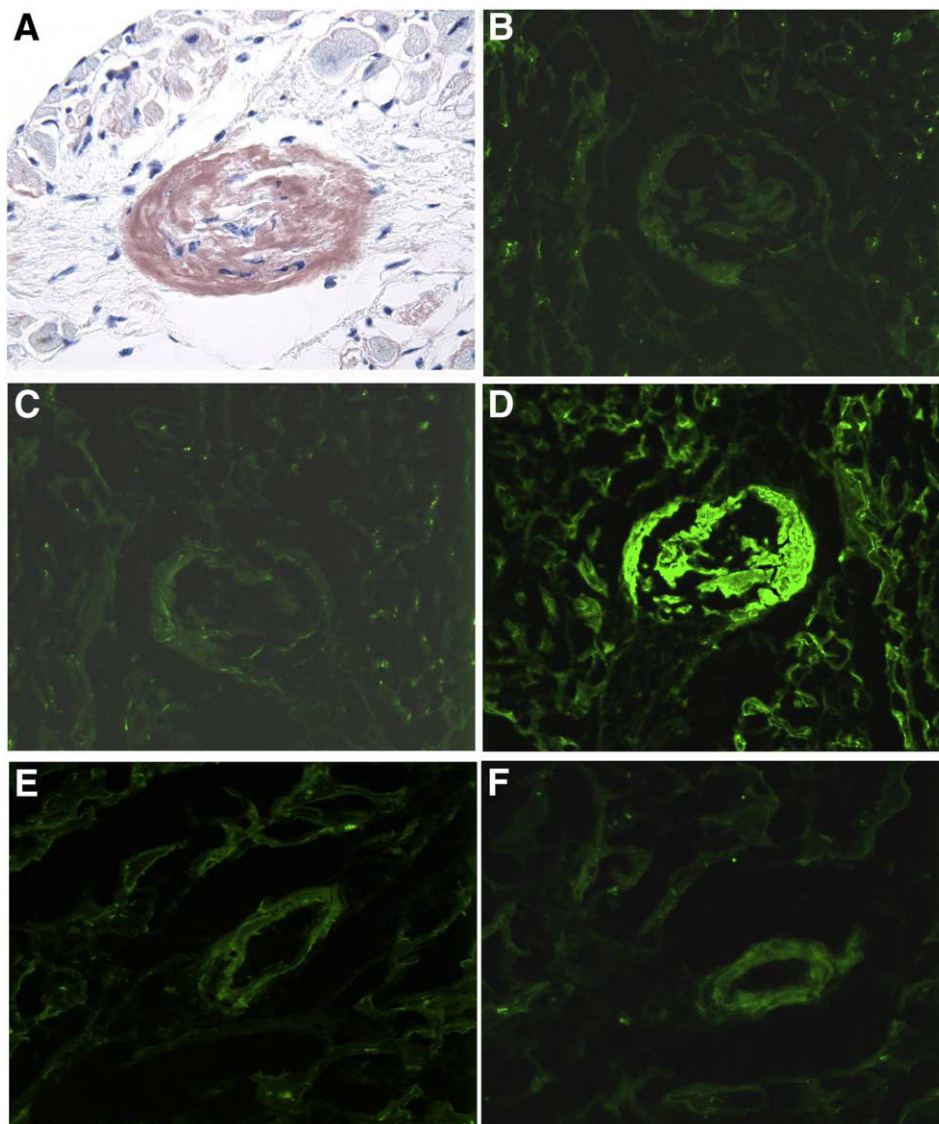


Fig. 3. AH Amyloid in a heart at autopsy. (A) Congo red-stained section showing amyloid with a predominant vascular pattern. Immunofluorescence staining of frozen myocardium showing staining for (B) IgA heavy chain, (C) IgM heavy chain, and (D) IgG heavy chain, (E) κ light chain, and (F) λ light chain with more intense staining of the amyloid for IgG heavy chain compared with the other antigens. Original magnification 400 \times .

318 by the immunoperoxidase technique revealed a κ/λ ratio of
 319 0.82 (Table 5), which differs in a statistically significant
 320 manner from the expected value ($P=0.01$). In these three
 321 studies, the κ/λ ratio ranged from 0.78 to 1.00, in contrast to
 322 all of the other studies listed in Table 5, in which the κ/λ ratio
 323 ranged from 0.00 to 0.43. The amyloid cases in our study
 324 were not assessed by immunoperoxidase staining as we feel
 325 this method to be unreliable for subtyping amyloid.

326 4. Discussion

327 4.1. Subtyping cardiac amyloid by immunofluorescence

328 In this initial cardiac series, which was dominated by AL
 329 amyloidosis, the amyloid deposits were successfully sub-

330 typed in 82% of cases, which was similar to our success rate
 331 with kidney specimens (87%). Both of these numbers are in
 332 good agreement with previously reported success rates for
 333 subtyping amyloid in kidney specimens by immunofluores-
 334 cence, which have ranged from 67% to 88% [64,66,67]. For
 335 all AL amyloid cases in this study, in which there was
 336 independent assessment of the light chain clonality, the
 337 immunofluorescence determination of the light chain restric-
 338 tion (κ vs. λ) was confirmed by the independent assessment.
 339 From these results, immunofluorescence appears to be useful
 340 for subclassifying immunoglobulin-derived amyloid in
 341 diagnostic cardiac specimens. Establishing the utility of this
 342 approach for subclassifying senile and hereditary amyloid
 343 deposits in the heart will require additional cases.

344 The classification of one case of cardiac amyloid as IgG
 345 AH amyloid in a patient with a κ light chain containing

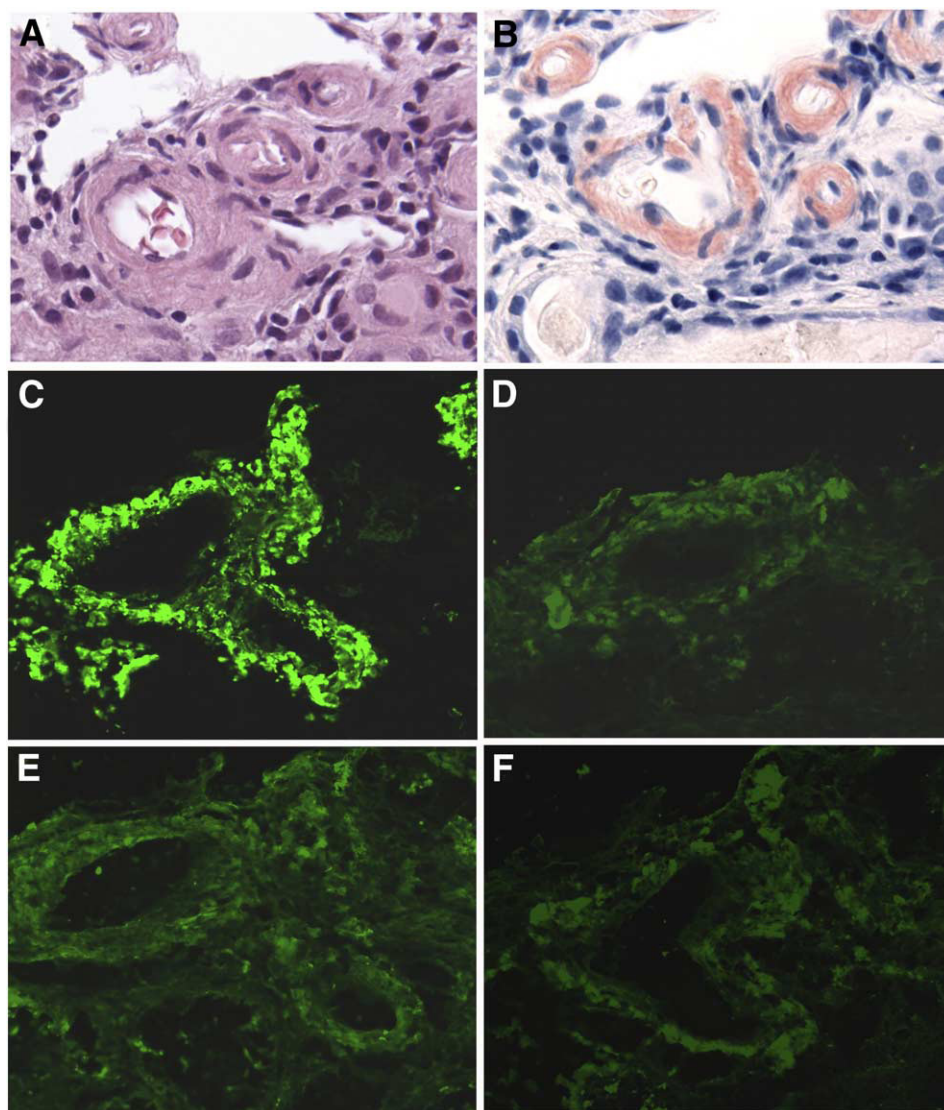


Fig. 4. AA Amyloid in a renal core needle biopsy. Hematoxylin and eosin (A) and Congo red-stained (B) sections depict a small blood vessel containing amyloid. Immunofluorescence staining of frozen tissue showing staining for (C) serum amyloid A, (D) IgG heavy chain, (E) κ light chain, and (F) λ light chain reveals more intense staining of the amyloid for serum amyloid A than the other antigens. Original magnification 400 \times .

346 paraprotein is particularly interesting. Immunoglobulin
347 heavy chains, particularly IgG, have been reported to form
348 amyloid, referred to as AH amyloid. Less than 10 cases have

been reported, and in only two of these previous cases was 349
cardiac involvement documented [9–12]. Certainly, one 350
potential pitfall in classifying amyloid as AH by immuno- 351

t5.1 Table 5

t5.2 Assessing the reliability of amyloid subtyping by immunoperoxidase and immunofluorescence

t5.3	Tissue	Antibodies used	Method ^b	<i>N</i>	κ/λ ratio ^c	<i>P</i> vs. expected ^d	References
t5.4	Autopsies	Proprietary ^a	IP	76	0.31	NS	[50–54]
t5.5	Kidney surgicals	Commercial	IF	26	0.24	NS	[64–66]
t5.6	Kidney surgicals	Commercial	IF	16	0.23	NS	This work
t5.7	Heart surgicals	Proprietary	IP	4	0.00	NS	[21]
t5.8	Heart surgicals	Commercial	IP	31	0.82	0.01	[55–57]
t5.9	Heart surgicals	Commercial	IF	10	0.11	NS	This work

t5.10 ^a Study employed commercial antibodies obtained prior to 1990.

t5.11 ^b IP indicates immunoperoxidase on formalin-fixed paraffin-embedded tissue; IF, immunofluorescence on frozen tissue.

t5.12 ^c The number of κ -AL cases divided by the number of λ -AL cases.

t5.13 ^d By the exact binomial test. Expected ratio is ≤ 0.33 as detailed in the text. NS indicates not statistically significant.

352 fluorescence could be failure of the appropriate light chain
353 antibody to recognize the light chain fragment comprising an
354 AL amyloid deposit, which may have some associated heavy
355 chain. However, previously the concept of AH amyloid has
356 been supported not only by immunofluorescence, but also by
357 additional studies including immunoelectron microscopy and
358 fibril extraction with biochemical sequencing [9–12]. It is
359 presently not clear whether or not AH amyloid patients have a
360 clinical course distinct from that of AL amyloid patients,
361 although cardiac involvement in AH amyloidosis may be less
362 prominent compared with that seen in AL amyloidosis. AH
363 amyloid deposits are notoriously difficult to classify by
364 immunohistochemical techniques; thus the true prevalence of
365 this condition may currently be underestimated.

366 4.2. Comparison of immunofluorescence 367 and immunoperoxidase

368 Currently, there is much disagreement in the literature
369 concerning the utility of immunoperoxidase staining to
370 subtype amyloid. While several studies have reported high
371 amyloid subtyping success rates with immunoperoxidase
372 staining, other studies have reported the technique to be not
373 reliable and to even result in misdiagnoses [47,48,59–62]. In
374 contrast to the relatively uniformly high success rates for
375 subtyping amyloid achieved by immunofluorescence dis-
376 cussed above, the success rate for subtyping amyloid by
377 immunoperoxidase staining has been as low as 38% [47].
378 The analysis depicted in Table 5 appears to document the
379 lack of reliability of immunoperoxidase staining for subtyp-
380 ing amyloid when the current commercially available
381 antibodies are utilized.

382 Based on large clinical databases with well over 1000 AL
383 amyloid patients, λ light chain is more common in this
384 condition than κ light chain by a ratio of 3:1 [68,69]. Thus,
385 when assessing a series of AL amyloid cases, it is expected
386 that the ratio of the number of κ -AL cases to the number of
387 λ -AL cases (the κ/λ ratio) will be ~ 0.33 in autopsy series
388 and ≤ 0.33 in kidney and cardiac surgical specimens. Older
389 autopsy studies employing immunoperoxidase staining with
390 mostly proprietary antibodies, and in one case commercial
391 antibodies obtained prior to 1990, document a κ/λ ratio of
392 0.31, in good agreement with the expected ratio [50–54].
393 Thus certain proprietary antibodies may be capable of
394 accurately subtyping amyloid by immunoperoxidase stain-
395 ing. Likewise, utilizing commercially available antibodies by
396 immunofluorescence with frozen kidney surgical specimens,
397 both in our series and in the sum of three previous studies,
398 resulted in κ/λ ratios of 0.23 and 0.24, respectively, in good
399 agreement the expected value (Table 5). Thus the current
400 commercially available antibodies do appear to be adequate
401 to subtype the majority of amyloid cases when used by
402 immunofluorescence. Correspondingly, in the cardiac surgi-
403 cal specimens described here, this approach resulted in a κ/λ
404 ratio of 0.11, in agreement with the expected value. This
405 result is also in agreement with one study from a group with a

great deal of experience with immunoperoxidase staining of 406
amyloid, in which proprietary antibodies were used with 407
cardiac biopsies to classify four cases of AL amyloid as λ 408
and none as κ [21]. 409

Since frozen cardiac tissue is often not systematically 410
obtained in clinical settings, attempts have been made to 411
subtype amyloid in cardiac biopsies using the current 412
commercially available antibodies with the immunoperox- 413
idase technique. As indicated in Table 5, analysis of three 414
such studies reveals this approach results in a κ/λ ratio of 415
0.82, which differs significantly from the expected value 416
($P=.01$). For many of the cases in these three studies, the 417
amyloid subtype was not correlated with an independent 418
assessment of the light chain restriction. While it is 419
possible that a confounding variable resulted in an 420
unusually high proportion of κ -AL cases being evaluated 421
in these three studies, a much more likely and troubling 422
conclusion is that at least some of the amyloid cases were 423
misclassified by reliance on immunoperoxidase staining 424
with commercial antibodies. 425

4.3. Alternative methods to subclassify amyloid deposits 426

Due to the limitations of more conventional immunohis- 427
tochemical techniques for subclassifying amyloid, much 428
effort has gone into alternative approaches. One such 429
approach is to analyze amyloid deposits with immunogold 430
labeling and transmission electron microscopy [71]. This 431
approach has the theoretical potential to reduce the 432
interference of background staining by more precisely 433
localizing the labeling to the amyloid fibrils. Another 434
antibody-dependent approach is to extract the amyloid 435
fibrils, isolate them by centrifugation, and then subject the 436
fibrils to electrophoresis and immunoblotting [72,73]. This 437
approach has the potential to reduce the high background 438
staining primarily observed with formalin-fixed tissue. 439
However, both of these antibody-based approaches still 440
require the use of a panel of antibodies. 441

Additional approaches forgo the need for antibodies 442
altogether. The amyloid fibrils can be extracted from the 443
tissue, even from formalin-fixed paraffin-embedded tissue, 444
and subjected to biochemical sequencing [59,73]. This 445
approach typically requires isolation of the amyloid 446
fibrils by liquid chromatography and/or electrophoresis. 447
These biochemical approaches have clearly been shown 448
to be successful in cases where immunohistochemical 449
methods were inconclusive, even with small diagnostic 450
specimens. In addition, biochemical sequencing has the 451
capability to identify novel types of amyloid. However, 452
biochemical sequencing relies on the ability to success- 453
fully isolate the actual amyloid fibrils. In addition, 454
amyloid fibrils are not uncommonly blocked at the 455
amino terminus, requiring proteolytic digestion in order 456
to be amenable to biochemical sequencing. 457

The newest approach to subtyping amyloid is to use 458
tandem mass spectrometry to identify the components in the 459

460 amyloid deposit. Tandem mass spectrometry, typically
 461 coupled with liquid chromatography, is a very powerful
 462 technique for identifying proteins in complex mixtures and
 463 lies at the heart of modern proteomic advances [74,75].
 464 Tandem mass spectrometry has superior sensitivity compared
 465 with traditional biochemical sequencing and has been
 466 used in place of immunoblotting and biochemical sequencing
 467 to analyze amyloid fibrils extracted and isolated from
 468 formalin-fixed paraffin-embedded tissue [4]. Additionally,
 469 tandem mass spectrometry can be applied to total tryptic
 470 digests of proteins from amyloid deposits isolated by laser
 471 microdissection [76]. It must be remembered that tandem
 472 mass spectrometry is a relatively new technique, and the
 473 number of amyloid deposits currently reported to have been
 474 characterized by this methodology is relatively small. Thus
 475 the actual sensitivity and specificity of the approach are not
 476 known. It should also be remembered that while tandem
 477 mass spectrometry is very powerful in terms of identifying
 478 proteins in complex mixtures, the technique has shortcomings
 479 in terms of absolute quantitation. Thus upfront amyloid
 480 fibril isolation techniques may still be necessary
 481 with this approach.

482 4.4. Practical considerations for amyloid subtyping in the 483 clinical setting

484 Pathologists are under increasing pressure to subtype the
 485 amyloid deposits in their specimens. Much consideration
 486 should go into establishing a procedure to achieve this goal.
 487 Immunoperoxidase staining on paraffin-embedded tissue is
 488 often an early consideration, and there exist proprietary
 489 antibodies that may be useful toward this end. However,
 490 based on concerns reported in the literature and the analysis
 491 in Table 5, it is our recommendation that clinical diagnostic
 492 laboratories not utilize current commercially available
 493 antibodies by immunoperoxidase to subtype amyloid in
 494 cardiac specimens, as this method is in our opinion not
 495 reliable. Since many factors influence the reliability of
 496 immunoperoxidase staining, it is possible that conditions
 497 may yet be established that enable reliable use of these
 498 antibodies in this fashion. Alternatively, much effort is
 499 currently being devoted to developing newer antibodies that
 500 may be more reliable for subtyping amyloid in formalin-
 501 fixed paraffin-embedded tissue [77–80].

502 Like immunoperoxidase staining, immunofluorescence
 503 can be performed in most pathology departments located in
 504 large medical centers. The major limitation of immunofluorescence
 505 is the requirement for frozen tissue. This is frequently a
 506 serious limitation, as amyloid is often only discovered
 507 after all of the material has been fixed in formalin and
 508 paraffin embedded. While some proteolytic salvage
 509 techniques have been reported for allowing immunofluorescence
 510 to be performed on formalin-fixed material [65], the
 511 reliability of these salvage immunofluorescence approaches
 512 is not entirely clear. Although necessity for frozen tissue is a
 513 serious limitation of the immunofluorescence approach, it is

also a limitation that is readily overcome by communication
 between pathologists and the clinical services obtaining the
 biopsies. Thus the immunofluorescence approach can be
 implemented now in most major medical centers. Like
 essentially all methods for subclassifying amyloid, evaluation
 of immunofluorescence is subject to interpretative error,
 and misclassification could still occur with this approach.

The alternative approaches discussed above are all
 associated with limitations that are not so easily overcome.
 Immunoelectron microscopy is simply cost prohibitive for
 the routine clinical setting. In our institution, the cost of an
 immunoelectron microscopy panel to subtype amyloid
 would be 5–10 times that of the immunofluorescence
 panel, and at present there is no evidence that immunoelectron
 microscopy yields a higher success rate for subtyping
 amyloid than obtained with immunofluorescence. The
 amyloid fibril extraction and isolation techniques along
 with biochemical sequencing and/or mass spectrometry are
 now relatively routine for biochemistry research laboratories;
 however, these methodologies are very complex for a
 clinical diagnostic laboratory, and the laboratories performing
 these techniques most commonly do not have complete
 regulatory approval for diagnostic testing. It is unlikely that
 amyloid fibril extraction and isolation will become a routine
 procedure in a large number of diagnostic laboratories, but
 the establishment of a small number of readily accessible
 clinical reference laboratories is feasible. As with immunoelectron
 microscopy, there is currently no evidence that the
 immunoblotting approach has any advantage over immunofluorescence,
 other than being applicable to formalin-fixed paraffin-embedded
 material. However, for the minority of amyloid cases that cannot
 be subtyped by immunofluorescence panel, biochemical sequencing
 or potentially tandem mass spectrometry in a properly regulated
 clinical reference laboratory may be appropriate.

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