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Tracing of *Helicobacter pylori* in the middle ear and mastoid mucosa of patients under 18 years of age with chronic otitis media (with and without cholesteatomas)

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Abstract

Objective. It has been estimated that about 5 million people of those affected with otitis media have cholesteatoma, however, its pathophysiology is unclear. In this study we aimed to detect *Helicobacter pylori* via polymerase chain reaction and real-time polymerase chain reaction in young patients with chronic otitis media.

Methods. Patients included in our prospective cross-sectional study had middle-ear/mastoid inflammation and underwent surgical procedures. Middle-ear mucosa samples were collected, and genomic DNA was extracted for *H pylori* detection by polymerase chain reaction and real-time polymerase chain reaction analyses. Sociodemographic data and gastroesophageal reflux symptoms were analysed.

Results. We included 49 patients with mean age of 12.7 ± 3.8 years. Twenty per cent of the patients were diagnosed with cholesteatoma. No increase in *H pylori*-amplified fluorescence was observed, indicating absence of *H pylori*.

Conclusion. Due to the absence of amplification for *H pylori* and the fact that albumin was amplified in all samples, we conclude that *H pylori* does not appear to be a causal factor.

Introduction

Chronic otitis media manifests as persistent middle-ear inflammation due to anatomical, physiological and microbiological factors. Over 20 million people are estimated to be afflicted with otitis media worldwide.[1] Of these, one-fourth (about five million) have cholesteatoma.[2] The pathophysiology of cholesteatoma is unclear, but chronic inflammation is a common factor. In recent years, multiple studies have shown the presence of *Helicobacter pylori* in exudates from patients with chronic otitis media resistant to medical treatment. This bacterium promotes the overregulation of mucus production and induces a continuous inflammatory response, thus suggesting its importance in the etiopathogenesis of cholesteatomas.[3–6] We are not aware of any studies that demonstrate the presence of *H pylori* in patients with cholesteatoma. Since cholesteatoma occurs in patients with persistent otitis media, *H pylori* could be an aggravating, predisposing or etiological factor. In this context, we aimed to detect *H pylori* via polymerase chain reaction and real-time polymerase chain reaction in young patients with chronic otitis media.

Materials and Methods

A prospective cross-sectional study was carried out from January 2019 to October 2021 in the Otorhinolaryngology Department of the Hospital Universitario del Valle. All patients who consulted and were under 18 years of age were considered. The inclusion criteria were as follows: (1) patients with symptoms and signs of chronic otitis media after history and clinical examination and (2) patients with the diagnosis confirmed by imaging. The exclusion criteria were as follows: (1) patients who received treatment for *H pylori* in the last three months and (2) patients who did not consent to participate in this study.

Patients were examined for symptoms (hearing loss, ear fullness, tinnitus, ear pain and balance disturbance) and signs (modification of the tympanic membrane, air-fluid levels and effusion) caused by inflammation in the middle ear. In addition, patients were evaluated for symptoms and signs related to gastroesophageal reflux disease using the symptom index of reflux (a score ≥ 13 was considered pathologic). Audiological and imaging evaluation was

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performed. Informed consent was obtained from each patient. All procedures performed with human participants were ethically reviewed and approved by the Institutional Review Board and the Research and Ethics Committee, Universidad del Valle.

Sampling

All patients underwent surgical procedures under general anaesthesia, including tympanoplasty, mastoidectomy, tympanostomy, or any combination of these procedures. Middle-ear mucosa samples were collected from the protympanum of the middle ear (the area where the opening of the Eustachian tube is located). A minimum of 3 × 3-mm samples were collected, which were stored and transported in the medium indicated by the laboratory.

Genomic DNA extraction from the middle-ear samples

Genomic DNA was extracted from each sample using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The extraction was performed following the manufacturer's instructions, with two changes in the preparation of the tissue lysate: (1) the samples were collected in 1.5-ml microcentrifuge tubes with 180 µl of the PureLink Genomic Digestion kit lysis buffer and were stored at −20°C until extraction, and (2) the samples were incubated at 37°C overnight to optimise the lysis.

The samples were resuspended in 100 µl of the PureLink Genomic Elution Buffer and were quantified by spectrophotometry using the NanoDrop™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) 2000 spectrophotometer to evaluate the concentration and quality of the extracted DNA. We extracted DNA from all collected samples. Most of the samples were collected twice, and their replicates were stored at −80°C in the RNAlater buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The average genomic DNA concentration was 13,342 ng/µl ± 11,048. The quality index A260/A280 presented an average value of 1.954 ± 0.315. Likewise, the quality index A260/A230 gave an average value of 1,752 ± 2,687. These average values did not include two of the samples (OMTC008 and OMTC016) because their concentrations were less than 2 ng/µl, and erroneous measurements could have been obtained given the lower detection limit of the equipment used (NanoDrop 2000).

Helicobacter pylori detection

The primers 5′-GCT CTC ACT TCC ATA GGC TAT AAT GTG-3′ and 5′-GCG CAT GTC TTC GGT TAA AAA-3′ were used for *H pylori* detection.[7] A pilot test was conducted using 10 samples, a positive control for *H pylori* diagnosed by pathology in a gastric cancer sample and a negative control. Similarly, the samples were evaluated for the presence of the albumin gene (5′-CTG CAT TGC CGA AGT GGA A-3′ and 5′-CAA ACA TCC TTA CTT TCA ACA AAA TCA-3′)[7] to verify the quality of the extracted genomic DNA and to serve as an internal control (i.e., *H pylori* was not amplified due to a technical error (false negative)). In this case, the control gene (albumin) was amplified, but not the gene for *H pylori* detection. This procedure was initially performed via conventional polymerase chain reaction and verified through agarose gel electrophoresis. We obtained a negative result for *H pylori* in all samples. Thus, we decided to complete the amplification using a more sensitive molecular test, such as real-time polymerase chain reaction using SYBR Green as the fluorescent molecule, which provided the same result.

The PowerUP SYBR Green Master Mix Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to detect *H pylori* via real-time polymerase chain reaction under the following polymerase chain reaction conditions: 95°C for 2 minutes; 46 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and fluorescence reading. A high-resolution dissociation curve protocol was performed at the end of amplification to verify whether the amplified fragments corresponded to the target region or were nonspecific products. For this, the temperature was gradually raised from 60°C to 95°C, with increments of 0.5°C every 5 seconds, and the fluorescence reading was performed at the end of each increment. This procedure was carried out using the CFX96 Real-time Polymerase Chain Reaction Kit (Bio-Rad Laboratories, Hercules, California, USA). Reactions were performed using positive and negative controls. Only the positive control and the OTMC005 sample showed signals (Figure 1).

A high-resolution dissociation curve test was performed to verify the identity of the *H pylori*. The maximum fluorescence peak was 79 for the positive control and 73 for OTMC005, indicating that the OTMC005 signal was due to a nonspecific product and not the detection of *H pylori*. Sample OTMC005 was resubjected to real-time polymerase chain reaction for confirmation, revealing negative *H pylori* amplification results. Therefore, all samples tested were negative for *H pylori*. Amplification of the human albumin gene was performed to ensure that the absence of *H pylori* amplification was not due to technical problems. Likewise, the identity of the amplified albumin gene was confirmed via High Resolution Melt analysis. All the amplified ones presented with a maximum peak between 77.5 and 78°C, which indicates that these amplicons were fragments of the gene of interest.

Results and analysis

We included 49 participants (30 males, 19 females) with a mean age of 12.7 ± 3.8 years old. All patients had positive gastroesophageal symptoms; 10 patients had a moderate middle-ear/mastoid disease, with 5 patients having a compromised ossicular chain. All patients had some degree of hearing loss. No increase in *H pylori*-amplified fluorescence was observed, indicating the absence of *H pylori*.

In our study, all patients had moderate gastroesophageal symptoms, all with a chronic active ear infection or refractory to management. However, *H pylori* was not identified in the samples studied, even though the method used for its study was real-time polymerase chain reaction, which is a test with high sensitivity and specificity, to demonstrate the presence of bacterial DNA.[8]

Of the sample of patients, all had histopathological findings compatible with chronic inflammation, and 20 per cent were diagnosed with chronic otitis media and cholesteatoma. This high percentage is most likely secondary to the fact that patients with persistent and difficult-to-manage pathologies were chosen that ultimately required surgical intervention, all with some degree of hearing impairment.

Discussion

Our study was designed to assess the relationship between chronic middle-ear disease caused by *H pylori* and chronic inflammation identified as the aetiology of acquired

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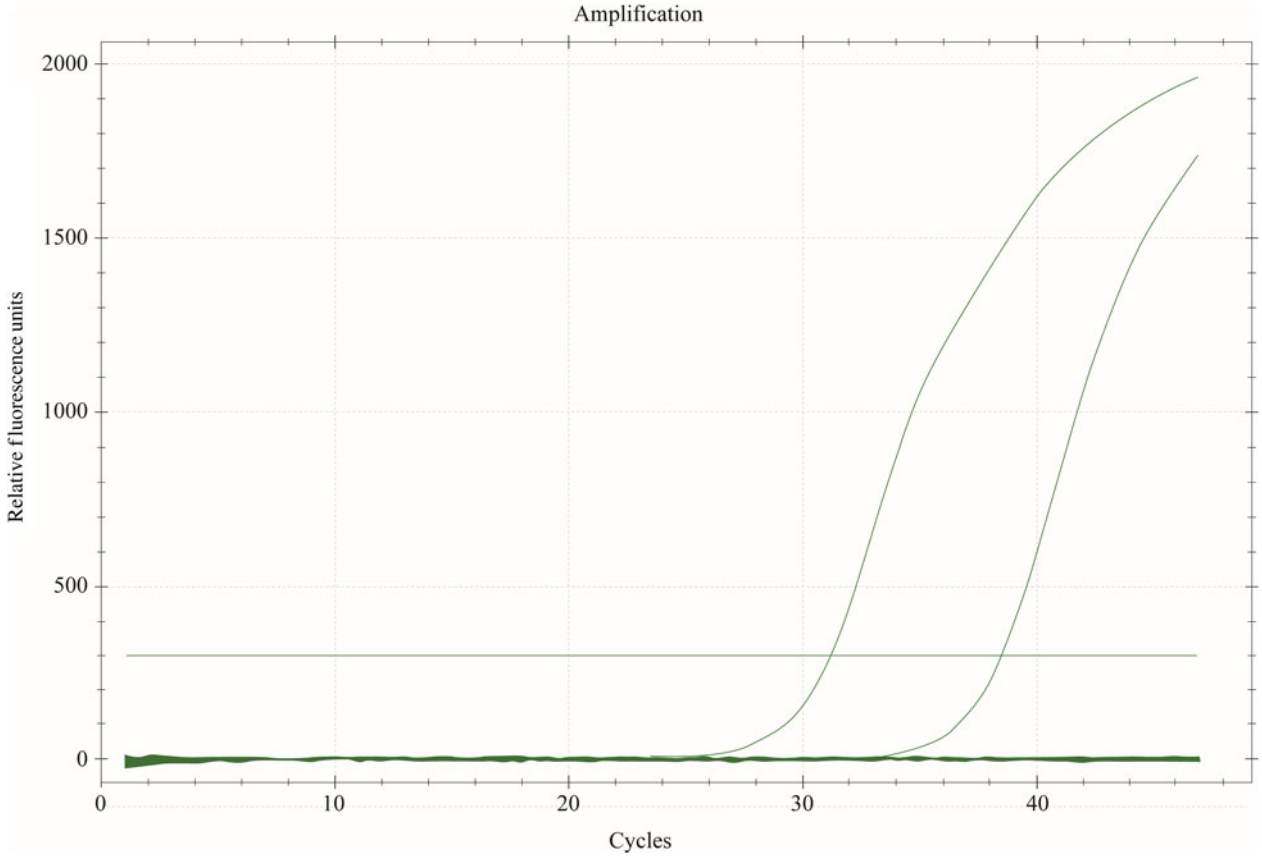

Figure 1. Detection of *Helicobacter pylori* via real-time polymerase chain reaction.

cholesteatoma, both highly prevalent pathologies in our population and more frequent in developing countries with low socioeconomic conditions and limited access to health services. Our population is homogeneous and representative of the group of patients suffering from this disease in our community. The lack of *H pylori* in tissue samples from patients with chronic otitis media with and without cholesteatoma might suggest that *H pylori* is not a causal factor. However, a metagenome analysis should be carried out to validate our results further.

The prevalence of *H pylori* is 70 per cent in the general population.[9] This bacterium has been identified mainly in stomach mucosa and cultured in different samples of the aerodigestive route, such as oropharynx, nasopharynx, and middle-ear content.[10] Through a proinflammatory mechanism that positively regulates the production of mucins MUC1 and MUC5AC, *H pylori* has been associated with inflammatory pathology of the middle ear.[4]

Tasker *et al.*[11] demonstrated the presence of gastric reflux content in the middle ear as a potential cause of chronic otitis media, finding that 83 per cent of investigated patients were positive for pepsin or pepsinogen at concentrations one-thousand times higher than those identified in serum, using different methods of immunohistochemistry. Gastroesophageal reflux would produce inflammation of the nasopharynx and the Eustachian tube, inducing obstruction and dysfunction which reduces intratympanic pressure due to the reabsorption of oxygen from the middle ear; negative pressure within the tympanic cavity is maintained and results in transudation of fluid from the microvasculature. This results in oedema of the mucoperiosteum and decreased oxygen diffusion that chronically leads to irreversible changes in the mucosa, thus predisposing to the development of chronic otitis media and even secondary cholesteatoma.

Helicobacter pylori is highly localised to its ecological niche and needs special growth conditions.[12] Certain factors in the middle ear may favour the presence of this bacterium, such as a microaerobic environment (e.g. 10 per cent CO_2). But the pH of intraluminal mucus and middle-ear secretions has been identified between 5 and 9, which is a condition that does not favour the growth of *H pylori*. Therefore, it has been considered that the presence of *H pylori* in tissues other than the stomach could be explained by its existence as a transient pathogen.

Variable results have been obtained from different studies that have attempted to identify *H pylori* in middle-ear secretions by other culture methods. For example, polymerase chain reaction – enzyme-linked immunosorbent assay test for *Campylobacter*-like organisms found 3–66 per cent positive results.[5,13,14] Dagli *et al.*[15] aimed to investigate the presence of *H pylori* by using a *Campylobacter*-like organisms test in the middle ear of patients with chronic otitis media, and found positive results in 53.6 per cent of them. Kutluhan *et al.*[16] also addressed this topic, and found that *H pylori* was present in 7.9 per cent of chronic otitis media patients. Likewise, the study by Yilmaz *et al.*[17] found that in 67 per cent of the chronic otitis media patients with effusion there was *H pylori* presence detected by real-time polymerase chain reaction.

In contrast with those studies but in line with our findings, Jeyakumar *et al.*[18] evaluated 48 middle-ear fluid samples in patients with otitis media with effusion without evidence of *H pylori* by polymerase chain reaction. Likewise, Bitar *et al.*[19] could not detect *H pylori* in the 28 middle-ear effusion samples from 18 children with otitis media using culture and polymerase chain reaction, and Shishegar *et al.*[20] did not detect the presence of *H pylori* in the middle-ear effusion of 40

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patients with otitis media with effusion by using polymerase chain reaction. Our findings show that while chronic inflammation may play a role in cholesteatoma formation, *H pylori* does not appear to be a causal factor because *H pylori* was not identified in any cholesteatoma samples.

- The study aimed to assess the relationship between *H pylori*-related middle-ear disease and cholesteatoma in young patients with chronic otitis media
- According to our research, although chronic inflammation may contribute to the development of cholesteatoma, we found no evidence linking *H pylori* as a causal factor
- All patients displayed histopathological results indicative of inflammation, and 20 per cent of them received diagnoses of chronic otitis media and cholesteatoma
- All patients displayed moderate symptoms related to gastroesophageal issues, and all patients had either a chronic active ear infection or a condition that did not respond to treatment
- Studies show variable results regarding *H pylori* identification in middle-ear secretions, but metagenomic analysis using gene sequencing may provide more accurate results

A metagenomic analysis could identify more precisely the presence of *H pylori* because it is a more sensitive method that uses 16S ribosomal RNA gene sequencing to identify bacteria in patients.[21] However, it is worth noting that we know of no other studies that have yet found *H pylori* in their samples, even with this method.[22,23]

A limitation of our study is the size of the sample. We initially proposed to treat 150 patients; however, after processing one-third of the samples and seeing no positive findings for the presence of *H pylori*, it was decided to conclude the project. Nonetheless, it corresponds to one of the most significant samples that has ever been studied and reported in the literature. Another limitation is the study type. It would be possible to draw more accurate conclusions from a randomised, controlled approach. Furthermore, in this study we limited our population to underage patients considering that young age has been reported as a factor associated with acute otitis media and that cholesteatoma is considered to be more aggressive in children than adults. However, it could be beneficial to include a wider age range in future studies.

Conclusions

This is the first study to conduct *H pylori* testing in the middle-ear mucosa via real-time polymerase chain reaction and the most extensive sample analysed to date. Due to the absence of amplification for *H pylori* and the fact that Albumin amplified all samples, we conclude that this bacterium could not be detected in the samples. Therefore, an analysis of the metagenome is being carried out.

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Data availability statement. The authors confirm that the data supporting the findings of this study are available within the article.

Competing interests. None declared

Ethical standards. The authors assert that all procedures performed with human participants were ethically reviewed and approved by the Institutional Review Board and the Research and Ethics Committee and compliant with the Helsinki Declaration of 1975, as revised in 2008.

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