

Isolation in Endothelial Cell Cultures of *Chlamydia trachomatis* LGV (Serovar L2) from a Lymph Node of a Patient with Suspected Cat Scratch Disease

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An inguinal lymph node, removed from a 21-year-old Romanian man suspected of having cat scratch disease, was sent to our laboratory for *Bartonella* culture. Lymph node specimens were inoculated on blood-enriched agar and in an endothelial cell culture system using the centrifugation shell vial technique. Bacteria were grown in cell monolayers and detected as positive with an anti-*Bartonella henselae* rabbit serum. However, such bacteria were identified as *Chlamydia trachomatis* biovar LGV serovar L2 by PCR sequencing techniques. Pathological examination of tissue biopsies was compatible with either lymphogranuloma venereum or cat scratch disease. The shell vial system is suitable for isolation of intracellular pathogens responsible for chronic lymphadenopathies, including *C. trachomatis*, *Bartonella* species, *Francisella tularensis*, and mycobacteria. However, care should be taken when identifying *Chlamydia* spp. and *Bartonella* spp. using polyclonal antibodies, since species of both genera have common antigens which are responsible for cross-reactions.

As a reference laboratory for rickettsial diseases, we have developed a centrifugation cell culture system in shell vials which we use routinely for isolation of rickettsial pathogens in a biosafety level 3-equipped laboratory. Species of the genus *Rickettsia* are grown in Vero cells and are usually recovered from blood and skin biopsy specimens (16, 20). *Coxiella burnetii* is cultured in human embryonic lung fibroblasts and may be grown from blood, liver biopsy specimens, and cardiac valve specimens removed from patients with Q fever endocarditis (19). *Bartonella* species are grown in endothelial cells (ECV 304) and have mainly been recovered from blood, lymph nodes (especially in patients with cat scratch disease), biopsy specimens from cutaneous bacillary angiomatosis lesions, and cardiac-valve specimens from patients with endocarditis (15, 21). However, using the same endothelial cell system in shell vials, we recently isolated other pathogens, such as *Francisella tularensis* (6), *Legionella pneumophila* (14), and *Mycobacterium* sp. (unpublished data) from lymph node specimens, which were originally cultured in an attempt to recover *Bartonella henselae*, the agent of cat scratch disease. We presently report the isolation of *Chlamydia trachomatis* biovar LGV, using the same shell vial system, from inguinal lymph node specimens removed from a patient with typical stage 2 lymphogranuloma venereum (LGV).

MATERIALS AND METHODS

Case patient. A 21-year-old immunocompetent man from Romania was admitted to a hospital in Marseille, in the south of France, on 3 July 1998, while visiting for the soccer World Cup, because of pain in the right inguinal region with a low-grade fever. The patient had undergone surgery for appendicitis when he was 6 years old. On admission, his body temperature was 38.5°C, his arterial pressure was 130/70 mm of Hg, and his pulse rate was 92/min. Examination of the right inguinal region revealed inflammation of the skin with the presence of a swollen painful inguinal mass on palpation. Surgery was performed because of

suspicion of incarcerated inguinal hernia, and it revealed the presence of multiple lymph nodes which were excised for pathological examination and culture.

Pathological examination of lymph node tissue. The lymph node specimens were fixed in 10% formol saline, embedded in paraffin, and sectioned at 5- μ m intervals. The sections were stained with hematoxylin-phloxin-saffron and examined under light microscopy.

Culture of lymph node tissue. The lymph node specimens were homogenized in 1 ml of brain heart infusion broth and inoculated on blood-enriched Columbia agar and Lowenstein-Jensen medium (BioMérieux, Lyon, France). Alternatively, tissue homogenization was performed in Eagle minimum essential medium supplemented with 4% fetal calf serum and 2 mM L-glutamine, and the suspension was inoculated into endothelial cells (ECV 304) grown in shell vials, as previously described for isolation of *Bartonella* species (15, 21). The inoculated shell vials were centrifuged at 700 \times g for 1 h at 22°C and then incubated at 35°C in a CO₂-enriched atmosphere. In this culture system, bacterial growth is usually detected after a 15-day incubation of cultures, either by Gimenez staining of cell monolayers or by an immunofluorescence technique using locally prepared polyclonal rabbit anti-*Bartonella* sp. antibodies and a goat anti-rabbit immunoglobulin (Life Technologies, Merelbeke, Belgium).

Serology. A serum sample was collected at the time of hospitalization. The following serologies were performed: *C. trachomatis*, using the reference microimmunofluorescence technique; *Bartonella* sp., using an immunofluorescence technique described previously (4); human immunodeficiency virus using two enzyme-linked immunosorbent assays (Sanofi Pasteur and Ortho Clinical Diagnostic, Paris, France); *Treponema pallidum*, using both Venereal Disease Research Laboratory (Diagast, Lille, France) and microhemagglutination assay tests (Bayer, Paris, France); and *F. tularensis*, using an immunofluorescence technique with a strain isolated in our laboratory as the antigen (6).

PCR sequencing. DNA was extracted from infected cell cultures, using the QIAmp tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. These extracts were used as templates in different PCR assays: (i) a *Bartonella*-specific PCR assay incorporating the primers QHVE1 and QHVE3, derived from the sequence of the intergenic spacer region (ITS1) of a *Bartonella* species (24); (ii) a PCR assay incorporating primers fD1 and rP2, which allow amplification of 16S rRNA genes from a wide variety of bacterial taxa but not *Chlamydia* species (30); and (iii) a PCR assay incorporating primers fD4 and rD1, derived from 16S rRNA gene sequence, currently recommended for amplification of *Chlamydia* species (30). The 5' end of the amplified fragment obtained with primers fQ4 and rD1 was sequenced using primers fD4 and 536r (GTATTACCGCGGCTGCTG). Sequencing reactions were carried out with a DNA sequencing kit (dRhodamine Terminator cycle-sequencing ready-reaction with AmpliTaq polymerase FS) (PE Applied Biosystems, Washington WA1, 4SR, United Kingdom), and electrophoresis was performed with the ABI PRISM 377 DNA sequencer (Perkin-Elmer). The sequence that was determined was compared, using BLAST software, with sequences deposited in GenBank. Because the amplified sequence showed 100% similarity with that of the *C. trachomatis* 16S rRNA gene, a new PCR sequencing assay was performed in order to determine the biovar and serovar of our strain (32). This new PCR incorporated primers 5'-MOMP and BFP-2, which allow amplification of vari-

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able domains (VD) I and II of the *C. trachomatis* major outer membrane protein (MOMP)-encoding gene, allowing differentiation of serovars (32). The amplified DNA fragment was sequenced with the same primers, as previously described.

RESULTS

Pathological examination of lymph node tissue. Pathological examination of lymph node tissue revealed the presence of central abscesses containing neutrophils and necrotic debris surrounded by epithelioid cells, macrophages, and a few multinucleated giant cells. Although not specific, histological findings were compatible with either cat scratch disease or LGV.

Culture of lymph node tissue. The Gram stain performed on tissue homogenates was negative. Standard cultures and mycobacterial cultures remained sterile after 15 days and 2 months of incubation of culture media, respectively. In contrast, Gimenez staining of cell monolayers, performed on the 15th day of incubation of the shell vials, revealed the presence of intracellular bacteria. Such bacteria were also revealed by the immunofluorescence technique, using anti-*B. henselae* or anti-*Bartonella quintana* antibodies. However, after the identification of these bacteria as *C. trachomatis* by the PCR sequencing technique, subcultures performed using the same ECV304 endothelial cells allowed detection of bacterial growth within 48 h of incubation of the shell vials, which is compatible with the intracellular development cycle of this species (18).

PCR sequencing. No PCR amplification was obtained with primers QHVE1 and QHVE3 or fD1 and rP2. In contrast, the PCR assay using primers incorporating fD4 and rD1 yielded a 500-bp DNA fragment, which was sequenced. The sequence was compared, using BLAST software, with sequences deposited in GenBank and showed 100% similarity with the *C. trachomatis* 16S rRNA gene (accession number M59178). The PCR assay incorporating the primers 5'-MOMP and BFP-2 allowed amplification of a 490-bp DNA fragment including *C. trachomatis* VDI and VDII. The sequence of this fragment showed 99% identity with a MOMP-derived DNA fragment previously described for *C. trachomatis* biovar LGV serovar L2 (32), with 100% homology in the VDI region, whereas variation was noted in base position 490 in VDII, where an adenine was replaced by a guanine, leading to replacement of an asparagine by an aspartic acid as amino acid 142.

Serology. Patient's serum collected at the time of hospitalization displayed anti-*C. trachomatis* antibodies, with immunoglobulin G (IgG) and IgA titers of 1:64 and 1:16, respectively, by microimmunofluorescence. Anti-*B. henselae* antibodies were also detected in patient's serum, with an IgG titer of 1:50 by microimmunofluorescence. Serologies for HIV, *T. pallidum*, and *F. tularensis* were negative.

DISCUSSION

C. trachomatis biovar trachoma and serovars D, E, F, G, H, I, J, and K are responsible for oculogenital infections with worldwide distributions (3). In Europe and North America, these serovars are responsible for the majority of bacterial sexually transmitted diseases. In contrast, LGV, due to *C. trachomatis* biovar LGV and serovars L1, L2, and L3, is a sexually transmitted disease restricted to India, southeastern Asia, sub-Saharan Africa, South America, and the Caribbean. Sporadic cases are diagnosed in other areas (3), including exceptional cases reported in France (26). Serovar L2 is most frequently isolated in areas where the disease is not endemic, whereas serovar L1 is uncommon (2). The disease is characterized by three clinical stages. The first stage corresponds to a

primary genital lesion, usually a small papule or a herpetiform ulcer, which often remains unnoticed. LGV is most often recognized at the second stage, which corresponds to a local lymphadenopathy (most often inguinal), which may evolve to a local abscess (or bubo), which ruptures in approximately 30% of cases, and fistulize to the skin. This stage may include systemic manifestations, such as fever, headaches, and myalgia. The third stage correspond to late fistula and stricture formation.

A specific diagnosis of LGV is established by isolation of *C. trachomatis* from bubo pus in 30% of cases, or occasionally from the cervix in women or the urethra in men, and rarely from systemic sites (3). However, isolation of this pathogen remains difficult, and it can only be done in laboratories with specially equipped biohazard facilities and personnel experienced in cell culture. Only a few isolates have been obtained in the last 20 years, especially in the United States (2) and in Europe (8, 26). More recently developed molecular biology techniques, including PCR-based techniques, may be useful (9, 11). Such techniques are not used routinely for diagnosis of LGV and are not available in most areas where LGV is endemic. Thus, serology using the complement fixation test or the microimmunofluorescence technique remains the diagnostic method most frequently used (3). Antibody titers of $\geq 1:64$ by the complement fixation test or $\geq 1:512$ by microimmunofluorescence techniques are considered highly indicative of LGV in patients with typical clinical presentation (3) but are not fully specific because of cross-reactions among different *Chlamydia* species, biovars, and serovars.

We report a patient with second-stage LGV, a 21-year-old Romanian man infected with *C. trachomatis* biovar LGV serovar L2. The patient presented with an inguinal lymphadenopathy for which cat scratch disease was a possible etiology. Thus, lymph node specimens, as well as a serum sample, were sent to our laboratory for *Bartonella*-specific culture and serology. We used a previously described centrifugation shell vial method for isolation of *Bartonella* species (15, 21). A direct immunofluorescence assay using rabbit polyclonal anti-*Bartonella* sp. antibodies revealed fluorescent bacteria within the cell cultures. However, the use of a PCR sequencing technique led to the unexpected identification of these bacteria as *C. trachomatis* biovar LGV rather than *Bartonella* sp. Such strain identification cannot be considered misinterpretation due to cross contamination, since *C. trachomatis* biovar LGV was never sequenced before in our laboratory. A serum sample collected at the time of hospitalization revealed the presence of anti-*C. trachomatis* antibodies (IgG titer, 1:64; IgM titer, 1:16 [by microimmunofluorescence]) but also low-titer anti-*B. henselae* antibodies (IgG titer, 1:50 by microimmunofluorescence).

Interestingly, our strain was obtained in endothelial cell cultures, whereas McCoy cells are usually recommended for isolation of this species from clinical specimens (3). *C. trachomatis* has been grown in vitro in McCoy cells (28, 29, 31), HeLa cells (13, 23), and BGMK cells (10, 12), including in shell vial systems (10, 29), but to our knowledge this is the first time growth in endothelial cells has been reported for diagnostic purposes. This is compatible with a recent demonstration that *Chlamydia pneumoniae* and also *C. trachomatis* can infect human umbilical vein endothelial cells in vitro (7).

Cross-reactions between *Chlamydia* spp. and *Bartonella* spp. have been previously described (4, 17) and recently characterized (17). These cross-reactions have led to misinterpretation of *Chlamydia* sp. serology in the past. *Chlamydia* species were proposed as probable etiological agents of cat scratch disease (5) because sera from many patients with cat scratch disease

reacted with chlamydial antigens. Recent investigations have shown that *B. henselae* is the major etiological agent of this disease (1, 22), whereas serum reactivity against *Chlamydia* antigens in fact represented cross-reactions. Only 27 *Chlamydia*-related endocarditis cases have been reported in the literature (17, 25). In one case, a specific diagnosis was established by culturing *Chlamydia psittaci* from blood cultures (27), whereas in another case, *C. pneumoniae* DNA was detected by PCR from a left aortic leaflet (25). For all 25 remaining *Chlamydia*-related endocarditis cases, diagnosis was based solely on serology. Using cross adsorption and Western blot procedures, we have recently demonstrated that eight of these cases were probably caused by *Bartonella* sp. (17). Sera from other patients were not available to us, and *Chlamydia* endocarditis should not be considered until the possibility of such antigenic cross-reactions has been eliminated. Thus, the possibility of cross-reactions between species of the genera *Bartonella* and *Chlamydia* should be considered when interpreting results obtained with immunological methods. Both *Bartonella* and *Chlamydia* serologies should be performed concomitantly, especially for clinical manifestations in which species of both genera may be involved. Likewise, direct immunofluorescence technique to detect *Bartonella* or *Chlamydia* species in clinical samples and in cultures should be considered cautiously when using polyclonal antibodies or monoclonal antibodies for which the possibility of reactivity to both *Chlamydia* and *Bartonella* antigens has not been tested.

The present report, together with previous reports from our laboratory (6, 14, 15, 21), reemphasizes the usefulness of the centrifugation shell vial system as a versatile culture system allowing isolation of fastidious facultative or obligate intracellular pathogens.

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