

## Review

# Increasing trend of metronidazole resistance in the treatment of *Helicobacter pylori* infection: A global challenge

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*Helicobacter pylori* are gram negative spiral bacteria that colonize the human stomach. Infection with *H. pylori* is associated with chronic gastritis, peptic ulcer, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Antibiotic resistance is an ever increasing problem with the treatment of most microbial infections including *H. pylori*; and has become a growing problem worldwide with the eradication of this organism. In recent years, several treatment regimens have been proposed for *H. pylori* eradication. However, the only conditions for which such treatment is strongly recommended on the basis of unequivocal supporting evidence are peptic ulcer disease and low grade gastric MALT lymphoma. Success of antimicrobial regimens for *H. pylori* eradication depends on patient compliance and lack of antimicrobial resistance. Metronidazole (Mtz) containing regimens have been shown to limit effectiveness because of increasing prevalence of resistance to this drug. A high prevalence (> 90%) of Mtz resistance in *H. pylori* has been reported especially in developing countries. Mtz resistance may be mediated through an inability of Mtz-resistant strains to remove oxygen from the site of Mtz reduction, thereby preventing Mtz activation. This has been attributed to a mutation on the *frxA* and/or *rdxA* genes resulting in strains of the organism with defective nitro-reductases coded by these genes. Infection by Mtz or amoxicillin resistant strains is an important factor leading to treatment failure; subjecting all *H. pylori* clinical isolates to susceptibility testing most especially to Mtz is recommended. If not possible, a program to survey the prevalence of resistance should be implemented in a given area or population. This increasing emergence of antimicrobial resistance in *H. pylori* treatment poses serious public health problems and is therefore necessary that new drug regimens be examined.

**Key words:** *Helicobacter pylori*, drug resistance, metronidazole, gene mutations, public health.

## INTRODUCTION

*Helicobacter pylori* are gram negative spiral bacteria that colonize the human stomach. Infection with the organism is associated with chronic gastritis, peptic ulcer, gastric

adenocarcinoma and gastric MALT lymphoma (Suerbaum and Michetti, 2002; Ndip et al., 2004, 2008). Simple blood, breath and stool tests can determine if one is infected with *H. pylori*. The most accurate way to diagnose *H. pylori*, however, is through upper endoscopy of the oesophagus, stomach and duodenum (Ables et al., 2007). Eradication of the organism has been shown to result in ulcer healing, prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high risk populations (Sepulvedo and Coelho, 2002; Tanih et al., 2009).

Antibiotic resistance is an ever increasing problem with

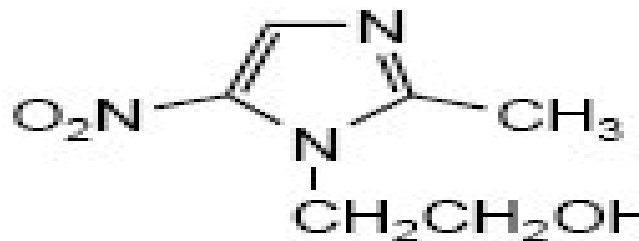
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**Abbreviations:** MALT, Mucosa-associated lymphoid tissue; Mtz, metronidazole; PPIs, proton pump inhibitors; MIC, minimum inhibitory concentration; CLSI, clinical laboratory standard institute; Mtz<sup>S</sup>, Mtz sensitive; Mtz<sup>R</sup>, Mtz resistant.

the treatment of most microbial infections including *H. pylori* infection (Hoffman, 1999; Thyagarajan et al., 2003). New antimicrobial agents are therefore being developed to overcome the problem of antibiotic resistance in bacterial pathogens, such as combination of antibiotics with plant extract and other natural products that possess antimicrobial activity (Chaudhuri et al., 2003; Ndip et al., 2007a,b; Tanih et al., 2009). Combinations of drugs have often been used for the treatment of drug resistant infections as this takes advantage of different mechanisms of action (Chaudhuri et al., 2003).

Antibiotic resistance with regard to *H. pylori* eradication has become a growing problem both in the developing and in developed countries. Mtz was initially used against a diversity of anaerobic microorganisms but was later established to have activity against certain microaerophilic organisms such as *H. pylori*. At present, Mtz is a cornerstone of many triple-therapy formulations for the eradication of *H. pylori*. However, resistance approaches 90% in many developing countries and even in Western Europe it ranges from 5 to 50% (Alarcon et al., 1999). In some countries, the prevalence of Mtz resistant strains approaches 70-100% and is associated with prior exposure to the drug (Noach et al., 1994; Goddard and Logan, 1996; Ndip et al., 2008). While several triple and quadruple-therapy formulations use more than one antibiotic, Mtz resistance has a profound effect on the efficacy of these regimens (van der Wouden et al., 1997, 2000, 2001).

Based on findings of several studies, the currently recommended therapy for the eradication of *H. pylori* infection include proton pump inhibitors (PPIs) such as omeprazole, lansoprazole, rabeprazole, or pantoprazole together with clarithromycin and either Mtz or amoxicillin (Salcedo and Al-Kawas, 1998; Veldhuyzen et al., 1998; Chaudhuri et al., 2003; Ndip et al., 2008). Success rates of cure with the use of these combination therapy ranges from 85 to 95%. However resistance to Mtz or clarithromycin results in an increased failure rate of therapies (Megraud, 1997). The emerging resistance to Mtz limits its use in the treatment of infections; and this problem is encountered more in Africa (Asrat et al., 2004; Ndip et al., 2008; Tanih et al., 2009). The resistance mechanisms in anaerobic organisms and *H. pylori* vary. Mtz, a synthetic nitroimidazole (Figure 1), is a prodrug and becomes active when reduced in the cytosol of the microorganism to a toxic metabolite. Unstable Mtz radicals react rapidly with proteins, RNA and DNA, eventually resulting in cell death. Under the conditions of low-redox potential in anaerobic organisms, drug activation can be catalyzed by nitroreductases such as pyruvateflavodoxin reductase by means of a single electron transfer event. *H. pylori* possesses this enzyme, but owing to its microaerophilic nature, molecular oxygen is also present and can compete with the Mtz radical for electrons in a futile cycle that restores the prodrug along with super oxide. Instead, a separate mechanism seems to account for most Mtz sensitivity in *H. pylori* (Goodwin et al., 1998). A non-



**Figure 1.** The chemical structure of metronidazole (Menz and Megraud, 2002).

oxygen-sensitive NADPH nitroreductase encoded by the *rdxA* gene reduces Mtz by a two-electron transfer step into a toxic metabolite that cannot be retransformed to its parent by molecular oxygen. The vast majority of clinically isolated (Tankovic et al., 2000) or experimentally induced (Jenks et al., 1999) Mtz-resistant clones contain a mutation somewhere in the *rdxA* coding sequence. However, there have been reports that mutation of a second reductase NAD(P)H-flavin oxidoreductase encoded by *frxA* could also confer low-level Mtz sensitivity in some strains (Kwon et al., 2001) and a role for oxygen sensitive reductases has not been formally excluded. Sequencing candidate genes, such as the reductases (mentioned above), in sensitive and resistant isolates has provided support for the idea of these genes playing a role in resistance, but reports also show that frameshift mutations in *frxA* occur with similar frequencies in sensitive and resistant strains (Chisholm and Owen, 2004). Jeong et al. (2000), concluded that most Mtz resistance in *H. pylori* depend on *rdxA* inactivation, of which mutations in *frxA* can enhance resistance, and that genes conferring Mtz resistance without *rdxA* inactivation are rare or nonexistent in *H. pylori* populations. Although null mutations in a *rdxA* gene that encodes oxygen-insensitive NAD(P)H nitroreductase was reported in Mtz-resistant *H. pylori*, an intact *rdxA* gene has also been reported in Mtz-resistant *H. pylori*, suggesting that additional Mtz resistance mechanisms exist in *H. pylori* (Kwon et al., 2000a). In this paper, we present an overview of Mtz resistance as well as the roles of the putative genes *frxA* and *frxA* implicated in Mtz resistance.

## TREATMENT OF *H. PYLORI* INFECTION

In recent years, several treatment regimens have been proposed for the eradication of *H. pylori*. However, the only conditions for which such treatment is strongly recommended on the basis of unequivocal supporting evidence are peptic ulcer disease and low grade gastric MALT lymphoma (Graham, 1998). Success of antimicrobial regimens for *H. pylori* eradication depends on patient compliance and lack of antimicrobial resistance. Thus, complicated regimens or those associated with side effects or both may result in non-compliance and failure

to eradicate the organism (Lesbros-Pantoflickova et al., 2007; Ndip et al., 2008). Theoretically, therapies have improved such that regimens should demonstrate 85 to 95% efficacy. Single-agent therapy should not be used because of the unacceptably low eradication rates. Clinical trials have shown that multidrug regimens (triple therapy) are the most effective treatment with eradication rates of up to 96% (Lamouliatte et al., 1996; Salcedo and Al-Kawas, 1998; Kwon et al., 2001; Aboderin et al., 2007). Despite these high eradication rates, the problem of drug resistance is still well established. The regimen of choice is a PPI or ranitidine bismuth citrate (Tritec) plus two antibiotics for 14 days (Megraud and Lehours, 2007; Ndip et al., 2008). Mtz, clarithromycin, amoxicillin and tetracycline are the major antibiotics prescribed for *H. pylori* eradication (Goddard et al., 1996; Kobayashi et al., 2001; Megraud, 2004). These agents have superior efficacy to and are better tolerated than the standard bismuth based triple therapy consisting of bismuth subsalicylate, metronidazole (flagyl) and tetracycline hydrochloride (Graham et al., 1991; Meurer and Bower, 2002).

## METRONIDAZOLE

Mtz is a nitroimidazole used principally for the treatment of anaerobic and parasitic infections. Mtz is stable at a low pH and is actively secreted into the gastric juice (Edwards, 1993). Active secretion of Mtz is reduced when it is given with a proton pump inhibitor (Goddard et al., 1996) and has been reported to have a half-life of 8 to 12 h. The most common adverse effects of Mtz are a metallic taste in the oral cavity, nausea and epigastric discomfort. Mtz has been reported to produce a disulfiram like reaction when taken in combination with alcohol. In the past two decades, Mtz has become a stronghold in the treatment of *H. pylori* infection. In Western countries where Mtz use is very minimal, more than 70% of *H. pylori* isolates are sensitive to it (Glupczynski, 1992); however, more recent data has indicated an Mtz resistance of 10-50% of all adult patients infected with *H. pylori* in the developed world (Lopez-Brea et al., 1997; Osato et al., 1999; Adamek et al., 1998). In fact, in developing countries where Mtz use is more common, there have been reports that more than 80% of *H. pylori* isolates are Mtz resistant (Bell et al., 1992; Tanih et al., 2010). *H. pylori* eradication is rarely achieved when it is administered as a single agent. Therefore, Mtz is always given in combination with one or more antibiotics, that is, multiple therapies (Chowdhurjer et al., 2002). Mtz resistance may be mediated through an inability of Mtz - resistant strains to remove oxygen from the site of Mtz reduction, thereby preventing Mtz activation. This has been attributed to a mutation on the *frxA* and/or *rdxA* genes resulting in a strain of *H. pylori* with defective nitroreductases coded by these genes (Cederbrant et al., 1992; Kwon et al., 2000a; Paul et al., 2001). Resistance

is defined by a cut off value, which depends on the minimum inhibitory concentration (MIC) of the strains and on the concentration of antibiotic that can be achieved in the tissue by a given therapeutic dose (Megraud and Doermann, 1998; Thyagarajan et al., 2003). Although the Clinical Laboratory Standard Institute (CLSI) has not designated a MIC breakpoint for Mtz, values of  $\geq 8 \mu\text{g/ml}$  have been proposed (Vasques et al., 1996; Osato, 2000; CLSI, 2007). In a recent study in the Eastern Cape Province of South Africa, we reported a MIC value of  $> 10 \mu\text{g/ml}$  for Mtz (Tanih et al., 2010).

## TRENDS IN METRONIDAZOLE RESISTANCE

A systematic review of the data on antibiotic resistance published in the last 5 years highlighted regional differences in resistance pattern for Mtz and clarithromycin (Megraud, 2004). Mtz containing regimens have been shown to limit effectiveness because of increasing prevalence of resistance to this drug (Ching et al., 1996; Wang et al., 2000; Al-Quarashi et al., 2001). In a study in Egypt, a universal high-level primary Mtz resistance in children, compared to lower resistances to other selected antibiotic was reported by Sherif et al. (2004), which is consistent with other reports in Africa (Wang et al., 2000; Al-Quarashi et al., 2001; Ndip et al., 2008; Tanih et al., 2009). Very high resistances to Mtz and amoxicillin have been reported in Nigerian, Kenyan and Cameroonian patients (Abdulrashed et al., 2005; Lwai-lume et al., 2005; Aboderin et al., 2007; Ndip et al., 2008). Eradication failures in children are mostly due to non compliance because of adverse effects or due to resistance to Mtz and clarithromycin. In Cameroon, Ndip et al. (2008) revealed a very high antimicrobial resistance rate of 93.2% for Mtz. A similar study in Western Nigeria documented 100% resistance of *H. pylori* strains to Mtz (Smith et al., 2001). Equally, in a study conducted in Ethiopia, Asrat et al. (2004) found 76% of their strains resistant to Mtz.

Mtz containing regimens have been shown to limit effectiveness because of increasing prevalence of resistance to this drug (Wang et al., 2000; Al-Quarashi et al., 2001; Abdul et al., 2001). Patients originating from Africa have been reported to harbour resistant strains significantly more than those from other parts of the world (Megraud, 2004). Resistance rates as high as 75 - 98% have been reported in some areas of South Africa (Wong et al., 2000; Tanih et al., 2010) and up to 100% in Ethiopia. The problem of Mtz resistance was thought to be ameliorated when the drug was used in combination with clarithromycin (Hardin and Wright, 2002). However, high resistance to clarithromycin has been reported in patients originating from Africa (Loffeld et al. 2003). Banatvala et al. (1994) in their study documented that women born in the United Kingdom, were more likely to harbour Mtz-resistant *H. pylori* strains than men (54 v 18% respectively) and more likely to have a history of previous nitroimidazole ingestion (41 v 11% respectively);

and patients previously exposed to either Mtz or tinidazole were more likely to harbour resistant strains (84 v 41%).

Mtz consumption appears to be an important risk factor for this resistance. A marked difference has been found between the rate of resistance to nitroimidazoles in developed and developing countries. This difference may be linked to the high level of general use of the drug in developing countries to treat parasitic infections such as amoebiasis (Alarcon et al., 1999) because it is inexpensive and also commonly sold in the street corners heralding untold resistances to it. The cause of this resistance may also be linked to the use of these compounds for genital infections, especially trichomoniasis and therefore, strains isolated from women are more likely to be resistant than strains isolated from men (Banatvala et al., 1994; Alarcon et al., 1999; Cameron et al., 2004; Ndip et al., 2008). Another possible cause may be the use of these compounds to treat dental infections (Megraud, 1997).

Studies of *H. pylori* antibiotic resistance in South Africa are lacking. This could be a serious problem owing to the fact that susceptibility patterns are changing globally, and rapidly too. As a result, eradication failures will be frequent as some of the drugs given to the patients may fail to produce the desired effects and yet left on the shelf. However, we reported an Mtz-resistance of 95.5% in a recent study we conducted in South Africa (Tanih et al., 2010) which seems to corroborate earlier reports made in other African countries, thus, confirming high Mtz-resistance in the developing world.

### THE ROLE OF *RdxA* NITROREDUCTASE IN METRONIDAZOLE RESISTANCE

The elemental discovery two decades ago by Goodwin et al. (1998) that *H. pylori* Mtz resistance may be a result of loss of activity of an oxygen independent NADPH nitroreductase encoded by the gene *rdxA* marked the establishment of a renewed and intense interest in gaining a full understanding of the causes of resistance to this drug. Mutational inactivation of *rdxA* was initially proposed as the cause of naturally acquired Mtz resistance in *H. pylori* (Goodwin et al., 1998), and various features of *rdxA*, such as the polypeptide it encodes, were suggested as the molecular bases for a low redox potential capable of reducing Mtz (Kwon et al., 2000b). However, the results of further studies to verify this hypothesis revealed other enzyme activities with biological functions different from nitroreductases that appear to modulate Mtz reduction and were thus proposed as potential candidates to cause resistance to this drug in *H. pylori* (Jeong et al., 2001). As a follow up to these data, successive modifications were proposed to the initial hypothesis, with the result that the association of *rdxA* in susceptibility to Mtz is accepted currently, but its specific role is unclear to many researchers (Kwon et al., 2001). The principal role

on the involvement of *rdxA* nitroreductase in the Mtz sensitive (Mtz<sup>S</sup>) phenotype of *H. pylori* and the specification of *rdxA* inactivation as the necessary and sufficient cause of Mtz resistance in this bacterium, were proposed by Goodwin et al. (1998).

Goodwin et al. (1998) demonstrated that intrinsically resistant *E. coli* transformed with functional *H. pylori rdxA* became sensitive to Mtz, and assays of the reduction of the drug by one of the clones demonstrated the functionality of the protein expressed in *E. coli*. The study showed that introduction of *rdxA* into Mtz resistant (Mtz<sup>R</sup>) *H. pylori* rendered them Mtz<sup>S</sup>, that isogenic *rdxA* knockout *H. pylori* mutants of an Mtz<sup>S</sup> parent became Mtz<sup>R</sup> and that *rdxA* genes originating from matched pairs of Mtz<sup>S</sup> and Mtz<sup>R</sup> strains differed from one another by several base substitutions (Goodwin et al., 1998). It was also demonstrated that in *rdxA* alleles that showed extensive deletions, insertions of transposable elements (Debets-Ossenkopp, 1999; Tankovic et al., 2000; Kwon et al., 2000b) or mutations leading to stop codons which could result in premature translation, termination and truncated polypeptides (Jenks et al., 1999; Tankovic et al., 2000), would lead to a situation whereby the corresponding *rdxA* will not be expressed or functional. Several studies have also demonstrated the large heterogeneity of point mutations present in the *rdxA* genes of resistant strains, with no particular nucleotide mutation or amino acid substitution that could be linked to Mtz resistance with the exception of mutations generating stop signals (Goodwin et al., 1998; Debets-Ossenkopp, 1999; Jenks et al., 1999; Tankovic et al., 2000; Kwon et al., 2000a; Solca et al., 2000; Jeong et al., 2000; Kwon et al., 2001; Paul et al., 2001; Jorgensen et al., 2001). Other studies have also revealed the existence of unchanged *rdxA* in sensitive and resistant isolates and of different *rdxA* in Mtz<sup>S</sup> strains (Jenks et al., 1999; Tankovic et al., 2000). In contrast with resistance mechanisms for other antibiotics, the resistance mechanism of Mtz is complex (Mendz and Megraud, 2002; Chisholm and Owen, 2003). Clearly, alterations of the *rdxA* gene are of prime importance but it has not been possible to identify a clear panel of point mutations which could explain the phenomenon. Moreover, research data still question the unequivocal linkage between *rdxA* mutations and the resistant phenotype. Additionally, other genes such as *frxA* also seem to be involved (Marais et al., 2003).

### THE ROLE OF *FrxA* NITROREDUCTASE IN METRONIDAZOLE RESISTANCE

Studies of the role in resistance of other enzymes potentially capable of reducing Mtz have yielded mixed results (Hoffman et al., 1996; Kaihovaara et al., 1998; Kwon et al., 2000b). The discovery of frameshift mutations in the gene *frxA*, encoding an NAD (P) H: flavin oxidoreductase, suggested the potential role of *frxA* in Mtz resistance in

*H. pylori* (Kwon et al., 2000b).

In a study by Kwon et al. (2000b), confirmation was obtained by transforming Mtz<sup>S</sup> strains with the mutated *frxA*, and by inactivation of this gene in sensitive strains. Furthermore, an *E. coli* resistant strain transformed with the *frxA* gene of an *H. pylori* Mtz<sup>S</sup> strain was capable of reducing Mtz, and was rendered sensitive to the drug. Several studies have demonstrated that inactivation of *rdxA* or *frxA* led to increase in the MIC for Mtz and simultaneous inactivation of both genes led to higher MIC than the inactivation of either gene (Kwon et al., 2000a), thus providing a possible explanation for the heterogeneity of resistance observed in the wide range of MIC for Mtz measured in *H. pylori* strains. These findings have motivated several studies designed to verify the role of *frxA* in Mtz resistance and a synergy of the interpretations of the data obtained for *frxA* and *rdxA*, revisiting the methods used in the studies of *rdxA* (Jeong and Berg, 2000; Kwon et al., 2001; Jeong et al., 2000, 2001). Interestingly, there is general agreement that both *rdxA* and *frxA* have roles in Mtz resistance, but there are some differences in two important points:

The roles of *frxA* and *rdxA* in Mtz resistance, and their relative contributions to the MIC for Mtz. In one analysis, deficiencies in either *rdxA*, *frxA* or both explained the resistant phenotype, even though with mutations in *frxA* alone yielding low-level Mtz resistance in clinical isolates, and moderate-to-high-level Mtz resistance in laboratory mutants with a disrupted gene (Jeong et al., 2000).

In contrary to this, it has been observed that *H. pylori* strains become resistant to Mtz in two ways: by inactivation of *rdxA* (type I) or by inactivation of both *rdxA* and *frxA* (type II), and rarely, if ever by inactivation of *frxA* alone, disruption of *rdxA* alone can produce Mtz resistance at all levels, and mutations in *frxA* can enhance the level of resistance of type I strains (Jeong et al., 2000; Jeong and Berg, 2000; Jeong et al., 2001). In spite of the common argument shared by both views, there are significant differences in the data and their interpretations; it has not been possible to identify a clear panel of mutations which could explain the phenomenon (Chisholm and Owen, 2003).

## CONCLUSION

Infection by Mtz or amoxicillin resistant strains is an important factor leading to treatment failure; subjecting all *H. pylori* clinical isolates to susceptibility testing most especially to Mtz is recommended (Megraud, 2004). If not possible, a program to survey the prevalence of resistance should be implemented in a given area or population. This increasing emergence of antimicrobial resistance in *H. pylori* treatment poses serious public health problems and is therefore necessary that new drug regimens be examined. Studies are currently underway in our group to study the role of Mtz in the treatment of *H. pylori* in our

environment (Tanih et al., 2010). We have already determined the prevalence of Mtz resistance and successfully amplified the two putative resistance genes (*rfxA* and *frxA*) implicated in Mtz resistance. Sequencing of these genes, which is our next line of experiments, is expected to add to existing global knowledge the types of mutations involved and consequently the roles of these genes in Mtz resistance in *H. pylori* strains circulating in the Eastern Cape Province of South Africa.

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