



Quantitative Morphological Analysis of Infectious Endocarditis by the SAMBA System

Authors

Mabika Barthelemy¹, Ngoua Meye Misso Rick-Léonid², Lepidi Hubert³, Didier Raoult³

¹Laboratory of Anatomical Pathology, University of Health Sciences, Libreville, Gabon

²Laboratory of Biochemistry Research, University of Sciences and Technology of Masuku, Franceville, Gabon

³Unité Des Rickettsies, CNRS UMR 6020, Faculte de Medecine, Universite de la Mediterranee

*Corresponding Author

MABIKA Barthélemy

Laboratory of Anatomical Pathology, University of Health Sciences, Libreville, Gabon

Email: barthelemy.mabika@yahoo.com

Abstract

The aim of this work was to determine the criteria for histologically differentiating infectious endocarditis from degenerative valve processes on native valves, bioprostheses and mechanical valves using the SAMBA technique.

The Cardiac Surgery Service of the Center hospital-universities of Timone provided 630 valves. Histological sections stained with hematoxylin eosin saffron (HES) were reviewed. The histological lesions analyzed on slides stained with HES were vegetation, fibrosis and calcifications. Those analyzed after immunostaining were the cells of the inflammatory response (neutrophils labeled with the CD15 antibody, the CD68 macrophages and the CD3 lymphocytes). For endothelial cells, Factor VIII antibody was used. White slides from each case were used to practice immunohistochemistry techniques to mark the different cells of the inflammatory response and the endothelial cells of the neovascular valve.

Of the 630 valve devices examined, 350 were native valves, 200 were bio-prostheses and 80 were mechanical prostheses. Quantitative analysis with the SAMBA technique has shown that infectious endocarditis always has vegetation associated with a multicellular inflammatory infiltrate. In addition, the composition of this inflammatory infiltrate was practically superimposable on the three types of valves. In contrast, the inflammatory response accompanying valvular pathologies other than infectious endocarditis (aortic valve stenosis, chronic rheumatic valvulopathy), was almost composed of mononuclear cell elements. The vegetation was absent. While the phenomena of repair accompanied the two varieties of inflammatory infiltrates.

These results open up new perspectives in the management of patients with infectious endocarditis with negative blood cultures, where the pathologist can, by the technique of quantitative analysis of the valvular histological lesions, minimize the risk of diagnosing an infectious endocarditis because of the evidence of an inflammatory infiltrate.

Keywords: SAMBA Quantitative Analysis, Valvular Devices, Inflammation and Infectious Endocarditis.

Introduction

Infectious endocarditis is currently one of the most serious and rapidly life threatening diseases in the absence of appropriate antibiotic therapy for the causative pathogen. It is defined by the invasion of the heart valves by bacterial or fungal microorganisms^[1]. Infectious endocarditis can pose diagnostic problems and in about 10% of cases it is endocarditis with negative blood cultures, that is to say without microbial identification^[2]. The histological study of cardiac valves represents an excellent diagnostic approach to infectious endocarditis^[3]. The definition of an infective endocarditis, which is the infection of the endocardium, implies for the pathologist the *in situ* visualization of microorganisms within the valvular tissue through the use of histochemical staining or immunohistochemical revelation^[4,5]. This last condition is not always fulfilled and histologically two major lesions can guide the diagnosis or strongly suspect it: it is the presence of valvular inflammation and valvular vegetation (inflammatory thrombus). Other valvular lesions contributing to the diagnosis of infective endocarditis are foci of necrosis, fibrosis, calcification and neovascularization^[3].

One of the main histological difficulties of the pathologist is to differentiate the inflammatory infiltrates accompanying an infectious process from those, which are of a reactive nature. These are very frequently found in many lesional processes: degenerative valve pathology, prosthetic valve material. Degenerative valve pathologies are by far the most common causes of valve dysfunction. They are characterized histologically by a fibrous and often calcified degeneration of the heart valves. It is not uncommon for these degenerative lesions to be accompanied by inflammatory changes that should not be confused with an infectious process.

In order to obtain precise histological criteria necessary to diagnose infective endocarditis during an anatomopathological examination of the heart valves, we performed a quantitative morphological analysis of several histological parameters on a large number of cardiac valves. The purpose of this

work is to determine criteria for histologically differentiating infectious endocarditis from degenerative valvular processes. For this study, a computerized image analysis system called the automatic scanning microphotometric analysis system (SAMBA) was used.

Material and Methods

Biological material

630 heart valves from La Timone's cardiac surgery department were studied.

Treatment of valve devices

As soon as the valve was surgically removed, specimens were taken from the Rickettsia Unit of the Faculty of Medicine. The remainder of the specimen was fixed in 10% formaldehyde and then embedded in paraffin. Serial sections 5 µm thick were made from the paraffin blocks and then stained with hematoxylin eosin saffron (HES) and white slides reserved for other special stains.

Macroscopic study

The management was different depending on whether it was a native valve, a bio-prosthesis or a mechanical prosthesis. For the native valves, the size of the piece has been specified, its various components such as valvular tissue, ropes, pillars and papillary muscles. Subsequently, samples were taken along the long axis of the cusps and according to the identified lesions.

For bio-protheses, the same procedure as for the native valves was carried out with in addition a careful examination for the peri-annular area.

For the mechanical valves, only the annular ring area was sampled in search of foci of infection. When calcification foci existed, a decalcification step was performed to facilitate microtome tissue cutting.

Histological study

The diagnosis of infectious endocarditis begins with the identification of identified tissue alterations on tissue section stained with HES (Table 1).

Table 1: Diagnosis of Infective Endocarditis

Major criteria	Minor criteria
Vegetation	Neovascularization
Active endocarditis: cellular polymorphism of the inflammatory infiltrate	Mononuclear inflammatory infiltrate (lymphocytes or macrophages)
	Necrosis
Demonstration of micro-organisms	Fibrosis
	Calcification

Diagnosis of infectious endocarditis in the native valves

Infectious endocarditis was diagnosed by two main alterations including vegetation and inflammation.

a. Etiology of infectious endocarditis

Etiology of infectious endocarditis was determined by the size and appearance of the vegetation. Infectious endocarditis of fungal etiologies has a large vegetation compared to those produced by bacterial etiologies^[6]. Q fever has been identified by very small or absent vegetation^[1].

b. Evolution of the infectious process

The evolution of the infectious process was determined by the presence of different types of inflammatory cells (neutrophils, macrophages and lymphocytes). Indeed, the presence of neutrophils corresponds to an active lesion whereas their absence associated with the identification of macrophages or lymphocytes on a fibrous background orients towards cicatricial lesion. The characterization of the germs was achieved through the use of special histochemical stains. Acute infectious endocarditis has been associated with high virulence germs such as *Staphylococcus aureus*. Valvular destruction is rapid and important, associated with many polynuclear and few fibroblastic elements. The vegetation is often large and friable. Subacute infectious endocarditis has been associated with less virulent germs such as hemolytic α streptococci, which causes slowly progressive lesions. Mononuclear cells associated with fibrosis of variable importance dominate the inflammatory elements.

Beside these two main lesions, others occurring in a variable way can be found. These are tissue necrosis, neovascularization, fibrosis and calcification. The normal valve is avascular. Neovascularization

occurs during the inflammatory process. Fibrosis and calcification may be responsible for the diffuse thickening of the valve and its retraction.

Diagnosis of infectious endocarditis in valve prostheses

The diagnosis of infective endocarditis in valve prostheses required a different approach depending on whether it was a bio-prosthesis or a mechanical prosthesis.

For mechanical prostheses, the diagnosis of infectious endocarditis was made by the inflammation of the peri-prosthetic tissue more precisely to the insertion areas of the device with the possibility of development of ring abscess. The infection did not destroy the mechanical valves but could cause their dysfunction and even their disinsertion.

For bioprostheses, the diagnosis of infectious endocarditis was made by the presence of organized thrombus, fibrin, calcification or perforation, by the presence of the infectious process at the sutures in the annular zone, the presence of large vegetations were and foreign body granulomas frequent especially at the insertion areas of the prosthesis were independent of the infectious process of the endocardium.

Quantitative analysis

Quantitative analysis was performed using a SAMAP (automatic scanning microphotometric analysis system) according to Lepidi et al^[2]. The SAMBA technique includes an optical microscope connected to a camera that transmits digitized histological images to a computer thanks to the different image analysis systems. Adapted interactive programs allow, under visual control, the analysis of the different histological sections. For each series of observations, the area of the parameter studied is measured on ten fields taken at random or on the totality of the tissue section at a given magnification. Quantification is defined as the ratio of the average of the areas studied to the total tissue area.

The histological parameters studied with HES staining were fibrosis, calcification and vegetations. With immunohistochemistry techniques, the studied parameters are: neo-vascularization and inflammatory response.

For the evaluation of the neovascularization we apply on deparaffinized tissue sections a monoclonal anti-factor VIII antibody (Dako, diluted to 1/200).

To analyze the inflammatory response, we quantify the different cellular components. For macrophages we used CD68 Dako monoclonal antibody at a concentration of 1:50. For T cells the polyclonal CD3 antibody at 1/25. Finally, for polynuclear CD15 Dako monoclonal antibody (kit ready for use). With these last two cell types a step of antigenic restoration by heat was used (microwave waves, 20 minutes at 750W in a citrate buffer at pH 6).

For neovascularization and inflammatory response, the revelation was carried out using the Dako LSAD kit, including a peroxidase neutralization step (3% oxygenated water for 10 min) and endogenous biotin (Dako biotin blocking system). The chromogen used was diaminobenzidine with counterstaining with Mayer's hematoxylin for 3 min.

Etiological diagnosis of infectious endocarditis

Histochemical staining

The etiological diagnosis of infectious endocarditis is rarely performed using HES staining. In case of inflammatory infiltrate a Gram stain was systematically performed. The Gram method is based on the ability of certain bacteria to retain a crystal-iodine violet complex when subjected to basic differentiation and counterstaining. Gram-positive bacteria appear blue and Gram-negative bacteria appear red. However, if the wall of the bacterium is damaged, this coloring will be of no help. In the case of negativity, other complementary stains were performed: Schiff periodic acid staining (SPA) and Gomori-Grocott silver staining for a fungal agent.

Stainings from Warthin Starry and Giemsa helped identify Bartonella and Rickettsia bacteria. For chlamydia, the use of Machiavello staining was

more appropriate. While Gimenez staining was used to identify *C.burnetii*, *bartonella* and *legionellasp*. The rarely used Ziehl-Neelson stain was used to detect acid-fast bacilli, especially mycobacteria. But these are rarely involved in infectious endocarditis. All histochemical stains usually employed are shown in Table 2.

Immunohistochemical methods

With immunohistochemistry techniques, the parameters studied were neovascularization and inflammatory response.

For evaluation of neovascularization, a monoclonal anti-factor VIII antibody (Dako diluted 1/200) was applied to deparaffinized tissue sections.

To analyze the inflammatory response, different cellular components were quantified: For macrophages, the CD68 Dako monoclonal antibody at the concentration of 1/50 was used. For T cells, polyclonal CD3 antibody at 1: 25 was used and for polynuclear CD15 Dako monoclonal antibody (kit ready for use). With these last two cell types, a step of antigenic restoration by heat was used (microwaves, 20 min at 750W in a citrate buffer at pH 6).

For neovascularization and inflammatory response, the revelation was carried out using the Dako LSAD kit, including a peroxidase neutralization step (3% oxygenated water for 10 min) and endogenous biotin (Dako biotin blocking system). The chromogen used was diaminobenzidine with counterstaining with Mayer's hematoxylin for 3 min. The histopathological account of a heart valve operated for an infectious endocarditis included the response items recorded in Table 2

Table 2: Elements of a histological report of heart valve

Elements	Heart valve
Operated valve seat	Aortic valve
	Mitral valve
	Tricuspid valve
Valve type operated	Native valve
	Bio prosthesis
	Mechanical prosthesis
Histological lesions	Vegetation
	Fibrosis
	Calcification
	Inflammation

Special histochemical stains	Giemsa
	Orcein
	Gram
	SPA
	Grocott
	Warthin Starry
	Other
Quantitative analysis of inflammatory response and neovascularization	Neutrophils (CD 15)
	T Lymphocyte (CD3)
	Macrophages (CD68)
	Endothelial cells (Factor VIII)
Microorganisms identified	Cocci
	Bacillus
	Other
Conclusion and comments: Code	

Statistical analysis

The Mann and Whitney U test were used to perform a comparison of inflammatory infiltrates accompanying infectious endocarditis and other valvular pathologies, respectively. With this test, the difference is significant when $P < 0.005$.

Results

Of the 630 valve devices analyzed, 350 were of native valves, 200 of bio prostheses and 80 of mechanical prostheses. Their seat was exclusively aortic and mitral.

For all types of valve devices we analyzed, a code was assigned to each conclusion of the histological report. Table 3 summarizes the different conclusions reached where each corresponds to an encrypted code.

Table 3: Different conclusions asked where each corresponds to an encrypted code.

Type of anatomic-pathological finding	Code
Certain infectious endocarditis with visible germ or positive immunohistochemistry	1
Certain infectious endocarditis without visible germ or negative immunohistochemistry	2
Infectious endocarditis possible	3
Degenerative changes with reactive inflammatory infiltrates	4
Non-inflammatory degenerative changes	5
Marathon endocarditis or non-bacterial thrombotic endocarditis	6
Other (myxoid degeneration)	7

The type of valve examined, the various histological parameters analyzed, the code, the valve seat, the mean age of the patients and the sex ratio are shown

in Table 4. We identified for the native valves, the bioprostheses and the mechanical valves three subsets corresponding to degenerative, inflammatory lesions without identified microorganisms and infectious endocarditis with evidence of the pathogen.

The histological parameters studied during the morphological analysis with the SAMBA technique are: fibrosis, calcifications and vegetations on the one hand, whose analysis is done on slides stained with hematoxylin eosin saffron. The analysis of the inflammatory response and neo-vascularization is done on slides treated with immunolabeling techniques. We used anti mouse monoclonal antibodies directed respectively against macrophages (CD68), neutrophils (CD15), lymphocytes (CD3) and endothelial cells (Factor VIII) for the inflammatory response, respectively.

For the distribution of the average age of patients according to the type of valve device analyzed, for the native prostheses, the average age of the patients was 60.51 ± 14.13 years, the extremes varying from 52 to 78 years and a sex ratio H / F of 0.7.

For the bioprostheses these values were 60.63 ± 11.63 years, the extremes of 59 to 75 years and the ratio M / F of 0.8. For mechanical valves, the mean age was 62.89 ± 10.12 years, the extremes of 78 and 60 years with a sex ratio of H / F of 1.2.

Table 4: Results of quantitative morphological analysis of valve devices

	Native valves(N=350)			Bioprosthesis (N=200)			Mechanical Prosthesis(N=80)			
	NoInflam.	Inflam	Infect.Endoc	NoInflam.	Inflam	Infect.Endoc.	NoInflam.	Inflam	Infect.Endoc	
N	224 (64%)	52 (14.86%)	74 (21.14%)	102 (51%)	83 (41.50%)	15 (7.50%)	49 (61.25%)	22 (27.50%)	9 (11.25%)	
Fibr	65.13±8.61	59.44±5.36	13.29±2.27	0	0	0	0	0	0	
Calci	13.20±4.92	9.67±3.56	1.85±9.94	17.54±6.11	16.89±7.43	8.48±4.34	3.85±3.01	1.06±2.38	1.39±2.63	
Vegt	0	0	30.93±4.87	0	0	22.07±5.15	0	0	0	
CD68	0	0.82±0.44	5.72±1.58	0	2.82±0.80	4.42±2.14	0	1.68±1.03	3.70±2.33	
CD15	0	0.26±0.17	8.85±2.66	0	0.12±0.08	5.26±2.49	0	0.15±0.06	7.78±3.06	
CD3	0	1.53±0.91	3.98±1.71	0	0.51±0.19	2.62±1.85	0	1.89±0.78	3.39±2.14	
FVIII	0	0.42±0.29	1.34±0.39	0	0	0	0	0.49±0.18	1.82±0.52	
Code	5=90.54 7=9.46	3=86.79 4=13.21	1=82.67 2=17.33	5=100	3=4.81 4=95.18	1=73.33 2=26.67	5=100	3=223.73 4=77.27	1=22.22 2=77.77	
Valv	A	45.50	75.47	58.67	44.12	30.12	26.66	46.94	63.64	66.66
	M	54.50	24.53	41.33	55.88	69.88	73.33	53.06	36.36	33.33
Age	66.27±11.09	61.12±14.79	54.14±16.57	61.05±12.89	62.04±12.44	58.8±9.55	59.82±14.3	69.12±9.23	59.75±6.83	
Sexe	M	53.60	66.04	68	49.02	54.22	33.33	48.98	40.91	77.77
	F	46.40	33.96	32	50.98	45.78	66.66	51.02	59.09	22.22

N=nombre, Fibr= fibrosis, Calci= calcifications, Vegt= vegetations, Valv A, M= Aortic, Mitral valve, No Inflam= No inflammatory, Inflam=inflammatory, Infect. Endoc= Infectious endocarditis

Discussion

During this work we proceeded to the morphological quantitative analysis of the histological parameters identified on routine staining (hematoxylin eosin saffron) or on slides treated with the immunolabeling techniques. This analysis system comprises various programs allowing the analysis of each of the parameters^[2,7]. The histological signs analyzed on HES stained section are: vegetation, fibrosis and calcifications. The parameters analyzed after immunostaining are the inflammatory response and the neovascularization. Vegetation is an almost constant sign of infectious endocarditis. It consists of a platelet fibrin aggregate infiltrated with inflammatory elements and sometimes microorganisms. In a recent work, Lepidi et al^[3] noted that during Q endocarditis the vegetation was small. For infectious endocarditis on mechanical valves, the absence of vegetation could be explained by the fact that the infectious agents do not attack the inert constituents of these prostheses. With respect to the inflammatory infiltrate, quantitative analysis allowed us to distinguish on the three types of valves an inflammatory infiltrate with endocarditis and an inflammatory infiltrate without endocarditis. The respective composition of these two types of infiltrates was almost superimposable regardless of the type of valve being examined. With the Mann and Whitney U test,

the comparison of these two types of inflammatory infiltrates showed a significant difference ($P<0.005$). Neovascularization is a histological sign that accompanies any valvular inflammatory process^[8]. It is not specific for infectious endocarditis. Although it is sometimes absent on mechanical prostheses and bioprostheses, this is due to their intrinsic constitution. Infectious involvement is possible on the collar of the fibrous ring and on the suture son.

The diagnosis of infectious endocarditis is made when isolating and characterizing the infectious agent from the blood culture. This is therefore an activity that is primarily the responsibility of microbiology laboratories. However, this diagnostic activity can be tricky when infectious endocarditis is associated with negative blood cultures. It is precisely during this type of situation, currently representing 10% of cases in the daily exercise^[9] where the input of the pathologist can be fundamental. In fact, the macroscopic and histological study makes it possible to identify a certain number of signs confirming the infectious reality of the disease. The pathological changes may be ulcerations, vegetations, fibrosis, calcifications, inflammatory infiltrate or neovascularization. The pathologist through the use of special histochemical stains can identify a microorganism located in a tiny territory of valvular tissue. The same is true of the use of immunolabeling techniques, which involves a

monoclonal antibody directed against a specific germ to characterize it.

Among the histological signs encountered with infectious endocarditis is inflammatory infiltrate, which from time to time leads to misdiagnosis^[3]. This is why the quantitative analysis of this histological parameter should be generalized because, as we saw in the results of our work, the inflammatory infiltrate that accompanied the infective endocarditis in each type of valvular device presented a composition stackable.

Conclusions

The results provided by this technique are objective and reproducible, which could make it possible to carry out multicenter studies and to be able to make comparisons between different working groups.

Conflict of Interest

The authors declare that there are no competing interests. All the authors read and approved the final version.

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