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EDITORIAL

Mechanisms of *Helicobacter pylori* antibiotic resistance: An updated appraisal

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Abstract

Helicobacter pylori (*H. pylori*) antibiotic resistance is the main factor affecting the efficacy of the current eradicating therapies. The aim of this editorial is to report on the recent information about the mechanisms accounting for the resistance to the different antibiotics currently utilized in *H. pylori* eradicating treatments. Different mechanisms of resistance to clarithromycin, metronidazole, quinolones, amoxicillin and tetracycline are accurately detailed (point mutations, redox intracellular potential, pump efflux systems, membrane permeability) on the basis of the most recent data available from the literature. The next hope for the future is that by improving the knowledge of resistance mechanisms, the elaboration of rational and efficacious associations for the treatment of the infection will be possible. Another auspicious progress might be the possibility of a cheap, feasible and reliable laboratory test to predict the outcome of a therapeutic scheme.

INTRODUCTION

The discovery of *Helicobacter pylori* (*H. pylori*) infection and its role in different diseases from chronic gastritis to gastric cancer has radically changed the management of patients with this condition. Unfortunately, the goal of achieving a cure in all treated patients at the first therapeutic approach, as generally occurs for common infective diseases, has not been achieved for *H. pylori*. Indeed, it has been immediately evident that only a few antibiotics are active against such a bacterium in the acidic environment of the stomach.

The initial susceptibility of *H. pylori* to both clarithromycin and imidazoles, key drugs for triple first-line therapies, has progressively been undergoing a marked reduction and the eradication rate following therapy regimens including these antibiotics is decreasing[1]. Similarly, the low *H. pylori* resistance rate towards quinolones, mainly used for second-line therapy, observed in the past has increased during the last decade, whilst both amoxicillin and tetracycline resistance rates seem to have remained low[2].

Alternative molecules, such as furazolidone, bismuth salts and rifabutin are not available worldwide and they are not free of significant side-effects. All these observations highlight the crucial role of antibiotic resistance in the management of *H. pylori* infection[3,4]. Therefore, the knowledge of resistance mechanisms may contribute to elaborate more rational antibiotic combinations with the aim of improving treatment success.

We reviewed the mechanisms of *H. pylori* antibiotic resistance towards the drugs mainly used, including clarithromycin, metronidazole, levofloxacin, amoxicillin and tetracycline.

CLARITHROMYCIN

Clarithromycin remains the currently available most powerful antibiotic against *H. pylori* with minimal inhibitor concentrations (MICs) being the lowest as compared to the other molecules. Indeed, MIC values as low as 0.016-0.5 mg/L are generally reported, antibiotic resistance being recognized with MIC values ⩾ 1.0 mg/l (range: 2-256 mg/L)[5,6]. The bacteriostatic activity of clarithromycin depends on its capacity to inhibit the protein synthesis by binding to the 50S bacterial ribosomal subunit. Extensive studies by PCR-based tools have demonstrated that point mutations in the peptidyltransferase region encoded in domain V of 23S rRNA are responsible for the bacterial resistance to macrolides[7]. These mutations are able to inhibit the binding between clarithromycin and the ribosomal subunit dedicated to the specific antibiotic related protein synthesis[7,8]. The more frequent mutations associated with clarithromycin resistance are the transition adenine to cytosine in 2143 and 2142 positions of rRNA, whilst the substitution of adenine with cytosine in 2142 position is less frequent. These mutational events are responsible for more than 90% of clarithromycin resistance in developed countries[9]. In detail, the mutation at position 2143 seems to be associated with different resistance levels rather than an on/off behavior, with MIC values widely ranging from 0.016 to 256 mg/L. Conversely, the mutations at position 2142 are associated with more restricted MIC values, close to 64 mg/L[10,11]. Of note, we found that the presence of the A2143G point mutation, rather than the A2142G or A2142C mutation, markedly reduces *H. pylori* eradication rate[12]. These data should suggest that a mutational event detected *in vitro* does not precisely predict *in vivo* results[13].

Several other point mutations have been identified such as A2115G, G2141A, T2117C and T2182C, T2289C, G224A, C2245T, C2611A. Besides the low frequency, the clinical relevance of the A2115G, G2141A, T2117C T2289C, G224A, C2245T mutation is still not proven, their role not being consistently reported[14,15], whilst the T2182C and C2611A have been associated with low resistance levels[16,17].

Another relevant mechanism for macrolide resistance is ascribed to the efflux pump system. At least 5 conserved families of drug efflux mechanisms are associated to bacterial species, including Small Multidrug Resistance, Multidrug and Toxic Compounds Extrusion proteins, the Major Facilitator Superfamily, the ATP-Binding Cassette Superfamilies and the Resistance-Nodulation Cell Division[18]. The Resistance-Nodulation-cell Division (RND family) is responsible for macrolide intrinsic resistance in several Gram negative bacteria and it has been recently proposed also for *H. pylori*. In detail, it has been observed that 4 RND gene clusters (HP0605-HP0607, HP0971-HP0969, HP1327-HP1329, HP1489-HP1487) in the efflux pump system play a role in promoting multidrug *H. pylori* resistance[19]. These systems of excretion can be inhibited by the administration of specific Efflux Pump Inhibitors (EPI), such as Phe-Arg-β-naphthylamide (PAβN). Indeed, EPI-administration is associated with a relevant intracellular entrapment of antibiotic and a significant decrease of MIC values. In detail, a dose-dependent reduction of MIC values in 15 rRNA point mutate resistant strains has been demonstrated by using PAβN. Increased intracellular antibiotic concentrations able to compensate the reduced drug affinity for the mutate ribosomal site have been postulated as a possible mechanism. This effect is constantly associated with the HP0605-HP0607 cluster gene. Interestingly, a different effect of EPI administration on MIC values is observed between susceptible and rRNA mutate strains. A possible explanation is that, in susceptible strains, clarithromycin binds preferentially to the ribosomal subunits rather than the efflux pumps. Consequently, the excretive activity of efflux pumps becomes irrelevant, the effect of PAβN on MIC value modifications vanishing. On the contrary, in the rRNa mutate strains, clarithromycin is preferentially excreted by the efflux pumps because of its low affinity with the mutate ribosomal site, with the more relevant impact of efflux pumps inhibition by PAβN on MIC values[20]. Based on these findings, it is reasonable to hypothesize that PAβN (or PAβN-like molecules) administration could improve the eradicating efficacy of the clarithromycin-based therapies by increasing its intracellular entrapment.

The possible interaction between the RND efflux pump system and proton pump inhibitors (PPIs) due to structural analogies is also of clinical interest. Besides the deep gastric acid inhibition, PPIs may inhibit the activity of bacterial efflux pumps, similar to EPI drugs. Interestingly, MIC values of clarithromycin, as well as metronidazole, amoxicillin and furazolidone, are decreased 4-fold and 3-fold in *H. pylori* multi-resistant strains by using rabeprazole and pantoprazole respectively, whilst no significant effect is observed with omeprazole, esomeprazole and lansoprazole[21]. These differences should be considered when choosing the PPI in eradication regimens.

METRONIDAZOLE

Mechanisms of metronidazole resistance have been extensively investigated and new information has been recently obtained[22]. In *H. pylori* strains, MIC values of 0.5-2 mg/L are reported, antibiotic resistance being recognized with MIC values ⩾ 8 mg/L (range: 16-128 mg/L)[5,23]. Bactericide activity of metronidazole depends on the reduction

of its nitro-groups in anionic radicals, nitroso-derivates and hydroxylamines which are able to damage the DNA-helicoidal structure. These reactions of reduction are strongly dependent on the intracellular redox potential of components of electron transport chain which needs to be effectively negative. In detail, electrons are produced by the Pyruvate Oxido Reductase complex (POR) and are passed to either ferredoxin or flavodoxin which, in turn, reduce other molecules as metronidazole[24]. This process is particularly active in anaerobe species which are highly susceptible to metronidazole. The acquisition of antibiotic resistance depends on the reduction or abolition of activity of the electron carriers. On the contrary, the high intracellular redox potential of aerobe species prevents the metronidazole reduction-activation and is responsible for the intrinsic resistance of these bacteria[25].

A further action mechanism of metronidazole against anaerobe bacteria in aerobe conditions consists in the production of oxygen-free radicals. In this case, the oxygen acts as the last acceptor of electrons from reduced metronidazole, leading to the regeneration of the parent inactive antibiotic (futile cycle) and the production of oxygen-free radicals which are toxic for DNA structure[26]. In resistant strains, such a bactericide effect is neutralised by a catalase-superoxide dismutase system with final water production. This enzymatic system increases its activity in the presence of metronidazole[25,27]. The intracellular redox potential/oxygen tension also plays a relevant role in the resistance of microaerophilic species, such as *H. pylori*, in which catalase/superoxide dismutase is not present. Of note, the pre-exposure of *H. pylori* resistant strains to anaerobic conditions is associated with a loss of resistance and restoration of metronidazole susceptibility[28]. In this event, a NADH oxidase acts as an 'oxygen scavenger' assuring low redox potential/oxygen tension and maintaining the antibiotic in the active form. An inactive mutate NADH oxidase and intracellular higher redox potential/oxygen tension have been found in *H. pylori* resistant strains[29].

Different mutations involving the rdxA gene which encodes for an oxygen insensitive NADPH nitro-reductase have been identified in metronidazole resistant strains. These mutations are recognized as the main mechanism conferring metronidazole resistance in *H. pylori*[30]. In the susceptible strains, NADPH nitro-reductase reduces several compounds, including metronidazole, by 2 electrons transfer and generating toxic nitro-derivates for DNA. For example, the activation of NADPH in E. coli, which is usually resistant to metronidazole, generates susceptible strains. Besides these mutations, other and more complex genetic events (insertions and deletions of transposons, missense and frameshift mutations) could be simultaneously present in the rdxA gene. These events complicate metronidazole resistance assessment by bio-molecular tools[31-33].

More recently, the inactivation of other reductases, encoded by genes as frxA (for NADPH flavin oxidoreductase) and frxB (for ferredoxin-like enzymes), has been investigated. There is evidence that these point mutations are able to increase bacterial resistance exclusively in the presence of rdxA gene mutations[23,34-35]. Indeed, the rare cases of metronidazole resistant strains in the absence of rdxA mutations have been attributed to mutations involving genes outside the rdxA which can, in turn, down-modulate its expression[36].

A role for a complex efflux system responsible for metronidazole in *H. pylori* strains has been recently reported. In detail, the presence of Outer membrane Efflux Proteins (OEP) in *H. pylori* which interact with several intracellular translocases and regulate secretion of different antibiotics has been found. Of note, the inactivation of 2 OEPs (HP0605 and HP0971) in a double-knockout mutant strain significantly increased susceptibility towards metronidazole, supporting a significant role for this efflux pump system in metronidazole resistance[19].

LEVOFLOXACIN

The use of levofloxacin for *H. pylori* eradication is increasing worldwide because of its role in 'rescue therapy' regimens following the failure of clarithromycin-based treatments. MIC values of 0.25-0.50 mg/L are generally reported, antibiotic resistance being recognized with MIC values $\geqslant$ 1 mg/L (range: 4-32 mg/L)[37,38]. Fluoroquinolones exert a dose-dependent bactericide effect by binding the sub-unit A of DNA gyrase (topoisomerase Ⅱ), an essential enzyme for the maintenance of DNA helicoidal structure. In susceptible strains, levofloxacin stops DNA and, at high doses, even RNA synthesis. Surprisingly, when the dose is further increased, quinolones become bacteriostatic agents.

Point mutations in Quinolones Resistance-Determining Region (QRDR) of gyrA prevent binding between the antibiotic and the enzyme, conferring antibiotic bacterial resistance[39]. Different studies found the involvement of the following *H. pylori* loci: (1) position 91 (Asp91Gly, Asn, Ala, or Tyr); (2) position 87 (Asn87Lys); and (3) position 88 (Ala88Val)[39-41]. Mutations in both 91 and 87 position have been observed in the 100% of levofloxacin resistant isolates and a new mutation consisting in the substitution of Asn with Tyr in position 87 has been additionally identified[37]. Rare mutations involve the position 86 (Asp86Asn) which, in turn, is usually associated with the mutations at 87 and 91 positions[37], lowering its role on MIC values. Similarly, the constant association between the gyrB with the gyrA 87-91 mutations most likely minimize the role of the gyrB mutations in quinolone resistance[42]. Indeed, gyrA and gyrB gene mutations involvement in levofloxacin resistance has been observed in 83.8% and 4.4% respectively[43].

Other factors involved in levofloxacin resistance are an amino acidic polymorphism in the codon 87 of gyrA, consisting in the presence of different asparagyne-threonine residues. In detail, the complete sequencing genome of 2 strains, i.e. the 26695 and the J99, allowed identifying the presence of threonine in the J99 strain and asparagyne residues in the 26695 strain associated with a higher antibio-

tic susceptibility. Interestingly, the presence of threonine residue at 87 codon is also conserved in other. Helicobacter types thus indicating the possibility of a "philogenic" evolution of Helicobacter species[37].

AMOXICILLIN

Amoxicillin is a β-lactam antibiotic included in all current therapeutic regimens for *H. pylori* eradication[4]. MIC values ranging from 0.06 to 0.25 mg/L are generally reported in susceptible strains, antibiotic resistance being recognized with MIC values ⩾ 1 (range: 1-8 mg/L)[5,44]. Amoxicillin acts by interfering with the peptidoglycan synthesis, especially by blocking transporters named penicillin binding proteins (PBP)[5]. This drug has been the first antibiotic used in *H. pylori* therapy because of a presumed absence of resistance. Nevertheless, the evidence of stable amoxicillin resistant strains, with a MIC of 8 mg/L, has been reported[45]. Moreover, an instable amoxicillin resistance has been described in *H. pylori* isolates, the resistance being peculiarly lost upon freezing the culture at -80°C. Such an unusual condition has been defined as 'amoxicillin tolerance' rather than resistance[46].

Different mechanisms have been invoked in the stable amoxicillin resistance. The Penicillin Binding Proteins (PBPs) are enzymes involved in the synthesis of the peptidoglycan layer of the bacterial wall by a glycosyl transferase-acyl transpeptidase activity. This enzymatic activity is located in the C-terminal region, in 3 distinct motifs ($SKN_{368-371}$, $SNN_{433-435}$, $KTG_{555-557}$) of PBPs. The first motif occupies a central position in the catalytic cleft whereas second and third motifs are dislocated on the outside. PBP-1, PBP2, PBP3 are reported as high molecular PBPs whilst PBP4 is reported as low molecular protein. The β-lactam binding to PBPs motifs leads a bactericide effect by synthetic interruption of the peptidoglycan layer, as well as an osmotic bacterial shock. Production of β-lactamase, i. e. the main mechanism of penicillin resistance in other bacteria, has been consistently found to be inactive in *H. pylori*[47-49].

Several investigations indicate that multiple point mutations in pbp1 gene are the major mechanism of amoxicillin resistance, leading to a loss of affinity between amoxicillin and PBP-transpeptidase[44,50]. It has been observed that the Ser_{414} to Arg substitution, adjacent to the SKN motif in PBP1, is responsible for amoxicillin resistance with a significantly increased MIC (> 0.5-1 mg/L)[49]. Another study reported the substitution of Asn_{562} aminoacid with a Tyr residue in proximity to KTG motif of PBP1. Such a point mutation is able to confer high resistance to all strains *in vitro* and is considered the main mutation conferring resistance. Other substitutions (Ala_{369} to Thr, Val_{374} to Leu, Leu_{423} to Phe, Thr_{593} to Ala) not constantly associated with Asn_{562}-Tyr seem to play an additive role in increasing MIC values of the resistant strains similarly to point mutations in PBP2, in PBP3 and PBP4[48,51]. Interestingly, *H. pylori* resistant strains obtained by transformation *in vitro* of susceptible naive strains, exhibit MIC values 5-10 fold lower than naïve resistant strains[49], suggesting that several and concomitant mechanisms are probably involved in conferring the high levels observed in natural antibiotic resistance.

The outer bacterial membrane constitutes a first barrier for accounting for an intrinsic and not specific resistance. Indeed, the variable fluidity of lipopolysac caridic layer is able to limit the diffusion of several lipophilic compounds. Recent findings indicate that "porin" narrow channels, encoded in *H. pylori* by hopB and hopC genes, regulate the penetration of small solutes. Point mutations in hopB and a deletion in hopC gene are associated with reduced amoxicillin accumulation in all naïve mutant and transformed strains, with a consequent increase of MIC values (250 mg/L for hopB gene and 125 mg/L for hopC gene)[44]. When point mutations either in hopB or in hopC are associated with mutations in PBP1 gene (triple mutants), a further increase of MIC values (400 mg/L) is observed. These findings could suggest that channels and PBP1 mutations are factors able to support the resistance[44,52]. It has been reported that several encoding "porin" genes could be over-regulated (omp25 porin gene) or down-regulated (omp32 porin gene) by antibiotic exposure leading to alterations in the membrane permeability. Comparable alterations of permeability are likely associated to variable expression of genes involved in import/export/binding of metals[53].

Finally, the efflux of molecules is a frequently reported event in bacteria as a protective process from the toxic effect of environmental compound accumulation. Nevertheless, it seems unlikely that amoxicillin resistance is sustained by these mechanisms because amoxicillin shows a very low hydrophobicity which is an indispensable requirement for substrates of multidrug efflux pumps[54,55].

TETRACYCLINE

Tetracycline is a fundamental antibiotic in quadruple regimens for *H. pylori* eradication. MIC values 0.25-2 mg/L[56] are generally reported, antibiotic resistance being recognized with MIC values ⩾ 4[5]. Bacterial resistance towards such a drug, although still rare, appears to be increasing. Tetracycline acts as a bacteriostatic against either Gram positive or Gram negative species by inhibiting codon-anticodon link at level of 30S ribosomal subunit and blocking the attachment of aminoacyl-tRNA to the acceptor site. Resistant strains show wide range of MIC values (2-256 mg/L). Recent studies have identified 2-6 possible sites for antibiotic-ribosome interaction at high affinity, whilst several biochemical investigations reported multiple, likely hundreds, sites at low affinity[57,58]. Simultaneous triple point mutations from the 965 to 967 position in loop of helix 31 - i.e. the crucial part of primary acceptor site (site P) is recognized as the major mechanism of tetracycline resistance. The main point mutation is a substitution of an AGA with a TTC triplet[59,60] and it reduces the affinity of 24%-52%[61]. Levels of resistance are proportional to the number of changes in the AGA 965-967. Single and double point mutations are associated with low and inter-

Table 1 Minimal inhibitory concentrations of the different antibiotics (left side) and main mechanisms of resistance induction for each antibiotic (right side)

Antibiotics	MIC in susceptible strains	MIC in resistant strains	Mechanisms of resistance
Clarithromycin	0.016-0.50 mg/L	⩾ 1 mg/L (2-256 mg/L)	Point mutations in rRNA Efflux pumps system (RND family)
Metronidazole	0.5-2 mg/L	⩾ 8 mg/L (16-128 mg/L)	Mutate NADPH reductase Mutate NADH oxidase Other efflux pumps
Levoxacin	0.25-0.50 mg/L	⩾ 1 mg/L (4-32 mg/L)	Point mutations in QRDR of gyrA gene Polymorphism in 87 codon of gyrA
Amoxicillin	0.06-0.25 mg/L	⩾ 1.0mg/L (1-8 mg/L)	Point mutations in pbp genes Point mutations in hpB and hpC genes Point mutations in omp25 and omp32 porin genes
Tetracycline	0.25- 2 mg/L	⩾ 4 mg/L (2-256 mg/L)	Point mutations in primary binding Tet-P site Point mutations in Tet-4 secondary inding site Alterations by oxidoreductase

MIC: Minimal inhibitory concentrations.

mediate MIC values whilst high resistance levels are observed in the presence of a triple mutation from AGA 956 to 957. In detail, among the possible mutations in AGA triplet, the substitution involving the Guanine in the central position is associated with higher MIC values, suggesting that purine base plays a more consistent role in the configuration of the primary site. Purine-rich sequences in the loop of helix 31 are more frequently observed in susceptible strains, whilst pyrimidine-rich loops are in the resistant strains. It is possible that pyrimidine-rich sequences in helix 31 are not compatible with tetracycline conformation, leading to a decreasing affinity[60]. Another study found a deletion of G942 in all resistant strains. This guanine base is located in Tet-4 site, in proximity of primary *P* site. Since the affinity of tetracycline for Tet-4 site is significantly lower than those for primary *P* site, Tet-4 may be considered an accessory site for the antibiotic activity in susceptible strains. Therefore, the loss of affinity due to a deletion G492 in such a site may exert a marginal role in the increasing bacterial resistance[62] (Table 1).

Serial exposures of susceptible strains on antibiotic are unable to confer resistance whereas the exposition to mutate resistant DNA leads easily transformation. These data indicate a horizontal spread of mutate genome rather than a vertical or parental transformation[62]. Of note, resistant transformants from susceptible strains exhibit intermediate MIC values between parental susceptible strains (4-8 mg/dL) and natural resistant strains (> 32 mg/dL)[61,63]. Such a finding would indicate that factors other than point mutations in 30S ribosomal subunit may work in concert for the tetracycline resistance development. Indeed, resistant strains without point mutations have been observed[61].

Another mechanism of tetracycline resistance is attributed to ribosomal protection by the soluble protein Tet (O). Such a protein removes the antibiotic from ribosome preventing the arrest of protein synthesis[64]. In addition a chemical modification of tetracycline by an oxidoreductase NADP-dependent may interfere in the binding between antibiotic and the ribosomal site[58].

Decreased membrane permeability and a reduced intracellular accumulation of tetracycline were observed in tetracycline resistant strains, which are also cross-resistant to amoxicillin. This finding suggests an identical profile of outer protein for both antibiotics. Finally, the possible role of a specific tetracycline efflux pumps system affecting intracellular drug concentrations has been investigated with discordant results. Indeed, pre-exposure of resistant strains to a de-energizing agent such as cyanide m-chlorophenylhydrazone (CCCP) leads to variable reductions of MIC values[21,58]. However, the role of either specific pumps unaffected by CCCP or a variable expression of not specific multidrug efflux pumps, such as the MexAB-OprM system, cannot be excluded and should be further investigated[62,63].

CONCLUSION

The amount of data we have reported in this editorial reveals that the knowledge about *H. pylori* antibiotic resistance is a topic with a rapidly and constantly increasing interest. Future perspectives hope for new information aimed at elaborating novel and rational antibiotic associations very effective for *H. pylori* infection cure in clinical practice. Another "fascinating challenge" could be a feasible, cheap and not time consuming laboratory investigation able to predict the treatment outcome and address the best therapeutic choice case by case.

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