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Detection of *Chlamydia pneumoniae* in Cholesteatoma Tissue: Any Pathogenetic Role?

*Francesco Ronchetti, †Roberto Ronchetti, †Francesco Guglielmi, *Ilaria Chiappini, ‡Carlo Contini, *Roberto Filipo, §Iolanda Santino, *Rosario Cerruto, *Daniele Bernardeschi, and *Maurizio Barbara

**Departments of Neurology and Otorhinolaryngology, †Pediatrics, and §Public Health, University "La Sapienza" I, Rome, and ‡Section of Infectious Disease, Department of Clinical and Experimental Medicine, University of Ferrara, Italy.*

Background: Acquired cholesteatoma is a complication of chronic otitis media that is usually associated with an intense local inflammatory reaction. Cholesteatoma probably arises from epithelial migration close to an ongoing host inflammatory response attributable to a chronic bacterial infection. *Chlamydia pneumoniae* is an intracellular microorganism associated with several pathologic conditions originally considered noninflammatory, including asthma, atherosclerosis, and Alzheimer disease. To investigate a possible relationship between *C. pneumoniae* and the development of cholesteatoma, tissue was studied in three different layers by polymerase chain reaction analysis. The results were compared with those relative to other two common middle-ear pathogens, *Mycoplasma pneumoniae* and *Haemophilus influenzae.*

Methods: Cholesteatoma specimens were collected from 32 patients undergoing middle ear surgery. A series of 5-μm-thick specimens were obtained at three different tissue levels, internal (matrix), intermediate (perimatrix), and external (granulation tissue), and processed by polymerase chain reaction for detection of *C. pneumoniae*, *H. influenzae*, and *M. pneumoniae.* Fragmentation and polymerase chain reaction amplification were carried out using two substantially different techniques.

Results: *C. pneumoniae* was detected with either polymerase chain reaction techniques in the internal layers in 16 of the 32 cholesteatomas (50%), associated with a positive finding in the intermediate layer in two cases and in the external layer in one case. Four specimens contained *H. influenzae*, always in the external layer, whereas none contained *M. pneumoniae*.

Conclusions: The close relationship between cholesteatoma and *C. pneumoniae* demonstrated by the findings of this study could suggest a direct cause and effect link between the pathogen action and the clinical manifestations. Otherwise, a facilitated colonization by *C. pneumoniae* and chronic pathology of the ear could both take origin from a peculiar immunologic background of the host.

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Cholesteatoma of the middle ear is an accumulation of desquamated keratin in the tympanic cavity from a multilayered ectopic epithelial mass. Histology shows stratified keratinized squamous cell epithelium and cholesterol (matrix), covered by connective tissue (perimatrix) and surrounded by inflammatory (granulation) tissue. Classic definitions distinguish primary from acquired cholesteatomas (1). The pathogenesis of the acquired form, by far the most frequently observed, comprises two fundamental events involving, in the early stages, the entrapment of the multilayered and keratinized tissue of the external canal within the middle ear, starting from retraction pockets, tympanic membrane perforations, or microperforations with subsequent reclosure (2). Released cell mediators then lead to chronic inflammation, which sustains the mass and causes its progressive growth (3).

Why cholesteatoma tends to recur, however, is still unclear. Both in the early stages and during the later growth stage, an individual predisposition seems essential for the disorder to develop (4,5) and for its tendency to recur, even after careful surgical removal.

The microorganisms isolated from cholesteatoma and from chronic, acute, or secretory otitis media are not the same (6,7). More specifically, the species that seem especially favored by the microenvironment associated with a cholesteatoma (*Pseudomonas aeruginosa*, *Bacteroides species*, and *Staphylococcus aureus*) lack features that could explain the persistent and typical inflammatory state associated with a cholesteatoma (8). Most of the relevant microbiologic research dates back many years, and, to our knowledge, the relationship between cholesteatoma and intracellular microorganisms has never been specifically studied.

Address correspondence and reprint requests to Professor Barbara Maurizio, Ospedale S. Andrea, V. di Grottarossa 1035-1039 00198, Rome, Italy. Email: maurizio.barbara@uniroma1.it

C. pneumoniae is an intracellular pathogen that is purportedly linked with several chronic inflammatory disorders, including asthma, atherosclerosis, Alzheimer's disease, and cardiovascular diseases, conditions heretofore regarded as noninfective in etiology (9–12). It has therefore been thought useful to design the present study to analyze the presence of *C. pneumoniae* in different areas of the cholesteatoma tissue. The data have then been correlated with those obtained for two other microorganisms: *H. influenzae*, a pathogen commonly found during acute otitis media, and *M. pneumoniae*, an intracellular bacterial agent with epidemiological affinity and action similar to *C. pneumoniae*.

MATERIALS AND METHODS

Samples of pathologic tissue were taken from 32 consecutive patients (16 males and 16 females; age range, 3 to 67 years) who underwent tympanoplasty for removal of cholesteatoma from December 1999 to May 2000. Cholesteatoma was surgically removed under sterile conditions, and the material was excised as far as possible en bloc together with the matrix. Samples were stored at −80°C and cut by cryostat into 5-μm-thick sections relative to granulation tissue, perimatrix, and matrix. Tissue specimens of a first series of 18 cholesteatomas were analyzed for the presence of *C. pneumoniae* (primers for membrane protein detection). After the first results became available, some of the material already examined (at least one sample per case) of the first 18 cases and three samples from each of the following 14 cases were also sent to a second laboratory, where cholesteatoma specimens were processed by fine ultrasonic fragmentation (sonication) and analyzed not only with the primer already used for the first series but also with a primer for a *C. pneumoniae*–specific ribosome gene sequence. All cholesteatomas were tested once or more in both laboratories. At least four tissue fragments from each cholesteatoma were analyzed overall in the two laboratories, with a total number of 168 samples tested for *C. pneumoniae*.

In all specimens, *M. pneumoniae* was searched in three different areas (external, intermediate, and internal) and *H. influenzae* was searched in a fragment of the specimen, without site distinction.

Sample preparation and DNA extraction

The samples were broken up into small pieces with a sterile scalpel and vortexed in 1 mL of solution containing 10 mmol/L TRIS and 0.5% sodium dodecyl sulfate. With the first technique, the samples were vortexed for 30 seconds, centrifuged at 15,000 rpm for 15 minutes, and then incubated overnight at −80°C to obtain thermal shock and cellular lysis. In the second technique, after incubation for 1 hour at 37°C, the resulting preparations were sonicated for 10 to 30 seconds (according to the size) at low speed in a Biosonick Sonicator (Bronson Sonic Power Company, Danburg, Connecticut) and rapidly put on ice. In both techniques, samples were then centrifuged at 10,000 rpm for 1 hour at 4°C and pellets resuspended in 100 μL lysis solution containing 10 mmol/L Tris-Hcl, pH 8.3, 2.5 mmol/L $MgCl_2$, and 100 μL containing 10 mmol/L Tris-Hcl, pH 8.3, 2.5 mmol/L Mg Cl_2, 1% Tween 20, 1% Triton X-100, and proteinase K 200 μg/mL. To optimize lysis, the amount of proteinase K was increased twofold or threefold, and the time of incubation was increased from 4 hours to overnight at 56°C, according to the size of the pellets. During incubation, samples were thoroughly vortexed several times according to Held (13).

For *H. influenzae*, the specimens were identified and serotyped. DNA was extracted from overnight cultures as already described (14). For analysis of *M. pneumoniae*, specimens were centrifuged at 2,000 rpm for 15 minutes in an equal volume of phosphate-buffered saline (PBS), pH 7.2. The pellet was resuspended in 1 mL of PBS and stored until polymerase chain reaction (PCR) evaluation was performed. The samples were lysed according to the method of Kellog and Kwok (15) and finally heated at 95°C for 10 minutes in a water bath and then cooled at room temperature. DNA aliquots of 10 mL were used for PCR analysis.

Amplification protocol

C. pneumoniae DNA was extracted by phenol-chloroform-isoamyl alcohol followed by ethanol precipitation. *C. pneumoniae*–specific primers encoding for major outer membrane protein (*MOMP)* and 16s rRNA gene targets were used. PCR first round was performed by a *Touchdown* PCR according to Don et al. (16) and to a personal modification. For MOMP gene *n*-PCR, *MOMP2* primers were as follows: sense, 5′-TGC CAA CAG ACG CTG GCG T-3′ and *MOMP2*r antisense, 5′-AGC CTA ACA TGT AGA CTC TGA T-3′ (17). For the second technique also 16s rRNA gene targets were used, with *TW50* sense, 5′-AGT CCC GCA ACG AGC GCA-3′ and *TW51* antisense, 5′-GCT GAC ACG CCA TTA CTA-3′ primers (18). PCR reaction buffers, Ampli*Taq* Gold DNA polymerase, and oligonucleotide primers were provided by Perkin-Elmer Applied Biosystems, Buckinghamshire, England, U.K. DNA was amplified according to Mahony et al. (19). The results of PCR were confirmed by Southern blot analysis using gene-specific probes with the chemiluminescent method (Amersham, International PLC, Buckinghamshire, England, U.K.).

Analysis of H. influenzae

Two different primer sets were used. The first was derived from the published sequence for Bex A, a capsulation-associated protein (20); the second from the published sequence for the outer-membrane protein P6 of *H. influenzae* (21). Oligonucleotides were selected from these sequences by excluding homologous sequences of a similar protein in *Escherichia coli* (22). The amplification procedure with both primer sets was based on the method described by Saiki et al. (23) and performed in a final volume of 100 μL.

Analysis of M. pneumoniae

Initially, 2-μL portions of undiluted and 10-fold dilutions of each sample were amplified with human globin-specific primers (GLO5/GLO6, 989 base-pairs [bp]) to test cell lysis (23). Plasmid pBR322 DNA (Promesa, Madison, WI, U.S.A.) was added to each sample and PCR was performed with primers pBRl/pBR2 (719 bp) to identify any inhibition of amplification caused by the presence of inhibitors in the sample (24). Mycoplasmas were predetected with genus-specific primers (MGSO/GPO1, 717 bp), fol1owed by amplification of positive samples with species-specific primers for *M. pneumoniae*, *M. genitalium*, *M. hominis*, *M. salivarium*, *M. orale*. *M. pirum*, *M. fermentans*, *M. penetrans*, and *U. urealyticum* (25).

PCR mixtures (50 μL) contained 50 mmol/L KC1, 10 mmol/L Tris HC1, pH 8.3, 1.5 mmol/L $MgCl_2$, 25 μmol/L tetramethylammonium chloride (Aldrich, Milwaukee, WI, U.S.A.), 40 pmol of each primer, 0.2 mmol/L dNTPs, and 1 U of *Taq* DNA polymerase (Promega) and were overlaid with two drops of mineral oil. Sample DNA (2 μL) was added through

the oil. PCR conditions comprised initial denaturation for 15 minutes at 95°C, followed by 30 cycles of 30 seconds at 95°C, 90 seconds at 58°C, and 90 seconds (increased s/cycle) at 72°C, with a final extension at 72°C for 10 minutes (24).

Analysis of amplified DNA

Portions (20 μL) of amplified products were electrophoresed in agarose 0.8% wt/vol gels in Tris acetate buffer (40 mmol/L Tris acetate, 1 mmol/L EDTA, pH 8), together with positive and negative controls, and DNA bands were visualized under ultraviolet illumination after ethidium bromide staining.

RESULTS

The first PCR technique allowed to detect *C. pneumoniae* DNA in 6 of the 32 cases tested (18%), three times in the internal layer and three times in the intermediate layer (Table 1). In one cholesteatoma, PCR yielded a positive result for the internal and external layers.

By using the second PCR technique, *C. pneumoniae* was found in 11 of the 32 specimens tested (34%). In 3 cases, PCR positivity involved two cholesteatoma areas; in one, *C. pneumoniae*–positive sites were located one in the internal and one in the external layer, whereas in the other, two positive specimens were located in the intermediate and internal layers.

In one case only, the positive result yielded by the first PCR technique was confirmed also by the second technique. Overall, both techniques detected *C. pneumoniae* in 16 of the 32 cholesteatoma specimens (50%). Assay of *H. influenzae* yielded a positive reaction in 4 of the 32 specimens tested (8%). In one case, the positivity of *H. influenzae* was associated with the positivity to *C. pneumoniae*. Assay of *M. pneumoniae* yielded no positive reactions in any of the samples tested.

DISCUSSION

This study represents the first demonstration of the presence of *C. pneumoniae* in human cholesteatoma tissue. In samples independently prepared and analyzed by two substantially different PCR methods in two different laboratories, *C. pneumoniae* was detected in 50% of the surgical specimens.

In the first series of 18 cholesteatoma specimens tested, PCR analysis conducted by the first laboratory detected, with one exception, the pathogen in only one of the three fragments obtained by manually sectioning each specimen. Because the pathogen enveloped within triturated fragments might have escaped PCR detection, a second experimental method was set up to process each fragment examined by fine ultrasonic fragmentation (sonication) and to amplify a second *C. pneumoniae* antigen sequence. Although this second technique notably increased the number of positive fragments (16.6% against 7.7% of the first technique), the analysis gave a positive result for more than one fragment in only two occasions. Hence, despite being detected in 50% of the cases analyzed, *C. pneumoniae* seems to reside in well-defined areas of the cholesteatoma tissue.

Another interesting finding is that *C. pneumoniae* was isolated almost exclusively from the internal fragment layers (13 positivities over 64 fragments analyzed, i.e., 20.3%). In only four cases, however, the positivity of the intermediate layer fragment for *C. pneumoniae* was not accompanied by positivity elsewhere . Only in one cholesteatoma (1 of 32) was the pathogen isolated from the external layer. In that case, however, *C. pneumoniae* was isolated also from a fragment of the internal layer. Hence, it would seem likely to assume that this microorganism is associated with the early stages of formation of the cholesteatoma mass.

In all the laboratory procedures, particular care was taken to avoid tissue contamination. Evidence showing that contamination played no part in our results is that *C. pneumoniae*, a nonubiquitous infective agent, was isolated almost exclusively from the internal part of the cholesteatoma, i.e., the most protected portion of the sample. The fact that no *M. pneumoniae* and relatively few *H. influenzae*, an agent present far more frequently than *C. pneumoniae* in acute otitis media (26), were detected supports the specificity of our PCR findings for *C. pneumoniae*.

To our knowledge, this is the first study describing the frequent presence of *C. pneumoniae* in cholesteatoma tissue. Our findings could thus engender new theories to explain this association. One possible explanation envisages a causal relationship. Another considers the association between the pathogen and cholesteatoma as consequence of some host immunologic characteristic, which creates a peculiar inflammatory microenvironment within the middle ear that favors both the development of the cholesteatoma and its colonization by this specific infective agent. We are in favor of the latter.

TABLE 1. *C. pneumoniae DNA detection in surgically excised cholesteatomas*

	Technique 1			Technique 2			Total		
	No.	Positive	%	No.	Positive	%	No.	Positive	%
CS	32	6*	18	32	11*	34	32	16	50
IF	32	3	9.3	32	10	31.2	64	13	20.3
INF	26	3	11.5	25	3	12	51	6	11.7
EF	32	1	3.2	25	0	0	57	1	0.17
Total	90	7†	7.7	82	13[c]	15.8	172	20	11.6

*One case positive for internal fragment (technique no. 1) + internal fragment (technique no. 2).

C. pneumoniae is classically considered an important respiratory agent, responsible for at least 10% of all cases of pneumoniae (27) but also for numerous respiratory upper airways inflammatory disorders, including sinusitis, pharyngitis, otitis, and bronchitis (28). *C. pneumoniae* is widely distributed in persons of all ages, even during the early years of life (from 5% to 10% of the general population are healthy carriers). However, persons producing *C. pneumoniae* IgG antibodies are exceedingly rare under the age of 5 years, and the proportion of IgG antibody producers slowly reaches 50% of the general population toward 40 years, with no additional increase at older ages (29). The peculiar epidemiological behavior depends on the type of immunologic characteristic of the human response to *C. pneumoniae.*

C. pneumoniae is a microorganism that enters the cell and can survive for a long time as a reticulated corpuscle (30). Eliminating these corpuscles means producing elevated amounts of immunologically active substances that not all cell populations or persons are able to express (31).

According to an immunologic paradigm popular in the 1990s, each individual is born with an activity of the T helper lymphocytes (Th) predominantly polarized toward the Th_2 activity that induce tolerance to antigens, produce interleukin-4, and favor IgE antibody production. After the immunologic stimulation an individual receives at birth, Th_1 responses then develop the Th_1 cell–mediated response that produces interferon-γ, thus eliminating the incompatible cellular clones (inhibition of autoimmunity) and infected cells (defense against infection). Ultimately, a satisfactory balance is reached between the Th_2-Th_1 activity lymphocytes. In *C. pneumoniae*–infected cells, eliminating the germ nevertheless require a strong Th_1-type cytokine response. This response is obviously lacking when Th_2-type responses predominate, as they do in asthma and atopic diseases (32,33). This schematic view is in reality very complex and involves other cell populations i.e., Ts ($CD8^+$). In fact, in genetically modified (knockout) mice deprived of T-lymphocyte suppressor cells ($CD8^+$), a notable facilitation to *C. pneumoniae* infection develops.

In these knockout mice, a *Chlamydial* inoculation no longer controlled by T-lymphocyte suppressor cells elicits a severe *Chlamydial* infection and a simultaneous production of typical Th_2-cell mediators (34,35). Hence, despite being a respiratory infective pathogen, *C. pneumoniae* can be also considered a microorganism that readily colonizes subjects with a Th_2-predominant immune response. As a matter of fact, present research strongly suggests that the presence of these pharyngeal germs or serum antibodies is also associated with the onset or recurrence, or both, of asthma and disorders characterized by bronchospasm (36).

C. pneumoniae has also been linked to the pathogenesis of atherosclerosis (37), being an agent capable of interfering with lipid metabolism (38). Epidemiologic studies conducted in patients with coronary artery atheromatous disease have reported the presence of these microorganisms in atheroma (39), and experimental studies have described an association between *C. pneumoniae* and the development of fat deposits and atheroma (40). *C. pneumoniae* also has a conspicuous ability to grow in human vascular cell cultures, thus inducing production of cytokines, chemokines, and adhesion molecules (41).

Atherosclerosis, a disease in which plaques form owing to the slow formation of a fibrin-rich tissue in a cellular setting with increased metabolic activity able to alter the lipidic metabolism, in some way resembles the process of cholesteatoma formation (42). *C. pneumoniae* has been associated with Alzheimer disease (12), with certain types of arthritis (43), and even with sudden infant death syndrome (44). All of the foregoing data underline the possible association of *C. pneumoniae* with chronic diseases.

However, rather than having a causative meaning, the various associations of *C. pneumoniae* with chronic diseases might instead provide evidence that the pathogenesis of these diseases and the ready colonization of the organism by *C. pneumoniae* (as well as its persistence in the circulation and in the tissue) both depend on a Th_2-predominant immunologic response. From this viewpoint, the cholesteatoma could be added to the list of chronic or metabolic diseases having a proven association with *C. pneumoniae.*

In summary, although a causative role of *C. pneumoniae* in the pathogenesis of cholesteatoma cannot be ruled out, it is possible to hypothesize that the personal characteristics of subjects prone to cholesteatoma formation and to easy recurrence can also facilitate middle ear colonization by *C. pneumoniae* and its envelopment in the keratinized tissue at the core of the cholesteatoma. This uneven tissue distribution of the pathogen and its presence in small fragments, especially those from the internal layers of surgically excised specimens, may suggest that colonization by *C. pneumoniae* is favored in the initial stages of a cholesteatoma and its growth continues in that manner over time. Our findings strongly support this interpretation. Hence, prospective clinical studies are needed to be conducted on the different clinical forms of cholesteatoma (primary, secondary, recurrent, and residual; young patients and adults), along with investigations designed to study the immune status of the affected patients.

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