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Chlamydial antigen and nucleic acid detection in liver biopsies from patients with chronic cholelithiasis

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- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
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Summary

Background:

It is known that chlamydial species can propagate in hepatocyte cell lines. Moreover, some clinical cases of chlamydial infection involve liver abnormalities. This study was to clarify whether chlamydial markers (protein and nucleic acids) could be detected in liver biopsies from patients with calculous cholecystitis.

Material/Methods:

Liver biopsies were obtained from 39 patients during cholecystectomy and analyzed with immunohistochemical, nucleic acid amplification and serological protocols. Liver specimens from 8 trauma victims served as controls.

Results:

It was shown that from 39 patients with cholecystitis 19 gave considerable signal generated by antibodies against *C. trachomatis* (15 patients) or *C. pneumoniae* (4 patients). 10.2% (4/39) of the samples contained detectable 16S rRNA genomic sequence from *C. pneumoniae* while amplifiable fragments of 16S rRNA and pLGV cryptic plasmid from *C. trachomatis* were found in 20.5% (8/39) of DNA specimens. The control group had a zero detection rate for chlamydial genetic markers in the liver. Simultaneous detection of genetic and immunohistochemical markers validated by positive serological status took place in a very limited number of the patients (4 cases for *C. trachomatis* and 2 cases for *C. pneumoniae*). Moreover, it was shown that *C. trachomatis* and *C. pneumoniae* can efficiently propagate in freshly isolated rat primary hepatocytes forming infectious progeny.

Conclusions:

Identification of chlamydial markers in liver biopsies along with the ability of the chlamydial pathogens to propagate in native hepatocytes may suggest the possible involvement of chlamydial species in inflammatory hepatobiliary disease.

Key words:

***C. trachomatis* • *C. pneumoniae* • liver biopsies • cholelithiasis • PCR • immunohistochemistry**

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BACKGROUND

C. trachomatis and *C. pneumoniae* are two major human pathogens from the genus Chlamydia [1]. It was postulated long ago that chlamydial species have a strict tissue tropism. *C. pneumoniae* preferentially targets epithelial cells of the respiratory system, whereas *C. trachomatis* is known to infect epithelial cells of the urogenital tract and conjunctivae [2,3]. However, some chlamydial strains can easily penetrate submucosal membrane and infect lymphocytes, spreading through human body via regional lymphatic and blood vessels [4]. Among the different serological variants of *C. trachomatis* at least the L1, L2 and L3 serotypes are believed to have distinct invasive properties [5]. In contrast, other serovars (ocular A-C and genital D-K) restrict their propagation to mucosal epitheliocytes [5,6]. The invasive properties of *C. trachomatis* can explain the appearance of the pathogen in some extragenital tissues and fluids of the human body – liver, synovial exudates, ascitic fluid and respiratory secretion fluid [7–10].

Invasiveness is also an unquestionable feature of *C. pneumoniae*. Its isolates have been obtained from respiratory secretion fluid as well as nasal, tracheal and lung tissue of patients [11–13]. There are numerous reports on detection of *C. pneumoniae* in atherosclerotic plaques [14], myocardium [15], brain [16] cerebrospinal fluid [17] and joints [18].

Chlamydial species target different organs since there are a remarkable variety of eukaryotic cells supporting chlamydial growth. Chlamydia can efficiently propagate in mononuclear cells [18] as well as in astrocytes, microglia, muscle cells and myocardiocytes [19–23]. Thus, the striking ability of chlamydial pathogens to accomplish their developmental cycle in non-epithelial cells is likely to be a crucial determinant for generalization of chlamydial infection *in vivo*.

Among cells recently discovered to be capable of supporting the chlamydial life cycle are hepatocytes [24,25]. We have recently reported that *C. trachomatis* and *C. pneumoniae* can efficiently propagate in a human hepatoma cell line – HepG2 cells. Chlamydial growth in a HepG2 cell line affects transcription of some liver-specific genes and leads to the formation of infectious progeny [26]. However, immortalized hepatoma cell lines have a very remote resemblance to the phenotype of “native” hepatocytes due to irreversible dedifferentiation [27]. Therefore, neither the effect of infectious agents on hepatic function nor their developmental cycle in liver can be accurately studied using hepatoma cells [28]. Primary hepatocytes whose phenotype can be efficiently preserved in the short term [29] might be a much better option for acute *in vitro* experiments with hepatotropic pathogens.

Our recent paper also originates from the assumption that molecular markers of chlamydial pathogens might be detected in human liver biopsy material obtained from patients with inflammatory hepatobiliary disease.

Here we report that *C. trachomatis* and *C. pneumoniae* can efficiently propagate in freshly isolated rat primary hepatocytes forming infectious progeny. Both pathogens can be detected in liver biopsies obtained from patients with cholelithiasis using specific immunochemistry and nucleic acid amplification protocols.

MATERIAL AND METHODS

Patients

The clinical work was conducted at the Razumovsky Medical University and Institute of Cardiology (Saratov, Russian Federation) from January 2007 to January 2008. The study protocol was approved by the local Ethical Committee. All patients were informed about the purpose of the study and have given written consent regarding participation in the study. The major group of the study included 39 patients who underwent open cholecystectomy due to symptomatic chronic calculous cholecystitis (mean age 52.4±6.2; range 38–64 years; 21 females, 18 males). Liver biopsy specimens were obtained during surgery from the hepatic areas adjacent to the gall bladder. Serum specimens were collected from all patients before surgery and stored at –80°C for retrospective determination of antibodies specific to *C. trachomatis* and *C. pneumoniae* in addition to PCR analysis. The study did not interfere with pre-operative therapeutic options or have an impact on post-operative treatment chosen by physicians for each consenting individual. All patients included in the study were negative for features of pelvic inflammatory disease and Fitz-Hugh-Curtis syndrome.

Control specimens (liver and blood) were collected using aseptic technique from 8 trauma victims with uncompromised medical anamnesis within 12 hours of death (aged from 29 to 53 years, 4 females, 4 males). No macro-microscopic evidence of cholelithiasis or other hepatobiliary pathology has been found among specimens added to the control group.

Specimen handling

All specimens were collected in the operating room under sterile conditions. Liver specimens approximately 4×4 mm in length were placed in microcentrifuge tubes. Transport vials were sealed in the operating room and opened only in the laminar air flow safety cabinet at the Department of Medical Microbiology in the Gamaleya Institute of Epidemiology and Microbiology (Moscow, RF). All specimens were kept at –70°C until processing. Dissected tissue was homogenized using a sterile glass grinder. Chromosomal DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method according to the DNA Miniprep protocol of Wilson (30). This method is known to remove complex polysaccharides interfering with PCR amplification.

Reagents and bacteria

All reagents were purchased from Sigma-Aldrich unless mentioned otherwise. The *C. trachomatis* strain L2/Bu434 and the *C. pneumoniae* strain *Kajjani-6* were used as reference cultures. Both of them were kindly provided by Prof. P. Saikku (University of Oulu, Finland). *C. pneumoniae* *Kajaani-6* strain was propagated in *Mycoplasma*-free HL cells whereas *C. trachomatis* *Bu434* strain was propagated in *Mycoplasma*-free McCoy cells grown in RPMI-1640 medium supplemented with 2 mM l-glutamine (Invitrogen), 5% fetal bovine serum, 50 µg/ml of gentamicin sulfate and 1 mg/ml of cycloheximide. Infectious elementary bodies were isolated [31] from McCoy cells by sonication, washed in phosphate buffered saline, purified by Renografin gradient centrifugation and

kept frozen at -80°C in SPG buffer (pH 7.2; 250 mM sucrose, 10 mM sodium phosphate, 5 mM l-glutamic acid). Chlamydial titers were determined by infecting host cells with 10-fold dilutions of thawed stock suspension.

Bacteriological assay

After overnight transportation biopsy specimens were cut into ~100 mg segments and homogenized separately with a Heidolph Silent Crusher M (Germany) in 1 ml of RPMI-1640 at 4°C . Equal parts of the resulting suspensions were used for PCR and cell culture. Infection of the host cell monolayers (HL and McCoy cells) was performed by centrifugation of 24 well plates at 1500 g for 30 min. Supernatants were replaced with fresh RPMI-1640 containing 1 $\mu\text{g}/\text{ml}$ cycloheximide and plates were incubated at 37°C with 5% CO_2 for 3 days. Cells were harvested for DNA extraction. Chlamydial growth was evaluated by comparison of bacterial loads in inocula and harvested monolayers by TaqMan-PCR.

Serological evaluation

Chlamydial antibody titers (IgG, IgM and IgA) were measured according to the standard microimmunofluorescence (MIF) protocol [32]. In brief, chlamydial particles grown and purified from HL (*C. pneumoniae*) or McCoy cells (*C. trachomatis*) were filtered and resuspended in 0.02% formalin in Dulbecco solution. Bacterial suspensions normalized in protein content were kept frozen at -80°C until the assay was performed. Chlamydial antigens were spotted in a 15-circle area on glass slides (ICN Biomedicals, UK), dried and fixed with acetone. Diluted sera and anti-human isotype-specific FITC-labeled antibodies were applied to the glass slides. For *C. trachomatis* IgG titers $\geq 1:64$ or a collective increase in IgG $\geq 1:64$ and IgM (or IgA) $\geq 1:8$ were considered as evidence of positive serological status. *C. pneumoniae* IgG titers $\geq 1:128$ alone or IgG $\geq 1:64$ combined with IgM (or IgA) $\geq 1:8$ were assumed to witness the seropositivity of the patients.

In addition, IgG titers specific to *C. trachomatis* cHSP60 protein were measured in serum specimens using a ChlamiBest cHSP60-IgG kit (Vector-Best Inc, RF).

Immunohistochemistry

Deparaffinized and rehydrated 7–10 μm liver sections were blocked in PBS with 1% FCS overnight at 4°C . Monoclonal antibody against lipopolysaccharide of *C. trachomatis* or polyclonal antibody specific to the major outer protein (MOMP) of *C. pneumoniae* (both from NearMedic, RF) were used for immunohistochemistry analysis. After 2 hours incubation with FITC-labeled primary antibodies (5 $\mu\text{g}/\text{ml}$, 37°C) the sections were washed in PBS 3 times and analyzed using a Nikon Eclipse 50i microscope.

Assessment of infective progeny

In order to assess the infective progeny accumulation in rat primary hepatocytes after a 48 hour cultivation period, infected hepatocytes were harvested, frozen and thawed, as described elsewhere. Serial dilutions of lysates were inoculated onto monolayers of HL cells or McCoy cells to verify the growth of *C. pneumoniae* or *C. trachomatis* respectively. The plates were centrifuged for 0.5 hour at 1500 g. The

infected cells were visualized with anti-chlamydial genus-specific monoclonal FITC-labeled antibodies (NearMedic, RF) after 48 hours.

Primary hepatocyte isolation

Primary hepatocytes were isolated from Sprague-Dawley rat liver of non-fasted rats by the collagenase perfusion method as described [33]. Animals 6–8 wks old were obtained from Pushino Animal Breeding Facility (Moscow, RF) and kept in the animal facility in compliance with the “Declaration of Helsinki and Guiding Principles in the Care and Use of Animals” under an approved protocol at the Gamaleya Institute for Epidemiology and Microbiology (Moscow, RF). Livers of halothane-anesthetized rats were perfused *in situ* through the portal vein with warmed (37°C) Liver Perfusion Medium and later with Liver Digest Medium (Gibco/BRL, UK). Livers were excised and the hepatic capsule disrupted with needles in Digest Medium. The resulting cell suspension was filtered and washed twice by low-speed centrifugation (20 g, 3 min, 4°C) in ice-cold DMEM with 10% FCS and penicillin/streptomycin (100 $\mu\text{g}/\text{ml}$ each). Remaining non-hepatic cells were eliminated by pre-absorption on 100 mm plastic dishes at 37°C for 20 min. Viability and purity of unattached cells were determined before plating. Cell suspensions with a viability rate $\geq 90\%$ in the trypan blue exclusion test were used. Purified primary hepatocytes were plated onto BD BioCoat™ collagen-coated cover slips. After 3 hours attachment cell monolayers were washed with serum-free DMEM containing 0.4% glucose and 2 $\mu\text{g}/\text{ml}$ cycloheximide. 6-well plates with inserted cover slips were infected with *C. trachomatis* or *C. pneumoniae* by centrifugation at 1500 g for 30 min at multiplicity rate 2. After incubation at 37°C for 48 hours (95% O_2 , 5% CO_2) cover slips were fixed with acetone. Permeabilized cells were stained by direct immunofluorescence using anti-chlamydial genus-specific FITC – conjugated monoclonal antibody (NearMedic, RF). Inclusion-containing cells were visualized using a Nikon Eclipse 50i fluorescence microscope at $\times 1350$ magnification.

DNA isolation

Extraction of total nucleic acids was conducted with a NucliSENS® easyMAG® automated system (BioMerieux Inc., Netherlands). Briefly, ~50 mg of biopsy specimens were homogenized with 1 ml of lysis buffer (BioMerieux) containing 0.25 mg/ml proteinase K (Promega, USA). After 3 hours of incubation at 55°C digested specimens were loaded onto the NucliSENS® easyMAG® platform. Loading of samples, reagents and disposables were the only manual steps during the DNA extraction procedure using the NucliSENS® easyMAG® platform. Up to 24 samples were analyzed in one BioMerieux automated run. DNA was eluted from the cartridges with 50 μL of BioMerieux elution buffer. Bacterial load in serum specimens and bioptates is shown below in genome equivalents of per ml of serum or in genome equivalents of the pathogens referred to 10^6 copies of eukaryotic β -actin (liver specimens). Calibration standards were prepared using amplified fragments of 16S rRNA from *C. pneumoniae*, 16S rRNA and pLGV440 from *C. trachomatis*, or eukaryotic β -actin and cloning them in the pGEM-T plasmid vector (pVU56) using a TA cloning kit (Invitrogen, San Diego, CA) similarly to Broccolo’s protocol [34].

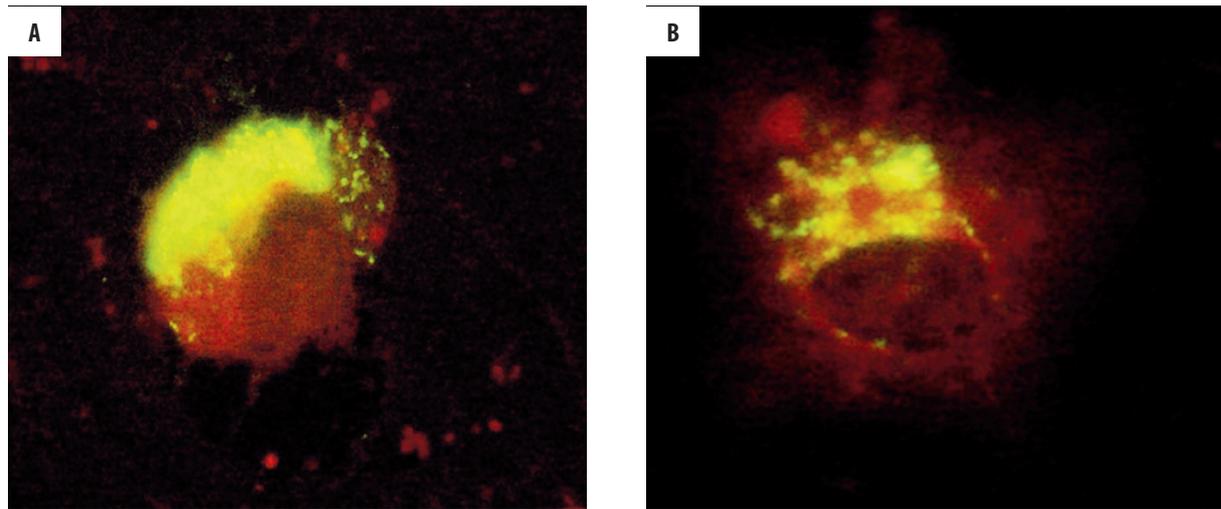


Figure 1. Immunofluorescent staining in rat primary hepatocytes infected with *C. trachomatis* (A) and *C. pneumoniae* (B). Rat primary hepatocytes we isolated, plated and infected with *C. trachomatis* (A) and *C. pneumoniae* (B) and stained with FITC-labeled genus-specific antibody against chlamydial lipopolysaccharide as described in the "Material and Methods". The slides were visualized and photographed using 90 × immersion objective.

Quantitative TaqMan-PCR

For quantification purposes, real-time PCR for 16S rRNA of *C. pneumoniae* and for 16S rRNA and cryptic plasmid of *C. trachomatis* was conducted. PCR primers and TaqMan probes for 16S rRNA *C. pneumoniae* (GenBank accession number), for 16S rRNA of *C. trachomatis* (GenBank accession number AM884176) and cryptic plasmid of *C. trachomatis* (GenBank accession number X06707.3) were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA) and synthesized by Syntol Inc. (Moscow, RF). Designed primers and TaqMan probes were: for 16S rRNA of *C. pneumoniae* forward primer, 5'-GGTCTCAACCCCATCCGTGTCGG-3'; reverse primer, 5'-TGCGGAAAGCTGTATTTCTACAGTT-3'; and TaqMan probe, ROX-TCCAGGTAAGGTCCTTCGCGTTGCATCG-BHQ2; for 16S rRNA of *C. trachomatis* forward primer, 5'-GGCGTATTTGGGCATCCGAGTAACG-3'; reverse primer, 5'-TCAAATCCAGCGGGTATTAACCGCCT-3'; and TaqMan probe R6G-TGGCGGCCA ATCTCTCAATCCGCCTAGA-BHQ2; for cryptic plasmid of *C. trachomatis* forward primer, 5'-GGGATTCCTGTAACAACAAGTCAGG-3'; reverse primer, 5'-CCT CTCCCCAGAACAATAAGAACAC-3'; and TaqMan probe ROX-CTCCCAGAG TACTTTCGTGCAAGCGCTTTGA-BHQ2. The predicted sizes of the generated PCR products were 194 bp, 316 bp and 206 bp respectively. An additional BLAST search analysis was conducted to ensure specificity of the primers and probe. Real-time PCR was performed with the iCycler IQ system (Biorad, USA). 2 µl of the extracted DNA was analyzed with the PCR mixture in a total volume of 25 µl. The PCR mixture consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 1,5 mM MgCl₂, 200 µM of each dNTPs, 2,5 U of ThermoStar *Taq* DNA polymerase (Syntol, Moscow, RF); and 5 pmol of both forward and reverse primers and 3,5 pmol probe. The real-time PCR run was 10 min at 95°C, and 50 repeats of 20 sec at 95°C and 50 sec at 62°C. All samples were analyzed in triplicates. A sample was considered positive if three out of three assay results were positive in the triplicate test and if the average value for the PCR runs was greater than or equal to 1.0.

The cycle threshold (C_T) values, defined as the number of cycles at which the fluorescence of the reporter dye first exceeds the calculated background level, were automatically estimated by the instrument for each reaction. C_T values of specimens were plotted against calibration standards of cloned DNA fragment. Gel mobility of amplification products and their sequencing were performed to confirm identity of pathogens in some positive specimens. Specimens with cycle threshold (CT) values exceeding 35 were considered as negatives.

RESULTS

Chlamydial infection in rat primary hepatocytes

Figure 1 shows immunofluorescence (IF) in rat primary hepatocytes after inoculation with *C. trachomatis* (Figure 1A) and *C. pneumoniae* (Figure 1B). IF signal became visible after 20 hours of the postinfection period when some parts of the hepatocyte perinuclear area started to appear slightly opalescent with punctuate and granular structures. Typical inclusion morphology started to emerge in the 48 hour hepatocyte cultures. *C. trachomatis* inclusions were large and had homogeneous IF staining resembling those traditionally observed in McCoy cells. In contrast, multiple granular particles were seen within the *C. pneumoniae* inclusions. These were smaller and had less intense IF signal as compared to *C. trachomatis* infected cells. Formation of chlamydial inclusion bodies within hepatocytes led to nucleus dislocation especially in the case of *C. trachomatis* infection. At later stages (72 h) infected hepatocytes were enlarged, poorly attached and tended to come off the collagen-covered slips. Some of the cells appeared to be ruptured with most of the chlamydial endosomes released. Under the conditions used, successful chlamydial infection has been observed in ~50% of primary hepatocytes regardless of the pathogen type. Lysates obtained from primary hepatocytes infected with chlamydial pathogens were capable of inducing new rounds of chlamydial infection and specific immunostaining in McCoy (*C. trachomatis*) and HL (*C. pneumoniae*) cells (results not shown).

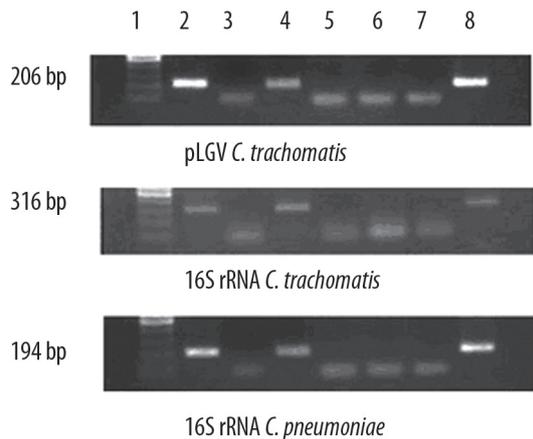


Figure 2. Amplicon size verification by gel electrophoresis. Recovery of PCR products in amplification reactions with different primers (pLGV *C. trachomatis*, 16 S rRNA *C. trachomatis* and 16 S rRNA *C. pneumoniae*) using DNA extracted from liver biopsies and rat primary hepatocytes infected with the chlamydial pathogens: 1 – molecular size standards, 2 – Liver from positive patient V, 3 – Liver from negative patient K, 4 – Primary hepatocytes infected with *C. trachomatis* or *C. pneumoniae*, 5 – uninfected primary hepatocytes, 6 – PCR negative control, 7 – DNA isolation control and 8 – positive control (plasmids with corresponding specific inserts). All procedures were performed as described in the “Material and Methods”.

Moreover, as can be seen in Figure 2 we were able to amplify specific chlamydial genetic markers – pLGV (*C. trachomatis*) and 16S rRNA (*C. trachomatis* and *C. pneumoniae*) in DNA extracted from primary rat hepatocytes infected with each particular chlamydial pathogen.

Serological evaluation of the patients

Table 1 shows the results of serological status of the patients. As can be seen, IgG seropositivity for *C. pneumoniae* seems to be quite a common finding affecting 69.2% of the patients with cholelithiasis. In contrast, only 20.5% of the patients had detectable IgG levels for *C. trachomatis*. Seroprevalence of anti-HSP60 IgG specific to *C. trachomatis* was in good agreement with the IgG detection rate. Control serum specimens showed a remarkably lower incidence of seropositivity to chlamydial antigens. Isotype-specific response has been mostly limited to the IgG class of immunoglobulins in both groups.

Immunohistochemistry

Immunohistochemistry analysis of liver biopsies revealed that from 39 patients with chronic cholecystitis 19 have had considerable signal originated by preincubation of the sections with antibodies against *C. trachomatis* (15 patients) or antibodies against *C. pneumoniae* (4 patients). Inclusions visualized with antibody against *C. trachomatis* were numerous and large (Figure 2), while inclusions seen in the sections preincubated with *C. pneumoniae* – specific antibodies were much smaller and less abundant (Figure 3). Among control liver biopsies positive immunostaining for *C. pneumoniae* was not detected in any specimens and signal generated with *C. trachomatis* antibodies was not seen in any specimens either.

In all sections immunohistochemistry signal had no clear association with hepatic vascular topography.

PCR analysis

The nucleic acid amplification protocol used in our study revealed that liver DNA obtained from 10.2% (4/39) of patients with cholelithiasis contained detectable 16S rRNA genomic sequence of *C. pneumoniae*. No positives were detected in the control group.

On the other hand, amplifiable fragments of 16S rRNA and pLGV cryptic plasmid of *C. trachomatis* were found in 20.5% (8/39) of DNA specimens extracted from liver biopsies of cholelithiasis patients. Simultaneous detection of genetic and immunohistochemical markers was found in 12.8% (5/39) of patients. The control group had a zero detection rate of chlamydial pathogen genetic markers in the liver. Amplification products were routinely analyzed in gel electrophoresis with all relevant controls (Figure 4). Amplicons derived from RT-PCR reactions with hepatic DNA matched up in their gel mobility to the amplification products derived from reference cultures and primary rat hepatocytes infected with chlamydial pathogens. Randomly chosen positive PCR reactions (5 total) were subjected to sequencing and confirmed the identity of amplicons and the specificity of PCR analysis.

All attempts to quantify bacterial load in liver tissue were complicated to some extent by variations in triplicates and some differences in β -actin counts in hepatic DNA specimens. However, our best estimate of the bacterial load for chlamydial pathogens in the liver tissue is very low with a median value for *C. trachomatis* $\sim 8.5 \times 10^2$ copies/ 1×10^6 copies of β -actin. The corresponding value for *C. pneumoniae* was $\sim 5.5 \times 10^3$ copies/ 1×10^6 copies of β -actin.

Bacteriological assay

We failed to obtain culturally retrievable isolates of the chlamydial pathogens from the liver specimens. PCR quantification of the genetic markers in the inocula and the post-cultivation DNA specimens showed no significant difference in the amounts of pLGV cryptic plasmid for *C. trachomatis*. In 4 cases (cholelithiasis group) there was a measurable increase in the amount of 16S rRNA for *C. pneumoniae* after cultivation of liver biopsy material in HL cells.

DISCUSSION

Liver cell heterogeneity predetermines the remarkable diversity of hepatic functions. Parenchymal cells (hepatocytes) as well as non-parenchymal cells (Kupffer cells, stellate cells and hepatic endothelial cells) are reported to be involved in the innate immune response to different pathogens. Yet their involvement in the pathogenesis of chlamydial infection remains unknown. As we have published previously [25,26], *C. trachomatis* and *C. pneumoniae* can efficiently propagate in an immortalized hepatic cell line (HepG2 cells). However, HepG2 cells do not display the whole array of hepatic markers and functions. Hepatoma cell lines are known to have abnormal gene expression, uncontrolled proliferation, anomalous signaling and atypical receptor turnover [35]. Therefore, it was essential for us to explore

Table 1. Serological variables.

Group	MIF – <i>C. pneumoniae</i>			MIF – <i>C. trachomatis</i>			ELISA <i>C. trachomatis</i> HSP60 IgG
	IgG	IgM	IgA	IgG	IgM	IgA	
Control (n=8)	2	0	0	1	0	0	0
Cholelithiasis (n=39)	27	0	1	8	0	0	9

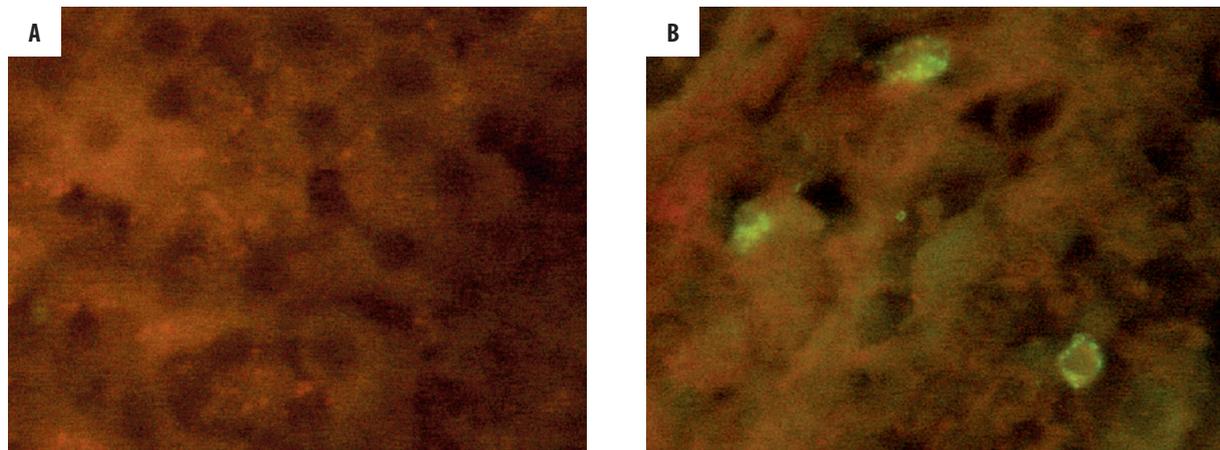


Figure 3. Immunohistochemical staining of liver biopsy with non-immune IgG (A) *C. trachomatis* – specific antibody (B) liver biopsies were obtained, processed and hepatic sections were immuno-stained with non-immune IgG (A) and monoclonal antibody against monoclonal antibody against lipopolysaccharide of *C. trachomatis* (B) as described in the “Material and Methods”.

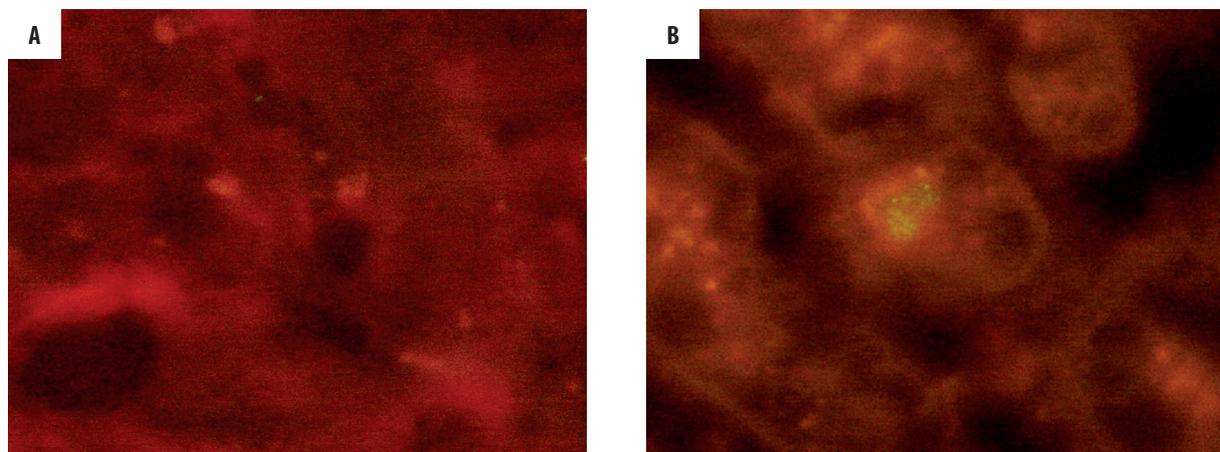


Figure 4. Immunohistochemical staining of liver biopsy with *C. pneumoniae* antibody. Liver biopsies were obtained, processed and hepatic sections were immuno-stained with non-immune IgG (A) and polyclonal antibody specific to the major outer protein (MOMP) of *C. pneumoniae* (B) as described in the “Material and Methods”.

at this stage whether chlamydial biovars could propagate in freshly isolated primary hepatocytes whose phenotype remains relatively well preserved on a collagen matrix in the short term [36].

Herein we show that freshly isolated rat hepatocytes provide perfect support for the full developmental cycle of both chlamydial pathogens. Primary hepatocyte infection caused by *C. trachomatis* or *C. pneumoniae* is productive and leads to the formation of infective progeny. Overall dynamic and morphological features of the chlamydial infectious

cycle in the primary hepatocytes remarkably resemble those seen in the classical cell lines used for chlamydial research (McCoy and HL cells).

Secondly, and most importantly, in the recent work we have shown that chlamydial antigens as well as chlamydial genetic markers can be detected in the human liver of patients suffering from cholelithiasis. Immunostaining analysis for chlamydial antigens is known to produce a high positivity rate among patients (37) which was the case for the liver sections in our study. A smaller number of the patients were

positive in TaqMan PCR. It is essential that the PCR signal in human liver was attributable to the chlamydial primary rRNA sequences which are indicative of active infection since extrinsic chlamydial DNA is rapidly degraded by restriction endonucleases [37,38]. There was no reasonable agreement between the detection rate of chlamydial markers in liver biopsies and serological status of the patients especially in case of *C. pneumoniae*. Simultaneous detection of genetic and immunohistochemical markers validated by positive serological status took place in a rather very limited number of the patients (4 cases for *C. trachomatis* and 2 cases for *C. pneumoniae*).

Even though our results may present an obvious step forward in the understanding of chlamydial diseases, there are some significant limitations in the relevance of our data to clinical practice. To begin with, although immunostained cells in the hepatic sections noticeably resemble hepatocytes, our current results do not disclose clearly what types of hepatic cells have positive immunostaining for chlamydial antigens in liver tissue. Theoretically, besides hepatocytes, whose ability to support chlamydial growth is shown in our previous and recent work, some other hepatic cells such as Kupffer and endothelial cells can bear viable chlamydial pathogens [39,40]. Although immunohistochemical study on cell type identification is now under way in our lab and might be extremely valuable for future therapeutic strategies, this question is of secondary significance to the current paper. It is rather more important to us to report at this stage the fact that hepatic biopsies may contain chlamydial immunohistochemical and genetic markers. This suggests that the liver might be a target organ for chlamydial infection, harboring active pathogens in the human body.

Another concern arises from the very low values for bacterial load in biopsy material revealed by both the nucleic acid amplification protocol and the immunohistochemistry method. However, a low copy number is rather a common problem for human specimens. Detection of *C. pneumoniae* in atherosclerotic plaque often approaches the sensitivity limit of the RT-PCR assay [41]. A low number for *C. trachomatis* bacterial load has been also reported for synovial fluid from patients with reactive arthritis [42].

At first sight there is a worrisome discrepancy between infection rate seen in primary rat hepatocytes and liver biopsy specimens from cholelithiasis patients. However, such disagreement may originate from, the use of a centrifugation protocol to infect primary hepatocyte monolayers. Although centrifugation remains a main conventional tool in infecting cultured cells with chlamydial species, such a highly artificial procedure has no analogy *in vivo*. Centrifugation may force attachment of chlamydial particles to the cell membrane despite low affinity of the pathogen for the host cell.

However, our previous data revealed that there might be a highly-specialized and exclusive mechanism for chlamydial entry to the hepatocytes. We have shown that *C. trachomatis* and *C. pneumoniae* bind to ApoB-containing lipoproteins boosting infectivity rate of chlamydial particles in a hepatoma cell line. As a result, LDL and VLDL receptors can facilitate the entry of *Chlamydia* into hepatocytes [25]. Therefore, even random attachment of chlamydial particles to the cell membrane is likely to be followed by receptor-facilitated

entry of the pathogens into the hepatocytes. We have assumed previously [25] that abnormalities of cholesterol homeostasis associated with the increase of ApoB-containing lipoproteins (VLDL and LDL) may promote enhanced uptake of chlamydial particles in the liver. In this regard it becomes essential that intrahepatic cholestasis and cholelithiasis are known to be accompanied by dyslipidemia with an increased level of ApoB [43]. Thus, it is conceivable that the appearance of chlamydial markers in hepatic biopsies of patients with cholelithiasis takes place due to pro-atherogenic changes in plasma lipoprotein profile.

Moreover, our results might reflect a possible link between chlamydial infection and liver diseases. Although additional studies are required to back up such an assumption, there are a small number of clinical observations supporting our finding. In particular, immunohistochemical detection of *C. pneumoniae* and/or *C. trachomatis* has been reported previously in liver specimens from patients with prolonged fever, Fitz-Hugh syndrome and biliary cirrhosis [44–46]. In broader terms, a possible role of *C. pneumoniae* in the pathogenesis of the primary biliary cirrhosis has been extensively discussed previously [46]. We also realize that our results do not establish any causal relationship between hepatobiliary diseases and chlamydial pathogens. Fulfillment of Koch's postulates is required to make such a claim [47]. Nevertheless, any assumption regarding the possible role of chlamydial species in the pathogenesis of cholelithiasis would be premature with the exception of their likely contribution to the inflammatory background of the disease.

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