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PCR TEST FOR *HELICOBACTER PYLORI* DETECTION AND CLARITHROMYCIN RESISTANCE PREDICTION ON FECAL SAMPLES: PREDICTION POWER AND CORRELATION WITH ERADICATION RATE.

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Abstract

Background: *Clarithromycin-based regimens are commonly used as a first line therapy in Helicobacter pylori positive patients, however treatment failures associate with resistance to clarithromycin are increasing worldwide.*

Objectives*: to evaluate the use of stool samples to detect the presence of Helicobacter pylori DNA, while concurrently detecting mutations associated with the clarithromycin resistance.*

Methods*: 292 Helicobacter pylori negative and positive patients were included. DNA was extracted from raw stools. TaqMan real-time PCR amplification was used to detect the presence of Helicobacter pylori as well as predict the genotype of the organism and the related outcome of patients treated with clarithromycin. Patients were also tested by Urea breath test and subjected to esophagogastroduodenoscopy followed by histology, culture and rapid urease test, to obtain a consensus patient infection status.*

Results*: Out of 292 total stool samples, 228 were deemed true positives. The sensitivity and specificity for Helicobacter pylori detection by PCR was 92.8% and 77.6% respectively.* Out of 239 samlples that resulted Hp positive with PCR, 213 were also sequenced*, 36% showed point mutations associated with CLA resistance (A2142C, A2142G, A2143G). The final correlation of genotype and eradication was 83.5%.*

Conclusions*: Helicobacter pylori DNA can be detected in human stool specimens with a high sensitivity, and therefore can be used to determine its presence without obtaining biopsy sample. Moreover, the genotypic resistance to clarithromycin can be detected without obtaining a biopsy sample in order to choose the right therapeutic approach.*

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Chapter 1- Introduction

1.1 Helicobacter pylori infection epidemiology and colonization

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that has colonized the human stomach for 58.000 years. During this period it has developed highly specific and sensitive mechanisms of adaptation to survive in the acid environment of the stomach that is usually inhospitable for other bacteria (1). *H. pylori* infects over 50% of the world population and it has been demonstrated that the predominant composition of its molecular structure is different among different geographic areas and that its distribution follows known human migration patterns (2). Currently there are two main epidemiological patterns and their distribution follows the level of economic and social development of each country. In more developed countries (e.g. Western Countries) there is an increasing prevalence of the infection with age ranging from 5% in the first decade to 50-60% in the sixth decade. Less developed countries (e.g. Asia and Africa) show a high prevalence of the infection already present in the first decade (50-60%) and only a little increase with age (3). The prevalence of the infection also varies with a low prevalence (30%) in industrialized countries compared to high prevalence in less industrialized countries (80-90%) (4). The infection is acquired during childhood but the route of transmission is still unclear. The age of acquisition depends on the prevalence of the infection in the country considered and relates to social conditions and public health structure. Children from developed countries show a higher age of seroconversion (when the infection becomes visible in blood samples) and the acquisition rate is lower than in developing countries. These differences in age of acquisition relates to different social and environmental conditions such as low standard of hygiene, overcrowding and promiscuity. The same risk factors are also related to the probability of reinfection which is higher in developing countries than in developed countries (70% vs 1-2% respectively).

Despite multiple attempts to find the route of transmission of this infection, the exact mechanism is still unknown. Humans, animals and environment are all possible responsible for *H. pylori* transmission. The first route, from human to human, is generally recognised as the most important source of infection and it is believed that the organism is acquired in childhood from family members. In countries with a poor public hygiene a role could be played by the environment (contaminated water or food) or animals, although there is a very little evidence that supports the latter (5).

1.2 Helicobacter pylori infection and metabolism

H. pylori enters the body via the mouth. There are many factors that can limit bacteria colonization in the stomach such as acidity, host innate and adaptive immunity, peristalsis and nutrient availability. *H. pylori* has developed numerous ways to deal with these problems and survive in an hostile environment.

Once in the stomach the bacterium, due to its particular shape and motility, is able to move toward higher pH zones (less acid) and bind to receptors on the surface of gastric epithelial cells in order to penetrate the gastric mucosa. *H. pylori* is also able to bind the gastric cells due to the presence on his surface of lipopolysaccharides (LPS) called “adhesins”. Many adhesins have been identified in this process but the principal one is called BabA (blood group antigen-binding adhesin) and it is able to bind a specific group antigen (Lewis b blood group antigen) that is located on gastric tissue leading to a strong adhesion of the pathogen to the gastric mucosa. This molecule has a structure that mimics that of some host antigen and therefore it can escape the control of the immune system (6). The adhesion of BabA to the gastric epithelium facilitates the secretion of intracellular proteins that are able to damage the gastric cells with an effect that is called “cytotoxic” leading to *H. pylori* survival. At the same time the pathogen produces a large amount of a particular enzyme called *urease* that is responsible of acid neutralization. This enzyme breaks down the urea in the stomach to carbon dioxide (CO_2) and ammonia. The latter, who is alkaline, contributes to neutralising the stomach acid, therefore the bacterium can survive and replicate. *H. pylori* is therefore able to live in a hostile environment such as the stomach and is also capable of reducing the host immune response by secreting several proteins and to compete with other bacteria with a complex system that facilitates the uptake of nutrients (7).

All these mechanisms explain how the pathogen is able to survive in the gastric environment but they are not useful to understand the mechanisms of action that leads to many gastric pathologies that are related to it. In fact, its ability in determining gastric

lesions seems to be linked to the presence of a DNA fragment located in the inner part of the bacteria chromosome called Cag PAI (cytotoxin associated pathogenicity island). One of the gene that is present in this “isle” is called CagA (cytotoxin associated gene A) and codifies for the homonymous protein cagA. It has been demonstrated that this protein has an important role in activating the host immune response. Subjects that are cagA positive develop a higher immune response and therefore are at higher risk of developing gastric pathologies (e.g. peptic ulcer or gastric adenocarcinoma). In association with cagA, *H. pylori* can express another protein that presents a high cell toxicity called “vacA” (vacuolizing-associated gene A). The expression of the cagA protein on the surface of *H. pylori* defines two different type of bacteria with different ability of creating damage in the stomach: *H. pylori* cagA positive and vacA positive (type I) that creates a bigger inflammation and is highly associated with peptic ulcer and colonises the gastric epithelium with high bacterial density. Whereas *H. pylori* cagA negative (type II) although it can express vacA on its surface, it is not toxigenic for gastric epithelium and it is less related to the presence of gastric pathologies (8).

The host response to the presence of the infection contributes to the damage of the gastric epithelium itself: it activates the immune cell response and the local releases of toxic substances (e.g. oxygen radicals) that contributes to the tissue damage.

1.3 Helicobacter pylori clinical features

Since its discovery in 1982, interest in the role of this bacterium in determining human pathologies has grown and it has been recognised as one of the key factor responsible for many gastric and extra-gastric pathologies. Stomach colonization is usually acquired during childhood and, if not treated, persists for the entire life. The route of transmission of the infection is unclear but some evidence has suggested that oral-oral, gastric-oral, and faecal-oral could be plausible ways of transmission (9).

In some individuals *H. pylori* colonises the stomach for many decades without consequences but, more often, it is responsible for pathological changes in the gastric mucosa that lead to several diseases including peptic pathologies (gastric and duodenal ulcer) and malignancies such as non-cardia gastric adenocarcinoma and gastric mucosal associated lymphoid tissue (MALT) lymphoma (10). The causal link between the infection and the occurrence of peptic ulcer disease is supported by the correlation of its eradication and the resolution of ulceration achieved after the treatment, which has been shown not only to heal ulcers but also to prevent their recurrence. The same close relationship can be seen between long term colonization by the pathogen and the development of gastric malignancies (especially MALT lymphoma) that, if detected at an early stage, can regress after *H. pylori* eradication (11). While these relationships are well established, there is still an ongoing debate about the role of the pathogen and the real benefit that is achieved after its eradication in other clinical conditions such as esophageal reflux disease and non-ulcer dyspepsia. However, it is now clear that the role of *H. pylori* as a causal agent of many diseases is not limited to gastric pathologies but can also be related to infection and respiratory disorders such as Chronic Obstructive Pulmonary Disease (COPD), asthma, cardiovascular diseases, immune-mediated allergic disorders, hematological and endocrine disorders (9).

1.3.1 Gastric disorders

1.3.1.1 Peptic ulcer disease

The first association with *H. pylori* and gastric pathologies was discovered in 1983, then the pathogen was recognized as the causal agent of gastritis and ulceration (12). Peptic ulcer is a lesion of the gastrointestinal lining (mucosa) that can be localized in the stomach (gastric ulcer) or in the duodenum (duodenal ulcer). Several factors have been demonstrated to be linked to the development of these diseases: smoking habits, genetic predisposition and gender (males seem to be more susceptible than females). Moreover, the role played by external factors such as the chronic administration of anti-inflammatory drugs (FANS) can increase the risk of developing peptic ulcers and the risk of bleeding in presence of pre-existing gastrointestinal lesions (13).

The leading mechanism in the development of an ulcer is the loss of balance between gastric protective factors (e.g. production of mucus) and elements that are able to attack the gastrointestinal mucosa (e.g. gastric acid). The presence of *H. pylori* can facilitate this disequilibrium and determine the occurrence of peptic ulcers. There are no specific symptoms that can be surely related to the presence of the pathogen, however epigastric pain or burning, bloating and dyspepsia (a feeling of slow digestion) are common in patients with non-complicated peptic ulcers. Sometimes subjects can develop a massive active bleed that can be seen both as hematemesis (the vomiting of blood) and/or melena (dark feces with digested blood).

However, not every person infected will develop clinical symptoms, only about 20% of patients with *H. pylori* infection will develop peptic ulcer disease while the rest of persons colonized by the pathogen will remain symptom free (9).

1.3.1.2 Gastric cancer

In 1994 the International Agency for Research on Cancer (IARC) classified *H. pylori* as a class I carcinogen, they admit that a long term colonization can be responsible of the development of gastric malignances such as adenocarcinoma and MALT lymphoma (14). The presence of *H. pylori* constitutes a chronic inflammatory stimulus on the gastric layer that lead to the development of an immune response that consists in small aggregates of inflammatory cells (lymphoid aggregates). Those immune aggregates progressively acquire an autonomous growth potential through the accumulation of genetic alterations leading to the development of tumor tissue. In some cases the presence of the infection and MALT lymphoma is associated with other haematological malignancies (e.g. diffuse large B-cell lymphoma) (15) suggesting that other neoplastic lesions can originate from a previous *H. pylori* infection following a prolonged contact. Moreover, recent studies demonstrates a correlation between *H. pylori* eradication and the a complete resolution of MALT lesions and diffuse large B-cell lymphoma with concomitant MALT lesions confirming a close relationship between the three and the importance to test and treat subjects with suspected or known gastric lymphoid pathology (16).

1.3.2 Extra-gastric disorders

Several attempts have been made to explore the relationship between *H. pylori* infection and extra-gastric disorders, however studies gave contradictory results so far (17). An example is given by the presence of conflicting data on the relationship between *H. pylori* and esophageal diseases. The uncertainties about the real effect of the pathogen derive from different effects that can be determined on gastric acid production related to its localization in the gastric mucosa and its effect on acid production. Studies suggested that the presence of *H. pylori*, the subsequent development of chronic changes in gastric mucosa (*gastric atrophy*) and the related reduced acid production are protective factors for the development of esophageal pathologies such as esophagitis and reflux disease.

Therefore its eradication and the consequent healing of the gastric mucosa are a primary cause of esophageal pathologies, whereas other studies showed that its eradication only reveals a pre-existing condition (18). In recent years several studies claimed a role for the pathogen in the development of clinical conditions of many different extra-gastric systems (e.g. respiratory, cardiovascular, immune and endocrine systems) however, there are still conflicting results on the real impact of the infection outside the gastric environment (9).

1.4 Helicobacter pylori genome

The *H. pylori* genome is about 1.60 Mbp long, constituted by numerous genes coding outer membrane proteines and putative adhesions, testifyng to the importance of adhesion in its biology. Many adhesins have been identified, but the principal is Bab A, which binds the Lewis b blood group antigen (Leb) expressed on gastric tissue. In addition to Bab A, also the Sialic acid-binding adhesin (Sab A) is important to adhesion because it bends sialyl-Lewis x blood group antigen (sialyl-Lex) which is expressed after chronic inflammation induced by *H. pylori.* The interaction between gastric sialyl-Le^x and SabA determines the colonization density of patients who do not express gastric Le^b or who express it weakly.

Other functions of *H. pylori* genome is to modulate the urease activity in order to obtain a correct modulation of the bacterial intracellular pH while transiently present in an acid environment. Urease is one of the most abundant proteins produced by *H. pylori*, comprising about 10-15% of the total protein content (9).

Another important area of the *H. pylori* genome is the Vac A gene, coding for the vaculating cytotoxin A protein. This is present in nearly all strains of *H. pylori* but only half of them secrete Vac A as a mature protein. As previously pointed out, Vac A is released by *H. pylori* as a free solubile toxin and is activated by exposure to acid. Vac A affects different host cells like gastric epithelial cells, macrophages neutrophilis, mast cells, T cell, and goblet cells. It interacts with several cellular compartiements resulting in cell apoptosis or necrosis, with the patophysiological effects depending upon cell type affected. Cag A also interferes with host cell signaling, leading to altered morphology, cell division or apoptosis. The presence of the Cag A denotes susceptibility to a more severe pathology, therefore *H. pylori* strains are classified into Type I (Cag PAI rich) and Type II (lacking Cag PAI) (9).

H. pylori has a high degree of genetic diversity, which has been attributed to horizontal transfer, high mutations rate and impaired DNA repair (19). The bacteria is capable to develop antibiotic resistance trough several mechanisms; one of them leads to amoxicillin (AMX) resistance by the expression of active efflux pumps that excrete drugs and point mutationsn the pbp1A gene that may contribute to the mechanisms of resistance to beta-lactams.

Several mutations have been detected also for Clarithromycin (CLA) resistance; it has been found that three point mutations—namely A2143G, A2142G and A2142C—are responsible for >90% of cases of primary CLA resistance in *H. pylori* strains isolated in Western countries (20). The *H. pylori* proteome suggests that all its evolution went through implementation of adhesive mechanisms (eg. BabA) and resistance mechanisms, and further investigations of his host-pathogen interaction will yeld other important informations on its bioechology.

1.5 Helicobacter pylori diagnosis

At least 50% of the world's human population has *H. pylori* infection (21). Whilst only a proportion of ulcer patients actually develop serious and costly complications, many are afflicted by recurrent episodes of ulcer relapse which results in ongoing demands on the health care system. Hence the potential economic burden of peptic ulceration is considerable, both in terms of the direct costs of providing medical care and in terms of the indirect costs resulting from lost productivity (22). Accurate diagnostic tests are thus important in order to set up targeted intervention (e.g. antibiotic therapy). *H. pylori* can be diagnosed by using invasive techniques such as endoscopy and biopsy for histology, culture and rapid urease test and non-invasive techniques like serology, ^{13}C-urea breath test (^{13}C-UBT) and stool antigen tests (SAT) (23). The choice of the right test should be based on the prevalence of the infection in the population, the presence of alarm symptoms, the "likelihood ratio" for a positive and a negative test and the costs and availability of the test in different settings.

1.5.1 Endoscopy

Upper gastrointestinal endoscopy is an expensive and unpleasant procedure that carries the risk of hemorrage and perforation, but is still the only procedure that permits the definitive diagnosis of the infection and its complications. The European Helicobacter Study Group (EHSG) Maastricht V guidelines recommended that a "test and treat" strategy (TTI: testing for colonization by non-invasive screening and treating if colonization is confirmed) is optimal in patients with uninvestigated dyspepsia in areas of high Helicobacter prevalence (24).

Unfortunately, TTI approach can't define a definitive diagnosis of ulceration as opposed to non ulcerated dyspepsia; therefore endoscopy should be considered only in patients with alarm symptoms (weight loss, bleeding, anemia) of any age and patients aged more than 55 years (25). In recent years there has been increasing interest in the goal of endoscopic

“in vivo” histology using narrow-band imaging, chromoendoscopy and confocal laser endomiscroscopy (26). In fact the mucosal surface and subsurface can be examinated in detail for the presence of characteristic pathological features and detection of *H. pylori* based on the assumption that the endoscopic appearence of the normal stomach is of a regular arrangements of epithelial cells, gastric pits and subepithelial capillaries while in *H. pylori* gastritis, the collecting venuoles cannot be seen or the gastric pits are enlarged with surrounding erithema and the normal capillary network is lost.

1.5.2 Rapid Urease Test (RUT)

Urease is one of the most abundant proteins produced by *H. pylori*, comprising about 10-15% of the total protein content, testifying its importance to the organism. Urease is found on the surface of the organism. The gastric biopsy test is based on the activity of the *H. pylori* urease enzyme, which splits the urea test reagent to form ammonia. Ammonia increases pH, which is detected by the indicator phenol red. Many different commercial RUT test are available (gel-based, paper-based and liquid-based) results are available after 1 h or up to 24 h depending in part on the format of the test and the number of bacteria in the biopsy specimen. All the commercial RUT tests have specificities of 95-100% but the sensitivity is slightly less at 85-95%. The sensitivity is affected mainly by the number of bacteria in the biopsy, but also presence of blood, achlorhydria and proton pump inhibitors (PPI) therapy.

1.5.3 Culture

H. pylori can be cultured from gastric biopsies. Biopsies collected for bacterial culture are immediately streaked onto commercial selective medium Pylori Agar and the plates are incubated under microaerobic conditions at 37°C for 72 h. Once incubated, the colonies

resembling *H. pylori* are identified by Gram stain and by oxidase, catalase and urease tests. Suspensions from the primary plates are prepared in sterile saline solution to McFarland opacity standard 4 (approximately 10^8 cells/mL) to perform an E-test. An agar plate is streaked in three directions with a swab dipped into each bacterial suspension to produce a lawn of growth, an E-Test strip is placed each onto a separate plate, which is immediately incubated in a Microaerophilic atmosphere at 37°C for 72 h. Isolated strains are tested for primary Clarithromycin (CLA), Metronidazole (MTZ) and Levofloxacin (LVX) resistance using break points for the minimal inhibitory concentration (MIC) of >0.5, >8 and >1 mg/L, respectively, according to the updated recommendations of the European Committee on Antimicrobial Susceptibility Testing (27).

As the prevalence of antibiotic resistance increases globally there is a strong argument on performing culture and sensitivity testing after the first treatment failure (to prevent emergence of double resistance to CLA and MTZ) and certainly after the second; indeed, some would argue that it should be performed at the initial diagnosis in areas of high resistance prevalence. Moreover, it has to be emphasized that susceptibility to a full range of antibiotics can only be tested by cultures. However, culture needs qualified and skilled operator to reach high performance and it is quite invasive and expensive.

1.5.4 ^{13}C-Urea Breath test (^{13}C-UBT)

The 13C-Urea Breath Test (^{13}C-UBT) is one of the main non-invasive methods for *H.pylori* infection diagnosis.^{13}C-UBT can detect the presence of *H. pylori* indirectly by demonstrating the presence of marked carbon dioxide in subject's breath by somministrating ^{13}C labelled urea. In fact the presence of urease due to *H. pylori* elicit the splitting of urea and carbon dioxide which diffuses into the epithelial blood vessels and appears in the patient's breath. The isotopic labelled urea is usually given to the patient with a test meal to delay gastric empting and increase contact time with the mucosa. ^{13}C-

UBT has a very high sensitivity and specificity, ranging from 95 to 97 %; however it may not be reliable in assessing patients who have had a gastric surgery or in patients who have been on proton pumps inhibitors. ^{13}C and ^{14}C-urea breath test are similar, except that ^{13}C is a non radioactiive isotope of ^{12}C hence usable for children and pregnant women (28).

1.5.5 Stool antigen test (SAT)

An enzimatic immunoessay that detects the presence of *H. pylori* antigen in stool specimens has recently become available. This test is comfortable for the patient and very useful with the pediatric patients. Recent transition from polyclonal to monoclonal has improved considerably this technique. A metanalysis showed that Monoclonal SAT is an accurate noninvasive method both for the initial diagnosis of *H. pylori* infection and for the confirmation of its eradication after treatment. The monoclonal technique has higher sensitivity than the polyclonal one, especially in the post-treatment setting (29).

Most of the studies have suggested that the accuracy of this test is similar to that of the ^{13}C-UBT for initial diagnosis of infection and are both recommended by both the National Istitute for Health and Clinical excellence (NICE) in the UK and in the EHSG Maastrict V Consenus Statement. A positive stool test 7 days after completion of treatment is predictive for failed eradication (30).

1.6 Helicobacter pylori management

Eradication of *H. pylori* prevents development of pre-neoplastic changes (atrophic gastritis and intestinal metaplasia) and reduces the risk of gastric cancer as evidenced by non-randomized controlled studies in animals and humans. Several studies show regression of pre-cancerous lesions, or at least, slower progression as compared with control groups after *H. pylori* eradication. Moreover, MALT lymphoma shows strong epidemiological association with *H. pylori* infection and the eradication of *H. pylori* can reverse this condition (11). A TTI strategy is appropriate for uninvestigated dyspepsia. This approach is subject to regional *H. pylori* prevalence and cost-benefit considerations. It is not applicable to patients with alarm symptoms or older patients. In areas of low *H. pylori* prevalence (<20%), PPI empirical therapy or a TTI strategy are considered to be equivalent options. Screening for *H. pylori* may not be appropriate when the population prevalence of *H. pylori* decreases to 10% as this may result in a significant proportion of false positives, leading to unnecessary treatments. This is more likely to occur with the less sensitive and specific serology tests than with the ^{13}C-UBT (31) .

In patients on long-term acid suppressive therapy, it is advisable to eradicate *H. pylori* in case there is development of atrophic gastritis caused by bacterial overgrowth. Moreover, the relationship between *H. pylori* Infection and non-steroidal anti-inflammatory drugs (NSAIDs) in gastroduodenal pathology is complex: the risk of ulcer bleeding when both factors are present increases, but results of *H. pylori* eradication in NSAIDs users are conflicting. However, in patients with *H. pylori* infection who are naive NSAIDs users, the eradication therapy is superior to placebo in preventing peptic ulcer and upper gastrointestinal bleeding. Instead for high risk patients the eradication of the organism should be mandatory.

1.6.1 Antibiotics

H. pylori is very sensitive to many antibiotics, although over the past two decades there has been an increasing problem with the development of resistance. Antibiotic sensitivity testing is important to check sensitivity before treatment and to assess local, national or international resistance rates. Several antibiotics are involved in *H. pylori* eradication regimens, most of them are now less effective due to the development of antibiotic resistance that is in turn related to the over-mis use of antibiotics therapies worldwide. Eradication regimen are based mainly on regimens containing: penicillin, macrolide, fluorquinolone and imidazole:

- Amoxicillin (AMX) is a B lactam antibiotic that binds to cell wall transpeptidase, inhibiting the making of cell wall. Resistance to B lactam antibiotics is due to B lacatmasase production or an altered penicllin-binding protein.

- Clarithromycin (CLA) is another key antibiotics in *H. pylori* eradication regimens, it is a macrolide and inhibits protein synthesis by binding to the 23SrRNA component of the 50S subunit of the ribosome. CLA resistance is due to point mutations in 23S rRNA gene. Since now several point mutations have been identified which include A2143G, A2142G, A2142C, A2115G, A2142T, A2223G, C2147G, C2694A, C2611A C2195T, C2245T, G224A, G2141A, T2190C, T2182C, T2117C, T2289C. The first three above mentioned mutations (A2143G, A2142G, A2142C) represent >90% of the observed mutations with confirmed clinical relevance (32).

- Metronidazole (MTZ) is also involved in eradication of the bacteria. It is a 5-nitroimidazole that is activated by *H. pylori* nitroreductase enzyme, and his resistance varies between 15-78%.

- Levofloxacin (LVX), a fluoroquinolone used in rescue therapeutic regimens, interacts with type II topoisomerases preventing the unwinding of DNA and DNA replication. It is used normally as a second line treatment.

1.6.2 Proton pump inhibitors (PPI)

These agents comprehend omeprazole, esomeprazole, lansoprazole, pantoprazole and raberprazole. PPI are always associated to antibiotics in eradication regimens. Their mechanism provides antisecretory activity by blocking the H/K ATPase proton pump in parietal cells (33). PPI also enhance the pharmacodynamics of antibiotics due to their inhibitory effect on acid secretion. Plus, PPI are capable also of antimicrobial activity themselves against *H. pylori*. A consideration must be made upon metabolism of PPI which is dependent upon polymorphisms in the cytochrome P450 2C19, leading to high or low metabolizers. High metabolizers (patiens with a rapid eliminarion of PPI) presents a lower eradication rate, this is the reason why high dose PPI are usually suggested in association with antibiotic therapy.

1.6.3 Current therapies

EHSG suggests one week triple therapy (PPI, CLA plus AMX or *MTZ*) for seven day in those areas where CLA resistance is lower than 15-20 % (34), and is being currently prescribed by 85%, 84% and 67% physicians in Italy, Israel and USA.

A prolonged 14 day regimen or a 10-14 day quadruple therapy (PPI + Bismuth + MTZ + Tetracycline administered for 7-10 days) should be administered when bacterial resistance is higher (34). Unfortunately a metanalysis has shown that the 14-day triple therapy offers only a modest improvement (87.7% of eradication) over the 7 day regimen (79.7% of eradication) in terms of *H. pylori* eradication rates (35). One of the main reasons for the poor performance of treatments is the increasing number of *H. pylori* strains resistant to antibiotics especially CLA and MTZ. Indeed antimicrobial resistance is responsible for the declining rates of *H. pylori* eradication seen in many countries (36).

However the situation changed in 2000 with the incoming of a new sequential treatment regimen as alternative to CLA triple therapy, reaching an average of 92% eradication rate

(37). The sequential regimen is a simple dual therapy (a PPI plus AMX 1g, both given twice daily) for the first 5 days, followed by a triple therapy (PPI, CLA 500 mg and tinidazole, all given twice daily) for the remaining five days (37).

The reason for this sequence is that dual therapy administered for less than 7 days is able to achieve a cure rate up to 50%, affecting the bacterial load which is inversely related to the efficacy of triple therapy, that needs a low bacterial load to favour its efficacy. Moreover, AMX is able to prevent selection of secondary CLA resistance by weakeing bacterial cell wall and preventing the development of efflux channels (38).

A recent systematic review and meta-analysis compared sequential therapy to established and newer therapies. The overall eradication rate for sequential therapy was 84.3% (95% CI, 82.1–86.4%), confirming its high efficacy despite a decrease in eradication rate during years due to the increasing antibiotic resistance. Sequential therapy was superior to 7 days of CLA triple therapy (RR 1.21; 95% CI, 1.17–1.25). However, sequential therapy was only marginally superior to 10 days of CLA-based triple therapy (RR, 1.11; 95% CI, 1.04–1.19) and was not superior to 14 days of CLA-based triple therapy (RR, 1.00; 95% CI, 0.94– 1.06) or 10–14 of bismuth quadruple therapy (RR, 1.01; 95% CI, 0.95–1.06) (39). Ultimately, another study from Taiwan suggested that eradication rates might be improved by extending the duration of sequential therapy to 14 days (40).

1.6.4 approach to resistance

In settings with high CLA resistance the efficacy of triple and sequential therapy is undermined; plus MTZ resistance undermines the efficacy of sequential therapy, and dual CLA and MTZ resistance undermines the efficacy of sequential, hybrid and concomitant therapy(24). Therefore, the choice of therapy should be based on the frequency of MTZ and dual CLA and MTZ resistance.

In geographical areas where MTZ resistance is almost negligible (eg, Japan), replacing CLA for MTZ in triple therapy (ie, PPI-MTZ-AMX) still shows excellent cure rates (41).

In regions with high CLA resistance (15–40%) but low to intermediate MTZ resistance (<40%) (a pattern common for most central and southern European countries and the USA) (42)(43), non-bismuth quadruple concomitant therapy, prescribed for 14 days (44), can be an effective alternative.

Non-bismuth quadruple therapy or “concomitant” therapy is the combination of PPI-CLA-AMX-nitroimidazole administered together. A recent meta-analysis of randomized trials showed that the eradication rate with non-bismuth quadruple therapy was 90% compared to 78% with triple therapy (45). Therefore concomitant therapy seems to be a valid alternative to triple therapy in those areas where bismuth is not available.

In areas of high dual CLA and MTZ resistance, bismuth quadruple therapy (BQT) is the recommended first-line treatment. Ideally, CLA should be avoided and a combination of alternative antibiotics for which resistance does not become problematic (eg, amoxicillin, tetracycline, furazolidone, rifabutin) or can be successfully overcome with increasing doses, dosing interval and duration (eg, MTZ) should be recommended (24). If bismuth is not available in high dual CLA and MTZ resistance areas, LVX, rifabutin, and high dose dual (PPI+AMX) treatments can be considered (24).

1.6.5 Other factors affecting treatment response

1.6.5.1 Patient’s adherence to treatment

In addition to antimicrobial resistance, several other factors affects the eradication rate; patients taking less than 80% of their treatment regimen have a high rate of treatment failure, which is strongly associated with other anti-microbial resistances. It should always be emphasized to the patient that successful eradication depends on full compliance with the treatment. A least a short time should be taken to counsel the patient, explaining the

procedures involved in taking complicated drug therapies and describing the side-effects to improve the outcome.

1.6.5.2 Side effects and smoking habits

Up to 50% of patients have mild side-effects while taking *H. pylori* treatment and less than 10% of patients stop treatment because of side- effects. Some of the most common side-effects are metallic taste, especially by using MTZ or CLA, constipation while taking bismuth based treatments, and diarrhea or stomach cramps. Smoking reduces the success rate of standard triple therapy mainly altering antibiotics delivering into the gastric mucosa. Indeed, reduction in gastric blood flow and mucus secretion or an increased acidsecretion can reduce the antibiotic activity.

1.7 Aim and objective

H. pylori infection is a major cause of gastric ulcer disease and gastritis in humans and is a risk factor for the development of gastric cancer. Current guidelines recommend that all patients with documented *H. pylori* infection should be treated with an appropriate antibacterial therapy. CLA-based triple therapy is commonly used as a first line treatment; however, resistance of *H. pylori* to CLA has been gradually increasing worldwide (36) (39). Treatment failures associated with resistance to CLA were reported and genetic analysis of resistant strains isolated from patients who failed primary CLA-based treatment, identified mutations in the 23S ribosomal RNA (rRNA) as the predominant cause of resistance. In particular, point mutations in positions 2143 (A to G) and 2142 (A to G/C),(46). A lower prevalence of A2142G/C mutation has been described, but these mutations are also commonly found in patients in whom CLA-based treatment failed to eradicate the infection, highlighting that these mutations are due to selective pressure under antibiotic therapy. Testing for antibiotic sensitivity has been complicated for *H. pylori* due to the necessity to perform culture and chemosusceptibility test, which require

additional biopsies and specific laboratory competence. The approach of using a stool specimen in a molecular test for CLA resistance has therefore been proposed (47). To date, there is very limited data on use of such approach in clinical research. It appears that, while the specificity of the molecular test has been consistently very high (more than 95%), the achievement of a high sensitivity remains very challenging.

Therefore, the aim of this study was to evaluate the feasibility of using stool samples to detect the presence of *H. pylori* DNA, while concurrently detecting the presence of mutations associated with the resistance to CLA.

Chapter 2- Methods

2.1 Samples and extraction method

Stool samples were acquired from a population of patients tested for *H. pylori* infection in Bologna, Italy. Samples were collected, frozen and kept at -20 °C prior to testing. Patients were diagnosed as *H. pylori* positive or negative according to concordant results of the composite reference method (CRM: ^{13}C-UBT, RUT, histology and culture) as the gold standard (48)(49). Total DNA was extracted from 292 raw stool samples using the QIAamp Fast DNA Stool Kit (QIAgen) where the workflow matched the protocol for human DNA isolation (instead of the pathogen protocol) as provided by the manufacturer's instructions. A qPCR exogenous control (Bioline Cat #BIO-11025) made up of a known concentration of bacterial DNA was spiked into the lysis buffer just prior to extraction. The exogenous control was applicable for high annealing temperature Polymerase Chain Reaction (PCR) and was added during the lysis step of the DNA extraction to monitor extraction efficiency and the presence of inhibitors. Extracted DNA was stored at -20 °C prior to qPCR testing.

2.2 DNA extraction and purification

The collected stools were defrosted naturally, 200mg of the specimen were put in 1ml of InhibitEX buffer, after a strong passage into vortex and 1 min in the centrifuge, proteinaseK was used to to purify nucleic acid preparations by digesting the contaminating proteins.

Buffer AL was added and throughout an incubation at 70°C for 10 min and the subsequent addition of ethanol, the DNA has been released in the solution making possible his purification and isolation (through successive washes with buffers AW1 and AW2) in order to amplify it with PCR.

2.3 Molecular detection

TaqMan real-time PCR amplification was used to detect the presence of *H. pylori* as well as predict the phenotype of the organism and the related outcome of patients treated with CLA. Extracts were amplified on the QIAgen Rotorgene.

The PCR master mix was comprised of *H. pylori*/Resistance primer set (Meridian Bioscience; Cat#ASR100), *H. pylori* probe (Meridian Bioscience; Cat#ASR101) and *H. pylori CLA* Resistance (HPCR) probe (Meridian Bioscience; Cat#ASR102) and was assembled into a final PCR reaction as follows: 100nM of each TaqMan probe targeting the *H. pylori* 23S rRNA in the green (Emission 510nm) and red (Emission 660nm) channels; 400nM of ASR primers; 40nM of control primers (Bioline Cat #BIO-11025 includes primers and probe) and 100nM control probe (Emission 610nm), 1x SensiPLUS mastermix (Bioline Cat #BIO-11021).

Typically, 20 µL of purified DNA sample was added to the amplification mixture. Molecular grade water was added to make up a total volume of the PCR reaction to 50 µL. The PCR amplification was performed with the following parameters: Initial hold temperature 95 ^{o}C for 10 min, then 50 cycles of 95 ^{o}C for 20 sec and 65 ^{o}C for 60 sec; followed by a melting protocol measuring fluorescent intensity of the red probe (emission 660nm) in the temperature range of 52 ^{o}C-70 ^{o}C; (10 second wait between intervals; 1^{o}C increase each cycle).

2.4 Analysis of PCR

PCR results for detection of *H. pylori* was calculated based on CRM as the comparator method. The CLA resistance prediction due to the presence of 23S rRNA mutations was performed in parallel with detection of *H. pylori*. Amplification observed in the green channel translated to the presence of *H. pylori* in the purified DNA from stool. Presence of the amplification in the red channel was used for making a prediction of CLA resistance or

susceptibility. Two alternative approaches were employed to analyse amplified DNA and make a determination of CLA susceptible or resistant for each sample:

1) The first approach comprised of using **differential fluorescent signal** analysis and finding the derivate function for both the green and red signal for every cycle. The difference between the green and red signal determined the resistance prediction. If the red signal had an attenuated result and, therefore, a positive delta from the green signal, the sample was called as containing mutation conferring clarithromycin resistance. If the red signal was the same or, in most cases, stronger than the signal in the green channel, the result would be a negative delta from the green signal. The sample was then deemed as not containing targeted mutations and susceptible to CLA**.** **Figure 1** shows an example of a sample that was predicted to be resistant to CLA and a sample that was predicted to be susceptible to CLA.

2) The second approach was to analyse the *H. pylori* amplified target and make a prediction of resistance was by melting curve analysis in the temperature range of 52°C-72°C. A 10 second wait was programmed between intervals and temperature increased 1°C with each cycle. When a single peak was observed at temperatures lower than 60°C, typically 54-56°C, the sample was deemed as containing mutations conferring resistance to CLA. When a single peak was observed above 60°C, typically 63-65°C, the sample was deemed as lacking targeted mutations and susceptible to CLA**.** **Figure 2** shows examples of typical melt profiles produced by the analysis of samples comprising wild type sequences and different CLA-resistant mutations. Samples demonstrating amplification in green channel and no amplification in red channel were called as *H. pylori* positive and indeterminate for the presence of CLA-resistant mutations.

2.5 Purification of PCR products and sequencing

PCR products were purified using Macherey-Nagel's NucleoSpin Gel Purification kit (Cat #740609). The identity of amplified PCR products was confirmed by Sanger Sequencing. GeneWiz [NJ, USA] performed bi-directional sequencing to confirm the genotype.

2.6 Reference testing

Patients were tested, in parallel to molecular testing, by ^{13}C-UBT and subjected to esophagogastroduodenoscopy (EGD) followed by RUT, histology and culture, obtaining a consensus patient infection status.

PCR data were compared to the outcome of the eradication treatment for a subset of patients treated with clarithromycin as part of a sequential therapy (PPI plus AMX 1g, both given twice daily for the first 5 days, followed by a triple therapy (PPI, CLA 500 mg and tinidazole), all given twice daily for the remaining five days. All patients underwent a standard ^{13}C-UBT after an overnight fast at the beginning and 4 to 6 weeks following the end of antibiotic therapy to assess the eradication status (48).

2.7 Statistical analysis

Means and their 95% confidence intervals were calculated. Comparisons among patient subgroups were performed using the chi-square. Test accuracy was calculated with "2x2" table method (MEDCALC 17.2). A *P* level less than .05 was considered significant.

Chapter 3- Results

Stool specimens were collected from August 2016 to March 2017 and tested by PCR within a month of collection. The optimal amount of stool producing the best sensitivity of detection was determined as approximately 200mg (180-220mg, same as manufacturer's instructions) from preliminary experiments.

3.1 PCR versus CRM

Out of 292 total stool samples from *H. pylori* patients, 228 were positive by CRM and by the novel PCR test. Two samples were invalid by PCR and were excluded. PCR resulted in approximately 93.8% (CI: 90.0-96.5%) (228/243) sensitivity in comparison with the CRM. Specificity was approximately 77.6% (CI:63.3-88.2%) however, the current study targeted predominantly positive patient population in order to establish clinical sensitivity of the stool PCR method, therefore before drawing conclusions regarding clinical specificity a higher number of negative samples should be included. According to the previous results the positive predictive value (PPV) was 95.4% (CI:92.5-97.2%) while the negative predictive value was 71.7% (CI:60.2-80.8%) with an overall accuracy of the PCR test of 91.1% (CI:87.2-94.1%). The diagnostic outcome for *H pylori* detection for PCR are shown in Table 1.

3.2 PCR versus Sequencing for Clarithromycin susceptibility prediction

The CLA susceptibility prediction from PCR was verified by sequencing that established the identity of nucleotides in positions 2142 and 2143 of the 23S rRNA gene. The genotype for all samples positive by PCR that produced definitive sequencing results was therefore analysed. In total 239 samples were considered (228 positive at CRM and PCR and 11 negative at CRM but positive at PCR). However 26 samples were not able to be included in the genotypic analysis due to unevaluable results from either PCR and/or sequencing: 7 sample were not sequenced or resulted in a failed sequence; 4 sequences were unreadable; 9 samples were deemed indeterminate or containing mixed alleles by

sequencing; 4 samples were discrepant by melt and differential signal analysis using PCR determination; 2 were indeterminate for genotype by PCR. Therefore 213 samples were considered in the final comparison between PCR performance and sequencing (Table 2).

Out of 213 remaining positive samples, 77 (36.1%) contained mutations and 136 (63.8%) contained the wild type sequence. PCR correctly identified 133 out of 136 samples (97.8%) containing wild type (CLA sensitive) sequence and 76 out of 77 (98.7%) samples containing mutations with a total accuracy of 98.1% (CI:95.2-99.4%).

Among the mutations, 3 samples contained A2142C sequence (genotype CAA), 19 samples contained A2142G sequence (genotype GAA), and 55 samples contained A2143G sequence (genotype AGA). One sample that was predicted by PCR to be a wild type (genotype AAA), contained a mutation at the A2143G position (genotype AGA). Three samples that were predicted by PCR to be resistant were wild type (genotype AAA). In our population the most frequent mutation was A2143G that was observed in 55 cases; 54 out of 55 were correctly identified by PCR. The A2142G mutation was detected in 19 cases, all of them were correctly identified by PCR. The A2142C mutation was detected in 3 cases in concordance with PCR. A discrepancy between sequencing and PCR results was detected only in 4 cases. The PCR test total accuracy was 98.1% (Table 3).

3.3 Correlation between genotype prediction by PCR and phenotype

168 culture were available out of 213 samples with PCR prediction and sequencing results. PCR sensitivity (ability to identify resistant strains) and specificity (ability to identify susceptible strains) were 60.4%(CI:50.4-69.7%) and 93.5% (CI:84.3-98.2%) respectively (Table 4). Considering culture as the gold standard, PCR accuracy was 72.6% (CI: 65.2%-79.2%).

3.4 Correlation between genotype prediction by PCR and eradication

Eradication results were available for 121 patients treated with therapeutic regimens containing clarithromycin (Table 5).

Correlation between genotype prediction by PCR and eradication of patients treated with clarithromycin was 83.5% .

Potential cross-reactivity of the PCR toward different *Campylobacter* species commonly found in non-diarrheal stool were also evaluated. At the concentration of 10^6 genomic copies in the reaction analytical specificity was confirmed with the various Campy species.

Chapter 4- Discussion

According to our findings, these data demonstrate that *H. pylori* DNA can be detected in human stool specimens with high sensitivity and therefore this test can be used to determine the presence of *H. pylori* with a performance similar to that of other direct assays, such as the SAT or UBT (48). More importantly, genotypic resistance to clarithromycin can be detected, enabling the right therapeutic approach to be chosen without a biopsy sample obtainded during an invasive procedure such as gastroscopy. Moreover, culture and antibiotic susceptibility test are not always feasible and skilled biologists are needed. Culture test is also difficult to perform, expensive and time consuming (5-7 days to obtain results) and nearly 20% of cultures are not diagnostic (no growth or overgrowth of other bacteria and fungi). This finding confirms previous reports describing the use of PCR detection and assessment of clarithromycin sensitivity using stool specimens (27).

The design for this PCR test allows for multiple ways to analyze the data. Melting of the red probe targets the mutation sites associated with resistance. The proprietary nature of the TaqMan probes improves melt resolution, enabling a CLA susceptibility prediction (49). The prediction based on a melting curve can be determined immediately upon analysis with a specific software. Alternatively, resistance can be predicted using fluorescent signal analysis differentiation as described in “Materials and Methods” (50). Discrepancies between sequencing results and PCR prediction of resistance may be due to nonspecific traces and biologists interpretation. This study also shows a significant correlation between the detection of 23S rRNA mutations conferring resistance to clarithromycin and the final eradication status of the patient. Taking into account the expected bias of this study due to the fact that the majority of the patients predicted to be resistant were not treated with clarithromycin because they were previously treated with first line eradication therapy for *H. pylori* eradication (also called “not-naïve” patients), the drop in the response rate from 93% (93 out of 100 patients with predicted CLA-sensitive

organisms were eradicated) to 62% (13 out of 21 patients with 23SrRNA point mutations were eradicated) suggests the beneficial impact of the PCR test in the selection of the appropriate therapy. This PCR test using stool specimens is capable of sensitive detection of *H. pylori* DNA in stool while simultaneously detecting mutations causing clarithromycin resistance. This is very important as there is a significant correlation between the presence of such mutations and the outcome of clarithromycin-based eradication treatment. The results of this study suggest that stool can be used to determine resistance with a high correlation to eradication status.

This assay can be used as a stand-alone screening tool, since it can detect the presence of *H. pylori* as well as resistance, but is most cost-efficient once negative patients are eliminated by a stool antigen test or UBT. Both approaches quickly eliminate negative patients from further analysis. (48) (51)

This stool/PCR test has also the potential to reduce health care costs with an updated algorithm. A biopsy sample requires the patient to return to the health care provider for an invasive procedure if treatment is unsuccessful (24). The PCR test option with the noninvasive sample can significantly reduce the need for biopsy samples, currently used to determine resistance in patients who do not need to undergo upper endoscopy according to clinical guidelines (52).

However, further clinical studies with a more balanced patient population and with investigators blinded to the outcome of the resistance determination, would define the true clinical value of the stool PCR test. The high analytical specificity and clinical sensitivity of this molecular *H. pylori* resistance assay provide a strong likelihood of accurately predicting eradication of infection by CLA therapy.

In conclusion this PCR test using stool specimens is capable of sensitive detection of *H. pylori* DNA in stool while simultaneously detecting presence of mutations causing clarithromycin resistance. There is a significant correlation between the presence of such mutations and the outcome of clarithromycin-based eradication treatment but furhter clinicaltrials are needed to strengthen these findings.

Definitions

Term	Explanation
AMX	Amoxicillin
BabA	blood group antigen-binding adhesion
Cag A	cytotoxin associated gene A
Cag Pai	cytotoxin associated pathogenicity island
CLA	Clarithromycin
CO2	Carbon Dioxide
CRM	Composite Reference Method
^{13}C-UBT	^{13}C-Urea Breath Test
EGD	Esofagogastroduodenoscopy
EHSG	European Helicobacter Study Group
IARC	International Agency for Research on Cancer
Le^b	Lewis b blood group antigen
sialyl-Le^x	Sialyl-Lewis x blood group antigen
LVX	Levofloxacin
LPS	Lipopolysaccharide
MALT	Mucose Associated Limphoid Tissue

MIC	Minimal Inhibitory Concentration
MTZ	Metronidazole
NSAIDs	Non-steroidal anti-inflammatory drugs
PCR	Polymerase Chain Reaction
PPI	Proton Pump Inhibitor
RUT	Rapid Urease Test
Sab A	Sialic acid-binding adhesin
SAT	Tool antigen Test
TTI	Test and treat
Vac A	Vacuolizing associated gene A

Tables

	CRM+	CRM-	TOT
PCR+	228 (93.8%)	11 (22.4%)	239 (81.8%)
PCR-	15 (6.2%)	38 (76.6%)	53 (18.2%)
	243	49	292

Table 1: diagnostic outcome of PCR for *H. pylori* detection compared to CRM.

Concordance: 91.1% Cohen's K concordance 0.69 (good)

	Seq R	Seq S	TOT
PCR R	76 (98.7%)	3 (2.2%)	79 (37.1%)
PCR S	1 (1.3%)	133 (97.8%)	134 (62.9%)
	77	136	213

Table 2: diagnostic outcome of PCR for *H. pylori* detection compared to sequencing.

Concordance: 98.1% Cohen's K concordance 0.96 (excellent)

No. of samples	Genotype	PCR prediction
133 (62.4%)	AAA	susceptible
54 (25.3%)	AGA	resistant
19 (8.9%)	GAA	resistant
3 (1.4%)	CAA	resistant
3 (1.4%)	**AAA**	**resistant**
1 (0.5%)	**AGA**	**susceptible**

Table 3: The CLA susceptibility prediction from PCR verified by sequencing that established the identity of nucleotides in positions 2142 and 2143 of the 23S rRNA gene**.**

	Culture R	Culture S	TOT
PCR R	64 (60.4%)	4 (6.5%)	68 (40.5%)
PCR S	42 (39.6%)	58 (93.5%)	100 (59.5%)
	106	62	168

Table 4: Correlation between genotype prediction by PCR and phenotype (culture).

Concordance: 72.6% Cohen's K concordance 0.48 (moderate)

	Not eradicated	Eradicated	TOT
PCR R	8 (53.3%)	13 (12.3%)	21 (17.3%)
PCR S	7 (46.7%)	93 (87.7%)	100 (82.7%)
	15	106	121

Table 5: Correlation between genotype prediction by PCR and eradication.

Concordance: 83.5 % Cohen's K concordance 0.35 (moderate)

Figures

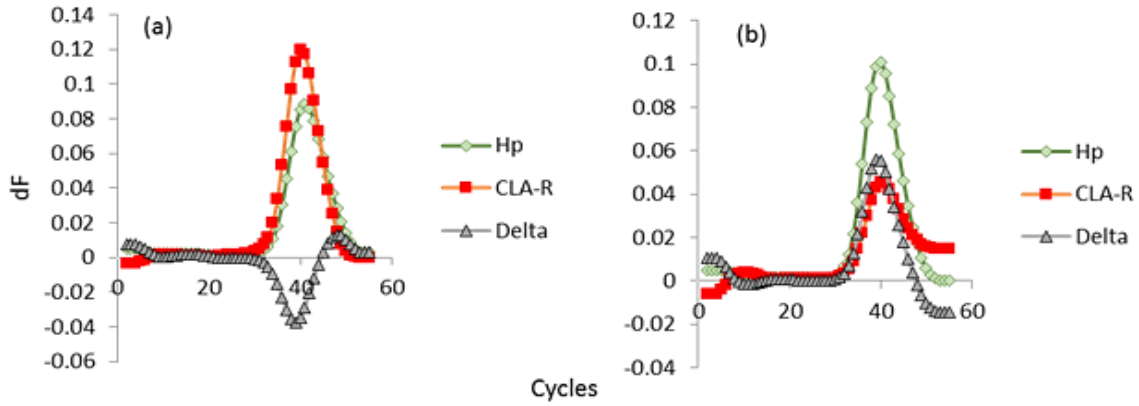

Figure 1: Fluorescent signal analysis for **(a)** wild type and **(b)** a mutant genotype.

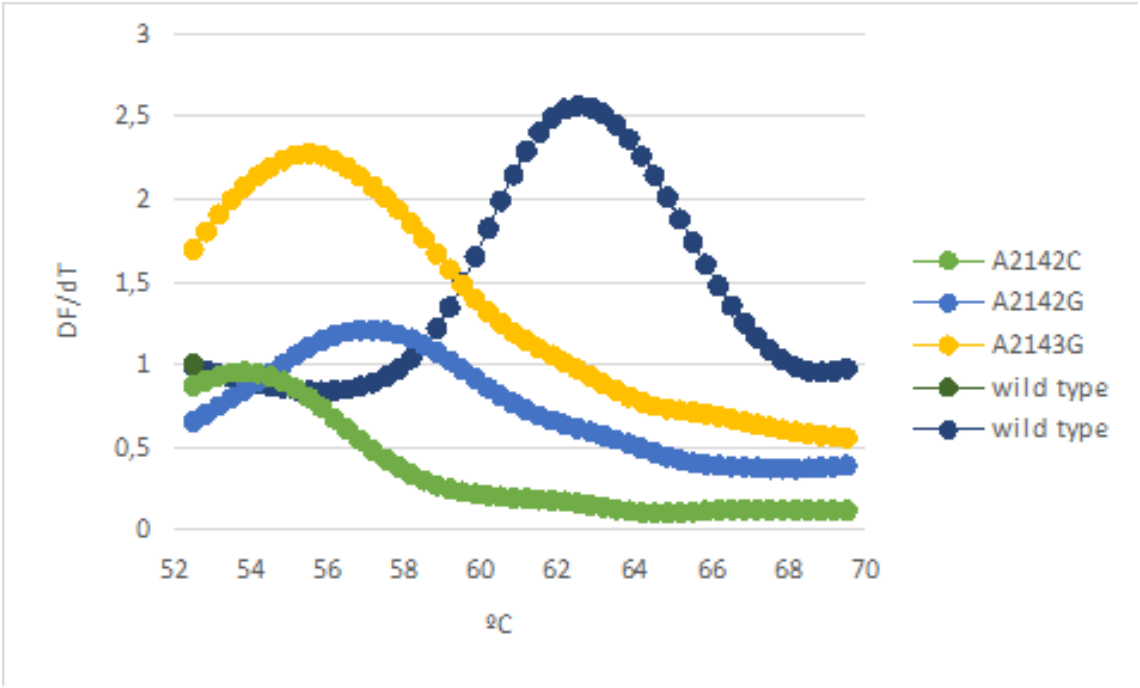

Figure 2: Melt curve analysis for wild type, A2141G, A2142C, and A 2143G mutant genotypes associated with resistance to clarithromycin.

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