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MAIN ARTICLE

Absence of *Helicobacter pylori* in healthy laryngeal mucosa

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Abstract

Objectives: To evaluate the presence of *Helicobacter pylori* in healthy laryngeal mucosa.

Design: Prospective analysis of *Helicobacter pylori* colonisation in healthy laryngeal mucosa, using the ^{13}C urea breath test and polymerase chain reaction analysis.

Subjects: Twenty randomly chosen men (28–78 years) without laryngeal pathology or gastrointestinal disease were investigated. All subjects were scheduled for elective operative procedures, under general, endotracheal anaesthesia. Cytobrush samples were taken for *Helicobacter pylori* DNA detection. Nested polymerase chain reaction testing was performed on DNA solutions using two primer pairs from the urease A gene of the *Helicobacter pylori* genome. The ^{13}C urea breath test was performed on two exhalation samples.

Results: Eight (40 per cent) of the subjects were positive for urease on urea breath testing; none were positive for *Helicobacter pylori* DNA on polymerase chain reaction testing.

Conclusion: Based on these results, we do not consider *Helicobacter pylori* to be a normal constituent of healthy laryngeal microflora.

Key words: *Helicobacter Pylori*; Microbiology; Bacteriology; Larynx; Polymerase Chain Reaction

Introduction

Helicobacter pylori is the most common cause of chronic gastric infection in humans. *Helicobacter pylori* infection has been reported throughout the world, with a mean prevalence of 50 per cent, which increases with age. After entering the gastrointestinal system, *H pylori* colonises the gastric epithelial cell surface. *Helicobacter pylori* infection of the gastrointestinal system is a major factor in the development of chronic mucosal inflammation, and has also been identified as a carcinogen promoting gastric carcinoma. The outcome of infection depends on the bacterium's potential to pass through the physicochemical gastric barriers, and on its replication and further dissemination. To date, research has focussed on the virulence factors which enable colonisation, persistence, disease induction, and dissemination to other organs and organ systems.

Isolation of *H pylori* is not an easy task and is affected by various factors, including certain physicochemical and biological properties of the bacterium, and the type of diagnostic procedure used (i.e. invasive or non-invasive).

Non-invasive diagnostic methods detect the presence of *H pylori* by demonstrating specific bacterial activity (primarily via the activity of urease), or by identifying specific antibodies in the serum and/or saliva, or a specific antigen in the stool. The sensitivity and specificity of the ^{13}C urea breath test exceeds 90 per cent. This test is effective in the diagnosis of *H pylori* in dyspeptic patients, and is also useful in monitoring the therapeutic outcome of *H pylori* eradication in patients with non-ulcerative dyspepsia and duodenal ulcer.[1,2] Despite the high specificity and sensitivity of the urea breath test for *H pylori*, other urease-producing bacteria in the oral cavity and stomach can produce false positive results. So far, five bacterial species with urease activity have been isolated from the oral cavity and/or stomach: *Proteus mirabilis, Citrobacter freundii, Klebsiella pneumoniae, Enterobacter cloacae* and *Staphylococcus aureus*.[3]

Recently, several molecular techniques have been introduced into the *H pylori* diagnostic procedure. The presence of *H pylori* DNA has been demonstrated in biopsy specimens of gastric mucosa and other tissues, using polymerase chain reaction analysis. This technique can also be used to demonstrate and identify various bacterial strains and subtypes.[4–6]

The present study aimed to assess the presence of *H pylori* in clinically healthy laryngeal mucosa, using

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urea breath testing and polymerase chain reaction analysis.

Materials and methods

This human study was appropriately reviewed and approved by the ethics committee of the Dr Josip Benčević General Hospital in Slavonski Brod, Croatia.

Twenty volunteers were randomly selected, and each gave written, informed consent.

The absence of pathological laryngeal changes was confirmed from the subjects' medical history and clinical examination. The selected individuals had no history of chronic laryngitis, Reinke's oedema, vocal nodules, polyps or malignant tumours. In addition, they had not suffered any kind of gastrointestinal disease for the past three years. These subjects were scheduled for elective surgery for repair of bone fracture (eight subjects), pilonidal sinus (seven) or inguinal hernia (five). All subjects were men aged between 28 and 78 years (median, 53 years).

The ^{13}C urea breath test was performed on two exhalation samples: the first obtained immediately after a test meal, using 75–100 mg of ^{13}C- or ^{14}C-labelled urea, and the second 30 minutes thereafter. If present in the host, bacterial urease converts urea to bicarbonate and ammonia ions. The $^{13}CO_2$ or $^{14}CO_2$ released by bicarbonate breakdown is then detected in the exhaled breath, giving a positive result.

All subjects were scheduled for elective operative procedures under general, endotracheal anaesthesia. Samples for *H pylori* DNA detection were obtained using the cytobrush technique, upon anaesthesia induction and immediately before intubation. After sampling, the brushes were immersed in Eppendorf microtubes containing 1.5 ml of sterile digestion buffer (50 mM Tris–HCl, pH 8.5, 1 mM ethylene diamine triacetic acid (EDTA) and 1 per cent Tween 20) and frozen at −20°C until analysis. Cells were removed from the brushes by vigorous shaking. Proteinase K was added to a final concentration of 100 μg/ml. Samples were incubated, with gentle shaking, for 10 hours at 37°C. The genomic DNA was extracted by two phenol chloroform extractions and precipitated at −20°C for 10 hours, with the addition of 1/10 volumes of 10 M ammonium acetate and 2.5 volumes of absolute cold ethanol, as previously described.[5] After a 10-minute centrifugation at 14 000 rpm at room temperature, the residual pellets were washed with 70 per cent ethanol, air-dried and dissolved in 30–50 μL Tris–EDTA buffer, pH 8.0. Nested polymerase chain reaction was performed as previously described.[5] All reactions were performed in triplicate, with positive and negative controls, according to standardised laboratory practice.

Results and analysis

The test results are summarised in Table I.

The ^{13}C urea breath test revealed that eight (40 per cent) subjects were *H pylori* positive and 12 (60 per cent) were *H pylori* negative. Positive results were recorded in 25 per cent of subjects aged 50 years or less, and in 75 per cent of those older than 50 years.

Polymerase chain reaction analysis failed to detect the presence of *H pylori* DNA in any patient.

TABLE I
HELICOBACTER PYLORI TEST RESULTS*

Test result	Subjects† (*n*)
UBT +ve	8
UBT −ve	12
PCR +ve	0
PCR −ve	20

*In healthy laryngeal mucosa. †Total = 20. UBT = urea breath test; +ve = positive; −ve = negative; PCR = polymerase chain reaction

Discussion

The prevalence of *H pylori* infection exceeds 50 per cent of adults worldwide, and is considered a major cause of pathology. *Helicobacter pylori* has been primarily described as causing gastrointestinal infection, and the species has been classified as having slow pathogenicity. *Helicobacter pylori* actively modifies the host's immune response, enabling a sustained state of balance between bacterial replication and the host's defence mechanisms. This explains the relatively mild chronic inflammatory reaction characteristic of *H pylori* chronic gastritis (prevalence, 60–100 per cent) and *H pylori* duodenal ulceration (prevalence, 90–100 per cent).[7,8] In addition, *H pylori* has been identified as a significant carcinogen for gastric carcinoma.[9]

Given the evidence of high *H pylori* seroprevalence and infection rates, this organism may also play a role in the aetiology of extra-gastrointestinal diseases such as respiratory disorders (e.g. chronic obstructive pulmonary disease, bronchiectasis, lung cancer, pulmonary tuberculosis and bronchial asthma), vascular disorders (e.g. ischaemic heart disease, stroke, primary Raynaud's phenomenon and primary headache), autoimmune disorders (e.g. Sjögren's syndrome, Henoch–Schönlein purpura, autoimmune thrombocytopenia, autoimmune thyroiditis, Parkinson's disease, idiopathic chronic urticaria, rosacea and alopecia areata) and other conditions (e.g. iron deficiency anaemia, growth retardation, liver cirrhosis and nasal polyposis).[10–13]

Helicobacter pylori colonisation has been detected in the healthy oral cavity and also in the presence of various types of oral pathology, including ulcerative stomatitis, atrophic glossitis, dental plaque and chronic tonsillitis.[12,14,15] Despite numerous studies on the route of *H pylori* colonisation, the role of *H pylori* in the oral cavity remains obscure. Is *H pylori* part of the normal oral microflora, or is it only transiently present in the oral cavity? Gall-Trošelj *et al.* have demonstrated that there is no preferential site for *H pylori* colonisation in healthy oral cavity mucosa. On the other hand, mucosal changes

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may make the environment more acceptable for such colonisation.[15] *Helicobacter pylori* reaches the oral cavity via occasional reflux from the gastric reservoir; it can also be introduced via oral–oral or faecal–oral transmission, and then swallowed into the stomach.[16] *Helicobacter pylori* has been commonly detected in the oral cavity of patients with dyspeptic symptoms, but gastric reinfection does not occur in these patients despite persistent oral *H pylori* colonisation.[14] A possible explanation is that these patients had only a low level of *H pylori* organisms in their oral cavity, which does not reach the critical threshold that would make colonisation of the stomach possible. However, in a more favourable environment the number of viable bacteria may increase to a level sufficient to cause gastric infection or reinfection.[17]

- **This study assessed the presence of *Helicobacter pylori* in normal laryngeal mucosa**
- **Polymerase chain reaction analysis and ^{13}C urea breath testing were used**
- **These tests gave 0 per cent and 40 per cent positivity, respectively**
- ***Helicobacter pylori* is not a normal commensal in healthy laryngeal mucosa**

Helicobacter pylori has a unique way of adapting to the gastric environment. It penetrates through the mucous layer to infect gastric epithelial cells, and produces enzymes which break down substances in the gastric juice. The most important of these enzymes is urease, which converts urea from saliva and gastric juice into bicarbonate and ammonia. The by-product, carbon dioxide, is then absorbed into the circulation and exhaled.[18]

We believe that there are other *H pylori* transmission modes besides the oral route, and that healthy laryngeal mucosa may be one of these. Our study aimed to assess the presence of *H pylori* in healthy laryngeal mucosa, by testing 20 individuals without laryngeal pathology and with no gastrointestinal disease over the previous three years. Despite variable ^{13}C urea breath test results, polymerase chain reaction analysis showed that these subjects' laryngeal mucosa was free of *H pylori*. These negative polymerase chain reaction results suggest that normal laryngeal mucosa acts as a barrier to *H pylori* infection. However, impaired laryngeal mucosa has been shown to be a favourable medium for *H pylori* colonisation.

Recently published findings have suggested the importance of *H pylori* infection in chronic laryngeal mucositis.[19] However, Akbayir *et al.* failed to detect *H pylori* in carcinomatous and benign lesions of the laryngeal mucosa, using histology and immunohistochemistry.[20] Fang *et al.* reported laryngeal *H pylori* colonisation in patients with vocal polyps, but concluded that the role of *H pylori* as an aetiological factor for vocal polyps remained inconclusive.[21] Rubin *et al.* found an increased titre of *H pylori* antibodies in patients with chronic laryngeal dysplasia and in those with head and neck carcinoma; they concluded that the presence of gastroesophageal reflux was a significant pathological process which may cause laryngopharyngeal bacterial colonisation, and which may be a cofactor in the development of various inflammatory processes that may be a basis for carcinogenesis.[22] Titiz *et al.* used polymerase chain reaction methodology to identify *H pylori* in laryngeal carcinoma and mucosal tissue; they obtained positive results in 80.9 per cent of their cancer patients, but in none of their patients with benign mucosal neoplasias (e.g. polyps and nodules).[23] However, Cvorovic *et al.* detected *H pylori* in the nasal polyps of patients who also had *H pylori* in their stomach.[24] Zhuo *et al.* conducted a meta-analysis of 15 published studies on the impact of *H pylori* on the development of invasive laryngeal carcinoma.[25] Finally, Grbesa *et al.* reported that *H pylori* can be present in laryngeal squamous cell carcinoma, but that its presence does not seem to influence the IGF2 (Insulin like growth factor)/H19 (gen for a long noncoding RNA, this gen seems to have a role in some forms of Cancer) imprinting status.[26]

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