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Antibiotic Resistance of Helicobacter pylori Strains in the United Arab Emirates and its Relation to the Gene Associated to Gastric Cancer "Cag A Gene"

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A thesis
Submitted to

United Arab Emirates University
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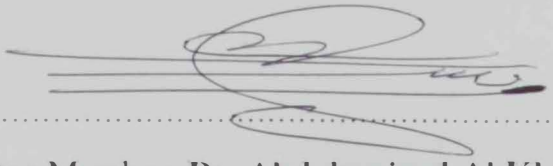
2008 -2009

The Thesis of **Wafa Ali Al-Tayyari** for the Degree of Master of Science in Environmental is approved.



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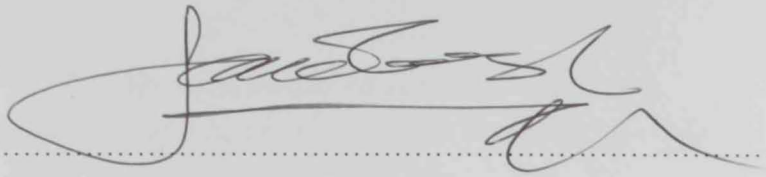

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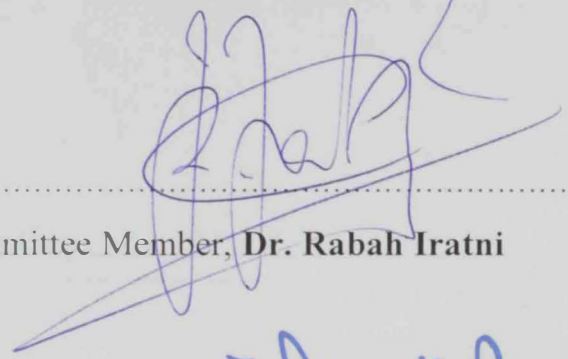

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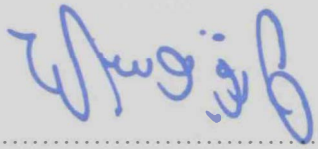

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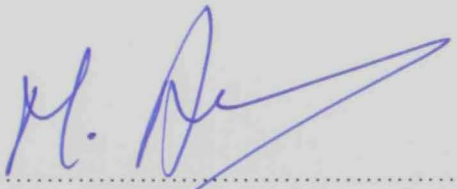

Acting Dean, College of Science, **Prof. Mohammed N. Anwar**

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"To research means to see what all people have seen and to think what no body has thought"

Dedicated to my Beloved Parents

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Who devoted himself to the building of the country and citizens....
knowledge and duty.... to whom the peoples' hearts beat with love and pray long life for him.

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Abstract

Helicobacter pylori is a flagellated corkscrew, slow growing neutrophilic gram negative ureolytic organism. It has an extraordinary ability to establish infection in human stomach that can last for years or decades. Its eradication remains an important public health challenge especially in light of broadening indications and increasing antimicrobial resistance.

AIM: i) Identification of *H. pylori* in UAE patients ii) Determination of the prevalence of antibiotic resistance genes (mutation in *23S rRNA* gene in clarithromycin, and deletion in *RdxA* gene in metronidazole) among *H. pylori* strains isolated from U.A.E patients by using molecular methods, iii) Ascertain whether *Cag A*- positive *H. pylori* strains correlates with the antibiotic resistance strains or not and iv) Screening for new *H. pylori* strains in UAE through the phylogenetic analysis of the *23S rRNA* middle region of the gene.

METHODS: The identification of *H. pylori* in UAE patients was carried on by primary screening for *H. pylori* using CLO test while the confirmation test were done using PCR technique. The prevalence of antibiotic resistance genes (mutation in *23S rRNA* gene in clarithromycin, and deletion in *RdxA* gene in metronidazole) among *H. pylori* strains isolated from U.A.E patients and *Cag A* gene relation between this gene and antibiotic resistant genes were studied by PCR and sequencing technique. Phylogenetic analysis of the *23S rRNA* gene was analyzed by using the software ClustalX, version 2. Reference sequences used in the alignment was obtained from NCBI data base for all *23s rRNA* from different *H. pylori* strains.

RESULTS: 26 out of 90 biopsy samples were positive for *H. pylori* using PCR whereas only 22 were positive when tested by CLO test. Resistance to clarithromycin and metronidazole was detected in 9 and 3 of strains, respectively. Of the clarithromycin resistant strains, 2 strains had the A2142G mutation in the *23S rRNA* gene, 5 strains in A2143G, 1 strain in A2143C and 1 strain of highly changed in sequence. Of the metronidazole resistant strains, deletion in *rdxA* gene was detected in 3 strains which were negative for CLO test. DNA sequence phylogenetic analysis of the *23S rRNA* middle region of the gene indicates that the strains from UAE harbor a unique *23S rRNA* sequences that is common among isolates from the UAE patients and different than other strains published in the NCBI database.

CONCLUSION: This study is the first time done in the UAE where a significant proportion of gastric mucosal biopsies obtained in the UAE are positive for Genes associated with Clarithromycin and Metronidazole resistance (mainly in Clarithromycin). A2143G remains the most prevalent point mutation involved, thus suggesting that new therapeutic strategies are needed.

Key Words: *H. pylori*; Antibiotic resistant genes; *Cag A*; Phylogenetic Analysis of *23S rRNA*.

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List of Abbreviations

C. jejuni	:	*Campylobacter jejuni*
H. pylori	:	*Helicobacter pylori*
DNA	:	Deoxy Ribose Nucleic Acid
Cag A	:	*Cytotoxin associated gene A*
PPI	:	Proton Pump Inhibitor
LPS	:	Lipopolysaccharide
PAI	:	Pathogenicity Island
GERD	:	Gastroesophageal reflux disease
MALT	:	Mucosa-associated lymphoid tissue
CLO	:	Campylobacter Like Organism
PCR	:	Polymerase chain reaction
ATPase	:	Adenosine triphosphatase
RBC	:	Ranitidine bismuth citrate
NI	:	Nitroimidazole
POR	:	pyruvate oxidoreductase
NADH	:	Reduced form of Nicotinamide adenine dinucleotide
MIC	:	Minimal inhibitory concentration
RFLP	:	Restriction fragment length polymorphism
OLA	:	Oligonucleotide ligation assay
DEIA	:	DNA enzyme immunoassay
LiPA	:	Line probe assay
PHFA	:	Prefrential homoduplex formation assay

CHAPTER I

INTRODUCTION

1.1. Overview

In the early 1980s, Drs. Barry Marshall and Robin Warren from Australia discovered bacteria in the stomach lining of patients with chronic gastritis and peptic ulcers (Marshall and Warren, 1984). It was originally named *Campylobacter pyloridis* because it was structurally similar to other *Campylobacter* species, such as *C. jejuni* (gut pathogen which has the ability to colonize the gastric mucosa of animals) (Holeston, 2004). *Campylobacter pyloridis* was renamed *Campylobacter pylori* to fit with the names of other enteric pathogens. In 1989, it was finally named *Helicobacter pylori* based on functional and enzymatic properties (Holeston, 2004). This bacterium was flagellated corkscrew, slow growing neutrophilic gram negative ureolytic organism (Sachs *et al.*, 2002; Roussos *et al.*, 2003). It has an extraordinary ability to establish infection in human stomach that can last for years or decades (Kersulyte *et al.*, 2000). In order to know more about *H. pylori*, DNA motifs at several informative loci in more than 500 strains of this bacteria from five continents were studied by PCR and sequencing, and thus to gain insights into the evolution of this gastric pathogen. Five types of DNA motifs such as deletion, insertion, and substitution were found at the right end of *H. pylori* cag Pathogenicity Island. As a result of a study, three motifs types were found to be common; type I, types II and type III (kersulyte *et al.*, 2000).

Walker and Edwards (2003) reported that *H. pylori* are mostly transmitted by oral-oral and faecal-oral routes. Many important risk factors are present and can cause infection by this bacteria; such factor are: low social class, overcrowding and home environment during childhood (e. g. bed-sharing). The prevalence of *H. pylori* in developed countries was compared with developing countries, and the result

showed a reduction in the infection with *H. pylori* in most developed countries, and is paralleled with low incidence of duodenal ulcer and gastric cancer.

Variety of extra digestive disorders in the past few years, including respiratory diseases, cardiovascular, skin rheumatic and liver diseases have been discovered to be associated with *H. pylori* infection. The pathogenetic mechanism of this bacteria is due to the activation of inflammatory mediators which underlying the observed associations between *H. pylori* infection and respiratory diseases. A small number of epidemiological and serological case control studies suggest that *H. pylori* infection may be associated with the development of chronic bronchitis. A frequent presence of pulmonary tuberculosis and *H. pylori* infection has also been found (Roussos *et al.*, 2003).

As it was mentioned before that *H. pylori* is an important factor of chronic gastritis disease (Normark *et al.*, 2003; Rintala *et al.*, 2004). Gastritis is a term which can be defined as an infiltration of the tissue with lymphocytes and plasma cells. These bacteria can make infection by secreting large amounts of urease, which helps it to survive in the highly acidic environment of the stomach. About 50 percent of all strains produce cytotoxins, some of which (e.g., *cag A* toxin) are associated with gastritis and ulceration **(Fig. 1.1)**. These cytotoxins cause local inflammation, resulting in a weakening of the gastric mucosal barrier that can lead to further tissue injury.

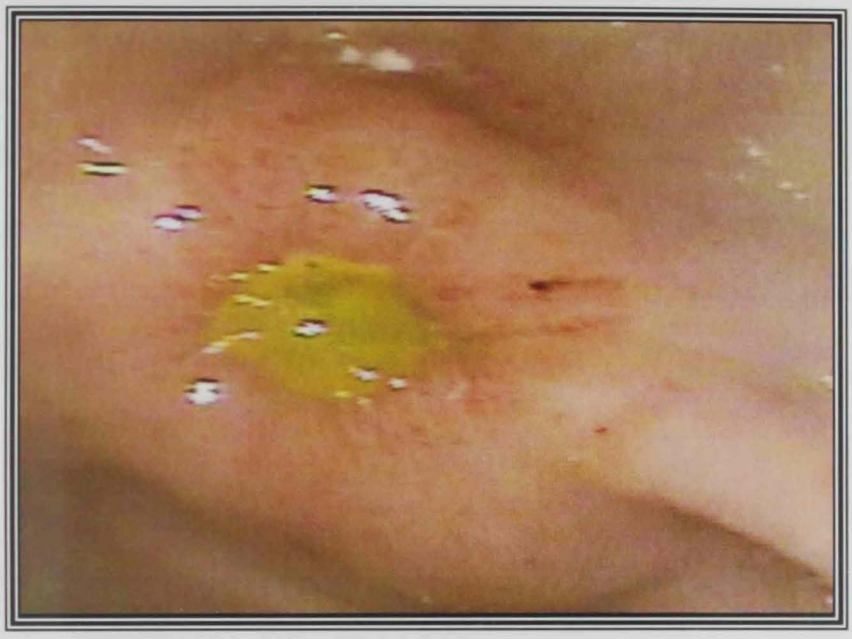


Fig. 1.1: Photograph of an ulcer in the stomach as seen on upper endoscopy.

Decreasing in gastric juice ascorbic acid levels, which are thought to be a predisposing factor for gastric ulceration and possibly gastric cancer, is also a result of *H. pylori* infection (Kleef *et al.*, 2003).

H. pylori eradication remains an important public health challenge especially in light of broadening indications and increasing antimicrobial resistance (Cavallaro *et al.*, 2006). Eradication of the organism play a role in the accelerating the ulcer healing and also prevents long-term ulcer relapse. Now a day, two antibiotics have been demonstrated to have a high eradication rates (greater than 90%) based on the eradication regimens using proton pump inhibitor (PPI). Amoxicillin, clarithromycin, and metronidazole are the most frequently used antibiotics for the treatment of *H. pylori* infection. However, antibiotic resistance frequently causes failure of eradication of *H. pylori* (Kato *et al.*, 2002). The resistance of *H. pylori* to the recently available antibiotic treatment regimens has been a growing problem. About 10% to 15% of the adult infected with *H. pylori* has been found in the developed countries as a metronidazole resistance, whereas virtually all strains have been found in the developing countries with high rate of resistant for this drug (Kato *et al.*, 2002). On the other hand, the rates of clarithromycin resistance were relatively low, ranging from 2% to 15%, where as nowadays clarithromycin resistance has increased (Kato *et al.*, 2002).

A few studies were done in the United Arab Emirates to study antibiotic resistance for *H. pylori*. One of these studies was done in Zayed Military Hospital (ZMH) and showed that: 65.5% of *H. pylori* were mutated for clarithromycin anitibiotic by Real time PCR technique, which indicate high percentage of resistance for this antibiotic (Al-Faresi *et al.*, 2007).

1.2. Pathogenesis of *H. pylori*

Helicobacter pylori have a unique set of virulence factors that allow it to colonize the stomach wall (**Fig. 1.2**). These factors include urease, helicoidal shape, flagella, adhesion and pro-inflammatory molecules (Bernard *et al.*, 2004).

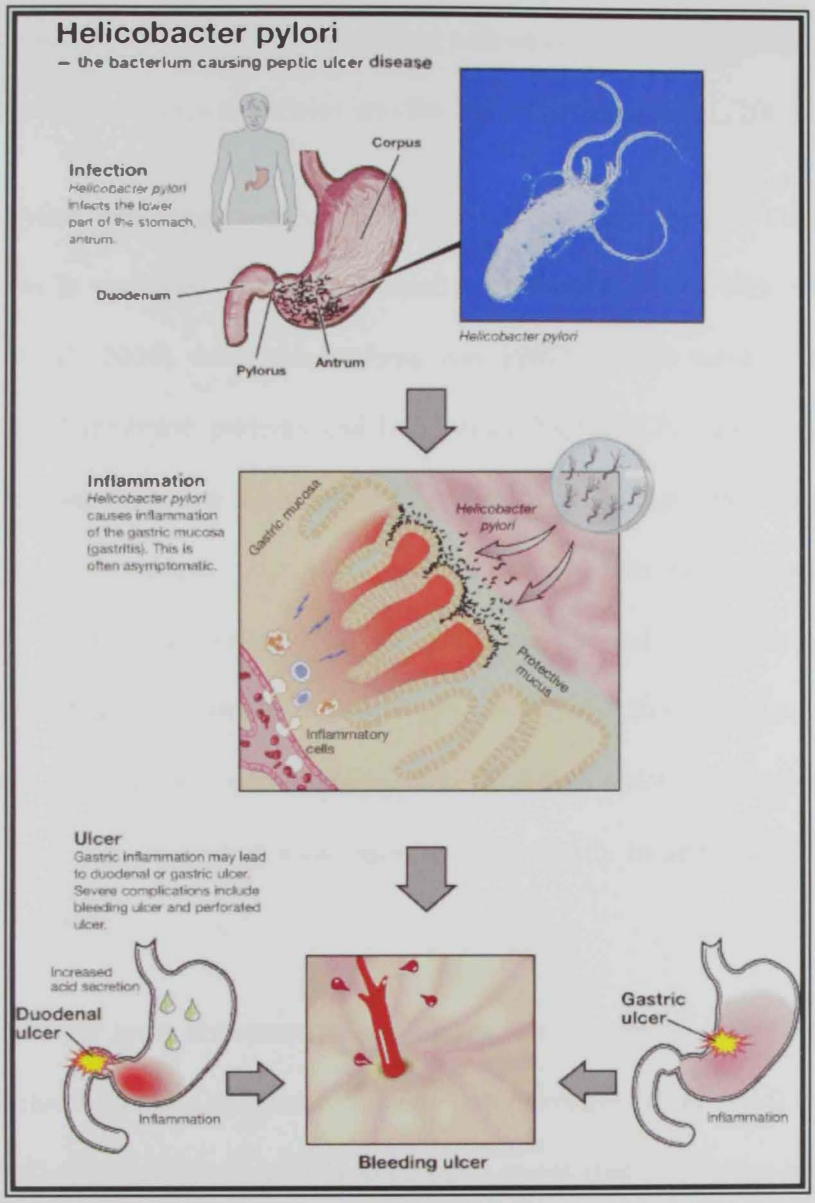


Fig. 1.2: Process of *H. pylori* pathogenesis

The ability of *H. pylori* to colonize the human stomach can be attributed to the production of specific bacterial products such as urease enzyme and several outer membrane proteins, including BabA, SabA, AlpA, AlpB, and HopZ. Beside these products flagella factor, permit bacterial motility, which allows bacterial penetration of the mucus layer **(Fig.1.3)** (Eaton and Krakowka, 1994; Eaton *et al.*, 1996) which lead to the activation of numerous signaling pathways that permit efficient delivery of toxins or other effector molecules into the cells (Guillemin *et al.*, 2002).

H. pylori has many mechanisms to evade immune system. One of these mechanisms is antigenic disguise in which bacteria are coated with host protein (Jonsson *et al.*, 2004), other mechanisms may involve phase variation of surface components. Membrane proteins and lipopolysaccharide (LPS) antigens has been reported as phase variation for multiple *H. pylori* surface components (Appelmelk *et al.*, 1999). For example, *H. pylori* strains express LPS O antigens that are structurally related to lewis blood group antigens found in human cells. This similarity in structure between *H. pylori* LPS and lewis blood group antigens may represent a form of molecular mimicry that allows *H. pylori* LPS antigens to be shielded from immune recognition because of similarity to self antigens (Algood and Cover, 2006).

One of the most important pathogenic factors present in *H. pylori* is 40 Kb region of chromosomal DNA known as *cag pathogenicity island* (PAI) (Akopyants *et al.*, 1998). The pathogenicity island contains genes that enable the bacterium to cause cellular damage (Yang *et al.*, 2004; Zhou *et al.*, 2004). Some strains contain an incomplete *cag* PAI (less than 40Kb in size) and in other strains the *cag* PAI is completely absent (Censini *et al.*, 1996). From different studies, they found that *cag*

PAI positive strains have a serious role in the stimulation of gastric epithelial cells to produce high level of pro inflammatory cytokines (Akopyyant *et al.*, 1998; Brandt *et al.*, 2005;

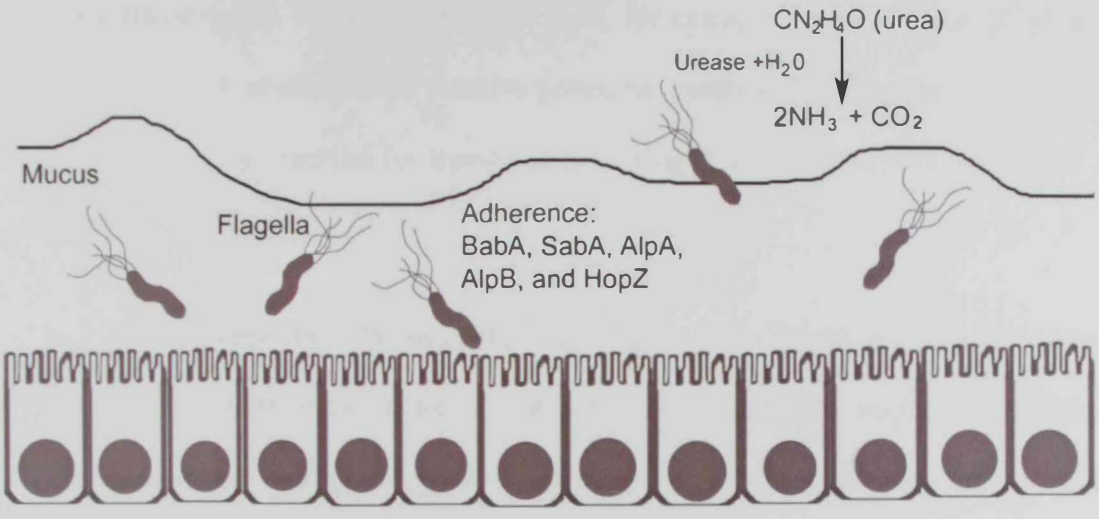


Fig. 1.3: Colonization factors of *H. pylori*. Multiple bacterial factors contribute to the ability of *H. pylori* to colonize the stomach. Urease contributes to the acid resistance of *H. pylori*. Flagella permit bacterial motility, which allows bacterial penetration of the mucus layer. Several outer membrane proteins, including BabA, SabA, AlpA, AlpB, and HopZ, can mediate bacterial adherence to gastric epithelial cells.

1.2.1. *Cag A* (*Cytotoxin Associated Gene A*)

Cytotoxin associated gene A (*Cag* A) is one of the most virulent product of 40kb DNA fragment corresponding to 25 genes in some strains and 27 genes in others, where these genes called the *cag* pathogenicity island (PAI). This bacterial effector protein (*Cag* A) which is encoded by *Cag* A gene (one gene of the *Cag* PAI) translocate into the gastric epithelial cells and induces numerous alterations in cellular signaling (Asahi *et al*., 2000; Higashi *et al*., 2002). Multiple other products of *cag* PAI have been found to play a role in secretion of *cag* A and in alteration of gene transcription in gastric epithelial cells, for example the *cag* PAI in *H. pylori 26695* strain is consists of 27 putative genes. Seventeen out of 27 genes were found to be absolutely essential for translocation of *Cag* A into host cells (Fischer *et al*., 2001).

Many researchers investigating *cag* A gene have shown that infection with positive *cag* A *H. pylori* strain increases the virulence of the bacterium. Striking evidence shows that 88% - 100% of patients with ulcers were infected by *cag A* positive strains; and about 50% - 60% patients infected by *cag A* negative strains developed ulcers. Also they found that patients with *cag A* antibodies, had the ability to develope gastric cancer (Holeston, 2004).

Although the actual role of the *cag A* gene is unknown, many studies currently show an increased risk for developing ulcers and gastric cancer associated with *cag A*-positive strains (Holeston, 2004).

1.3. Clinical Aspects of *H. pylori* Associated Disease

As a fact colonization with *H. pylori* is not considered as a disease, however, conditions that affect the relative risk of developing various clinical disorders of the upper gastrointestinal tract is a disease (Kusters et al., 2006).

Even though gastric colonization with *H. pylori* induces histologic gastritis in all infected individuals, only very few people develop any apparent clinical signs of this colonization. About 10% to 20% lifetime risk of developing ulcer disease and a 1% to 2% risk of developing distal gastric cancer have been estimated in *H. pylori*-positive patients (Kuipers *et al.*, 1995; Kuipers, 1999; Ernst and Gold, 2000). The risk of development of these disorders in the presence of *H. pylori* infection depends on a variety of bacterial strains, host, and environmental factors that mostly relate to the pattern and severity of gastritis **(Fig.1.4)** (Kusters et al., 2006).

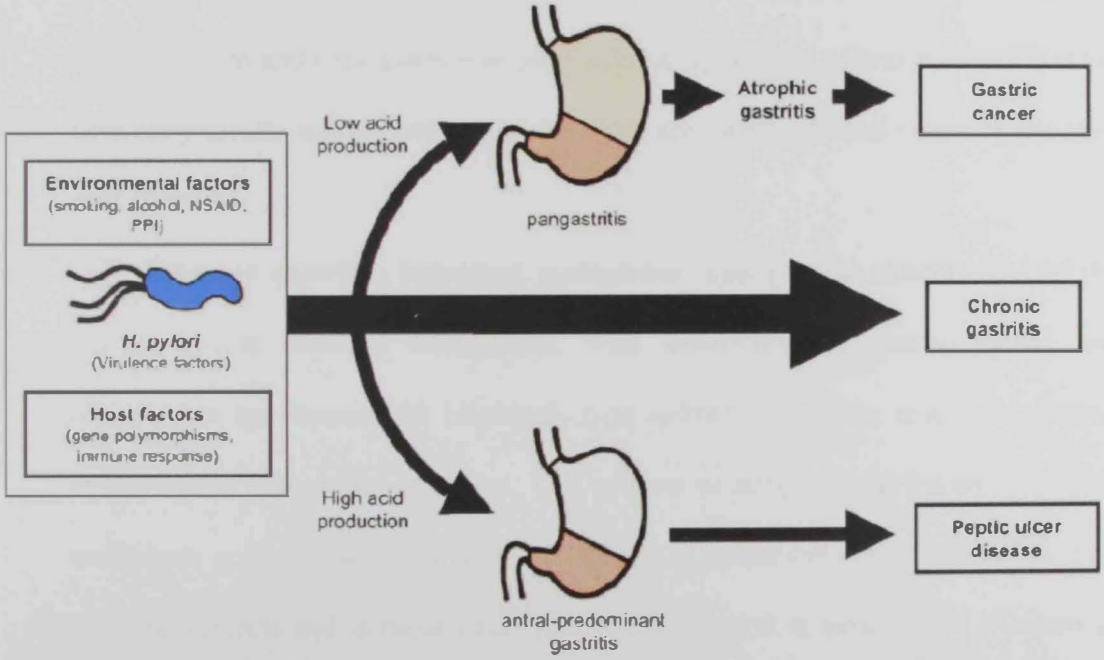


Fig. 1.4: Schematic representation of the factors contributing to gastric pathology and disease outcome in *H. pylori* infection.

Acute and chronic gastritis: gastritis can be defined as infiltration of the gastric mucosa in both antrum and corpus with neutrophilic and mononuclear cells as a result of *H. pylori* colonization (Kusters et al., 2006).

Peptic ulcer disease: gastric or duodenal ulcers (commonly referred to as peptic ulcers) are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa. Gastric ulcers mostly occur along the lesser curvature of the stomach, in particular, at the transition from corpus to antrum mucosa (Veldhuyzen van Zanten *et al.*, 1999). Duodenal ulcers usually occur in the duodenal bulb, which is the area most exposed to gastric acid (Kusters et al., 2006).

Non-ulcer dyspepsia: functional dyspepsia is defined as the presence of symptoms of upper gastrointestinal distress without any identifiable structural abnormality during diagnostic work-up, in particular including upper gastrointestinal endoscopy. Dyspeptic symptoms may have a reflux-like character, with heartburn and regurgitation as predominant signs; may appear dysmotility-like, with early satiety and nausea; or may be ulcer-like, with pain and vomiting (Kusters et al., 2006).

Atrophic gastritis, intestinal metaplasia, and gastric cancer: loss of the normal gastric mucosal architecture, with destruction of gastric glands and replacement by fibrosis and intestinal- type epithelium are the results of chronic inflammation induced by *H. pylori*. This process of atrophic gastritis and intestinal metaplasia occurs in approximately half of the *H. pylori*-colonized population, first in those subjects and at those sites where inflammation is most severe (Kuipers *et al.*, 1995b). The risk for atrophic gastritis depends on the distribution and pattern of chronic active inflammation. As such, subjects with decreased acid output show a

more rapid progression towards atrophy (Kuipers *et al.*, 1996) **(Fig. 1.5)**. Areas of gland loss and intestinal metaplasia extend with time; they increase the risk for gastric cancer by 5- to 90-fold depending on the extent and severity of atrophy (Sipponen *et al.*, 1985).

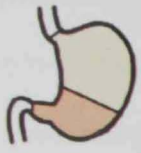
Pattern of gastritis	*Gastric histology*	*Duodenal histology*	*Acid secretion*	*Clinical condition*
 Pan-gastritis	• Chronic inflammation • Atrophy • Intestinal metaplasia	• Normal	• Reduced	• Gastric ulcer • Gastric cancer
 Antral-predominant	• Chronic inflammation • Polymorph activity	• Gastric metaplasia • Active chronic inflammation	• Increased	• Duodenal ulcer

Fig. 1.5: Acid secretion and the associated pattern of gastritis play an important role in disease outcome in *H. pylori* infection. The figure displays the correlations between the pattern of *H. pylori* colonization, inflammation, acid secretion, gastric and duodenal histology, and clinical outcome.

Gastroesophageal reflux disease (GERD): has long been considered to occur independently of *H. pylori* colonization, i.e., to occur with the same frequency and severity in *H. pylori*-positive and *H. pylori*-negative subjects. This opinion was based on cross-sectional observations which suggested that the prevalence of *H. pylori* among GERD patients was similar to that among controls (Werdmuller and Loffeld, 1997). However, further studies suggested that *H. pylori* might protect against the development of GERD and as such also be of benefit to their hosts (Kusters et al., 2006).

Gastric MALT lymphoma. The gastric mucosa does not normally contain lymphoid tissue, but MALT nearly always appears in response to colonization with *H. pylori*. In rare cases, a monoclonal population of B cells may arise from this tissue and slowly proliferate to form a MALT lymphoma (Kusters et al., 2006).

1.4. Diagnosis of *H .pylori*

H. pylori infection can be diagnosed by invasive techniques requiring endoscopy and biopsy (Ex: histological examination, culture, polymerase chain reaction) and by non-invasive techniques such as serology, urea breath test, urine/blood test, or detection of H .pylori antigen in stool specimen (Nakata et al., 2004).

1.4.1. Non-Invasive Tests

A number of noninvasive tests have been developed to establish the presence of *H. pylori* infection. Although polyclonal antibody-based stool antigen testing has a good sensitivity and specificity, it is less accurate than urea breath testing. Recently, a monoclonal antibody-based stool antigen test demonstrated an excellent

performance in diagnosing *H. pylori* infection in adults and in pediatric populations (Dore *et al.*, 2004). The two main techniques for this diagnosis are serology and urea breath test.

1.4.2. Invasive Tests

Invasive diagnosis requires endoscopy of a patient, during which biopsies are taken from multiple sites in the esophagus, stomach, and duodenum. The most popular test is the biopsy urease test. This test checks for the presence of the enzyme urease in the biopsy tissue sample. A Gram stain of a biopsy sample can be made. The Giemsa stain can also be used for detection of the organism. A biopsy also allows for a bacteriological culture of the sample. A culture allows for the determination of antibiotic sensitivity. Also we can use histology, bacterial culture as invasive test Giemsa stain demonstrating colonization of the gastric mucosa by *H. pylori* (Kim *et al.*, 2004; Nimish *et al.*, 2004).

1.4.2.1. Rapid Urease Test

It is a widely used test in endoscopy room for rapid detection of *H. pylori* infection (Hazell et al., 1987). The mode of action of this test is as follow: A gastric biopsy samples from antrum are placed in urea broth (5% urea solutions with phenol red). If there is any urease produced by *H. pylori* in biopsy specimen, the urease will then hydrolyze urea in broth to ammonium ion. This will raise the pH which can be detected by the color change from yellow to red due to the presence of the phenol red as indicator. In true positive results, 75% of the broth will turned red in 20 minutes and 90% within 3 hours. Commercial rapid urease test are available, the

first one known as CLO test (CLO= *Campylobacter* Like Organism) (Wong *et al.*, 1997).

Rapid urease test has a limited sensitivity (70-90%), this sensitivity reduced after eradication therapy. False positives may occur after 24 hours due to other urease positive organisms in the gastric biopsy. As a result this test should be read within 24 hours. Also false negative have been reported in patients with recent intake of proton pump inhibitors, bismuth, H2 receptor antagonists or antibiotics days before the test (Wong *et al.*, 1997).

1.4.2.2. Polymerase Chain Reaction

Polymerase chain reaction (PCR) can be defined as an enzymatic process in which specific region of DNA is replicated over and over again to yield many copies of a particular sequence. This molecular technique involves heating and cooling samples in specific thermal cycling pattern over 30 cycles **(Fig. 1.6)**. During each cycle, a copy of the target DNA sequence is generated for every molecule containing the target sequence. After 30 cycles billions copies of the target region on the DNA template have been generated and this was calculated theoretically **(Table 1.1)** (Butler, 2005).

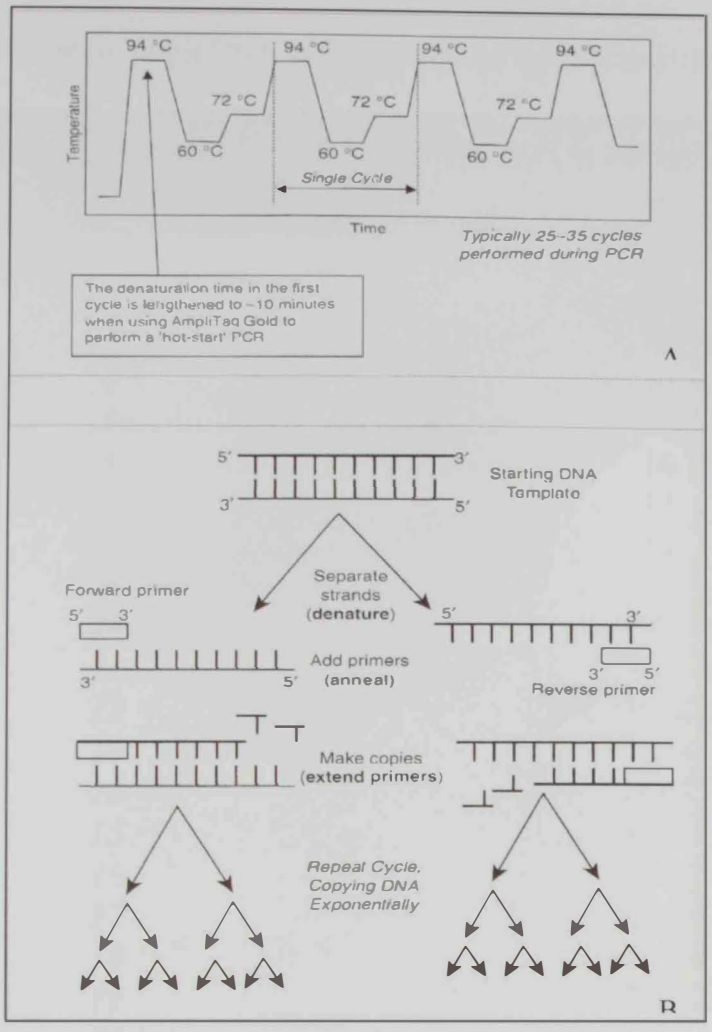


Fig. 1.6: PCR Process. A) Thermal cycling temperature profile for PCR: Thermal cycling involves three different temperatures that repeated over and over again 25-30 times. At 94^0C, the DNA strands separate, or denature, At 60^0C primers bind or anneal to the DNA template and target the region to be amplified. At 72^0C, The DNA polymerase extends the primers by copying the target region using the deoxynucleotide triphosphate building block. B) DNA amplification process within the polymerase chain reaction: In each cycle, the two DNA template strands are first separated by heat. The sample is then cooled to an appropriate temperature to bind "anneal" the oligonucleotide primers. Finally the temperature of the sample is raised to optimal temperature for the DNA polymerase and it extends the primers to produce a copy of each DNA template strand. For each cycle, the number of DNA molecules doubles.

Table 1.1: Number of target DNA molecules created by PCR amplification.

Cycle Number	*Number of Double Stranded Target Molecules (Specific PCR Product)*
1	0
2	0
3	2
4	4
5	8
6	16
7	32
8	64
9	128
10	256
11	512
12	1024
13	2048
14	4096
15	8192
16	16384
17	32768
18	65536
19	131072
20	262144
21	524288
22	1048576
23	2097152
24	4194304
25	8388608
26	16777216
27	33554432
28	67108864
29	134217728
30	268435456
31	536870912
32	1073741824

PCR has been known for its high sensitivity and specificity. It was used as diagnostic tools in clinical field. For example PCR is used nowadays for the detection of *H. pylori* DNA in bodily materials such as biopsies, feces and saliva. A specific segment of DNA from *H .pylori* can be amplified. The primers used routinely are derived from 26 KDa antigen gene, Urease C gene (Labigne et al., 1991). Other may use *Cag A* gene for PCR (Wong *et al.*, 1997).

1.5. *H. pylori* Eradication

Many antimicrobial agents have been studied for their efficacy in eradicating *H. pylori* infection either as a single agent or as a combination therapy. In fact single antimicrobial agent treatment schedules have not been sufficiently effective with eradication rates ranging from only 23% for amoxicillin to 54% of clarithromycin (Chiba *et al.*, 1992; Peterson *et al.*, 1993). Single drug regimens are not advocated due to potential for the development of antimicrobial resistance especially to macrolides and nitroimidazoles, which are the key agents in multi drug regimens for H. pylori (Kate. V and Ananthakrisshnan. N, 2001). Dual treatment combining a proton pump inhibitors (PPIs) with either clarithromycin or amoxicillin were popular a few years ago (Kate. V and Ananthakrisshnan. N, 2001). PPIs are drugs that covalently bind and irreversibly inhibit the H^+/K^+ adenosine triphosphatase (ATPase) pump, effectively inhibiting acid release. Omeprazole (Prilosec), lansoprazole (Prevacid), rabeprazole (Aciphex), and pantoprazole (Protonix) given in daily or twice-daily doses for 4 weeks heal 80-100% of gastric ulcers if *H pylori* infection is not present or has been eradicated (Walker. R and Edwards. C, 2003).

A lot of triple drug therapy had been used **(Table 1.2)** and the recent one is consist of giving ranitidine bismuth citrate (RBC), clarithromycin and metronidazole. These combination produce less acid suppression but provide the additional antimicrobial action of bismuth (McColm *et al.*, 1996).

Efforts have been made to achieve 100% eradication of *H. pylori* by using Quadruple therapy by addition of anti secretory agent to the classic bismuth based triple therapy **(Table 1.2)** (Kate. V and Ananthakrisshnan. N, 2001).

Table 1.2: Regimens for eradication of *H. pylori*

Regimens	Dosage	Duration	*H.pylori* eradication
Omeprazole	20 mg, *b.i.d.*	2 weeks	60-80%
Clarithromycin	500 *b.i.d.*		
Colloidal Bismuth Subcitrate (CBS)	120 mg, *q.i.d.*	2 weeks	30-95%
Tetracycline	500 mg, *q.i.d.*		
Metronidazole	400 *q.i.d.*		
Omeprazole*	40 mg, *o.d.*	1-2 weeks	75-90%
Amoxicillin	500 mg, *t.i.d.*		
Metronidazole	400 mg, *t.i.d.*		
Omeprazole*	40 mg, *o.d.* or 20 mg, *b.i.d.*	1-2 weeks	85-95%
Clarithromycin	250 mg, *b.i.d.*		
Metronidazole	400 mg, *b.i.d./t.i.d.*		
Omeprazole*	40 mg, *o.d.* or 20 mg, *b.i.d.*	1-2 weeks	85-95%
Amoxicillin	1 g *b.i.d.*		
Clarithromycin	250-500 mg, *b.i.d.*		
Omeprazole	20 mg, *o.d.* or *b.i.d.*	1 week	86-98%
CBS	120 mg, *q.i.d.*		
Tetracycline	500 mg, *q.i.d.*		
Metronidazole	400-500 mg, *q.i.d.* or *t.i.d.*		
Ranitidine bismuth citrate	400 mg, *b.i.d.*	7-14 days	> 90%
Clarithromycin	500 mg, *b.i.d.*		
Metronidazole	500 mg, *b.i.d./t.i.d.*		

1.6. Antibiotic Resistance

1.6.1. Metronidazole Mode of Action

Nitroimidazole (NI) such as metronidazole is widely used for *H. pylori* and other anaerobic bacteria treatment (Jenks and Edwards, 2002). It enter the cell by passive diffusion (Jenks and Edwards, 2002; Van Der Wouden *et al.*, 2000). The antimicrobial toxicity of this drug is dependent on the reduction of nitro moiety to other compounds like nitro anion radical, nitroso and hydroxyl amine derivates (Lindmark and Muller, 1976; Moreno *et al.*, 1983). These reduction products will cause DNA degradation and also it damaging other macromolecules (Jenks and Edwards, 2002).

Jenks and Edwards (2002) reported that the selective toxicity of metronidazole in anaerobic bacteria is due to the redox potential of the electron transport chain components which are negative to reduce the nitro group of this drug. Within these microorganisms electron can be produced by decarboxylation of pyruvate which passed on to ferredoxin or flavodoxin. (Smith and Edwards, 1995), these low redox electron carrier proteins will then reduce another component, mostly a proton, which work as terminal electron acceptor (Jenks and Edwards, 2002). In fact metronidazole has very low redox potential and was reduced by accepting electrons from reduced ferredoxin or flavodoxin (Edwards *et al.*, 1973; Edwards, 1993a). The continuing reduction of this drug maintains a favorable transmembrane metronidazole concentration gradient facilitating further diffusion of the drug to the cell (Igns *et al.*, 1974).

Mainly Metronidazole can be metabolized in anaerobic bacteria by two mechanisms: The first one known as oxygen-insensitive nitoreductase **(Fig.1.7)** (Van Der Wouden *et al.*, 2000).

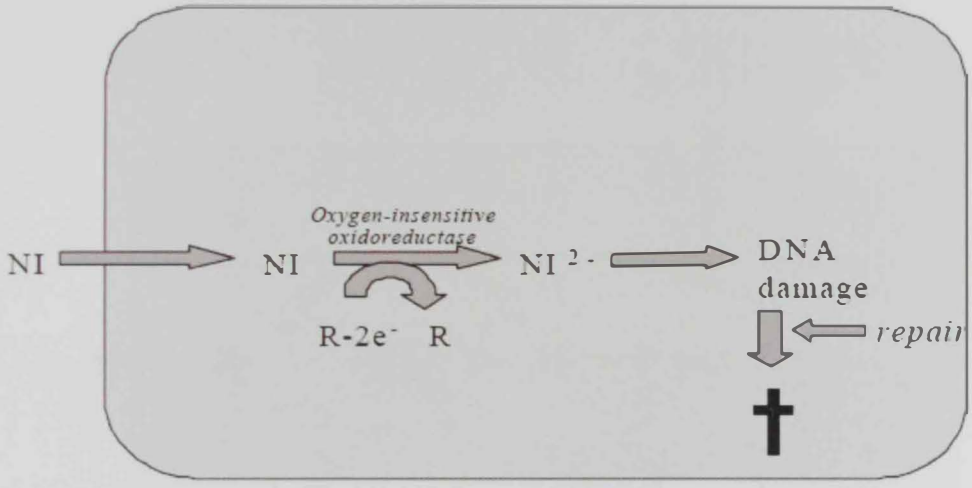


Fig.1.7: Metabolism of a nitroimidazole (NI) by an oxygen-insensitive nitroreductase.R represents the electron donor, e represents an electron.

Oxygen- insensitive denotes the reduction of metronidazole results in a nitroso derivates by simultaneous transfer of two electrons (Van Der Wouden *et al.*, 2000). The products which resulted from this step can't be reoxidized by molecular oxygen and the nitroreductase facilitating the two electron transfer step, therefore, called oxygen insensitive (Van Der Wouden *et al.*, 2000).

The second mechanism of action were it is considered as an alternative mechanism when anaerobic bacteria are exposed to aerobic atmosphere conditions is through oxygen sensitive nitroreductase **(Fig. 1.8)** (Jenks and Edwards, 2002).

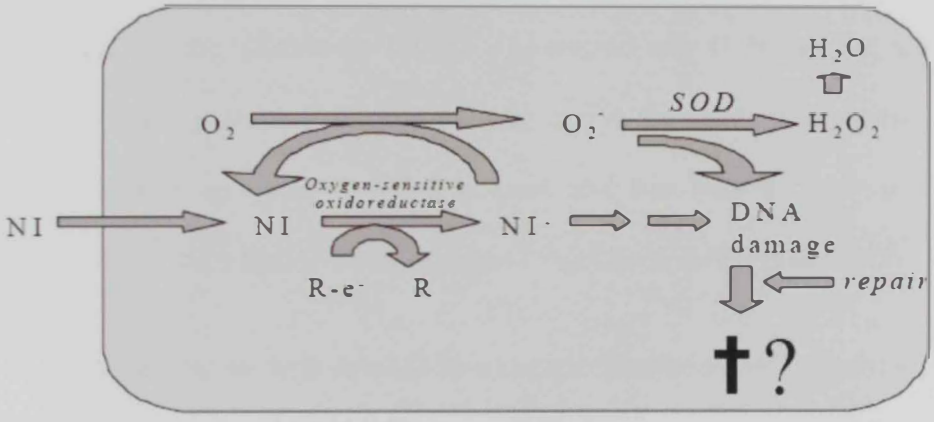


Fig. 1.8: Metabolism of a nitroimidazole (NI) by an oxygen-sensitive nitroreductase. R represents the electron donor, e represents an electron. SOD represents superoxide dismutase.

In this process metronidazole is reduced by one electron transfer step to toxic free radical anion that can be metabolized by one of the following two ways: 1) Free radical anion can be reoxidized to the original compound by molecular oxygen with production of superoxide. Thereby, molecular oxygen reverts the reduction step and these nitroreductase are therefore called oxygen-sensitive (Van Der Wouden *et al.*, 2000). This process of reduction and reoxidation is repeated endlessly and called "futile cycling" (Docampo and Moreno, 1986). Although this cycle results in the detoxification of the drug, it also generates superoxide radical anion that maybe toxic to the microorganisms. (Docampo and Moreno, 1986). The superoxide produced during this cycle can be eliminated by the action of superoxide dismutase which generates hydrogen peroxide which is further reduced to water by the catalase enzyme. (Edwards, 1993a). The second way to metabolize the toxic free radical anion is another electron transfer step in the presence of transition element such as iron and copper which convert the free radical anion to more toxic substances which lead to DNA damages (Van Der Wouden *et al.*, 2000).

Anaerobic bacteria develop resistance to metronidazole by reducing activity of element of this series of electron transport reaction POR and ferredoxin with appropriate modification of the normal fermentative pathway (Narikawa, 1986; Quon *et al.*, 1992; Edwards, 1993b; Townson *et al.*, 1996; Wassamann *et al.*, 1999). On the other hand the very low redox potential of metronidazole prevents its reduction by aerobic bacteria and this explains their intrinsic resistance to this drug (Jenks and Edwards, 2002).

1.6.2. Metronidazole Resistance in *H. pylori*

It was proposed that the reactive oxygen species generated by futile cycling were responsible for cell death in *H. pylori* and this is due to the environment conditions where these bacteria live (Lacey et al., 1993). A lot of research were devoted to demonstrate the presence of this process in *H. pylori* but all these efforts failed because no successful results were found (Smith and Edwards, 1995; Jorgensen *et al.*, 1998), where they found that superoxide dismutase and catalase (which are enzymes produced during oxygen sensitive process) are not induced by metronidazole and there is no correlation between enzyme levels and resistance patterns (Smith and Edwards, 1995). Therefore, little evidence that futile cycling contributes to the mode of action in *H. pylori*. On the other hand the observation of the pre-exposure of many metronidazole-resistant strains to anaerobic conditions which resulted in the loss of the resistant phenotype was the early indication that the activity of metronidazole in *H. pylori* dependent on reductive activation (Smith and Edwards, 1995; Cederbrant *et al.*, 1992). This means that the susceptibility to metronidazole may be restored at lower oxygen tensions through the activation of lower redox potential anaerobic reduction pathways which function less, or not at all under microaerobic environment. Furthermore, It was found that metronidazole resistant *H. pylori* have reduced activity of NADH oxidase (Smith and Edwards, 1997) which is thought to act as an oxygen scavenger, reducing the intracellular oxygen tension and maintaining a low redox status at the site of metronidazole reduction. Low level of NADH oxidase activity in resistant strains may compromise oxygen scavenging, allowing the redox potential to rise sufficiently to prevent activation of metronidazole (Jenks and Edwards, 2002).

Several studies on fresh clinical isolates indicated that metronidazole resistant mostly results from point mutation in the *rdxA* gene, a gene that encodes an oxygen-insensitive NADPH nitroreductase. High level of resistance to metronidazole is attributable to mutational inactivation of *rdxA* gene (Debets-Ossenkopp *et al.*, 1999). Although the knock out of the enzyme encoded by this gene results in resistance, the activity of other enzymes, as well as the ability of the strains to neutralize the toxic metabolites and repair DNA Damage may result in background variation in susceptibility. It is therefore, not unexpected that large variation in the minimal inhibitory concentration (MIC) are seen both in any susceptible and resistant isolates (Graham, 1998; Van Der Wouden *et al.*, 1999a; Van Der Wouden *et al.*, 1999b).

Finally it was found that the absence of functional *rdxA* encoded nitroreductase causes no survival disadvantage of microorganisms in the absence of this antibiotic (Goodwin *et al.*, 1998). So resistant mutants may persist for decades, often coexisting with susceptible organisms (Taylor *et al.*, 1995).

1.6.3. Clarithromycin Mode of Action

Macrolides mechanism of action has been studied for more than 30 years but is still unclear. All macrolides inhibit bacterial protein synthesis to varying extents (Mazzei *et al.*, 1993). One of these macrolides is clarithromycin which is active intracellularly, and its action is static or bactericidal, depending on the organism and antimicrobial agent concentration (Anderson *et al.*, 1993).

1.6.4. Clarithromycin Resistance in *H. plori*

Point mutation from adenine (A) to guanine (G) in the *23S rRNA* gene is the main reason for clarithromycin resistance in *H. pylori* **(Fig. 1.9)**. An adenine (A) to

guanine (G) transition mutation within a conserved loop of *23S rRNA* of *H. pylori* and its association with clarithromycin resistance was first identified by Versalovic *et al*, (Versalovic *et al.*, 1996).

The mutation occurs commonly at two positions 2058 and 2059 in *Escherichia coli-23S rRNA*, while in *H. pylori* the mutation occurs at position 2142/ 2143 formerly known as position 2143/2144 **(Fig 1.10)** (Megraud, 1998; Taylor *et al.*, 1997). Point mutations may occur at other positions, and can be a transition (A G) or a transversion (A C), but the transition is far more frequent (Megraud, 1998; Versalovic *et al.*, 1997; Hulten *et al.*, 1997). Furthermore, A2142G mutation was associated with a high level of resistance (MIC>64 mg/L) than the A2143G mutation as Versalovic *et al.*, 1997 found.

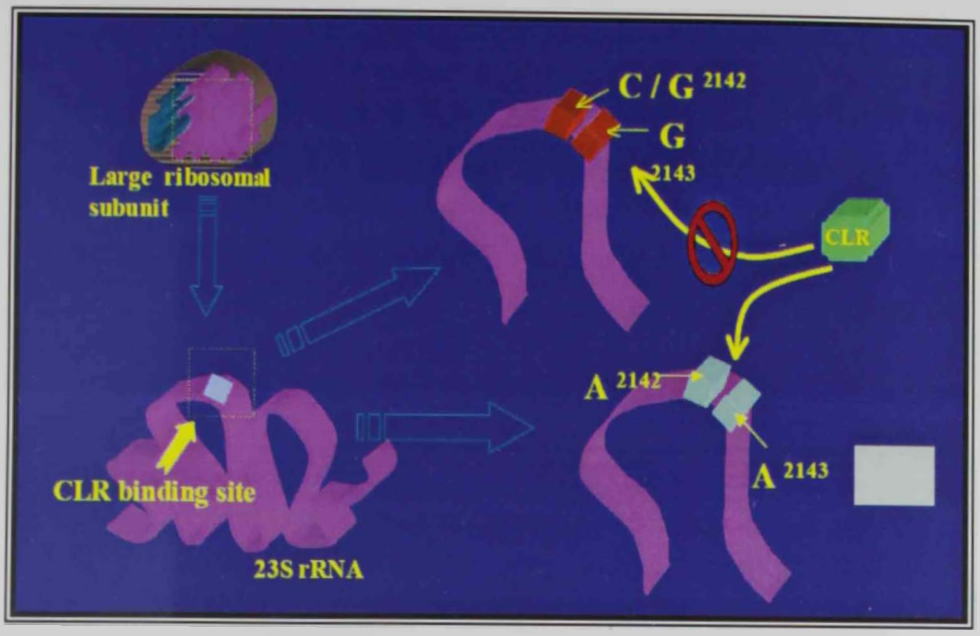


Fig. 1.9: Base substitutions in *23S rRNA* confer resistance to macrolides

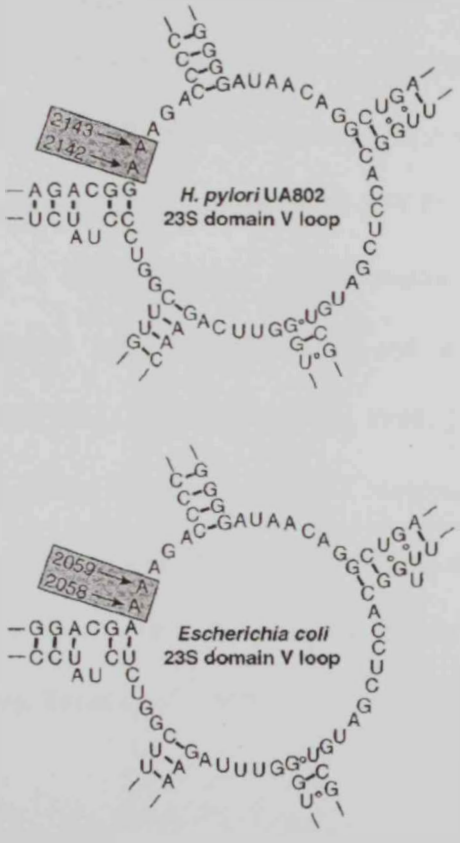


Fig. 1.10: Domain V loops of the 23S rRNA molecules from *H. pylori* UA802 and *E. coli*.

These observations are supported by others studies (Stone *et al.*, 1997; Wang and Taylor, 1998). Additionally, macrolide-resistance has been reported as unstable in some strains of *H. pylori in vitro* and in *vivo* where strains developed resistance post-treatment and then reverted to being susceptible after a period of follow up (Xia *et al.*, 1996; Versalovic *et al.*, 1996). Cross-resistance between macrolides in *H. pylori* has been observed (Midolo *et al.*, 1997; Xia *et al.*, 1996. Versalovic *et al.*, 1996). Commonly, *H. pylori* strains resistant to clarithromycin are also resistant to erythromycin and azithromycin (Wang and Taylor, 1998).

New approach for diagnosing macrolide resistant *H. pylori* strains have been started through the association between point mutations on the *23S rRNA* gene and macrolide resistance in *H. pylori*. Cycle DNA sequencing of the *23S rRNA* gene amplicons is considered as the reference method, however, simpler techniques have been developed (Megraud, 1997). These include polymerase chain reaction based restriction fragment length polymorphism (PCR-RFLP), an oligonucleotide ligation assay (PCR-OLA), a DNA enzyme immunoassay (PCR-DEIA), a reverse hybridisation line probe assay (PCR-LiPA), and a preferential homoduplex formation assay (PCR-PHFA) (Versalovic *et al.*, 1996; Taylor *et al.*, 1997; Stone *et al.*, 1997; Pina *et al.*, 1998; Maeda *el al.*, 1998). Molecular techniques such as PCR are quicker than micro biological susceptibility testing, and more importantly, they can be performed directly on gastric biopsies and gastric Juice (Maeda *et al.*, 1998; Bjorkholm *et al.*, 1998; Sevin *et al.*, 1998).

1.7 Objectives

The Objectives of the present study are:

1- Screening of peptic ulcer patients in UAE and testing for the presence of *H. pylori*.

2- Determination of the prevalence of antibiotic resistance genes (mutation in *23S rRNA* gene in clarithromycin, and deletion in *RdxA* gene in metronidazole) among *H. pylori* strains isolated from U.A.E patients by using molecular methods (PCR and sequencing).

3- To ascertain whether *Cag A*- positive *H. pylori* strains correlates with the antibiotic resistance strains or not.

4- We also screened for new *H. pylori* strains in UAE through the phylogenetic analysis of the *23S rRNA* middle region of the gene.

CHAPTER II

MATERIALS AND METHODS

2. Material and Methods

2.1. Sample Collection

From July 2008 to January 2009, a total of 90 Gastric biopsy samples were obtained from dyspeptic patients referred for endoscopy departments in Zayed Military Hospital (ZMH) in Abu Dhabi, UAE (mean age 40 years [range 19- 80]; 64 male [71.1%], 26 female [28.9%]). For each patient, one endoscopic biopsy specimen taken from antrum of stomach and directly frozen at -20°C for further processing. The research were conducted according to appropriate ethical guidelines and approved by Deputy Commander at ZMH.

2.2. DNA Isolation

DNA was isolated from 25-50 mg thawed biopsy tissue using QIAamp DNA Mini kit (QIAGEN, Cat No: 51306) according to the manufacture's direction. The isolated DNA was eluted in 60 µL elution buffer and stored at – 20°C until further analysis.

2.3. Identification of *H. pylori* in Biopsy Samples

All samples were initially tested for *H. pylori* infection with CLO test at the endoscopy unit in ZMH. To confirm CLO test results, PCR identification was carried out using species-specific antigen gene. The following primer pair was used: antigen specific forward and antigen specific reverse **(Table 2.1)**, primers were obtained from Operon. The amplification of DNA was done in 0.2 ml reaction tubes by PCR using a thermal cycler (PERKIN ELMER 2400). 50 µl reaction mixtures consisted 1 x PCR master mix (QIAGENE, Cat No: 201445), forward and reverse primer each at a concentration of 0.1 µM and 2 µl from extracted DNA as a template where the best bands resolution were at the concentration range from 100-200ng/µl.

Each reaction mixture was amplified as follows **(Table 2.2)** : denaturation at 94°C for 5 and then the sample was allowed to undergo 40 cycle which consisted in denaturation of DNA for 1 min at 94°C, annealing of primer for 1 min at 68°C and extension of primer for 1 min at 72°C. A final extension of DNA was carried out for 10 min at 72°C to ensure complete amplification. Sequenced sample from positive amplified PCR products served as positive control **(Fig. 2.1)**, where as, distilled water served as a negative control during the amplification. The amplified DNA product was analyzed by electrophoresis using agarose (Invitrogen, Cat No: 10975-035) prepared in TBE (Tris Borate-EDTA) buffer (1.5 g/ 100 ml). The size of the gel was 10 x 7 cm with a thickness of 0.3 cm. Samples were loaded on the gel and a voltage of 100V was applied. 100bp DNA ladder (Invitrogen, Cat No: 10488-058) was used as marker to know the size of the PCR product. The sample was allowed to run for 1 hr and the gel was stained with 0.5 mg /ml ethidium bromide for 30 min. The band was seen by illumination with UV light in a gel documentation system.

Table 2.1: Primers sequences used in this study

Gene	Forward primer	Reverse primer
Sspecies-Sspecific Aantigen Gene	5'-TGGCGTGTCTATTGACGACGAGC-3'	5'-CCTGCTGCGCATTCACCATG-3'
***rdxA* Gene**	5'-AATTTGAGCATGGGGCAGA-3'	5'-GAAACGCTTGAAAACACCCCT-3'
***23S rRNA* Gene**	5'AGTCGGGACCTAAGGCGAG-3'	5'TTCCCGCTTAGATGCTTTCAG-3'
***Cag A* Gene**	5'-ATAATGCTAAATTAGACAACTTGAGCGA-3'	5'-AGAAACAAAAGCAATACGATCATTC-3'

Table 2.2: PCR programs to amplify different genes

Gene	PCR Program (followed by a 10-min extension step at 72°C)	product size
Sspecies-Sspecific Aantigen Gene	40 cycles, denatured at 94 °C for 5 min , 94°C for 1 min, 68°C for 1 min, 72°C for 1 min	298-bp
***rdxA* gene**	35 cycles, denatured at 94 °C for 5 min , 94°C for 30 s, 55°C for 30 s, 72°C for 30 s	850-pb
***23S rRNA* gene**	40 cycles, denatured at 94 °C for 6 min , 94°C for 30 s, 50°C for 1 min, 72°C for 3 min	1400-bp
***Cag A* gene**	50 cycles, denatured at 95 °C for 3 min , 94°C for 1 min, 48°C for 45 s, 72°C for 45 s	128-bp

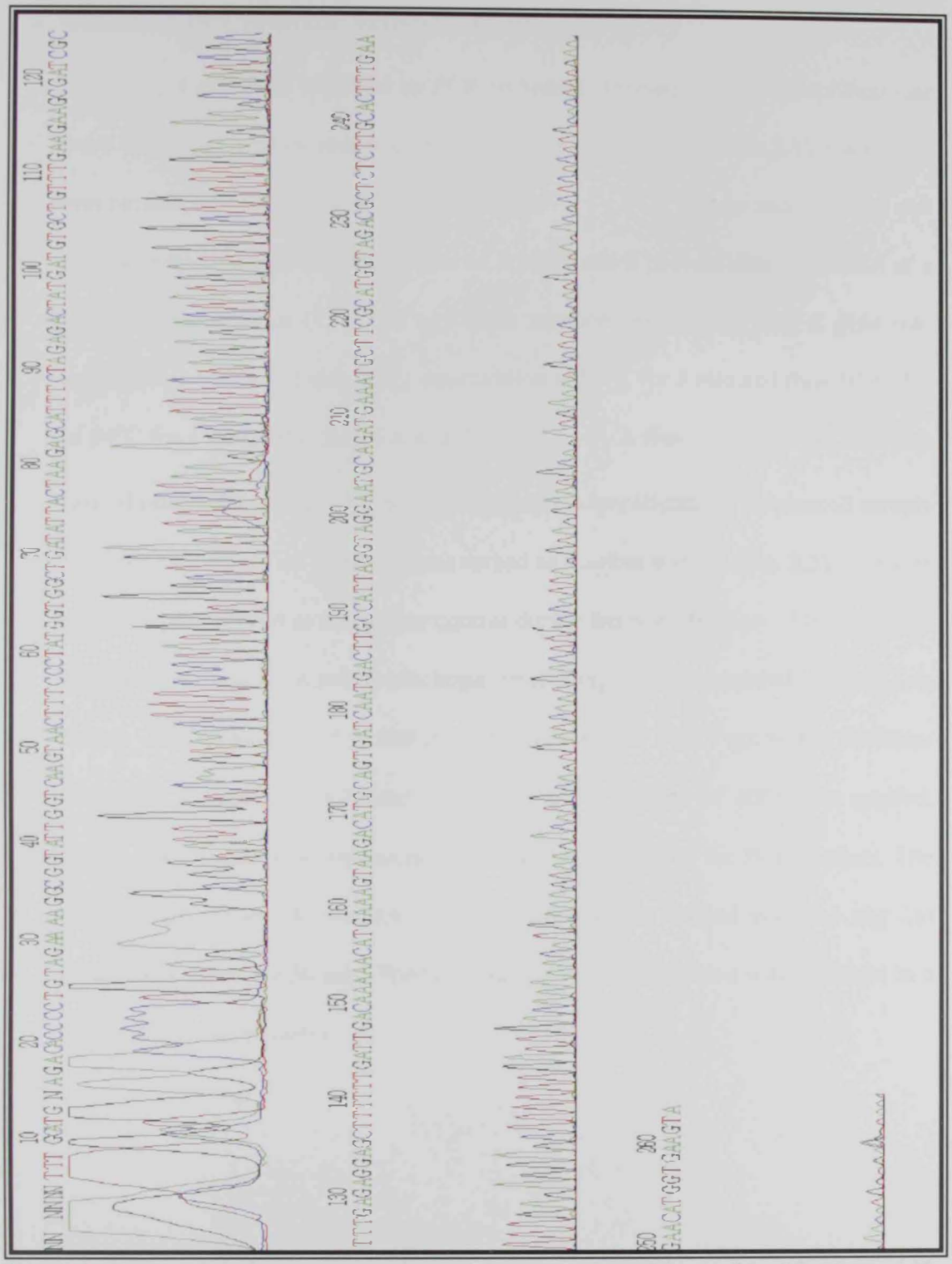


Fig. 2.1: Sequencing chromatogram for positive control used in the Identification of *H. pylori* by PCR.

2.4. Detection of *Cytotoxin Associated gene A* (*Cag A*)

Cag A gene was screened by PCR technique. Primers for PCR amplifications were designed based on published sequences for *cag A* gene **(Table 2.1)**. Each PCR was performed with a volume of 50 µl containing 1 x PCR master mix, forward and reverse primer each at a concentration of 0.1 µM and 2 µl from extracted DNA at a suitable concentration (15 to 20 ng). Each reaction mixture for *Cag A* gene was amplified as follows **(Table 2.2)** : denaturation at 95°C for 3 min and then 50 cycles of 94°C for 1 min, 48°C for 45 s, and 72°C for 45 s , A final extension of DNA was carried out for 10 min at 72°C to ensure complete amplification. Sequenced sample from positive amplified PCR products served as positive control **(Fig. 2.2)**, where as, distilled water served as a negative control during the amplification. The amplified DNA product was analyzed by electrophoresis using agarose prepared in TBE (Tris Borate -EDTA) buffer (1.5 g/ 100 ml). The gel size was 10 x 7 cm with a thickness of 0.3 cm. Samples were loaded on the gel and a voltage of 100V was applied. 100bp DNA ladder was used as marker to know the size of the PCR product. The sample was allowed to run for 1 hr and the gel was stained with 0.5 mg /ml ethidium bromide for 30 min. The band was seen by illumination with UV light in a gel documentation system.

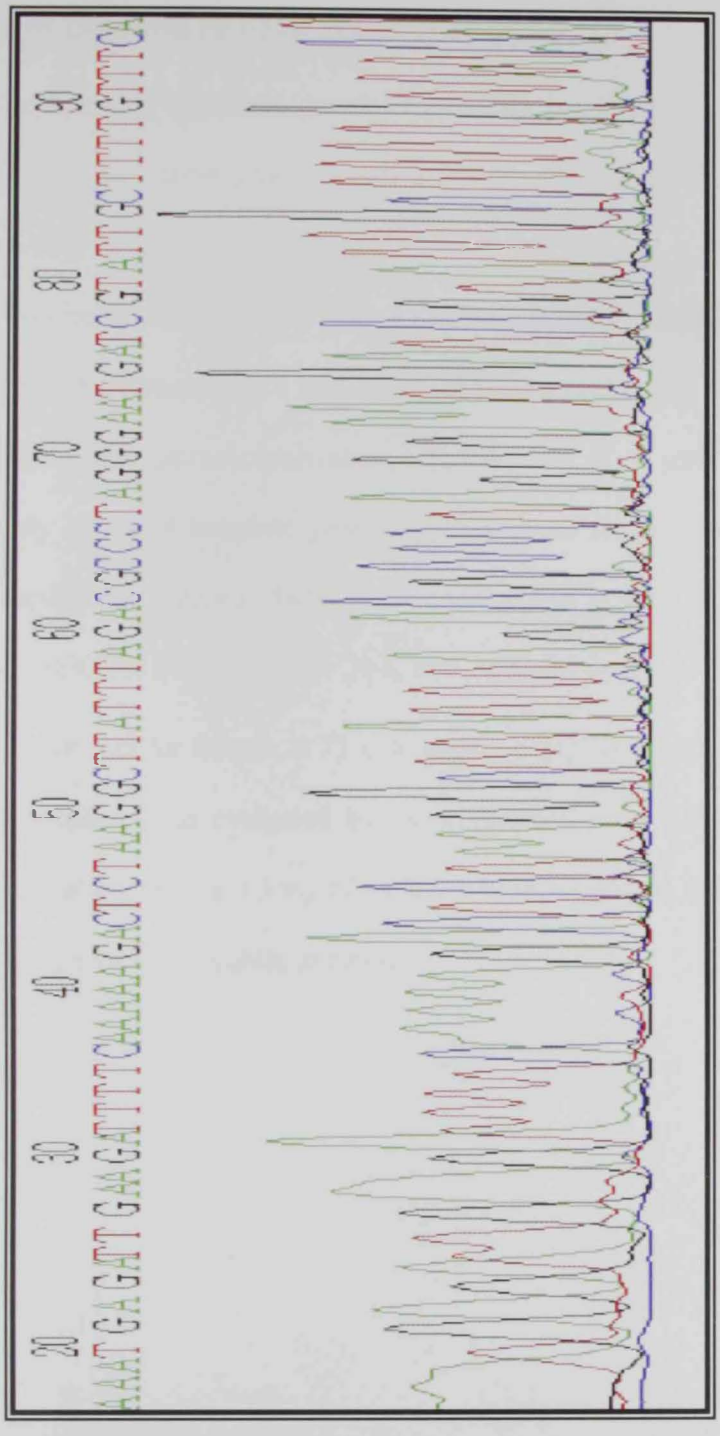


Fig. 2.2: Sequencing chromatogram for positive control used in the amplification of *Cag A* gene.

2.5. Detection of Deletion in *rdxA* Gene of *H .pylori*

For the detection of metronidazole resistant strains, deletion in the *rdxA* were screened, where the native gene yielded a 850-bp PCR product and its deletion resulted in a 650-bp fragment.

PCR was performed in an automated thermal cycler (PERKIN ELMER 2400), Each PCR was performed with a volume of 25 µl containing 1 x PCR master mix, forward and reverse primer each at a concentration of 1 µM and 2 µl from approximately 20 ng of template genomic DNA. Each reaction mixture for *rdxA gene* was amplified as follows **(Table 2.2)** : denaturation at 94°C for 5 min and then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s , A final extension of DNA was carried out for 10 min at 72 °C to ensure complete amplification. The size of the PCR products was evaluated by electrophoresis on a 1.5% agarose– Tris-borate-EDTA gel containing 0.5 mg of ethidium bromide per ml. PCR amplification result was compared to the public database.

2.6. Detection of Point Mutations in the *23S rRNA* Gene of *H .pylori* by Sequencing

Point mutations at two position in *23S rRNA* gene (2142 and 2143) were identified by amplifying 1400 bp of the target gene encoding *23S rRNA* and then sequence it.

PCR amplifications were done by using oligonucleotide primers 18 and 21 **(Table 2.1)** complementary to conserved regions of the gene encoding *23S rRNA*. Each PCR was performed with a volume of 50 µl containing 1 x PCR master mix, forward and reverse primer each at a concentration of 1 µM and 2 µl from extracted DNA. Each reaction mixture was amplified as follows **(Table 2.2)** : denaturation at 94°C for 6 min and then 40 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 3 min, A final extension of DNA was carried out for 10 min at 72°C to ensure complete amplification.

PCR product was purified with Qiaquik purification kit (Qiagen) and a cycle sequencing reaction was performed with the same primers. DNA sequencing was performed In Macrogen Inc (Korea) under standard conditions. DNA sequence editing and analysis were performed by ClustalX, version 2 (Larkin,M.A *et al.*, 2007) softwear.

2.7. Phylogenetic Analysis of *23S rRNA*

2.7.1. Analysis of the nucleotide sequence. The nucleotide sequence reported in this study was analyzed by using the software ClustalX, version 2 (Larkin,M.A *et al.*, 2007). Reference sequences used in the alignment was obtained from NCBI data base for all *23S rRNA* from different Helicobacter pylori Strains.

2.7.2. Nucleotide Sequence Accession Numbers. The partial *23S rRNA* gene sequences obtained in this study were deposited in GenBank. The accession numbers are shown in Table 2.3. For comparison, published *23S rRNA* gene sequences were downloaded from GenBank **(Table 2.3)**.

2.7.3. Phylogenetic Tree. Tree view program was used to draw the phylogenetic tree. This program dose not creates those trees, it simply uses files created by clustalX to display and print the trees.

2.8. Statistical Analysis

SPSS software were used to calculate Chi-square which used to test for differences proportions between different groups, P value less than 0.05 was reported as statistically significant.

Table 2.3: Sequence accession numbers of the strains included in the study

strain	Source	GenBank accession no.
		23S rRNA gene
Helicobacter pylori J99	Reference strain	NC_000921.1
Helicobacter pylori 26695	Reference strain	NC_000915.1
Helicobacter pylori G27	Reference strain	NC_011333.1
Helicobacter pylori Shi470	Reference strain	NC_010698.2
Helicobacter pylori P12	Reference strain	NC_011498.1
Helicobacter pylori HPAG1	Reference strain	NC_008086.1
Helicobacter pylori HPKX_438_CA4C1	Reference strain	NZ_ABJP01000007.1
Helicobacter pylori HPKX_438_AG0C1	Reference strain	NZ_ABJO01000217.1
Helicobacter pylori HPKX_438_CA4C1	Reference strain	NZ_ABJP01003236.1
Helicobacter pylori Hpuae-1	Patient, UAE	FJ527516
Helicobacter pylori Hpuae-2	Patient, UAE	FJ527508
Helicobacter pylori Hpuae-3	Patient, UAE	FJ527521
Helicobacter pylori Hpuae-4	Patient, UAE	FJ527524
Helicobacter pylori Hpuae-5	Patient, UAE	FJ527509
Helicobacter pylori Hpuae-6	Patient, UAE	FJ527514
Helicobacter pylori Hpuae-7	Patient, UAE	FJ527511
Helicobacter pylori Hpuae-8	Patient, UAE	FJ527520
Helicobacter pylori Hpuae-9	Patient, UAE	FJ527513
Helicobacter pylori Hpuae-10	Patient, UAE	FJ527506
Helicobacter pylori Hpuae-11	Patient, UAE	FJ527515
Helicobacter pylori Hpuae-12	Patient, UAE	FJ527510

strain	Source	GenBank accession no.
Helicobacter pylori Hpuae-13	Patient, UAE	FJ527512
Helicobacter pylori Hpuae-14	Patient, UAE	FJ527523
Helicobacter pylori Hpuae-15	Patient, UAE	FJ527519
Helicobacter pylori Hpuae-16	Patient, UAE	FJ527517
Helicobacter pylori Hpuae-17	Patient, UAE	FJ527505
Helicobacter pylori Hpuae-18	Patient, UAE	FJ527518
Helicobacter pylori Hpuae-19	Patient, UAE	FJ527522
Helicobacter pylori Hpuae-20	Patient, UAE	FJ527507

Continued Table 2.3: Sequence accession numbers of the strains included in the study

CHAPTER III

RESULTS AND DISCUSSIONS

3. Results and Discussion

3.1. Sample collection

From July 2008 to January 2009, a total of 90 Gastric biopsy samples were obtained from dyspeptic patients referred for endoscopy departments in Zayed Military Hospital (ZMH) in Abu Dhabi, UAE. The mean age were 40 years [range 19- 80] (**Fig. 3.1**); 64 male [71.1%], 26 female [28.9%]). Within the patients population 67 [74.4%] were UAE nationals and the rest are non locals (**Fig. 3.2**). Presences of *H. pylori* are not linked to certain age group and not concern for locals only; it is randomly distributed within groups.

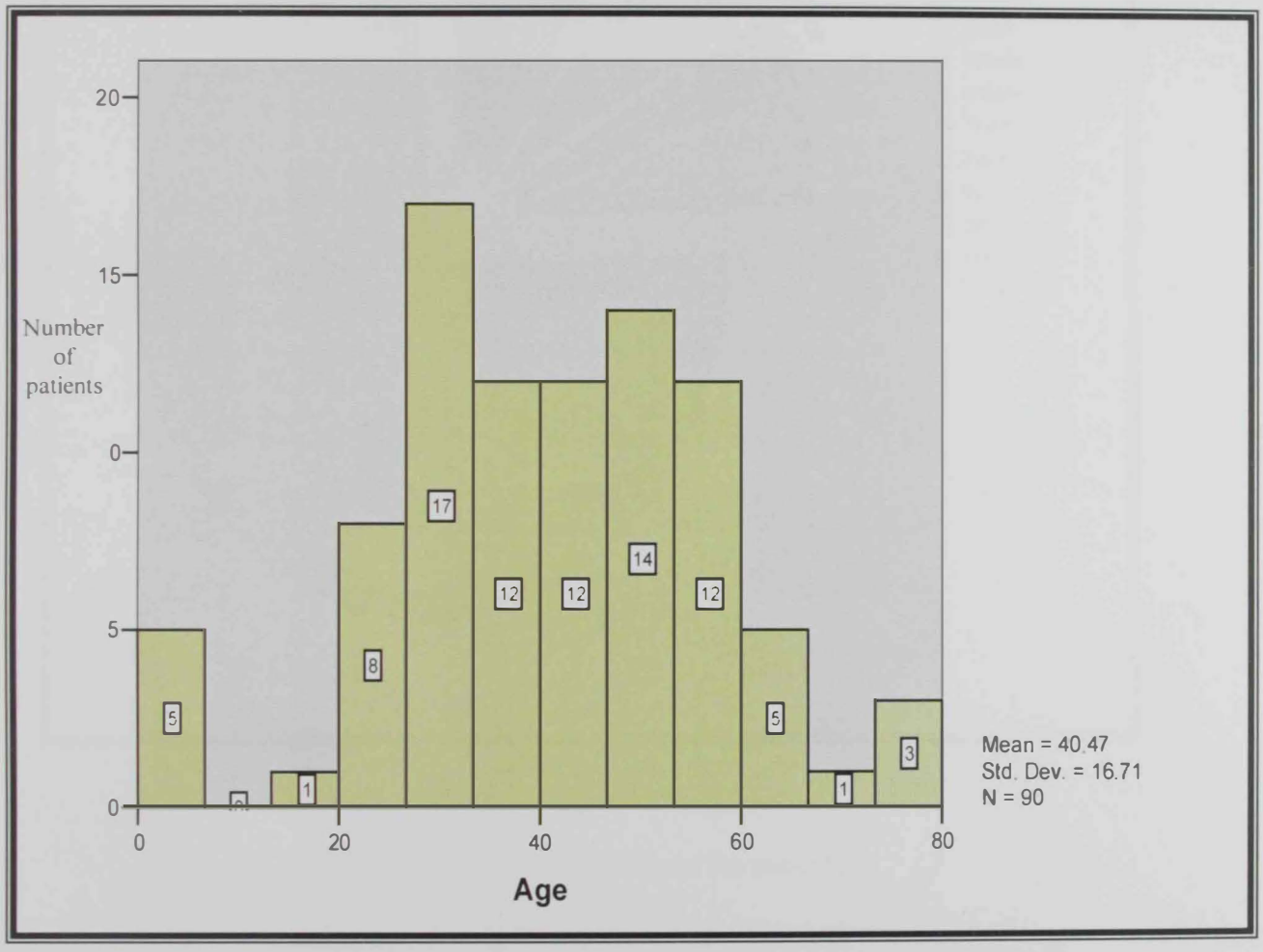


Fig. 3.1: Mean age of patients and range of distribution.

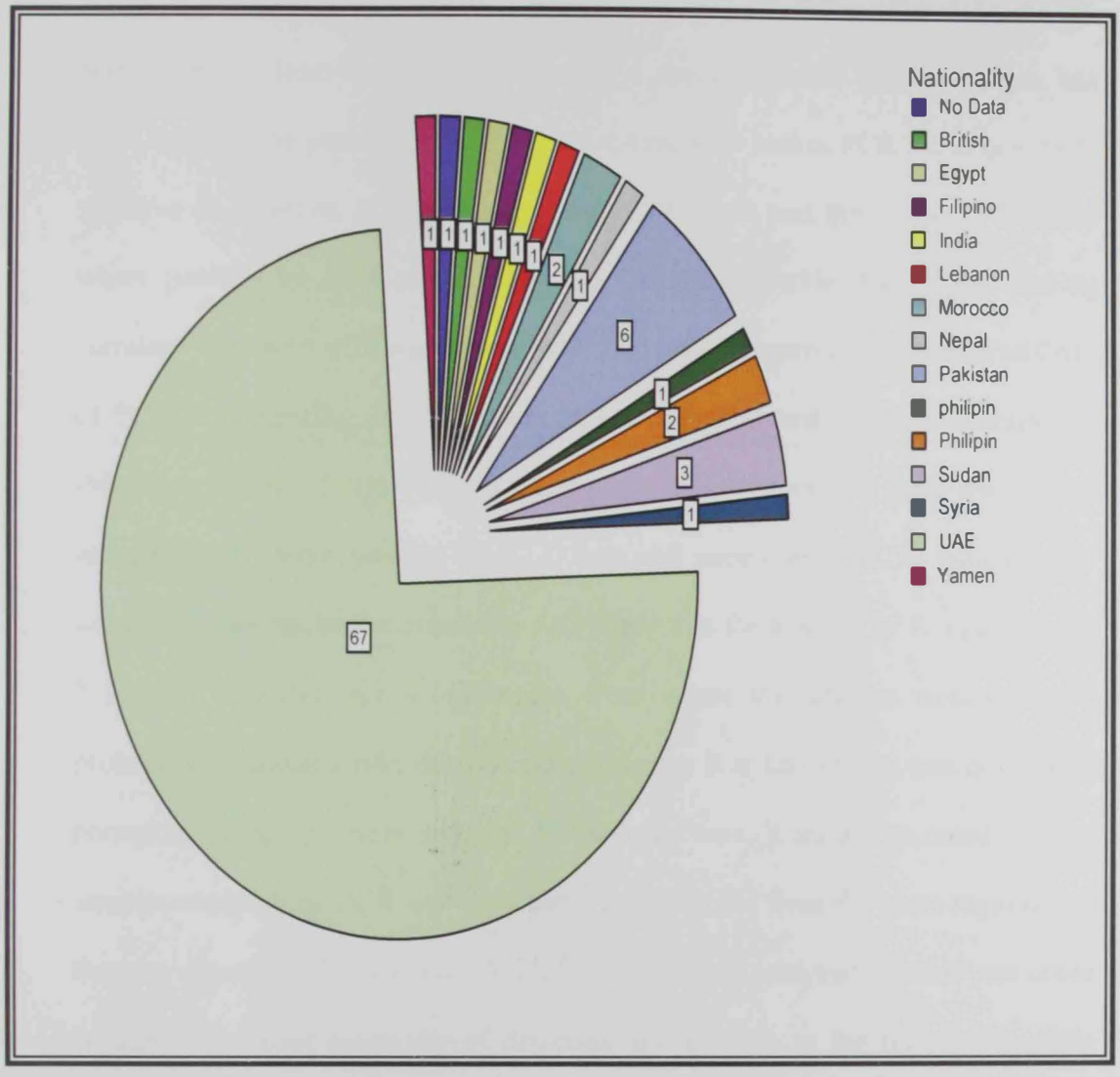


Fig. 3.2: Different nationality of the patients.

3.2. Detection of *H. pylori* in Gastric Biopsy Samples.

Ninety antral biopsy samples were collected from patients suffering from dyspeptic symptoms. Primary screening was done using CLO test while conformation were done by PCR method. The present study has indicated that among the 90 patients, 22 [24.4%] showed the presence of *H. pylori* by CLO test while 26 [28.9%] by PCR method with a band size of 298bp **(Fig. 3.3)**. As the primer derived from the DNA sequence of a species-specific protein antigen has been shown to be present only in *H. pylori*, this what makes PCR technique more sensitive in detecting *H. pylori* compared to CLO test and this is why 7 samples where positive by PCR and negative by CLO test **(Table 3.1)**. These finding correlates very well with many other published results regarding the high sensitivity of PCR in comparing to other methods such as CLO test and Urea breath test (Murugesan *et al.*, 2005). Although our results also shows that there are 7 other samples which were positive by CLO test and negative by PCR, which might contradict with our earlier comment, we believe that the absence of *H. pylori* in the 7 samples could be due to the region from where the samples were collected probably not infected with this microorganism, as it is known that this microbe is present in patches in these patients. At the same time, it should be noted that the samples analyzed by PCR and CLO test were collected from the same regions and thus the absence of *H. pylori* in 7 out of 22 patients as analyzed by CLO test could be due to the poor sensitivity of detection and not due to the region of sample collection.

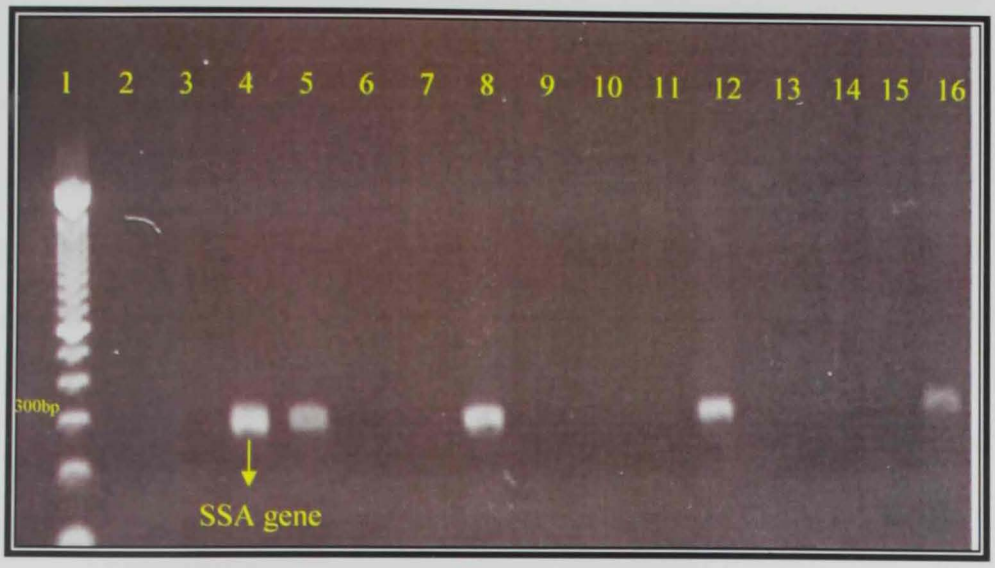


Fig. 3.3: 1.5 % Agarose gel electrophoresis of amplified 298-bp PCR product from cloned target sequence of species- specific antigen (SSA) gene (p26KDa); lane 1 shows 100bp marker, Lane 16: positive control ; lane 15: negative control, lanes 4, 5, 8, and 12 show positive PCR assay performed on a gastric biopsy sample.

Table 3.1: PCR and CLO test results

CLO Test	PCR		Total
	positive	**negative**	
positive	15	7	22
no data	4	7	11
negative	7	50	57
Total	26	64	90

3.3. Detection of *Cytotoxin Associated Gene A* (*Cag A*)

H. pylori infection is the major causative agent of chronic active gastritis, which plays a central role in the etiology of peptic ulcer disease and is a risk factor for development of gastric cancer. One of the most virulent factors that is thought to play a role in development of gastric cancer is *cytotoxin associated gene A* (*Cag A*). *Cag A* gene was present in 16 (61.54%) of the 26 samples that was positive for *H. pylori* by PCR **(Fig. 3.4)** with a band size of 128bp **(Fig. 3.5)**. Several studies have shown that the prevalence of *Cag A* gene in *H. pylori* isolates range from 80% to 100% (Cover et al., 1990; weel et al., 1996; Uemura et al., 2001), where geographical differences in the prevalence have been found, as it has been reported in western countries that *Cag A* gene is present in 95% of *H. pylori* infected patients. The same observation was published for eastern countries, where *Cag A* gene expressing *H. pylori* was found in 89.3% of the 28 isolates (Cover et al., 1990; weel et al., 1996; Uemura et al., 2001). Although our positive rate of *Cag A* gene (61.5%) is lower than published reports from other nation (Cover et al., 1990; weel et al., 1996; Uemura et al., 2001), our results is acceptable as this might be due to strain variation and geographical differences as it was mentioned before. By comparing with data published earlier in the UAE, our finding is similar to previous studies showing the presence of *H. pylori* strains in UAE with lower percentage of having *Cag A* gene.

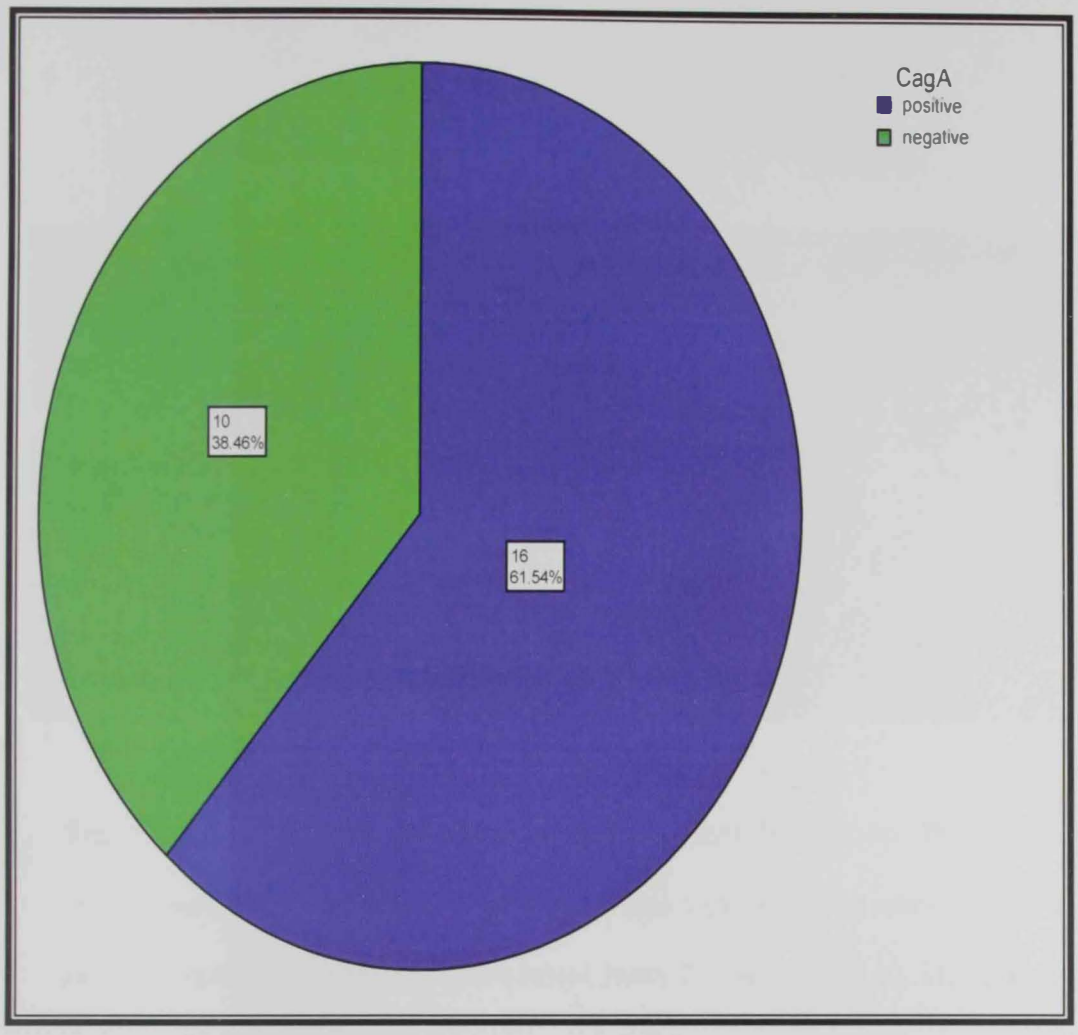


Fig. 3.4: Percentage of *Cag A* positive and negative in the 26 *H. pylori* strains

Fig. 3.5: 1.5 % Agarose gel electrophoresis of amplified 128-bp PCR product from cloned target sequence of *Cag A* gene; lane 1 shows 100bp marker, Lane 14: positive control ; lane 15: negative control, lanes 2, 3, 4, 6, 7, 8, 10, 11, 12 and 13 show positive PCR assay performed on a gastric biopsy sample.

3.4. Detection of Deletion in *rdxA* Gene of *H .pylori*

In recent years, *rdxA* gene analysis of the fresh samples showed that the metronidazole resistance is mainly attributed to the mutations in this gene including gene deletion (Debets-Ossenkopp et al., 1999). In this study only 3 (11.5% of total) strains out of 26 were found to carry a 200 bp deletion in the *rdxA* gene. All three samples were negative for identification by CLO test **(Table 3.2)** and also are characterized by the absence of *Cag A* gene **(Table 3.3)**. This absence of functional *rdxA* encoding for nitroreductase causes no survival disadvantage to microorganisms in the absence of this antibiotic (Goodwin *et al.*, 1998). So, resistant mutants may persist for decades, often co-existence with susceptible organisms and this is what we found in some of the samples where two band sizes expressed in the same strain **(Fig 3.6D)**. There is a strong association between the presence of *Cag A* gene and the deletion in the *rdxA* gene ($p = 0.02$). Relation between *Cag A* and *rdxA* 200bp deletion are not clear. Further researches with more samples are needed to confirm these results.

Table 3.2: CLO test results and its relation to metronidazole susceptible and resistant strains

rdxA gene	CLO Test			Total
	positive	**no data**	**negative**	
Resistant	0	0	3	3
Susceptible	16	2	5	23
Total	16	2	8	26

Table 3.3: Presence of *Cag A* gene among metronidazole (*rdxA*) susceptible and resistant strains

rdxA gene	Cag A gene		Total
	positive	**negative**	
Resistant	0	3	3
Susceptible	16	7	23
Total	16	10	26

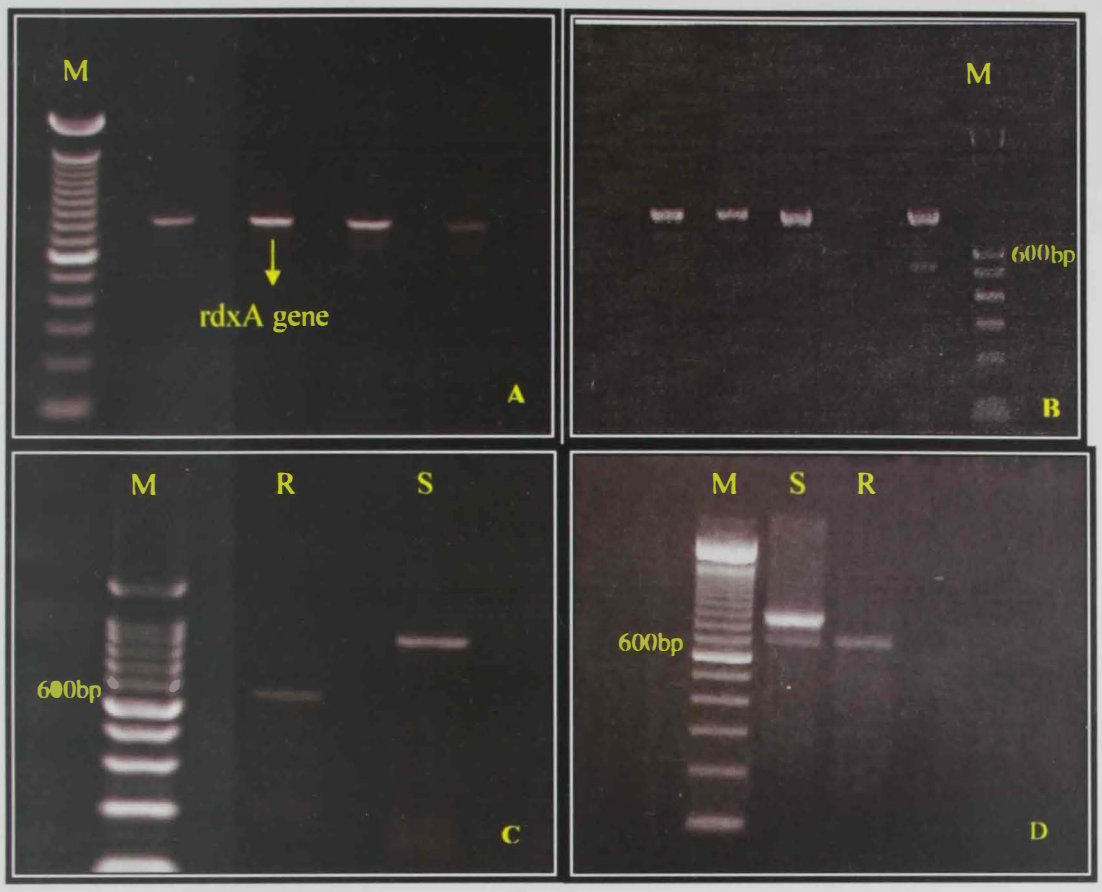


Fig. 3.6: 1.5 % Agarose gel electrophoresis of amplified PCR product from cloned target sequence of *rdxA* gene; (A) and (B) shows susceptible strains (S) for metronidazole with a band size of 850bp, While (C) and (D) Shows resistant strains (R) with a band size of 650bp and susceptible one (850bp).

3.5. Detection of Point Mutations in the *23S rRNA* Gene of *H .pylori* by Sequencing

The prevalence of clarithromycin resistance varies from 1% in Norway to 65.5% in United Arab Emirates as Al-Faresi reported (2007). Recent data from Western Europe indicate that greater than 60% of failed clarithromycin based treatments are associated with clarithromycin resistant *H. pylori* isolates (Blaser, 1997; Cover *et al.*, 1990; Van Doorn *et al.*, 1998, Arents *et al.*, 2001; Mukhopadhyay *et al.*, 2000).

The present study did not examine cultured isolates of *H. pylori* from biopsies. Rather it examined the biopsies directly using molecular techniques Depending on the published data, clarithromycin binding site located in the V domain of peptidyltransferase-encoding region of the *23S rRNA* gene (Uemura *et al.*, 2001). **(Fig 3.7).** Mutations at this site alter the mechanism of binding making the bacteria resistant to the antibiotic. Three point mutations in two positions of the binding site have been described in which an adenine residue is replaced by guanine or a cytocine residue at adjacent positions: A2142C, A2142G and A2143G.

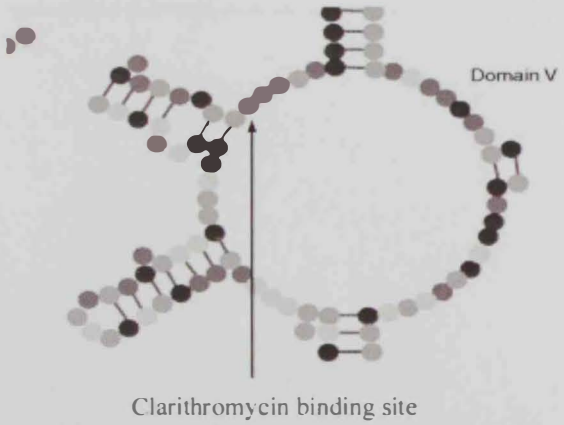


Fig. 3.7: Clarithromycin binding site.

All DNA samples were positive by the PCR assay for the amplification of the 1400bp of *23S rRNA* gene **(Fig. 3.8)**. Sequencing analysis showed that 9 out of 26 (34.6%) strains were mutated (susceptible strains showed in **Fig. 3.9**). In this group 2 strains [22.22%] had the A2142G **(Fig. 3.10)**, 5 strains [55.56%] A2143G **(Fig. 3.11)**, 1 strain [11.11 %] A2143C and 1 strain [11.11%] with highly changed in sequence **(Fig. 3.12)**. The prevalence of the point mutation A2142/43G among our patients is higher than what other countries reported (29% in Japan (Kato *et al.*, 2002)), and still lower than 65%, what Al-Faresi reported (2007). In addition there was no relation between the presence of *Cag A* gene and the clarithromycin resistance (P = 0.07) **(Table 3.4)**.

Table 3.4: Presence of *Cag A* gene among Clarithromycin (*23S rRNA*) susceptible and resistant strains

23S rRNA gene	Cag A gene		Total
	positive	**negative**	
Resistant	8	1	9
Susceptible	8	9	17
Total	16	10	26

Fig. 3.8: 1.5 % Agarose gel electrophoresis of amplified 1400bp PCR product from cloned target sequence of *23S rRNA* gene; lane 1 shows 100bp marker, lane 5: negative control, lanes 1, 2, 3 and 4 show positive PCR assay performed on a gastric biopsy sample.

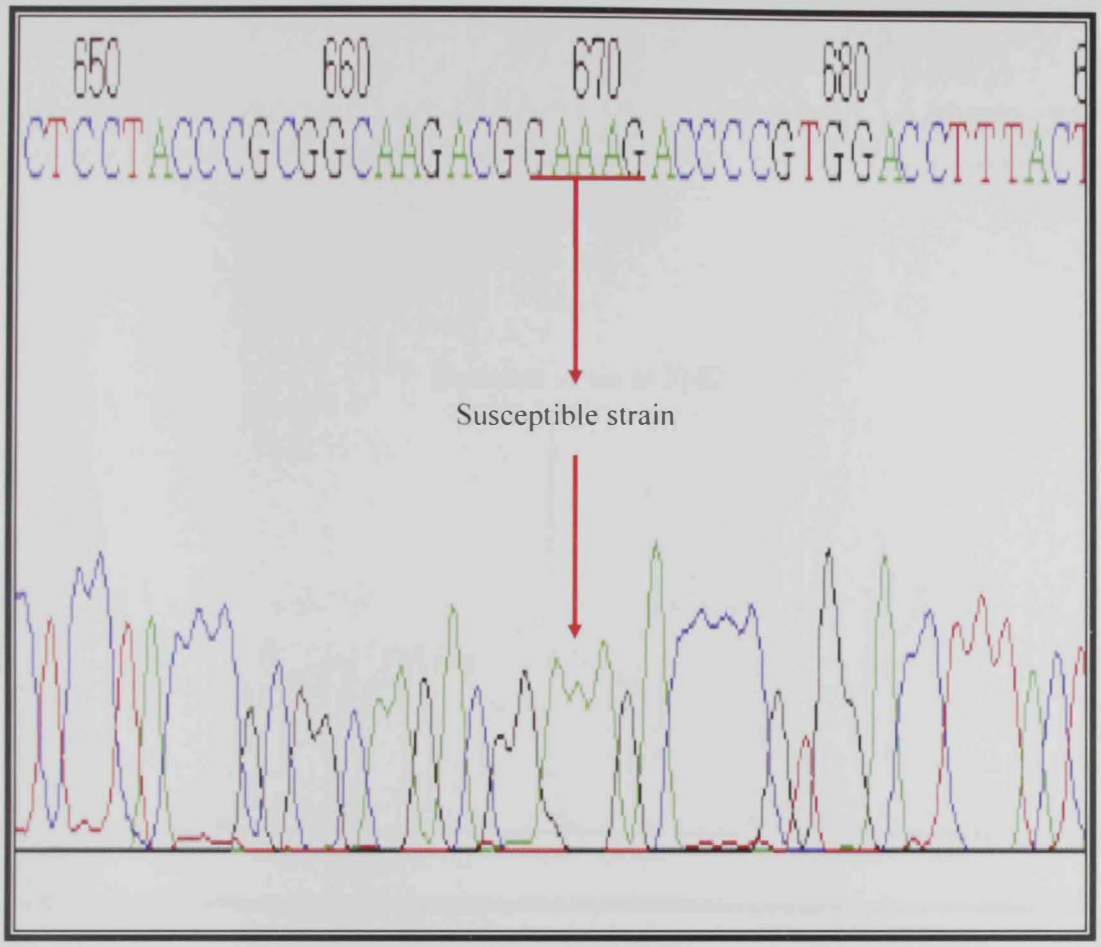


Fig. 3.9: Sequencing chromatogram for clarithromycin susceptible strain isolated from UAE patients

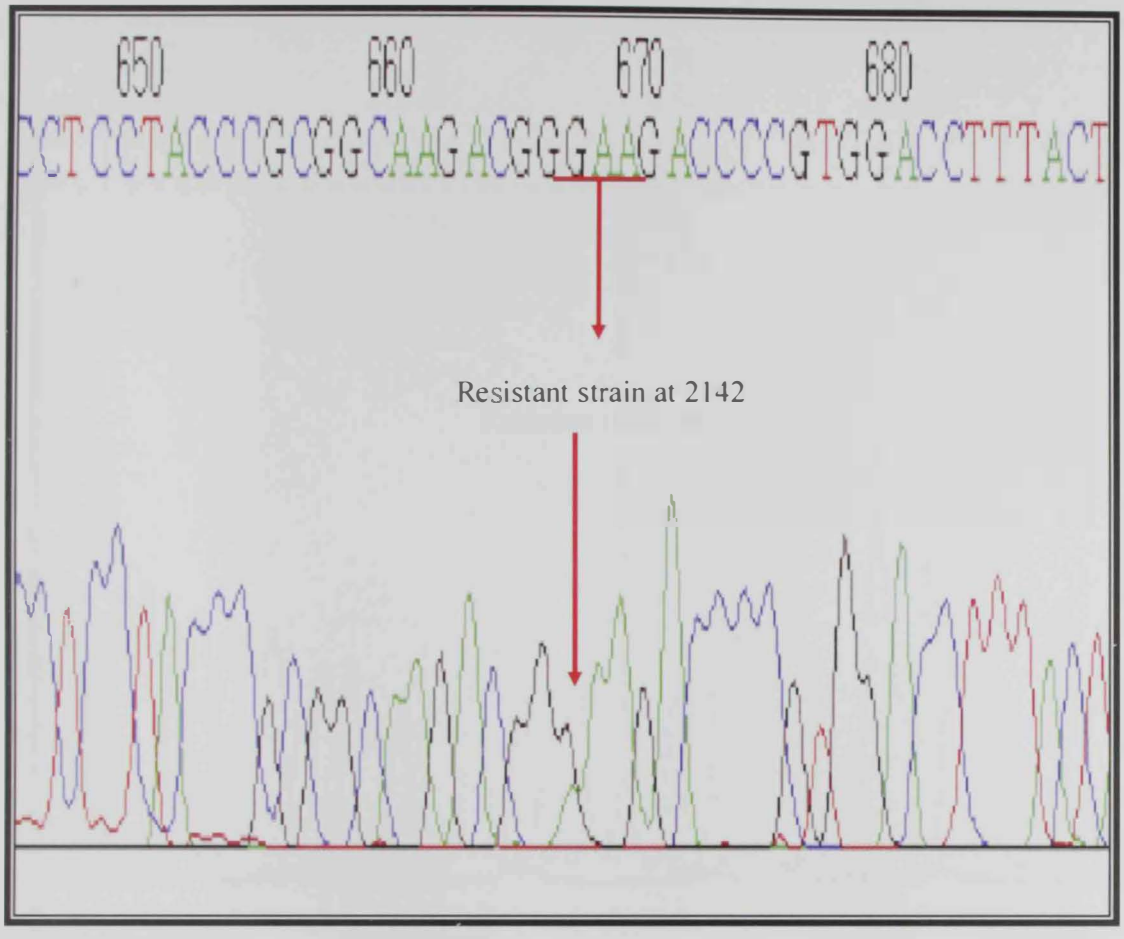


Fig. 3.10: Sequencing chromatograms for clarithromycin resistant strain with nucleotide alterations from A to G at 2142 position isolated from UAE patient.

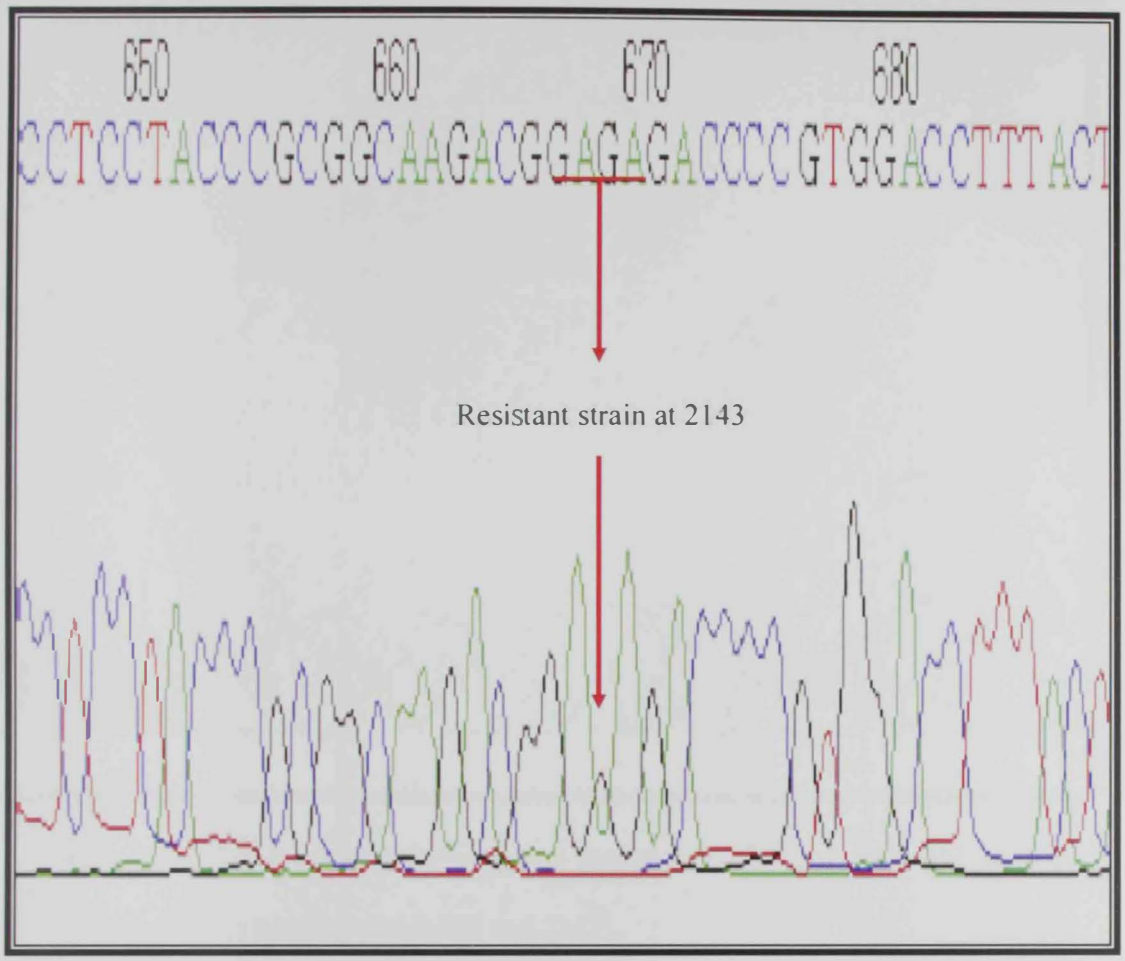


Fig. 3.11: Sequencing chromatograms for clarithromycin resistant strain with nucleotide alterations from A to G at 2143 position.

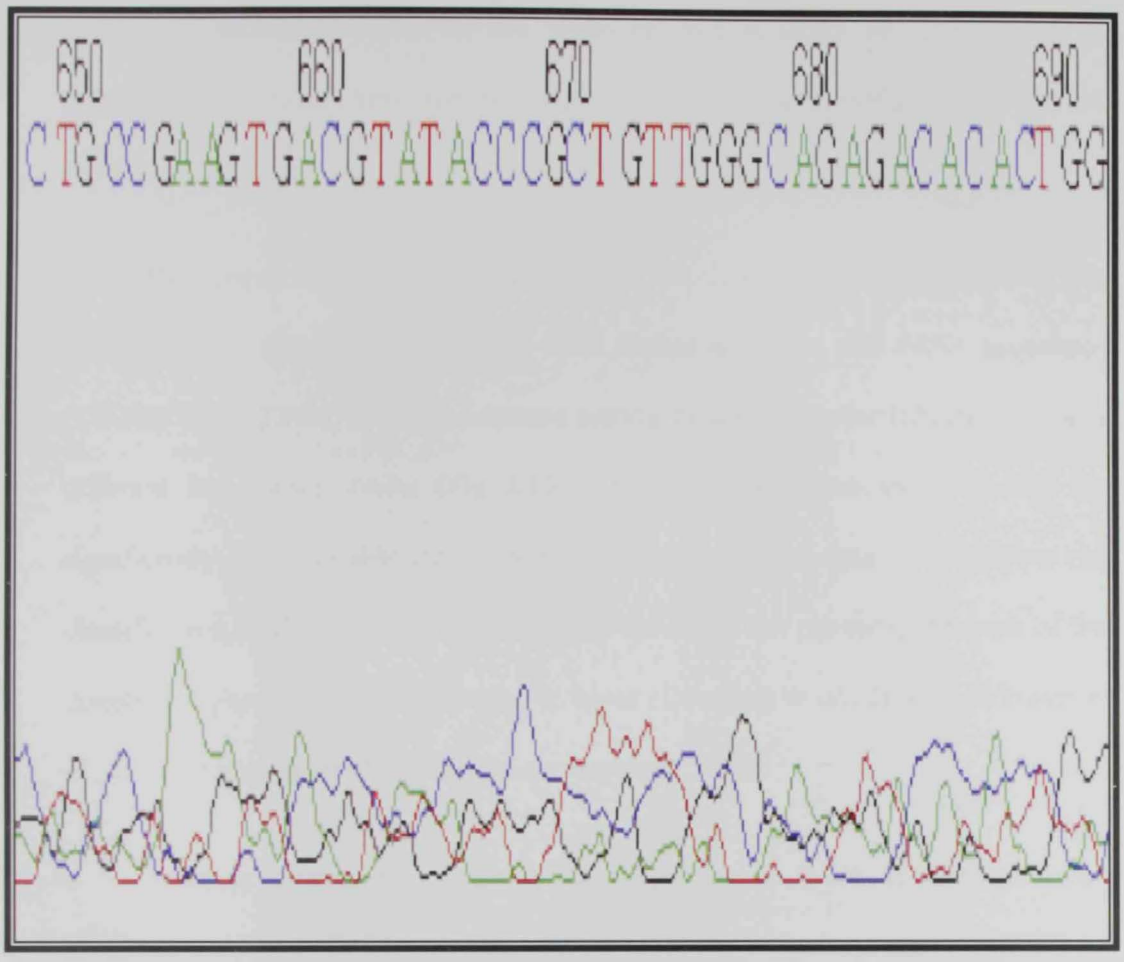


Fig. 3.12: Sequencing chromatogram for highly changed sequence at the clarithromycin binding site (2142 and 2143).

3.6. Phylogenetic Analysis of *23S rRNA*

H. pylori strains from UAE were studied to gain new insight into the population genetic structure of the pathogen and to study the implication of genotype in disease. Although *H. pylori* infection occurs worldwide, there are significant differences in its prevalence within and between countries.

DNA sequence phylogenetic analysis of the *23S rRNA* middle region of the gene indicates that the strains from UAE harbor a unique *23S rRNA* sequences (TTGGTTATATTCCA) that is common among isolates from the UAE patients and different from other strains **(Fig 3.13)**. The *23S rRNA* gene sequence data are significantly more reliable than 16S rRNA gene sequence data for identification, classification, and phylogenetic analysis of helicobacters primarily because of the threefold-higher number of informative bases (Dewhirst *et al.*, 2005; Mikkonen *et al.*, 2004; Hanninen *et al.*, 2003; *Vandamme et al.*,2000).

Genomic Comparison between *H. pylori* strains were done by software ClustalX, version 2. Reference sequences used in the alignment was obtained from NCBI data base for all 23s rRNA from different *Helicobacter pylori* Strains. The *H. pylori J99* (GenBank accession no. NC_000921.1), *H. pylori* 26695 (GenBank accession no. NC_000915.1), *H. pylori* G27 (GenBank accession no. NC_011333.1), *H. pylori* P12 (GenBank accession no. NC_011498.1), *H. pylori* Shi470 (GenBank accession no. NC_010698.2), *H. pylori* HPAG1 (GenBank accession no. NC_008086.1) and *H. pylori HPKX_438* genomes were downloaded. Homology searches were conducted against the genomes sequences of *H. pylor*i on the nucleotide level by using the BLAST software package. The BLAST output was

processed further to determine syntenic regions between two genomes (**Fig 3.14, 3.15** and **3.16**).

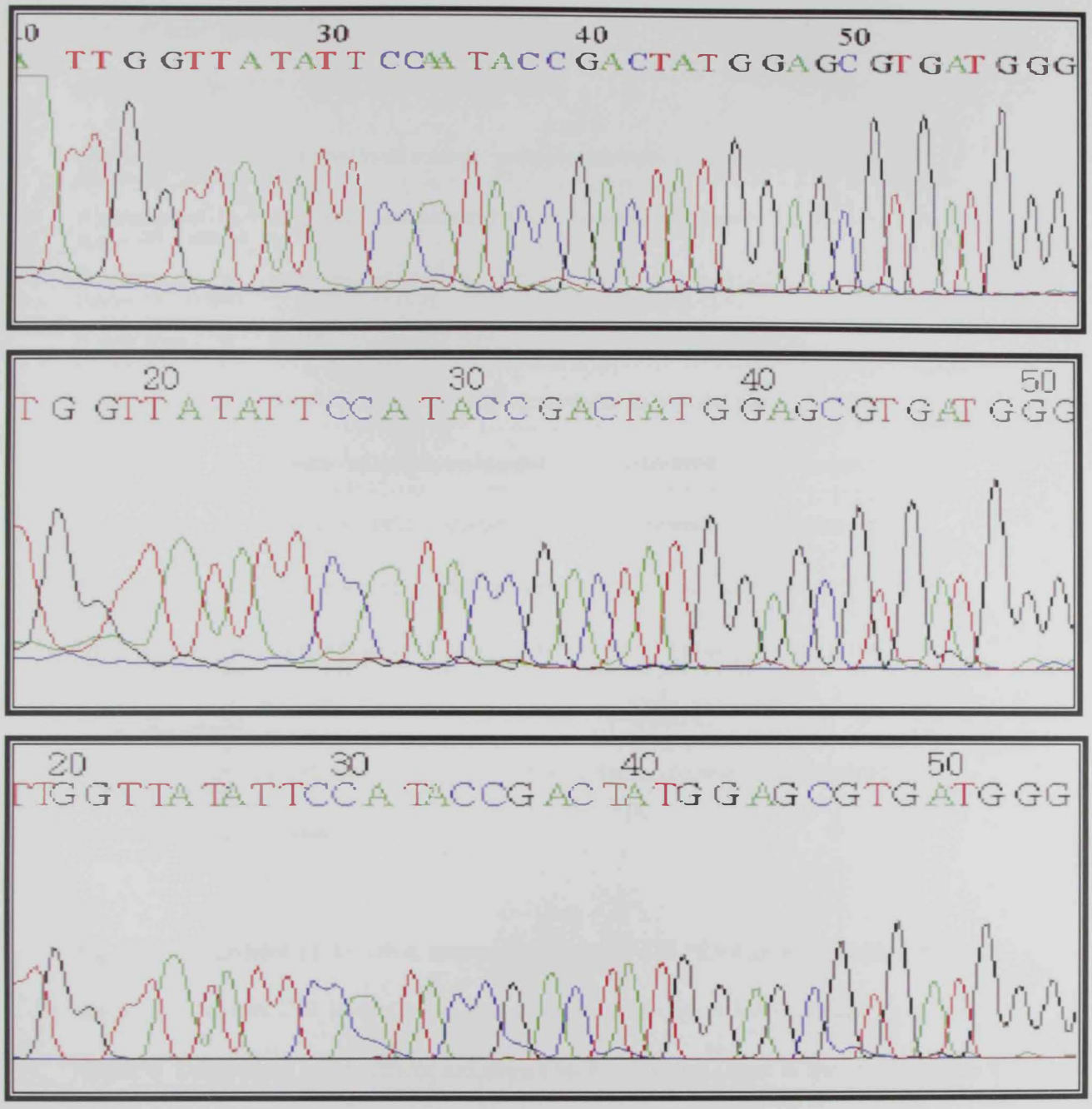


Fig. 3.13: *H. pylori* sequences from different strains isolated from UAE patients' shows the unique position (TTGGTTATATTCCA) for this strains which differentiate them from other *H. pylori* strains.

Helicobacter pylori J99

Identities = 669/671 (99%), Gaps = 1/671 (0%)

```
H. pylori Hpuae-5  1       TGGTT-ATATTCCAATACCGACTATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGAG  59
H.pylori J99       1059039 .....A.....................................................  1058980

H. pylori Hpuae-5  60      CTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCTG  119
H.pylori J99       1058979 ............................................................  1058920

H. pylori Hpuae-5  120     TATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTTAGGACAAAGGGAGAATCGCTGATAC  179
H.pylori J99       1058919 ............................................................  1058860

H. pylori Hpuae-5  180     CGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACACA  239
H.pylori J99       1058859 ............................................................  1058800

H. pylori Hpuae-5  240     GGTAGATGAGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCAAA  299
H.pylori J99       1058799 ........G...................................................  1058740

H. pylori Hpuae-5  300     CTAGCACCGTAAGTTCGCGATAAGGTGTGCCACAGCGATGTGGTCTCAGCAAAGAGTCCC  359
H.pylori J99       1058739 ............................................................  1058680

H. pylori Hpuae-5  360     TCCCGACTGTTTACCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGT  419
H.pylori J99       1058679 ............................................................  1058620

H. pylori Hpuae-5  420     GACGCCTGCCCGGTGCTCGAAGGTTAAGAGGATGCGTCAGTCGCAAGATGAAGCGTTGAA  479
H.pylori J99       1058619 ............................................................  1058560

H. pylori Hpuae-5  480     TTGAAGCCCGAGTAAACGGCGGCCGTAACTATAACGGTCCTAAGGTAGCGAAATTCCTTG  539
H.pylori J99       1058559 ............................................................  1058500

H. pylori Hpuae-5  540     TCGGTTAAATACCGACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGA  599
H.pylori J99       1058499 ............................................................  1058440

H. pylori Hpuae-5  600     TTCAGTGAAATTGTAGTGGAGGTGAAAATTCCTCCTACCCGCGGCAAGACGGAAAGACCC  659
H.pylori J99       1058439 ............................................................  1058380

H. pylori Hpuae-5  660     CGTGGACCTTT  670
H.pylori J99       1058379 ...........  1058369
```

Fig. 3.14: Alignment of the DNA sequences of partial *23S rRNA gene* (670bp). Reference strain is *H. Pylori J99* (Red Color) and isolated strain from UAE patient is *H. pylori Hpuae*-5. Differences in sequences are shown with red color. Gaps in the DNA sequence are marked with dashes, and identical nucleotides are indicated by dots.

Helicobacter pylori 26695

Identities = 668/671 (99%), Gaps = 1/671 (0%)

```
H. pylori Hpuae-5  1       TGGTT-ATATTCCAATACCGACTATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGAG  59
H. pylori 26695    446742  .....A.....................G.....................................  446801

H. pylori Hpuae-5  60      CTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCTG  119
H. pylori 26695    446802  ..........................................................  446861

H. pylori Hpuae-5  120     TATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTTAGGACAAAGGGAGAATCGCTGATAC  179
H. pylori 26695    446862  ..........................................................  446921

H. pylori Hpuae-5  180     CGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACACA  239
H. pylori 26695    446922  ................T.........................................  446981

H. pylori Hpuae-5  240     GGTAGATGAGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCAAA  299
H. pylori 26695    446982  ..........................................................  447041

H. pylori Hpuae-5  300     CTAGCACCGTAAGTTCGCGATAAGGTGTGCCACAGCGATGTGGTCTCAGCAAAGAGTCCC  359
H. pylori 26695    447042  ..........................................................  447101

H. pylori Hpuae-5  360     TCCCGACTGTTTACCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGT  419
H. pylori 26695    447102  ..........................................................  447161

H. pylori Hpuae-5  420     GACGCCTGCCCGGTGCTCGAAGGTTAAGAGGATGCGTCAGTCGCAAGATGAAGCGTTGAA  479
H. pylori 26695    447162  ..........................................................  447221

H. pylori Hpuae-5  480     TTGAAGCCCGAGTAAACGGCGGCCGTAACTATAACGGTCCTAAGGTAGCGAAATTCCTTG  539
H. pylori 26695    447222  ..........................................................  447281

H. pylori Hpuae-5  540     TCGGTTAAATACCGACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGA  599
H. pylori 26695    447282  ..........................................................  447341

H. pylori Hpuae-5  600     TTCAGTGAAATTGTAGTGGAGGTGAAAATTCCTCCTACCCGCGGCAAGACGGAAAGACCC  659
H. pylori 26695    447342  ..........................................................  447401

H. pylori Hpuae-5  660     CGTGGACCTTT  670
H. pylori 26695    447402  ...........  447412
```

Fig. 3.15: Alignment of the DNA sequences of partial *23S rRNA gene* (670bp). Reference strain is *H. Pylori 26695* (Red Color) and isolated strain from UAE patient is *H. pylori Hpuae*-5. Differences in sequences are shown with red color. Gaps in the DNA sequence are marked with dashes, and identical nucleotides are indicated by dots.

Helicobacter pylori HPKX_438_AG0C1 NZ_ABJO01000217

Fig. 3.16: Alignment of the DNA sequences of partial *23S rRNA gene* (670bp). Reference strain is *H. pylori HPKX_438_AG0C1* (Red Color) and isolated strain from UAE patient is *H. pylori Hpuae*-5. Differences in sequences are shown with red color. Gaps in the DNA sequence are marked with dashes, and identical nucleotides are indicated by dots.

The *23S rRNA* sequences for the twenty strains listed in **Fig 3.17** was determined by using sequencing methods. Sequences were entered in *23S rRNA* databases and aligned based on secondary structure. Phylogenetic trees were constructed using the neighbor-joining method. The neighbor-joining tree constructed from the partial *23S rRNA* gene sequences is shown in **Fig. 3.18** and **Fig. 3.19**. The more detailed similarity analysis of the partial *23S rRNA* gene sequences among *Helicobacter* pylori strains is shown in Table **3.5** and **3.6**. The similarities of the *23S rRNA* sequences of *H. pylori* strains isolated from UAE patients to those other published sequences were 99% except for *H. pylori HPKX_438_CA4C1* strain (NZ_ABJP01003236.1) **(Table 3.5)**.

Two main strains are found in UAE patients, one is closely related to *Helicobacter pylori G27* and the other one to *Helicobacter pylori J99* just the differences in two or three nucleotides **(Fig. 3.17)**. *H. pylori Hpuae-5* **(Fig. 3.20)** is an example of one group of strains which is closely related to *H. pylori J99*, where it has two nucleotides difference **(Fig. 3.14)**; Also it is similar to *H. pylori 26695* with three nucleotides difference **(Fig 3.15)**. *H. pylori HPKX_438_AG0C1* strain is excluded from this study because of missing nucleotides **(Fig 3.16)** On the other hand *H. pylori Hpuae-20* **(Fig. 3.21)** is an example of second group which is closely related to *H. pylori G27* but different in two nucleotides. All isolated strains sequences showed in **APPENDIX 2**.

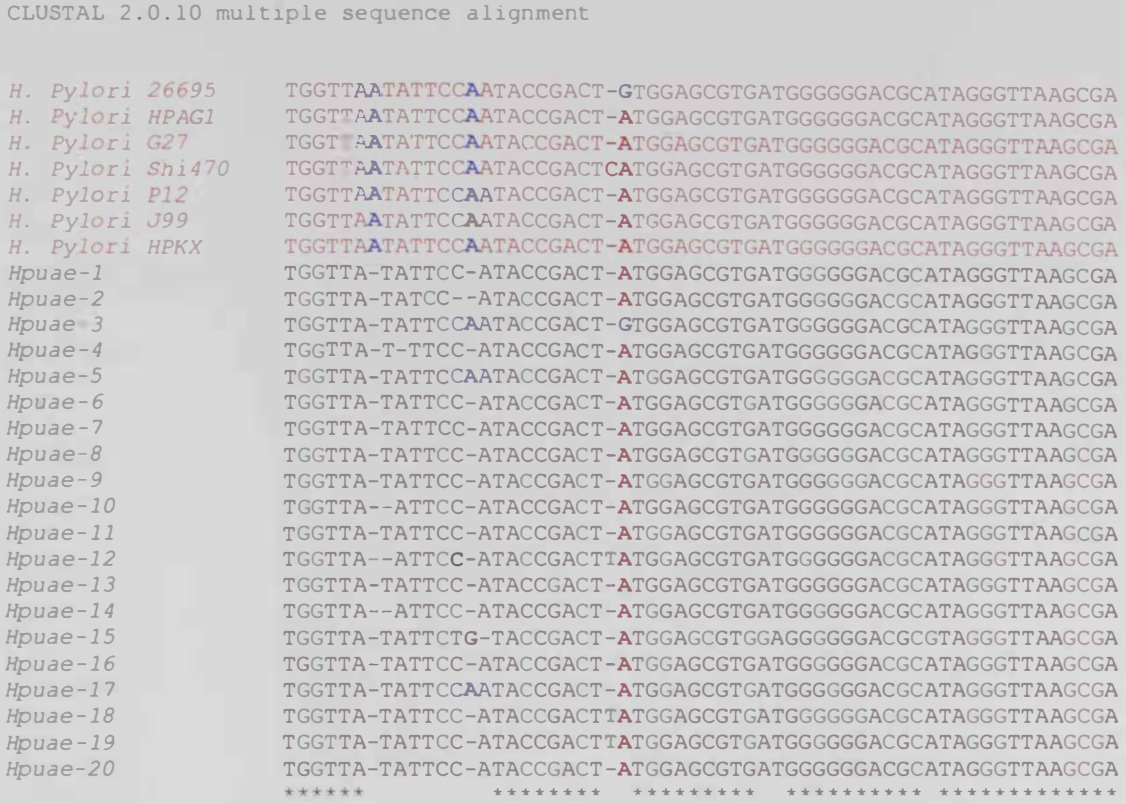


Fig. 3.17: Alignment of the DNA sequences of partial *23S rRNA gene* (670bp). Reference strains used in the alignment are in Red Color (*H. Pylori 26695*, *H. Pylori HPAG1*, *H. Pylori G27*, *H. Pylori Shi470*, *H. Pylori P12*, *H. Pylori J99*, *H. Pylori HPKX*), while the sequences of strains isolated from UAE patient are in black color with *H. pylori Hpuae* name. The differences in sequences are shown with different colors with boldface. Gaps in the DNA sequence are marked with dashes, and identical nucleotides are indicated by stars. **See Appendix 1**.

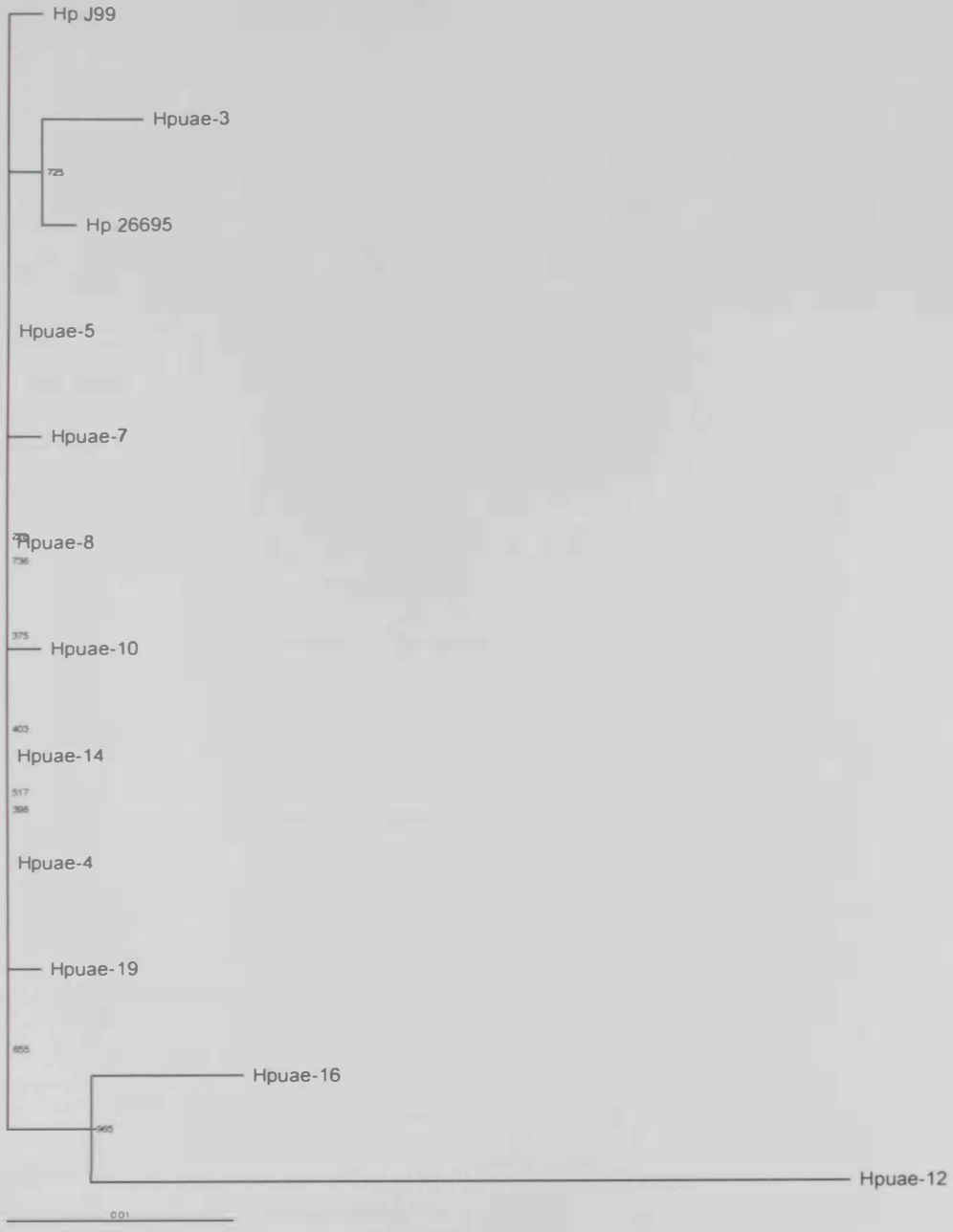


Fig. 3.18: Neighbor-joining tree based on partial 670-bp *23S rRNA* gene sequences. The tree was rooted with *H. pylori J99*. The number at each branch point represents the percentage of bootstrap support calculated from 1,000 trees. The scale bar represents the sequence divergence.

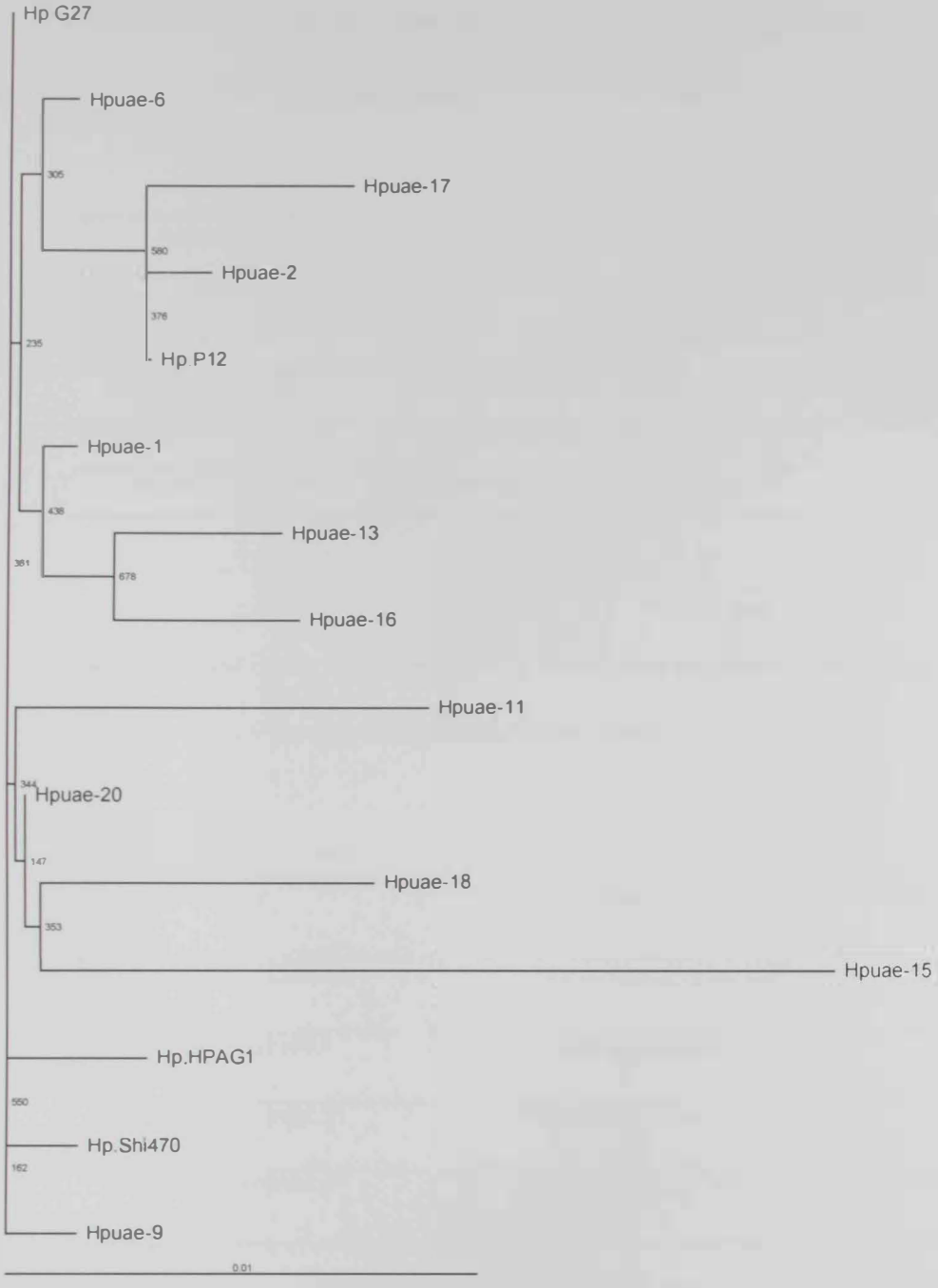


Fig. 3.19: Neighbor-joining tree based on partial 670-bp *23S rRNA* gene sequences. The tree was rooted with *H. pylori G27*. The number at each branch point represents the percentage of bootstrap support calculated from 1,000 trees. The scale bar represents the sequence divergence.

Table 3.5: Similarity of the partial *23S rRNA* gene sequence isolated from *H. pylori Hpuae-5* among other *Helicobacter* pylori strains.

Accession	Description	Max ident
NC_000921.1	Helicobacter pylori J99	99%
NC_000915.1	Helicobacter pylori 26695	99%
NZ_ABJP01000007.1	Helicobacter pylori HPKX_438_CA4C1	99%
NZ_ABJO01000217.1	Helicobacter pylori HPKX_438_AG0C1	99%
NZ_ABJP01003236.1	Helicobacter pylori HPKX_438_CA4C1	89%

Table 3.6: Similarity of the partial *23S rRNA* gene sequence isolated from *H. pylori Hpuae-20* among *Helicobacter* pylori strains

Accession	Description	Max ident
NC_011333.1	Helicobacter pylori G27	99%
NC_011498.1	Helicobacter pylori P12	99%
NC_010698.2	Helicobacter pylori Shi470	99%
NC_008086.1	Helicobacter pylori HPAG1	99%

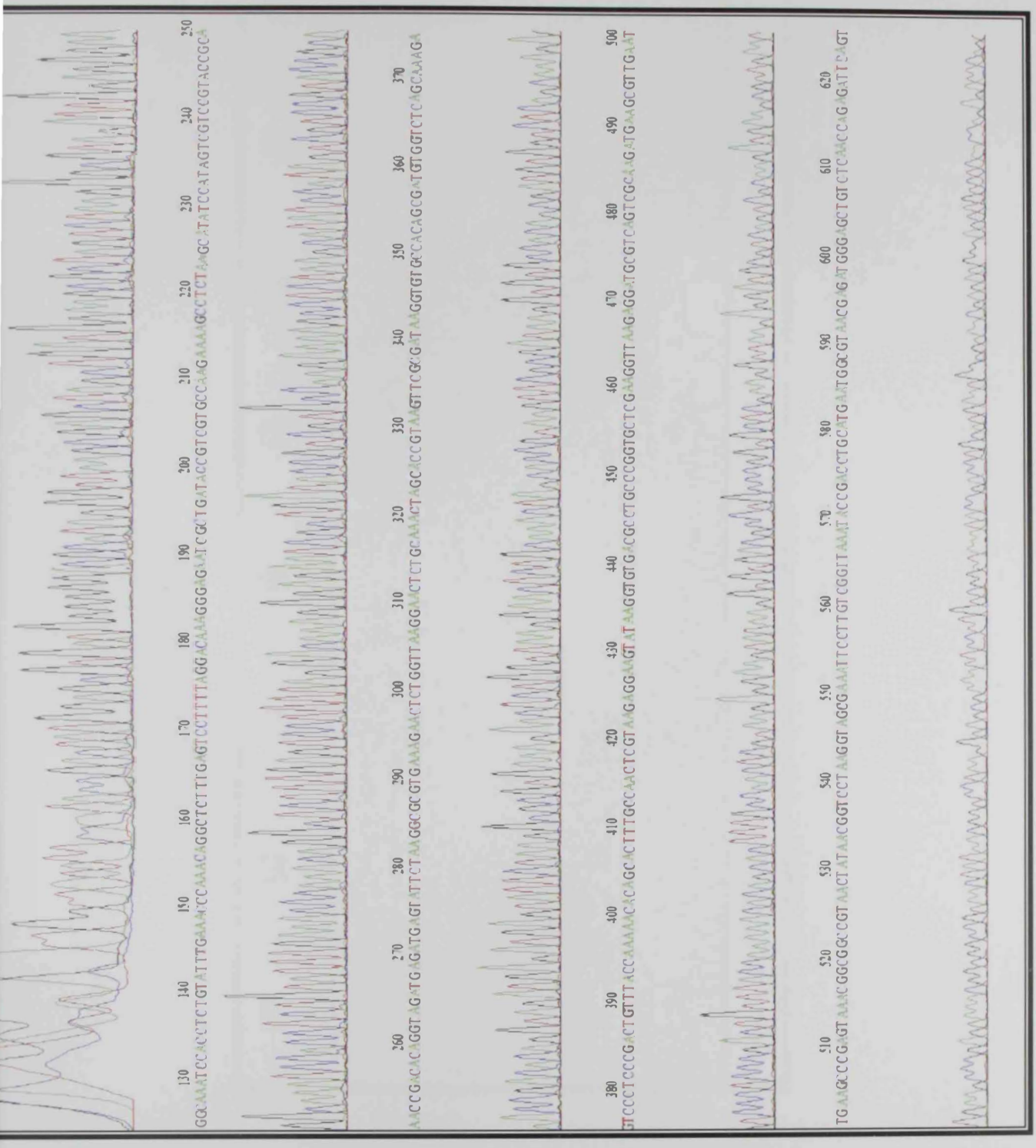
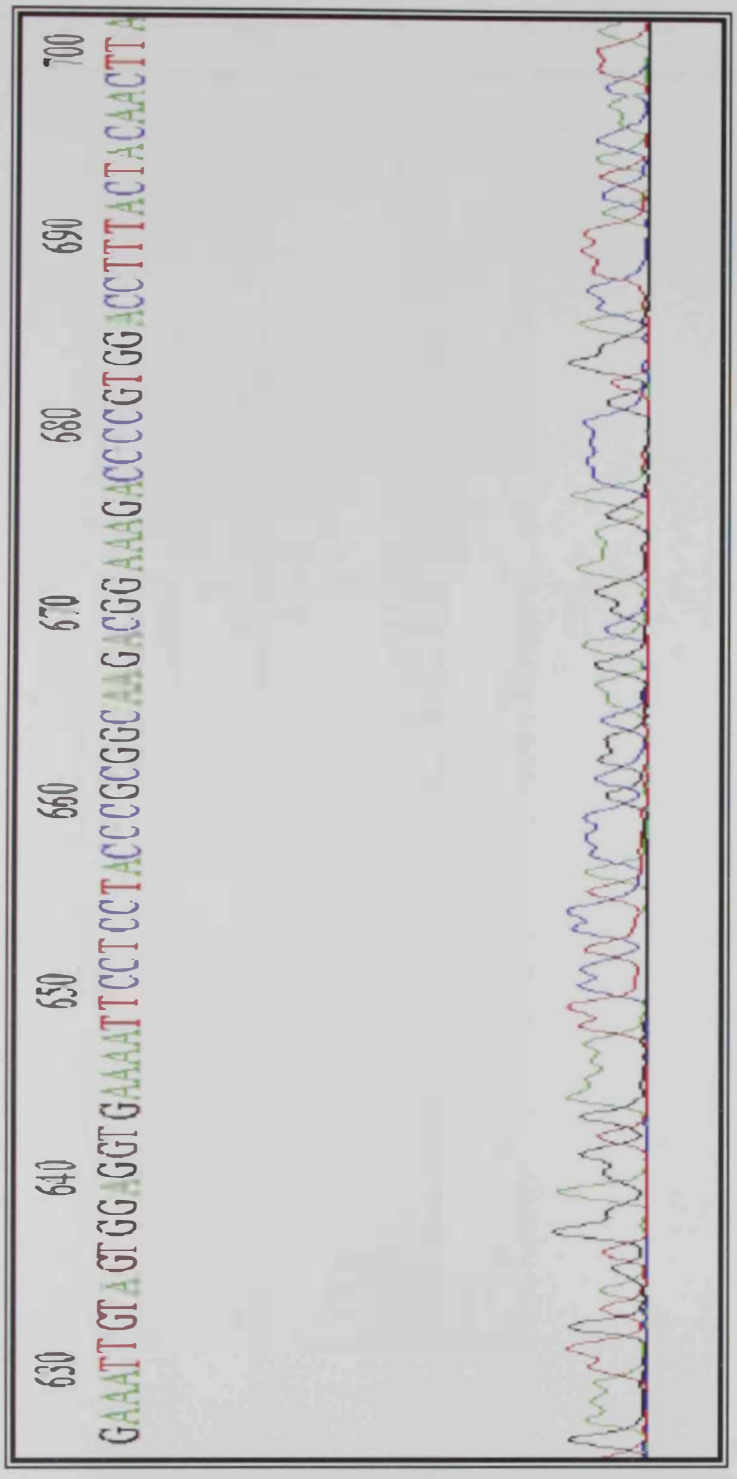


Fig. 3.20: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-5*



Continued **Fig. 3.20**: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-5*

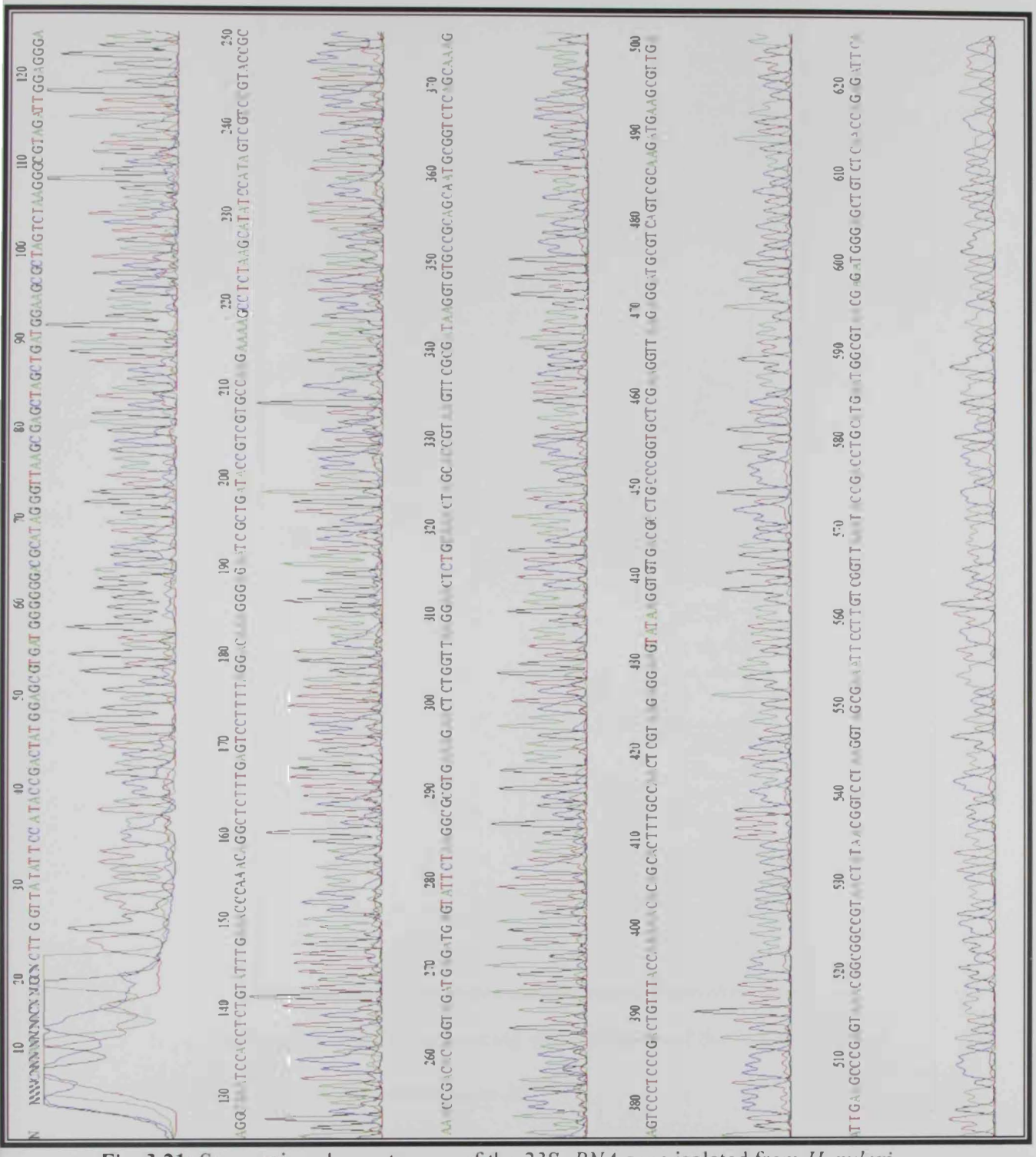
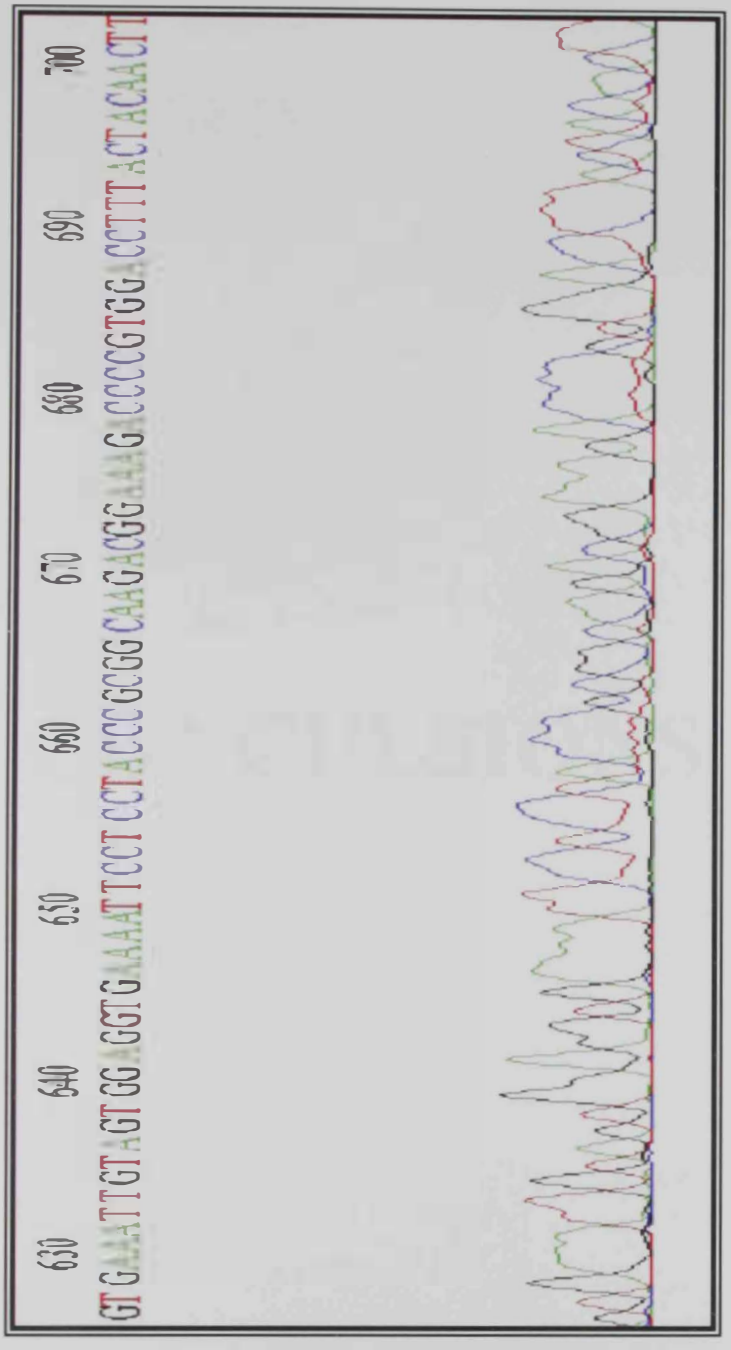


Fig. 3.21: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-20*



Continued **Fig. 3.21**: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-20*

CHAPTER IV

CONCULSIONS

4. Conclusions

Several conclusions could be drawn from the presented study are as follow:

(i) The relationship between deletion of 200bp in *rdxA* gene (gene which is responsible for highly resistance for metronidazole) and *Cag A* gene needs further researches at the molecular fields with high number of samples to make the relation more clear.

(ii) The finding of a high rate of resistance to clarithromycin, which is most likely due to the vast consumption of erythromycin in cases of upper respiratory infections, calls for an effective eradication program and disqualifies clarithromycin as an effective regimen. These results further confirm the essence of continuous monitoring of antibiotic resistance patterns in order to reduce the rate of eradication failure in UAE or any other target population. These data may provide the basis for treatment recommendations for *H. pylori* eradication in UAE.

From this study: full genomic sequencing for the new *H. pylori* strains which have been found in the UAE patients (locals and non locals) with unique sequences in the *23S rRNA*, is recommended.

Also I highly recommend using PCR technique in all clinical laboratories and hospitals in UAE for detecting *H. pylori*, and this is due to the sensitivity of PCR more than other methods in the identification of this bacteria, beside the ability of the PCR assay which has the advantage of detecting low numbers of bacteria after successful or unsuccessful therapy or prior to relapse.

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APPENDIX I

APPENDIX I

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CLUSTAL 2.0.10 multiple sequence alignment

H. Pylori 26695     TGGTTAATATTCCAATACCGACT-GTGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
H. Pylori HPAG1     TGGTTAATATTCCAATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
H. Pylori G27       TGGTTAATATTCCAATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
H. Pylori Shi470    TGGTTAATATTCCAATACCGACTCATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
H. Pylori P12       TGGTTAATATTCCAATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
H. Pylori J99       TGGTTAATATTCCAATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
H. Pylori HPKX      TGGTTAATATTCCAATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-1             TGGTTA-TATTCC-ATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-2             TGGTTA-TATCC--ATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-3             TGGTTA-TATTCCAATACCGACT-GTGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-4             TGGTTA-T-TTCC-ATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-5             TGGTTA-TATTCCAATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-6             TGGTTA-TATTCC-ATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-7             TGGTTA-TATTCC-ATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-8             TGGTTA-TATTCC-ATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-9             TGGTTA-TATTCC-ATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
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Hpuae-16            TGGTTA-TATTCC-ATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
Hpuae-17            TGGTTA-TATTCCAATACCGACT-ATGGAGCGTGATGGGGGGACGCATAGGGTTAAGCGA
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H. Pylori HPAG1     GCTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
H. Pylori G27       GCTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
H. Pylori Shi470    GCTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
H. Pylori P12       GCTAGCTGATGGAATAGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
H. Pylori J99       GCTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
H. Pylori HPKX      GCTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
Hpuae-1             GCTAGCTGATGGAAGTGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
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Hpuae-3             GCTAGCTGATGGAAGTGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
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Hpuae-19            GCTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGGCAAATCCACCTCT
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H. Pylori G27       GTATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTTAGGAC-AAAGGGAGAATCGCTGAT
H. Pylori Shi470    GTATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTCAGGAC-AAAGGGAGAATCGCTGAT
H. Pylori P12       GTATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTTAGGAC-AAAGGGAGAATCGCTGAT
H. Pylori J99       GTATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTTAGGAC-AAAGGGAGAATCGCTGAT
H. Pylori HPKX      GTATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTTAGGAC-AAAGGGAGAATCGCTGAT
Hpuae-1             GTATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTTAGGAC-AAAGGGAGAATCGCTGAT
Hpuae-2             GTATTTGAAACCCAAACAGGCTCTTTGAGTCCTTTTAGGAC-AAAGGGAGAATCGCTGAT
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H. Pylori HPAG1     ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
H. Pylori G27       ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
H. Pylori Shi470    ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
H. Pylori P12       ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
H. Pylori J99       ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
H. Pylori HPKX      ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
Hpuae-1             ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
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Hpuae-4             ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
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Huaep-6             ACCGTCGTGCCAAGAAAAGCCTCTAAGCATATCCATAGTCGTCCGTACCGCAAACCGACA
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H. Pylori HPAG1     CAGGTAGATGAGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCA
H. Pylori G27       CAGGTAGATGAGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCA
H. Pylori Shi470    CAGGTAGATGAGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCA
H. Pylori P12       CAGGTAGATGAGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCA
H. Pylori J99       CAGGTAGATGGGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCA
H. Pylori HPKX      CAGGTAGATGAGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCA
Hpuae-1             CAGGTAGATGAGATGAGTATTCTAAGGCGCGTGAAAGAACTCTGGTTAAGGAACTCTGCA
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H. Pylori G27       AACTAGCACCGTAAGTTCGCGATAAGGTGTGCCGCAGCAATGCGGTCTCAGCAAAGAGTC
H. Pylori Shi470    AACTAGCACCGTAAGTTCGCGATAAGGTGTGCCGCAGCAATGCGGTCTCAGCAAAGAGTC
H. Pylori P12       AACTAGCACCGTAAGTTCGCGATAAGGTGTGCCGCAGCAATGCGGTCTCAGCAAAGAGTC
H. Pylori J99       AACTAGCACCGTAAGTTCGCGATAAGGTGTGCCACAGCGATGTGGTCTCAGCAAAGAGTC
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H. Pylori G27        CCTCCCGACTGTTTACCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGT
H. Pylori Shi470     CCTCCCGACTGTTTACCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGT
H. Pylori P12        CCTCCCGACTGTTTACCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGT
H. Pylori J99        CCTCCCGACTGTTTACCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGT
H. Pylori HPKX       CCTCCCGACTGTTTA-----------------------------------------
Hpuae-1              CCTCCCGACTGTTTACCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGT
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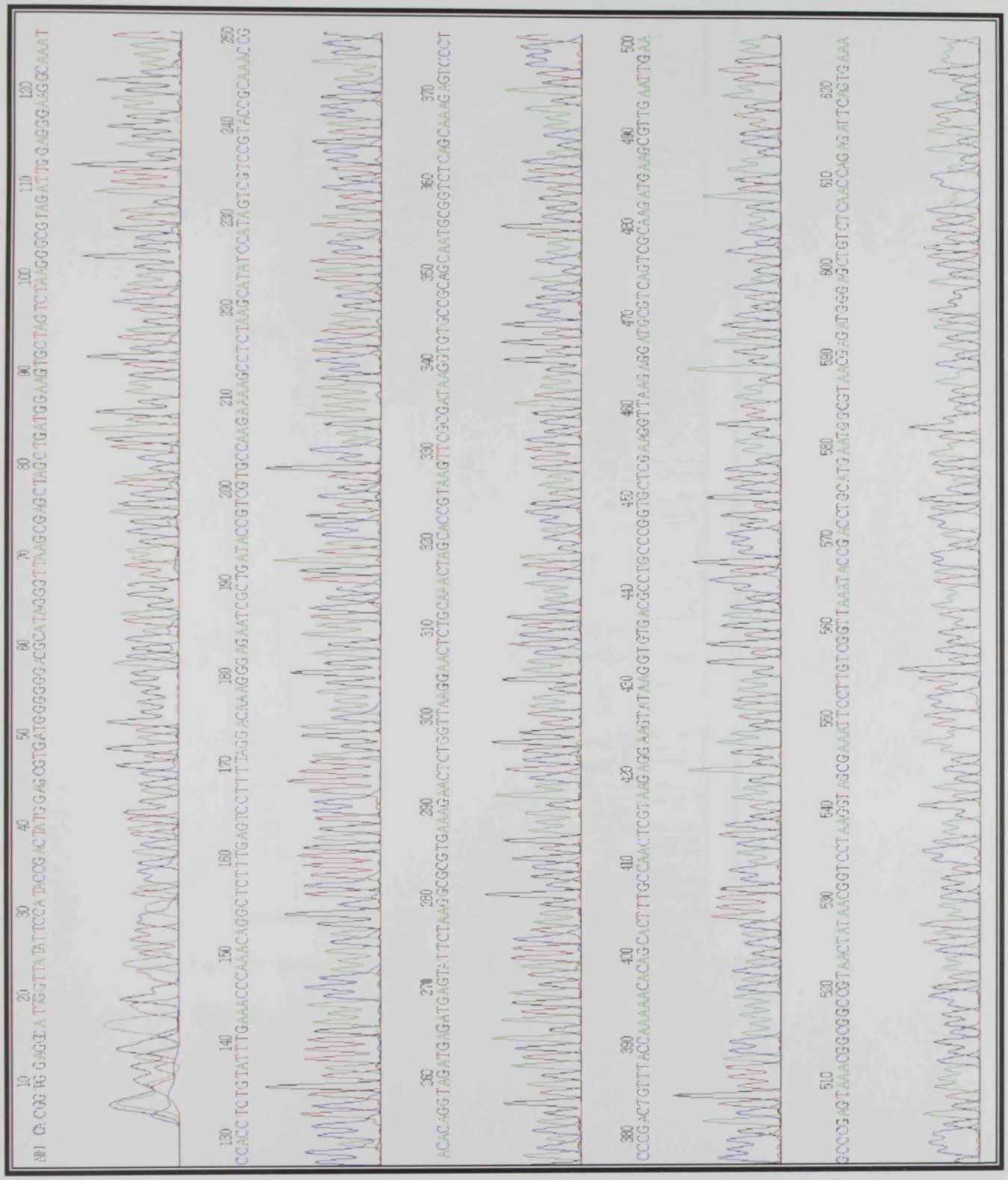
Alignment of the DNA sequences of partial *23S rRNA gene* (670bp). Reference strains used in the alignment are in Red Color (*H. Pylori 26695*, *H. Pylori HPAG1*, *H. Pylori G27*, *H. Pylori Shi470*, *H. Pylori P12*, *H. Pylori J99*, *H. Pylori HPKX*), while the sequences of strains isolated from UAE patient are in black color with *H. pylori Hpuae* name. The differences in sequences are shown with different colors with boldface. Gaps in the DNA sequence are marked with dashes, and identical nucleotides are indicated by stars.

APPENDIX II

APPENDIX II

H. pylori Hpuae-1



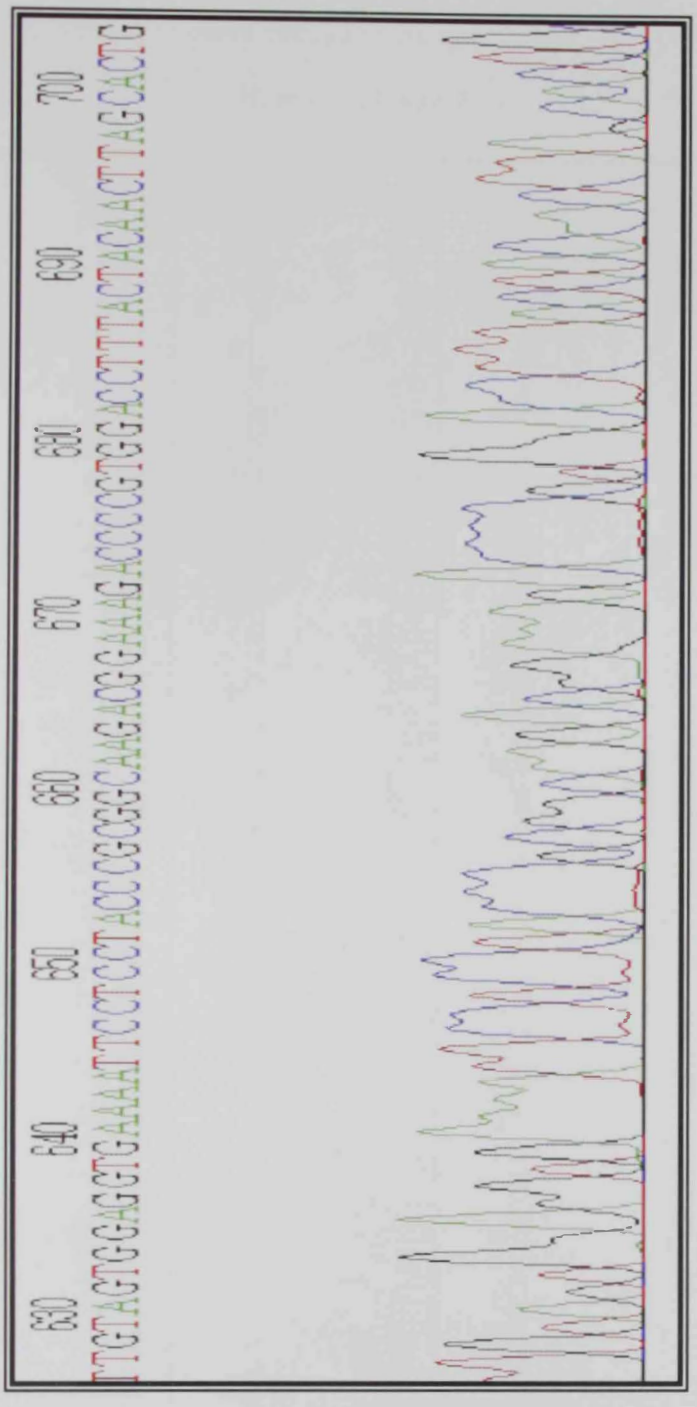
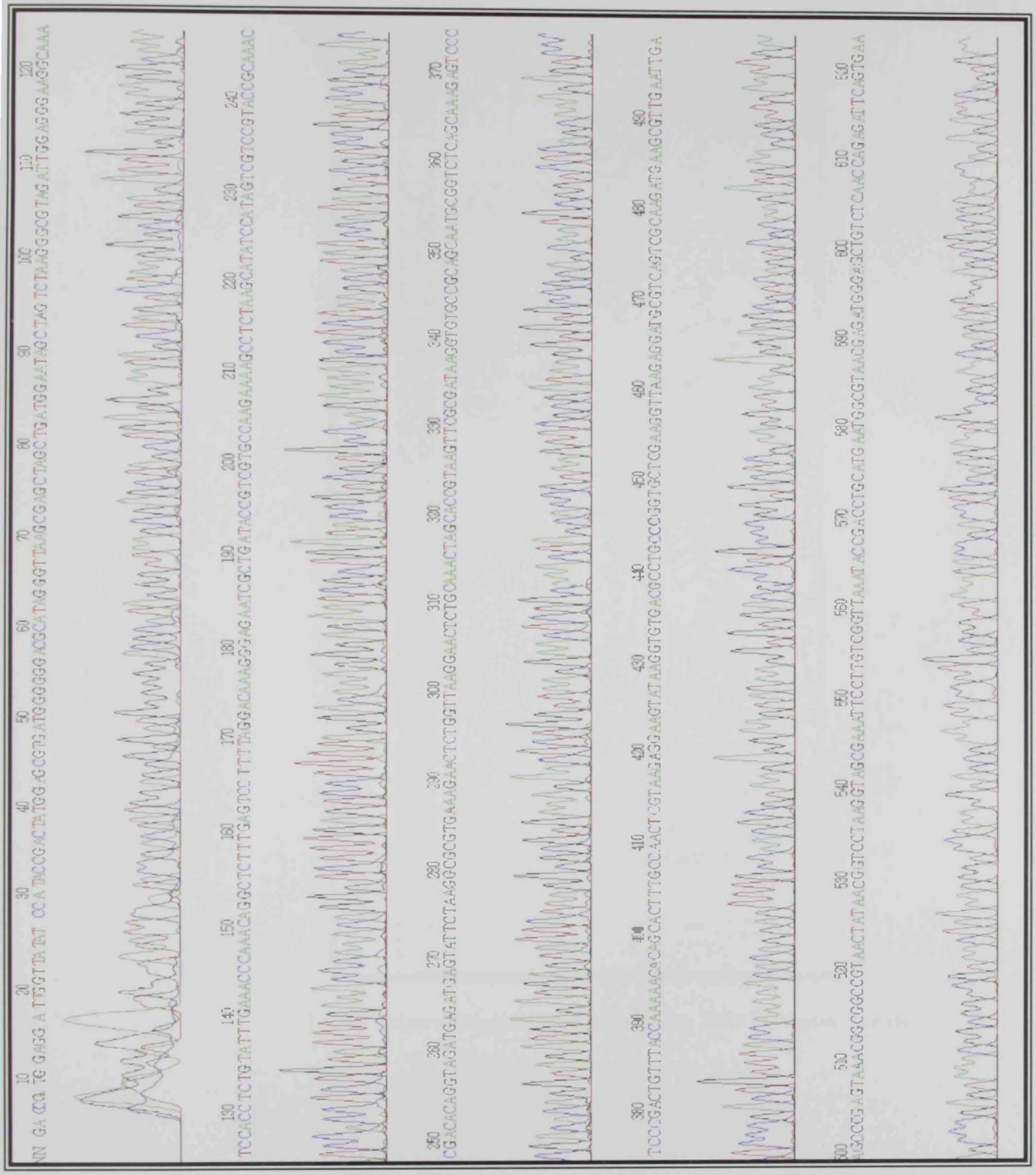


Fig.1: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-1*

H. pylori Нрпае-2



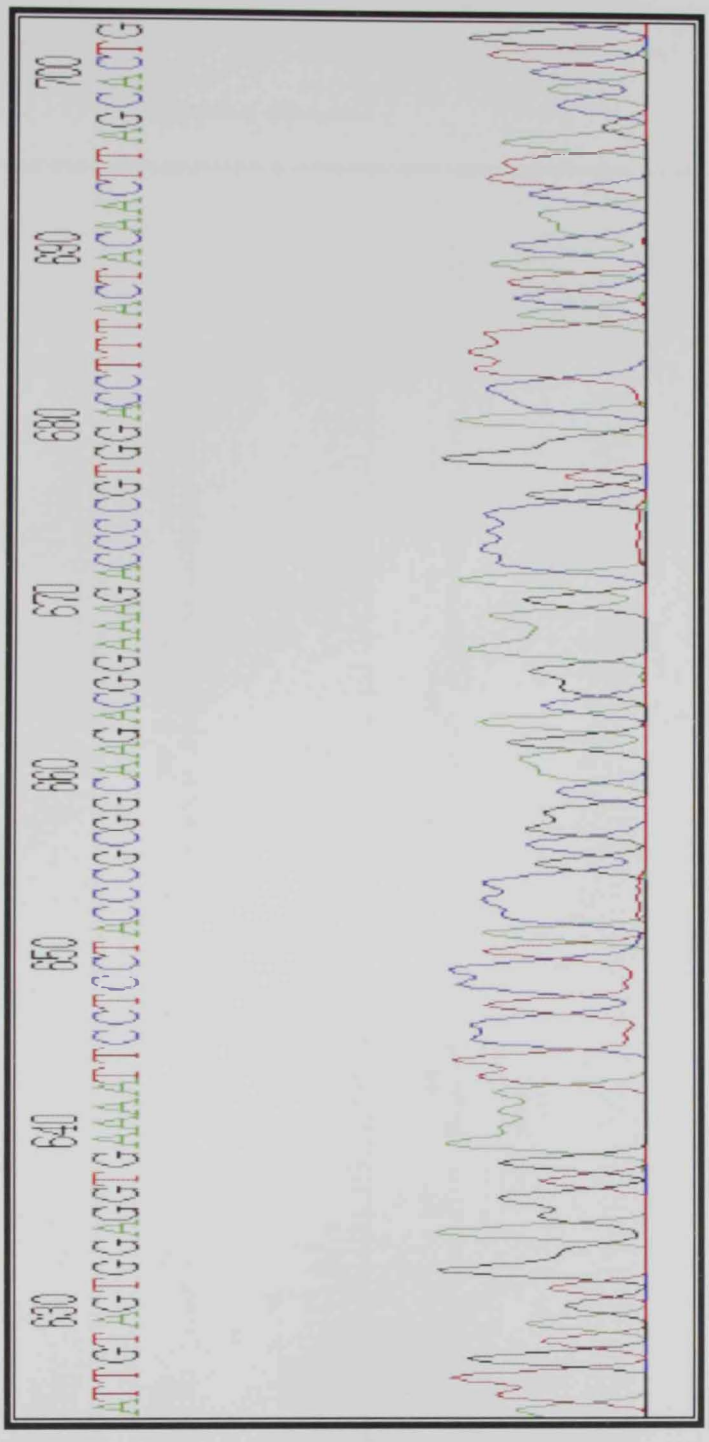
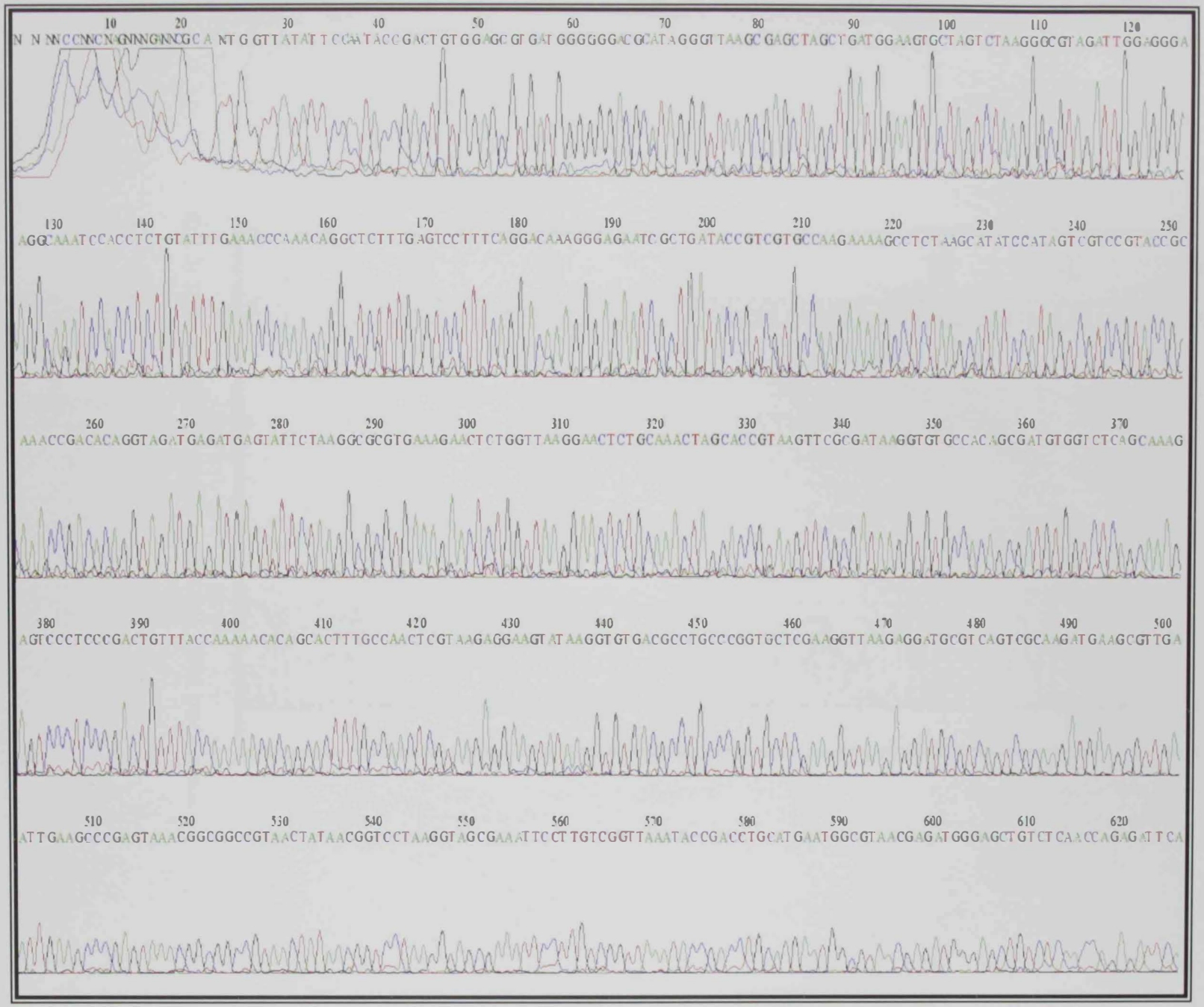


Fig. 2: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-2*



H. pylori Hpuae-3

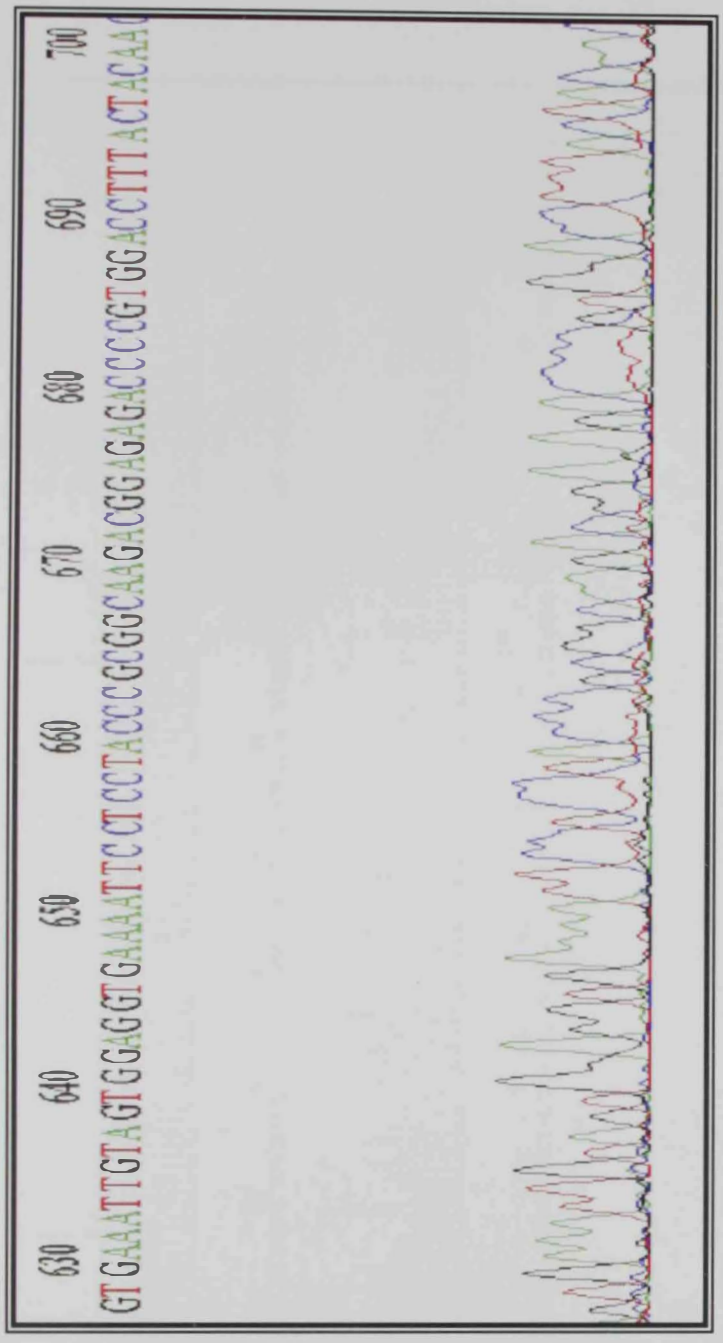
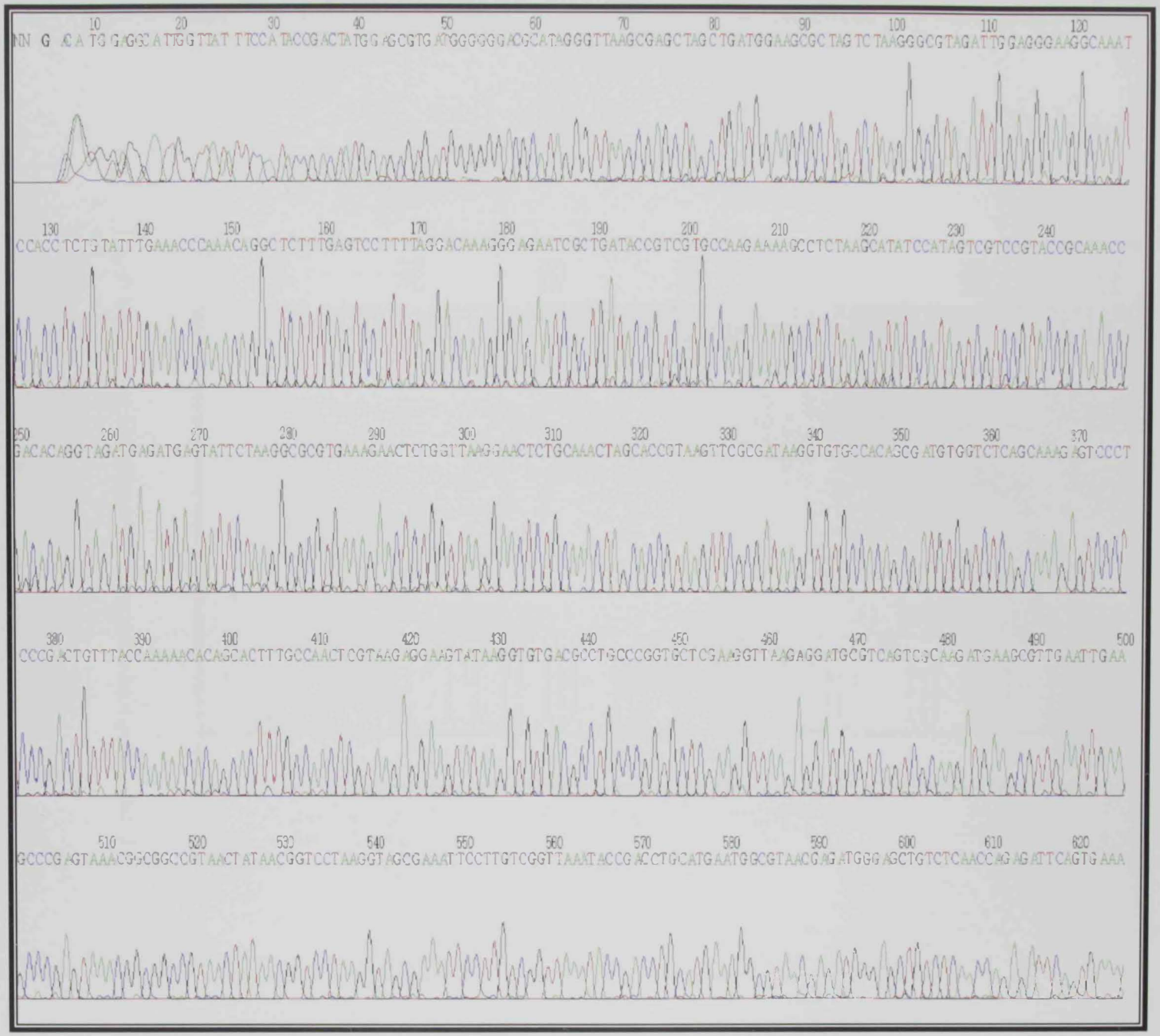


Fig. 3: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-3*



H. pylori Hpuae-4

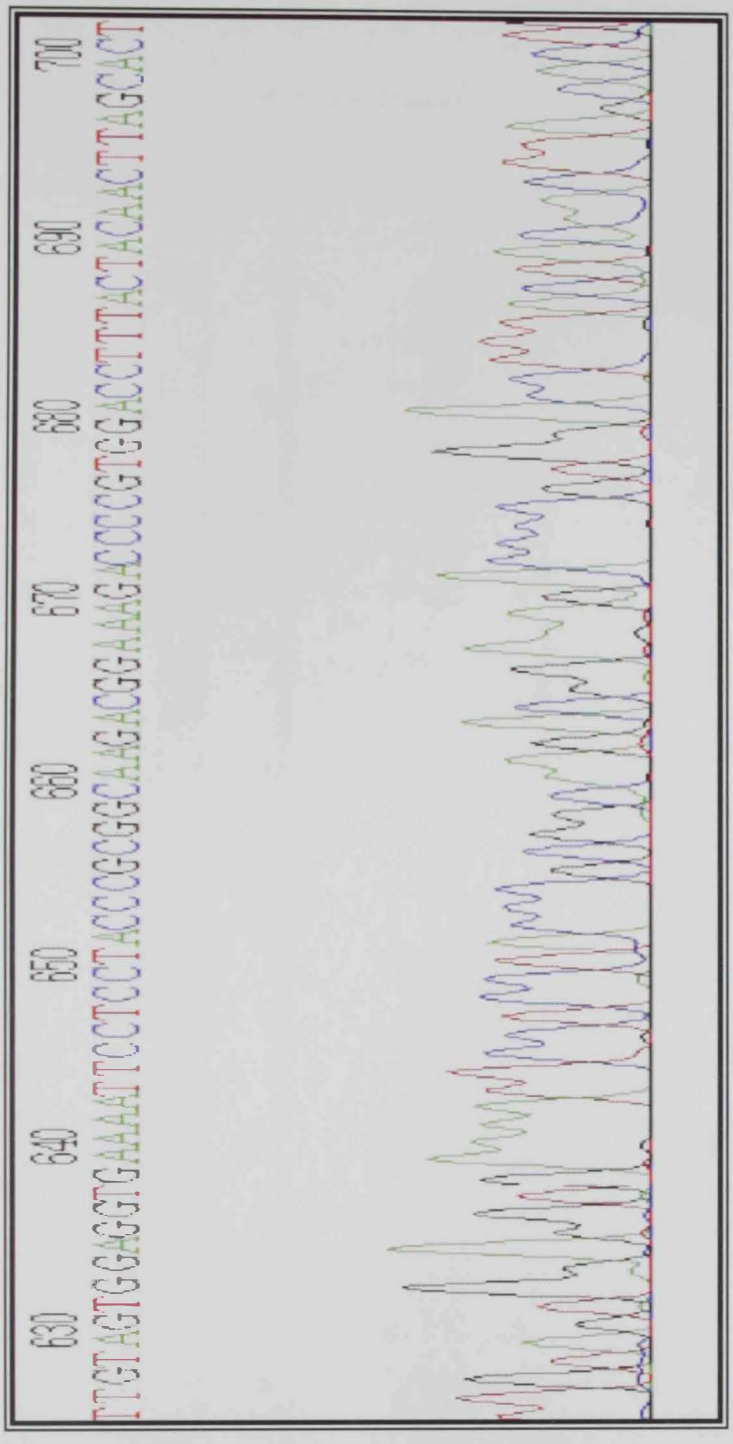
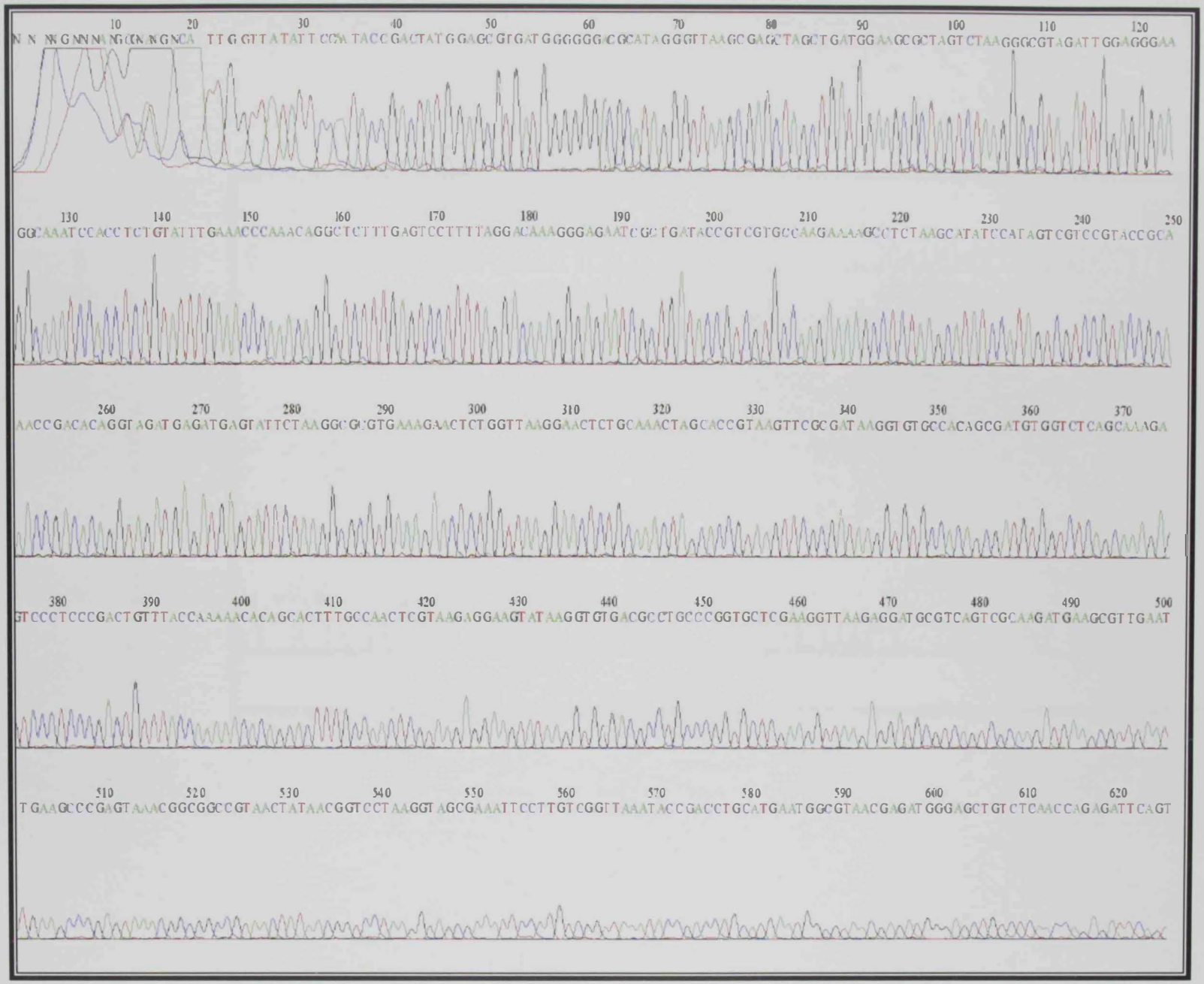


Fig. 4: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-4*



H. pylori Hpuae-5

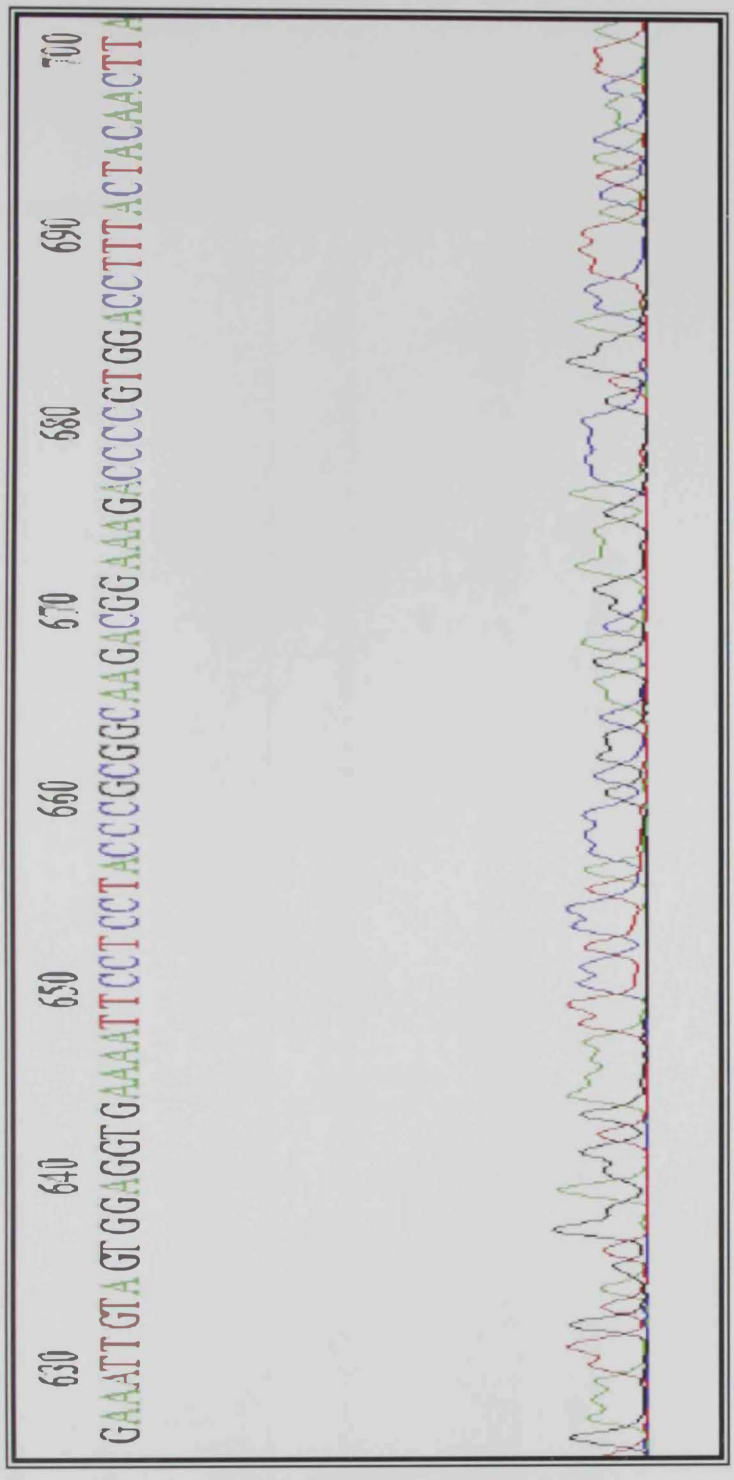
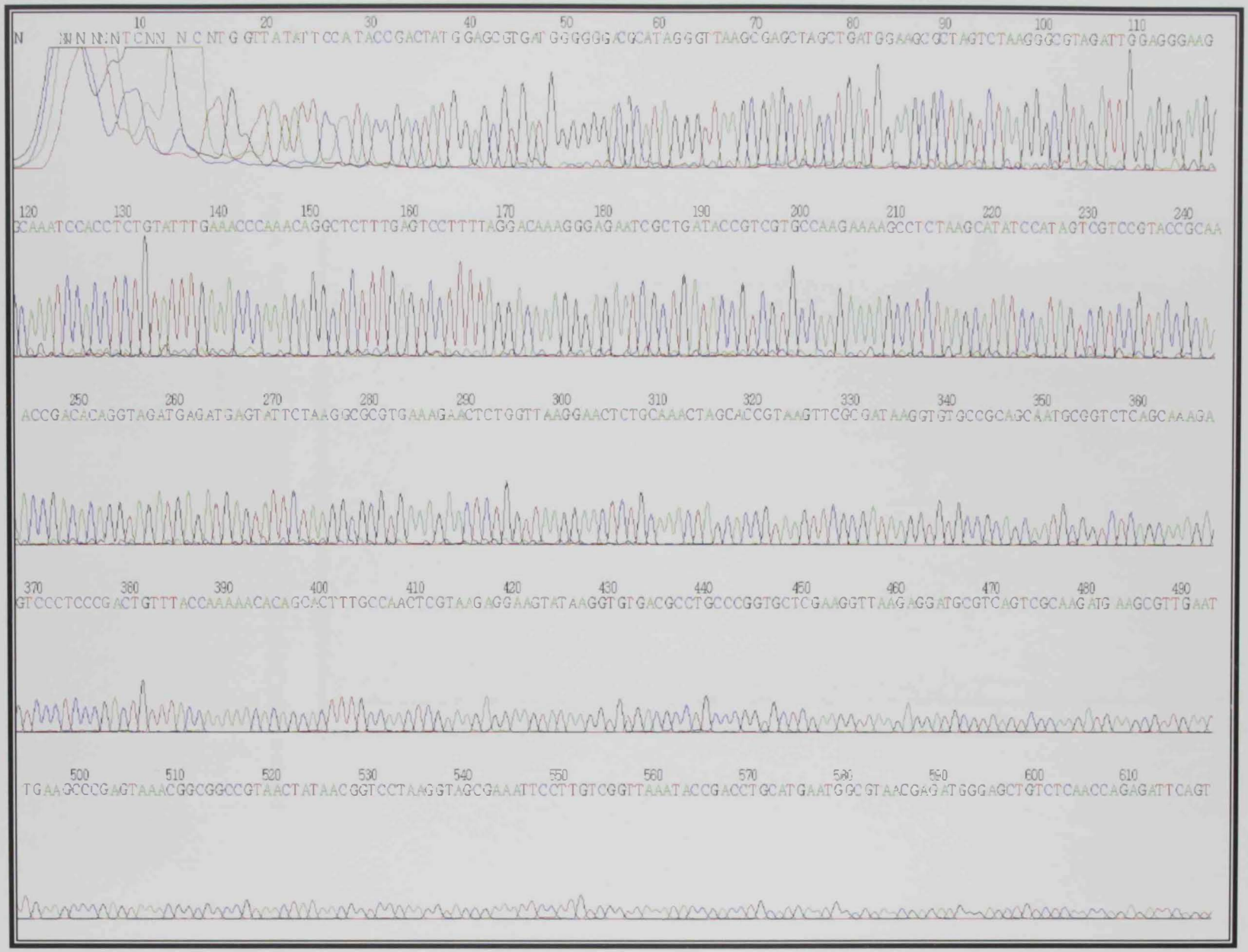


Fig. 5: Sequencing chromatogram of the *23S rRNA* gene isolated from *H. pylori Hpuae-5*



H. pylori Hpuae-6

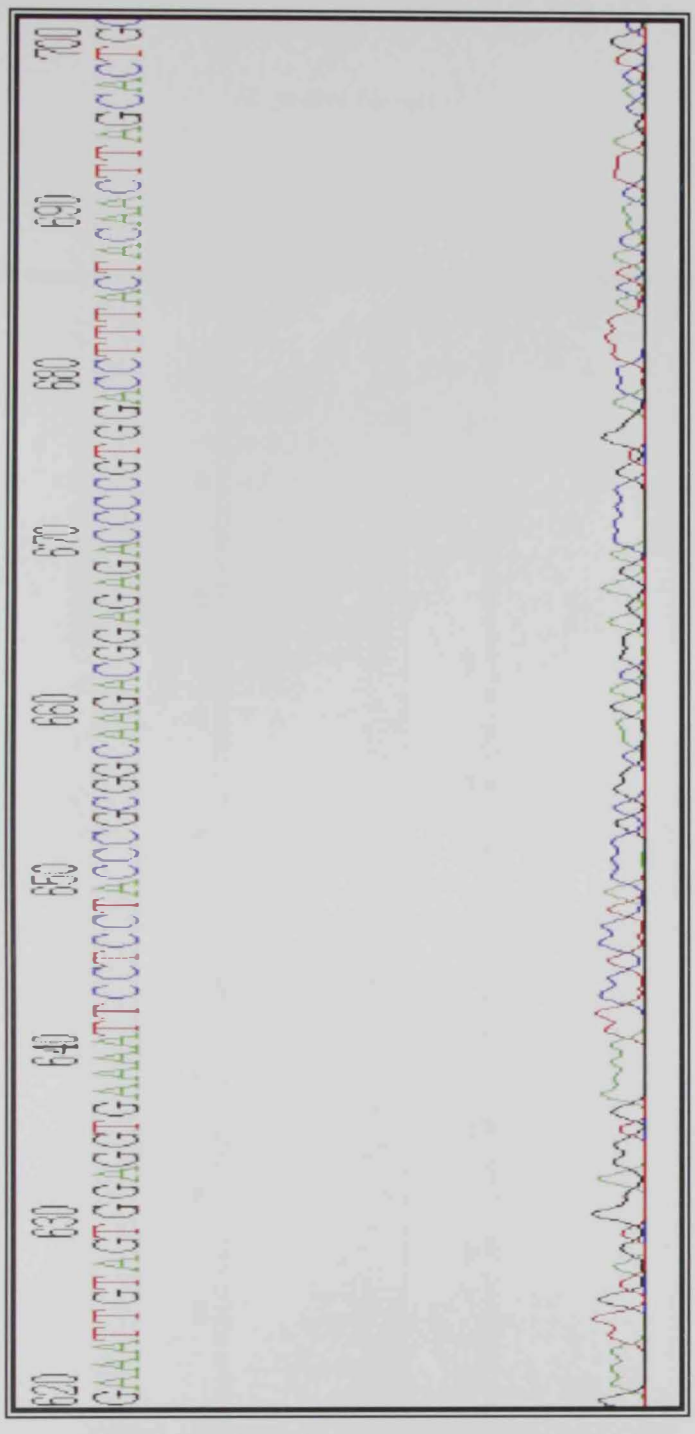
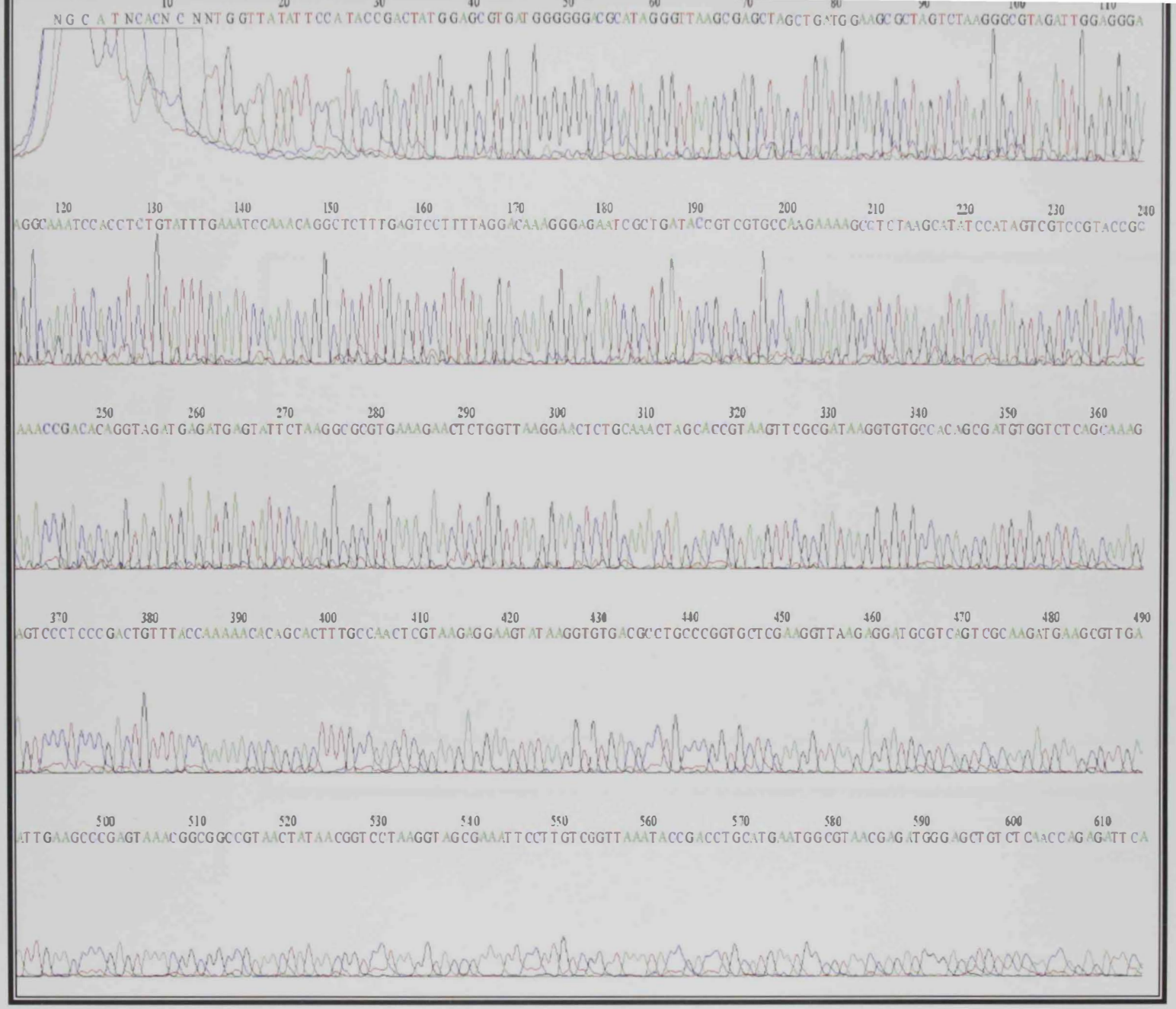


Fig. 6: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-6*



H. pylori Hpuae-7

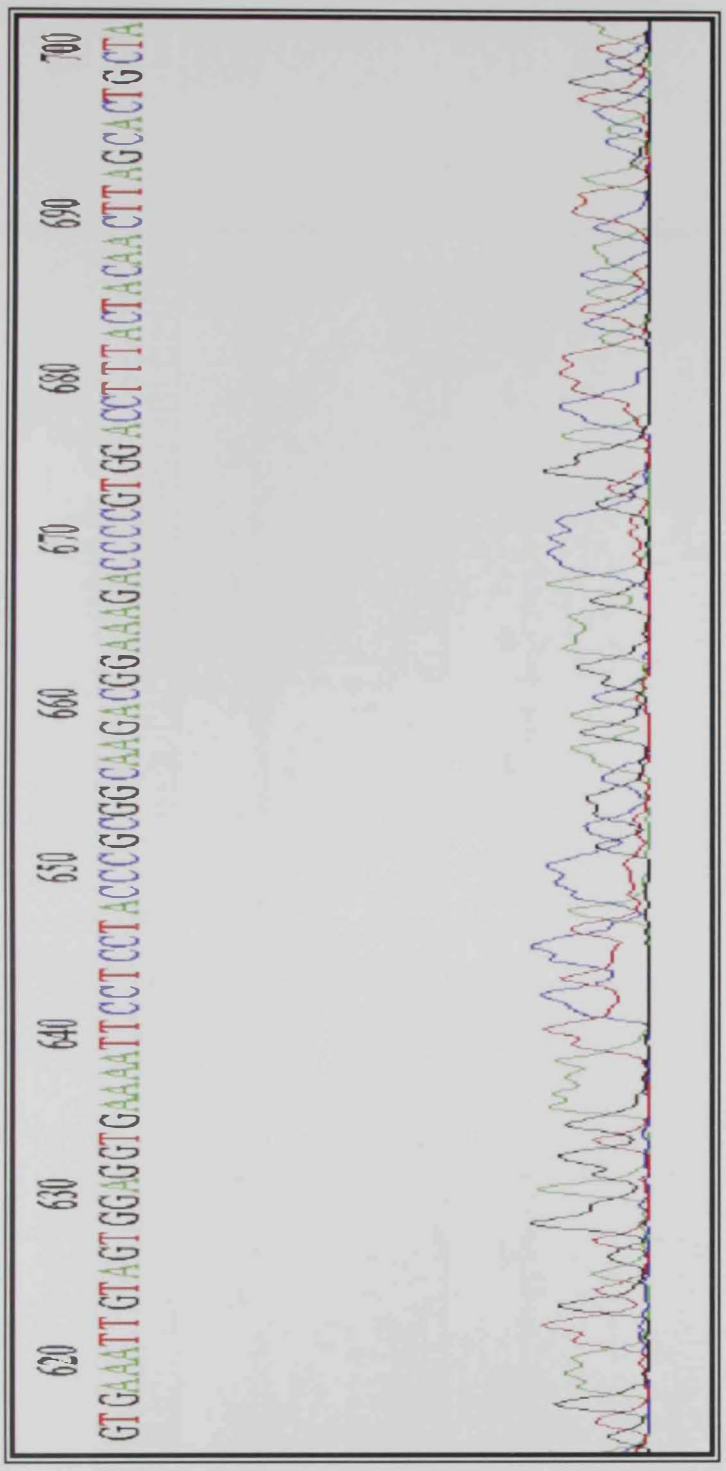


Fig. 7: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-7*

H. pylori Hpuac-8

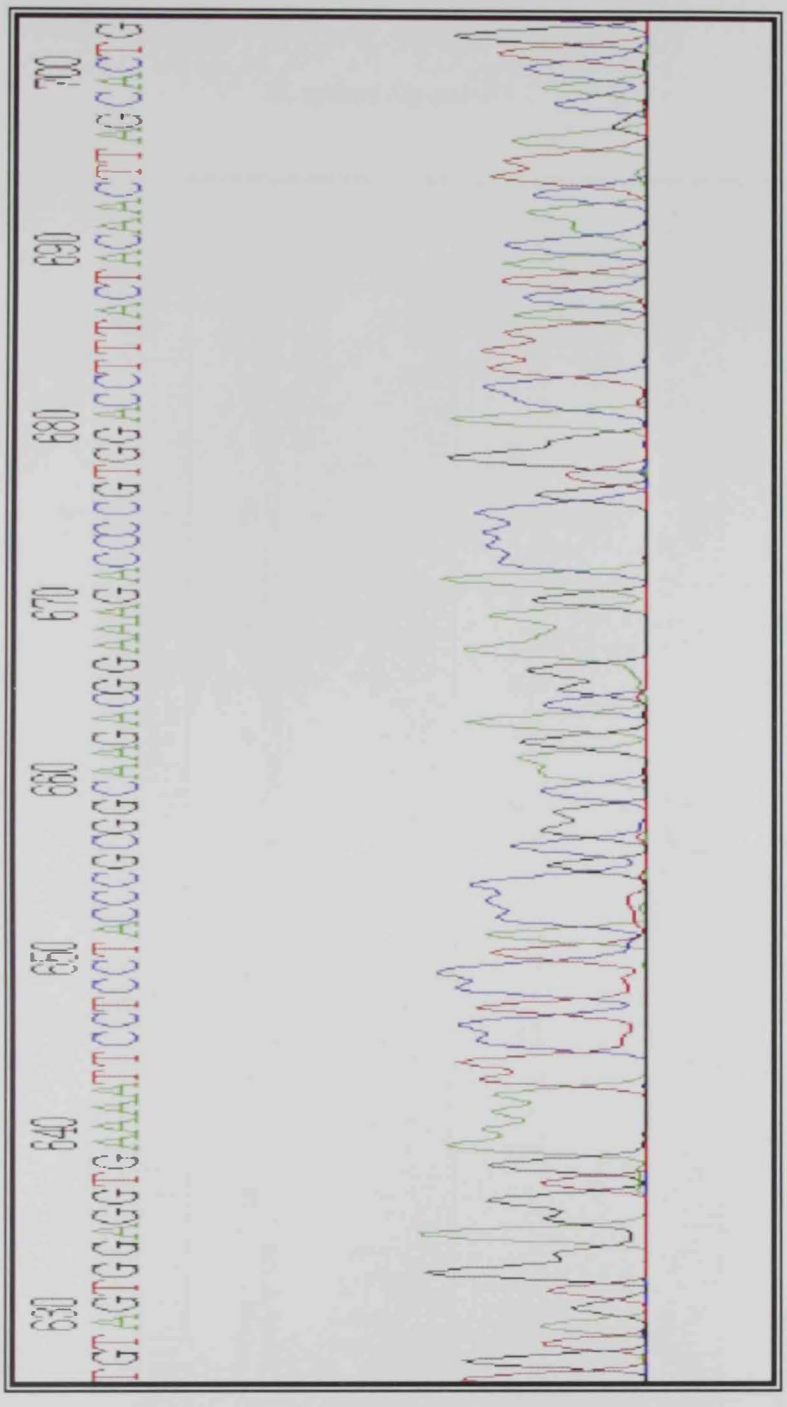
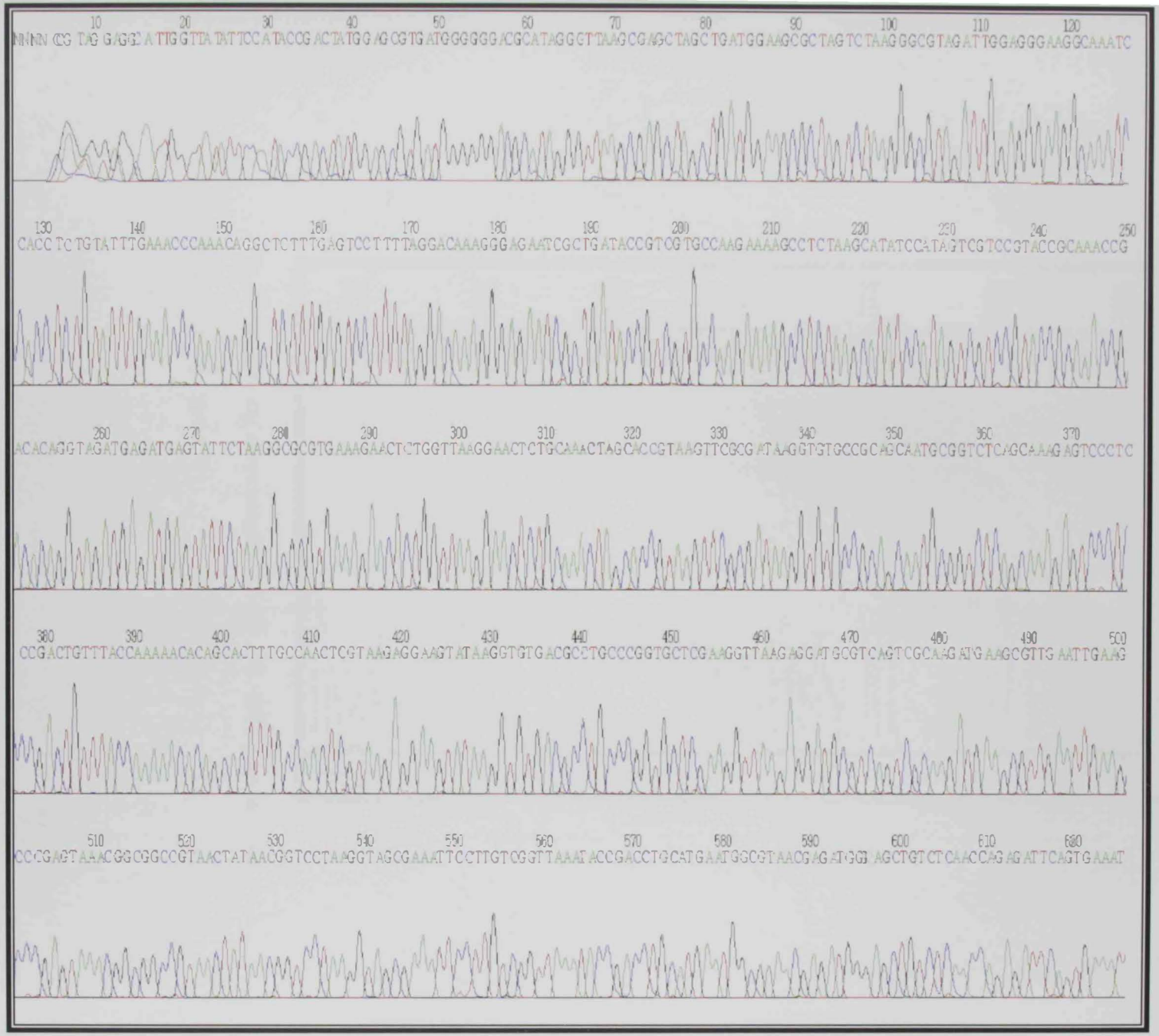


Fig. 8: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-8*



H. pylori Hpuae-9

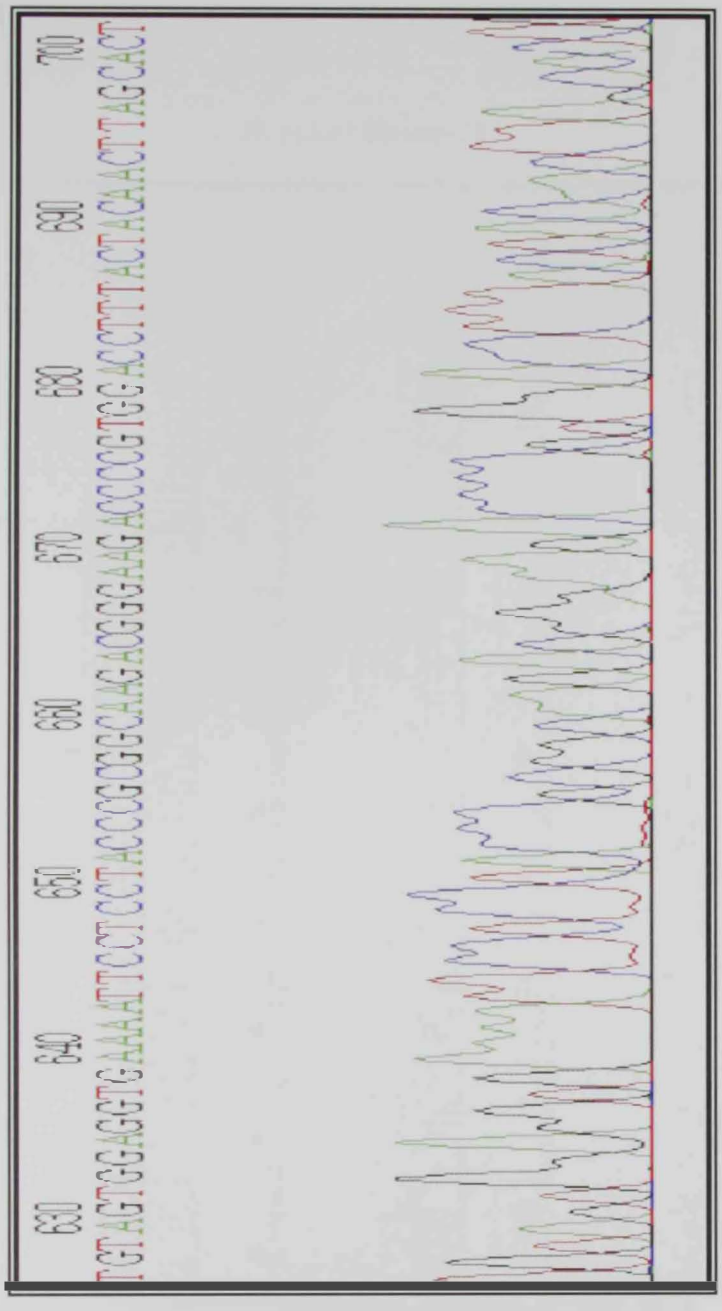
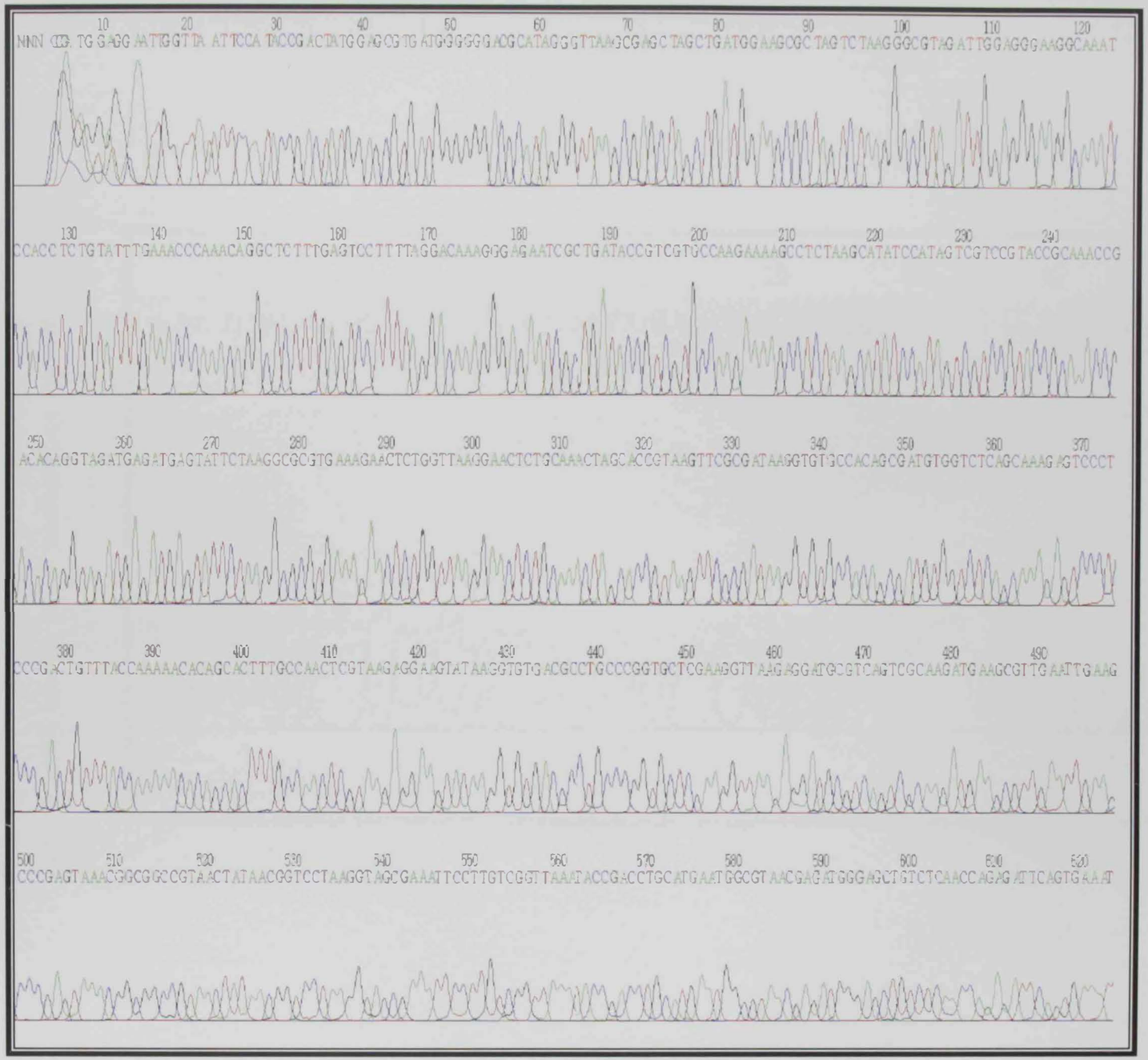


Fig. 9: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-9*



H. pylori Hpuure-10

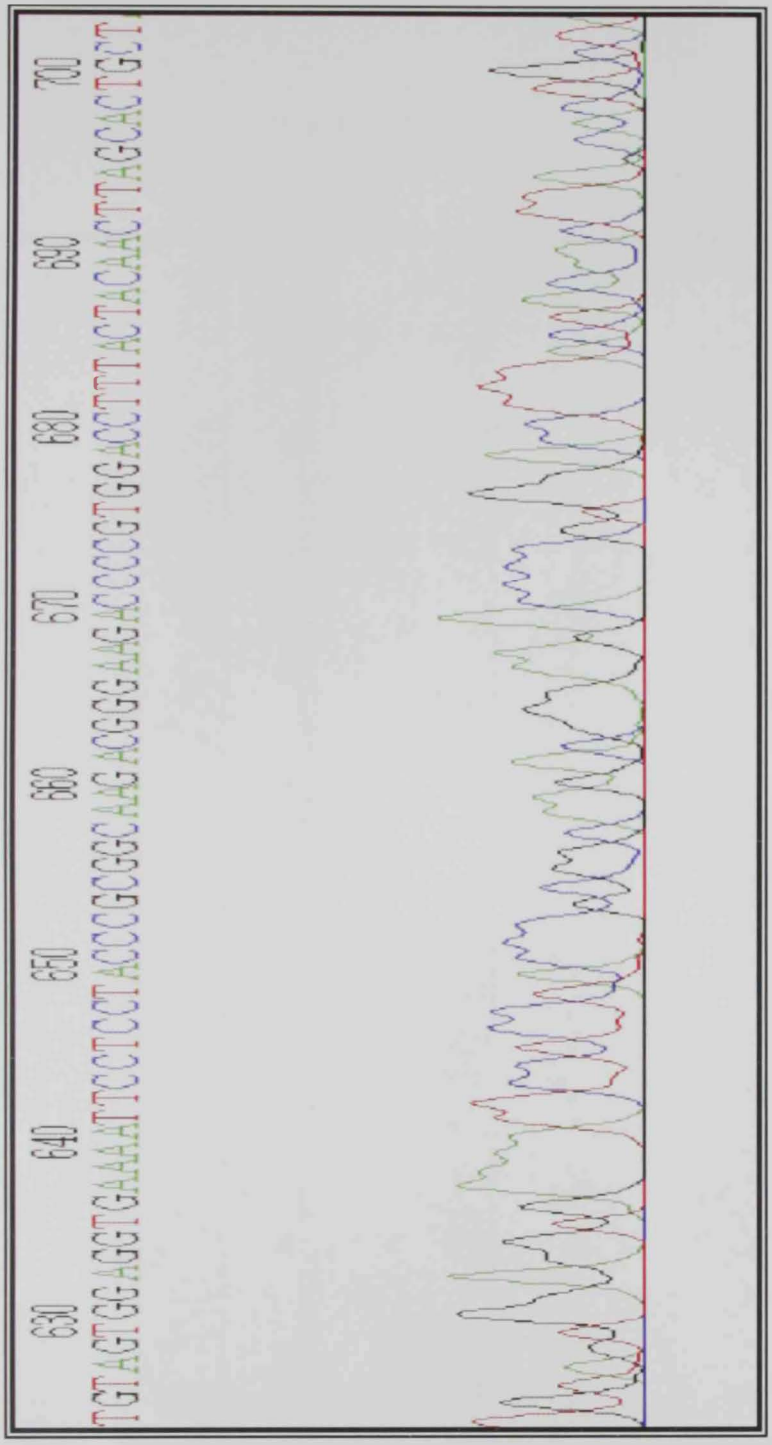
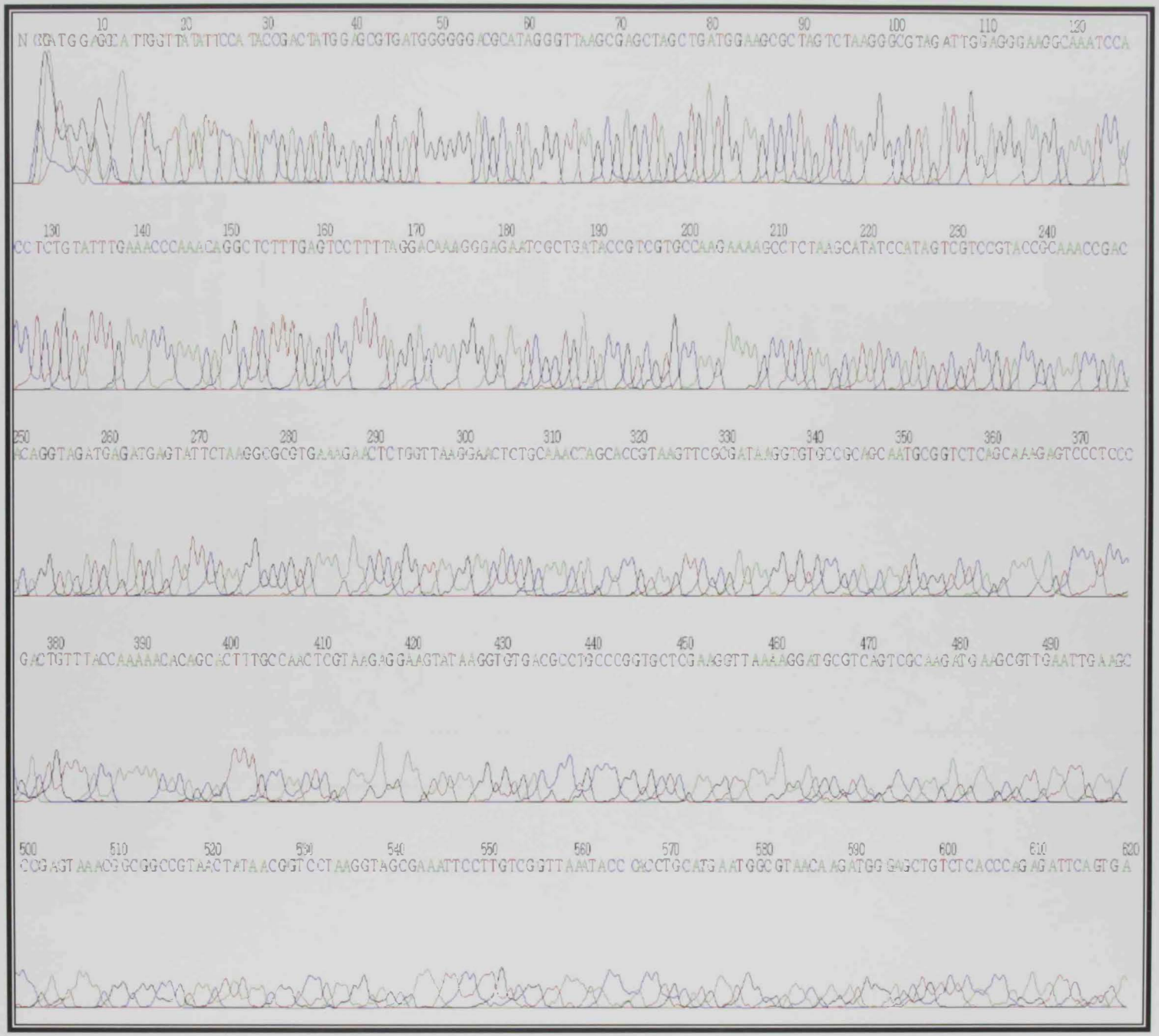


Fig. 10: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-10*



H. pylori Hpuae-11

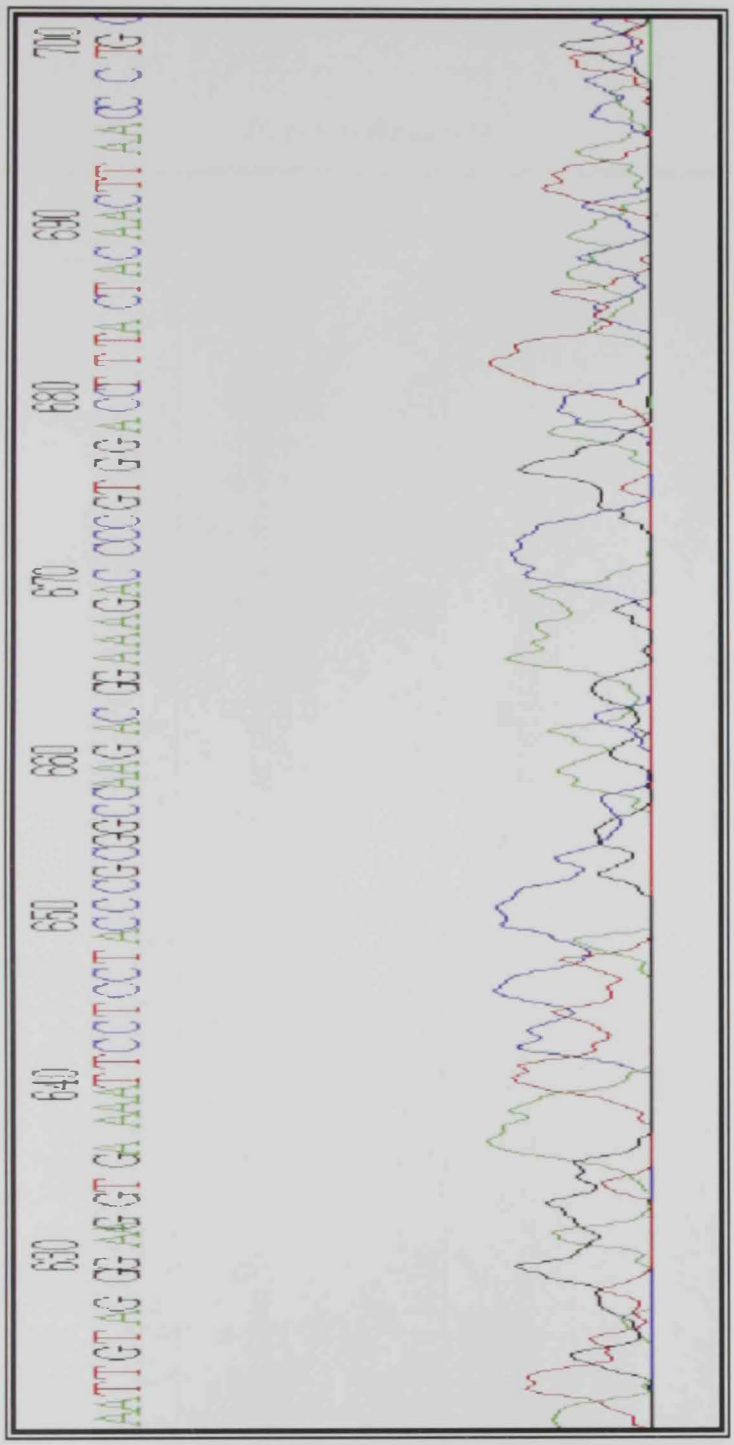
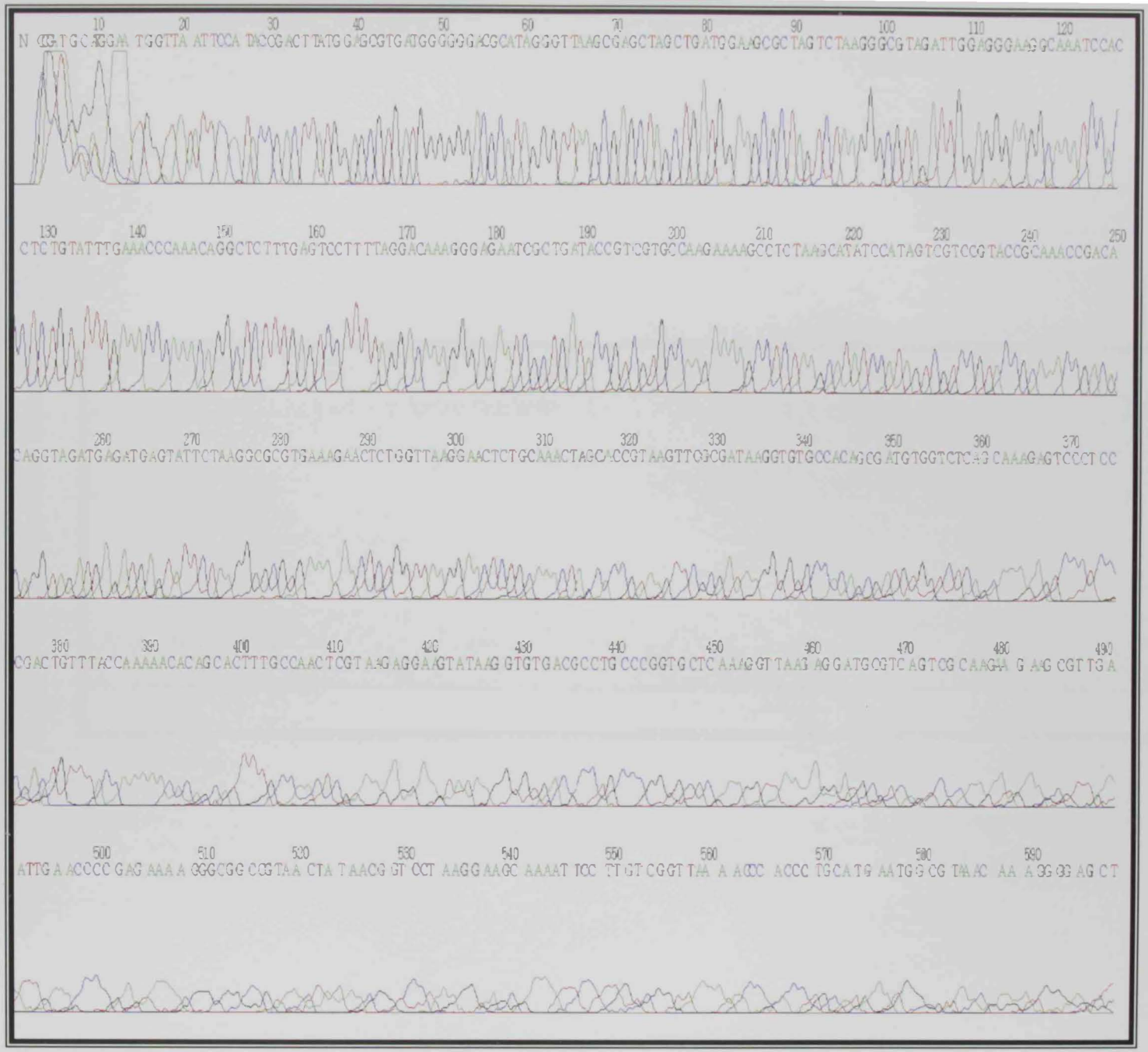


Fig. 11: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-11*



H. pylori Hpuae-12

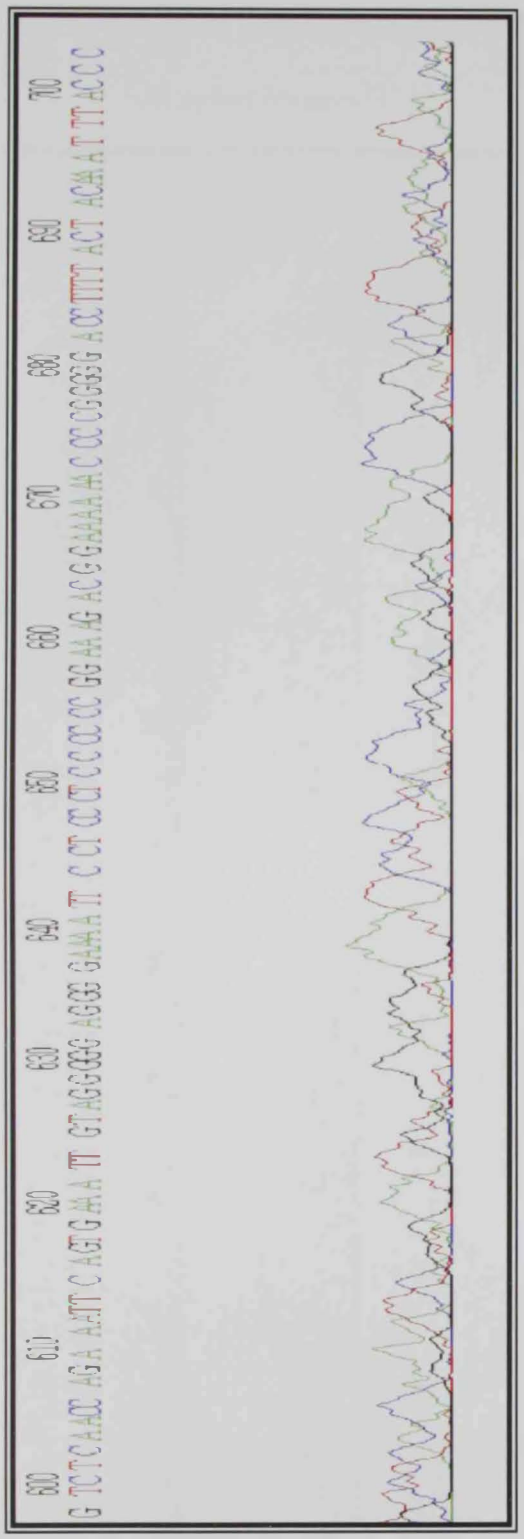
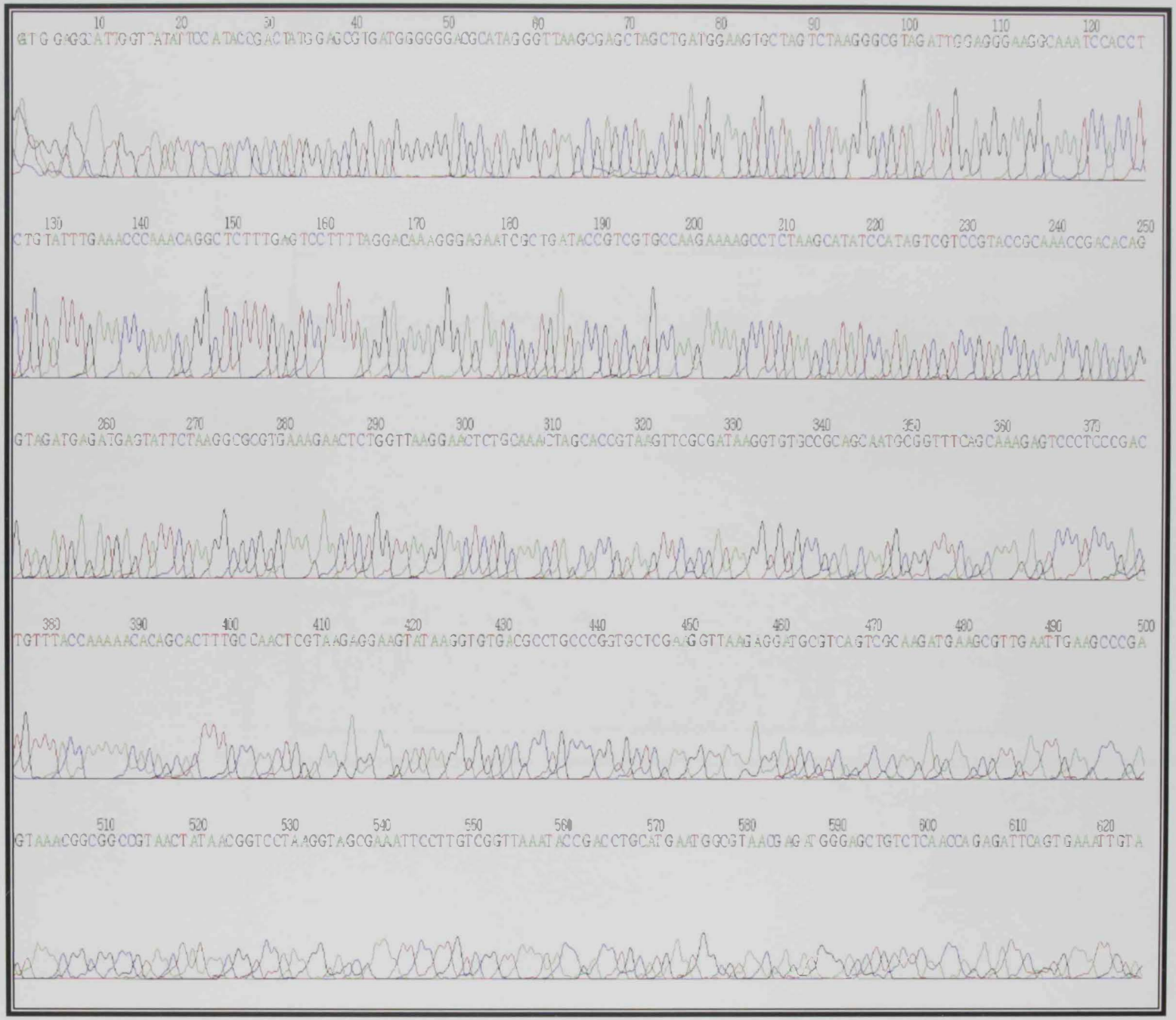


Fig. 12: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-12*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260 270 280 290 300 310 320 330 340 350 360 370
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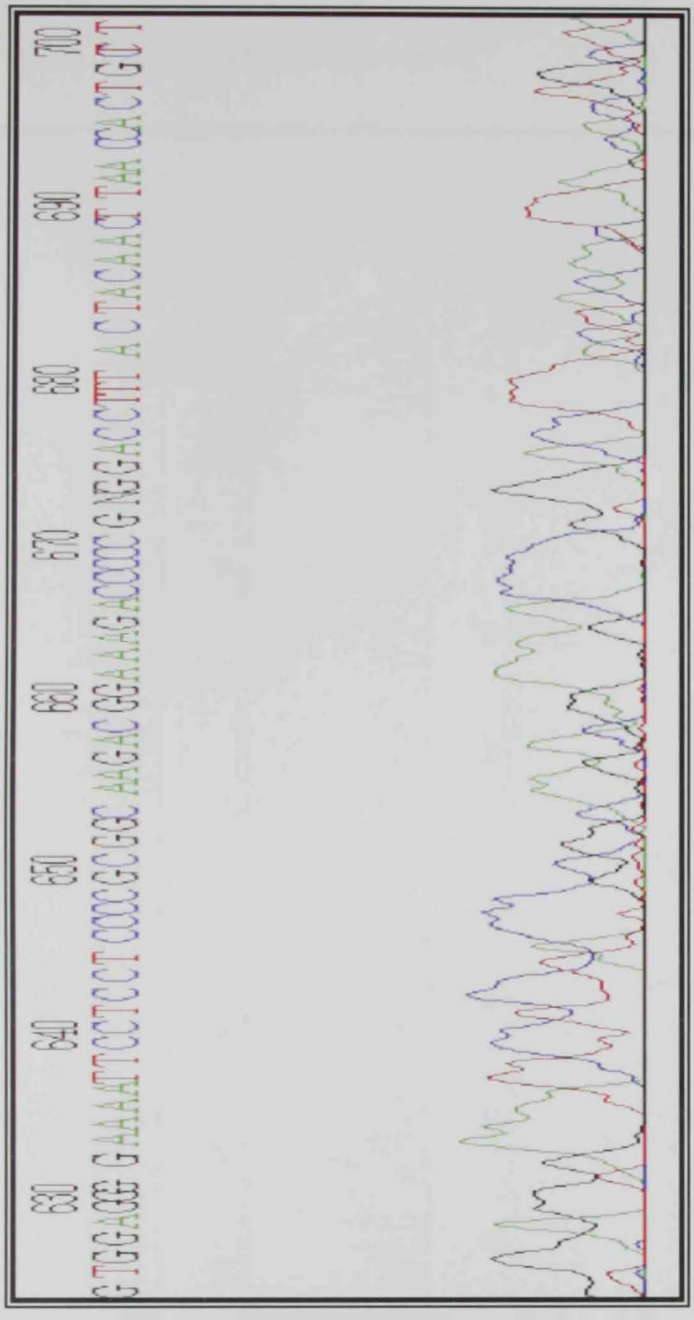
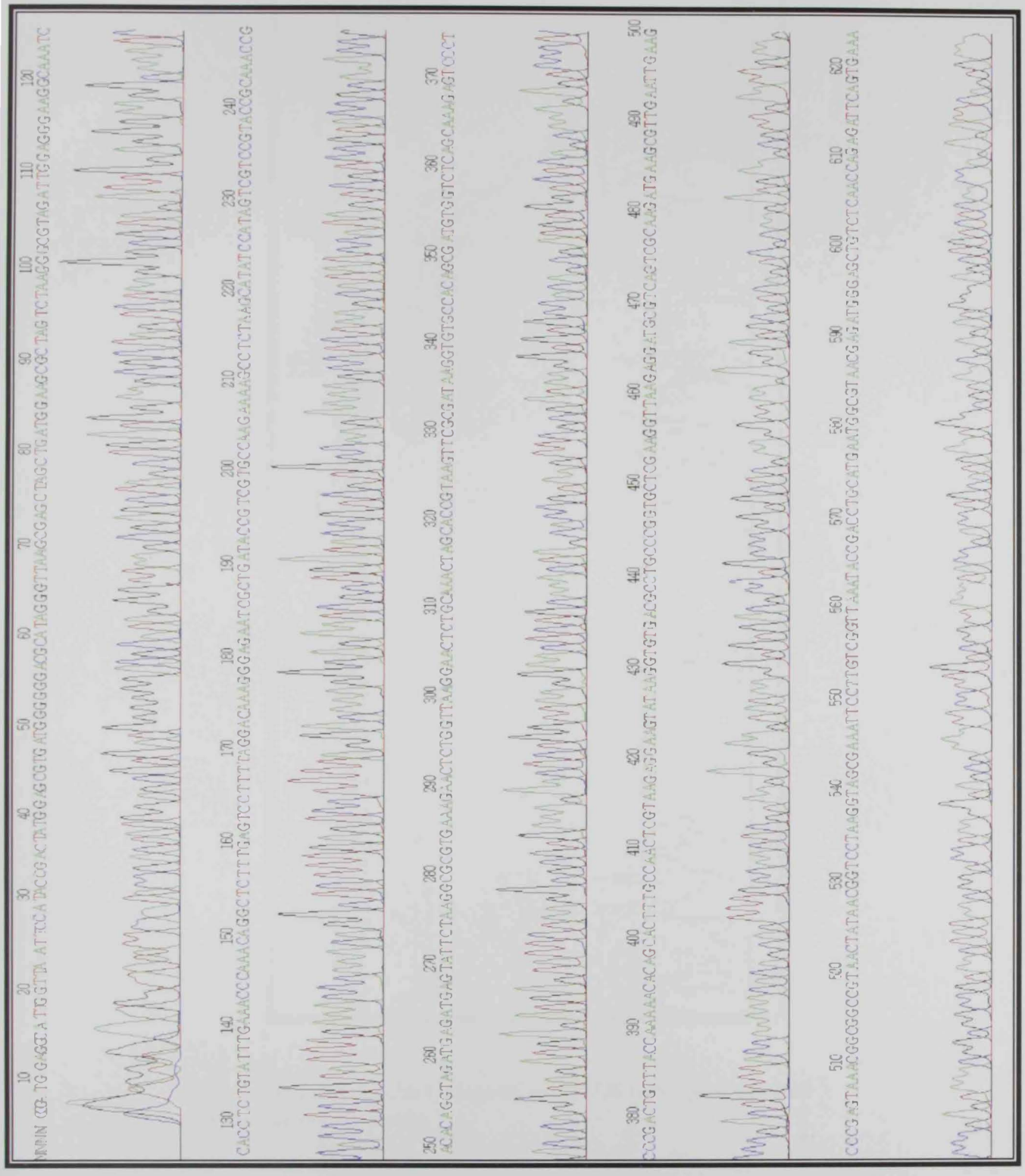


Fig. 13: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-13*

H. pylori Hpuae-14



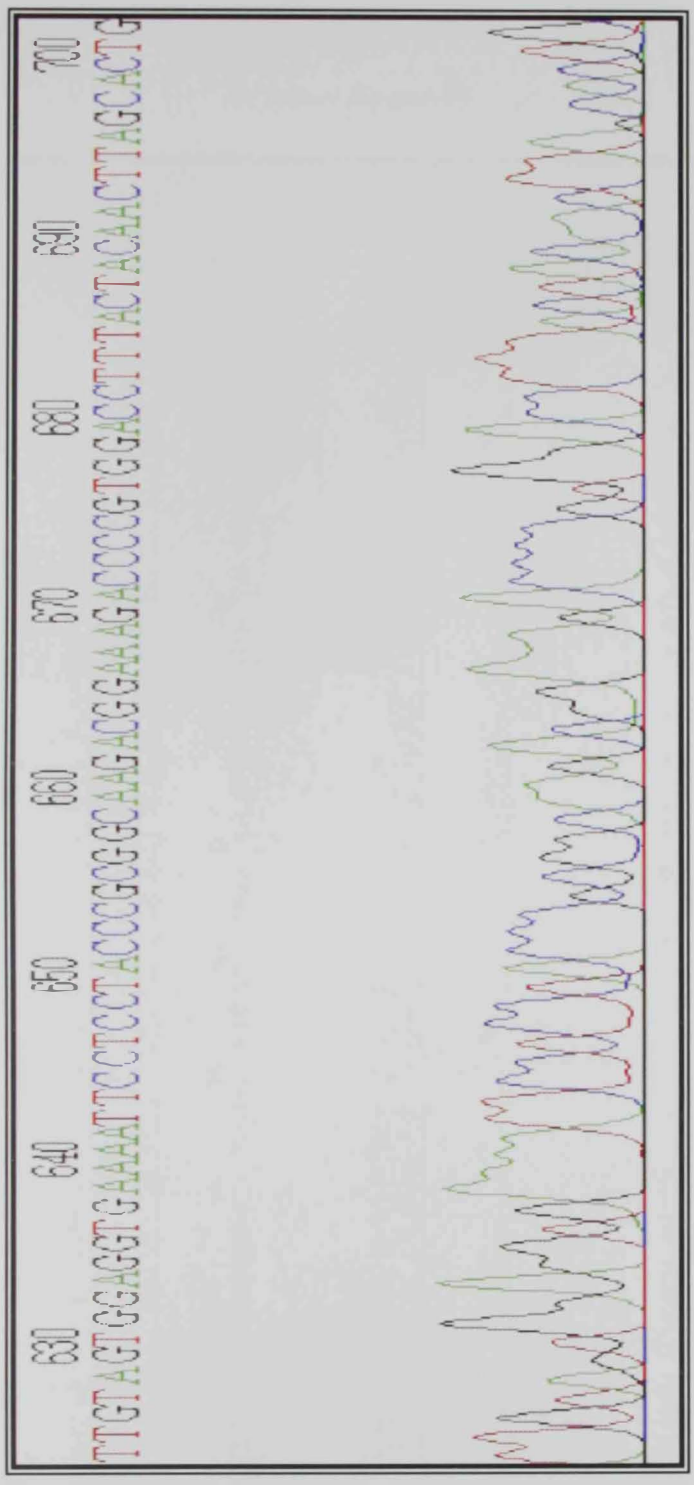
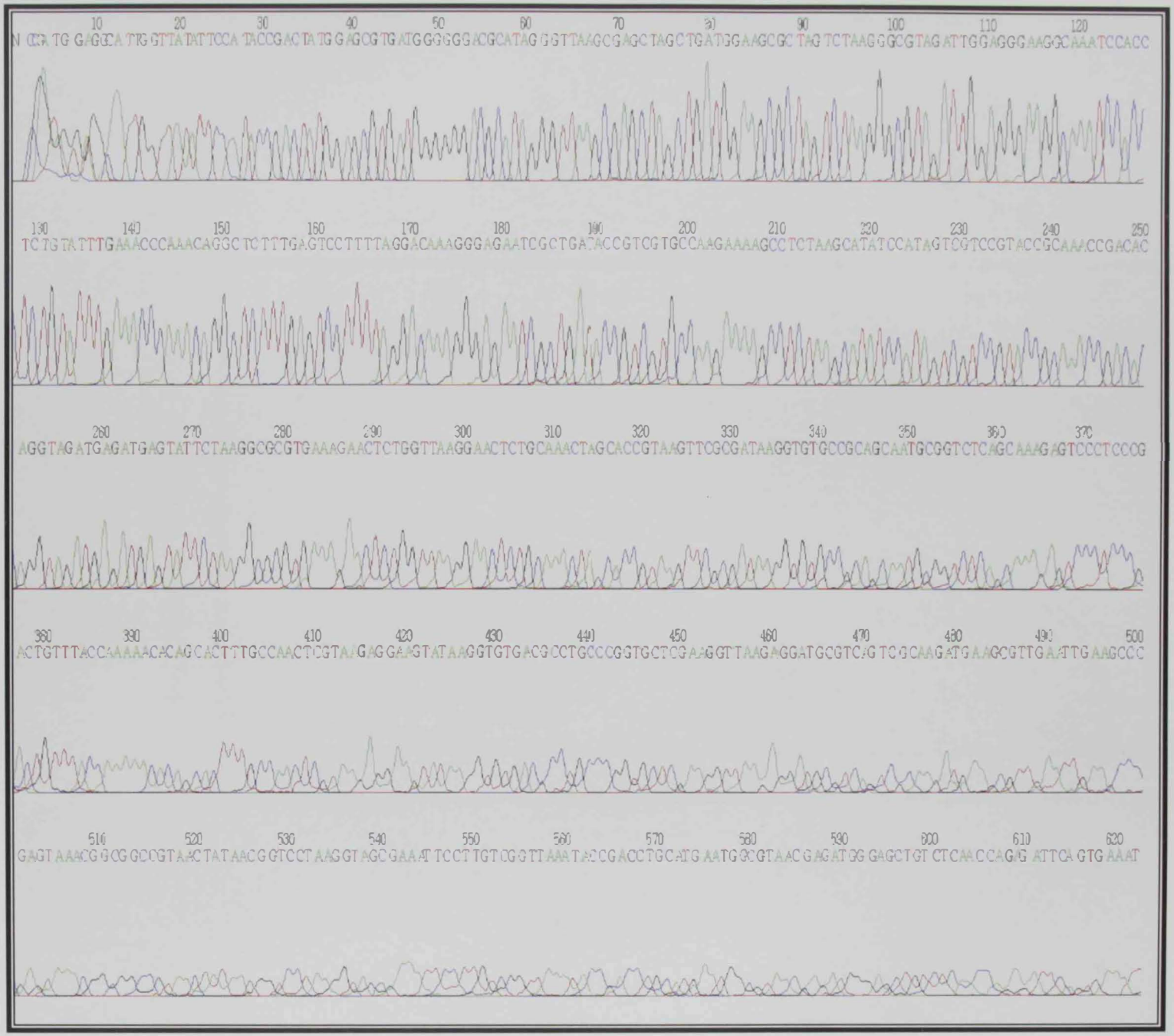


Fig. 14: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-14*



H. pylori Hpmae-15

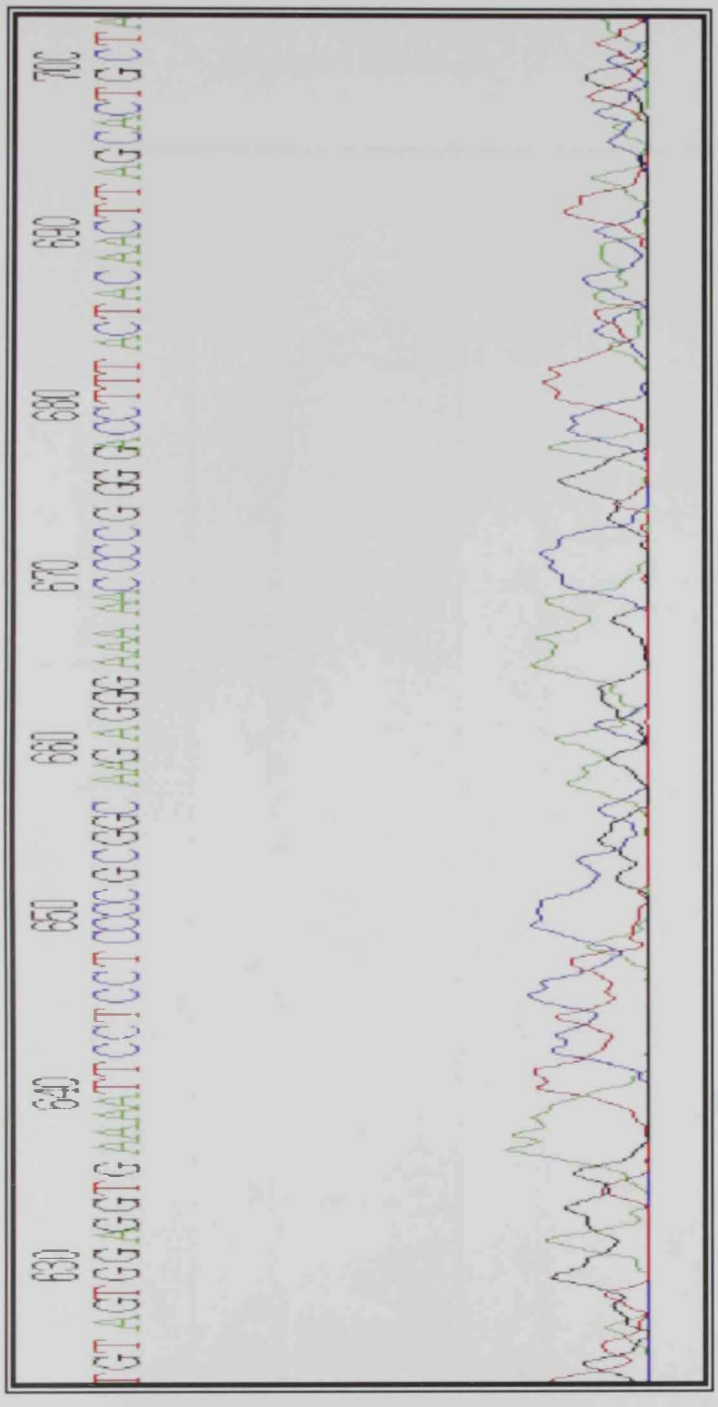
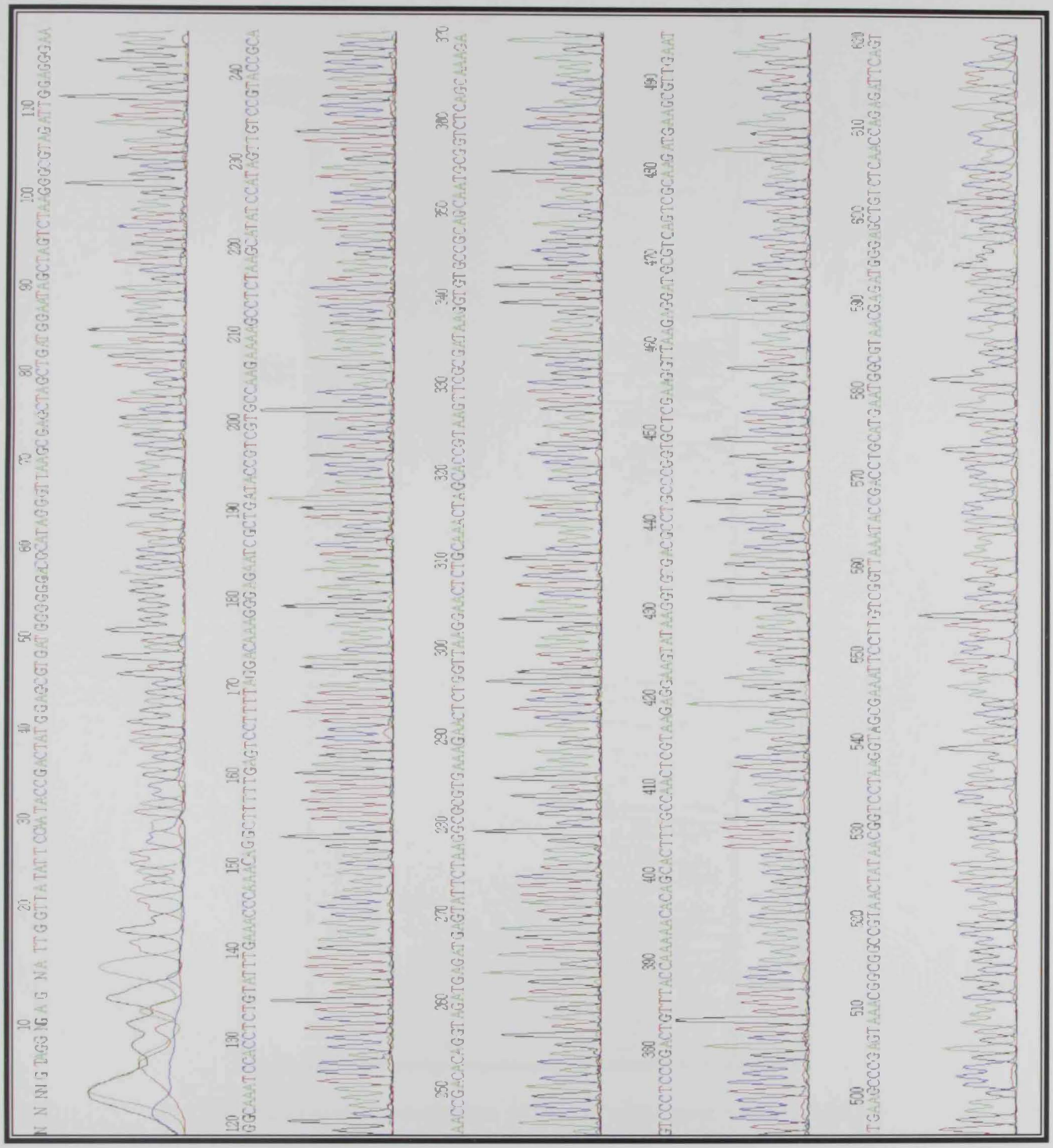


Fig. 15: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-15*

H. pylori Hpuae-16



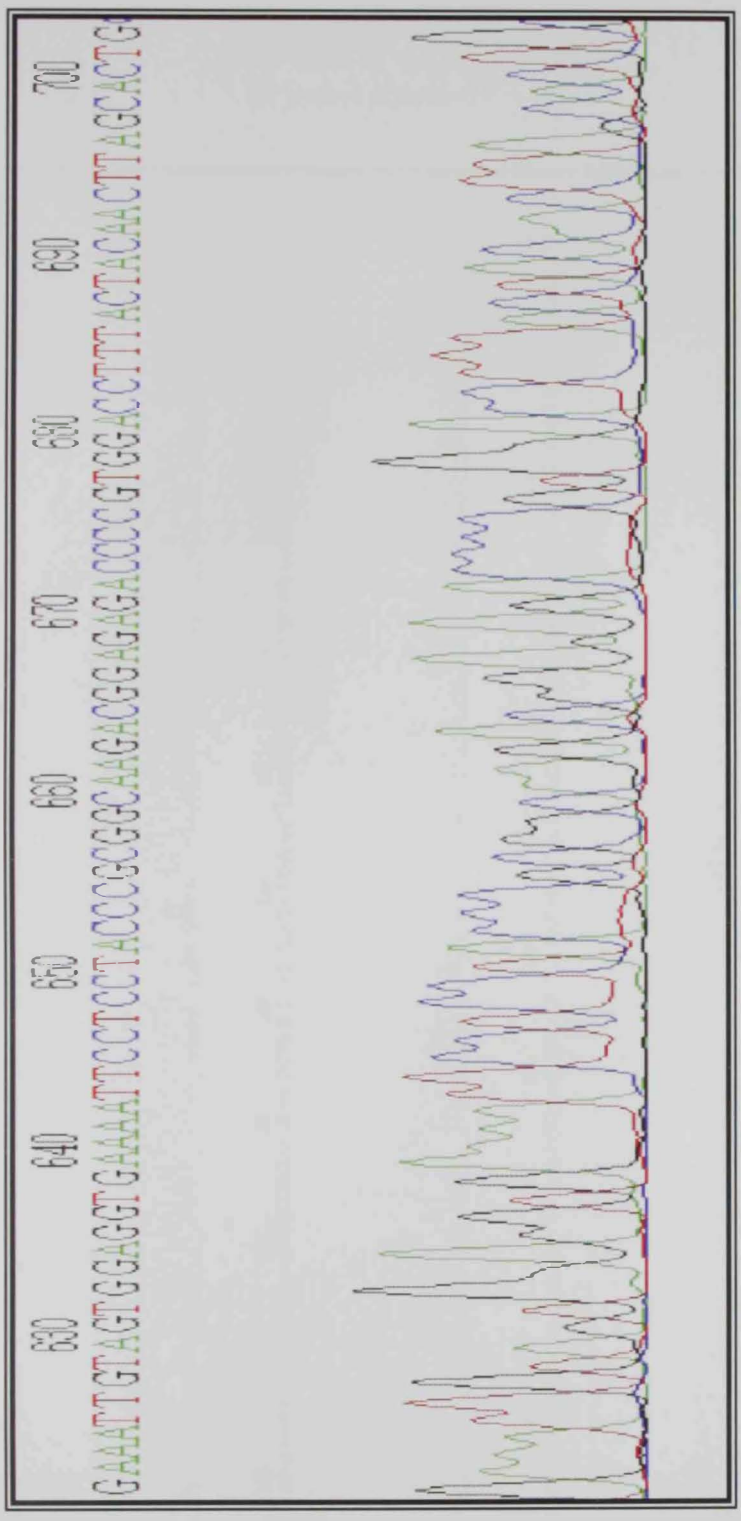
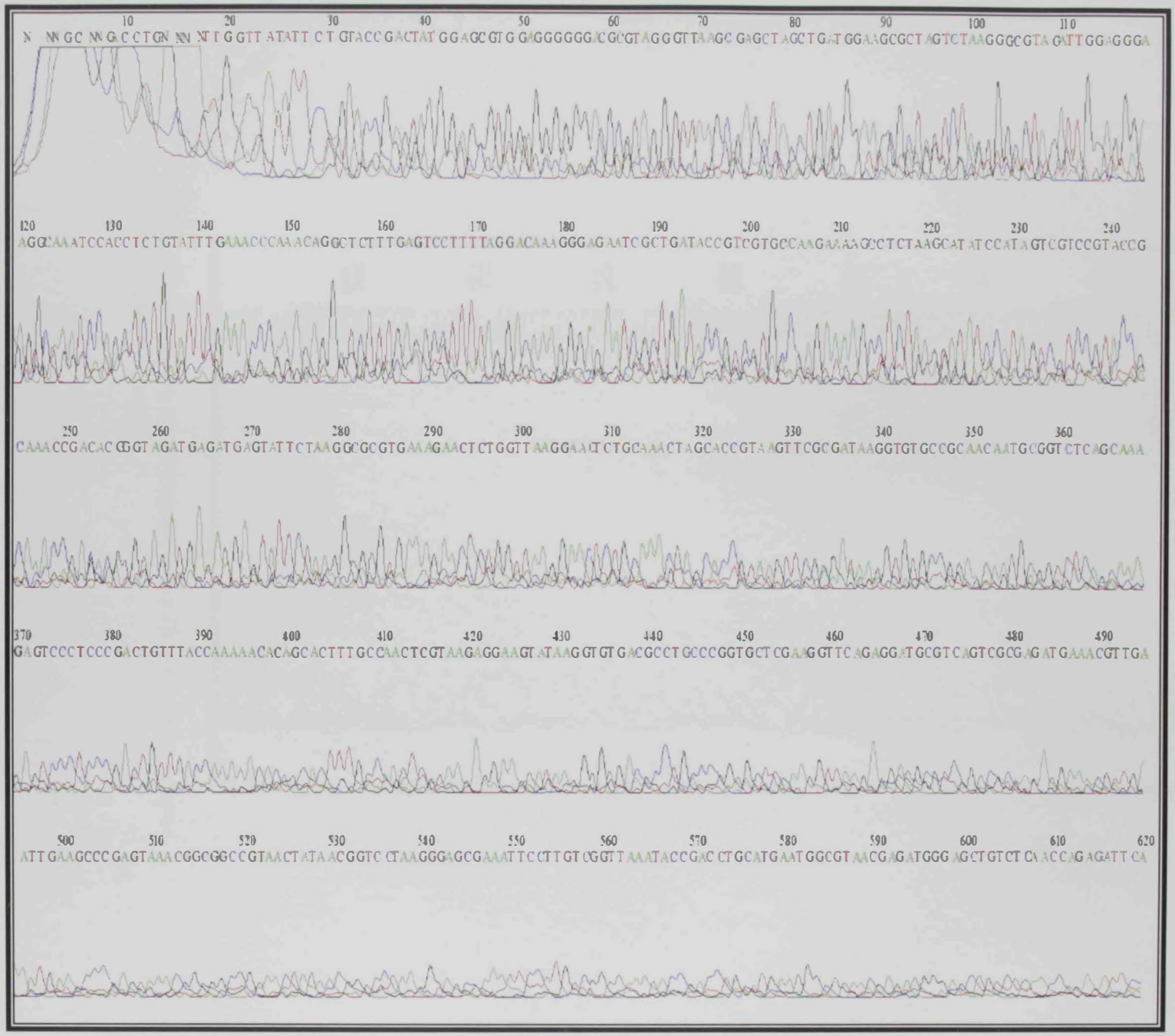


Fig. 16: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-16*



H. pylori Hpuae-17

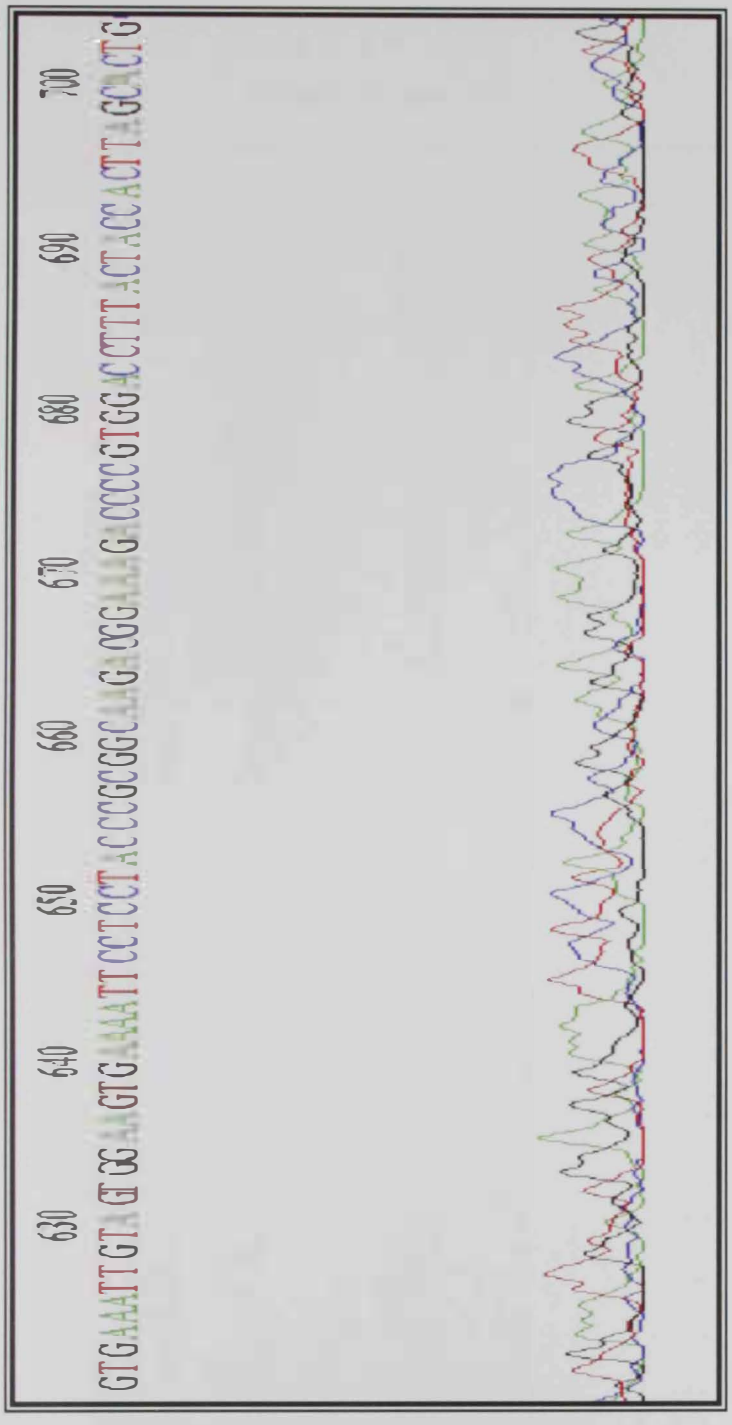
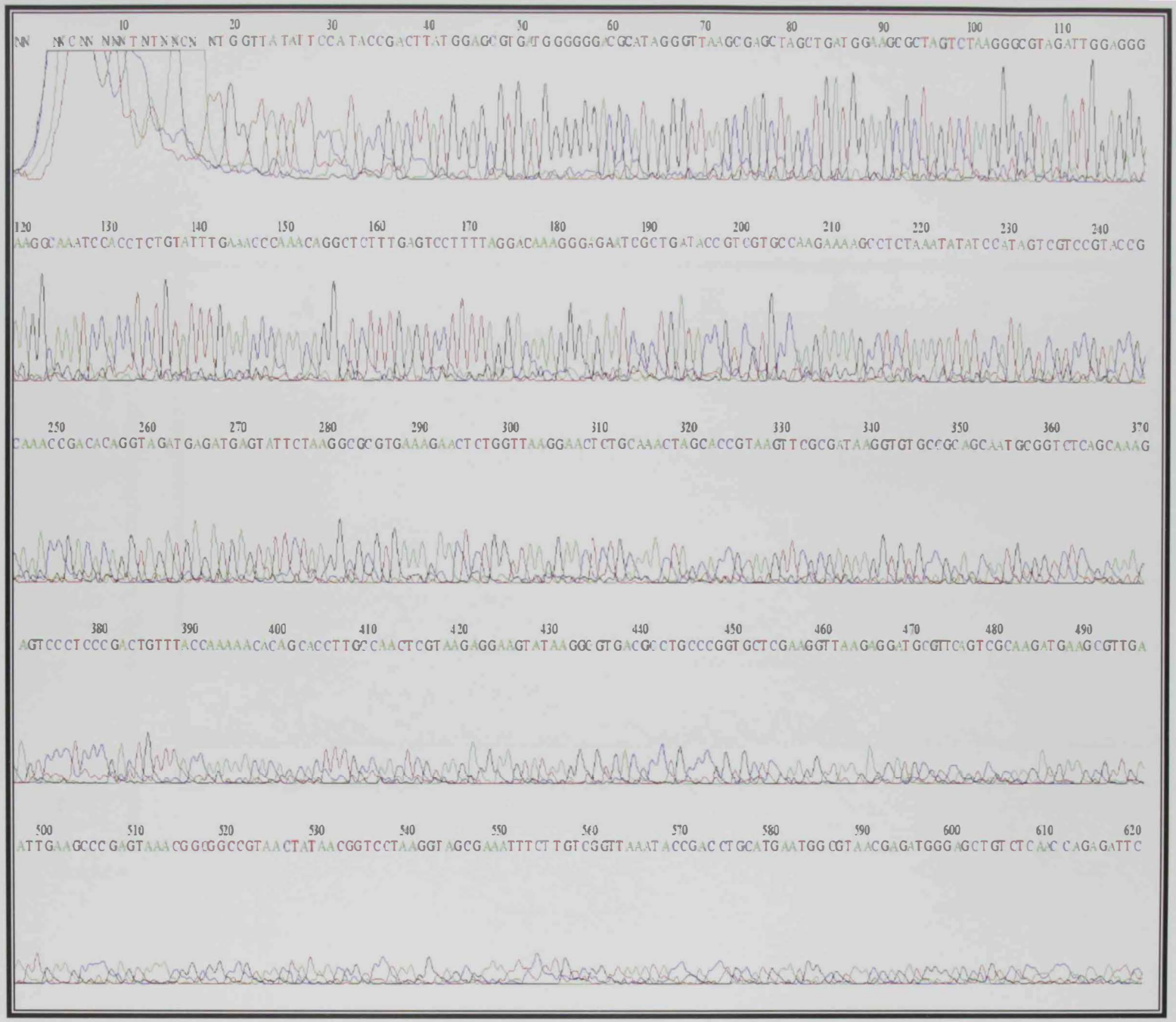


Fig. 17: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-17*



H. pylori Hpuac-18

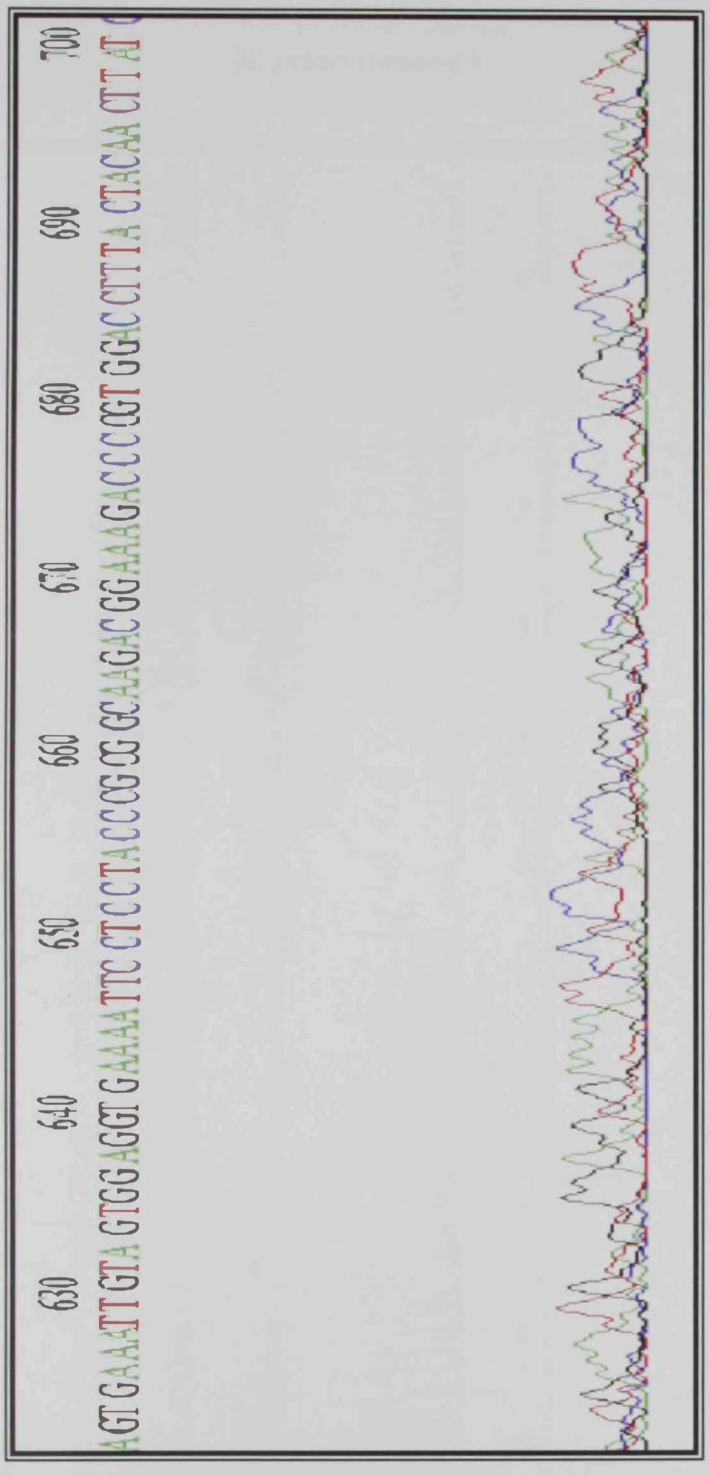
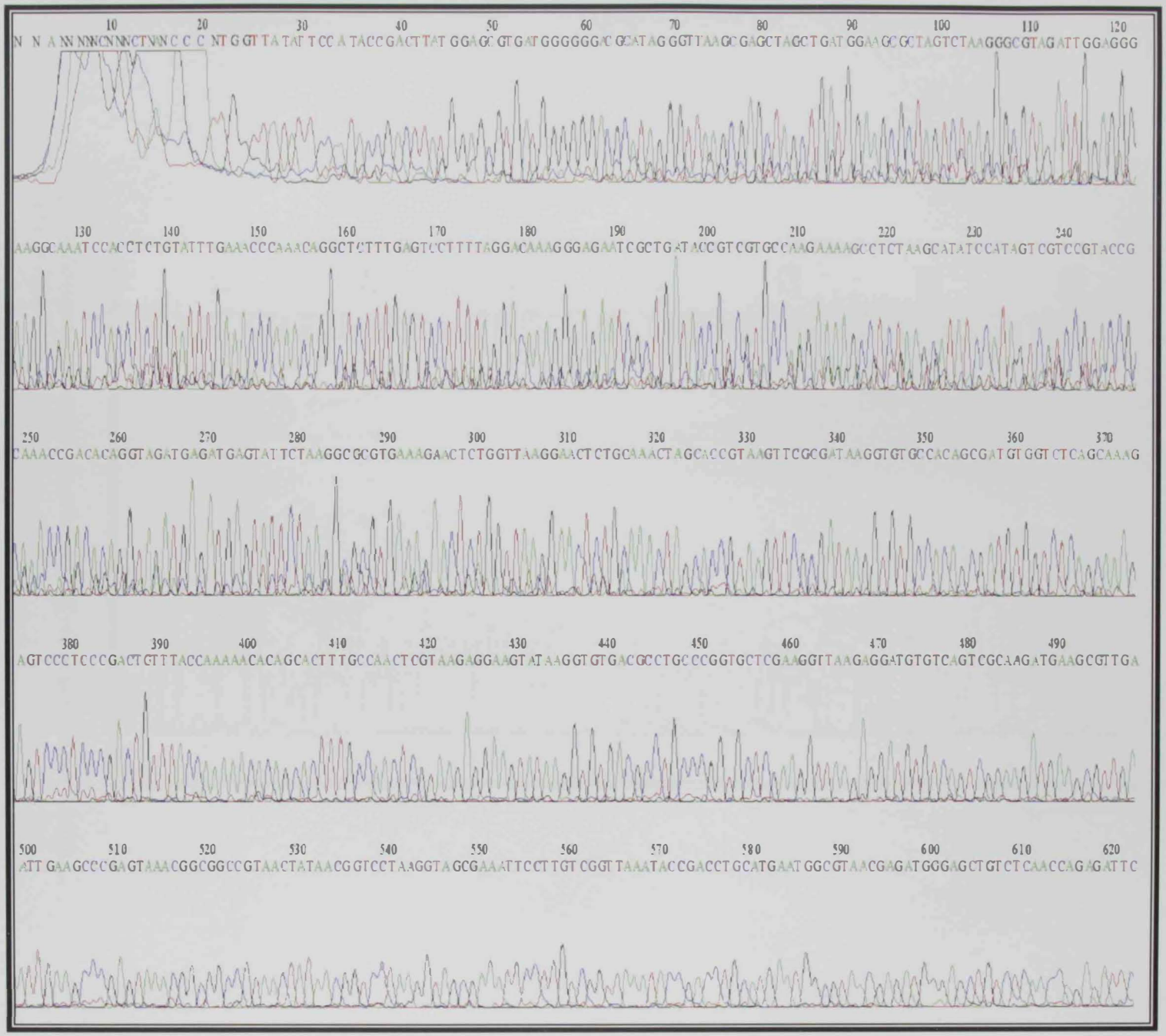


Fig. 18: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-18*



H. pylori Hpuae-19

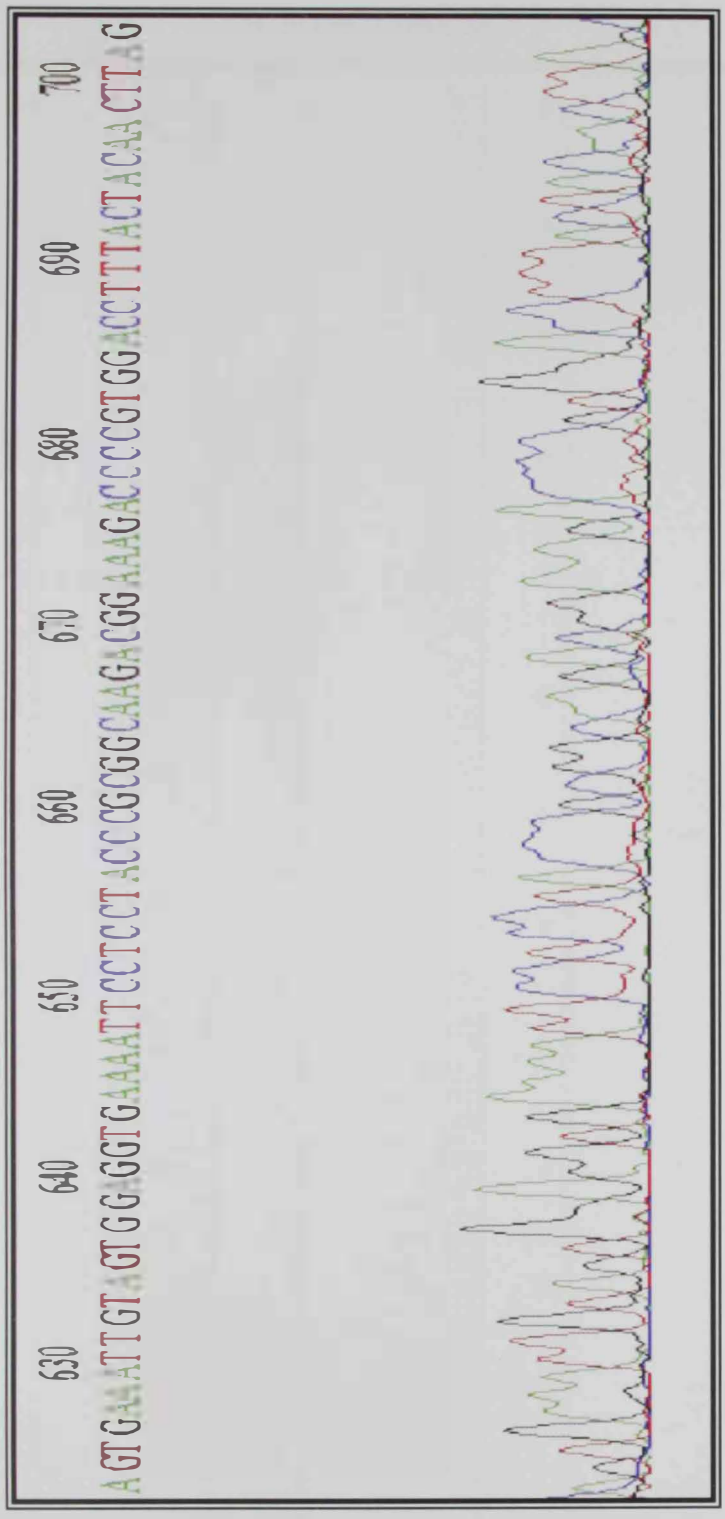
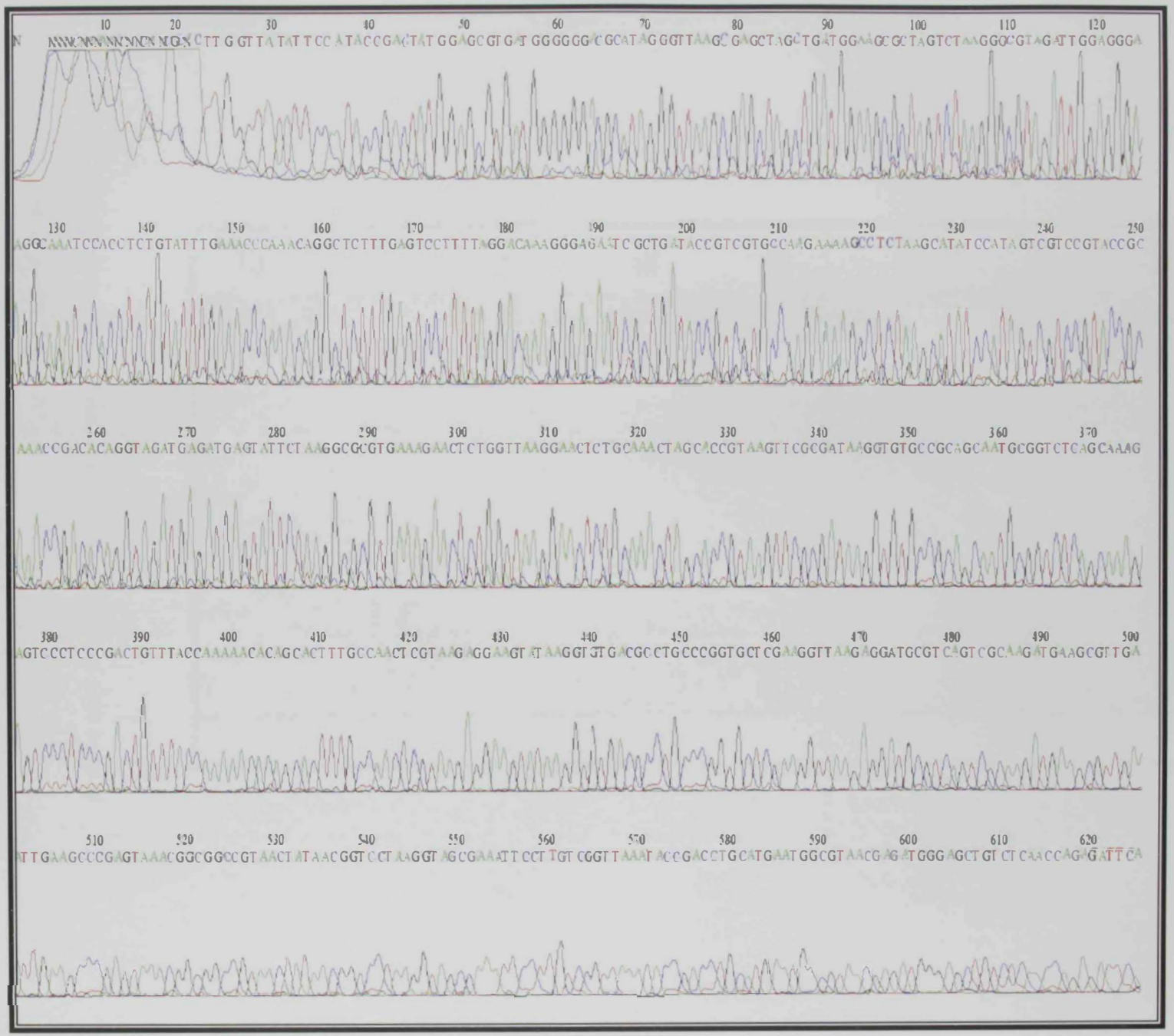


Fig. 19: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-19*



H. pylori Hp uae-20

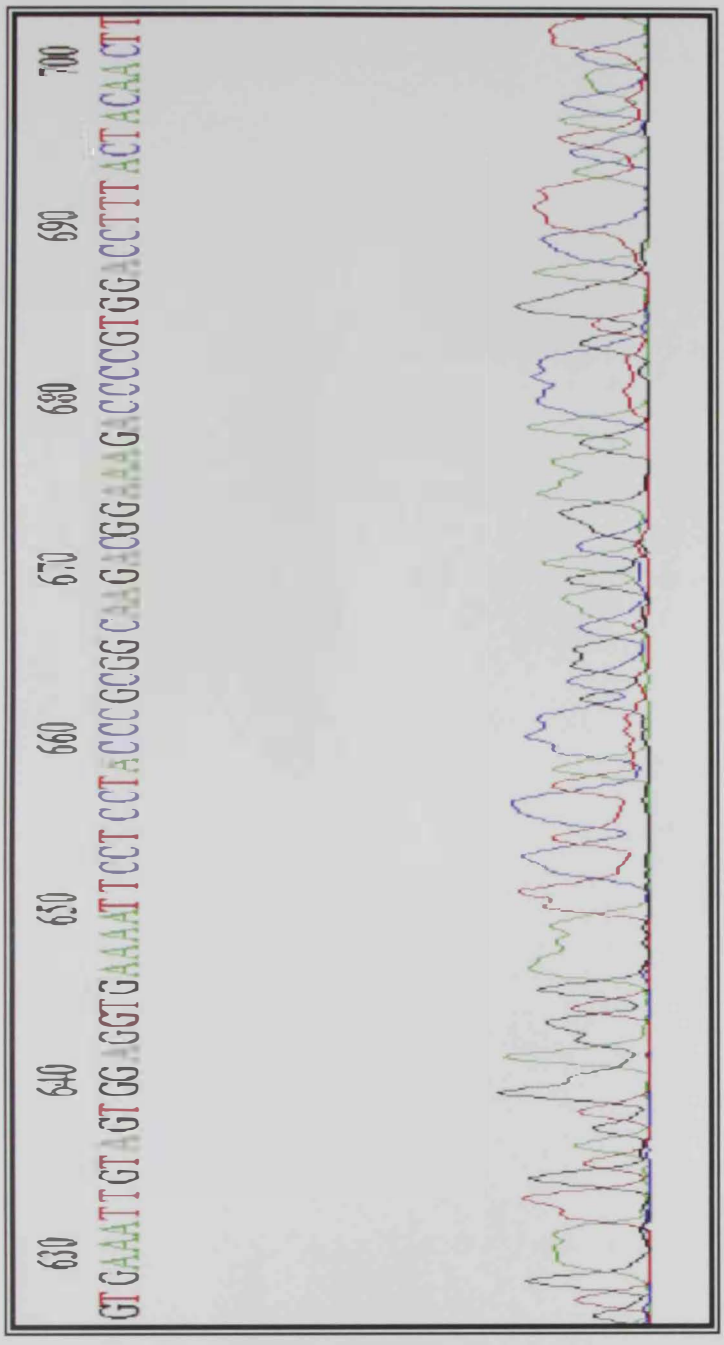


Fig. 20: Sequencing chromatogram of the *23S rRNA* gene isolated form *H. pylori Hpuae-20*

الملخص

جرثومة المعدة *هيليكوباكتر بيلوري* هي عبارة عن بكتيريا حلزونية لولبية الشكل، بطيئة النمو، و محبة للوسط المتعادل. تمتلك هذه البكتيريا انزيم يعرف باليوريز ووظيفته هو تحطيم مادة اليوريا و تحويلها إلى مادة الأمونيا وثاني أوكسيد الكربون (CO_2)، فبتحطيم هذه البكتيريا لمادة اليوريا تكون قد شكلت طبقة قاعدية واقية حولها.

وتعتبر هذه البكتيريا التي تعيش في بطانة المعدة من أشد أنواع البكتيريا مقاومة لنظام المناعة الجسدي و مقاومة أيضا للمضادات الحيوية. و تظهر أعراض المرض الناشئ عن هذه البكتيريا بنسبة قليلة في بداية العدوى، إلا أن التقدم البطيء للمرض يؤدي إلى تحويل الالتهاب إلى قرحة مزمنة و من ثم قد يتحول إلى سرطان المعدة.

و تكمن سر مقاومة البكتيريا *هيليكوباكتر بايلوري* للعلاج هو أن هذه البكتيريا تشل نظام المناعة «المحلي» في المعدة حيث تطلق هذه البكتيريا العنيدة نوعا من السموم الكفيلة بوقف آلية تحفيز الخلايا المناعية حيث يعد السم البروتيني *CagA* من أخطر هذه السموم المفرزة. و من المعروف ان هذا النوع من البكتيريا الحامل للجين المسؤول عن انتاج السم البروتيني *Cag A* هو أكثر عدائية والتهابية من بقية أنواع البكتيريا *هيليكوبكتر بايلوري*.

كما كشفت العديد من الدراسات أن البكتيريا المحتوية على الجين المسؤول عن انتاج السم البروتيني *CagA* هي أكثر مقامة للعلاج من بقية أنواع *هيليكوباكتر بايلوري*، فالبرغم من تقدم أنواع المضادات الحيوية لهذه البكتيريا وجد أن البكتيريا أكثر مقاومة من ذي قبل.

خلال هذه الدراسة تم البحث عن وجود البكتيريا الحلزونية *هيليكوباكتر بايلوري* في معدة المرضى في دولة الإمارات العربية المتحدة.

أما المرحلة الثانية هدفت إلى تحديد مدى انتشار الجينات المقاومة للمضادات(طفرة في الجين المسمى ب *23S rRNA* المسؤول عن مقاومة البكتيريا للمضاد الحيوي كلاريثروميسن، و طفرة في الجين المسمى ب *rdxA* المسؤول عن مقاومة البكتيريا للمضاد الحيوي ميترونيدانول) الحيوية بين السلالات المعزولة من المرضى في دولة الإمارات عن طريق استخدام الأساليب الجزيئية (PCR and Sequencing) .

وفي المرحلة الثالثة تم التأكد مما إذا كان هناك ثمة أي ارتباط إيجابي بين السلالات المقاومة للمضادات الحيوية و بين وجود السم البروتيني *Cag A* المسؤول عن تطوير القرحة المعدية إلى سرطان معدة.

أخيرا تم البحث عن وجود أي سلالات جديدة لهذه البكتيريا في دولة الإمارات وذلك من خلال تحليل ذو علاقة بالتطور النوعي للمنطقة الوسطى للجين المسمى بـ *23S rRNA* .

و جاءت نتائج البحث كالتالي:

- تم العثور على البكتيريا الحلزونية *هيليكوباكتر بيلوري* في 26 عينة من أصل 90 عينة باستخدام اختبار PCR في حين أن 22 فقط من هذه البكتيريا تم العثور عليها عندما تم الاختبار بواسطة CLO.
- وجد أن 9 (34.6%) من السلالة المعزولة من المرضى في الإمارات جاءت مقاومة للمضاد الحيوي كلاريثروميسن بينما 3 (11.5%) وجدت مقاومة للمضاد الآخر المعروف بميترونيدازول.
- من خلال هذه الدراسة تم التوصل إلى أن البكتيريا المقاومة للمضاد الحيوي ميترونيدازول مرتبطة ارتباط قوي بالجين المسؤول عن انتاج السم البروتيني *Cag A* في حين لم توجد هذه العلاقة بالنسبة للمضاد الحيوي كلاريثروميسن.
- من دراسة التطور النوعي للمنطقة الوسطى للجين *23S rRNA* من خلال تحليل تسلسل الحمض النووي تم العثور على سلالات فريدة في الإمارات مختلفة عن غيرها من السلالات المنشورة في قاعدة البيانات NCBI وسميت هذه السلالة الجديدة بـــ *هيليكوباكتر بيلوري الإمارات* "*H. pylori uae*" .

جامعة الإمارات العربية المتحدة
عمادة الدراسات العليا
برنامج ماجستير علوم البيئة

الكشف عن الجينات المقاومة للمضادات الحيوية في البكتيريا الحلزونية *هيليكوباكتر بيلوري* و علاقة هذه الجينات بالجين المسبب لمرض سرطان المعدة في دولة الإمارات

رسالة مقدمة من/
وفاء علي راشد الطيار

مقدمة إلى/
جامعة الإمارات العربية المتحدة
استكمالاً لمتطلبات الحصول على درجة الماجستير في علوم البيئة

2009 - 2008