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Original Article

Mutational Analysis of Clarithromycin and Levofloxacin Resistance in *Helicobacter pylori* from Gastric Biopsy Specimens in a Tertiary Care Hospital in Dhaka, Bangladesh

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Abstract

Background: Clarithromycin and Levofloxacin are most frequently included in the standard triple therapies for *H. pylori* eradication in our country. Resistance to clarithromycin and fluoroquinolones are particularly related with treatment failure.

Objectives: The objective of this study was to detect, clarithromycin and levofloxacin resistance associated with gene mutations in *H. pylori* directly from gastric biopsies using an allele specific primer-PCR (ASP-PCR) assay.

Materials and Methods: Gastric biopsy specimens were collected from 143 adult dyspeptic patients, from Department of Gastroenterology, BSMMU and Dhaka Medical College Hospital (DMCH), during the period of March, 2018 to February, 2019. *H. pylori* was identified by rapid urease test, *ureC* gene by PCR, histological staining and culture. ASP-PCR was used to identify 23S rRNA gene and *gyrA* gene mutation predictive of clarithromycin and levofloxacin resistant *H. pylori* respectively.

Results: *H. pylori* positive cases were 32.9% based on the case definition used in the study. Among 42 *ureC* positive *H. pylori* cases, point mutations in 23Sr RNA gene for clarithromycin resistance were detected only at A2142G position in 9 (21.4%) cases and *gyrA* gene mutations for levofloxacin resistance were detected in 16 (38.1%) cases. Only 1 (2.4%) case had mutation both in 23Sr RNA and *gyrA* gene.

Conclusion: Those findings may guide toward the therapeutic choices in our country. PCR based diagnostic assays can be the alternative approach for rapid detection of antibiotic resistances of *H. pylori* directly from gastric biopsies, where culture and susceptibility tests are not routinely performed.

Key words: Clarithromycin resistance, levofloxacin resistance, allele specific primer PCR

Introduction

Helicobacter pylori (H. pylori) specifically colonize the epithelium of the human stomach, in particular the gastric antrum.[1] Half of the world's population and as many as 80% of developing country residents carry *H. pylori* in their gastric mucosa. Only about 10-20% of infected persons become symptomatic.[2] Prevalence are higher in developing than in developed countries, due to diverse contributing factors including socioeconomic status, geographical or living conditions, ethnicity and location of each population.[3] In Bangladesh, *H. pylori* infection rate was 47% in adult dyspeptic patients in the year 2016.[4] *H. pylori* has been linked to gastritis, duodenal ulcer, gastric carcinoma and mucosa associated lymphoid malignancies.[5] Treatment for *H. pylori* infection is recommended in all symptomatic individuals in order to prevent the development of gastric adenocarcinoma and for the successful treatment of *H. pylori* induced mucosa-associated lymphoid tissue (MALT) lymphoma.[6]

Historically, the first truly effective therapy for *H. pylori* infection comprised bismuth, tetracycline and metronidazole (MNZ), but was of limited use because of side effects, low patients compliance and inconsistent and low eradication rate.[7,8]

Now a days, triple therapy containing a proton pump inhibitor and two antibiotics, amoxicillin (AMX) and clarithromycin (CAM) or MNZ, is the standard first-line treatment regimen in populations with less than 15-20% clarithromycin resistance for eradication of *H. pylori* infection.[6] The standard CAM-based triple regimen for 7-14 days is still the first option as *H. pylori* eradication therapy in Bangladesh.[9] After failure of first line therapy, levofloxacin containing triple therapy is

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recommended as second line treatment in our country which is an alternative approach for patients with macrolid allergy and /or treatment failure.[9,10]

Antibiotic resistance is a key factor in the failure of eradication therapy and recurrence of *H. pylori* infection. Several clinical trials have shown that the most commonly used first line therapies may fail in up to 20% of patients.[11] larithromycin resistance rates have been reported to be 58.8% and 36.0% in India and Pakistan respectively.[12,13] High resistance rates of *H. pylori* to levofloxacin (73.2%) has been reported in north India.[14] In Bangladesh 39.3% clarithromycin and 66.1% levofloxacin resistance had been reported in patient with gastritis.[15]

The more frequent mutations associated with clarithromycin resistance (>90%) are, a single spontaneous point mutation, the adenine to guanine transition at 2143 and 2142 positions of 23S rRNA, whilst the transversion of adenine with cytosine in 2142 position is less frequent.[16] Several other point mutations have been identified such as A2115G, G2141A, T2117C, T2182C, T2289C, G224A, C2245T and C2611A.[17]

In *H. pylori* (as in several other bacterial species), levofloxacin resistance is caused by point mutations in the quinolone resistance determining region (QRDR) of the *gyrA* gene, which encodes subunit A of DNA *gyra*se.[18] The amino acid substitutions observed in clinical strains have been primarily reported at position 87 (Asparagine to Lysine) or 91 (Aspartic acid to Glycine, Aspartic acid to Asparagine, or Aspartic acid to Tyrosine), although resistant strains lacking these mutations have also been described.[19,20,21]

In routine practice, the detection of antibiotic resistance is mainly based on phenotypic methods: disk diffusion method, agar diffusion using E-test or the agar dilution method.[22] Conventional phenotypic susceptibility testing is time consuming (10-14 days), and challenging due to slow growth and fastidious growth requirements of *H. pylori*, low bacterial load and overgrowth of contaminating organism reduce the sensitivity of culture testing method.[23] PCR has been used extensively for the diagnosis of *H. pylori* from gastric biopsy specimens, saliva, stool, gastric juice and variable specimens.[24]

ASP-PCR method can determine point mutations, which consists of the specific primers for mutations within a short period by a PCR amplification alone without digestion with restriction enzyme or direct sequencing.[25] Using agar dilution as the "gold standard", the sensitivity and specificity of ASP-PCR of 23S rRNA gene were reported to be 100% and for *gyrA* gene 100% and 92.7% respectively.[21, 25-26]

The present study was designed to detect the two most prevalent point mutations; A2143G and A2142G in 23SrRNA gene and point mutations in *gyrA* gene, for clarithromycin and levofloxacin resistance respectively by ASP-PCR. Periodic monitoring of the antibiotic susceptibility may define the resistance pattern, and will reduce the proportion of treatment failures and the cost of treatment. The information on in vitro *H. pylori* susceptibility tests and robustness of ASP-PCR for detecting the mutation is limited.

Materials and methods

This cross-sectional observational study was conducted at the Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh from March, 2018 to February, 2019.

Sample collection and processing

Gastric biopsy samples were collected from 143 adult dyspeptic patients who attended outpatient department of Gastroenterology, Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Medical College Hospital (DMCH) and were selected for endoscopy as per inclusion and exclusion criteria's. Patients presented with the symptoms of dyspepsia for more than 1 month were included in this study.[27] Individuals having severe medical or surgical illness such as asthma, COPD, previous gastric surgery etc and history of intake of proton pump inhibitors, non-steroidal anti-inflammatory drugs, colloidal bismuth compounds, or antibiotics for eradication of *H. Pylori* over the past four weeks of enrolment were excluded from this study.[28] After taking written informed consent from each patient, four endoscopic gastric biopsy tissue were collected from lesion and surrounding sites in gastric antrum and body. First specimen was inoculated immediately into a screw capped bottle containing rapid urease test media to detect the presence of *H. pylori* and Second biopsy specimen was collected in Stuart transport media for culture. Third biopsy specimen was collected in PBS for PCR assay. Fourth specimen, from margins of lesion or gastric antrum were fixed in 10% buffered formalin and transported to the Department of Pathology, BSMMU for histopathological examination. For PCR assay, all the biopsy samples were stored at 20°C for 2 to 4 weeks.

A case was considered as *H. pylori* positive case, if Positive *H. pylori* culture and / or at least two of the following tests were positive i) Rapid urease test (RUT), ii) *ureC* gene PCR and ii) Histopathology for *H. pylori*.[29,30]

Laboratory procedure

Rapid urease Test

Rapid urease test was done according to Ramis et al (2012).[31] Urease positive organism Proteus spp was used as a positive control and urease negative organism *E. coli* was used as a negative control for the test. Controls were collected from the stock culture preserved at the Department of Microbiology, BSMMU.

Culture[32-34]

Biopsy specimen was minced manually using a sterile scalpel in a sterile glass slide. Then the tissue materials were plated on brain heart infusion agar (Oxoid Ltd, Basingstoke, Hampshire, United Kingdom) supplemented with 10% sheep blood, Vitamino Growth Supplement (Twin Pack) (Hi Media, India) and Campylobacter selective supplement (Hi Media, India) for the culture of *H. pylori*. A gas generation pack (CO_2 Gen Sachet™, Oxoid, USA) was used to produce a microaerophilic condition. The anaerobic jar containing the inoculated plates was then incubated at 37˚C for 3-10 days and growth of H. pylori were examined on day 3 and colonies were identified based on their typical morphology (small, round, convex, translucent colonies), positive characteristic appearance on Gram staining (curved rod), positive catalase, urease and oxidase test. If no growth observed on day 3, the plates were incubated for another 7 days and checked on every alternative day for the growth of *H. pylori.*

DNA extraction

DNA extraction was performed on the gastric tissue specimens preserved at -20˚C using QIAmp DNA mini kits (Qiagen, Hilden, Germany) in PCR laboratory of Department of Microbiology and Immunology, BSMMU.

PCR analysis of *ureC* gene[35]

The *ureC* primer sequences were - *ureC*-F 5′-AAGCTTTTAGGGGTGTTAGGGGTTT-3′, ureC-R 5′-AAGCTTACTTTCTAACACTAACGC-3′; using these primers, 294 base pair(bp) fragments were amplified. The PCR assay was performed as described by Lu *et al* (1999)[35]. The PCR amplification conditions was: initial denaturation at 94˚C for 5 minutes, 35 cycles containing of denaturation at 93˚C for 1 minute, annealing at 55˚C for 30 seconds, extension at 72˚C for 1 minute and final extension at 72˚C for 10 minutes. The amplified products were subjected to electrophoresis on 2% agarose gel.

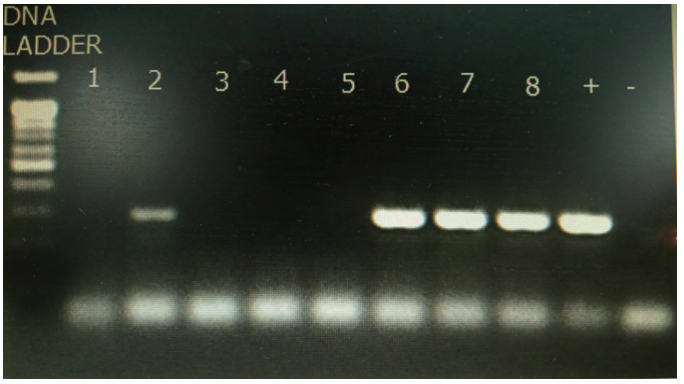

Figure-I: Agarose gel electrophoresis analysis showed amplified DNA product of *ureC* gene (294 bp). Lane 2, 6, 7, 8: *ureC* positive. Lane 1, 3, 4 and 5: *ureC* negative. Positive control: *ureC* gene positive *H. pylori*, Negative control: Amplified product of PCR without DNA)

ASP-PCR to determine 23S rRNA mutation of *H. pylori* for clarithromycin resistance[36]

ASP-PCR for 23S rRNA gene was performed with 4 primers. The primer sequences were FP-1 (5′-TC-GAAGGTTAAGAGGATGCGTCAGTC-3′), FP2143G (5′-CCGCGGCAAGACAGAGA-3′), RP-1(5′-GACTC-CATAAGAGCCAAAGCCCTTAC-3′), and RP2142G (5′-AGTAAAGGTCCACGGGGTATTCC-3′).The PCR was performed as previously reported in another study[25]. The amplification was conducted under the following conditions: 1 cycle at 94˚C for 2 min; 40 cycles of 98˚C for 10 s, 65˚C for 30 s, 68˚C for 20 s, with a final extension at 72˚C for 2 min. The amplified products were electrophoreses in 2% agarose gel, a band of 320 bp for both wild and mutant type, 238 bp for A2142G mutation, and 118 bp for A2143G mutation were obtained.

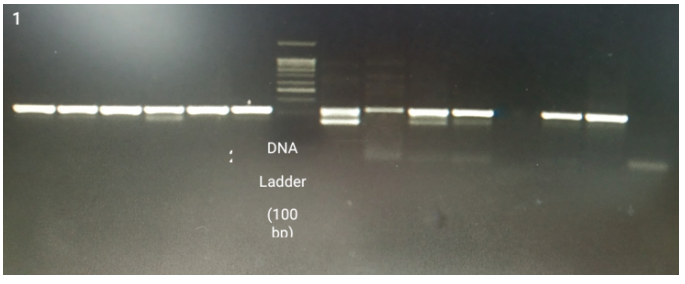

Figure-II: Agarose gel electrophoresis analysis showed amplified DNA product of multiplex ASP-PCR for clarithromycin resistance. Lane 1,2,3,4,5,6,7,8,9,10,12 and 13 indicate wild type of gene (band of 320bp). Lane 4,7,9 and 10 indicate point mutation at A2142G position (238bp), Negative control: amplified product of PCR without DNA.

ASP-PCR to determine *gyrA* gene mutation of *H. pylori* for levofloxacin resistance:

Table-I: Primer sequences of *gyrA* mutation in *H. pylori* for levofloxacin resistance

Primer name	Primer direction	Mutation	Sequence	Tm	%GC ratio	Amplicon size	Reference
F261A1	Forward	C261A	CCCCCATGGCGAGAAaG	57.2	64.71		36
F261G1	Forward	C261G	CCC CCA TGG CGAGAAgG	59.6	70.59	254 bp	
F271A5	Forward	G271A	GCGATAACGCGGTTTAGaA	55.2	47.37		
F271A9	Forward	G271A	GCGATAATGCGGTTTAGaA	53	44.11		
F271T9	Forward	G271T	GGCGATAATGCGGTTAATtA	54.3	40.0		
F272G1	Forward	A272G	GCGATAACGCGGTTTAGGgT	60.5	55.0		
F272G9	Forward	A272G	GCGATAATGCGGTTTAGGgT	58.4	50.0		
F87ATC	Forward	A260T, T261C	CCCCCATGGCGAGAtcG	59.6	70.59		
gyrA R	Reverse		GTTAGGCAGACGGCTTGGTARAAT	64.2	44.0		

A reaction mixture with total volume of 25 µl containing 10 µl of master mix (dNTP, $MgCl_2$ and reaction buffer) (Helini, biomolecules. INDIA), 0.2 µl Taq polymerase (Solis BioDyne Germany), 5.8 µL of primer mixture (0.8 µl of *gyrA* primer R, 0.2 µl of primer F261A1, 0.2 µl of primer F261G1, 0.8 µl of primer F271A5, 0.10 µl of primer F271A9, 0.6 µl of primer F271T9, 0.6 µl of primer F272G1, 0.6 µl of primer F272G9 and 0.6 µl of primer F87ATC) solution was prepared in a 0.2 ml PCR tube for each sample. The amplification was conducted under the following conditions: 1 cycle at 94°C for 2 min; 40 cycles of 98°C for 10 s, 65°C for 30 s, 68°C for 20 s, with a final extension at 68°C for 2 min. Samples were defined as positive for mutation of gyrA gene when 254-bp band was detected after electrophoresis in 2% agarose gel 110V for 42 minutes.

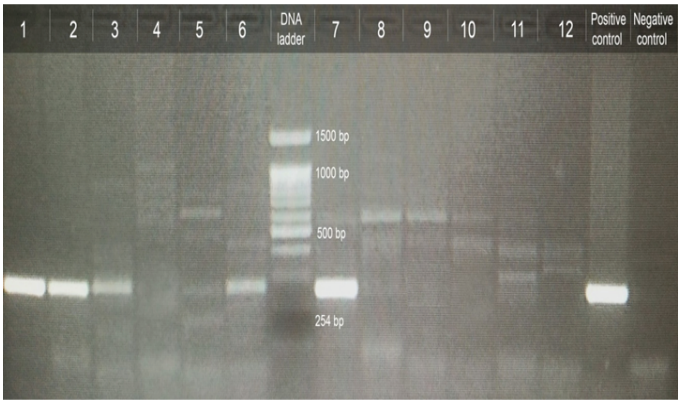

Figure-III: Agarose gel electrophoresis analysis showed amplified DNA product of multiplex ASP-PCR for levofloxacin resistance. Lane 1,2,3,6 and 7indicate point mutation of *gyrA* gene (band of 254bp). Lane 4, 5, 8, 9, 10, 11 and 12 indicates no mutation of *gyrA* gene. Positive control: levofloxacin resistant *H. pylori*, negative control: amplified product of PCR without DNA.

Data analysis:

The data were entered into Microsoft Excel 2010 and analyzed using SPSS software Version-23 (SPSS Inc., Chicago, IL, USA). Descriptive analyses of all relevant variables were done by using frequency, percentage, mean, table and figure etc.

Results

The study population (n=143) were between the ages 18-65 years, with the mean age 42.3 ± 14.3 years. The highest rate of *H. pylori* infection was found in the age group from 41-50 years (25.5%), while the lowest percentage of *H. pylori* infection (4.3%) was in the age group <20 years. Out of those 143 patients, 71 (49.7%) were female and 72 (50.3%) were male and the male female ratio (1.00: 1.01) was almost equal.

According to the case definition of *H. pylori* positive case used in this study, out of 143 patients, 47 (32.9%) were considered as *H. pylori* positive cases. Cultures for *H. pylori* were done only in 104 cases, as rest of the samples were not properly collected and transported for culture among them only 10 yield growth of *H. pylori*. Mutational analysis of clarithromycin and levofloxacin resistance were done on 42 (29.4%) *ureC* gene PCR positive cases, 5 cases were RUT and histopathology positive but *ureC* gene PCR negative.

Point mutation at A2143G and A2142G position of 23SrRNA gene for clarithromycin resistance were detected by ASP-PCR. Among 42 *ureC* gene positive *H. pylori* positive cases, only 9(21.4%) cases harbored point mutation at A2142G position and none had point mutation at A2143G position of 23SrRNA gene. Neither *H. pylori* positive cases harbored mutation at both A2143G and A2142G positions. Among ureC gene positive *H. pylori* positive cases (n=42) mutations in *gyrA* gene were detected in 16 (38.1%) cases by ASP-PCR. Only 1 (2.4%) case had mutation both in 23Sr RNA and gyrA gene, which confer multidrug resistant *H. pylori* strain. Out of 9 clarithromycin resistant H. pylori cases detected by ASP-PCR, only 2 (22.2%) cases were *H. pylori* culture positive, 5(55.6%) were RUT, *ureC* gene PCR and histology positive and in 2(22.2%) cases, were RUT and *ureC* gene PCR positive. Among 16 levofloxacin resistant cases detected by ASP-PCR, only 3 (18.8%) cases were *H. pylori* culture positive, 7 (43.7%) were positive by RUT, *ureC* gene PCR and histology, 5(31.3) cases were RUT and *ureC* gene PCR positive and 1 (6.2) case was positive by *ureC* gene PCR and histology.

Table-II: Distribution of clarithromycin resistant *H. pylori* and levofloxacin resistant *H. pylori* detected by ASP-PCR as per *H. pylori* positive case definition

Resistant *H. pylori*	Culture positive n (%)	Positive by methods other than culture			Total n (%)
		RUT, *ureC* gene PCR and histology n (%)	RUT and *ureC* gene PCR n (%)	*ureC* gene PCR and histology n (%)	
Clarithromycin resistant *H. pylori* (n=9)	2(22.2)	5(55.6)	2(22.2)	0(0)	9(100)
Levofloxacin resistant *H. pylori* (n=16)	3(18.8)	7(43.7)	5(31.3)	1(6.2)	16(100)

Discussion

In this study, prevalence of *H. pylori* was 47 (32.9%). This finding correlated with the finding of Niknam et al (2014) who reported 31% *H. pylori* positivity among adult dyspeptic population in Iran and Aftab et al (2018) reported 47% of *H. pylori* infection in adult dyspeptic patients in Bangladesh.[4, 37]

ureC gene was detected in 42 (29.4%) cases in this study. Similar finding was reported by Pandya et al (2017) where 31% *H. pylori* were positive in gastric biopsy by *ureC* gene PCR.[38] However, Mishra et al (2002) had reported 53% *H. pylori* positive cases detected by *ureC* gene PCR.[39] A variety of genes including the cagA gene, *ureC* (glmM) gene, *ureA* gene, 16S rRNA gene and 26-kDa species-specific antigen (SSA) gene have been used as potential targets to detect *H. pylori*. Several studies observed that PCR based amplification of *ureC* gene is the most appropriate target gene for detection of *H. pylori* from clinical samples.[35,40]

Only five (10.6%) cases were *ureC* gene PCR negative but positive by RUT and histopathology, may be due to insufficient amount of *H. pylori* DNA in those samples.[41] Presence of PCR inhibitory substance such as Taq polymerase inhibitors, prolonged transportation, inappropriate storage of specimen, also can give negative results.[29] All the *ureC* positive cases were either RUT or histological staining positive.

Among 42 *ureC* gene positive cases, Clarithromycin resistances were 9 (21.4%) by ASP-PCR. All 9 (100%) clarithromycin resistant cases had point mutation at A2142G position, no point mutation were found at A2143G position. Wani *et al* (2018) and Ubhayawardana et al (2015) also reported 65.2% and 100% point mutation at A2142G position of 23S rRNA gene.[42,43] Prevalence of the particular point mutations varies across geographical areas. On the contrary, Gehlot *et al* (2016) and Nakamura et al (2007) reported 87.5% and 99.0% point mutation at A2143G position.[26,44]

This result was consistence with the result of the studies conducted by Gehlot et al (2016) and Sun et al (2010) in which resistance to clarithromycin were 11.8% and 20.7% respectively.[44,45] Higher resistance rate of clarithromycin was reported by Aftab et al (2016) who detected 39.3% clarithromycin resistance by agar dilution method.[15]

This study was designed to explore two most common described mutations, A2142G or A2143G for clarithromycin resistance, other mutations which are less likely to cause resistance and treatment failure could not be detected due to time constrains. Additionally, the molecular test correctly classified all negative patients as no sample showed any amplification.

gyrA gene mutations were also detected by ASP-PCR for levofloxacin resistance. Among 42 cases, *gyrA* gene mutation was present in 16 (38.1%) cases. Similar to this finding, Miftahussurur et al (2016) reported 42.9 % levofloxacin resistance.[46] High prevalence (66.1%) of levofloxacin resistance was reported by Aftab et al (2016).[15] Trespalacios et al (2015) found 100% sensitivity and 92.7% specificity of ASP-PCR of *gyrA* gene mutation for levofloxacin resistance, using agar dilution as the gold standard.[36]

Only 10 *H. pylori* were isolated from culture, so antibiotic susceptibility testing could not be performed in all *H. pylori* positive cases by disk diffusion method. So, the agreement between ASP-PCR and disk diffusion method for the detection of resistance could not be detected and also it was not possible to observe any discrepancies between these methods to determine the reproducibility.

Out of 9 clarithromycin resistant cases detected by ASP-PCR, only 2 (22.2%) cases were *H. pylori* culture positive, 7 (77.8%) cases were *H. pylori* positive as per case definition by methods other than culture. All 16 levofloxacin resistant *H. pylori* cases detected by ASP-PCR were ureC gene PCR positive. Among them only 3 (18.8%) cases were culture positive, 7 (43.7%) cases were positive by RUT and histology, 5 (31.3%) cases were RUT and 1 (6.2%) case was positive by histology. Most of the resistant cases were detected by ASP-PCR directly from the gastric biopsy specimen rather than culture as *H. pylori* culture is difficult to perform and cultures do not always recover all the strains present in the sample.

Conclusion

All clarithromycin resistant cases detected by ASP-PCR, have point mutation at A2142G position. As most of the resistant cases are detected by ASP-PCR from the biopsy specimen rather than culture, it could be an alternative approach for detection of antibiotic resistance pattern of clarithromycin and levofloxacin where culture facilities are not available or culture cannot be

done. This may guide towards the therapeutic choices in our country where culture and susceptibility tests are not routinely performed.

Acknowledgments

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Disclosure

The author reports no conflicts of interest in this work.

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