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# Diagnosing Lymphocytic Myocarditis in Adult Autopsies Combining the Dallas Criteria with Immunohistochemical Stainings

Judith Fronczek[1*], Franklin RW van de Goot[1], Paul AJ Krijnen[1,2], Allard C van der Wal[3], Hans WM Niessen[1,2,4]

[1]Department of Pathology, Symbiant, Medisch Centrum Alkmaar, Alkmaar, The Netherlands

[2]The Institute for Cardiovascular Research of the Vrije Universiteit of Amsterdam (ICaR-VU), Amsterdam, The Netherlands

[3]Department of Cardiovascular Pathology, AMC, Amsterdam, The Netherlands

[4]Department of Cardiac Surgery, VU University Medical Centre, Amsterdam, The Netherlands

**Corresponding author:** Dr. Judith Fronczek, Symbiant, Medisch Centrum Alkmaar, Department of Pathology, Wilhelminalaan 12, 1815 JD Alkmaar, The Netherlands, Tel: +31-72-5483697; Fax: +31-72-5482184; E-mail: j.fronczek@symbiant.nl

## Abstract

**Aim:** Endomyocardial biopsies form the golden standard in diagnosing (borderline) lymphocytic myocarditis ((B)-LM) in living patients. Herein immunohistochemistry (IH) is mandatory. Diagnosing (B)-LM in autopsy hearts however is less defined. In this study we performed immunohistochemical stainings of heart slides in a cohort of consecutive adult autopsies that were evaluated subsequently with the Dallas criteria.

**Methods:** Sections of the myocardium of consecutive adult autopsies (n=107) performed at Symbiant, Pathology Expert Centre, from January 1, 2012, to December 31, 2012 were revised. The two Hematoxylin/Eosin (H&E) slides of the heart with the highest number of interstitial inflammatory cells were stained for LCA (leukocyte common antigen) and activated complement (C3d). The slides were then analysed for clusters (≥1) of LCA-positive lymphocytes, with or without cardiomyocyte adhesion. C3d staining was used to visualize myocytolysis.

**Results:** In H&E slides only, LM was found in 2 out of 107 cases (1.9%), but no B-LM was diagnosed. Using IH and applying the Dallas criteria, LM was found in 4 cases (3.7%) and B-LM in 4 additional cases (3.7%) out of 107.

**Conclusions:** Combining the Dallas criteria with immunohistochemical stainings, LCA (identifying lymphocytes) and C3d (visualizing myocytolysis), improves the diagnosis of (B)-LM in autopsy hearts.

**Keywords:** Lymphocytes; Autopsy; Immunohistochemical stainings; Monocytes; Lymphocytes; Myocytes; Cardiology; Atazanavir; Raltegravir

## Introduction

Lymphocytic myocarditis (LM) is a cardiac disease that is difficult to diagnose. In the 1980's the so-called Dallas Classification System was developed to diagnose LM, providing histopathological criteria for its diagnosis in endomyocardial biopsies (EMB). As per these criteria, myocarditis requires histological evidence of inflammatory infiltrates within the myocardium associated with myocyte necrosis of non-ischemic origin. In the so-called borderline myocarditis (B-LM) there is no microscopical evidence of myocytolysis (dissolution of myocytes) and/or the inflammatory infiltrate is less intense [1]. In LM the inflammatory infiltrate mainly consists of lymphocytes. The cause of LM is most often viral and/or postviral autoimmune-related [2]. Through the years the Dallas criteria, without immunohistochemical stainings, have shown not to be that adequate for diagnosing LM in EMB due to sampling error and/or inter-observer variety [3-5]. Additional criteria for the diagnosis LM in EMB since then have been developed [6-8]. In 2013 immunohistochemical quantification criteria, defining an abnormal inflammatory infiltrate as ≥14 leukocytes/mm$^2$ including up to 4 macrophages/mm$^2$ with the presence of ≥7 CD3-positive T-lymphocytes/mm$^2$, were proposed by the European Society of Cardiology (ESC) to diagnose LM in EMB [9].

Criteria for the diagnosis of LM in autopsy hearts however are not that clear yet. Previous autopsy studies of unselected patient groups using the Dallas criteria, thus without immunohistochemical analysis, found an incidence of LM ranging from 0.2 to 1% [10,11]. In selected patient groups of sudden unexpected death patients, an incidence of LM in respectively 11 out of 29 cases (38%, Yunnan province, China [12]) and 4 out of 48 cases (8.3%, coastal area of Tunesia [13]) was found using the Dallas criteria. Previous studies showed that interobserver variability in counting numbers of lymphocytes in EMB obtained at autopsy, can be drastically reduced when immunostaining with leukocyte common antigen antibody (LCA or CD45) (identifying B- and T-lymphocytes, but also macrophages [14]) is applied, compared with Hematoxylin/Eosin (H&E) staining only [15]. This could indicate the need for the use of immunohistochemistry (IH) in LM diagnosis of autopsy hearts also. Dettmeyer et al. [16] analyzed the role of IH in the diagnosis of LM in autopsies in cases of sudden infant death syndrome (SIDS). When the Dallas criteria were applied in the absence of IH, LM was diagnosed in only 1 out of these 20 cases. However, immunohistochemical staining (LCA and CD3) resulted in the diagnosis of LM in three additional cases, when LM was defined as >10 LCA-positive and >2 CD3-positive lymphocytes/HPF (high power field; magnification x400). An autopsy study by Feeley et al. [17] used

the immunohistochemical quantification criteria that were first suggested by the International Society and Federation Cardiology (ISFC) task force [18], which correspond with the criteria proposed by the ESC as stated above [9], but stained heart slides with CD45ro antibodies instead of CD3 (visualizing all T-lymphocytes) and CD68 (visualizing macrophages) as proposed by the ESC. They then found an incidence of LM of 0.6% in routine hospital autopsies. The relatively low incidence they found can be explained by the CD45ro antibody they used, as this marker only identifies activated or memory T-cells [19].

Recently Nielsen et al. [20] analyzed the cut-off value of CD3 immunostains (identifying T-lymphocytes), combined with morphological changes of myocytolysis to diagnose (B-)LM, in autopsy hearts that were previously diagnosed as (B-)LM by means of applying the Dallas criteria on H&E slides. The authors analyzed different putative cut-off values from 5 to 25 CD3-positive lymphocytes/mm$^2$ where after the specificity and sensitivity with corresponding 95% point-wise a confidence bound were calculated. The highest combination of sensitivity and specificity for diagnosing (B-)LM was observed at a cut-off value of 13 CD3-positive lymphocytes/mm$^2$. However this yielded a sensitivity of 68%, meaning that in their (B-)LM group 36 of the 112 (B-)LM cases would be missed. It has to be noticed, that the authors did also stain heart slides with CD68, as proposed by the ESC, but they didn't analyze these slides because of background staining and difficulty to identify individual positive cells.

Therefore controversies still exist related to the criteria that should be applied to diagnose LM in hearts at autopsy. Quantification of inflammatory cells only to diagnose LM in EMB as proposed by the ESC, neglects the patchy character of LM in whole heart slides. The Dallas criteria do correct for this provided that the myocardium is widely sampled [21]. To the best of our knowledge these Dallas criteria have never been applied on immunohistochemical stained slides of autopsy hearts. This we have now studied in a cohort of adult autopsies, in which we combined the use of a marker for myocytolysis (anti-complement C3d antibody) with LCA immunostaining.

## Materials and Methods

### Cohort

Sections of the myocardium from all adult autopsies performed at Symbiant Pathology Expert Centre, The Netherlands, from January 1, 2012, to December 31, 2012, were revised. Routinely five sections of the heart were taken at autopsy, namely left ventricular anterior-, posterior- and lateral wall, interventricular septum and right ventricular wall. These slides were retrieved from the archive and reviewed. Since we used archival pathology material, which does not interfere with patient care and does not involve the physical involvement of the patient, no ethical approval is required according to Dutch legislation (Medical Research Involving Human Subjects Act) [22]. Use of material for research after completion of a pathological examination is part of the patient contract in our hospital.

### Histologic analysis

Tissue blocks were fixed in 4% formaldehyde solution and embedded in paraffin. For histochemical analysis, 4 μm-thick tissue sections were stained with Hematoxylin/Eosin (H&E) according to standard methods. Of each autopsy two slides with visually the highest number of interstitial inflammatory cells in the H&E slide were selected by two pathologists (JF/FRWVDG) for immunohistochemical stainings (Figure 1).
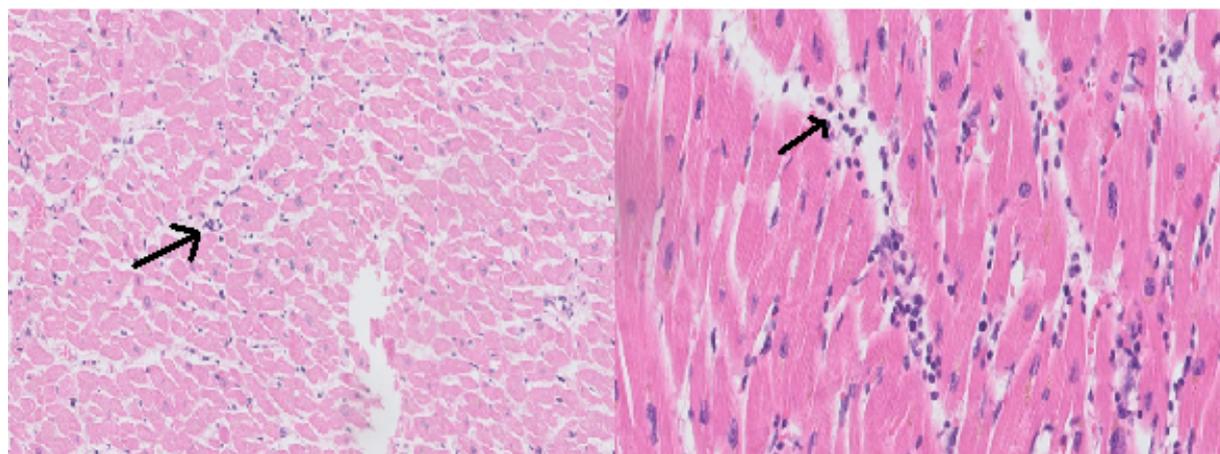


**Figure 1:** An increase of interstitial inflammatory cells in an H&E slide of the heart (arrow) and the interstitial inflammatory infiltrate consists predominantly of lymphocytes (arrow).

### Immunohistochemistry (IH)

IH was performed on 3 μm-thick tissue sections of formalin-fixed, paraffin-embedded tissue placed onto glass slides. For immunohistochemical analysis the Bond III autostainer with Bond Polymer Refine (Red) Detection system was used. Tissues were deparaffinated in the Bond III autostainer by using Bond dewax solution at a temperature of 72˚C and dehydrated with alcohol 100%. Antigen retrieval was performed by either boiling slides in the Bond III autostainer with citrate pH 6.0 (C3d) or Tris-EDTA pH 9.0 (LCA) solution for 20 minutes at 100˚C. Sections were incubated with a mouse anti-human LCA (1:300, LEICA) for 25 minutes or rabbit anti-human C3d (1:1000, DAKO) antibody for 15 minutes. To block endogenous peroxidases a blocking solution was used. As a secondary

antibody for LCA the Bond post primary rabbit anti-mouse solution was used and for C3d the Bond polymer Poly-AP anti-rabbit IgG solution was used. Staining was visualized using 3,3'-diaminobenzidine (0.1 mg/ml, 0.02% H2O2) (LCA) and Mixed Red Refine solution (C3d). Sections were then counterstained with haematoxylin, dehydrated and covered.

## Classification

Slides were analyzed using the Dallas criteria, namely clusters (≥1) of lymphocytes surrounding and/or adherent to cardiomyocytes with or without myocytolysis (≥1 cardiomyocytes), on H&E slides only, in the absence of microscopic ischemic changes in this area. Slides were then stained for LCA and C3d (Figure 2) and were scored for:

1. Clusters (>1) of LCA-positive lymphocytes surrounding and/or adherent to cardiomyocytes.

2. Complement (C3d)-objectified myocytolysis (>1 cardiomyocytes positive for C3d).

As macrophages can also stain with LCA staining, we only scored LCA-positive cells that morphologically recognized as lymphocytes.
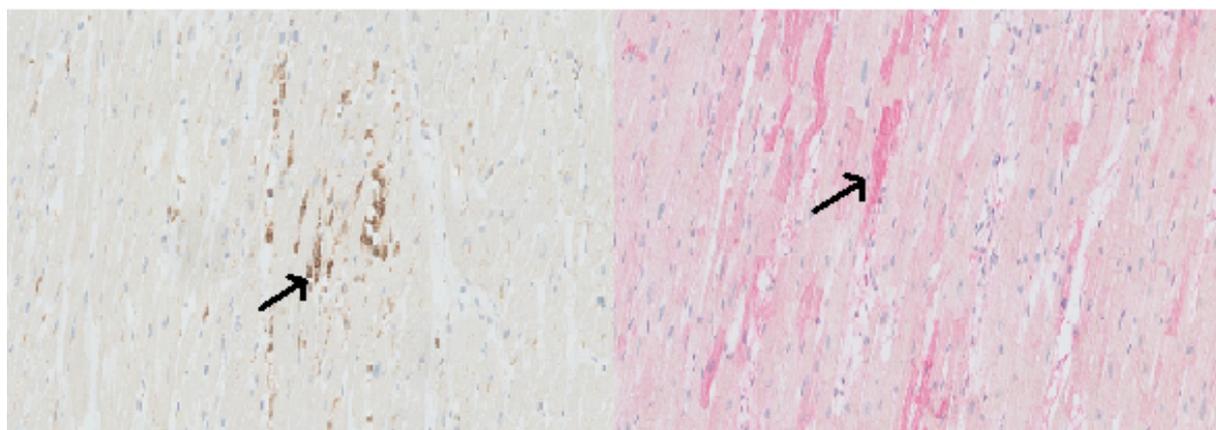


**Figure 2:** Immunohistochemical staining of heart slides: Clusters of LCA-positive lymphocytes (arrow) or epitopes surrounding cardiomyocytes and Complement (C3d)-objectified myocytolysis (arrow).

If both variables were present the case was classified as LM. When myocytolysis could not be objectified, the case was classified as B-LM. The slides were evaluated by one pathologist (JF). Equivocal cases were revised by a second pathologist (FRWVDG). In all cases a consensus was reached. In all myocarditis cases there was no direct relation with an ischemic event.

## Results

The cohort of 107 autopsies consisted of 66 males (61.7%) and 41 females (38.3%). Mean age was 71 years (range: 35-92 years). Using H&E staining and according to the Dallas criteria, LM was seen in 2 cases (1.9%). No positive cases were seen with B-LM. Using the Dallas criteria in combination with LCA /C3d immuno staining, resulted in 4 cases with LM (3.7%) and 4 additional cases with B-LM (3.7%). Male-female ratio in the 8 myocarditis cases was 1.7:1, and means age was 63 years (range: 42-78 years). In none of these cases LM or B-LM was considered clinically.

Other cardiac findings in the reviewed heart slides were old myocardial infarction (23.4%/ 25 cases), acute myocardial infarction (21.5%/ 23 cases), catecholamine-induced myocarditis (11.2%/ 12 cases), acute epicarditis (1.9%/ 2 cases) and cardiac amyloidosis (1.9%/ 2 cases).

In Table 1 other major pathological findings in the 8 cases of (B-)LM are depicted, showing that LM existed next to other major pathology. In one patient, with acute myocardial infarction caused by a ruptured coronary atherosclerotic plaque, B-LM was diagnosed in a myocardial section remote form the infarcted area.

| Patient | Sex | Age | Borderline LM/ LM | Major pathological findings |
|---|---|---|---|---|
| 1 | Male | 67 | Borderline LM | Necrotizing pancreatitis |
| 2 | Male | 76 | Borderline LM | Metastasized colon carcinoma |
| 3 | Female | 60 | LM | Fatty embolism |
| 4 | Male | 78 | Borderline LM | Pulmonary fibrosis, pneumonia |
| 5 | Male | 45 | Borderline LM | Myocardial infarction |
| 6 | Male | 54 | LM | Cerebrovascular accident |
| 7 | Female | 42 | LM | Pulmonary embolism |
| 8 | Female | 78 | LM | Metastasized pancreatic carcinoma |

**Table 1:** Other major pathological findings in the eight cases of (B-)LM.

## Discussion

We have studied the incidence of (B-) LM in a cohort of adult autopsies combining the Dallas criteria with immunohistochemical stainings for LCA (lymphocytes) and C3d (complement identifying myocytolysis), and diagnosed 8 cases (7.5%) of (B-)LM in immunohistochemical stained slides compared with only 2 cases

(1.9%) of LM and no B-LM when Dallas criteria were applied on H&E stained myocardial slides only.

Our finding of 1.9% LM in H&E slides only, based on the Dallas criteria, is comparable with the incidences of LM found in previous studies also analyzing unselected patient groups, namely ranging from 0.2 to 1% [10,11]. In two other studies that were reported in the literature, selected patient groups with sudden unexpected death in which LM was also diagnosed based on the Dallas criteria, the incidence of LM was much higher, namely in respectively 38% and 8.3% of the autopsied patients, as to be expected [12,13].

Our study shows that additional immunohistochemical staining with specific markers for inflammatory cells (LCA) and myocardial damage (C3d) can improve the diagnosis of LM in autopsy hearts, in a similar way as has been reported for EMB biopsies of living patients [23]. In a series of 20 patients with sudden infant death syndrome (SIDS), Dettmeyer et al. [16] reported only 1 case of LM when Dallas criteria were applied on H&E stained sections. In this study, LM was defined as > 10 LCA-positive cells/HPF and > 2 CD3-positive lymphocytes/HPF. Recently Nielsen et al. [20] suggested a cut-off value in the range of 11 to 16 CD3-positive lymphocytes/mm$^2$ in autopsy hearts for diagnosing LM, albeit this yielded a false negative percentage of almost 30%. Although in autopsy hearts, which allow to study full thickness sections of both left and right ventricular wall at several locations, there is clearly lower risk of sampling error compared with EMB [3-5], it has to be noticed that a quantification scoring system based on immunohistochemical stainings only still neglects the patchy character of the disease, thus theoretically can underdiagnose LM also in autopsy hearts, as suggested by Grasmeyer [24].

For this we combined LCA and C3d stainings with the Dallas criteria. We used C3d staining to visualize myocytolysis, as cell death of individual cardiomyocytes is not always discernable in H&E slides and can therefore be underscored [25]. We then detected (B-)LM in 7.5% of the cases in our unselected autopsy population. This is a much higher percentage compared to Feeley et al. [17] who described a relatively low incidence of LM in an unselected autopsy population, namely 0.6%. However, they stained heart slides with CD45ro, identifying activated or memory T-lymphocytes only [19], while we stained slides with LCA (i.e. CD45), identifying B- and T-lymphocytes [14]. Furthermore, while Feeley et al. scored the infiltrate based on the IFSC criteria (corresponding to the ESC criteria) for EMB, we applied the Dallas criteria, what could also explain the differences between Feeley's study and our results, as explained above.

We found (B-)LM in our group of clinical adult autopsies next to other major pathology. The diagnosis of (B-)LM is important in both clinical and forensic autopsies. Especially in forensic cases the pathologist wants to exclude a natural cause of death in cases without a clear cause of death. (B-)LM can be a cause of death as a result of vascular spasms and arrhythmias.

In conclusion, we propose a new method for diagnosing (B-)LM in autopsy heart slides, based on application of immunohistochemical stainings to identify B- and T-lymphocytes (LCA) and to visualize myocytolysis (C3d) which are evaluated by histomorphological parameters of the Dallas criteria.

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