Artículo Original

Chlamydophila pneumoniae infection is not associated with primary biliary cholangitis

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La infección por *Chlamydophila pneumoniae* no está asociada con colangitis biliar primaria

Antecedentes: La colangitis biliar primaria (CBP) es una enfermedad hepática inflamatoria crónica colestásica de causa desconocida. Varios patógenos virales y bacterianos han sido propuestos como factores que podrían gatillar una respuesta inmune por mimetismo molecular, o directamente estar relacionados en la persistencia del daño biliar. Existen reportes controversiales respecto al rol de *Chlamydophila pneumoniae* en la patogenia de CBP. **Objetivo:** Investigar marcadores de infección de *C. pneumoniae,* séricos y en hígado de pacientes con CBP. **Pacientes y Métodos:** Veinte pacientes diagnosticados con CBP y 20 pacientes control con otras enfermedades hepáticas crónicas no colestásicas fueron estudiados. Se determinaron anticuerpos séricos anti-*C. pneumoniae* (IgG). Se realizó detección inmunohistoquímica de antígenos de *C. pneumoniae* en hígado. Se extrajo DNA de hígado para amplificación de la secuencia específica de rRNA 16S de *C. pneumoniae* por PCR. Fueron usados controles de amplificación de DNA bacteriano y humano. Los pacientes firmaron consentimiento informado. Se realizó un metaanálisis de la diferencia de riesgo de CBP en pacientes infectados por *Chlamydophila pneumoniae* y en un grupo control. **Resultados:** Los anticuerpos séricos fueron positivos en 30% de los pacientes con CBP y 50% de los controles (p = NS). Antígenos de *C. pneumoniae* no fueron detectados en tejido hepático de pacientes con CBP ni de controles. No se amplificó ADN bacteriano en ninguna de las muestras. El metaanálisis de la diferencia de riesgo mostró gran heterogeneidad de los estudios, por lo que no se realizó una estimación de diferencia de riesgo agrupada. **Discusión:** No encontramos asociación entre infección por *C. pneumoniae* y CBP. En la evidencia actual, un estudio presenta resultados a favor de la asociación entre *C. pneumoniae* y CBP y tres estudios resultados en contra.
Palabras clave: Colangitis biliar primaria, *Chlamydophila pneumoniae*, inmunohistoquímica.

Abstract

Background: Primary biliary cholangitis (PBC) is a chronic cholestatic inflammatory liver disease of unknown cause. Several viral and bacterial pathogens have been proposed as factors that could either trigger an immune response by molecular mimicry or directly be involved in the persistence of biliary damage. There are conflicting reports respecting the role of *Chlamydophila pneumoniae* in the pathogenesis of PBC. **Aim:** To investigate markers of *C. pneumoniae* infection in serum and liver tissue from patients with PBC. **Patients and Methods:** Twenty patients with diagnosis of PBC and 20 control patients with other non-cholestatic chronic liver diseases were studied. Serum anti-*C. pneumoniae* antibodies (IgG) were determined. Liver tissue was available for immunohistochemistry detection of *C. pneumoniae* antigens. DNA was extracted from liver tissue and a specific sequence of *C. pneumoniae* 16S rRNA gene was amplified by CPR. Adequate controls of bacterial and human DNA amplification were used. Informed consent was obtained from patients. A meta-analysis of risk difference of PBC in Chlamydophila pneumoniae infected patients and in the control group was performed. **Results:** Serum antibodies were positive in 30% of patients with PBC and 50% of controls (p = NS). *C. pneumoniae* antigens were not detected in liver tissue neither of patients with PBC nor controls. Bacterial DNA did not amplify in any of the samples, despite good amplification of internal and external controls. Risk difference meta-analysis showed high heterogeneity between studies. Therefore, we did not estimate a pooled risk difference. **Discussion:** Our results do not support the association between *C. pneumoniae* infection and PBC. In the current literature only one study shows an association between *C. pneumoniae* and PBC, but other three studies do not support it.
Key words: Primary biliary cholangitis, *Chlamydophila pneumoniae*, immunohistochemistry.

Introduction

Primary biliary cholangitis (PBC) is a chronic cholestatic disease characterized by progressive inflammation and destruction of bile ducts[1]. Prevalence is greater among women than men, with a ratio of 10:1[2]. The clinical features are diverse, and may include fatigue, pruritus, cognitive impairment, sicca complex, and others. However, up to 50% of patients are asymptomatic at presentation[3]. Prevalence has been estimated in 6.7 to 940 cases per 1.000.000[4].

Ursodeoxycholic acid (UDCA) is the first-line therapy for PBC. It has been widely used, and has shown evidence of improving survival, improving liver biochemistry, and reduction in need of liver transplantation[5]. In case of inadequate response to treatment is recommended the addition of obeticholic acid (OCA), that showed reduction of biochemical parameters. However, efficacy studies are still under development[5,6].

Specific etiology of CBP remains unknown, hence actual treatments are not etiology-based, remarking the importance of searching new therapeutic targets for treatment of PBC, that might improve management of these patients[7].

Regarding pathophysiology, have been postulated a genetic susceptibility to immune reactivity against self-antigens[8]. Anti-mitochondrial antibodies (AMA) bind to a mitochondrial antigen (E2 component of pyruvate-dehydrogenase complex)[9], and consequently, immune response occurs against biliary epithelial cells[8]. Positive AMA titers are present in PBC patient's serum, with a sensitivity of 84.5% and a specificity of 97.8%[10].

For that, PBC has been considered an autoimmune disease. Indeed, over 50% of patients with PBC suffer from other concomitant autoimmune diseases (e.g. Sjogren syndrome, thyroid disorders, CREST, Raynaud, celiac disease, inflammatory bowel disease, and others)[11,12].

Autoimmune diseases are the result of immune reactions against self-antigens, which lead to tissue damage and organ failure[13]. Similarity between self-antigens and exogenous proteins is one of the main hypotheses explaining the "break" in immunotolerance, a phenomenon called "molecular mimicry"[14]. According with framework of the theory, there is evidence supporting the role of certain triggers, such xenobiotics[15], and infectious pathogens, for instance, mycobacterium[16], *E. Coli*, *H. pylori*, *P aeruginosa*, Cytomegalovirus, *H. influenzae*[17], and retroviruses[18]. Other microorganisms, like *Chlamydophila pneumoniae*, also have been studied[19-22].

Chlamydophila pneumoniae is a strictly intracellular gram-negative bacteria, distributed around the world which accounts for 6 to 22% of lower respiratory tract infections in children and adults[23]. It has been considered the etiologic agent in 7.8% of community acquired pneumonias in Chile[24]. Microbiological characteristics of *Chlamydophila pneumoniae*, together with the capacity to isolate its antigens and DNA from samples of human tissue, have driven investigation pointing towards its relationship with multiple pathologies, e.g. asthma[25], arteriosclerosis[26], as well as PBC[19]. However, actual evidence is controversial about a clear role or association between *Chlamydophila pneumoniae* and the pathogenesis of PBC.

The aim of this study was to evaluate markers of *C. pneumoniae* infection, in serum and liver tissue of patients with PBC in a medical center in Chile.

Material and methods

Patients/subjects

This study included a total of 40 patients, evaluated at Liver Disease Clinic in San Joaquin Medical Center from July 2004 until March 2005. Patients were divided into 2 groups depending on disease: PBC group included 20 patients while control group included 20 other hepatic disease patients.

Diagnosis of PBC was based on clinical features, liver biochemistry and biopsy. Subjects were excluded if they presented respiratory symptoms at moment of blood sampling for serology, or if diagnosis of PBC was uncertain, or if liver disease could be attributed to causes different from PBC.

This study was approved by the Ethics Committee of the Pontificia Universidad Católica. All subjects signed an informed consent.

Serology

Serology was performed in all 40 patients in study. Detection of antibodies against *Chlamydophila pneumoniae* was performed using microimmunofluorescence of serum samples from patients[27]. Titers greater than or equal to 1:64 were considered positive.

Immunohistochemistry

Immunohistochemistry was performed in 26 subjects (13 subjects from the PBC group and 13 controls). Paraffin was used to preserve samples for histological analysis. Specific monoclonal antibodies were used for immunohistochemical testing (ViroStat® Portland, ME). Antigen detection by inmunohystochemistry is based on recognition of specific antigens in tissue samples by monoclonal antibodies; these are fixed in the tissue and then recognized by a marked secondary antibody (polivalent, anti-goat and anti-rabbit) which gives the sample a brown color under optic microscopy. Positive reaction was determined by the presence of brown intracytoplasmic granules in hepatocytes. The examiner pathologist was not in-

formed about neither pathology nor serologic results.

PCR

PCR was performed in 10 subjects (5 subjects from the PBC group and 5 controls). Microtomy was used to obtain 4 to 5.5 µm thickness slices from each sample. A stove at 60° C was used to dry them for 1 hour. Then, pure Xilol was applied for 15 minutes to eliminate paraffin. Dehydration was achieved using descending alcohol concentrations every 10 minutes (100-100-100-95-70). Hematoxylin stain was applied for 2 minutes and then slices were left to dry.

Samples were microdisected with scalpel and then centrifuged at 14.000 rpm for 10 minutes for DNA extraction. K protease (Sigma) 200 mg/ml (100 µl per tube) were added to each sample before incubation in a stove set at 37°C for 48 hours; inactivation of the protease was then achieved by heating the samples at 100°C for 10 minutes. Finally, samples were centrifuged for one minute and then stored at 20°C[28].

Then 0.5 µM of the extracted DNA was amplify used a master mix. It included: 0.5 µM primers, 0.2 µM dNTPs (Invitrogen), 1×PCR buffer, 2.5 mM $MgCl_2$ and 2.5 units of Taq polymerase (Accuprime·Invitrogen). The following sequences were used as specific primers for amplification of the 16S rRNA gene for Chlamydia *pneumoniae*: 5'-TGACAACTGTAGAAATACAGC-3' (CpnA) and 5'-CGCCTCTCTCCTATAAAT-3' (CpnB)[29]. A thermocycler was used for the reaction (MJ Research INC, Model PTC 100). It was programmed for 5 minutes denaturation at 94°C, then 35 denaturation cycles (94°C, one minute each cycle), mating (56°C for one minute) and extension (72°C for one minute). The expected amplified fragment contains 463 base pairs. A purified extract from AR-39 strain *Chlamydophila pneumoniae* CE was used as positive control (Washington Research Foundation, Seattle). To assess integrity of the extracted DNA, parallel amplification of DNA from the 16S human ribosomal subunit gene was performed (Quantum RNA 18S Internal Standards, Ambion Inc. Texas, USA). The expected fragment contained 489 base pairs.

Meta-analysis

The literature databases PubMed, Web of Science (WoS), and SciELO were searched for observational studies evaluating association between markers of Chlamydophila pneumoniae infection and Primary Biliary Cholangitis. To identify studies in PubMed, the following search strategy was used: "Primary biliary cholangitis AND Chlamydophila pneumonia".

The following information was selected from each article: first author, year of publication, study location (country), sample size, risk in exposed/non-exposed group, and risk difference (RD) (Risk or proportion of PBC in Chlamydophila pneumoniae infected patients-risk or proportion of PBC in the control group).

Statistical analysis

For categorical variables, we compared relative frequencies using a Fisher's exact test or chi-squared test. Confidence intervals at 95% were estimated for frequencies/proportions (95% CI). To compare the prevalence of serum antibodies between PBC patients and control group the Acock´s test of proportions was applied (*prtest* command in STATA software). In order to estimate type II error a power analysis for a two-sample proportions test was carried out.

To calculate a pooled risk difference, we performed a random effects meta-analysis using the *metan* command in STATA software. Between-study heterogeneity was measured by I^2, which quantifies percentage of the variability in effect estimates due to heterogeneity rather than sampling error. For all analyses, a two-sided statistical significance was considered at 5% level. All statistical analyses were performed in STATA 14 (StataCorp. LP, College Station, TX).

Results

Patient characteristics are summarized in *Table 1*. The mean ages were 57.5 ± 10 years and 58.5 ± 14 years in PBC and control groups, respectively. All PBC patients were female, whereas 25% (5/20) patients in the control group were male.

Serology was positive in 30% (95% CI 9.9-50.1) of the subjects and 50% (95% CI 9.9-50.1) of the controls; this difference in proportions showed no statistical significance (p = 0.196). The statistical power for testing differences mentioned above related to sample size (n = 40) was 0.25. Immunohistochemistry resulted negative for both PBC and control patients. Results are summarized in Table 1.

Amplification of *Chlamydophila pneumoniae* DNA was negative in all samples. Parallel amplification used as a control (human ribosomal 18S subunit) was positive in all samples. Results are summarized in Table 1 and illustrated in Figure 1.

Meta-analysis

The literature search identified a total of 8 studies[19-22,30-33]. Following exclusion criteria, one study was excluded[19] because it did not report results of serology testing. Two studies met the inclusion criteria[20,22] and were analyzed with our current study, with a combined sample size of 361 patients. The characteristics of the studies are shown on Table 2. One study showed a positive and significantly high RD (55% more risk of PBC in CP+ group)[20]. However, in Montaño-loza et al., RD was negative. Finally,

Table 1. Patient's characteristics and results for serology, immunohistochemistry and bacterial DNA amplification

	PBC (n = 20)	Controls (n = 20)	p-value
Age years old (range)	57,5 ± 9,88 (43-79)	58,5 ± 14,29 (18-75)	
Gender male (%)	0 (0)	5 (25)	
Child-Pugh			
A (%)	16 (80)	12 (60)	
B (%)	4 (20)	7 (35)	
C (%)	0 (0)	1 (5)	
Autoantibodies serology			
Positive AMA (%)	19 (95)	2 (10)	
Positive ANA (%)	10 (50)	7 (35)	
Liver biopsy fibrosis stage			
1 (%)	15 (75)	-	
2 (%)	2 (10)	-	
3 (%)	2 (10)	-	
4 (%)	0 (0)	-	
Associated pathologies			
Thyroid disease (%)	6 (30)	-	
Sjögren (%)	9 (45)	-	
CREST (%)	3 (15)	-	
Raynaud (%)	4 (20)	-	
Etiology			
PBC	20 (100)	0 (0)	
Autoimmune hepatitis (%)	-	10 (50)	
Hepatitis C virus (%)	-	5 (25)	
NASH (%)	-	4 (20)	
Hemochromatosis (%)	-	1 (5)	
***C. pneumoniae* positive serology results**			
Total (%)	6 (30)	10 (50)	
Autoimmune hepatitis (%)	-	4/10 (40)	0,33
Hepatitis C virus (%)	-	3/5 (60)	
NASH (%)	-	3/4 (75)	
Hemochromatosis (%)	-	0/0 (0)	
Positive immunohistochemistry	0/13	0/13	NS
Positive DNA	0/5	0/5	NS

AMA: antimitochondrial antibody, ANA: antinuclear antibody, NASH: non-alcoholic steatohepatitis, PBC: primary biliary cholangitis.

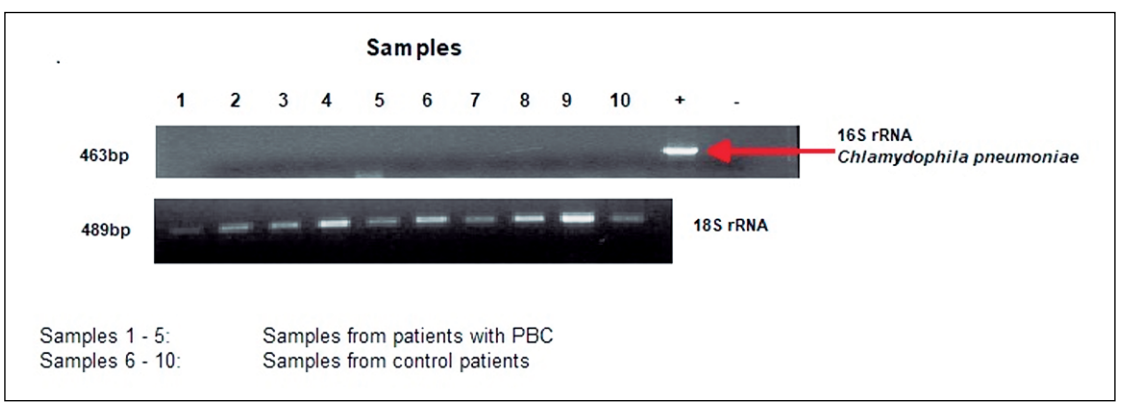

Figure 1. Results of amplification of *C. pneumoniae* DNA with polymerase chain reaction in liver tissue.

Table 2. Sample characteristics and differences in proportions (risk difference) of PBC by Chlamydophila pneumoniae serology results reported in studies selected

Study	Study location	Sample size	Risk of PBC in exposed group (CP +)	Risk of PBC in non-exposed group (CP -)	Risk difference	95% CI
Leung et al., 2003	USA	170	0.57	0.01	0.55	0.45–0.66
Montaño-Loza et al., 2006	Mexico	151	0.47	0.70	-0.25	-0.42– -0.08
Soza et al., 2019	Chile	40	0.30	0.50	-0.20	-0.50–0.10

PBC: Primary Biliary Cholangitis, CP: Chlamydophila pneumoniae.

a non-significant risk difference was observed in this current study. Risk difference meta-analysis showed high heterogeneity between studies (I2 = 97.6%). Therefore, we did not estimate a pooled risk difference.

Discussion

C. pneumoniae serology showed no statistically significant differences between subjects and controls, and seroprevalence results (30%) were lower than in the general population reported in a Chilean study (61.4%)[34]. Negative immunohistochemistry (IHC), must be interpreted as absence of bacterial antigens in samples studied, and negative amplification of bacterial DNA must be interpreted as absence of bacterial DNA in samples, confirming negativity of IHC.

Our results differ from those published by Abdulkarim et al[19], in which IHC was positive in 100% of the samples studied from PBC patients, and in-situ hybridization for *C. pneumoniae* 16S RNA was reported as positive in 86% of the PBC samples. However, other studies show no association between *C. pneumoniae* and PBC. Leung et al[20] found negative results for 16S RNA and IHC for *C. pneumoniae* in all individuals studied. Liu et al[21], showed an elevated specific *C. pneumoniae* IgG seroprevalence in PBC patients respect to healthy patients, although there wasn't a significant difference with post-hepatitis patients. Consequently, their results don´t support the role of *Chlamydophila pneumoniae* triggering the autoimmune disease. Another study, conducted by Montaño-Loza et al[22] didn't found association between serology titers of *C. pneumoniae* and PBC, though they concluded that probably the bacteria plays a role in severity of disease.

In conclusion, our results don't support the idea that *Chlamydophila pneumoniae* is related to the pathogenesis of CBP.

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