Antibiotic Resistance in Helicobacter pylori

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H. pylori is a “fastidious” microorganism; culture methods are time-consuming and technically challenging. The advent of molecular biology techniques has enabled the identification of molecular mechanisms underlying the observed phenotypic resistance to antibiotics in H. pylori.

Keywords: H. pylori antibiotic-resistance ; molecular methods ; next generation sequencing ; whole genome sequencing

1. Introduction

Antibiotic resistance is an increasing problem for *H. pylori* eradication therapies. Standard antimicrobial susceptibility testing (AST) can be used to prescribe appropriate therapies but is currently recommended only after the second therapeutic failure [1] because it requires an invasive test such as endoscopy, and culture methods are challenging and time-consuming [2]. The advent of molecular biology techniques has enabled the identification of molecular mechanisms underlying the observed phenotypic resistance to antibiotics in *H. pylori.*

2. Clarithromycin

Clarithromycin (CLA) is a bacteriostatic antibiotic, it belongs to the group of macrolides that bind reversibly to the peptidyl transferase loop of domain V of the 23SrRNA, interfering with protein elongation and blocking protein synthesis. Compared with other macrolides, clarithromycin is better absorbed in the gastric mucus layer and is more acid-stable [3]. Investigating clarithromycin resistance with traditional molecular methods, a high number of mutations have been detected in the macrolide-binding site of 23SrRNA gene, i.e., A2142G, A2142C, A2143G, T1942C, G1939A, C2147G, G2172T, T2182C, A2116G, A2144G/T, A2115G, G2111A, T2717C, T2289C, G2224A, and C2245T (in accordance to Taylor et al. numbering) [4][5][6]. In general, NGS has confirmed that clarithromycin resistance is mainly based on point mutations in nucleotide positions 2142 (A to G/C) and 2143 (A to G) in the 23SrRNA gene [7][8][9]. The commercially available qPCR (quantitative Polymerase Chain Reaction) assays for the detection of A2142C/G and A2143G, would be sufficient to monitor CLA resistance in clinical practice; but only a limited number of nucleotide position can be analysed with this tool, while whole genome sequencing (WGS) delivers a more comprehensive description of resistance determinants present in a clinical isolate and may detect new mutations potentially conferring drug resistance. In addition, the clinical relevance of new mutations can easily be assessed by retrospective analysis of WGS data [10]. In fact, mutations of insertion or deletion in *rpl22* gene (encoding a ribosomal protein that interacts with the 23SrRNA domains) and a “G to A”point mutation in *infB* gene (encoding a translation initiation factor, IF-2) were detected with WGS in some CLA-resistant *H. pylori* strains carrying a wild type 23SrRNA [11]. On the other hand, it has been reported that five conserved families of multidrug efflux pump transporters can contribute to antibiotic resistance in *H. pylori*. One of these, the resistance-nodulation-cell division (RND) family, forms a homotrimer with an outer membrane protein (TolC) and a periplasmic membrane fusion protein (AcrA) [12][13][14]. Currently, using WGS, four-gene clusters of efflux pump systems (hp0605-hp0607, hp0971-hp0969, hp1327-hp1329, and hp1489-hp1487) [15][16] have been identified as belonging to the RND family in *H. pylori*, and linked to the development of resistance to CLA [17]. With WGS analysis, Chen et al. (2018) [15] failed to find significant differences in gene mutations of RND family between CLA-susceptible and CLA-resistant phenotype. However, the number of mutations in the RND family was significantly higher in *H. pylori* strains carrying the A2143G point mutation of the 23SrRNA gene. Moreover, Iwamoto et al. (2014) [12], using the same type of approach, observed a significant difference in the number of SNVs (single-nucleotide variant) of the hp0605-hp0607 cluster between susceptible and resistant *H. pylori* strains.

3. Metronidazole

Metronidazole (MZ) and tinidazole are bactericidal chemotherapic compounds that belong to the nitroimidazole group of drugs, their antimicrobial activity is only marginally affected by low pH. Nitroimidazoles are prodrugs that need to be activated within the target cell by one or two electron transfer processes. This reduction leads to the formation of nitro-anion radicals and imidazole intermediates that cause lethal damage to subcellular structures and DNA [18][19][20]. The prediction of metronidazole resistance based on genotypic information still remains challenging. Various mutational changes affecting several genes have been shown or hypothesized as being involved in the development of MZ resistance in *H. pylori* [5][21][22]. Traditional molecular techniques identified *rdxa* (oxygen-insensitive NAD(P)H nitroreductase) and *frxa* (NAD(P)H flavin nitroreductase) genes [23] as putative MZ-resistance determinants. Whole genome NGS enabled the detection of complex variants in *gyrA* and *frxA* genes, as well as the study of mutations in other genes, i.e., *recA* (RecA protein for the repair and maintenance of DNA), *sodB* (superoxide dismutase B), *fur* (ferric uptake regulator protein), *mdaB* (NADPH:quinone oxidoreductase MdaB), *ribF* (Riboflavin biosynthesis protein), *omp11* (Outer membrane protein 11), and *rpsU* (30S ribosomal protein S21). All genes hypothetically related to MZ resistance encode putative MZ-reducing enzymes, DNA repair proteins, proteins regulating cell responses to oxidative stress, and outer membrane proteins [24]. Most metronidazole-resistant *H. pylori* strains carry multiple *rdxA* and *frxA* mutations. In particular, frameshift mutations (i.e., at codon positions 105, 149 or 192 in *frxA* and 18, 38 and 112 in *rdxA*) and point mutations resulting in amino acid exchanges (i.e., A67V, A68E, K64N, P106S, R90S and R16C/H in the *rdxA* gene) were observed only in metronidazole resistant strains [25][26]. Other mutations (i.e., at codon positions 18 in *frxA* and 62, 96 and 162 in *rdxA*) were distributed between resistant and susceptible strains [5][7][25]. Null mutations in *rdxa* functional sites (frameshift, premature stop, large deletion, large sequence insertions ending with a stop and point-mutations) leading to a loss of binding sites for FMN (flavin mononucleotide) are predictive of MZ-resistance. The same types of mutations were detected also in the *frxa* gene, but with lower predictivity for phenotypic resistance. The role of the other putative MZ-resistance genes is even less evident [27]. Metronidazole-resistant strains without mutations in *rdxA* and/or *frxA* were also reported, and downregulation of *rdxA* expression or mutations in other genes has been suggested [12][27][28][29]. Chua et al. (2019) [27], in fact, identified through whole genome sequencing four protein clusters harbouring a variable site in which the distribution of amino acid variants was significantly greater among the MZ-R strains: D85N in the inner-membrane protein RclC, V265I in a biotin carboxylase protein and A51V/T in HP0918 (hypothetical protein involved in tRNA biosynthesis). Overall, these observations suggest that MZ resistance is multifaceted and more studies are needed to investigate the association between gene polymorphisms and metronidazole resistance.

4. Levofloxacin

Levofloxacin (LEVO) is a broad-spectrum antibiotic which belongs to the third generation of the quinolones group, namely fluoroquinolones. They are bactericidal antibiotics that exert their antimicrobial activity by inhibition of type II topoisomerases (DNA gyrase and topoisomerase IV). *H. pylori* lacks topoisomerase IV, so fluoroquinolone resistance is likely due to mutations in the DNA gyrase. This enzyme is a tetramer that consists of two A subunits and two B subunits, encoded by the *gyrA* and *gyrB* genes. The main function of this enzyme is to catalyse the negative supercoiling of DNA [30]. Using conventional molecular methods, point mutations in the regions of *gyrA* gene encoding amino acids 87, 88, 91 and 97 had been proposed as putative levofloxacin-resistance determinants. In *gyrA* and *gyrB*, quinolone resistance-determining regions (QRDR) were identified. *GyrA* QRDR goes from codons A71 to Q110; *gyrB* QRDR goes from codons E415 to S454. With NGS techniques, mutations inside and outside the QRDR of both *gyrA* and *gyrB* genes were analysed and associated with levofloxacin resistance in *H. pylori* [5]. Amino acid exchanges at codon 87 and/or 91 (i.e., N87I/N87K/D91Y/D91N/D91G) in the QRDR of the *gyrA* gene were confirmed as determinants of the resistant status [5][25]. Strains with mutations in both positions generally show higher levels of resistance. Qumar et al. (2020) [31], using whole genome NGS, also identified a N-terminal extension of GyrA by five amino acid residues (QDNSV) that occurred solely in LEVO-R *H. pylori* strains. This insertion caused a conformational change in the GyrA protein, reducing its binding affinity to fluoroquinolone antibiotics. Mutations in the QRDR of *gyrB* gene (i.e., D435N and V437L), and mutations outside of it (i.e., R484K and R579C), are not so strictly correlated to the phenotypic resistance; and it appears that their presence has no synergistic effect with *gyrA* mutations [32]. On the other hand, it was suggested that genotypic and phenotypic LEVO-resistance statuses are highly concordant only when considering mutations of the QRDR sequence in both *gyrA* and *gyrB*. Moreover, a small portion of phenotypically resistant strains carrying wild type *gyrA* and *gyrB* genes were reported, suggesting the involvement of other mechanisms in the onset of LEVO-resistance [5]. In many Gram-negative bacteria, the presence of efflux pumps has been reported to confer the fluoroquinolone resistance [33], but this mechanism seems to be rather unlikely in *H. pylori* [16][34]. On the other hand, resistance to quinolone drugs may be

attributed to the change in membrane permeability, which may also play a role in multi-drug resistance (shown by a decrease in drug accumulation inside the cells) and alteration of the outer membrane protein (OMP) [35][36][37]. Overall, genotype analysis based on QRDRs of *gyrA* and *gyrB* genes gives a high predictivity of the LEVO-R status in *H. pylori* [5].

5. Amoxicillin

Amoxicillin (AM) is a bactericidal antibiotic that belongs to the penicillin group of drugs. The drug binds to penicillin binding proteins (PBPs) and interferes with bacterial cell wall synthesis, resulting in lysis of replicating bacteria. The antibacterial activity of amoxicillin is much the same as that of other penicillins, but amoxicillin is better released in the gastric juice, and displays increased stability in acidic conditions compared with other penicillins [38]. In *H. pylori*, there is no evidence that amoxicillin resistance is caused by beta-lactamase activity [39]. Many *H. pylori* isolates described as being amoxicillin resistant are often only tolerant to penicillins due the absence of PBP 4 [40]. Stable amoxicillin resistance in *H. pylori* is rare and seems to be mainly mediated by alterations to PBPs. Mutations in (or adjacent to), three motifs “SXN/SXXK/KTG” in PBP1A, PBP2 and PBP3 were identified as putative AM-resistance conferring determinants, together with mechanisms of reduced membrane permeability [36][41][42]. By using WGS, it was confirmed that structural alterations within the PBP1A were relevant for AMX-R, in particular in those belonging to three PBP-motifs (SAIK368_371, SKN402_404, KTG555_557, and SNN559_561) and at C-terminus codons (A474, T558, T593, and G595). Moreover, new putative AM-R genotypes were detected, i.e., F366L, S405N, A474T, T558S and N562H [5][43]. Tshibangu-Kabamba et al. (2020) [5] using WGS, also observed mutations at five codons (S402G, S414R, T556S, N562Y, and T593A/G/K/S) that were previously linked to AM-resistance by natural transformation. Saranathan et al. (2020) [7] detected, in AM-resistant strains, point mutations in genes *pbp1* (N107R, A201V, V250I, S543T), *pbp2* (I259T), and/or *pbp3* (D2N, A50S, F490Y, A541T, V374I) located near the AM-resistance motifs. With WGS, also other mechanisms were observed inducing in vitro high-level AM-resistance, they included mutations in *hofH* (outer membrane protein), *hefC* (RND pump protein) and *hopC* (outer membrane protein) genes. Overall, the analysis of *pbp1A*, *hofH*, *hefC* and *hopC* genes gives a good predictivity of the AM-resistant phenotype [44].

6. Rifabutin

Rifabutin (RIFA) belongs to the “rifamycin-group” of bactericidal antibiotics (together with rifamycin, rifampicin, and rifaximin) that bind the β-subunit of the DNA-dependent RNA polymerase, leading to the inhibition of the transcription. Rifabutin is a recommended drug for *H. pylori* rescue therapies in regions with high quinolone resistance rates, because its antibacterial activity is not affected by the low pH and is higher than that of rifampicin [1]. The β-subunit of the DNA-dependent RNA polymerase complex is encoded by the *rpoB* gene [45]. Although increasing, the resistance against rifamycins is very rare [46]. In *H. pylori*, resistance to rifampicin has been associated with amino acid exchanges in the rifampicin resistance determining region (RRDR) of the *rpoB* gene [25], mainly at codons 525 to 545, 547and 586. Lauener et al. (2018) [25], through WGS, observed that amino acid substitutions L525P and H540N were associated with rifampicin resistance (MIC >32 mg/L). By considering a 4 mg/L clinical breakpoint (instead of 1 mg/L, as established by EUCAST) [47], it was possible to find an agreement between the WGS results and phenotypic rifampicin susceptibility testing. Cross-resistance between rifabutin and rifampicin was frequently reported [48][49]. With NGS, Miftahussurur et al. (2019) [50] detected several mutations in *rpoB* gene associated with rifamycin resistance (i.e., I2619V, V2592L, T2537A, F2538L, K2359S6, K2594R7, D2381E8, T1540A, N2603D and E2809D) that were located outside the known RRDR and were not related to high-level resistance (MIC ≥ 16 mg/L). It was suggested that some of these mutations (i.e., V2592L, T2537A, and F2538 L) might be related to rifaximin, but not rifabutin resistance. In another study, using the same type of approach, these authors identified point mutations (i.e., I837V, A2414T, K2068R, Q2079K) leading to rifaximin—but not rifabutin—resistance, suggesting the lack of cross-resistance between rifaximin and rifabutin [51].

7. Tetracycline

Tetracycline (TC), a bacteriostatic antibiotic that binds to the 16SrRNA, inhibiting protein synthesis, has been intensively used since the 1950s, and many bacterial pathogens have acquired resistance to this antibiotic [52]. In most species, tetracycline resistance is obtained in two main modes of action: efflux systems, or ribosomal protection proteins [53]. In the case of *H. pylori*, TC resistance is not observed as frequently as in other bacteria, although its prevalence is slowly increasing [54]. In *H. pylor*i, resistance to TC seems to be conferred by mutations in the 16rRNA gene, in particular a triple mutation AGA965 to 967TTC (h31 loop) and a G942 deletion (*E. coli* numbering) both located in domain III, and several nucleotides in this region interact with tRNA molecules [55]. Genotype-based prediction of TC resistance is difficult, also with NGS, this is due to the low resistance rate to this antibiotic, moreover isolates without mutations at these nucleotide

positions in the 16SrRNA gene display a resistant phenotype [7][56]. Therefore, tetracycline resistance seems to be multifactorial, involving alterations in ribosomal binding, enzymatic degradation of antibiotics, a reduction of membrane permeability, and an active efflux, and need further investigation [25][57][58].

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