Improvement and optimization of the classical gastric biopsy culture technique for *Helicobacter pylori* diagnosis using trypsin

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*Helicobacter pylori* infection represents a key factor in the aetiology of various gastrointestinal diseases. *H. pylori* infection diagnosis is generally achieved using both invasive (e.g. biopsy of the gastric epithelium) and non-invasive methods. Therefore, cultivation on a growth medium becomes complex. Trypsin is a proteinase enzyme that plays a role in an early stage of tissue digestion. In this study, we used trypsin in order to improve the diagnostic sensitivity of the *H. pylori* cultivation technique. We used 46 duplicate antrum biopsy specimens, divided into trypsin-treated and non-treated groups. The tissues were seeded on a selective *H. pylori* growth agar medium. We demonstrated that the classic *H. pylori* culture technique misses the growth of a large number of *H. pylori* colonies. Significantly more colonies were found in the trypsin-treated specimens group.

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INTRODUCTION

*Helicobacter pylori* is a Gram-negative, spiral-shape bacterium. In the human body, these bacteria reside in the gastric mucosa and duodenum, and cause chronic inflammation that can develop into an ulcer (Blaser & Atherton, 2004). *H. pylori* infection represents a key factor in the aetiology of various gastrointestinal diseases, ranging from chronic active gastritis without clinical symptoms to peptic ulceration, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma (McColl, 2010; Polk & Peek, 2010). Epidemiological studies indicate that, in underdeveloped countries, the prevalence of *H. pylori* ranges from 70 to 90% as opposed to 25–50% in industrialized countries (Magalhães Queiroz & Luzza, 2006). However, the exact mechanism of transmission is still unknown. The most likely mode of transmission is the faecal–oral route in underdeveloped countries and gastro-oral in industrialized countries (McColl, 2010). Currently, there are several available methods for diagnosis of *H. pylori* infection, which can be classified into invasive and non-invasive techniques. Biopsy of the gastric epithelium is an example of an invasive technique that enables histological examination, culture and molecular tests consequent to diagnosis of the presence of *H. pylori*. Non-invasive methods are based on detection of an antigen or an antibody present in stool or blood samples.

Another non-invasive method is the urea breath test (Lin et al., 1992).

In recent years, use of the culture technique as a diagnostic tool has been abandoned in favour of non-invasive, sensitive and rapid methods. This transition occurred mainly due to difficulties that arise from the cultivation process of this pathogen, particularly the excessive time consumption of the classic culture procedure and lack of professional microbiological skills. However, recently a rise in bacterial resistance to antibiotic treatment modalities has been witnessed (De Francesco et al., 2010). Therefore, the culture technique proves useful as a diagnostic method that provides information on the antibiotic susceptibility profile and plays an important role in empiric antibiotic treatment and the management of refractive cases.

Several methods have been developed to cultivate this human pathogen; some include different growth media – solid or liquid – and a range of protocols that, among others, call for the use of an automated system for successful bacterial culturing (Hachem et al., 1995; Peretz et al., 2013; Sainsus et al., 2008).

One of the main difficulties encountered in *H. pylori* cultivation from gastric biopsy specimens is that these bacteria adhere tightly to the epithelial cells, complicating the
task of their release from tissues. As a result, bacterial cultivation on a growth medium becomes complex and could result in incorrect false-negative results characterized by poor or non-detectable bacterial growth. Trypsin is a proteinase enzyme that catabolizes peptide bonds at the carboxyl end of amino acids arginine and lysine. This enzyme digests proteins that form cell-to-cell bonds and/or extracellular matrix bonds. Trypsin is a widely used digestive agent in the field of cell culture: it plays a role in an early stage of tissue digestion for cell-line formation (PheLAN, 2007). Our primary goal was to utilize trypsin’s tissue-digesting characteristics in order to improve the diagnostic sensitivity of the H. pylori cultivation technique.

**METHODS**

**Patient characteristics.** Forty-six duplicates of stomach antrum biopsy specimens were obtained during gastroscopy procedures from a total of 46 patients [17 children (mean age 13.8 years) and 29 adults (mean age 42.5 years)]. All of the patients underwent a rapid urease test (CUTest; Temmler Pharma GmbH & Co.) to confirm the presence of H. pylori. None of the patients included in the study received antibiotic treatment of any kind in the month preceding the gastroscopy procedure.

**Culture.** All biopsy specimens were sent to the Laboratory of Clinical Microbiology within 30 min of the gastroscopy procedure. Specimens were placed in sterile Eppendorf tubes containing 1 ml sterile physiological solution (0.9% NaCl). One biopsy specimen from each patient was minced manually with a sterile scalpel and seeded afterwards on selective growth agar media (BD Diagnostics) in accordance with the relevant protocol. A second biopsy specimen was incubated for 3 h at 37 °C in a sterile Eppendorf tube containing 1 ml 2% trypsin solution. At the end of the 1 h incubation period, the trypsin-digested biopsy sample was separated from the trypsin solution by washing in a centrifuge for 3 min at 600 g, discarding the supernatant and subsequent washing with 1 ml physiological solution (0.9% NaCl). Processed tissue was then seeded, similar to the first biopsy specimen, on a selective H. pylori agar growth medium, in accordance with the relevant protocol.

Culture samples were incubated for a period of 10 days at 37 °C in a micro-aerobic atmosphere (5% O2 and 10% CO2) produced by a gas-generating system suitable for Campylobacter (CampyGen; Gamidor Diagnostics). H. pylori was identified using a Gram-staining procedure followed by positive oxidase, catalase and urease tests (Isonberg, 2004).

**Statistical analysis.** For comparison between the trypsin-treated and non-treated groups, a Wilcoxon test, and a χ² test were used. A P value of ≤0.05 was considered significant.

**RESULTS**

For the non-treated group, the calculated mean value was 5.2 H. pylori colony units with a median value of 5.0; in comparison, for the trypsin-treated group, the mean value was 11.9 H. pylori colony units with a median value of 11.0. Quantitative data analysis demonstrated higher mean and median values for the trypsin-treated group compared with the non-treated group. The difference was statistically significant, indicating that the mean number of H. pylori colony units following treatment with trypsin solution was significantly higher, with a P value of <0.0001 calculated using the Wilcoxon test and a value of 0.0059 using the χ² test (Table 1). The frequency calculation of any culture growth showed a statistically significant difference between the trypsin-treated specimens group (100% of specimens exhibited growth) and the non-treated group, where 15.2% of the specimens did not show any growth at all. These data suggested that trypsin has a positive effect, improves results of the culture technique and assists in achieving genuine results. The total frequency of colony growth was calculated for the following subgroups: (i) non-growth; (ii) one to eight colonies; and (iii) more than eight colonies. The most frequent subgroup for the non-treated specimens was that of one to eight colonies (69.6%); 15.2% of specimens did not show any kind of growth and in only 15.2% was growth of more than eight colonies seen. This analysis demonstrated that the classic H. pylori culture technique misses the growth of a large number of H. pylori colonies. For the trypsin-treated specimens group, the results were significantly higher, with 0% non-growth, 23.9% growth of one to eight colonies, and 76.1% growth of more than eight colonies (Fig. 1). This means that treatment with trypsin increases the probability to achieve true, similar to reality, H. pylori growth.

<table>
<thead>
<tr>
<th>Cultures results</th>
<th>No treatment</th>
<th>Trypsin treatment</th>
<th>P value from χ² test</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>π</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>15.2</td>
<td>0</td>
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<tr>
<td>Positive</td>
<td>39</td>
<td>84.8</td>
<td>46</td>
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**DISCUSSION**

H. pylori is a common bacterial infection that has been recognized by the World Health Organization as one of the most frequent and major acquired bacterial infections of the human digestive system. Generally, diseases caused by H. pylori develop a chronic nature, unless treated by a combination of two antibiotic agents with a proton pump inhibitor or H₂ blocker. H. pylori infection diagnosis is generally achieved using both invasive and non-invasive methods. Among the invasive methods is gastroscopy, followed by histological examination of gastric samples and culture. Non-invasive techniques are applied to peripheral samples, and include breath tests, detection of antibodies in blood, stool, urine or saliva, and urease activity.

In recent years, there has been a noticeable decline in exploitation of the method for H. pylori cultivation from biopsy specimens due to technical difficulties, such as
culture maintenance, a time-consuming cultivation process that requires specific physical conditions and a demand for skilled microbiology experts to confirm diagnosis. Alongside this tendency is an increase in H. pylori resistance to antibiotic treatment, justifying the importance of susceptibility profile determination in order to obtain information on future optimal antibiotic treatments and management of refractive cases. The susceptibility profile can be obtained only following H. pylori cultivation, supporting the genuine need for technique optimization.

Several studies have suggested improvement by applying different kinds of growth media, such as liquid or solid media with different properties. Other studies suggest changes in protocol for the period of incubation or physical conditions.

In the current study, we were able to demonstrate that utilizing trypsin’s digestive characteristics for tissue chemical degradation increases the H. pylori cultivation technique efficiency, which was expressed by an increase in the number of H. pylori colonies on a specific growth medium. Another benefit from an increased yield in H. pylori colonies obtained on the growth medium is the ability to shorten the work process for susceptibility profile determination, as the larger number of colonies enables an immediate sufficient suspension concentration (McFarland standard 3.0) (BSAC, 2013).

This process enables us to avoid the time-consuming process of increasing the microbial concentration through agar plate transfer. Another important issue is the false-negative cultures with no H. pylori growth obtained without the trypsin treatment. These same specimens were found to be positive, forming colonies of H. pylori after trypsin treatment.

In summary, we have found a simple and accessible technique for optimization of the known H. pylori culture method. This technique may become prevalent for H. pylori cultivation due to its benefits in terms of time shortening, low cost and more successful outcomes.

Fig. 1. Number of H. pylori colonies with no trypsin treatment and after trypsin treatment. Numbers are indicated above the columns.
REFERENCES


