

Failure To Detect *Chlamydia pneumoniae* in Brain Sections of Alzheimer's Disease Patients

JENS GIEFFERS,^{1*} ERICH REUSCHE,² WERNER SOLBACH,¹ AND MATTHIAS MAASS¹

Institute of Medical Microbiology and Hygiene¹ and Institute of Pathology,² Medical University of Lübeck, D-23538 Lübeck, Germany

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A recent North American study detected *Chlamydia pneumoniae* in 17 of 19 brains of Alzheimer's patients and supposed a *C. pneumoniae* infection to be a risk factor for Alzheimer's disease (AD). In this study, we analyzed paraffin-embedded tissue samples of 20 AD patients by nested PCR and immunocytochemistry with a panel of antichlamydial antibodies and could detect neither *C. pneumoniae*-specific DNA nor chlamydial antigens. From our data, the presence of *C. pneumoniae* in the brains of Alzheimer's patients is not a common phenomenon; an association remains questionable.

Chlamydia pneumoniae has recently been established as a third species of obligate intracellular chlamydiae. An epidemiological study revealed a seroprevalence in the adult population of approximately 80% (4). *C. pneumoniae* has been identified as a common cause of community-acquired pneumonia, pharyngitis, and bronchitis. Serological and molecular biological evidence suggests a strong association between a previous or persistent *C. pneumoniae* infection and arteriosclerosis. Viable *C. pneumoniae* could be recovered from atheromatous plaques (3). Recently, *C. pneumoniae*-specific DNA and antigens were reported in 17 of 19 brains of patients with Alzheimer's disease (AD) (1). Therefore, infection with this organism has been suggested to be a risk factor for late-onset AD, a degenerative condition of unknown etiology. In order to reevaluate these findings in a northern European population, we screened brain sections of AD patients for the presence of *C. pneumoniae* genomic DNA and three different antigens by PCR and immunocytochemistry protocols that had been previously evaluated for coronary artery tissue (3).

Formalin-fixed and paraffin-embedded tissue samples were collected from 16 female (mean age, 84 years; range, 73 to 91 years) and 4 male (mean age, 83 years; range, 62 to 91 years) AD patients. Specimens from the hippocampus as well as from different cortical and subcortical regions were evaluated by silver staining (8) and by immunocytochemistry for β A4 amyloid and tau protein (Dako, Hamburg, Germany). Diagnosis of Alzheimer dementia had been made according to criteria of the Consortium To Establish a Registry for Alzheimer's Disease (6). For *C. pneumoniae*-specific PCR, 100 mg of tissue of the hippocampus region was deparaffinized for 1 h in xylene and washed in 100 and 70% ethanol. DNA was extracted with phenol-chloroform according to standard protocols and divided into four aliquots. The yield of DNA extraction was controlled for each individual by amplification of the pyruvate dehydrogenase (PDH) gene (9). *C. pneumoniae* DNA was detected by nested PCR based on the species-specific HL-1 and HR-1 primer pair and on the nested IN-1 (5' AGTTGAGCA TATTCGTGAGG 3') and IN-2 (5' TTTATTTCCGTGTCG TCCAG 3') primer pair, which yield a 128-bp product, as previously described in detail for cardiovascular tissue (3).

Each PCR step consisted of 32 cycles of 1.5 min at 95°C, 1 min at 55°C, and 1.75 min at 72°C. For confirmation and enhancement of sensitivity and specificity, nonradioactive DNA hybridization was performed using the digoxigenin-labeled HM-1 oligonucleotide probe (3). In order to assess the sensitivity of the PCR protocol, 10-fold dilutions of a plasmid containing the target sequence (pGMP, 3,475 bp; Promega, Madison, Wis.) were used as templates. To disclose the presence of PCR inhibitors, for each individual, 10² copies of the plasmid were added to the genomic DNA and the nested PCR was repeated as described above. In addition to PDH gene amplification, a second DNA extraction control was included by adding 4 × 10² plasmid copies to a second tissue sample of each patient before the extraction procedure was performed. One-fourth of the extracted DNA was added to the PCR mixture as described above.

Four-micrometer-thick paraffinized sections were used to detect chlamydial antigens by immunocytochemistry using an indirect immunoperoxidase method. A *C. pneumoniae* species-specific anti-membrane protein monoclonal antibody (MAb), RR-402 (dilution, 1:100; Washington Research Foundation, Seattle, Wash.), a species-specific antilipopopolysaccharide MAb (1:250), and a genus-specific anti-heat shock protein 60 MAb (1:500; Affinity Bioreagents, Golden, Colo.), which have been proven effective for immunocytochemistry (1, 5, 7), were used as primary antibodies to incubate the slides overnight at 4°C. After being washed, the sections were incubated with a peroxidase-labeled goat anti-mouse antibody (1:100; Southern Biotechnology Associates, Birmingham, Ala.) for 1 h at room temperature. Tyramide signal amplification was used according to the instructions of the manufacturer (NEN Life Science Products, Boston, Mass.). Peroxidase was visualized with diaminobenzidine (Sigma, Deisenhofen, Germany). The sections were counterstained with Nuclear Fast Red (Sigma). To control the procedure, antichlamydial antibodies were replaced by an antibody against neuron-specific enolase (Dako), an antigen ubiquitous in brain tissue. Spleen sections of systemically *C. pneumoniae*-infected BALB/c mice were used as further positive controls.

Neither specific DNA sequences nor *C. pneumoniae* antigens were detected in the 20 AD patients. Negative PCR results could not be attributed to inhibition or lack of sensitivity. The sensitivities of nested PCR and hybridization ranged between 1 and 100 plasmid copies. DNA extraction was sufficient in all cases as indicated by successful PDH gene ampli-

* Corresponding author. Mailing address: Institute of Medical Microbiology and Hygiene, Medical University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. Phone: 49 (451) 500-2818. Fax: 49 (451) 500-2808. E-mail: gieffers@hygiene.mu-luebeck.de.

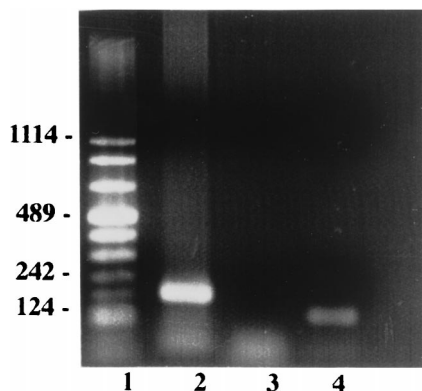


FIG. 1. Two percent agarose gel of the PCR products of a representative AD patient. Lane 1, Marker VIII (Boehringer, Mannheim, Germany); lane 2, DNA extraction control (185-bp amplification product of the PDH gene); lane 3, *C. pneumoniae* PCR (no amplification of *C. pneumoniae* genome by specific nested primers); lane 4, inhibitor control (128-bp PCR product of *C. pneumoniae* after addition of 10^2 copies of a plasmid containing the target sequence).

fication and amplification of target DNA that had been added before the extraction procedure in an amount just above the detection limit. The DNA preparations did not contain PCR inhibitors, as addition of 10^2 plasmid copies per reaction resulted in a positive signal (Fig. 1).

The suggestion of *C. pneumoniae* infection as a risk factor for AD is as yet based on few investigations. A Dutch study postulated an epidemiological relationship between AD and arteriosclerosis (2). Since arteriosclerosis is thought to be related to *C. pneumoniae* infection, a North American group investigated a potential contribution of *C. pneumoniae* infections to AD patients. They detected *C. pneumoniae*-specific DNA and antigens in 90% (17 of 19) of the brains from the AD patients tested and succeeded in the temporary cultivation of two *C. pneumoniae* isolates (1). In this study, we tried to revalidate these findings in a northern European population. We were unable to find specific DNA sequences or *C. pneumoniae* antigens in brain sections of a group of AD patients, who were comparable in size, sex, and age. Methodical shortcomings responsible for false-negative PCR results could be excluded as far as possible. Successful DNA extraction was ensured, since the PCR protocol used was previously evaluated for vascular tissue and proven to be substantially more sensitive than cell culture (3), which could not be performed due to the formalin pretreatment of the material. Inhibitors of the PCR, often reported from paraffin-embedded tissue, were absent. However, partial degradation of bacterial DNA could not be totally excluded, in spite of successful amplification of the PDH gene.

Therefore, we screened in addition for chlamydial protein antigens reported to be present in tissue for a longer period than genomic DNA is (5). None of the three chlamydial proteins was detected in the sections. One antibody was identical to the one used in the above-mentioned study (1), but protocols of immunocytochemistry, PCR, and sample processing differed. The lack of standardization of diagnostic methods in chlamydiology, supposed to be responsible for differing outcomes of studies of vascular tissue, may be the reason why this interesting finding was not reproduced in our hands. Nevertheless, some evidence suggests a neurotropism of *C. pneumoniae*. Recently, *C. pneumoniae* was reported to be cultivated from the cerebrospinal fluid of a patient with multiple sclerosis (10). However, the report of *C. pneumoniae* in the brains of AD patients remains an isolated finding and one to be reproduced by others. Further studies are required to establish an association between *C. pneumoniae* and AD.

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