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Original Article

Prevalence and association of mycoplasma infection in the development of coronary heart disease

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Abstract: Background: Patients that died due to acute coronary heart diseases had significant *Mycoplasma pneumoniae* infection. The objective of the study was to explore the hypothesis that *Mycoplasma pneumoniae* was present in the blood that leads to coronary heart diseases. Methods: Patients were divided into two groups. 181 patients were put in a group I who died due to acute coronary heart diseases and 211 patients were put in group II who died, but not due to severe coronary heart diseases. The study detected *Mycoplasma pneumoniae* infections by histopathology, immunohistochemistry, and *in situ* hybridization. *In vivo* rabbit model study was also done to endorse the hypothesis. The Wilcoxon test, Kruskal-Wallis test, and posthoc test were carried out for non-normal distribution of *Mycoplasma pneumoniae* in autopsy samples, immunohistochemistry studies, and *in situ* hybridization technique respectively at 5% significance level. Results: Mean % *Mycoplasma pneumoniae* were 9.3 ± 0.29 and 1.1 ± 0.019 *area*/square mm for necropsy specimen collected from the groups I and II. In *De novo* rabbit model study, total serum cholesterol was 1.79 ± 0.019 and 91 ± 1.91 mmol/L for without and with *Mycoplasma pneumoniae* infected rabbits even though rabbits were put on a cholesterol free diet. *Mycoplasma pneumoniae* infection showed more prolonged ST changes in the electrocardiogram in rabbits. Conclusion: *Mycoplasma pneumoniae* might play a key role in thrombosis plaque rupture leading to coronary heart diseases.

Keywords: Coronary heart diseases, forensic serology, immunohistochemistry, *in situ* hybridization, *Mycoplasma pneumoniae*

Introduction

In recent time coronary heart diseases have increased and it is considered as immune-mediated inflammatory diseases. Many factors have been found useful in coronary heart disease, e.g. heat-shock proteins, β2–glycoprotein-I, and oxidized low-density lipoproteins (oxLDL). The studies proved an association between atherosclerotic plaque vulnerability and plaque inflammation [1]. The studies gave evidence that infections lead to ischemic tissue damage which causes exposure of autoantigens to tissue, and finally, activation of autoreactive immune T cells and persistent infections may activate autoimmunity [2]. These discussions conclude that infectious agents might enable immune-mediated atherosclerosis. A correlation between infections and coronary heart disease was for the first time given in the late 1980s', and *in vivo* animal studies showed that significant amounts of *Mycoplasma pneumoniae* in atherosclerotic plaque lead to its vulnerability [3]. HDL (high-density lipoprotein), LDL (Low-density lipoprotein), VLDL (Very low-density lipoprotein) levels, and chylomicrons are crucial for the rupture of embolism. The studies proved that Antigens were also found significantly in the inflammation of the plaque which is symptomless but characterized by myalgia. The researchers have determined that the infection is proportionate to the degree of disease and inflammation of plaques and myalgia [4-6]. When many infections or antigen of *Mycoplasma pneumoniae* is found in blood, it leads to effects on the immunologic system of coronary heart diseases. Most pathological studies had suggested a correlation between *Mycoplasma pneumoniae* and coronary heart diseases like atherosclerotic plaques in general

Table 1. Selection of subjects for study

Group	Cause of death	Total numbers of subject (n)	Nos of males	Nos of females*	Mean age (years)	BMI (kg/m^2)	WHR
I	Due to acute coronary heart diseases	181	141	40	42 ± 2.1	29 ± 1.5	0.71 ± 0.08
II	Not due to coronary heart diseases	211	161	50	48 ± 3	24 ± 0.5	0.7 ± 0.08

*Non-pregnant female patients. Results presented as a mean ± standard deviation. BMI: Body mass index; WHR: Waist-to-hip ratio. None of the patients had WHR more than 0.85 and BMI more than 30. Patients were under treatment of full-time intensivist while indoor.

or the intensity of plaque inflammation. The clinical and serological evidence are favoring the pathogenesis conclusion. The characteristic of *Mycoplasma pneumoniae* is that the membrane is made of cholesterol. Therefore, it is required cholesterol to stay alive. The studies detected a significant amount of *Mycoplasma pneumoniae* inside lipid core of coronary heart.

Studies were made to develop a correlation between antibodies against *Mycoplasma pneumoniae* found in the blood and mean % of the area of DNA of *Mycoplasma pneumoniae* in acute coronary heart disease patient. Endorsement of the research was made via the study of the histopathology techniques, immunohistochemistry, and ISH (*in situ* hybridization).

Material and methods

3'3 diaminobenzidine, Harris' hematoxylin-eosin (HE) dye, Periodic acid-Schiff (PAS), propylene oxide, lead nitrate, sucrose-phosphate-glutamic acid, uranyl acetate, and biotin were purchased from BBI Solutions, China. Cholesterol-free and cholesterol-supplemented standard chow diets were purchased from Van Corporation, China. The other analytical grade reagents were used.

Subjects

The study initiated after obtaining approval from the Research Ethics Committee of Human and the study protocol at the Henan University of Science and Technology, China under reference number of ChiCTR-TRC-13003757.

Inclusion criteria: Patients of both sexes, aged 18 and older, were included in the study after signing the informed consent form by body claimed person. The two different groups were made as per **Table 1**.

Exclusion criteria: Patients that presented at least one of the following criteria were excluded: Difficulty to understand the informed consent form; Refusal to sign the informed consent form; Impossibility to return for the study follow-up by body claimed person; Age younger than 18 years of patients at death time.

The most of the coronary arteries of hearts collected at necropsies were transverse sectioned cut at 4-mm intervals and used for the study. Both groups were subjected to collect on necropsies performed, and angioplasty of thrombolytic agents. The samples were subjected to collect for autopsies performed from dead bodies within two days of death [7, 8].

Histopathology

Necropsy samples were treated with formalin, followed by paraffin and subjected to histopathological study. The *Mycoplasma pneumoniae* was visualized using HE and PAS staining [9].

ISH technique

The ISH method was used to mark *Mycoplasma pneumoniae*. 0.01 M citrate buffer (pH 6.0; Merk Specialty Ltd, Germany) solution was used to improve cell permeability processed through microwave oven respect to the standard protocol for the antigen. 21 µL of hybridization mixture was added to prepared materials. *Mycoplasma pneumoniae* (ATCC: ES1-5531; Gift sample; ATCC Bank, China) was used to make the probe of *Mycoplasma pneumoniae* followed by denaturation at 94 ± 6°C for 7 min. ISH did for 19 ± 0.1 h. Washings were carried out to discard unwanted hybrids with 0.21N SSC (Sigma-Aldrich, USA). The DAKO amplification system (DAKO Foundation, Canada) was used for the tempering of the signal. 3'3 diaminobenzidine and HE were used for development and stain [10].

Immunohistochemistry

The 55 × magnification and the thickest section of plaque with vasa vasorum were used to

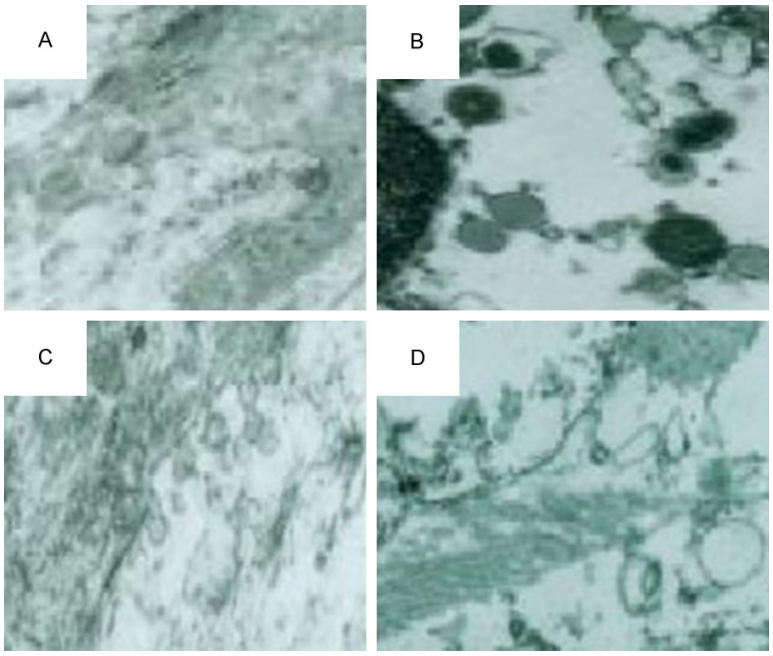

Figure 1. The histopathology of coronary arteries of a patient of group I. A: Microscopic view of vasa vasorum with enraptured plaque (45 ×). B: Microscopic view of vasa vasorum with enraptured plaque (450 ×) C: Microscopic view of cytoplasm (450 ×). D: Microscopic view of vasa vasorum with ruptured plaque (450 ×).

analyze 0.32 cm transverse sections of necropsies samples. One mm thick part was used for detection by immunoperoxidase. *Mycoplasma pneumoniae* specific antisera were used in the positive control (repeated sequence of Alu1/Alu2; DAKO Foundation, Canada). The omitting of the *Mycoplasma pneumoniae* specific antisera (a plasmid DNA marked with biotin) was used as negative controls [11]. Quantimet 500-Leica (Olympus, China) magnifying in 450 × was used for the study. Lymphocytes were counted per 450 × fields of the vasa vasorum then divided by area of the little field.

In vivo rabbit model study

Whole *in vivo* rabbit study followed the approval of the Institutional Animal Ethics Committee of China at Hospital and College of Clinical Medicine of Henan University of Science and Technology, under the reference number HAUST/CL/RP/14/61 and the Guidelines for Good Practice in Laboratory Animals Feeding and Management was followed [12]. One and a half-month-old female New Zealand White rabbits were selected for the study and fed cholesterol-free (groups 1 to 3) and standard chow diets (groups 4 and 5). Groups 4 and 5 were put on diet 0.6% w/w and 0.16% w/w peanuts. 1. 23 control rabbits were inoculated once by sterile carrier broth by way of the nasopharynx and sacrificed at 90 days. 2. 13 control rabbits were immunized three times with HEp-2 cells in the sucrose-phosphate-glutamic acid buffer at 15 days' time interval and sacrificed at 90 days from the first inoculation. 3. 31 rabbits were inoculated with *Mycoplasma pneumoniae* once by way of the nasopharynx and sacrificed at 90 days from injection. 4. 11 rabbits were fed 0.6% w/w of peanuts. All the rabbits were sacrificed at 90 days from the start of feeding. 5. 11 rabbits were fed 0.16% w/w of peanuts and sacrificed at 90 days from the start of feeding. *Mycoplasma pneumoniae* was cultured in chicken broth by incubation at 37°C in 5.5% v/v CO_2 for 96 h. The culture was diluted to get 1-2 × 10^7 CFU/ml in frizzed condition at -69°C [13]. The serology was performed for group 3 rabbits, before inoculation and at after scarification by serum complement fixation test using Mycoplasma CF (Johnson and Johnson, USA) as the control [14]. Control was used to exclude observation like anti-complementary activity [15-17]. Levels of total serum cholesterol were found by oxidase method on Ektachem 750 (Johnson and Johnson, USA) for all rabbits just before they were sacrificed [18]. Tissue sections from rabbits of group 3 were made by the immunoperoxidase method [19] in a dilution of 1:1,210. Both positive controls and negative controls were maintained during the study [20]. Electrocardiogram (ECG) was measured by ECG equipment (Remi equipment, China) for all rabbits just before they were sacrificed [20].

Statistical analysis

For non-normal distribution of *Mycoplasma pneumoniae* in necropsy sample collected from

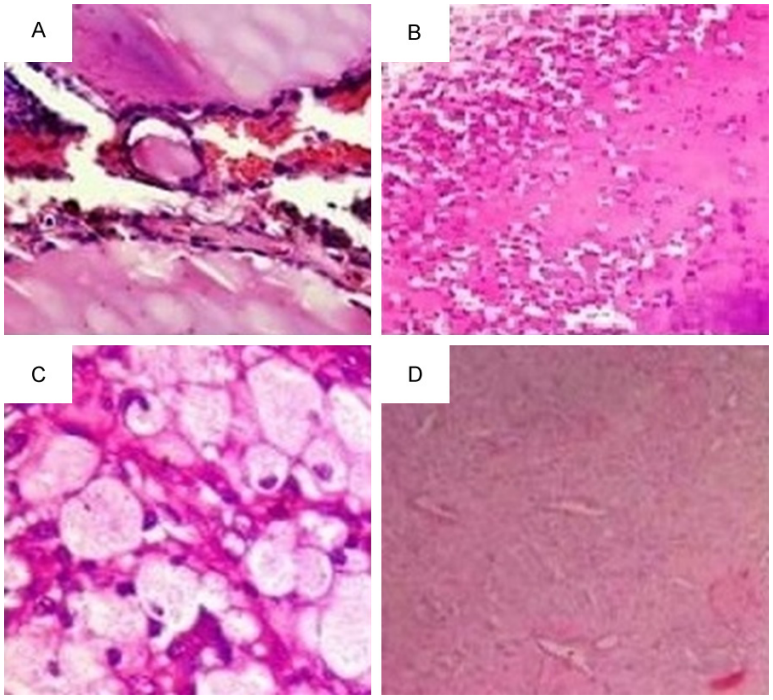

Figure 2. A: Histopathology of necropsy sample (450 ×, HE) of ruptured plaques collected from group-I. B: Histopathology of necropsy sample (450 ×, HE) of non-ruptured plaques collected from group-I. C: Histopathology of necropsy sample (450 ×, HE) of vasa vasorum collected from group-I. Small round or rod type structures are indicating *Mycoplasma pneumoniae.* D: Histopathology of necropsy sample (100 ×, HE) intima layer of sample collected from group-II.

Group I, the Wilcoxon test was carried out in between *Mycoplasma pneumoniae* counts of plaque samples and vasa vasorum sample [21]. For differences in counts of *Mycoplasma pneumoniae* in necropsy sample of a different group and immunohistochemistry study the Kruskal-Wallis test was carried out [22, 23]. The posthoc test was performed for significance difference between primary lymphocytes of ISH technique. All analysis was performed using SAS 9.4 (SAS Institute, Inc., NC) and SPSS Statistics 22 (IBM, Inc.). A multivariate regression analysis was performed in order to evaluate the role of confounding factors on final results. Statistical significance was set at a two-sided *p*-value of 0.05 or less [24].

Results

The histopathology of coronary arteries showed small round or rod type structures attached to the epithelium of vasa vasorum (**Figure 1A** and **1B**). Their morphology was identical with *Mycoplasma pneumoniae*. Results showed the presence of broken and damp mass in the cytoplasm (**Figure 1C**). Besides these, round granular materials were also present within vasa vasorum (**Figure 1D**). These were identified as *Mycoplasma pneumoniae. Mycoplasma pneumoniae* DNA was present in intima layer while more amount was present in ruptured plaques (**Figure 2A**) and non-ruptured plaques (**Figure 2B**) than vasa vasorum of the sample collected from group-I (**Figure 2C**) of the human subject (Wilcoxon test, $P < 0.05$, $n = 5$). Mean % *Mycoplasma pneumoniae* was 9.3 ± 0.29 and 1.1 ± 0.019 *area*/mm^2 for necropsy specimen collected from the groups I and II (**Figure 2D**) (Kruskal-Wallis test; $P < 0.05$).

Table 2 provided the quantitative results concerning the ISH method for detection of *Mycoplasma pneumoniae*. In the vasa vasorum, both the average numbers of percentage fatty area and the primary lymphocytes ($CD20^+$, $CD4^+$, $CD8^+$) per mm^2 were significantly higher in Group I than those in Group II ($P < 0.05$ for all of them; Posthoc test). The study showed the absence of neutrophils in group II in stable plaques and present in variable amounts in ruptured plaques. The neutrophils were mostly adjacent to the emboli but not as a main component of inflammation in group I. Macrophages were most frequent in inflammatory cells, in the ruptured plaques and stable plaques of group I, usually full of intracytoplasmic lipids, while almost absent in group II. Variable amounts of plasma cells were seen adjacent to the other types of lymphocytes, mainly in the vasa vasorum in both groups. The difference of plasma cells between groups was not significant ($P > 0.05$; Posthoc test). However, results were found not significant for the other parameters.

Table 3 showed results of staining for cellular markers and *Mycoplasma pneumoniae* antigen for human subjects. Kruskal-Wallis test showed significant differences between the values of

Table 2. Results of ISH technique

Counts/mm^2 of necropsy a sample of the coronary vessel	Group		Post-hoc test; *P*-value
	I	II	
% Fatty area	40 ± 1	5 ± 0.3	0.02
CD20$^+$	7 ± 0.7	1.3 ± 0.15	0.021
CD4$^+$	11 ± 0.5	4 ± 0.5	0.023
CD8$^+$	17 ± 1.2	7 ± 0.45	0.035
Neutrophils	29 ± 3	3 ± 0.05	0.009
Macrophages	38 ± 3	1.2 ± 0.05	0.01

The mean ± standard deviation from five independent experiments presented for the data. Results possessed for human subjects only.

Table 3. Immunohistochemistry study collected from human subjects

Cell type (counts/mm^2)	Group		Kruskal-Wallis test *p*-value
	I	II	
β-cells	16 ± 0.06	3.2 ± 0.04	0.049
T-cells	37 ± 2.1	6.5 ± 0.35	0.009
Macrophage	51 ± 3.5	7 ± 0.95	0.008
Smooth muscle	13 ± 0.35	9.9 ± 0.89	0.065
TNF-α	6.5 ± 0.35	1.25 ± 0.16	0.049
IL-1α	21 ± 0.85	3.2 ± 0.15	0.039
IL-6	32 ± 2.1	9 ± 0.35	0.038
TGF-β	37.5 ± 2.5	14 ± 0.24	0.047
PDGF	26 ± 2	3.1 ± 0.3	0.039
MDF-2	22 ± 1.9	17 ± 0.9	0.35

The mean ± standard deviation from five independent experiments presented for the data.

β-Cells, T-cells, macrophage, among the group I and II. The trial also demonstrated significant differences for TNF-α (tissue necrosis factor-α), IL (Interleukin)-1α, IL-6, TGF-β (transforming growth factor-β), and PDGF (Platelet-derived growth factor), counts/mm^2 ($P < 0.05$ for all of them). However, there was no significant difference between smooth muscle and MDF-2 (Myelopoiesis depressing factor-2) counts/mm^2 ($P > 0.05$ for both). The study demonstrated high positive monotonic correlations between the average percentage of the area of DNA of *Mycoplasma pneumoniae* and vessels area (mm^2) (correlation coefficient: $R^2 = 0.896$, Spearman rank correlation: $r_s = 0.88$; $P < 0.05$). It also revealed a high negative monotonic correlation between the average percentage of the area of DNA of *Mycoplasma pneumoniae* and mean CD4$^+$ T lymphocytes ($R^2 = 0.947$, $r_s = -0.723$, $P < 0.05$) and mean CD$^+$ 3/mm^2 ($R^2 = 0.969$; $r_s = -0.84$; $P < 0.05$).

Table 4 showed the results of staining for cellular markers, *Mycoplasma pneumoniae* antigen and total serum cholesterol for *in vivo* rabbit model. Kruskal-Wallis test showed a significant difference between values of β-cells, T-cells, macrophage, *Mycoplasma pneumoniae,* TNF-α, IL-1α, IL-6, TGF-β, PDGF counts/mm^2 and total serum cholesterol among of groups 1 and 3 ($P < 0.05$ for all of them). The Spearman rank correlation showed significant positive monotonic relationships for percentage mean of the area presented by *Mycoplasma pneumoniae* DNA in plaques and percentage vessel mean area between groups 1 and 3 ($P < 0.05$). Spearman rank correlation showed a significant negative monotonic correlation between *Mycoplasma pneumoniae* DNA in plaques and mean lymphocytes counts/mm^2 between groups 1 and 3 ($P < 0.05$). However, the study failed to provide a significant difference between smooth muscle and MDF-2 counts/mm^2 between groups 1 and 3. (Kruskal-Wallis test; $P > 0.05$ for both).

The study showed that *Mycoplasma pneumoniae* induced focal *periaortitis* (**Figure 3**). The total serum cholesterol was 1.79 ± 0.019 and 91 ± 1.91 mmol/L for rabbits in groups 1 and 3. Unlike all rabbits of Group 1 (**Figure 4A**), transitory and occasionally more prolonged ST changes in the electrocardiogram and early electrocardiograms were observed in all rabbits of Group 3 (**Figure 4B**).

Discussion

More presence of *Mycoplasma pneumoniae* was observed in intima layer and in ruptured plaques of all necropsy samples because there was a high quantity of fatty area. This is one evidence that there is more presence of *Mycoplasma pneumoniae* in autopsy sample collected from the group I than II. No previous study is available that providing the correlation between the quantitative presence of *Mycoplasma pneumoniae* and ruptured plaques [10].

ISH technique of detection for *Mycoplasma pneumoniae* DNA showed that group I had high

Table 4. Immunohistochemistry study for *in vivo* Rabbit model

Cell type (counts/mm^2)	Group				
	1	2	3	4	5
β-cells	12 ± 0.19	2.1 ± 0.02	1.95 ± 0.019	12 ± 1.45	15 ± 1.8
T-cells	11 ± 0.29	2.2 ± 0.03	2.4 ± 0.04	9 ± 1.2	13 ± 2.1
Macrophage	12 ± 0.05	13 ± 0.025	2.4 ± 0.05	8 ± 0.7	12 ± 2.1
Smooth muscle	2.1 ± 0.06	2.2 ± 0.03	3.5 ± 0.06	1.3 ± 0.06	1.8 ± 0.08
Mycoplasma pneumoniae	0	0	55 ± 3.5	0	0
TNF-α	16 ± 2.2	22 ± 0.03	1.3 ± 0.03	17 ± 2.1	28 ± 3.1
IL-1α	19 ± 2.2	18.5 ± 2.1	1.9 ± 0.04	20.1 ± 1.95	25 ± 1.95
IL-6	16.5 ± 1.45	22.5 ± 1.8	29 ± 1.45	16.5 ± 1.2	31 ± 1.8
TGF-β	17 ± 1.2	16 ± 2.6	1.8 ± 0.04	14 ± 2.2	14 ± 2.8
PDGF	14 ± 2.3	17 ± 1.5	2.3 ± 0.05	15 ± 0.35	14 ± 0.65
MDF-2	8 ± 0.5	9 ± 1.8	7 ± 0.8	11 ± 2.1	9 ± 1.2

The mean ± standard deviation from five independent experiments presented for the data. The study reported for the *De novo* Infection.

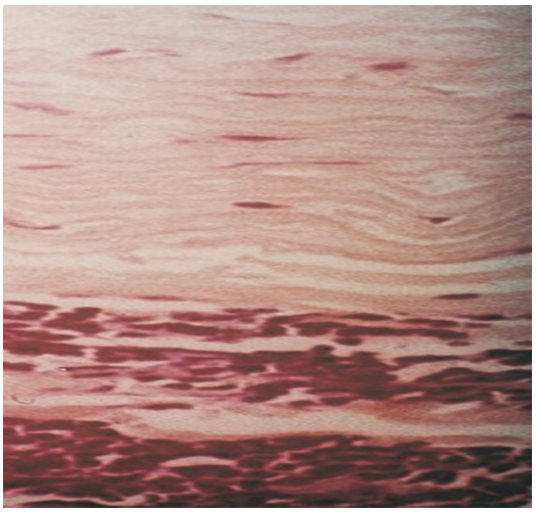

Figure 3. Histopathology of necropsy sample of the aorta of group 3 rabbit (60 ×, HE).

quantities of granule in extracellular and in the cytoplasm of xanthomata's cells. It was also demonstrated that *Mycoplasma pneumoniae* DNA was mostly present where the fatty areas of the plaques were found. The study also concluded that there was a high presence of *Mycoplasma pneumoniae* in necropsy sample from the group I than II [10].

T cells and macrophages were decreased more by *Mycoplasma pneumoniae* while *Mycoplasma pneumoniae* had the less negative correlation for MDF-2, the most negative correlation for TGF-β and PDGF.

Mycoplasma pneumoniae DNA was present in intima layer of vasa vasorum while more amount was present in ruptured plaques and non-ruptured plaques than vasa vasorum of sample collected from group-I because more amount of cholesterol was present at this place [10].

The post-mortem report of the hospital in group I and II showed the evidence that *Mycoplasma pneumoniae* is the primary cause of coronary heart disease.

Till date, *Mycoplasma pneumoniae* has been reported to cause pneumonia, arthritis, fatigue disorders, dysphonia, emphysema and renal diseases in human [25]. The study provided authentic evidence for the cause of coronary heart myalgia, coronary heart diseases like atherosclerosis due to *Mycoplasma pneumoniae* [26]. This study has presented a hypothesis for high oxLDL level, high TNF, cell proliferation, and inflammation caused by the presence of *Mycoplasma pneumoniae* inside ruptured plaques. This theory is hard to explain by the previous studies [7, 8]. Recent polymerase chain reactions (PCR) data were utilized to provide the correlation between *Mycoplasma pneumoniae* and coronary heart diseases, but the study provided accurate quantitative data like serological data, ISH technique, and immunohistochemistry to provide strong support to the hypothesis. PCR is comparatively costlier than ISH technique, and immunohistochemistry [27, 28].

Unlike previous studies, the theory succeeded in explaining apoptosis of coronary heart arteries cells [29].

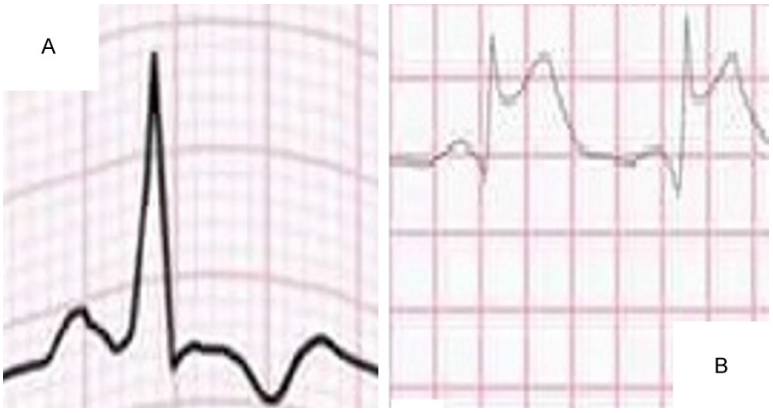

Figure 4. Electrocardiogram (ECG) of rabbits (25 mm/Sec; 10 mm/mV). A: ECG of rabbit belongs to group 1 just before sacrificed. B: ECG of rabbit belongs to group 3 just before sacrificed.

Group, 1 and 2 rabbits lacked high cholesterol level and no *periaortitis*. The lack of high cholesterol, clinical manifestation of *Mycoplasma pneumoniae* produces focal *periaortitis*, as well as a remarkable increase in cholesterol levels, was shown in group 3 rabbits compared to groups 4 and 5 leading to chances of atherosclerosis. Cholesterol was about 50-fold higher than without *Mycoplasma pneumoniae* infected rabbits even though rabbits were put on a cholesterol free diet. *Mycoplasma pneumoniae* precipitated atherosclerosis with an increase in cholesterol level without taking cholesterol in the diet. There was the significant correlation between mean antibodies in *Mycoplasma pneumoniae* animals with atherosclerosis or *periaortitis*. T cells, TGF-β, PDGF, and macrophages were decreased more by *Mycoplasma pneumoniae* while MDF-2 count had almost no effect.

The previous study demonstrated hypothesis on rabbit model, but necropsies samples were collected after 3-4 days of inoculation only providing rough means of evaluation and a casual approach. The study carried out autopsies from the rabbits at 90 days of vaccination. This study showed substantial evidence of hypothesis. Unlike this study, some previous studies also stated such hypothesis but did not use statistical data for substantial evidence. ST segment changes in ECG had been an indication of mild to moderated myalgia. The study had evidenced a decrease in lipid cholesterol level of group 3 when the rabbits were put on an antibiotic. These were because the infection was overcome [30].

Limitations

The study failed to establish a correlation between MDF-2, smooth muscle count, and plasma cells count against *Mycoplasma pneumoniae* infection.

Conclusion

The present clinical study concluded that the presence of a high number of *Mycoplasma pneumoniae* leads to the development of instability of plaque, the formation of embolism, and an increase in the number of inflammatory cells. The infection resulted in the accumulation of plaques, tempering of the immunological response which was characterized by a decrease in lymphocytes, development of inflammation, an increase in apoptosis, and finally, rupture of coronary artery plaques leading to symptoms of cardiac myalgia due to hypoxic conditions and death. *In vivo* rabbit model study concluded that a *de novo* respiratory tract *Mycoplasma pneumoniae* infection in rabbit led to cause atherosclerosis.

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Disclosure of conflict of interest

None.

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