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Morphometrical quantification of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* in human atherosclerotic abdominal aortic aneurysms

Quantificação morfométrica de Chlamydia pneumoniae *e* Mycoplasma pneumoniae *em aneurismas de aorta abdominal humana*

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Abstract

Objective: **Atherosclerotic inflammation with a possible role of infectious agents can contribute to the pathogenesis of abdominal aortic aneurysms (AAA). The finding of *Chlamydia pneumoniae* (CP) in these lesions in previous non-quantifying studies ranged from 0-100%. The objective is to quantify the presence of CP and *Mycoplasma pneumoniae* (MP) in AAA.**

Methods: **The thickness, and the number of cells positive for CP detected by the immunohistochemistry (immunoperoxidase, which is a type of immunohistochemical stain used in molecular biology, medical research, and clinical diagnostics), and the percentage of the area occupied by the *Mycoplasma pneumoniae* detected by *in situ* hybridization in three layers of the aorta were measured using an image-analysis system in 10 necropsies of abdominal aneurysmatic aortas. Three groups were used as controls: 1) samples of the same aortas, outside the aneurysms, except if the dilatation took the whole sub-renal portion of the artery (n=7); 2) aortas with severe atherosclerosis but without aneurysms (n=10); 3) aortas without or with mild atherosclerosis (n=10). All specimens were obtained at necropsies. Wald's test was used to compare groups; significance level was established at 5%.**

Results: **The tunica intima was thinner and the tunica media was thicker in the normal cases than in the other groups ($p<0.01$). Positive cells for CP were found in all groups, more frequently at the adventitia; no significant difference was detected between the groups ($p>0.05$). MP was also detected in all groups. This agent predominated in the group of patients with atherosclerosis, but without aneurysms at both tunica intima and adventitia; nevertheless, there were no significant differences between the groups ($p>0.05$).**

Conclusions: Our data suggested that the bacteria we focused to, does not play an important role in the pathogenesis of AAA.

Descriptors: **Aorta, pathology. Aortic aneurysm, abdominal. Chlamydophila pneumoniae. Mycoplasma pneumoniae. Atherosclerosis. Autopsy.**

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Resumo

Objetivo: **A inflamação aterosclerótica, com possível papel de agentes infecciosos, pode contribuir na patogênese dos aneurismas da aorta abdominal (AAA). O achado de *Chamydia pneumoniae* (CP) nessas lesões, em estudos prévios, sem quantificação, variou de 0-100%. O objetivo é quantificar a presença de CP e de *Mycoplasma pneumoniae* (MP) nos AAA.**

Método: **A espessura, o número de células positivas para CP detectadas por imunoperoxidase e a porcentagem de área ocupada por MP detectada por hibridização "*in situ*", nas três camadas da aorta, foram medidos com sistema de análise de imagens, em 10 aortas abdominais aneurismáticas. Usou-se três grupos-controle: 1) amostras das mesmas aortas, fora do aneurisma, exceto se a dilatação tomasse toda a porção sub-renal da artéria (n=7); 2) aortas com aterosclerose grave, mas sem aneurismas (n=10); 3) aortas sem aterosclerose ou com grau leve da doença (n=10). Todos os espécimes foram obtidos em necropsias. Usou-se o teste de Wald para comparar os grupos; fixou-se o nível de significância em 5%.**

Resultados: **A íntima era mais fina e a média mais espessa nos casos normais que nos outros grupos (p<0,01). Células positivas para CP foram encontradas em todos os grupos, mais freqüentemente na adventícia, porém sem diferença significativa entre eles (p>0,05). Também se detectou MP em todos os grupos. Este agente predominou no grupo de pacientes com aterosclerose, mas sem aneurisma na íntima e na adventícia; entretanto, as diferenças entre os grupos não foram significativas (p>0,05).**

Conclusões: **Nossos dados sugerem que os agentes enfocados não têm papel importante na patogênese dos AAA.**

Descritores: **Aorta, patologia. Aneurisma da aorta abdominal. Chlamydophila pneumoniae. Mycoplasma pneumoniae. Aterosclerose. Autopsia.**

INTRODUCTION

In the past few decades, increasing interest has also grown around the potential role of inflammation in atherosclerosis. The inflammatory process can modulate the rupture of the plaque (leading to thrombosis) and remodeling [1]. In the abdominal aortic aneurysm, commonly caused by atherosclerosis, the disruption of the extracellular matrix components, especially the elastic tissue, is considered an extremely important mechanism that influences the loss of both normal wall stiffness and dilation. This disruption can be induced by the proteases within the matrix including the elastase [2]; inflammatory cells are the main source of such enzymes. The individual variation in the inflammatory response could be an explanation to the fact that despite atherosclerosis being a common disease and aneurysms a relatively uncommon complication, only cases of atherosclerosis with prominent inflammatory infiltrate would present this pattern of arterial remodeling.

Several studies are trying to address the inflammation causal factors due to the importance given to it, essentially focusing on the research of infectious agents believed to be involved in the atherosclerosis process. Among others [3], the *Chlamydia pneumoniae* attracted the attention of many authors who believed in the role of the infectious agents in this disease, including the coronary arteries. Regarding the arteries, previous studies carried out in our laboratory have revealed not only the presence of this kind of bacterium, but also the *Mycoplasma pneumoniae* [4-7]. Such studies have found the *Mycoplasma pneumoniae* in almost all atheromas (atheromata), but the development of plaque instability is highly believed to be correlated with a large amount of both bacteria. It is well-known that the *Mycoplasma pneumoniae* co-infection by another bacterium enhances the virulence of both infectious agents [8, 9].

The fact that the *mycoplasma* requires cholesterol in order to survive because its membrane is built-up by this kind of fat, unique feature among the prokaryotes is according to the findings of these microorganisms in the atherosclerosis process. It has also been previously shown that the thrombosed atherosclerotic plaques are associated with a positive remodeling [10, 11]. The aortic aneurysms can be considered a positive segmental vascular remodeling, and they are followed by thrombosis in ulcerated fatty plaques. In other words, it is a process with similar characteristics to the vulnerable coronary lesions (injuries).

The association between *Chlamydia pneumoniae* and aortic abdominal aneurysms was investigated by several authors who have demonstrated variable degrees of correlation probably indicating the occasional causal role of this bacterium in such a disease [12-17]. However, morphological studies carried out either by electron microscopy or by immunohistochemistry or by *in situ* hybridization have only detected the agent without quantifying it. On the other hand, the presence of the *Mycoplasma pneumoniae* in these lesions (injuries) was les investigated. The data obtained through the polymerase chain reaction (PCR) technique do not indicate the association of this bacterium with the aortic disease [16, 18].

The aims of the present study are to verify whether these two agents are present in the abdominal aortic aneurysms using another approach for the diagnosis of *Mycoplasma pneumoniae*, and if its amount is largest in

aneurysms compared to the sites of atherosclerosis without such a complication.

METHODS

The present study was approved by the Research and Ethics Committee of the Heart Institute (InCor), University of São Paulo School of Medicine Clinics Hospital, São Paulo, Brazil.

Patients

A transverse section of 19 abdominal aorta atherosclerotic aneurysms located between the origins of the renal arteries and the aortic bifurcation randomly selected among the autopsies carried out at the Laboratory of Pathological Anatomy of the Heart Institute (InCor), University of São Paulo School of Medicine Clinics Hospital, São Paulo, Brazil were included in the study. These autopsies were always performed within a maximum 24-h period, in most cases, within 12 hours after the patients' death. These patients died due to either a ruptured aneurysm (two cases) or a heart ischemic disease. In one patient the heart ischemic disease was associated with rheumatic disease and infectious endocarditis. Just adult arteries were used. Cases were excluded if the abdominal aorta had any other pathological process, such as dissection.

The patients were divided into three control groups. The first one corresponding to the very same abdominal aortae of the cases abovementioned, excluding the aneurysm, once the dilation did not occupy the whole sub-renal portion of the artery (we named this group as "athero-aneu"). Samples of abdominal aortae from 10 patients with severe (acute) atherosclerosis, but without aneurysms, composed the second control group; dissection of the ascending aorta occurred in one patient, but the descending aorta (including the abdominal part) was not compromised by the disease. The third control group was composed by 10 samples of abdominal aortae which were either normal or has mild atherosclerosis (types I or II, according the American Heart Association).

Method

After formaldehyde fixation, all the fragments but the normal ones were decalcified in a saline solution 100d trisodium acid and 250 mL formic acid/L during 4 to 6 days. After that, the fragments were submitted to a routine histological processing and soaked in paraffin. A 3-µm thick sections were stained with hematoxylin and eosin by the Verhoeff method (without counterstain for collagen) to enhance the elastic fibers by limiting the vascular layers and for reactions of immunoperoxidases for *Chlamydia pneumoniae* detection. To display the *Micoplasma pneumoniae*, *in situ* hybridization reactions were performed in sections of 5 µm thick.

The plates stained by hematoxylin and eosin were used for general assessment of the lesions (injuries) and to choose both the section and the area around 1 cm in which the vessel was more preserved and could be better analyzed; the reactions for *C. pneumoniae* and *M. pneumoniae* were prepared in these regions.

Thickness determination of both tunica intima and media – These measures were performed in plates stained by the Verhoeff method using imaging analysis software (*Quantimet 500*) coupled to a microscope with a 10x objective. This thickness was measured in both the middle and margin of the sections; the measure considered for each vessel was the mean value of these three measures. In some cases, it was taken into consideration that here were atherosclerotic plaques that substantially shift this mean value, and they have not been included in the three measures aforementioned. In these cases, the thickness of this area was also computed as a fourth measure.

Quantification of the elastic fibers – In the same plates stained by the Verhoeff method, and with the same optic system utilized to determine the thickness of the layers, the percentage of the positive area for the elastic fibers by square micrometer was measured in both tunica media and adventitia.

Quantification of Chlamydia pneumoniae – The reactions of the immunoperoxidases were performed for this quantification. The monoclonal antibody for the prevailing protein in external membrane of *Chlamydia pneumoniae* (*Dako*, Denmark, clone RR-402) was used without dilution as a primary antibody with an approximately 16-hour incubation period ("*overnight*") at 4ºC, after antigenic recovery induced by heat in microwave (citrate buffer 0.01M/10 minutes), blockade of the endogenous peroxidase with H_2O_2 in a 3% TRIS buffer for a two-time period of 10 minutes each, and inhibition of the inespecific staining through incubation with a nonimmune fetal bovine serum diluted in a phosphate buffer solution (PBS) for one hour in a chamber at 37ºC. The secondary antibody was also provided by the DAKO Company, Denmark (E-354). Peroxidase-streptavidin (Amersham, United Kingdom) and diaminobenzidine conjugates (DAB, Dako, United States of America) were used to exposition and Harris hematoxylin for counterstaining.

The number of positive cells for *C. pneumoniae* by square micrometer was counted in each layer of the artery in a 1-cm length. The area was measured with same software of imaging analysis (*Quantimet 500*) coupled to a microscope with a 10x objective. Even though an optimization of the coincidence between the microscope imaging and the computer screen had been sought, it was impossible to avoid the brown color, corresponding to the

positivity, to be more effusive on the screen. Thus, the observer had to be very restrictive during the count. In any way, this difficulty was similar in all the cases; if there was any error, it was systematic and probably did not influence the comparison between both groups.

Quantification of the *Mycoplasma pneumoniae* – To perform the in situ hybridization reactions, the sections were heated in water-bath for 45 minutes, then, submitted to a peroxidase blockade similar to the one employed in the immunohistochemistry technique, soaked in a protein blockade without serum (Dako, Denmark) for 15 minutes, and then incubated with 20-µL biotin probe for *Mycoplasma pneumoniae* for each 80 µL solution with deionized formamid 50%, dextran sulfate, sodium citrate buffer 20x, Denhardt solution, salmon sperm deoxyribonucleic acid, ribonucleic acid of fungi transfer, poly-A and poly-C, and bidistilled water, at first at 95ºC for 6 minutes and then in humid chamber at 37ºC for about 16 hours ("*overnight*"). The signal was developed and amplified with the *GenPoint* system, and DAB was used to exposition (development) with counterstaining with Harry hematoxylin. Once the sections were prepared, they were analyzed with the same software aforementioned, and the percentage of the positive area for *Mycoplasma pneumoniae* was calculated also in each layer of each artery in a 1-cm length. The same previous considerations apply regarding the dissimilarities between the images observed on the microscope and in the screen.

Statistical analysis

As all the data involved both paired (matched) (areas inside and outside the aneurysms in the cases) and unpaired groups (the other two control groups), the outcome evaluation was planned to be performed using the Wald test. However, as the distribution of the values was abnormal, it was performed a scoring of the cases for each variable; the analysis was then performed considering the position on the scoring as a variable (the complacence to normality was positive) to be tested [19]. The level o significance was set at 5% and the SAS® system was used to perform statistic calculations.

RESULTS

Patients' age, gender and main disease and/or cause of death involved in the study are shown in Table 1. Examples of sections stained with Verhoeff method and submitted to reactions for *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are shown in Figures 1 to 3.

Table 1. Age, gender, and main disease and/or cause of the patients' death.

Groups	Age vrs	Gender	**Main disease**
Aneurysms	**59**	**M**	**Rheumatic disease, inffectious endocarditis**
e	**61**	**M**	**Ischemic heart disease, heart failure**
Athero/Aneu	**61**	**M**	**Ischemic heart disease (transplant)**
	62	M	Ruptured aneurysm
	70	M	Ischemic heart disease, cardiogenic shock
	72	F	Recent myocardium infarction
	72	**M**	**Ruptured aneurysm**
	77	**M**	**Ischemic heart disease, heart failure**
	78	**M**	**Recent myocardium infarction, cardiac tamponate**
	87	**M**	**Recent myocardium infarction, cardiogenic shock**
Atherosclerosis	41	F	Recent myocardium infarction, cardiogenic shock
	60	F	Recent myocardium infarction, cardiogenic shock
	60	F	Diseection of the descending aorta
	60	F	Endometrial cancer
	61	F	Recent myocardium infarction, cardiogenic shock
	63	F	Recent myocardium infarction, cardiogenic shock
	73	M	Chronic obstructive pulmonary disease (COPD)
	73	M	Intestinal infarction
	81	M	Degenerative valvar diease + arrhythmia
	82	F	Rheumatic disease
Normal	21	F	Chagas disease (transplant)
	29	M	Idiopatic dilated cardiomyopathy
	29	F	Rheumatic disease
	36	M	Pulmonary tuberculosis
	38	M	Pericarditis
	44	F	Breast cancer
	47	M	Rheumatic disease
	51	F	Ovarian cancero
	60	F	Rheumatic disease
	72	F	Degenerative valvar disease

M- male; F- female. The boldfaced cases are the ones in which the atherosclerotic samples of abdominal aorta could be obtained without aneurysms and composed the control group

As the statistical analysis was performed taking into consideration the order (scoring) of each measure, the tables show the average position of each group, besides the medians, averages, and standard deviations. In order to facilitate the visual recognition of the outcomes, the plots concerning to tunica media and intima thicknesses and the percentage of elastic tissue were made considering the averages.

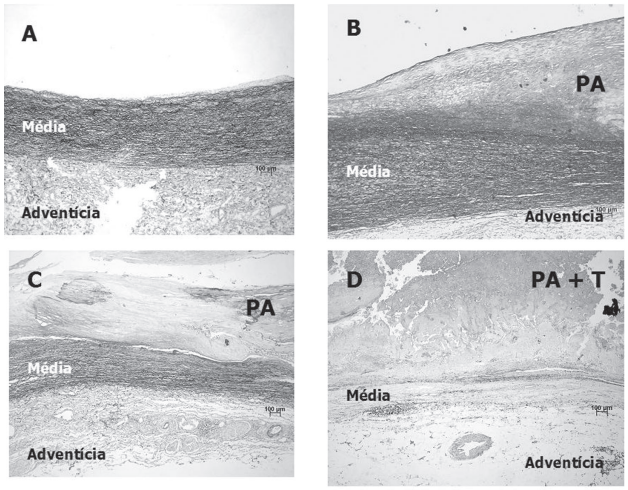

Fig 1 – Staining for the elastic system present in the abdominal aortae. A- Patient without significant atherosclerosis (normal); B- Patient with atherosclerosis but without aneurysms; C e D- Patient with aneurysm in the area outside (C) or inside it (D). AP – atherosclerotic plaque; T- thrombus. Staining by the Verhoeff method, magnified by 5x objective

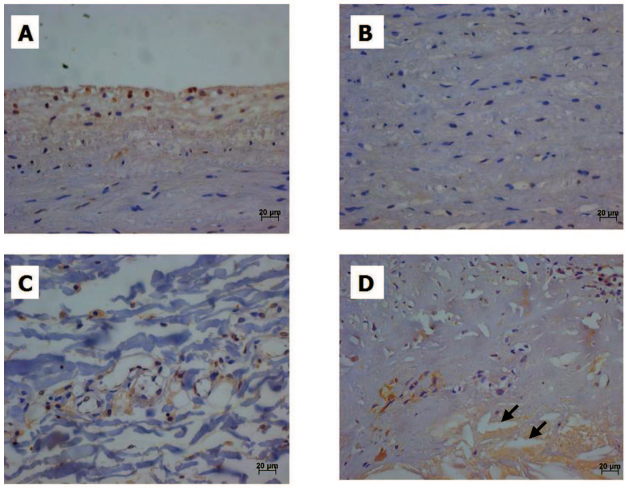

Fig. 2 – Immunohistochemistry for Chlamydia Pneumoniae in tunicae intima (A), media (B), and adventitia (C) of an aorta without significant atherosclerosis. D – The same reaction in atherosclerotic plaque inside an aneurysm. The positive cells are staining in brown. The arrows point to some of the cholesterol crystals located inside the plaques. Magnified 40x

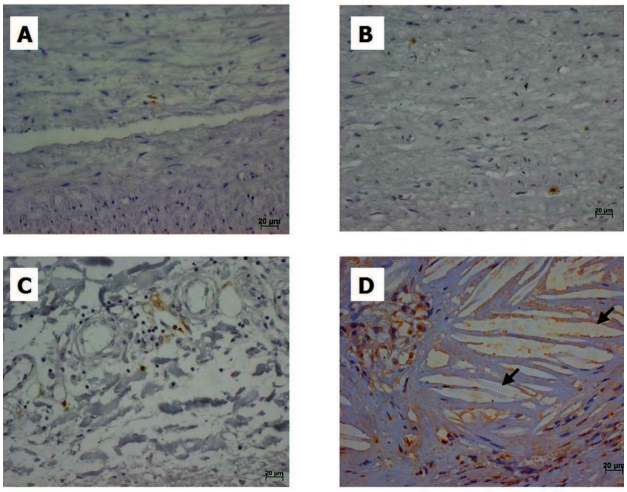

Fig. 3 – In situ hybridization for M. pneumoniae in tunicae intima (A), media (B), and adventitia (C) of an aorta without significant atherosclerosis. D – The same reaction in atherosclerotic plaque inside an aneurysm. The positive cells are staining in brown. The arrows point to some of the cholesterol crystals located inside the plaques. Magnified 40x

Table 2 and Figure 4 show the thickness of the tunica intima and media of each group. The aneurysmatic regions presented the thickest tunica intima and the thinnest normal aortae. The non-aneurysmatic atherosclerotic lesions (injuries), incases with and without aneurysms, occupied an intermediate position. However, it was a found a significant difference between the normal group and the other three groups (normal vs aneurysms; normal vs athero-aneu; and normal vs atherosclerosis, $p < 0.01$), but not in these latter ones among themselves (aneurysms vs athero-aneu, $p = 0.12$; aneurysms vs atherosclerosis, $p = 0.35$; and athero-aneu vs atherosclerosis, $p = 0.36$). In contrast, the normal aortae had the thickest tunica intima and the aneurysmatic the thinnest ones. Once more, the measure of the non-aneurysmatic atherosclerotic lesions (injuries) has been in-between the ones of the other groups. Regarding this layer, both atherosclerosis groups without aneurysm (athero-aneu and atherosclerosis) were similar to each other ($p = 0.89$). However, the regions with aneurysm were significantly thinner, and the normal arteries thicker than that of the other groups (all $p < 0.01$). Figure 5 shows the amount of elastic tissue in the tunicae media and adventitia. The areas with aneurysms had the lowest values compared to the same artery, without aneurysms($p = 0.01$), and the aortae of other patients with atherosaclerosis ($p < 0.01$) or without atherosclerosis ($p = 0.04$). On the other hand, it was found difference between athero-aneu and atherosclerosis ($p = 0.40$) suggesting that the disruption of the tissue is related to aneurysms rather than atherosclerosis.

Table 2. Average Scoring position, median, mean and standard deviation (SD), in millimeters of the tunicae intima and media thickness of the aorta within the four groups.

	Group	n	Average Scoring Position	Median (mm)	Mean (mm)	SD (mm)
Intima						
	Aneurysms	8	18.43	1.54	1.71	1.15
	Athero/Aneu	7	24.63	0.77	1.16	0.94
	Atherosclerosis	10	22.10	1.07	1.13	0.32
	Normal	9	5.33	0.15	0.19	0.10
Media						
	Aneurysms	10	20.14	0.15	0.17	0.14
	Athero/ Aneu	7	6.60	0.55	0.59	0.94
	Atherosclerosis	10	19.60	0.58	0.56	0.32
	Normal	10	30.00	0.89	0.87	0.10

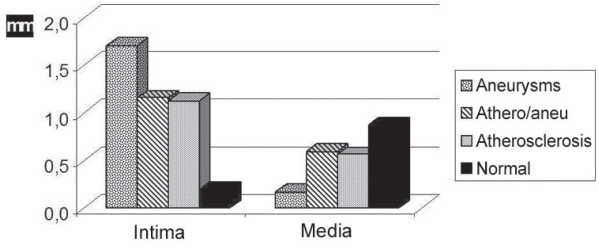

Fig. 4 – Mean thickness of the abdominal aortic layers in aneurysmatic areas (aneurysms) in patients with aneurysms – areas outside the lesion (athero/aneu); in patients with atherosclerosis, but without aneurysms (atherosclerosis); and in patients without significant atherosclerosis (normal)

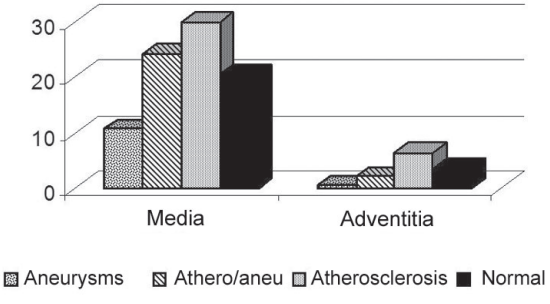

Fig. 5 – Elastic tissue average percentage in tunicae media and adventitia of the abdominal aorta – aneurysmatic areas (aneurysms), in patients with aneurysms in areas outside the lesion (athero/aneu); in patients with atherosclerosis, but without aneurysms (atherosclerosis); and in patients without significant atherosclerosis (normal)

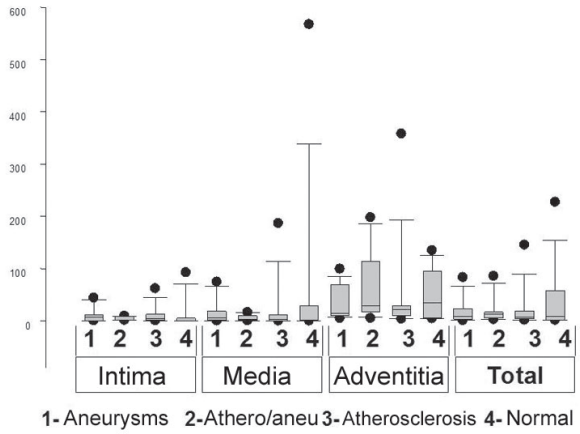

Fig. 6 – "Box-plot" showing the number of positive cells for Chlamydia Pneumoniae in the layers of tunicae media and adventitia, and in the whole vessel, in abdominal aorta in aneurysmatic areas (1 - aneurysms); in patients with aneurysms but in areas outside the lesion (2 - athero/aneu); in patients with atherosclerosis, but without aneurysms (3 - atherosclerosis); and inpatients without significant atherosclerosis (4 - normal)

The outcomes about the number of positive cells for *Chlamydia pneumoniae* are shown in Table 3 and Figure 6. In all groups, the finding of these bacteria was more frequent in the tunica adventitia; no significant difference was found among them (all groups, $p > 0.05$).

Table 4 and Figure 7 show the percentage of the area occupied by the *Mycoplasma pneumoniae* in each group. *Mycoplasma pneumoniae* was most prevalent in the group of patients with atherosclerosis but without aneurysm, i.e., either in the tunica intima or in the tunica adventitia, but the differences was not significant ($p > 0.05$).

Table 3. Average Scoring Position, median, mean and standard deviation (SD) for the number of positive cells for Chlamydia pneumoniae per square millimeter in each layer and in the total of the abdominal human aortae.

	p	Group	*n*	Average Scoring Position	Median	Mean	SD
Intima	0.27						
		Aneurysms	10	19.90	1.44	3.91	3.44
		Athero/ Aneu	7	20.43	2.31	9.17	13.85
		Atherosclerosis	10	22.30	3.51	10.39	16.18
		Normal	10	13.80	0.19	12.64	26.38
Media	0.83						
		Aneurysms	10	21.10	2.75	4.84	5.07
		Athero/ Aneu	7	18.71	4.46	15.35	22.22
		Atherosclerosis	10	18.20	2.51	21.38	48.93
		Normal	10	17.90	1.32	60.5	149.83
Adventitia	0.83						
		Aneurysms	10	16.80	24.79	54.2	62.62
		Athero/ Aneu	7	22.57	12.02	27.99	28.58
		Atherosclerosis	10	16.30	18.53	44.18	90.92
		Normal	10	21.40	29.75	43.86	40.35
Total	0.73						
		Aneurysms	10	18.80	11.04	17.75	24.43
		Atghero/Aneu	7	20.29	7.79	17.89	22.29
		Atherosclerosis	10	17.70	5.65	20.35	36.9
		Normal	10	19.60	7.63	37.99	59.29

Table 4. Average Scoring Position, median, mean, and standard deviation (SD) for the percentage of the area occupied by the *Mycoplasma pneumoniae* per square millimeter in each layer and in the total of the abdominal human aortae.

	p	Group	*n*	Average Scoring Position	Median	Mean	SD
Intima	0.53						
		Aneurysms	10	15.65	0.03	0.1	0.18
		Athero/ Aneu	7	17.86	0.03	0.14	0.19
		Atherosclerosis	10	23.65	0.09	1.34	2.72
		Normal	10	16.44	0.05	0.05	0.03
Media	0.76						
		Aneurysms	10	20.65	0.13	0.13	0.1
		Athero/ Aneu	7	18.29	0.03	0.5	1.02
		Atherosclerosis	10	19.9	0.03	1.38	3.91
		Normal	10	16.95	0.03	0.37	0.85
Adventitia	0.46						
		Aneurysms	10	16.25	0.1	0.1	0.1
		Athero/ Aneu	7	16.14	0.07	0.21	0.32
		Atherosclerosis	10	22.70	0.23	0.7	0.85
		Normal	10	20.05	0.11	0.53	1.32
Total	0.98						
		Aneurysms	10	17.80	0.08	0.12	0.15
		Atghero/Aneu	7	17.43	0.06	0.19	0.28
		Atherosclerosis	10	22.10	0.11	1.06	2.05
		Normal	10	18.20	0.07	0.42	1.01

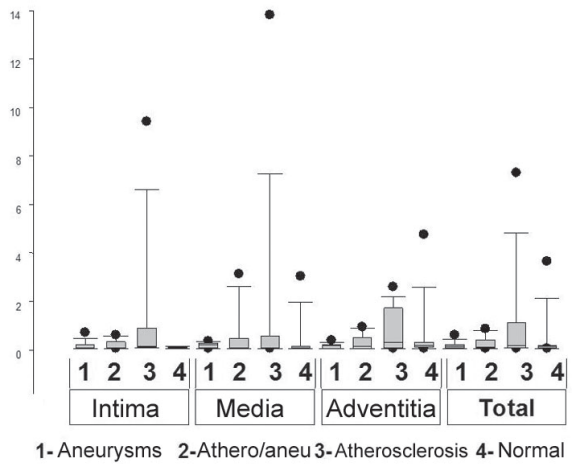

Fig. 7 – "box-plot" showing the percentage of the area occupied by the M. pneumoniae in the layers of tunicae intima, media, and adventitia, and in the whole vessel, in abdominal aorta in aneurysmatic areas (1 - aneurysms); in patients with aneurysms but in areas outside the lesion (2 - athero/aneu); in patients with atherosclerosis, but without aneurysms (3 - atherosclerosis); and inpatients without significant atherosclerosis (4 - normal)

DISCUSSION

Upon trying to get the most complete clinical picture as possible regarding the putative involvement of both *Chlamydia pneumoniae* and/or *Mycoplasma pneumoniae* in the pathogenesis of both atherosclerosisand aneurysms of abdominal aorta, we used three types of control: 1) the same abdominal aortae that constituted the study group, not considering the area affected by the aneurysm. This control group was included to reveal occasionally located changes which might explain why a specific area is affected by dilation, but not the rest of the vessel. This group had only 7 cases because 3 aneurysms affected all the abdominal aorta; 2) aortae with severe atherosclerosis without aneurysms. Because only a small percentage of the patients with severe atherosclerosis develop aneurysms, the aim of this comparison was to seek for factors that could distinguish these patients from the majority; 3) aortae without significant atherosclerosis were included as true controls, especially in order to verify the possible presence of bacteria focused in healthy people.

The present study did not aim at evaluating the patients' age and gender. As was expected, the ages of the cases with atherosclerosis (with or without aneurysms) were higher than that one of the normal group. A higher proportion of men among the cases of aneurysm could be expected according to the epidemiologic dada [20], even though in our study, we have had 90%, while in the literature the prevalence is not as high, ranging from 66% to 88% depending on the review [20]. Also we have a relatively high number of women with and without atherosclerosis in control groups.

The outcomes regarding the thickness of the aortic layers were in accordance with the expectation: normal aortae had thinner tunica intima and thicker tunica media, the opposite occurring with the aneurysms. Considering that the atherosclerosis is the built up of lipids and other substances in the tunica intima, it is easy to understand why the three groups of aortic segments with atherosclerosis had the greatest tunica intima. The differences between atherosclerotic lesions (injuries) with and without aneurysms were not significant. On the other hand, the tapering of the tunica media is a well-known secondary effect of the atherosclerosis. The disruption of the elastic tissue which is associated with the establishment of aneurysms turns this characteristic to be particularly profuse. These facts explain why the tunica media was thinner in the aneurysms, intermediate in atherosclerotic areas without them (regardless of the presence of the dilation of the artery) and thicker in the aortae without atherosclerosis.

Chlamydia pneumoniae and *Mycoplasma pneumoniae* were detected in all groups. Previous researches about *Mycoplasma pneumoniae* in abdominal aortic aneurysms using the polymerase chain reaction (PCR) method turned to be negative. Regarding the *Chlamydia pneumoniae*, the previous findings were controversial: the percentage of the positive cases ranged from 0% to 100% for PCR [21], in situ hybridization, or immunohistochemistry.

Such a variation between the data from different centers and the presence of bacteria in all groups in our study raises concerns whether the reactions could show a false-positive result. It has been suggested that the antibodies against the membrane of the *Chlamydia pneumoniae* used in the reactions of the immunoperoxidases (just like in Western blot technique which we did not employ) could have a cross-reaction with â hemoglobin chain [22], or heavy chain of immunoglobulin [23], and thus presenting an inespecific outcome. These studies, however, used antibodies prepared by their own authors rather than the commercial antibody used by our group and by the majority of the researchers, even though directed to the same external membrane of the *Chlamydia*.

The studies which have focused the abdominal aortic aneurysms did not measure comparatively the patients' level of infection. On one hand, as we have described in the aortae, previous researches of our laboratory have shown that even people without atherosclerosis can present some amount of bacteria in coronary arteries. Regarding the arteries, it was shown significant differences between the groups with and without atherosclerosis and between stable and unstable plaques [4-7]. These points strengthen

the necessity of a quantitative method to better analyze the role of both *Chlamydia* and *mycoplasma* in the diseases.

In the present article, using a quantitative method we did not show any difference between the groups regarding the two types of microorganisms contrary to what have been described by our laboratory concerning to coronary arteries. A possible explanation to this fact could involve the population of the studies. In other articles, only coronary arteries of patients with idiopathic dilated cardiomyopathy (IDC) or Chagas disease were enrolled in the control group [4-7], while in the present study, among patients without aortic atherosclerosis enrolled, there was cases of heart transplant, infectious diseases, neoplasia, and rheumatic disease (Table 1); one of the cases of aneurysm also had rheumatic diseases and infectious endocarditis. It is possible that these cases cause or are caused by some kind of immunologic imbalance, which could interfere with the patients' capacity to react to bacteria, we have focused, thus influencing our outcomes. This idea was strengthened by the observation that the patients with Chagas disease present less *mycoplasma* and *Chlamydia* in their tissues [24] which would raise yet more the differences between the control populations of both studies.

Some differences observed in the coronary arteries were detected in a number of patients similar to the present study [4]. However, the relatively small size of our sample reduces the statistical power of the comparison, and we cannot completely rule out the possibility that the real difference between both groups had been hidden by this reason.

Other differences have already been described in the atherosclerotic process among the aorta and the coronary arteries besides the evident lack of the role of the bacteria in the process, which contrasts with the previous studies performed in this laboratory. While in the coronary arteries the lipoid striae and the atherosclerotic plaques have the same distribution, in the aortae these two types of atherosclerotic lesions have different sites [25]. The time of appearance and evolution is different: in the aorta, the process begins in childhood and advances very slowly, while in the coronary arteries the lesions begin at puberty [26]. The lipid content of the lesions also varies according to the vascular site, at least in the lipoid striae [27]. Even in the aorta itself, the distribution is not homogenous: the impairment of the abdominal portion is much more severe than that of the thoracic portion. In another article written by our group, we have found the correlation between the intensity of the atherosclerotic lesions and the vascular diameter in the thoracic aorta, but not in the abdominal aorta [28]. In such a case, the differences relative to *Chlamydia* pneumoniae and *Mycoplasma pneumoniae* between the arterial beds can also represent a real distinction between the atherosclerotic processes in both sites. On the whole, our data suggest that the highlighted agents do not seem to have an important role in the pathogenesis of the abdominal aortic aneurysms.

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