ISOLATION AND IDENTIFICATION OF *Helicobacter pylori* SKP FROM SALIVA SAMPLE


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**ABSTRACT**

*Helicobacter pylori* infection is considered as one of the most common infectious diseases throughout the world, the carriage rate of *Helicobacter pylori* is reported to be 20-80% for adults in the developed world, and more than 90% in the developing world. It is also associated with peptic ulcer and gastric carcinoma and specially cofactor in the instance of happening again of aphthous ulceration, gastric colonization and mucosal attachment. In the present study the causal agent of human ulcer disease *Helicobacter pylori* SKP was isolated from patients of Government hospital, Mannargudi, Thiruvarur (Dt.), Tamil Nadu, India. The suspected bacteria isolated from the saliva samples from five patients with symptoms and signs suggesting gastritis was cultured on columbia blood agar. In routine practice the typical colonical and morphological appearances and rapid urease test is sufficient for establishing the identification of *Helicobacter pylori*.

**KEY WORDS:** *Helicobacter pylori*, saliva, peptic ulcer and urease.

**INTRODUCTION**

*Helicobacter pylori* is currently recognized as one of the most common chronic bacterial infections worldwide (Everhart, 2000). It is a gastric pathogen that chronically infects more than half of the world’s population, with a prevalence ranging from 25% in developed countries to more than 90% in developing areas (Blaser, 1997). This infection has a causative role in peptic ulcer disease, chronic superficial gastritis, non ulcer dyspepsia and gastric adenocarcinoma (McNulty et al., 2002). A peptic ulcer, sometimes called a stomach ulcer, is a sore that forms in the lining of the stomach or of the duodenum, which is the first part of the
small intestine. This common disease can happen to people of any age and both sexes. Almost 25 million people in the United States currently have peptic ulcers, and there are about 500,000 to 850,000 new cases of the disease diagnosed each year.

The prevalence of *Helicobacter pylori* infection is approximately 50% worldwide, and is as high as 80-90% in developing countries (Lacy and Rosemore, 2001). *H. pylori* is strongly associated with some pathologies such as gastric and duodenal ulcers and related to gastric cancer (Nomura *et al*., 1991). Eradication of *H. pylori* infection is widely recommended as the most effective treatment for peptic ulcer disease and substantially reduces the recurrence of gastroduodenal diseases (Rafeey *et al*., 2007). The objective of this study is to isolate and characterize strains *Helicobacter pylori* from ulcer patients saliva.

MATERIALS AND METHODS

Sample collection

The procedure for saliva collection was access from ulcer patients at Government hospital Mannargudi, Thiruvarur (Dt.), Tamil Nadu, India. Patients who underwent gastrointestinal including age and sex of the patients are presented in table 1. All studied patients signed an informed consent form and declared their willingness to allow the application of their anonymous data for research purposes. Saliva sample were collected from each patient. Saliva sampling was done in the morning. All patients were asked to wash their mouth with normal saline prior to saliva sampling. Saliva samples, in a volume of 2-3 mL, were collected using sterile toothpicks and filter paper. Samples were transported in sterile flasks containing digestion buffer [100 mmol NaCl, 10 mmol Tris-HCl (pH 8.0), 250 mmol ethylene diaminetetra acetic acid (EDTA) (pH 8.0) and 1% sodium lauryl sarcosine] on the day of sampling and were stored at -70°C (Silva, *et al*., 2010).

<table>
<thead>
<tr>
<th>Patients</th>
<th>Gender</th>
<th>Age (Year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Columbia Blood Agar Base medium (gm/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone, special</td>
</tr>
<tr>
<td>Corn starch</td>
</tr>
</tbody>
</table>
Sodium chloride - 5.000
Agar - 15.000
Final pH - 7.3±0.2

Added appreciate amount of Columbia Blood Agar Base to distilled water. Boiled to dissolve and sterilized by autoclaving at 121°C for 15 minutes. Aseptically add the contents to sterile Columbia Blood Agar Base cooled to approximately 50°C, Add 5% v/v sterile defibrinated sheep blood to sterile cool base and mixed well before pouring into sterile petri dishes. Addition of three antibiotics (vancomycin, polymyxin, trimethoprium) to make the media selective for the isolation of *H. pylori*.

**Incubation Conditions**

There are marked differences in the compositions of the microaerobic atmospheres used for culturing *H. pylori* (Kjoller et al., 1991). Concentrations ranging from 5-6% O₂, 7-12% CO₂, 10% H₂ and 0-85% N₂ have been reported. These can be achieved by using a gas generation kit, a variable atmospheric incubator or an anaerobic jar evacuated to 200mm Hg and refilled with a 10% CO₂, 10% H₂ and 80% N₂ gas mixtures. Although cultures should be incubated under microaerobic conditions at 37°C for at least four to five days, colony formation reported on Columbia blood agar plates after long term anaerobic incubation has been reported. Failure to detect the organism may be due to insufficient duration of incubation. It is important to maintain a humid atmosphere in the jars by placing moist filter paper or moist cotton wool at the bottom of the jar. However, this allows growth of fungi and other contaminant. The jars should be wiped with 70% alcohol between each use.

**Identification Of The Bacteria Through Colony Morphology And Staining Characteristics (Holt Et Al., 1994)**

The colonies were identified based upon their morphological characteristics like shape, size, structure, texture, appearance, elevation and colors. Further identification was done on the basis of staining. Differential staining i.e. Gram staining was performed.

**Biochemical tests**

The isolated organism was subjected to biochemical test for identification. There are several types of biochemical tests are available. Biochemical test were performed according to standing to standard methods the isolates are biochemically tested. The biochemical tests
include the following, Catalase, oxidase, Ureas, Citrate utilization, Nitrate reduction, Methyl red, Voges proskauer and Triple sugar iron test respectively.

RESULTS AND DISCUSSION

Robert warren, a pathologist and Barry Marshall a medical student at the time of the discovery received the Nobel Prize 2005 in medicine for their identification in 1982 of Helicobacter pylori. H. pylori are a gram negative, microaerophillic, rod-shaped bacterium that colonizes the human stomach. It resides beneath the gastric mucous layer, adjacent to the gastric epithelial cells; it causes inflammation of the gastric mucosa. Infection with this organism is now recognized as a serious, transmissible infectious disease, linked to duodenal and gastric ulcers and gastric carcinoma (Lee, 1994). H. pylori is the pathogen that infects humans, it is estimated 50% of the world population. It is a common cause of ulcer dyspepsia and peptic potentially curable.

H. pylori infection is one of the most common bacterial infections in man. The infection is widely accepted as an important cause of gastritis and is strongly associated with peptic ulcer disease and gastric cancer. The human stomach was considered to be the only reservoir for H. pylori until bacteria were discovered in the human dental plaque, in oral lesions or ulcers, in oral cavity, and in saliva (Madinier, et al., 1997).

In the present study, suspected bacteria isolated from the saliva samples from five patients with symptoms and signs suggesting gastritis was cultured on columbia blood agar plates and incubated for five days at 37 °C under microaerophilic conditions.

Parronet et al., (1998) reported that H.pylori was the most common infection caring agent in human. In this study, after isolating samples, immediately placed in transport media i.e. saline and samples brought to the laboratory within 2 hours and stored under cold conditions, same samples which are placed in urea broth, within 30 minutes colour of urea broth changes from yellow to pink. It indicates presence of H.pylori in isolated samples. For further conformation other biochemical tests such as catalase, motility, gram staining tests should be done. If the samples passed urease test, catalase test, motility test, gram-staining test, samples which are gram negative organism, having curved, rod shaped bacteria are considered as H.pylori organism. On one hand reports from across the globe suggest close association of H.pylori with gastric diseases. On the other, studies suggest fewer than 20% infected patients develop any clinical consequences (Blaser, 1997).
These organisms inoculated in brucella broth which is considered as liquid media, brucella broth is standardized by using Macfen solution. *H. pylori* in brucella broth streaked on Columbia blood agar which is selective media for *H.pylori*. Although success in *H.pylori* isolation and growth depends on many factors such as method of collection time, composition of culture media, transport conditions. Samples are incubated at 37°C under microaerophillic conditions, examined for 4 to 5 days, characteristic colonies of *H.pylori* were confirmed by Gram staining, Catalase and Motility tests. Several authors reported the presence of *H.pylori* in saliva and in esophagus (Quiding-Jarbrink *et al.*, 2009 and Suzuki *et al.*, 2008).

**Scientific Classification Of Helicobacter Pylori**

- **Class**: Epsilonproteobacteria
- **Order**: Campylobacterales
- **Family**: Helicobacteraceae
- **Genus**: *Helicobacter*
- **Species**: *pylori*

**Morphology characterization**

The identification and taxonomic position of the bacterial were determined microscopically based on morphology and shape under low and high power objectives. The morphology and the characters of bacterial isolates were identified (Plate-1).

**Cultural characteristics**

This was studied by observing the colony morphology of the bacterium. Here colonies were identified on the basis of morphology. The cultures showed typical morphology of *H. pylori* SKP they grew slowly, forming grey translucent colonies that looked like spreading fluid droplets.

**Microscopic appearance of Helicobacter pylori SKP**

**Motility test**

Hanging drop method carried out for motility test. This method is useful in observing the general shape of living bacteria and the arrangement of bacterial cells when they associate together. Organisms are observed in a drop that is suspended under a cover glass in a concave depression slide. *H.pylori* SKP was sluggishly motile. Bacteria are motile by means of flagella.
Gram staining test
The culture have been stained by primary stain and appeared stained in colour. It is a gram negative bacterium (Plate-1andTable-1). The gram stained \textit{H.pylori} SKP will have variable morphology including short rods, curved bacilli and characteristics U shaped spirals. A gram stain from a fresh clinical isolates is shown in Plate- 1.

\textbf{Plate -1 Morphological and Microscopic appearance of \textit{Helicobacter pylori} SKP}

\begin{center}
\includegraphics[width=\textwidth]{Plate1.png}
\end{center}

\textbf{Biochemical properties of \textit{Helicobacter pylori} SKP}
In the present study \textit{Helicobacter pylori} were isolated from saliva sample by using serial dilution spread plate method. The isolated bacteria were identified by using standard Bergey’s manual.

The cultures showed colour change from yellow to pink colour. Hence, it indicates urease positive. The key test is urease test. Effervescences were observed in strain this means that the result was catalase positive. Purple colour change was observed on oxidase sterile disc hence it indicates positive. In routine practice the typical colonical and morphological appearances and rapid urease test is sufficient for establishing the identification of \textit{Helicobacter pylori}.SKP. The \textit{Helicobacter pylori} SKP the following results were presented in Table -1. Similarly the urease hydrolysis of this bacterium convert urea into ammonia and bicarbonate in order to neutralize the acidity of the stomach, a way to adhere to mucus cells (Sobhani \textit{et al.}, 1995). These biochemical characteristics were demonstrated by Monteiro (1995). Cassel-Beraud \textit{et al.} (1996) found that after 60 min, the change in color of the medium urea-indole from orange to red indicates the presence of bacteria. Sobhani \textit{et al.} (1995) studied that the identification was made by evaluating the morphology of the bacteria,
biochemical characters such as oxidase, urease, catalase and H₂S production in TSI are also sought. After culturing, microscopic examination of a smear prepared from suspect colonies allowed us to observe for Gram-negative bacilli that can be *Helicobacter pylori*.

**Table -1 Morphological and Biochemical test for Helicobacter pylori**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Morphological and Biochemical test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Gram Stain</td>
<td>Gram Negative</td>
</tr>
<tr>
<td>2.</td>
<td>Shape</td>
<td>Helical &amp; Spiral Rods Shaped</td>
</tr>
<tr>
<td>3.</td>
<td>Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>4.</td>
<td>Methyl red</td>
<td>Positive</td>
</tr>
<tr>
<td>5.</td>
<td>Voges proskauer</td>
<td>Positive</td>
</tr>
<tr>
<td>6.</td>
<td>Citrate utilization</td>
<td>Negative</td>
</tr>
<tr>
<td>7.</td>
<td>TSI</td>
<td>H₂S production</td>
</tr>
<tr>
<td>8.</td>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>9.</td>
<td>Oxidase</td>
<td>Positive</td>
</tr>
<tr>
<td>10.</td>
<td>Urease</td>
<td>Very Strongly Positive Within 1-15 Minutes</td>
</tr>
<tr>
<td>11.</td>
<td>Nitrate reduction</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**CONCLUSION**

*Helicobacter Pylori* is a micro-organism that is associated with a Microbial and Bacterial infection. The suspected *Helicobacter pylori* SKP isolated from the saliva samples from five patients with symptoms and signs suggesting gastritis was cultured on columbia blood agar. It is a gram negative, rod shaped and sluggishly motile bacterium. In routine practice the typical colonial and morphological appearances and rapid urease test is sufficient for establishing the identification of *Helicobacter pylori* SKP. This infection responds to treatment by antibiotic, although in some patients the infection can be difficult to eradicate and there is a significant rate of recurrence. Whether the rate of recurrence can be reduced by concomitant emphasis on improving oral hygiene and treating periodontal disease remains to be clarified. Collectively, these data suggest that the oral cavity may be a reservoir for *H. pylori* in some individuals and transmission of the disease may be via an oral-to-oral route.

**ACKNOWLEDGEMENT**

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REFERENCES


