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Original Article

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

Prevalência e associação de infecção por micoplasma no desenvolvimento de doença arterial coronariana

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Abstract

Coronary heart disease (CHD) has been associated with significant morbidity and mortality worldwide. Although remain controversial, several studies have demonstrated the association of *M. pneumoniae* infections with atherosclerosis. We evaluated the possible association of mycoplasma infections in patients diagnosed with atherosclerosis by ELISA and PCR methods. Atherosclerotic tissue samples and blood samples were collected for the detection of mycoplasma antibodies (IgA) by ELISA from the 97 patients with coronary artery disease (CAD). *M. pneumoniae* specific IgA, IgG and IgM were measured by using the Anti-*M. pneumoniae* IgA/IgG/IgM ELISA. Detection of *M. pneumoniae* targeting the P1 adhesion gene was performed by PCR Acute infection of *M. pneumoniae* was diagnosed in 43.3% (42) of patients by PCR. The *M. pneumoniae* specific antibodies were detected in 36.1% (35) of patients. Twenty-five (25.8%) cases had IgG antibodies, 15 (15.5%) cases had IgM antibodies, 3 (3.1%) cases had IgA antibodies, 10 (10.3%) cases had both IgM + IgG antibodies and 1 (1%) case of each had IgM + IgA and IgG + IgA antibodies. None of the cases was positive for all three antibodies. A Pearson correlation coefficient analysis revealed an excellent correlation between the PCR and the serological results ($r=0.921$, $p<0.001$). A majority (17, 40.5%) of the *M. pneumoniae* positive patients are within the 41-50 years of age group, followed by 10 (23.8%) patients in the age group of 61-70 years and 2 (4.8%) patients were >70 years of age. Our study reported an unusually higher prevalence of *M. pneumoniae* by serological tests (36.1%) and PCR (43.3%). Although the hypothesis of the association of *M. pneumoniae* and CAD is yet to be proven, the unusually high prevalence of *M. pneumoniae* in CAD patients indicates an association, if not, in the development of atherosclerosis.

Keywords: coronary heart disease, mycoplasma, atherosclerosis, ELISA, PCR.

Resumo

A doença coronariana (DCC) tem sido associada a significativa morbidade e mortalidade em todo o mundo. Embora ainda sejam controversos, vários estudos têm demonstrado a associação de infecções por *M. pneumoniae* com aterosclerose. Avaliamos a possível associação de infecções por micoplasma em pacientes com diagnóstico de aterosclerose pelos métodos ELISA e PCR. Amostras de tecido aterosclerótico e amostras de sangue foram coletadas para a detecção de anticorpos contra micoplasma (IgA) por ELISA de 97 pacientes com doença arterial coronariana (DAC). IgA, IgG e IgM específicos para *M. pneumoniae* foram medidos usando o Anti-M. pneumoniae IgA / IgG / IgM ELISA. A detecção de *M. pneumoniae* visando o gene de adesão P1 foi realizada por PCR. A infecção aguda por *M. pneumoniae* foi diagnosticada em 43,3% (42) dos pacientes pela PCR. Os anticorpos específicos para *M. pneumoniae* foram detectados em 36,1% (35) dos pacientes. Vinte e cinco (25,8%) casos tinham anticorpos IgG, 15 (15,5%) casos tinham anticorpos IgM, 3 (3,1%) casos tinham anticorpos IgA, 10 (10,3%) casos tinham anticorpos IgM + IgG e 1 (1%) caso de cada um tinha anticorpos IgM + IgA e IgG + IgA. Nenhum dos casos foi positivo para os três anticorpos. A análise do coeficiente de correlação de Pearson revelou uma excelente correlação entre o PCR e os resultados sorológicos ($r = 0,921$, $p < 0,001$). A maioria (17, 40,5%) dos pacientes positivos para *M. pneumoniae* está na faixa etária de 41-50 anos, seguida por 10 (23,8%) pacientes na faixa etária de 61-70 anos e 2 (4,8%) pacientes tinham > 70 anos de idade. Nosso estudo relatou uma prevalência incomumente maior de *M. pneumoniae* por testes sorológicos (36,1%) e PCR (43,3%). Embora a hipótese da associação de *M. pneumoniae* e DAC ainda não tenha sido comprovada, a prevalência incomumente alta de *M. pneumoniae* em pacientes com DAC indica uma associação, se não, no desenvolvimento de aterosclerose.

Palavras-chave: doença cardíaca coronariana, micoplasma, aterosclerose, ELISA, PCR.

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1. Introduction

Coronary heart disease (CHD) has been associated with significant morbidity and mortality worldwide. Several well-recognized risk factors include smoking, diet, hypercholesterolemia, hypertension, diabetes and sedentary habits (Alviar et al., 2011). Atherosclerosis is a disease in which plaques build up inside the arteries and limits the flow of oxygen-rich blood to various parts of the body. The association of infectious agents with the atherosclerosis was first demonstrated in 1978 (Fabricant et al., 1978). It has been hypothesized that during atherosclerosis, several common bacteria and virus contributes to the inflammation of vascular heart wall and leads to the formation of atheroma (Ross, 1999; Ohayon et al., 2011). Although the role of infectious agents remains controversial, several studies demonstrated the association of viruses and bacteria in the development of atherosclerosis (Góis et al., 2006). Even though there is no usually acknowledged group of bacteria or technique available for evaluating infectious atherosclerosis, reports have associated *Mycoplasma pneumoniae* (Momiyama et al., 2004), *Chlamydia pneumoniae* (Hauer et al., 2006) and influenza viruses as a potential risk factor of CHD (Corrales-Medina et al., 2010).

M. pneumoniae are auto-replicating, the smallest microorganism that is devoid of the cell wall and requires cholesterol for its growth (Razin et al., 1998). They may also favor the proliferation of other infectious organisms by altering the host immune system (Higuchi et al., 2003). The association of *C. pneumonia* with atherosclerosis has been reported earlier (Danesh et al., 1997). Since there is a similarity in epidemiological behavior and antibiotic resistance pattern between *M. pneumoniae* and *C. pneumoniae*, it has been suggested that *M. pneumoniae* may also play a role in the development of atherosclerosis (Taylor-Robinson and Thomas, 1998). Higuchi et al. (2000), during the detection of *C. pneumonia* from necropsy specimens, demonstrated the presence of *M. pneumoniae* for the first time (Higuchi et al., 2000). Thereafter few other studies have also demonstrated the association of *M. pneumoniae* infections with atherosclerosis (Momiyama et al., 2004; Reunanen et al., 2005). In contrast, other studies reported discordant results regarding the role of *M. pneumoniae* in atherosclerosis pathogenesis. Thus, the association of *M. pneumoniae* is controversial and remains as unknown. The present study aims to evaluate the possible association of mycoplasma infections in patients diagnosed with atherosclerosis by ELISA and PCR methods.

2. Materials and Methods

2.1. Patients

A prospective study was conducted in 97 patients with coronary artery disease (CAD) admitted to the Department of Cardiology and underwent coronary artery bypass surgery (CABG) between February 2016 and November 2019. Fifty-eight (59.8%) patients were male and 39 (40.2%) were female. The age ranged from 38 to 73 years (mean (SD) 56.3 ± 7.8 years). An intraoperative endarterectomy was performed to obtain the atherosclerotic tissue sample during CABG. Blood samples were collected for the detection of mycoplasma antibodies (IgA) by ELISA from the CAD patients. Blood samples collected from 10 normal individuals whose baseline characteristics were similar to the patient group serve as a control for the ELISA test. Clinical characteristics of patients were obtained from the medical records. Tissue samples and serum separated from the collected blood samples were stored at -20^{0}C until further analysis. A written informed consent was obtained from each patient before surgery and the institutional ethics committee approved the study.

2.2. Serological analysis

Mycoplasma pneumoniae specific IgA, IgG and IgM were measured by using the Anti-*Mycoplasma pneumoniae* IgA/IgG/IgM ELISA Kit (Verion/Serion, Germany) as per the manufacturer instructions. Absorbance within 30 min of the addition of the stop solution was measured at 450 nm. As indicated by the manufacturer, a titer of IgA >14 U/ml, a titer of IgG >30 U/ml and a titer of IgG >1.1RU were considered as positive.

2.3. DNA extraction

DNA from the atherosclerotic tissues was extracted using the DNeasy® Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. For the PCR amplification, 100 ng of DNA was used. The purity and the quantification of DNA were checked using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). Extraction of DNA, preparation of PCR mix, PCR amplification, and analysis of PCR products were performed in separate laboratories.

2.4. Polymerase chain reaction

Detection of *M. pneumoniae* targeting the P1 adhesion gene was performed by PCR (Chaudhry et al., 2013). PCR was performed using a 25µl master mix containing 2.5µl of template DNA, 0.2µM (2µl) of each primer (Forward: 5'- CAAGCCAAACACGAGCTCCGGCC-3' and Reverse: 5'-CAGTGTCAGCTGTTTGTCCTTCCCC-3'), 12.5 µl of TEMPase Hot Start Master Mix (Ampliqon, Denmark) and 6µl of DNAse/ RNAse free water. The following PCR cycling conditions were used: initial denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension step at 72°C for 10 min. An expected amplicon size of 543 bp was resolved in 1.2% agarose gel electrophoresis and visualized under UV transilluminator.

2.5. Statistical analysis

Data with continuous values were represented as medians and ranges and categorical values as numbers and percentages. A Chi-Square test was performed to determine statistical significance and a Pearson correlation coefficient analysis was performed to determine the relationship of two variables using SPSS statistical software

(IBM® SPSS®, USA). A P value < 0.05 was considered to be statistically significant.

3. Results

A total of 97 blood samples were collected from patients with CAD who underwent CABG. The mean age of the patients was 56.3 ± 7.8 years. Twelve patients were in <40 years of age range, 36 patients were in 41-50 years of age range, 28 patients were in 51-60 years of age range, 17 patients were in 61-70 years of age range and 4 patients were in >70 years of age range (Figure 1). The predominant age group affected by CAD ranged from 41-50 years, followed by the 51-60 years. The distribution of CAD patients based on sex and other demographic details like family history and history of myocardial infarction is shown in Table 1. The clinical data of all the patients are presented in Table 2.

Acute infection of *M. pneumoniae* was diagnosed in 43.3% (42) of DNA samples using molecular detection of P1 adhesion gene. The anti-*M. pneumoniae* were positive in 36.1% of cases and negative in 63.9% of cases. There was no significant difference between the PCR and the serological results (p=0.358) (Table 3). Similarly, there were no significant differences in the detection of *M. pneumoniae* by PCR and serology between men and women (p>0.05). Twenty-five (25.8%) cases had IgG antibodies, 15 (15.5%) cases had IgM antibodies, 3 (3.1%) cases had IgA antibodies, 10 (10.3%) cases had both IgM + IgG antibodies and 1 (1%) case of each had IgM + IgA and IgG + IgA antibodies. None of the cases was positive for all three antibodies (Figure 2). A sub-group analysis of the 35 (36.1%) anti-*M. pneumoniae* seropositive patients revealed that 30 (35.7%) patients had hypertension, 29 (82.9%) patients had myocardial infarction, 27 (77.1%) patients had the total cholesterol of >250 mg/dl, 24 (68.6%) patients had LDL of >60 mg/dl, 22 (62.9%) patients had triglycerides of >150 mg/dl, 18 (51.4%) patients had HDL of <100 mg/dl and 15 (42.9%) patients had an urea level of >40 mg/dl.

Of the 35 (36.1%) samples positive for any of the tested antibodies, 31 (32.0%) samples were positive for *M. pneumoniae* by PCR. A Pearson correlation coefficient analysis revealed an excellent correlation between the PCR and the serological results (r=0.921, p<0.001). A sub-group analysis of the 42 (43.3%) patients positive for *M. pneumoniae* by PCR revealed that 37 (88.1%) patients had hypertension, 33 (78.6%) patients had myocardial infarction, 29 (69%) patients had the total cholesterol of >250 mg/dl, 26 (61.9%) patients had triglycerides of >150 mg/dl, 25 (59.5%) patients had LDL of >60 mg/dl, 21 (50%) patients

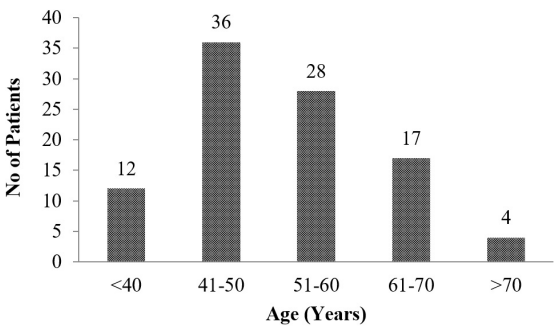

Figure 1. Distribution of age among the included patients.

Table 1. Demographic detail of CAD patients.

Demographic details	No of patients (%) (n=97)
Male	58 (59.8)
Female	39 (40.2)
Age (mean ± SD)	56.27 ± 7.83
Acute coronary syndrome	21 (21.6)
Family history of CAD	57 (58.8)
Diabetes mellitus	23 (23.7)
Hypertension	67 (69.1)
History of smoking	31 (32.0)
History of myocardial infarction	56 (57.7)

n: sample size; SD: Standard deviation

Table 2. Clinical characteristics of CAD patients.

Laboratory testing	Mean ± SD (n=97)	Range
CRP (<6 mg/L)	12.8 ± 1.7	10.5-15.0
LDL (<100 mg/dl)	128.6 ± 21.8	85-153
HDL (40-60 mg/dl)	57.2 ± 13.6	33-86
Urea (20-40 mg/dl)	40.8 ± 9.8	25-57
Total cholesterol (<200 mg/dl)	219.3 ± 50.4	132-309
Triglycerides (<150 mg/dl)	178.3 ± 30.1	120-213

n: sample size; SD: Standard deviation; CRP: C-Reactive protein; LDL: Low density lipoprotein; HDL: High density lipoprotein

Table 3. Molecular and serology results of *M. pneumoniae.*

Test	Positive (%)	Negative (%)
PCR	*42 (43.3)	55 (56.7)
Serology	35 (36.1)	62 (63.9)

*NS: Not significant; CHI-Square test DF = 1, P-Value = 0.358.

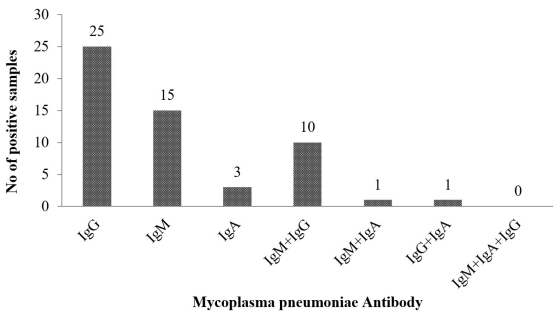

Figure 2. Serological test of *M. pneumoniae*.

had HDL of <100 mg/dl and 17 (40.5%) patients had an urea level of >40 mg/dl. There was no significant statistical difference in the sex and age proportions between the test and control groups ($p<0.05$). A majority (17, 40.5%) of the *M. pneumoniae* positive patients are within the 41-50 years of age group, followed by 10 (23.8%) patients in the age group of 61-70 years and 2 (4.8%) patients were >70 years of age.

4. Discussion

M. pneumoniae could be a possible candidate to play a role in the pathogenesis of atherosclerosis, as it has been related to cardiovascular disease and its ability to induce chronic inflammation (Higuchi et al., 2000). Even though *M. pneumoniae* accounts for 10%-30% of commonly acquired pneumonia, some studies have identified that *M. pneumoniae* is also present in atherosclerotic plaque (Higuchi et al., 2000). Furthermore, this bacterium is identified in the serological antibody test in patients with CAD (Momiyama et al., 2004). The association between *M. pneumoniae* infection and cardiovascular events remains undetermined. Therefore the present study was attempted to determine the prevalence of *M. pneumoniae* in adult patients with CAD using PCR and ELISA tests. The interpretation of serological results should be based on a combination of IgA, IgG and IgM antibodies' profiles. The presence of *M. pneumoniae* IgA, IgG or IgM in the serum generally reflects current/recurrent, past or current infection, respectively (Ngeh et al., 2004). In our study, *M. pneumoniae* antibodies were present in 36.1% of patients. Of these, 25.8% of patients had IgG antibodies, 15.5% of patients had IgM antibodies, 3.1% of patients had IgA antibodies, 10.3% of patients had both IgM + IgG antibodies and 1% of patient each had IgM + IgA and IgG + IgA antibodies. The overall seroprevalence of *M. pneumoniae* infection in our study was found to be higher than reported in other studies (Hauksdottir et al., 1998; Rastawicki et al., 1998; Tuuminen et al., 2000; Daxboeck et al., 2002). However, a study from Austria which investigated 91 patients with internal carotid artery (ICA) stenosis for specific IgA antibodies to *M. pneumoniae*, reported a higher seroprevalence rate (18.7%) than that reported (3.1%) in our study (Daxbock et al., 2011). Another study from Saudi Arabia, which demonstrated the association of *M. pneumoniae* with ischemic heart disease (IHD), reported a higher rate of M pneumonia IgG antibodies (31.3%) in IHD patients than that reported (25.8%) in our study (Yiallouros et al., 2013). A study from Japan evaluated the seropositivity for *M. pneumoniae* and *C. pneumoniae* in 396 patients with CAD, and 153 patients without CAD. The study reported that *M. pneumoniae* seropositivity (antibody titer ≥1/8 and ≥1/16) were significantly higher in patients with CAD than in patients without CAD, especially in those with myocardial infarction (Momiyama et al., 2004). Similarly, in our study higher number of (29, 82.9%) patients who were positive for *M. pneumoniae* antibodies had a myocardial infarction, which is in agreement with that reported by Momiyama et al. (Momiyama et al., 2004). Horne et al., in a case-control study, reported that a significantly higher number of patients with elevated levels of IgA (not IgG) for M. pneumonia had coronary heart disease (Watkins-Riedel et al., 2001). A recent study reported that a high level of anti-Mycoplasma pneumoniae IgM and IgG antibodies indicate a significant association of *M. pneumoniae* infection and history of this infection with increased risk for ischemic stroke (Roham et al., 2016). In our study, 10.3% of patients were sero-positive for both IgM and IgG antibodies, which indicates that these patients may be with an increased risk of ischemic stroke.

In our study, a total of 42 (43.3%) patients were positive for M. pneumonia by PCR. Maraha et al., investigated the presence of *M. pneumoniae* in 39 atherectomy specimens and in 64 degenerative heart valve (DHV) specimens. The study reported that only 2.5% of atherectomy and 3% of DHV specimens were positive for *M. pneumoniae* by PCR, which is much lower than that reported in our study (Maraha et al., 2000). Bayram et al., who investigated the presence of bacteria and viruses in atherosclerotic lesions of patients with CAD, reported that 6.7% atherosclerotic specimens and 3.3% on non-atherosclerotic vascular tissues samples were positive for *M. pneumoniae* by PCR (Bayram et al., 2011). In our study, the result of PCR is coherent with serology, which is concordant with the findings in other studies (Welti et al., 2003; Kumar et al., 2016). As stated before, *M. pneumoniae* is one of the suspicious agents which may be associated with atherosclerosis alone or in coexistence with other conventional risk factors (Guan et al., 2008). In this study, certain lipid risk factors (triglycerides, HDL and lipid tetrad index) were significantly higher ($p<0.05$) in the CAD patients who were positive for *M. pneumoniae* infection. The fascinating character of this bacterium is that they require cholesterol for survival because their membrane is constituted of cholesterol, a unique property among prokaryotes. Furthermore, this bacterium tends to be detected mainly in the lipid cores of coronary plaques in patients with MI (Higuchi et al., 2000; Higuchi and Ramires, 2002). A study by Momiyama et al., hypothesized that the combination of *M. pneumoniae* infection and hyperlipidemia may be an important cofactor for CAD. Though the above study concluded that they could not find any interaction between *M. pneumoniae* infection and hyperlipidemia for CAD, in our study the CAD patients with *M. pneumoniae* infection had hyperlipidemia (Momiyama et al., 2004). The non-inclusion of a parallel control group is one of the limitations, obtaining a tissue sample for non-atherosclerotic patients is practically difficult due to ethical considerations. Hence we could not include a parallel control group to compare the results obtained in this study. Secondly, this is a bi-center study and one should be cautious in interpreting this data to the general population.

5. Conclusion

Our study reported an unusually higher prevalence of *M. pneumoniae* by serological tests (36.1%) and PCR (43.3%). Although the hypothesis of the association of *M. pneumoniae* and CAD is yet to be proven, the unusually high prevalence of *M. pneumoniae* in CAD patients indicates an association,

if not, in the development of atherosclerosis. Hence larger studies establishing the relationship of *M. pneumoniae* in the development of atherosclerosis are required.

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