CHAPTER 3

Isolation, identification and characterization of Helicobacter pylori from human gastric biopsies
Chapter-3

ISOLATION, IDENTIFICATION AND CHARACTERIZATION

OF HELICOBACTER PYLORI FROM HUMAN GASTRIC

BIOPSIES.

3.1: Introduction:

Helicobacter pylori is Gram negative spiral bacterium and is a major cause of gastrointestinal diseases and mostly found in the stomachs of patients with gastro duodenal diseases, such as duodenal and gastric ulcers (Hansson, et al, 1996). In addition, H. pylori is thought to be one of the major causes of gastric cancer (Honda, et al, 1998).

Diagnostics of H. pylori is very important for the treatment of the infection by eradicating H. pylori. Culturing of the pathogen is considered to be as the “Gold standard” in terms of diagnostics, but since H. pylori is a fastidious organism which requires more than 36 hours for the growth, hence it is not easy in case of H. pylori infection (Cellini, et al, 1994) and the sensitivity of the H. pylori isolation method shows a marked variation (Chang-Young, et al, 2003).

The optimum temperature for the growth of H. pylori is 37°C and it takes almost 48 hours of incubation in microaerophilic conditions containing 5% oxygen, 10% carbon dioxide and 85% nitrogen (Henriksen, et al, 2000) with a pH range of 5.5-8.0 (Morgan, et al, 1987). It has been reported that H. pylori grows very well on Brain Heart Infusion agar (BHI) supplemented with 7% horse blood
serum and selective antibiotics with an isolation rate of about 99% (Hachem, et al, 1995). The two main prerequisites proposed to achieve the optimal growth, were the use of fresh media made with fresh blood and maintenance of adequate humidity (100%) throughout the incubation period.

Very tiny colonies of *H. pylori* measuring 1-2 mm, translucent in appearance occur after the primary culture. This bacterium changes from helical to curved and / or coccoied some times, which reflects an adaptation to a hostile environment generally resulted by the deprivation of nutrient, exposure to antibiotic or extended incubation. Biochemically the organism was most notable for its high urease activity, a good catalase and oxidase activity. The urease enzyme constitutes a simple and a rapid method for the detection and identification of *H. pylori* infection.

*H. pylori* has colonized itself in the stomachs of more than half of the world's population. Gastroduodenal site is supposed to be the major site of colonization. *H. pylori* localize it self under the mucus layer overlying the gastric epithelium. This bacterium possesses unusually high urease activity which helps it to colonize the viscous acidic environment by splitting urea present in the blood to ammonia and CO₂, this ammonia clouds helps lower down the pH up to 7.0. *H. pylori* is now accepted to be the major cause of duodenal ulcer (65-90%) and gastric ulcer (60-74%), but the majority of the infected subjects do not show any symptoms (45-50%) (Brown LM. 2000). In India the reported rate of *H. pylori* isolated from the asymptomatic subjects vary between 31-57% and 64-90% in peptic ulcer subjects respectively (Misra, et al, 1997). However despite the
commonality of infection between the symptomatic and asymptomatic subjects, the major difference was noted in the development of disease. Studies have attributed this difference in the establishment of disease to the presence of certain characteristic virulent factors.

Therefore the present study was designed to isolate the *H. pylori* individually from the dyspeptic subjects who have undergone upper gastrointestinal endoscopy.

3.2: Materials & Methods:

3.2.1: Collection of samples:

A total of 125 patients, who were suffering with various gastroduodenal diseases were enrolled for the study. Biopsies were collected from all the patients after taking informed consents, at Dept. of Gastroenterology, Deccan College of Medical Sciences & Allied Hospitals, Hyderabad, (A.P).

3.2.2: Transport of specimens:

Biopsy: A total of 4 biopsies were collected from the patients using fiber optic video gastroendoscope. Biopsies were taken from the antrum with sterile precautions. The first antral biopsy was collected in RUT broth for Rapid Urease Test, the second and third biopsies were collected in the transport medium (brucella broth) for culture and the fourth biopsy was collected in 10% buffered formalin for Histopathology (see the flow chart below).
Materials:

Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate dihydrate, phenol red, sodium azide, urea powder, sodium hydroxide.

Preparation of reagents:

1) Solution A (Sodium dihydrogen orthophosphate-\(\text{NaH}_2\text{PO}_4\)):

Sodium dihydrogen orthophosphate, 0.156 grams was dissolved in 100ml of distilled water to obtain a solution of 0.01M.

2) Solution B (Disodium hydrogen orthophosphate dihydrate-\(\text{Na}_2\text{HP}_4\)):

Disodium hydrogen orthophosphate, 0.141 grams was dissolved in 100ml of distilled water to obtain a solution of 0.01M. A working medium was prepared by adding 51ml of solution A to 49ml of solution B. To this solution 20mg of sodium azide, 2g of urea powder were added and the pH of the medium was adjusted to 6.8 with 0.1N NaOH. Finally 0.1 ml of phenol red indicator was added and the medium was sterilized at 5 lbs for 20 minutes. This medium was stored at 4°C for further use.

Procedure:

Liquid urease medium (approx. 200\(\mu\)L) was dispensed in 1.5 ml sterile eppendorf and the biopsy was inoculated in the endoscopy room. The reaction was observed for 1-4 hrs. Colour change from yellow to pink was considered positive for \(H.\ pylori\) and if there is no change in colour of the medium between 4-24hrs the reaction was considered as negative.
3.3.2: Culture:

Culture Medium:

For the primary isolation of H. pylori Brucella Agar supplemented with 7% sheep blood and the antibiotics \{Vancomycin (6mg/L), Amphotericin B (3mg/L), Polymixin B (2500 IU/L)\} were added to the prepared medium (Shanjana, et al, 1995).

Materials:

Antibiotics were procured from Hi-Media. Brucella agar from Becton, Dickinson & Co, Sparks, USA. Animal House, Deccan Medical College, kindly supplied sheep blood.

Preparation:

Brucella agar (BA), 4.3 grams was added to 93ml of distilled water and the medium was autoclaved at 15 lbs for 15 minutes. Medium was then cooled to 55°C, 7ml of freshly drowned sheep blood was added and kept at 85°C in a water bath for 20 min. Again cooled the medium to 55°C and selected antibiotics were added under sterile condition. The medium (approx. 20ml) was poured in sterile petri dishes and allowed to settle at room temperature. The plates were then stored at 4°C for further use.

Bacterial Culture:

For the primary isolation of H. pylori, biopsies transported in brucella broth medium were crushed between the two sterile slides and inoculated with the
help of the sterile loop on brucella agar plates. Plates were then incubated under microaerophilic environment (Candle jar) at 37°C for 72 hrs.

**Identification of H. pylori colonies:**

Characteristic grey, translucent, tiny (0.5-1 mm) colonies that were obtained have been used to identify *H. pylori*.

### 3.3.3: Histopathology:

Ten percent buffered formalin fixed antral biopsy specimens were used for histopathological analysis of *H. pylori* with the help of experienced pathologist. Presence of *H. pylori* was identified using Giemsa staining method.

### 3.4: Identification of *H. pylori* Culture:

#### 3.4.1: Morphology:

Bacterial smear was prepared by placing a single colony on a glass slide and a drop of sterile saline was added to it. Smear was allowed to fix the slide then processed for Gram staining. Presence of characteristic Gram-negative spiral or curved shaped organisms confirms the presence of *H. pylori*.

**Gram staining:**

Materials:

1. *Genta violet* (0.5% w/v): 0.5 grams of crystal violet was dissolved in 50ml of distilled water and the volume was made up to 100ml.
2. **Iodine** (1% v/v): 1% Iodine was prepared in water.

3. **Absolute Alcohol**

4. **Carbolfuschin (0.5% w/v)**: 0.5 grams of carbolfuschin was dissolved in 100ml of distilled water to attain a final concentration of 0.5%.

**Staining:**

The bacterial smear was initially stained with the 0.5% crystal violet solution for one minute, and subsequently with 1% iodine for 1 minute. The slide was washed in running tap water and decolourized with absolute alcohol. The smears were then rinsed thoroughly with distilled water and stained with 0.5% of carbolfuschin for 1-2 minutes. Finally the slide was washed in distilled water, dried and examined under the oil immersion for the presence of characteristic spiral or S-shaped Gram negative organisms.

**3.4.2: Identification by biochemical tests:**

To further substantiate the results from biochemical tests, as *H. pylori* is most notable for its high urease activity, along with oxidase and catalase activities. These tests help in the identification of *H. pylori* from other gram-negative bacteria specially the urease negative Campylobacters and other gastrointestinal pathogens.
a) **Oxidase:**

Reagent preparation:

N, N, N1, N1, tetramethyl-p-phenyl-ethylenediamine dihydrochloride (1 mg) was dissolved in 10ml of distilled water. This reagent was stored at 4°C in brown bottles.

Procedure:

The isolated colonies were tested for oxidase activity by transferring with a platinum loop onto the filter paper disks, presoaked in oxidase reagent. A positive reaction develops deep blue/black colour in the smeared area with in 10 seconds, is indicative of oxidase positive bacteria.

b) **Catalase:**

Three ml of hydrogen peroxide (H₂O₂) was mixed with 97 ml of distilled water to prepare 3% solution of H₂O₂.

Procedure:

Catalase activity was detected by placing a loopful of bacterial culture onto a drop of 3% H₂O₂ present on a glass slide. A positive reaction is seen by effervescence.

c) **Urease:**

As described above under biopsy rapid urease test, but here a loop full of culture was used.
3.4.3: Confirmation of isolated *H. pylori* strains by Polymerase Chain Reaction:

Isolated culture of *H. pylori* was then confirmed by Polymerase chain reaction (PCR). Primers have been synthesized for the species-specific gene of *H. pylori* i.e. 16S rRNA gene, iceA gene, and cagA gene as reported by Kauser *et al.*, in 2005. Following is the primer sequences of the same.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S F</td>
<td>5′TAAGATCAGCCTATGICC – 3′</td>
</tr>
<tr>
<td>16S R</td>
<td>5′TCCCACGCCTTAAGCGCAAT-3′</td>
</tr>
<tr>
<td>iceA1F</td>
<td>5′ TATTITCTGGAACTTGCGCAACCTGAT 3′</td>
</tr>
<tr>
<td>M.HpylR</td>
<td>5′ GGCTACAACCGCATGGATAT 3′</td>
</tr>
<tr>
<td>CycSF</td>
<td>5′ CGGCTGTAGGCACCTAAGCTA 3′</td>
</tr>
<tr>
<td>IceA2R</td>
<td>5′ TCAATCCTATGTGAAACAATGATCGT 3′</td>
</tr>
<tr>
<td>CagA-F1</td>
<td>5′ AACAGGACAAGTAGCTAGCC 3′</td>
</tr>
<tr>
<td>CagA-R1</td>
<td>5′ TATTAATGCCTGTGGCCTG 3′</td>
</tr>
</tbody>
</table>

Primers have been used for the amplification of 16S rRNA gene from the isolated DNA of the culture of *H. pylori*; overall method of PCR is as follows.

**Rapid method of DNA extraction:**

Approximately 4 loops of the bacterial culture is scraped of the culture plate and suspended in 1.5ml tubes containing 400 µl of sterile distilled water. Tubes were incubated at 96°C in a hot water bath for 15 min for lysis. The bacterial lysate was centrifuged at 12000 rpm for 15 min in a cool centrifuge and transferred the supernatant to a fresh tube for PCR.
**Reaction Mixtures for PCR:**

The reaction mixture /PCR mixture contained 2 μL of 10X buffer (mixed with 1.5 mM MgCl₂), 1 Unit of Taq DNA polymerase, 0.5 μL of 10mM dNTPs, 0.5 μL of both forward and reverse primers (20pM/μL) and 1μL of supernatant used as DNA. The final volume was made up to 20 μL by adding milliQ water. The amplifications were subjected to following conditions.

**PCR conditions:**

- 95°C for 5 min
- 94°C for 30 sec
- 56°C for 30 sec
- 72°C for 1 min
- 72°C for 10 min

**40 cycles**

**Electrophoresis conditions:**

The amplified PCR products were than loaded on a 1.5% agarose gel containing 4μL of ethidium bromide (20mg/ml) along with 100 Kb molecular weight marker so as to confirm the product size which is 534 bp for 16S rRNA gene.

**3.5: Preservation of Isolated cultures:**

Isolated strains were preserved in preservation vials of brucella broth with 10% Fetal Calf Serum (FCS) and 20% glycerol and maintained at -80°C.
3.6: Results:

Rapid Urease Test was done for a total of 125 biopsy specimens; from each patient, first antral biopsy was directly collected in an eppendorf tube containing rapid urease test (RUT) broth and was incubated at room temperature for 24 hrs to monitor the change in the colour of the medium.

Out of 125-biopsy specimens 103 (82.4%) of the biopsies showed the colour change in the medium from golden yellow to pink indicating the positive reaction (Tab-3.1 & Fig-3.1).

In individual diseases, out of 40 DU subjects 37 (92.5%) were positive, in 50 GU subjects 42 (84.0%) were positive, in 12 NUD subjects 8.0 (66.66%) were positive, in GERD patients 1 out of 8 patients (12.5%) were positive for RUT and in 15 GC cases 15 (100%) were positive for urease (Tab-3.2).

Isolation of \textit{H. pylori} from gastric biopsies:

For culturing of \textit{H. pylori} second and third antral biopsies were collected in the transport medium and taken to the Bacteriological laboratory with in one hour of the collection. \textit{H. pylori} colonies were identified by their typical morphology on chocolate agar plate (Fig-3.2). About 95 (76.0%) of the specimens were positive for the culture (Tab-3.1).

In individual diseases, out of 40 DU subjects 36 (90%) were positive, in 50 GU subjects 38 (76%) were positive, in 12 NUD subjects 8.0 (66.66%) were positive and in 15 GC cases 12 (80%) were positive for culture where as out of 8.0 GERD patients only 1 (12.5%) was found to be positive for \textit{H. pylori} culture.
Histopathology:

One section of fourth antral biopsy collected in buffered fixative formaline was stained with Giemsa stain and presence of *H. pylori* was identified microscopically (Fig-3.5). About 179 (87.7%) of the subjects were histopathologically positive (Tab-3.1).

In individual diseases, out of 40 DU subjects 32 (80%) were positive, in 50 GU subjects 37 (74%) were positive, in 12 NUD subjects 09 (75%) were positive, in 08 GERD patients nothing was positive (0%) and in 15 GC cases 15 (100%) were positive histologically.

Morphological identification of *H. pylori*:

The *H. pylori* colonies obtained on brucella chocolate agar plate were small, tiny, silver colour and translucent (Fig-3.2 & 3.3), which is the typical morphology of *H. pylori* on brucella chocolate agar hence confirming that the isolated bacterium is *H. pylori*.

Microscopy:

Prepared slides for gram staining when seen under oil immersion objective, Gram- negative spiral, curved and rod shaped bacteria were seen predicting the presence of *H. pylori*. 
Biochemical Tests:

**Oxidase:**

Oxidase test was done for the total isolates and a positive reaction was indicated by the appearance of purple colour in the smeared area within 10 seconds all the isolates (95) were positive for oxidase.

**Catalase:**

Catalase test was done for the complete isolates and the positive reaction was observed with effervescence and indicated the presence of catalase enzyme. All the isolates (95) were positive for catalase.

**Urease:**

Presence of urease enzyme is tested by rapid urease test (RUT) by inoculating the colonies in to 0.2 ml urea broth. The presence of urease is indicated by colour change from yellow to pink (Fig-3.1).

The bacterial culture was considered as positive for *H. pylori*, only if a positive reaction was obtained for all the biochemical tests and posses a characteristic spiral shaped morphology. Therefore we can conclude that all the 174 isolates tested were of *H. pylori*.

16S rRNA confirmation of the isolated strains:

All the isolated *H. pylori* strains were confirmed by using primers specific for 16S rRNA, *iceA* gene, and *cagA* gene of *H. pylori* to carry out the polymerase chain reaction (PCR). All the isolates (95) were found to be positive for the PCR
as they showed amplification of 534 bp (Fig-3.6) region, specific for *H. pylori*; this confirms that isolated strains were purely *H. pylori* without any doubt.

**Table No. 3.1: Overall prevalence of *H. pylori* in dyspeptic subjects**

<table>
<thead>
<tr>
<th>subjects</th>
<th><em>H. pylori</em> status</th>
<th>RUT</th>
<th>Culture</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>Positive</td>
<td>103</td>
<td>95</td>
<td>93 (74.4%)</td>
</tr>
<tr>
<td></td>
<td>(82.4%)</td>
<td></td>
<td>(76%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>22</td>
<td>30 (24%)</td>
<td>32 (25.6%)</td>
</tr>
<tr>
<td></td>
<td>(17.6%)</td>
<td></td>
<td>%</td>
<td></td>
</tr>
</tbody>
</table>

**Table No: 3.2: Prevalence of *H. pylori* by four different methods with respect to disease status.**

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>Total subjects</th>
<th>Urease (%)</th>
<th>Culture (%)</th>
<th>Histopathology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU</td>
<td>40</td>
<td>37 (92.5)</td>
<td>36 (90.0)</td>
<td>32 (80.0)</td>
</tr>
<tr>
<td>GU</td>
<td>50</td>
<td>42 (84.0)</td>
<td>38 (76.0)</td>
<td>37 (74.0)</td>
</tr>
<tr>
<td>NUD</td>
<td>12</td>
<td>08 (66.66)</td>
<td>08 (66.66)</td>
<td>09 (75)</td>
</tr>
<tr>
<td>GERD</td>
<td>8</td>
<td>01 (12.5)</td>
<td>01 (12.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>GC</td>
<td>15</td>
<td>15 (100)</td>
<td>12 (80)</td>
<td>15 (100)</td>
</tr>
</tbody>
</table>
3.7: Discussion:

The present study was carried out to evaluate the association of \textit{H. pylori} in various gastric disorders and to isolate the organism from these subjects. Detection of \textit{H. pylori} from patients suffering with various gastro duodenal disorders like Duodenal Ulcers [DU], Gastric Ulcers [GU], Non ulcer dyspepsia [NUD] and Gastric cancer [GC] has provided us much impetus for the significant association of \textit{H. pylori} with these diseases.

Traditionally, culturing the pathogen is considered the "gold standard" for the diagnosis of infectious diseases. However, diagnosing \textit{H. pylori} infection by culture alone may have certain limitations. Most importantly, there are possible false negatives due to sampling error because the culture, using biopsy specimens, can assess infection only at the biopsy sites (NIH Consensus Conference, 1994). Microscopic examination and the rapid urease test can be highly specific if strictly performed, but they are based on biopsy specimens and thus are theoretically prone to sampling error, as in the case of culture (Yoshida H, \textit{et al}, 1998). Hence in the present study we compared all the tests including histopathology.

All the 125 subjects were screened for the study. Biopsies taken during the upper gastrointestinal endoscopy were used for the isolation and identification of \textit{H. pylori}. The methods used for the detection of \textit{H. pylori} were rapid urease test (Fig-3.1), culture (Fig- 3.2 & 3.3), histology (Fig-3.5). Data showed that 82.4\% of the subjects were positive by urease (Tab-3.1), which is comparable with the result of Malfertheiner, \textit{et al}, (1996) who evaluated two commercial tests and suggested the comparable sensitivities and specificities of RUT and CLO.
(Campylobacter like organism) tests (RUT-93% and 100%, CLO-88% and 100%) but Borromeo, et al, (1987) reported that the urease test suffer from low sensitivity.

About 74.4% of the subjects were positive by histology. A study from Detroit (Vasundhara, et al, 2000) showed histology as 100% specific and sensitive. Some authors also found histology to be superior to the rapid urease test (Misra, et al, 1997) and considered to be the single most reliable test (Grove, et al, 1998). However, Deltenre, et al, (1989) suggested histology and urease are the best suited for the identification of H. pylori. Vasundhara, et al, (2000) reported 71% of culture positivity in their recent study. In our observation about 76% of the subjects tested were positive by culture, which is much better yield.

Infection with H. pylori is now accepted to be the major cause of duodenal ulcer i.e. 65-90% and gastric ulcer i.e. 60-74%, but the majority of the infected subjects do not show any symptoms i.e. 45-50% (Brown, 2000). However Misra, et al, (1997), reported 64-90% in peptic ulcer subjects. In the present data H. pylori is present in 92.5% in DU, 84.% in GU, 66.66% in NUD, 12.5% in GERD and 100% in GC by urease activity, while culture showed 90%, 76.0 %, 66.66 %, 12.5% and 80 % respectively. The organism was histologically detected in 80 % of DU subjects, 74 % of GU subjects, 75 % of NUD, 0% in GERD subjects and 100% of GC subjects (Tab-3.2).

The tests that have been employed in the present study are universally accepted as reliable and specific for the identification and isolation of the bacteria;
even though these tests are variable individually, but the combination of these three tests give more accuracy than the individual tests (Thijs, et al, 1996).

Weiss et al, (1994) demonstrated the specificity of unique \textit{H. pylori} 16S rRNA gene primers to identify the organism in paraffin-embedded gastric biopsy specimens. As PCR is a very specific and accurate test than the routine biochemical tests in the present study we applied it for culture and obtained 100% results.

Culturing of \textit{H. pylori} is a very laborious and time taking process because of the fastidious nature of the organism, but it is a very sensitive detection method with the advantages of specifically detecting the organism and making strains available for susceptible and virulence testing (Lage AP, et al, 1995). Here in our study the overall culture positivity was 85.3\% (Tab-3.1), where as in other reports the success rate was ranging from 60-90\%, even in some other studies it was greater than 95\% (Hachem CY, et al, 1995), this high rate of isolation in the latter study might be because of specialized CO\textsubscript{2} incubators or sophisticated desiccators with artificial CO\textsubscript{2} gas generators used for the isolation which are very expensive. Where as, we employed candle jar desiccators to maintain the appropriate conditions for the isolation of this fastidious organism, as it is reported by Morshed, et al, (1995).

Moreover this is the cost effective way of isolation of \textit{H. pylori} compared to the other methods generally in practice. However the success rate of the culture was reported to be influenced not only by the cultural conditions but also by several other factors like, transportation of the biopsies to the laboratory within
one hour of the collection, duration of the incubation, type of enrichment used in the media, type of cultural medium used, load of the bacteria in the biopsy, site of the biopsy, etc, (Henriksen, et al, 1995).

Hence we can conclude from the above study that isolation of Helicobacter pylori in candle jar desiccators is a more liable and flexible method, which gives more than 80% of accuracy. Moreover this is the cheapest method for the culturing of H. pylori, as far as developing countries like India is concern, the combination of Urease test, Culture and Histopathology combining together will give more accuracy than the individual test along with the conformation by polymerase chain reaction.
Fig-3.1: Typical photograph showing Rapid Urease Test (RUT) of *Helicobacter pylori* (pink colour indicates positive for RUT)
Fig-3.2: Typical photograph showing primary culture of *Helicobacter pylori*

single colonies of *H. pylori* were picked up from this plate and streaked on
another plate to obtain pure single colony isolated culture of *H. pylori.*
Fig-3.3: Typical photograph showing subculture of *Helicobacter pylori* from the primary culture, this culture is scraped and used for the DNA isolation for the genetic studies.
Fig-3.4: Typical photograph showing Gram negative spiral bacterium *Helicobacter pylori* under oil immersion objective [100X] + 1X digital camera magnification
Fig-3.5: Tufts of spiral shaped *Helicobacter pylori* seen after staining the gastric biopsy by Giemsa stain.
Fig-3.6. Gel image showing 16S rRNA amplification of *H. pylori* (Lane 1, 2, 3, and 5 represents 16S rRNA amplification of *H. pylori* DNA isolated from the culture at 534 bp. Lane 6 represents positive control ATCC 26695; Lane 4 represents 100 bp Molecular weight marker)
Fig 3.7: A typical microphotograph showing *H. pylori* in the crypt. (Haematoxylin & Eosin stain)
Figure 3.8: Gel image showing cagA gene amplification of *H. pylori* (Lane 3-10 represents cagA amplification of *H. pylori* DNA isolated from the culture at 756 bp. Lane 2 represents positive control ATCC 26695; Lane 1 represents 100 bp Molecular weight marker)
Figure 3.9: A typical gel image showing iceA allele amplification
(Lane 1, 2, 3, 5, 6 and 7 represents iceA1 and Lane 4 represents iceA2 allele, where as 
Lane M represents 100 bp molecular weight marker)
Figure 3.10: Typical photograph showing scanning electron microscopic photograph of *Helicobacter pylori* (arrows indicate flagellum)