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RESEARCH ARTICLE

Antimicrobial Activity of Ketoconazole and Fluconazole against Metronidazole Resistance Strains of *Helicobacter pylori*

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ABSTRACT

Resistance to drug such as metronidazole is one the commonest causes of treatment failure while eradicating *Helicobacter pylori*. Considering the safety of ketoconazole and fluconazole and their inhibitory activity on biosynthesis of fatty acids from cholesterol in cell membrane of *H.pylori*, the idea of their efficacy against *H.pylori is raising*. The aim of this study is to evaluate susceptibilities of metronidazole-resistant strains of *H.pylori* against two antifungal drugs, ketoconazole and fluconazole. In this prospective cross-sectional study, 35 isolates of *H.pylori* from patients with digestive disorders were recruited. Plates were incubated microaerobically. Resistance to metronidazole, minimum inhibitory concentrations (MIC) of ketoconazole and fluconazole for *H.pylori* isolates were determined by two methods: disc diffusion and agar dilution. Disc diffusion method indicated that metronidazole resistance was seen in 11 strains out of 35. Ketoconazole and fluconazole MICs were 8 and 40$^{mg/lit}$, respectively, which was confirmed by agar dilution method. Ketoconazole and fluconazole showed an excellent *in vitro* activity against the *H.pylori* isolates. However, *in vivo* activity of these drugs should be evaluated in controlled clinical trials.

Keywords: *Helicobacter pylori, Metronidazole resistance, Ketoconazole, Fluconazole*

Helicobacter pylori is currently recognized as one of the most common chronic bacteria infections worldwide [1]. It is a gastric pathogen that chronically infects more than half of the world's population, with a prevalence ranging from 25% in developed countries to more than 90% in developing areas [2]. This infection has a causative role in peptic ulcer disease, chronic superficial gastritis, non ulcer dyspepsia and gastric adenocarcinoma [2, 3]. Many groups, including the US National Institutes of Health, the *H.pylori* European Study Group and the European Society of Primary Care Gastroenterology [4, 5] now recommend treatment of *H.pylori* in all patients with duodenal ulcer, as it is well established that eradicating *H.pylori* infection cures ulcer disease [6, 7] and it is a cost-effective strategy [8].

It seems that H.pylori infection can be cured by antibiotics, however, the ideal anti-*H.pylori* treatment has yet to be found [9, 10].

By the way, the most commonly used treatments are triple therapies based on a proton pump inhibitor with clarithromycin and metronidazole or amoxicillin or quadruple therapy with a proton pump inhibitor, bismuth compound, tetracycline and metronidazole [11-15]. *H.pylori* successfully was treated in about 90% of cases; however, 10% of patients remained *H.pylori* positive [16]. Many factors have been implicated in treatment failure, including ineffective penetration of antibiotics into the gastric mucosa, antibiotic inactivation by low gastric PH, lack of compliance and emergence of acquired antibiotic resistance by *H.pylori* [17, 18]. One the most common causes of treatment failure is *H.pylori*'s resistance against metronidazole, which is estimated up to 54% [19] and 77.9% in some areas [20]. Nowadays, the eradication rate of *H. pylori* infection is low, mainly due to a considerable number (37%) of metronidazole-resistant strains [21]. Despite the increasing metronidazole resistance strains of *H.pylori* and it's side effects, usage of metronidazole is still considerable for eradication of *H.pylori*, therefore, it is necessary to try new drugs in common treatment protocols.

Since fatty acids, namely cholesteryl glucosides, have been found in the cell membrane of Helicobacter species, investigators have speculated that imidazole antimycotics such as ketoconazole might interfere with

the biosynthesis of these fatty acids from cholesterol [22]. The dual activity of ketoconazole against both *H.pylori* and fungi might be valuable because oral yeasts have been proposed as potent reservoirs as well as protective vehicles for transmission of *H.pylori* to the human gastrointestinal tract [23]. Thus design of chemotherapeutic regimens containing ketoconazole and fluconazole will benefit patients by eradicating *H.pylori* as well as reducing the number of yeast microflora harbouring *H.pylori*.

Therefore, the aim of this study is to evaluate the sensitivity of metronidazole resistance strains of *H.pylori* against antifungal drugs, ketoconazole and fluconazole.

METHODS AND MATERIALS

Patients & Samples

This was a prospective cross-sectional study. Thirty five isolates *H.pylori* were obtained from patients with digestive disorder referred to Hazrat-e-Rasoul hospital of Iran University of medical sciences in Tehran, Iran. Patients with digestive symptoms were undertaken endoscopy and the ones with antrum gastritis or peptic ulcer plus positive Rapid Urea Breath Test (UBT) were selected. Four specimens from antrum and body of stomach were taken through a biopsy procedure.

Endoscopic biopsied specimens were cultured in the microbiological center of Tehran University of medical sciences. Biopsies were cultured on selective brucella agar (Merck) containing blood under microaerobic conditions. Bacterial isolates were identified as *H.pylori* on the basis of Gram's stain, showing Gram-negative spiral forms, and positive urease, oxidase and catalase tests.

H.pylori Culture

1- *Transport media:* It was used to convey biopsy specimens from endoscopy center to microbiology laboratory. It is consist of 0.85^{gr} NaCl and 0.16^{gr} agar in 100^{cc} normal saline under 121^{0c} otoclave and $15^{pound/inch}$ pressure for 15 minutes. This media was kept in 4^{0c} refrigerator.

2- *Selective brucella blood agar:* This culture media were used to dissociate *H.pylori* strains from endoscopic biopsied specimens. Adequate antibiotics such as vancomycin, trimethoprim and polymixin to prevent other bacterial growth. After sterilization, the plates were stored in 4^{0c} refrigerator.

MIC & Zone Size Determination

After 48 hours, the colonies which were confirmed to be *H.pylori* with special exams, was undergone gram staining. After the observation of gram negative bacillus, the strains prepared to be tested. Antibiotics sensitivity of *H.pylori* was determined by two methods: disc diffusion and agar dilution.

1- *Disc diffusion method (DDM):* Blank metronidazole discs were deposited on the agar. Plates were incubated microaerobically and examined for the visualization of inhibition zones, after 48 hours. Suggested zone sizes with disc diffusion for metronidazole in brucella agar plus 7-8% blood are resistant no zone, and susceptible any zone.

2- *Agar dilution method (ADM):* National Committee for Clinical Laboratory Standards (NCCLS) agar dilution protocol (which has been considered the gold standard) defines the break point minimal inhibitory concentration (MIC) as the lowest concentration of antibiotic showing no growth, a haze, one discrete colony, or multiple tiny colonies; and where there is persistent slight growth, the MIC is read as the concentration at which a marked change in the appearance relative to that of the control plate occurs [24-26].

The resistance of *H.pylori* strains to metronidazole (Sigma) was assessed by ADM according to NCCLS guidelines. Metronidazole in ethanol was added to Mueller–Hinton agar (Merck) plates containing blood, to reach final dilutions. Aliquots (5^{mL}) of bacterial suspensions with turbidities equivalent to that of a no.2 McFarland standard were spot-inoculated on the surface of agar. Plates were examined for growth or inhibition after 3 days of appropriate incubation.

The MIC of metronidazole was determined as $>8^{mg/L}$. The antibacterial effectiveness of ketoconazole and fluconazole against *H.pylori* isolates was assessed, using ADM and DDM. In ADM, ketoconazole in DMSO was added to Mueller–Hinton blood agar. Aliquots (5^{mL}) of bacterial suspensions with turbidities equivalent to that of a no.2 McFarland standard were spot-inoculated on the surface of agar plates. Plates were examined after 3 days of microaerobic incubation and MICs were determined. Similar bacterial suspensions of Escherichia coli were spot-inoculated on Mueller–Hinton agar plates with serial dilutions of ketoconazole. In DDM, serial dilutions of ketoconazole were prepared in DMSO. Aliquots (100^{mL}) of bacterial suspensions were surface-inoculated on Mueller–Hinton blood agar. Each ketoconazole dilution was introduced into paper discs on the surface of the agar. After microaerobic incubation, growth inhibition zones were measured. Strains with inhibition zone diameters (IZDs) of 17–21 and $>21^{mm}$ were considered as susceptible and highly susceptible, respectively. The same procedures were performed for fluconazole.

On the other hand, the ketoconazole MICs range from 2 to $64^{mg/lit}$ were described as a susceptible one, whereas, ketoconazole resistance was defined as MIC $>64^{mg/lit}$. About fluconazole no antimicrobial sensitivity were seen in the MIC of = or < $128^{mg/lit}$. Therefore, it was evaluated in the MIC of even more than $128^{mg/lit}$.

RESULTS

In this cross-sectional study, the susceptibility of metronidazole resistance strains of *H.pylori* to fluconazole and ketoconazole were evaluated. Out of biopsies, 35 specimens were culture positive for *H.pylori*.

Susceptibility to metronidazole was evaluated using disc diffusion method for all these 35 specimens. The resistibility to metronidazole was seen in 11 strains (31.4%). All these 11 metronidazole resistance strains

Table 1. Means of inhibition zone diameters for H.pylori straines obtained with different dilutions of ketoconazole

Ketoconazole (mg/L)	Mean diameter (mm) ± SD
20	29.87±1.3
14	27.02±0.8
10	26.39±0.9
8	24.14±1.6
6	17.05±1.8
4	12.34±1.1

were incubated microaerobically in blood agar medias, containing 4,6,8,10,14 and 20$^{mg/lit}$ ketoconazole for 5 days. Susceptibility to ketoconazole for 10/11 (90.9%) isolates recruited in ADM was determined at MIC 8 $^{mg/L}$, although 5/11 (45.4%) were also inhibited at MIC $\leq 4^{mg/L}$ and one (9.1%) was inhibited at an MIC of 16 $^{mg/L}$. Four out of 11 isolates that were resistant to metronidazole showed high susceptibility to ketoconazole. Control *E.coli* exhibited growth on plates containing 128 $^{mg/L}$ ketoconazole. In DDM, the means of IZDs for *H.pylori* isolates at different dilutions of ketoconazole were determined (Table 1).

Susceptibility of bacterial isolates to ketoconazole was determined according to IZDs at 8 $^{mg/L}$. Thirteen out of 24 non-metronidazole resistance isolates (54.2%) were susceptible and 11 isolates (45.8%) were highly susceptible to ketoconazole.

Among 11 metronidazole-resistant strains, 7 (63.6%) were susceptible and 4 (36.4%) were highly susceptible to ketoconazole. The ketoconazole MIC was 8$^{mg/lit}$.

On the other hand, these strains were also incubated microaerobically in blood agar medias containing 4,6,8,10,14,20,24,30,34 and 40$^{mg/lit}$ fluconazole for 5 days. The fluconazole MIC was 40$^{mg/lit}$. Two out of 11 isolates that were resistant to metronidazole showed high susceptibility to fluconazole.

By the way, susceptibility to ketoconazole and fluconazole were evaluated in non metronidazole resistance strains of *H.pylori*, too. The results was the same as resistance ones.

DISCUSSION

H.pylori resistance to antimicrobial is of particular concern because it is a major determinant of eradication regimen failure [27]. The universal high-level primary resistance of *H.pylori* to metronidazole is nowadays seen in many studies [28].

Studies in the Middle East estimated that metronidazole resistance was between 60 and 80% [29-31]. The occurrence of metronidazole-resistant strains may be the consequence of increased consumption of this antibiotic in the community [29]. And it seems that metronidazole resistance was significantly associated with Asian ethnicity and female sex [32].

Several chromosomal loci have been implicated in resistance to this drug. Saturation transposon mutagenesis of the *H.pylori* genome revealed inactivation of the rdx A gene as uniquely able to confer metronidazole resistance [33].

Therefore, it is necessary to utilize new agents in order to eradicate *H.pylori*, such as ketoconazole and fluconazole.

Ketoconazole is an imidazole derivative used as a broad spectrum antifungal agent and fluconazole is a triazole which also prescribed as an antifungal drug.

In our study, ketoconazole and fluconazole, showed an excellent in vitro activity against the *H.pylori* isolates, and their MICs were 8$^{mg/lit}$ and 40$^{mg/lit}$, respectively.

However, Von Recklinghause et al in 1993 showed that the nitroimidazole MICs range from 2 to 64$^{mg/lit}$, with ticonazole, miconazole, bifenazole and ketoconazole as the most active substances, whereas, the results of their study added that fluconazole was ineffective at concentration < or =128$^{mg/lit}$ [34]. Also, in our study ketoconazole was more effective than fluconazole too.

All the 35 isolates were inhibited by ketoconazole in our study, although *E.coli* was highly resistant. The MIC of ketoconazole was determined as 8 $^{mg/L}$ in both ADM and DDM. Similarly, inhibitory concentrations of the antimycotic miconazole (MIC 2–32 $^{mg/L}$) against *H.pylori* have been reported [34].

Ketoconazole can be considered as a substitute for metronidazole. Regarding the safety of ketoconazole, it is proposed that the dose required to inhibit mammalian cells is much higher than that required for fungi [35] or bacteria [34]. Since the intracellular existence of *H.pylori* in yeast plays an important role in the persistence of *H.pylori* in the human oral cavity [23], administration of ketoconazole not only leads to eradication of *H.pylori*, but might also reduce the chance of recurrence of bacterial infection by affecting colonization of yeasts in the gastrointestinal tract.

However, it seems that the efficacy of ketoconazole and fluconazole against *H.pylori* should be tested in vivo in a controlled clinical trial on human regarding ethical considerations.

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