

Metronidazole resistance in *Helicobacter pylori*: magnitude, mechanism and implications for India

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Monotherapy with agents like metronidazole (Mtz) or clarithromycin, or dual therapy generally fail to eradicate *Helicobacter pylori* infection, whereas triple or quadruple drug combinations yield better results, particularly in patients whose strains are susceptible to all the agents used.^{1,2} Most anti-*H. pylori* regimens in current use consist of one or more antimicrobial agents (e.g., Mtz, clarithromycin, amoxicillin, tetracycline), proton pump inhibitors (PPI) and/or bismuth salts in various combinations, duration and dosages. But, even with the best available regimens, *H. pylori* eradication rates are usually below 90% and vary in different geographic regions. Many *H. pylori* strains show resistance to one or more antimicrobial agents *in vitro*; in general, such resistance correlates with failure of eradication.³⁻⁷

Mtz use and abuse in India

Mtz was developed in 1959 as a synthetic anti-parasitic agent for treatment of *Trichomonas vaginalis* infection. Its use increased considerably when it was found to be effective against *Entamoeba histolytica* and *Giardia lamblia*, and later against anaerobic bacterial infections.^{8,9} Mtz has been used widely in India since the 1970s; its use increased dramatically as it was prescribed as an antidiarrheal agent, especially following reports that iodoquinolones (hitherto used against diarrheal disease) might cause eye damage. Being relatively cheap and easily available, it is used for several diseases, besides its empirical self-administration for diarrheal illnesses. It is also used to treat irritable bowel syndrome and other functional bowel disorders; some of these patients are asymptomatic cyst passers, a condition incorrectly regarded by many physicians as warranting anti-amebic therapy.¹⁰⁻¹³ In fact, inappropriate Mtz use is far more common than its medically indicated use,^{14,15} and probably accounts for the high level of Mtz resistance observed in *H. pylori* strains prevalent in India. In addition, because Mtz in sub-inhibitory doses induces mutations,¹⁶ its widespread use may hasten the development of resistance to other drugs and evolution of virulence.

Mtz resistance – epidemiology and mechanism

Mtz is a key component of many combination regimens

against *H. pylori*.⁶ Unfortunately, however, many *H. pylori* strains are at least moderately resistant to Mtz. The frequency of resistant strains varies geographically, ranging from about 10% in Japan, where Mtz is not much used, to more than 50% in many developing countries, a pattern that generally correlates with local frequencies of Mtz use against other ailments.^{7,17-22} We found that about 90% of strains from Calcutta were Mtz-resistant (MtzR).²³ Others have found that over two-thirds of all strains from their regions (Lucknow, Mumbai) are MtzR.¹⁷ Such pre-existing Mtz resistance constitutes a major cause of failure of Mtz-based therapy against *H. pylori*.²⁴

Nitroimidazoles like Mtz are pro-drugs whose antimicrobial activity depends on activation by low redox active systems to biologically-active nitroso derivatives and hydroxylamines.²⁵ Our recent studies with *H. pylori* indicate that Mtz is activated by one, or in some strains two, nitroreductases, the two related genes named as *rdxA* and *frxA* (Fig 1); Mtz resistance results from loss of function mutations in one or both of these genes.

Molecular genetic analysis and basis of Mtz susceptibility and resistance in *H. pylori*

The first major insight into the mechanism of Mtz resistance came from identification of the gene *rdxA*, which encodes a nitroreductase that acts on Mtz, and demonstration of a nonsense mutation of this allele in an MtzR clinical isolate of *H. pylori*,²⁶ leading to a truncated non-functional nitroreductase protein. The *rdxA* gene from an MtzR isolate differed from that of its Mtz-sensitive (MtzS) relative by one or a few point mutations. This indicates that Mtz resistance can result from *de novo* mutation, rather than transfer of resistance genes from other pre-existing resistant strains,²⁶ an outcome confirmed with a dozen strains from France and North Africa.²⁷

We amplified *rdxA* gene from 50 MtzR strains from various geographic regions, including 12 from Calcutta, using polymerase chain reaction, and tested the ability of these PCR products to transform an MtzS strain to Mtz resistance. With each such MtzR *rdxA* DNA, transformation to MtzR was observed at frequencies of about 10⁻², i.e., nearly 100-fold higher frequency as compared

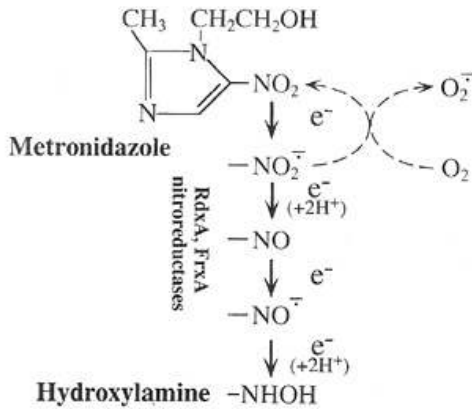


Fig 1: Mechanism of Mtz susceptibility and resistance in *H. pylori*. Nitroreductase enzymes encoded by chromosomal genes *rdxA* and *frxA* carry out series of electron transfers, ultimately resulting in hydroxylamine, which is bactericidal and also mutagenic. Resistance generally involves loss of function mutations in *rdxA*. In many strains (exemplified by strain 26695) this is sufficient to generate a moderately resistant (16 µg/mL) phenotype, with mutational inactivation of relatively quiescent *frxA* gene conferring increased resistance if strain is already mutant in *rdxA* (e.g., to 32 µg/mL) (*frxA* inactivation does not affect intrinsic susceptibility to Mtz in strains retaining functional *rdxA* genes). In other strains such as SS1, with more active *frxA* genes, resistance is achieved only if both *rdxA* and *frxA* have been inactivated

to transformation with control DNA from MtzS strains.²⁸ Further studies showed that most MtzS clinical isolates from various parts of the world, including India, could be rendered MtzR by simple *rdxA* inactivation (Fig 2). Thus, loss of function mutations in *rdxA* were associated with MtzR in nearly all cases studied, and conversely mutational inactivation of *rdxA* was sufficient to cause Mtz resistance in most MtzS strains. Further studies showed that expression of a functional *rdxA+* allele on a shuttle plasmid or a chromosomal site restored Mtz susceptibility to MtzR strains, and that expression in *E. coli* of the cloned *rdxA* gene similarly rendered this normally MtzR species MtzS.^{26,28}

Additional analyses indicated that simple *rdxA* inactivation was not sufficient to explain all the phenomena related to Mtz resistance.^{28,29,30} Inspection of the *H. pylori* genome sequence identified *frxA* as a gene whose inferred product had 25% amino acid sequence identity to *rdxA* and that might also act as a nitroreductase. Inactivation of *frxA* itself (essentially as in Fig 2), however, did not affect the intrinsic Mtz susceptibility. In contrast, *frxA* inactivation of *rdxA*-deficient mutants increased the level of resistance from 16 µg per mL to 32 µg per mL.²⁸ These results suggested that *frxA* might be less well expressed than *rdxA*. This inference was confirmed by RT-PCR and Northern blot analyses.^{29,30}

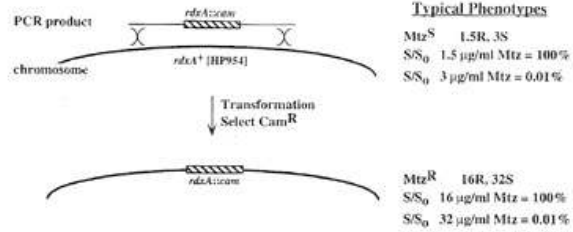


Fig 2: Generalized strategy for mutational inactivation of chromosomal genes in *H. pylori*. Mutated plasmid DNA or comparable mutant PCR product is mixed with young exponentially growing *H. pylori* cells (with or without enhancement by electric shock; “electroporation”), the mixture is incubated together for several hours or overnight, and then the bacteria are respread on medium containing chloramphenicol to select transformants. The structure depicted (the result of recombination with complete replacement of wild type allele by engineered mutant allele) is easily demonstrated by PCR.

In key to right, “1.5R 3S” designates ability of single cells to form colonies with near 100% efficiency on medium with 1.5 µg Mtz per mL, and significant (several orders of magnitude) reduction in capacity to form colonies on medium with 3 µg Mtz per mL. Similarly, 16R, 32S designates colony formation on medium with 16 µg Mtz per mL but not with 32 µg Mtz per mL. S/S₀ designates colony count on medium with indicated Mtz concentration, relative to that on Mtz-free control medium (survival)

Direct biochemical tests indicate that there are numerous nitroreductases in *H. pylori*,²⁶ and it is likely that some of them also act on Mtz, albeit inefficiently. Supporting this view are findings that although complete inactivation of *rdxA* and *frxA* results in resistance to just 32 µg of Mtz per mL under our assay conditions, many clinical isolates are resistant to at least 64 µg of Mtz per mL. In addition, it is easy to generate new hyperresistant mutants of standard *rdxA*- and *frxA*-null mutant strains that tolerate at least 64 µg of Mtz per mL in laboratory experiments. Separate studies on the mutagenicity of Mtz¹⁶ support the view that these Mtz-hyperresistant derivatives have additional mutations in other genes that allow Mtz activation, essentially as in Fig 1.

Certain MtzS strains become MtzR only if both *rdxA* and *frxA* are inactivated.^{28,29,30} The existence of this second type of MtzS strain was suggested by findings of such strains that mutated to MtzR only at extremely low frequencies, in the range of ~10⁻⁸ instead of 10⁻⁴. Such differences in frequency suggested that inactivation of two, rather than just one, genes was required to achieve resistance. Parallel transformation experiments using defined *rdxA*::*cam* and *frxA*::*kan* knockout mutant DNAs (and selection only for CamR or KanR, as appropriate) showed that Mtz resistance in these special “type II” strains resulted from inactivation of both genes.

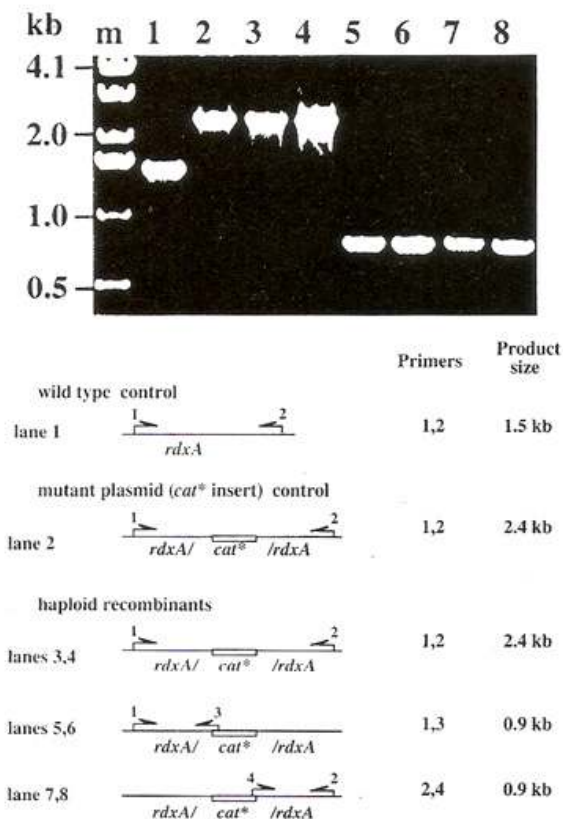


Fig 3: PCR tests to document allelic replacement. In example shown, *rdxA*-containing gene segment defined by PCR primers 1 and 2 is 1.5 kb long. Mutating it by simple insertion of chloramphenicol acetyltransferase (*cat*, or *camR*) gene cassette into this segment lengthens it by ~900 bp. This is seen by PCR using starting mutated plasmid from *E. coli* (lane 2), and also *CamR* transformants of the originally *CanS* *H. pylori* strain (lanes 3, 4). The exact position of *cat* cassette can also be determined or verified by PCR using appropriate combinations of *cat*- and *rdxA*-specific transformants (lanes 5-8)

Inactivation only of *rdxA* or only of *frxA* did however increase the ease of generating new MtzR mutants from $\sim 10^{-8}$ to $\sim 10^{-4}$.

These type II strains accounted for only about 10% or fewer MtzS strains in several parts of the world (none has been found among the seven MtzS Indian strains tested). It is noteworthy, however, that among type II strains are SS1 and X47,³⁰ two strains with the unusual ability to infect mice. Several workers have focused exclusively on type II strains;^{31,32} this is possibly because of the stability of MtzS phenotypes of these strains.³⁰ Northern blot analyses showed that *frxA* mRNA levels are much higher in type II MtzS strains than in type I strains.³⁰ Inspection of sequences upstream of *frxA* does not support models in which type I vs. type II strains differ in strength of the *frxA*

promoter. Rather, we hypothesize that this difference reflects differences in a metabolic regulatory molecule (repressor or inducer) that affects the transcription of *frxA* and perhaps other genes as well. In addition, we note that mouse-colonizing *H. pylori* strains are rare. The coincidence that each of the two tested to date is type II (*frxA* and *rdxA* both active) will make it interesting to test whether interstrain differences in the regulation of *frxA* expression affect parameters such as the capacity for growth in mice, and by extrapolation, parameters in human infection as well.

It should be noted that the genetic basis of Mtz resistance in *H. pylori* – loss of function mutations in normal chromosomal genes – differs markedly from that of most other forms of drug resistance in other bacterial species. We have found no evidence that Mtz resistance results from the gain of new resistance genes on R factor plasmids, transposons or other mobile DNA elements, as is so common with resistance to other antimicrobial agents. Unfortunately, this finding, coupled with the fact that mutations almost anywhere in *rdxA* or *frxA* can inactivate these genes, means that it is not worthwhile to try to develop a PCR-based diagnostic test for Mtz resistance, equivalent to those developed recently for clarithromycin resistance.

Mutagenicity of Mtz

Studies of mechanisms of Mtz susceptibility and resistance predicted that Mtz activation would lead to hydroxylamine and related compounds that are mutagenic (Fig 1) as well as bactericidal.^{26,28} This inference was tested by measuring frequencies of mutation to rifampicin resistance (*Rif^R*) in cells grown on medium with various levels of Mtz. The results showed that exposure of *H. pylori* to levels of Mtz sufficient to kill a fraction of the cells also caused a 10- to 100-fold increase in frequency of *Rif^R* mutants among survivors. Mtz-induced mutation was evident in strains with null mutant alleles of *rdxA* and *frxA* nitroreductase genes, as well as in their wild-type fully-MtzS ancestor, in each case at Mtz concentrations that partially inhibited growth. This implied that there are additional nitroreductases that can activate sufficient Mtz for antibacterial activity (mutagenicity or killing) when Mtz concentrations are high. A mutagenic effect of Mtz was also seen in *E. coli* carrying a cloned *H. pylori* *rdxA* gene.¹⁶ These results indicate that recurrent exposure of *H. pylori* to Mtz during chronic infection (e.g., when used in other infections) induces as well as selects for resistance. The mutagenicity of Mtz also probably contributes to the abundance of MtzR strains among clinical isolates in many populations. These studies further emphasize that inadvertent exposure of resident *H. pylori* strains to Mtz in regimens that do not eradicate infection may speed up the development of secondary drug resistance

(e.g., to clarithromycin), the adaptation of these strains to host defences, and perhaps also the evolution of virulence.

Superimposed on base substitution mutagenesis seen in studies of Rif resistance, the products of Mtz activation also induce single DNA strand breaks, high levels of which may cause most of the toxicity of Mtz to *H. pylori*. Lower (sublethal) DNA strand breakage may stimulate recombination between duplicate and divergent sequences within a genome, and perhaps also interstrain gene exchange during mixed infection. Such recombination events may also favor the development of host-specific adaptation and the evolution of virulence. Many species of the normal intestinal bacterial flora have enzyme systems that could activate Mtz, and hence these bacteria may be susceptible to mutagenesis on sublethal exposure to this drug.

It was important to test for possible effects of inadvertent Mtz exposure on host cells in proximity to nitroreductase-producing gastrointestinal microbes. A "bystander" experiment was developed for this purpose. A high-density mixture of MtzS and MtzR bacteria was grown on a medium with sufficient Mtz to induce mutation in the susceptible strain, and the level of newly induced mutations in both strain types was quantitated. Mutations were observed only in the MtzS bacteria.¹⁶ Consistent with this, Touati *et al*³³ did not find any evidence of Mtz-induced mutation in gastric epithelial cells of *H. pylori*-infected mice. This may be explained by the fact that Mtz is activated by intracellular bacterial enzymes, and its mutagenic derivatives may be so highly active that they are consumed before diffusing into neighboring cells.¹⁶ This result is comforting, since it indicates that Mtz activation by resident *H. pylori* strains (or other microbes) probably does not directly affect the frequency of mutation or neoplastic transformation in the human epithelial tissues near which these bacteria reside.

Technical note

Two considerations indicate that special care is needed when Mtz susceptibility and resistance are to be scored accurately. One stems from the mutagenic effects of Mtz activation just discussed. The second emerged while seeking to resolve disagreements in the literature,^{28,32} which we interpret as probably reflecting inadvertent use of strains that require inactivation of both *rdxA* and *frxA* ("type II") to achieve a resistant phenotype.³⁰

Traditionally, drug susceptibility has been determined by monitoring zones of inhibition of growth in dense bacterial lawns (starting with at least 10^7 cells per 150 mm diameter petri plate) on which have been placed filter paper disks containing fixed concentrations of drugs of interest ("disk diffusion"), or "E test" strips that

contain concentration gradients of the drug. An alternative method involves monitoring growth vs. non-growth after spotting dense bacterial suspensions (10^6 cells) on media with different fixed concentrations of drug ("agar dilution"). We cannot recommend using either of these methods for reliable scoring of Mtz resistance, at least in the setting of a research laboratory. Rather, we recommend spotting bacterial suspensions of higher dilution (e.g., approximately 10^3 viable bacterial cells) on Mtz-containing and Mtz-free (control) media; a strain is then considered resistant to the given level of Mtz only if the numbers of colonies on the two media are equivalent.

The data in Fig 4 illustrate that valuable insights can be gained from estimating frequencies of mutants resistant to particular levels of Mtz. Such estimation can be done efficiently and with sufficient accuracy by spotting aliquots of a series of 10-fold dilutions of bacterial suspension on Mtz-containing and Mtz-free medium. When MtzR mutants are very rare (e.g., one per 10^7 cells or less), suspensions should be spread over larger surfaces, to avoid inhibitory effects of crowding on bacterial colony formation.

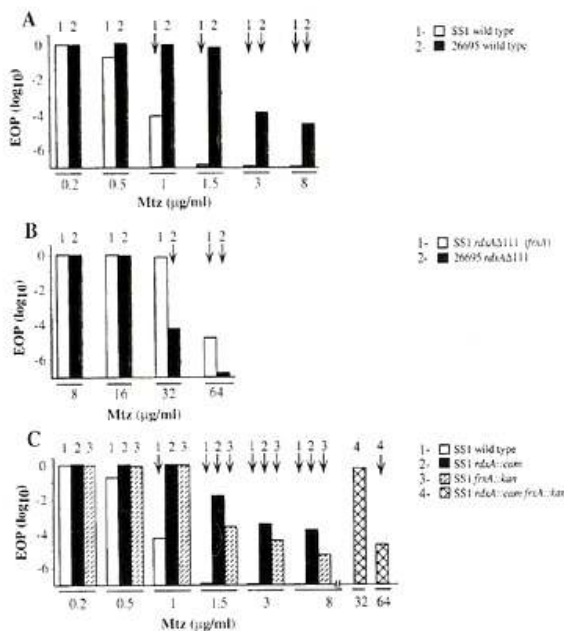


Fig 4: Metronidazole sensitivity profiles and impact of *rdxA* and *frxA* mutation on strains 26695 (type I strain) and SSI (type II strain). In SSI strain, both *rdxA* and *frxA* need to be inactivated to produce resistant phenotype²⁹

The value of such careful titrations was illustrated by re-examination of an agar dilution-based study,³¹ which had led the authors to conclude that inactivation of either *frxA* or *rdxA* is sufficient to cause MtzR. We believe that this conclusion was in error,²⁹ and our reconstruction experiments have now shown that this group had used type II strains (in which inactivation of both nitroreductase genes was needed to obtain an MtzR phenotype).³⁰ Inactivation of either gene alone increased the frequency of new MtzR mutant colonies in cultures by approximately 10⁴-fold (from about 10⁻⁸ to about 10⁻⁴) (Fig 4). They had found growth of their mutants on Mtz-containing agar after inactivation of just one nitroreductase gene, but this appears to have been due to their spotting enough cells (>10⁵ cells) to ensure (with *frxA* or *rdxA* single gene knockout strains) that each spot would contain many new MtzR mutants. The growth of such new MtzR mutants was then scored as full resistance, even though tests with more dilute suspensions would have shown that more than 99.9% of the cells spotted were MtzS.

Where do we go from here?

Molecular studies have shown that *H. pylori* is genetically an extremely diverse species. Variations in bacterial and host factors may explain why this microbe causes various overt diseases in some infected individuals, while maintaining a more benign, and possibly symbiotic, relationship in others. The remarkable persistence of this infection in the face of antimicrobial defense mechanisms must reflect often-subtle interactions between the products of many different genes, whose expression is tightly controlled but flexible, and which are susceptible to mutations. Subtle quantitative differences in the expression of these genes may become important in chronic infection, lasting for time periods extending over decades. The disease phenotypes that clinicians deal with may thus represent the end-point of years of accumulated damage. Studies on the mechanism of Mtz susceptibility and resistance in relation to population genetics, evolution and interaction of this organism with the human host will advance our understanding of this infection further.

Mtz resistance in *H. pylori* also provides some general lessons about the relationship of microbes and antimicrobial drugs. Unindicated or casual administration of antimicrobial agents such as Mtz for one group of infections leads to the development of resistance to that drug in pathogens that had not been targeted and against which it possesses only suboptimal activity. In the case of antimicrobials like Mtz, which can be mutagenic, such exposure may also speed up mutation and gene exchange at other loci, and thereby facilitate the emergence of secondary resistance to other drugs and enhance the virulence of various pathogens. For such

drugs, which induce as well as select for resistance, it is important that potential mutagenic effects and phenotypic instabilities be recognized; this can be achieved by using cultures that have been diluted sufficiently to allow scoring of colonies formed by single cells.

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