International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (3), pp. 001-005, March, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

The role of *hp1165* gene on the efflux-mediated resistance to tetracycline in clinical isolates of *Helicobacter pylori*

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Accepted 04 January, 2018

Tetracycline-resistant (Tet^R) *Helicobacter pylori* isolates have emerged in many parts of the world. We have previously demonstrated that among the mechanisms involved in the resistance of *H. Pylori* to Tet, its active efflux may be an important mechanism. This work, aimed to determine whether presence and/or expression of *hp1165* are associated with efflux-mediated resistance to Tet in clinical strains of *H. pylori*. Twenty five Tet^R strains including seven low level-resistant, eight intermediately- resistant, and ten high-level resistant strains/or mutants, of which 21 displayed the active efflux ability for Tet, were investigated. They were screened for the presence of mutation (s) in *16S rRNA* at 965- 967 position and for the presence of *hp1165* gene. Detection of *hp1165* gene transcription /or gene expression was performed by RT-PCR Two low-level Tet^R strains displaying no efflux ability, that contained mutation (s) at 965-967 position of *16S rRNA*. Ten out of 21 Tet^R strains displaying active efflux abilities, that contained hp1165 gene. Their PCR product was similar to that of 26695 standard strains, susceptible to Tet. RT -PCR was positive for five out of them however; their product size was approximately 100 bps smaller than that of 26695 strains. Regarding to the results of PCR and RT- PCR, *hp1165* plays a role in the active efflux of Tet in resistant strains. A post-transcriptional regulation step may be involved in the expression of *hp1165* gene in Tet^R strains.

Key words: *Helicobacter pylori*, tetracycline, resistance, efflux, *hp1165* gene.

INTRODUCTION

Helicobacter pylori isolates resistant to Tet have been reported in many parts of the world especially in Asian countries (Dailidiene et al., 2002; Khan et al., 2008; Ribeiro, 2004; Trieber, 2002). Multiple mechanisms participate in the development of resistance to Tet in both gram negative and gram positive bacteria (Chopra et al., 2001; Aminove et al., 2001). In *H. pylori*, the most frequently reported mechanism have been mutations in the 16S rRNA tetracycline-binding site that corresponds to the triple base-pair substitution AGA (965–967) to TTC (Trieber and Taylor, 2002; Dailidiene et al., 2002; Gerrits et al., 2003; Ribeiro et al., 2004). Presence of such mutation affects the affinity of the drug-ribosome interaction and reduces the efficacy of tetracycline as a translational inhibitor. Other mechanism involved in the resistance of *H. pylori* to Tet may be defect in its uptake, and/or increase in its efflux (Gerrits et al., 2003; Glocker et al., 2005; Li and Dannelly, 2006; Wu et al., 2005; Bina et al., 2000). There are several classes of Tet efflux pumps including single subunits containing multiple transmembrane segments that are specific for the active transport of Tet (Chopra et al., 2001; Aminove et al., 2001; Nelson, 2002; Agerso and Guardabassi, 2005).

A putative gene for Tet efflux pump of this kind, hp1165, has been identified in the genome of *H. pylori* 26695. The *hp1165* gene displays 98% identity to the *jhp1092* gene in *H. pylori* J99 and 40 to 44% homology to the multidrug transporters in *Campylobacter jejuni* and 49.8% homology with tetracycline efflux gene *tetA (P)* in

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Clostridium perfringens (Kennan et al., 1997; Bannam et al., 2004). Furthermore, previous works have suggested that *hp1165* is prevalent in *Helicobacter* laboratory strains and clinical isolates (Li and Dannelly, 2006). However, the real role of HP1165 in tetracycline resistance of *H. pylori* remains unknown. Using clinical Tet^R isolates and high-level-resistant mutants, the authors have previously reported that proton motive force- dependent efflux plays an important role in the resistance of clinical isolates of *H. pylori* to Tet (Anoushiravani et al., 2009). In this work, they sought to determine whether the presence and/or expression of *hp1165* are associated with efflux-mediated resistance to Tet in low-, intermediate-, and high-level Tet^R clinical strains of *H. pylori.* In these strains presence of mutation (s) in the *16S rRNA* gene at AGA (965–967) position was also evaluated.

MATERIALS AND METHODS

H. pylori strains and growth conditions

Bacteria were routinely cultured on modified Campy-blood agar (Merck), as described previously (Falsafi et al., 2004; Falsafi et al., 2007). The clinical strains used in this work included 20 Tet^R strains (MIC above 2 mg L^{-1}), and two strains susceptible to Tet (MIC $\leq$ 2 mg L^{-1}). Five Tet^R mutants obtained in our previous work were also included in this study. The *H. Pylori* 26695 standard strain was used as control for detection of *hp1165* gene. Based on the results of MIC obtained by agar dilution method, these strains have been classified into three groups of: low-level (MIC 4 to 8 mg L^{-1}), intermediate-level (MIC 16 mg L^{-1}), and high- level (MIC 32 to 64 mg L^{-1}) Tet^R strains (Anoushiravani et al., 2009).

Measurement of antibiotic accumulation

Accumulation of Tet was measured in the presence and absence of carbonyl cyanide-m chlorophenyl hydrazone (CCCP) as described previously (Anoushiravani et al., 2009). Briefly, two days cultures were harvested and resuspended in sodium phosphate buffer (50 mM, pH 7.2) with a density approximating 1.2×10^9 by CFU. Antibiotic uptake assay was initiated by addition of TET to bacterial cell suspension at a final concentration of 20 µg/ml. At 10 min after antibiotic addition, CCCP (200 µM) was added to one–half of the reaction mixture and the other half was used as control. After 20 min, the collected samples were diluted in 1.5 ml ice–cold sodium phosphate buffer and centrifuged. The supernatants were discarded and the pellets were resuspended in 0.1 M glycine hydrochloride (pH 3.0), and shaken at 25°C to extract the accumulated antibiotics. After 16 h, it was centrifuged and the supernatant was used to measure the fluorescence of antibiotics with a Shimadzu RF 5000 spectrofluorometer (Shimadzu Scientific Instruments, Inc., Colombia, Md) at excitation and emission wavelengths of 400 and 450 nm. The concentration of Tet in the supernatant was obtained by comparison with a standard curve of Tet in 0.1 M glycine hydrochloride (pH 3.0). The results were expressed as nanograms of Tet per milligram (wet weight) of bacteria. An increase of $\geq$ 2 in the concentration of accumulated Tet in the presence of CCCP was interpreted as presence of active efflux ability.

DNA preparation and PCR

Bacterial DNA was prepared according to the previously described procedure (Falsafi et al., 2009). The detection of *16S RNA* gene mutation was performed by using the specific primers (Faza Biotech Inc, Iran) based on published sequences of *H. pylori* such that the 3´ -end of the forward primer exactly matched with three nucleotides in 965-967 position of 16S rRNA gene. They were: 5´-GCA TGTGGTTTAATTCGAAGA -3´ (Forward) and 5´-CTTTGTGCACCCCATTGTA G-3´ (Reverse). The reaction was held at 95°C for 5 min for 1 cycle, followed by 40 cycles with parameters of 94°C for 30 s, 57°C for 1 min, 72°C for 1 min, and 1 cycle of 72°C for 10 min as final extension. The expected product size was 300 bp, and the absence of this product suggested that the 3´-end of forward primer could not match with the tetracycline binding site in *16S rRNA* gene due to a (the) mutation (s) in this position.

The detection of *hp1165*gene was performed by using the specific primers (Faza Biotech Inc, Iran) based on published sequences of *H. Pylori*. These primers were designed for detection of the middle sequences of the gene as described previously (Li and Dannelly, 2006). Their sequences were: 5´-AGGGAGTTCTTTGGGATCGT-3´ (Forward) and 5´-TCCAGACTGAGCGATAA-3´ (Reverse); the predicted product size was 624 bp. The PCR reaction was held at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 56°C for 1 min, 72°C for 1 min and 1 cycle in 72°C for 10 min.

Evaluation of *hp1165* gene expression by RT-PCR

To perform RT-PCR, the concentration of high-pure RNA prepared according to the manufacturer's recommendations (Roche, Germany), was measured at 260 nm and the cDNA was synthesized using 1st- strand cDNA synthesis kit for RT-PCR (Roche, Germany). The reaction for cDNA synthesis was held at 25°C for 10 min, 42°C for 60 min and 99°C for 5 min followed by PCR as described above. The PCR-products were visualized by electrophoresis on 1% agarose gel and 15% acrylamide gel with a 100 bp ladder size-marker (Cinnagene, Iran). RT-PCR analysis of *hp1165* was performed on tetracycline resistant, susceptible and 26695 *H. pylori* strains.

RESULTS AND DISCUSSION

PCR reaction for the detection of *16S rRNA* gene was positive for all of the strains except 10II, and 58II, two low-level Tet^R strains (Table 1 and Figure 1). This suggested that in these strains the 3´-end of forward primer could not match with the tetracycline binding site in the *16S rRNA* gene due to a (the) mutation (s) in this position which could affect efficacy of tetracycline binding. DNA prepared from the strains displaying active efflux ability for Tet, amplified normally for *16S rRNA* gene. This suggested that their resistance to Tet was most probably due to their active-efflux ability.

PCR reaction for the detection of *hp1165* gene was positive for seven Tet^R strains and three high-level resistant mutants. Association was observed between the presence of *hp1165* gene in these strains and the ability to pump-out actively Tet (Table 1 and Figure 2). The *hp1165* gene was not detected in the control susceptible strains (1 and 17B), and in strains with no-efflux ability. However, it was also absent in eleven Tet^R strains/or mutants capable to display active-efflux ability for Tet. This suggests that other determinant(s) than *hp1165* may

Table 1. Relationship between Tet-MIC, active efflux ability and *hp1165* in low-, intermediate-, high-level Tet^R and Tet-susceptible strains.

Strains	MIC to Tet	Active efflux [a]	PCR	RT-PCR
			Hp1165	Hp1165
12M	8	+	+	-
15M	8	-	-	-
27M	8	+	-	-
10II[b]	8	-	-	-
18B	8	+	-	-
58II[b]	8	-	-	-
23M	4	+	+	-
19M	16	+	-	-
20M	16	+	+	-
24M	16	+	+	+
25M	16	+	+	-
26M	16	+	+	+
28M	16	+	+	+
21D	16	+	-	-
2N	16	-	-	-
17M	32	+	-	-
18M	32	+	-	-
21M	32	+	-	-
1N	32	+	-	-
3N	32	+	-	-
25M-mut[c]	32	+	+	+
21M-mut[c]	64	+	-	-
23M-mut[c]	32	+	+	-
20M-mut[c]	64	+	+	+
18M-mut[c]	64	+	-	-
1B[d]	0.5	-	-	-
17B[d]	1	-	-	-
26695	2	-	+	+

a: Active efflux ability evaluated as ≥ 2 fold increase in amount of Tet in presence of CCCP. b: strains with mutation in 16S rRNA. c: high-level resistant mutants. d: negative controls.

be involved in the active-efflux of Tet in *H. pylori*. The *hp1165* gene was detected in low-level resistant, intermediately resistant and in high level-resistant strains/ mutants so, its presence may not be related to the level of resistance to Tet. Comparison of the PCR product between *hp1165*-positive Tet^R strains and 26695 Tet-susceptible strain, revealed that the same size, suggesting that similar DNA sequences were present in them (Figure 2).

RT-PCR was performed for all of the Tet^R strains and for 26695 Tet-susceptible *H. pylori* strain as control for presence of hp1165 gene. The reaction was positive for five out of ten Tet^R strains containing *hp1165* including low, intermediate and high-level resistant ones. This suggested that this gene may not be expressed in all of the strains containing *hp1165* gene, and its expression

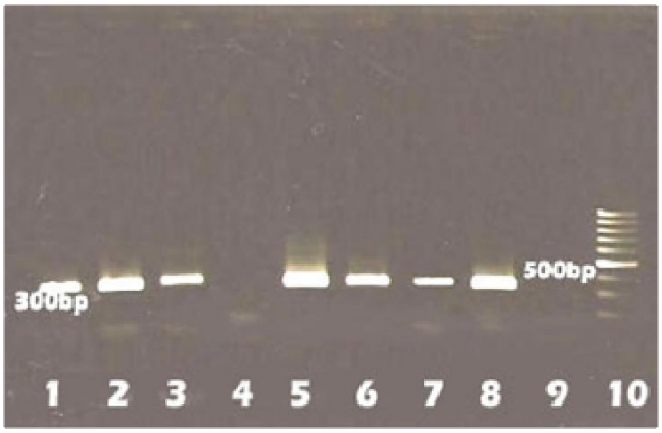

Figure 1. PCR detection of 16S-rRNA gene on 1% agarose gel. **Note:** 1. 12 M, 2. 15 M, 3. 17 M, 4. 17B (susceptible strain), 5. 18 M, 6. 19 M, 7. 20 M, 8. 26695 strain, 9. 10II, 10. Size marker.

may be induced under certain conditions. Furthermore, the transcription/and or expression of *hp1165* gene may not be related to the level of resistance. The size of RT-PCR product for 26695 susceptible strains was 624 bps, similar to that of PCR whereas the size of RT- PCR product for the Tet^R strains was approximately 500 and 100 bps smaller than that of their PCR product in 15% gel (Table 1 and Figure 3). Using 26695 laboratory strain and its mutant, Li and Dannelly (2006) have first studied the role of *hp1165* gene in the tetracycline resistance. They have proposed that *hp1165* gene is involved in the inducible tetracycline resistance in *H. pylori.*

Regulation of efflux-mediated resistance to Tet in Gram-negative bacteria is usually due to presence of *tetR*, which encodes a repressor protein and blocks *tetA/R* gene transcription in the absence of Tet (Speer et al., 1992; Chopra et al., 2001; Aminove et al., 2001). Several studies have confirmed non involvement of TetR kind of repressor in regulation of efflux genes in *H. pylori* (Nelson et al., 2002; Kutschke and Jonge, 2005; Liu et al., 2008; Morrison et al., 2003). Concerning the regulation mechanisms in *H. pylori*, a post- transcriptional regulation has been observed in the case of flagellin synthesis in *H. pylori* (Douillard et al., 2009), suggesting that this kind of regulation may be frequent in *H. pylori*.

Li and Dannelly (2006) have proposed that the expression of *hp1165* gene may be regulated at translational or posttranslational level. However, they could not observe a difference between the susceptible 26695 strain and its resistant-derivative concerning the result of RT-PCR. The results of this work may suggest the occurrence of a post-transcriptional regulation for the expression of *hp1165* efflux gene in Tet^R strains. As conclusion, the hp1165 gene exhibits a role in the active efflux of Tet in resistant *H. pylori* strains. A post-transcriptional regulation step may be involved in the expression of *hp1165* gene in Tet^R strains. An

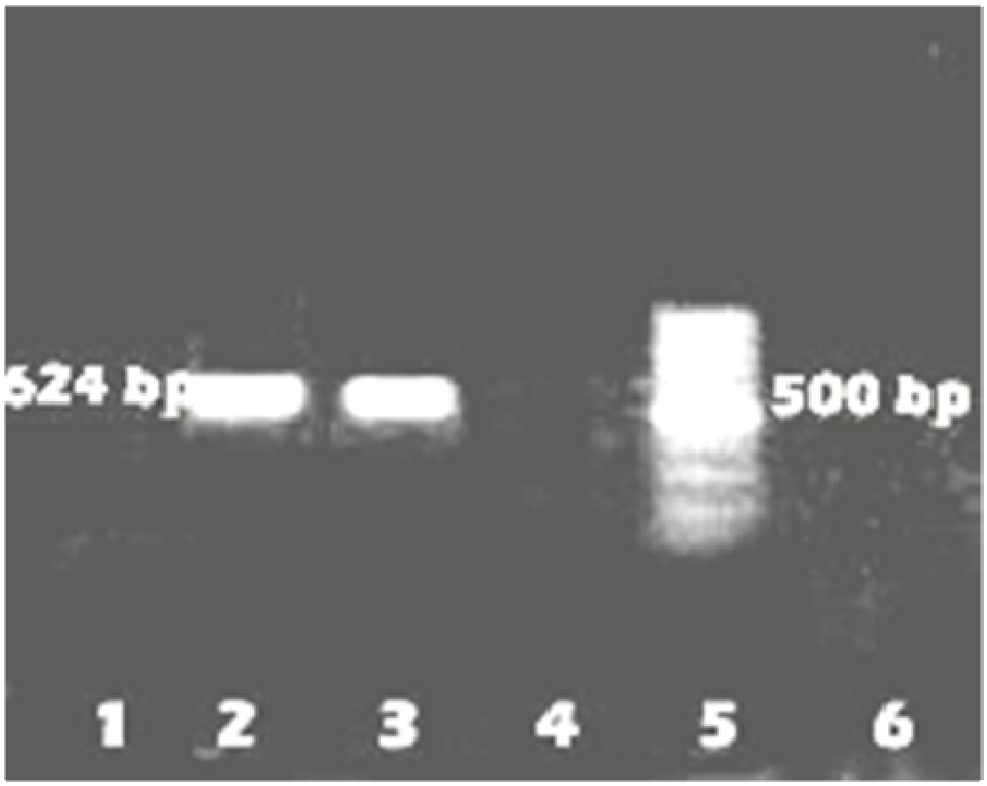

Figure 2. PCR detection of 1165 gene on 1% agarose gel. Note: 1.control, 2. 26695 strain, 3. 25M, 4. 17M, 5. size marker, 6. 10II strain.

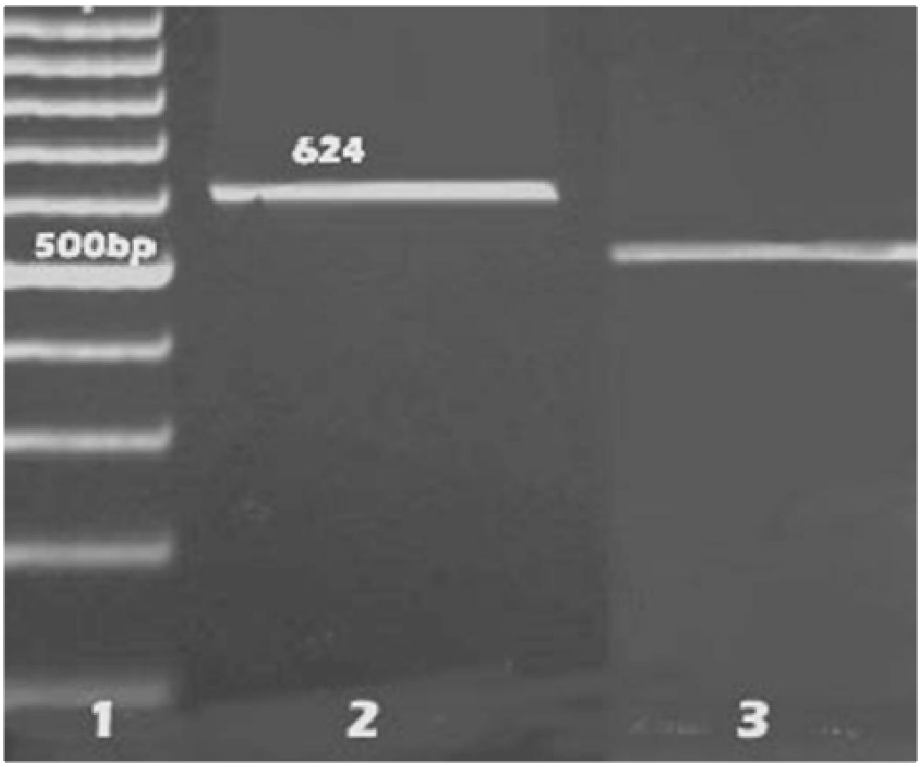

Figure 3. Presentation of the size difference of the RT-PCR products on 15% polyacrylamid gel. Note: 1. size marker, 2. 26695 standard strains, 3. 25M-mutant strain.

investigation of the HP1165 protein will help to understand its function and to identify the complete functional molecule involved in the active efflux of Tet in *H. pylori*.

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