



Basic study in molecular noninvasive estimation of Clarithromycin resistant *Helicobacter pylori*: Set up of ASP-PCR in differentiation of *infB*, and PCR-sequencing to evaluate 23S rRNA, and *rpl22* related mutations

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Authors

Atena Abedi Maghami¹,
Ashraf Mohabati Mobarez^{2*}, Abbas
Yadegar³,
Maryam Nikkhah⁴,
Amir Sadeghi⁵,

1,2 Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

3 Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

4 Department of Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

5 Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical, Sciences

*Corresponding authors:

Ashraf Mohabati Mobarez, PhD.
Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University, Al-e Ahmad Exp. Tehran, Iran.

mmmobarez@modares.ac.ir

ABSTRACT

Designation of the local profile of Clarithromycin resistant (CAM-R) in *Helicobacter pylori* (*H. pylori*) positive patients with phenotypic testes consequently evaluation of probable agreement between resistance phenotypes to genotypes is the necessity of accessing rapid molecular noninvasive tests. So, we designed ASP-PCR and PCR-sequencing methods to evaluate *infB* (G160A), and 23S rRNA (A2142C/G, A2143C/G) and *rpl22* (GTG deletion or TTCCATGTA insertion) nucleotide polymorphisms from stool of patients with symptoms of *gastritis*. Urea tubes were used to transport 96 gastric biopsies to the laboratory. **Methods.** Agar dilution method was performed to assess CAM-R strains. Besides the phenotypical identification, stool samples were collected and stored at -80° C. Molecular identity was characterized by amplification of 23S rRNA target gene. In evaluation of non-invasive genotypical molecular tests in detection of corresponded mutations, ASP-PCR was performed to isolate *infB* G160G wild-type strains and PCR-sequencing in determining 23S rRNA and *rpl22* polymorphisms. **Results.** Molecular isolation of *H. pylori* positive-patients was reported to be 34/54(62%). Among 35/96 (36%) phenotypically characterized *H. pylori*-positive infected patients, 16/35(45%) were considered for CAM-R strains. Distribution of point mutations between resistant isolates have been revealed to be (1/16) for A2143C, (4/16) for *infB* G160A (PCR negative patients), (2/16) *rpl22* for 3bp deletion, and (16/16) for *rpl22* 9bp insertion. **Conclusion.** We are honored to introduce *rpl22*-related point mutations as the potential marker in designing noninvasive molecular method in Clarithromycin resistant infected patients screening.

Keywords: Noninvasive molecular tests; Clarithromycin resistance; 23S rRNA; *rpl22*; *infB*; *Helicobacter pylori*

Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic pathogen bacterium and the most causative agent in the formation of gastritis and progressive gastric diseases e.g., gastric and duodenal ulcers, mucosal-associated lymphoid tissues (MALT) lymphoma, and carcinoma [1, 2]. Thereby, bacterial eradication directly influences clinical outcome and prevent progressive disorders [2]. According to various combination therapy, standard triple therapy (STT) is one of the most popular prescriptions which consist of proton pump inhibitors (PPIs), amoxicillin (AMX), and Clarithromycin (CAM) or metronidazole (MTZ) [3]. Erythromycin derivative macrolide, Clarithromycin is the main antibiotic in STT until the efficiency of eradication becomes $\geq 90\%$. Actually, in areas where the level of Clarithromycin resistance (CAM-R) infected patients over pass $\geq 15\%$ cure rate significantly becomes concerning [4]. Formation of CAM-R *Helicobacter pylori* is one of the main causes of treatment failure, which is naturally selected in subjects through high dosage in prescription and long duration of consumption [1]. Clarithromycin interferes with protein synthesis through reversible binding with low affinity to the peptidyl-transferase region (domain V) ribosome subunit 50S, blocking the peptide bond formation and peptidyl tRNA translocation from the A to P-site which leads to premature dissociation of peptidyl tRNA with the accumulation of truncated peptides [5]. Patient's routine *H. pylori* phenotypically antibiotics susceptibility tests in screening, are challenging, intricate, and in the best situation reported with low sensitivity [3, 6]. Thereby, based on Maastricht I/ Florence consensus reports, implementation of the potential alternative molecular tests in local management of Clarithromycin resistant patients improve the efficiency of standard triple therapy [7]. In Western countries, the majority of mechanisms in Clarithromycin resistant $\geq 90\%$ related to dominant point mutations in 23S rRNA 2142C/G that well known for MIC ≥ 64 mg/L, whereas the A2143G/C substitution reported for MIC < 64 mg/L, which accounted different in Asian countries [8, 9]. Recently, participation of two novel target genes mutations, translation initiation

factor IF2 *infB* (G160A) and ribosomal protein L22 *rpl22* (3bp deletion in 226 or 9bp insertion nucleotide location 295) become strongly desired in CAM-resistance evaluation. According to Binh et al., linkages between *rpl22* GTG deletion and TTCCATGTA insertion were introduced in formation of Clarithromycin -resistant *H. pylori* with MIC $\geq 2,4$ mg/L. They have also revealed that the synergism effect between 23S rRNA A2143G point mutation coupled with *infB* and *rpl22* promotes the minimal inhibitory concentration to the highest range ≥ 256 mg/L [10]. In 2016, Miftahussurur et al., discussed both *infB* silent point mutations and *rpl22* 19bp deletion at position 535 that resulted in low MIC range, and revealed the synergism effect among *infB*, *rpl22*, and 23S rRNA 2143 A to G in higher MIC values. They also suggested that "*rpl22* point mutations could be independent causes of CAM resistance" [11].

Therefore, in probable concordance between CAM-R genotypes to phenotypes, this study aimed to characterize details of CAM-R-related point mutations in raised target genes from the feces of the patients.

2. Materials and methods

From April 2020 to September 2020 a total number of 96 subjects, aged 14 to 88 years old, were recruited for this study. The patients had been excluded with the history of previous consumption of antibiotics, H₂ receptor blockers, or PPIs within two weeks before the Endoscopy procedure. The project was approved by the ethics committee of Tarbiat Modares University of Medical Sciences (IR-MODARES.REC.1398/019), Tehran, Iran.

2.1 Phenotypical Identification

2.1.1 Bacterial cultivation

Two pieces of antral biopsies were obtained from patients and delivered to *H. pylori* laboratory by Christenson urea tube within 5 hours. To achieve the optimum growth condition, tissues were homogenized in BHI broth containing FCS 10% (v/v), and the suspension was immediately introduced on Selective culture media Brucella Agar S1 (Merck, Darmstadt, Germany) containing 7% sheep blood (v/v), 10% fetal calf serum (FCS), Amphotericin B 2.5 mg/L Sigma-Aldrich (St.

Louis, MO, USA) and supplemented with Skirrow (Quelab, Montreal, Quebec, Canada). The plates were incubated under micro-aerobic conditions generated by using CO₂ incubator (Heraeus, Germany) at 37°C for 7-10 days. The morphological appearance was checked by gram staining, activities of urease, oxidase, and catalase enzymes [12].

2.1.2 Clarithromycin susceptibility testing

To archive CAM-R strains, agar dilution method was performed by replacement of *H. pylori* culture positive samples turbidity equipped to McFarland opacity standard of 2 on Brucella agar Sl plates with Clarithromycin concentration range between 0.016–256 mg/L under micro-aerobic condition for three days. Based on clinical and laboratory standards institute (CLSI) MICs ≥ 1 mg/L were considered to be CAM-resistance.

2.2 Stool sampling

A week after endoscopy procedure the same individuals' stool specimens were collected in plastic cups, and transported in cold chain condition, then stored at -80°C.

2.3 Assessment of candidate point mutations: 23S rRNA, *rpl22*, and *infB* in stool specimens of patients

2.3.1 Primer designing for detection of 23S rRNA, *infB*, and *rpl22*

We used the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/genbank>) in booking all the complete sequences of 23S rRNA, *infB*, and *rpl22* sequences of diverse *H. pylori* strains. consequently, by pooling all the strains with ClustalW (<http://www.ebi.ac.uk/clustalw/>), we focused on the conserved region of the renounced targets that cover the putative 23S rRNA mutations, *infB* mismatches and *rpl22* deletion and insertion, The specificity of both forward, and reverse primers were controlled with NCBI nucleotide blast database (<http://www.ncbi.nlm.nih.gov/blast/>). We used Oligo Calc: Oligonucleotide Properties Calculator software in the evaluation of self-complementarity of the primers. Site score of each primer was checked with Primer3 Input (version 0.4.0). NCBI primer blast

(<http://www.ncbi.nlm.nih.gov/Primer-BLAST/>) was used to organization of the amplification size.

2.3.2 DNA extraction

DNA extraction from stool samples was performed by following the manufacturer's directions of Favarogene DNA extraction kits (Taiwan), and the extracted DNA was preserved at -20°C.

2.3.3 *Helicobacter pylori* molecular isolation

23S rRNA target gene was considered in amplification by performance of first polymerase chain reaction (PCR) on the total number of 54 feces collection. DNA was amplified in a final volume of 20 μ L :10 μ l of 2dH₂O, 7 μ l of 2x Master Mix (Amplicon, Spain), 2 μ l 23S rRNA F-5' ATGAATGGCGTAACGAGATGG3' and 23S-rRNA R-5'CGCATGATATCCCATAGCAGT3' primer mixture (Metabion Germany), 1 μ l of stool analyte in each reaction tube. Amplification was performed in the Thermocycler T100 with the cycling condition: 95°C for 5 minutes, 42 cycles of amplification that performed at 95 °C for 60 s, 56°C for 35 s, and 70°C for 60 s. 1.0% agarose gel in a TAE buffer (1x), 60 minutes for 90 v was utilized for molecular verification of *H pylori* 147bp amplified positive strains.

2.3.4 ASP-PCR in *infB* G160G detection

To evaluate *infB* novel point mutation, we designed set primers to isolate wild-type *infB H. pylori* by ASP-PCR. Mixture prepared in the final volume of 20 μ L: 1 μ l of stool DNA extraction, 9 μ l of DDW, 8 μ l of 2x Master Mix (Amplicon, Spain), 2 μ l of 100 μ M set primers (Metabion Germany), the primers are shown in Table 1. Cycling conditions involved an initial denaturation at 95 °C for 5 min, followed by 42 cycles, each consisting: denaturation at 95 °C for 60 s, 58°C for 35 s, and 60 sec for extension stage at 70°C. The size of the product was monitored by electrophoresis in 2.0% w/v agarose gel, in a TAE buffer (1x), 110 minutes for 65 v.

2.3.5 PCR -sequencing of 23S rRNA, *rpl22* target genes

One directional sanger-sequencing in selection of candidate mutations among all 54 CAM-susceptible or resistant patients was conducted at Pishgam Company, Tehran, Iran. Targets amplification carried out in the separate tubes for 23S rRNA and *rpl22* genes, containing 20 μ L of the reagents: 1 μ L of stool analyte, 7 μ L of master mix (Amplicon, Spain), by using 1 μ L of each F/R set primers (Metabion Germany) Table 1, 10 μ L of DDW per mixture, under the cycling conditions, initial denaturation at 95 °C for 5 min, 42 cycles of amplification: denaturation at 95 °C for 60 s, annealing temperature of 23S rRNA, and *rpl22* at [56°C and 60°C] for 35s, consequently 70°C for extension step for 60 s.

4. Statistical Analysis

The SPSS Statistics for Windows (version 21.0, IBM Corp, Armonk, NY, USA) and Chi-square and Fisher's exact tests were applied to determine the statistical significance of the differences between categorical variables $P = 0.001$ was considered to be highly significant.

5. RESULTS

5.1 Phenotypical and Molecular Assessment of *Helicobacter pylori*

According to patient's Histopathological report as the gold standard, the rate of *H. pylori*-positive isolation was indicated to be 65% (N=63/96). 35/96(36%) of the patients were phenotypically characterized by bacterial culturing with the sensitivity and specificity [55.6%(CI),42.48% - 68.08% and 100% (CI), 89.42%-100%], $P= 0.001$ in detection, ordinarily.

The frequency of molecular identified *H. pylori* by first PCR from stool samples reported to be 34/54(62%). Sensitivity and specificity in the detection were reported to be 63% (CI),48.74% - 75.70% and 100%, $P= 0.001$.

5.2 Clarithromycin Susceptibility Testing

Sixteen out of 35 *H. pylori* culture positive subjects (45%) were considered to be Clarithromycin-resistant strains with minimal inhibitory concentration ≥ 2 mg/L; 4/16 with MICs ≥ 2 mg/L, 2/16 with MIC ≥ 4 mg/L, and 10/16 for MIC ≥ 8 mg/L. In this investigation, we

did not have any CAM-R patients with full level of Clarithromycin-resistant minimal inhibitory concentration. The lowest concentration of Clarithromycin in inhibition was reported to be ≤ 0.43 mg/L.

5.3 Identification and analysis of 23S rRNA, *infB*, and *rpl22* related point mutations Out of the sixteen CAM-R *Helicobacter pylori*-positive isolates there were only one (6.2%) presented mutation A to C at position 2143 with minimal inhibitory concentration of ≥ 8 mg/L. However, the possibility that a mutation is present at nucleotide position A2143C in the 23S rRNA gene and resulted in a Clarithromycin resistant phenotype was 100%, P value of ≤ 0.457 . Multiple replicates of ASP-PCR were performed in the total 54 stool samples of the subjects which were phenotypically categorized in Clarithromycin susceptible or resistance groups; the mutations were analyzed via PCR Figure 1. The total number of 6/54(11%) were *infB* PCR negative samples that considered mutated isolates for transition substitution at G160A. Two out of six *infB*G160A were determined among CAM-sensitive patients with MIC ≤ 0.32 mg/L and MIC ≤ 0.36 mg/L. In addition, 4/16 (25 %) were determined in CAM-R isolates; one of them with MIC ≥ 4 mg/L, and the other three were observed with higher MICs ≥ 8 mg/L. Sensitivity and specificity in detection were reported to be 25.0% [CI],7.26% - 52.37% and 89.5% [CI],66.86%-98.69%.

In this investigation, there was only one isolate characterized with A2143C mutation that coupled with *infB* G160A transition with MIC ≥ 8 mg/L, (*infB* G160A substitution beside 23S rRNA A2143C mutation).

In all the sixteen CAM-resistant patients *rpl22* TTCCATGTA insertion was distributed within CAM-R strains with low or high minimal inhibitory concentration, Table 2. Two CAM-resistant patients out of 16 (13.3%) were considered for *rpl22* GTG deletion with MIC ≥ 8 mg/L, sensitivity in detection reported to be 13.3% CI, [1.65% -40.46%]. In detail, one variant with the dominant 23S rRNA mutation A2143C, containing *infB* G160A and *rpl22* (9bp insertion and GTG deletion nucleotide) with a minimal inhibitory concentration of ≥ 8 mg/L, and the second 3bp deletion was found in clinical

isolate harboring *infB* G160A with MIC \geq 8 mg/L. The rest of CAM-resistant patients with MICs \geq 2, 4, 8 mg/L were with GTG nucleotides.

Discussion

According to the World Health Organization (WHO), the emergence of *Helicobacter pylori* drug-resistant has become one of the major barriers to eradicating the infection. Erythromycin derivative macrolide; Clarithromycin, is the key antibiotic in standard triple therapy in *H. pylori* eradication regime [13]. De Francesco V et al., renounced that the Clarithromycin resistant strains with high MIC values MIC \geq 8 mg/L, are causative marker for predicting the treatment failure [14]. During the decade 2006-2016, library for observational studies revealed the lower efficient STT, sequential therapy, and concomitant therapy along with increase of CAM-R in patients with the rate of 18% in Bangladesh, China 26%, India 17%, Japan 19%, Nepal 21%, Saudi Arabia 16%, South Korea 17%, Turkey 27%, Vietnam 34%, and Pakistan 37% [15]. Thereby, designing and setting alternative molecular methods in facing less sensitive phenotypically tests in resistant patients screening seems to be inevitable [3, 16].

The number of *Helicobacter pylori*-infected patients is relatively high in Iran with a general population percentage of 40% to 90% [17]. The highest observed in the northwest of Iran, in the city of Ardabil and its surroundings with a percentage towering at 89% [18]. According to the recent Iranian meta-analysis the mean average of CAM-resistant patients in Tehran, Shiraz, Ahvaz, Isfahan, Tabriz, Kashan, Mashhad, Yazd, Lahijan, Sari, Rasht, have been revealed to be 25.3%, that khademi in Iranian subgroup analysis the highest rate referred to Chaharmahal va bakhtiary 63.6% and the lowest reported in 5.5% in Rasht [19].

Among the limited studies in the evaluation of *H. pylori* CAM-resistant related mechanisms such as polymorphic human-gene variants *CYP2C19*2* (681G>A) and *CYP2C19*3* (636G>A) correlated in patients proton pump inhibitor (PPI) metabolization rate, efflux systems MFS (1181), RND transporter (*hef* ABC) along with HP0939, HP 0497 and HP0471

the transporters which is highly expressed in *H. pylori* biofilms and MDR strains; dominant point mutations inside of 23S rRNA; peptidyl transferase region of domain v are positively reputed in CAM-R infected patients screening [20-23]. Pooling the patients CAM-resistant sequences within 23S rRNA related point mutations demonstrated that A2143G has been more reputed than 2142G and 2142C in Asian countries that counted for 23%, in counterpart with those from Europe and North America that are responsible for 90% of the cases with the primary report of clarithromycin resistant rate [24]. In Iran according to khademi metanalysis; 59.1% of the CAM-R strains harbored A2143G, 17.8% with 2142G, 8.8% for 2142C, 6.2% 2144G although there was no report of 2143 A to C substitution among the resistant patients [8].

In our study, we observed high proportion of the infection 62% among the subjects which 45% were considered for Clarithromycin resistant with minimal inhibitory concentration of \geq 2mg/L (62% with MIC \geq 8 mg/L). In fact, the average resistance rate was near to Saniee et al., between 2010-2017 [25]. According to our results, the report of resistance rate was closest to Alavifard recent published datas in Tehran, that they have mentioned the rate of 43.9% in their population base study [26]. According to our analysis, out of the sixteen CAM-R *H. pylori*-positive infected patients, there was only one isolate 6.2% that demonstrated the rare mutation of A to C at 2143 nucleotide location with minimal inhibitory concentration of \geq 8 mg/L. According to Alavifard variability of 23S rRNA mutations in CAM-R patients recorded to be 6.2% for 2142G, and 8.3% for 2143G [26]. In Kashan, the variety of 23S rRNA related mutations reported to be 66.7% for 2143G, 33.3% for 2142G [27], and in Bushehr 1.5% for 2143G, and 6.7% for 2144G [28]. Already, the report of the rare mutation of A to C substitution at position 2143 was in line with the limited reports in the world in Germany and France [29, 30]. F Mégraud in 1999 mentioned that the characterization of point mutations within the resistant patients remarkably related to 23S rRNA 2144G, 2143G, and 2143C mutations [30]. In Germany between 2015-2018 among 1851 subjects, the overall resistant rate was pointed to be 11.3%. The participation of the dominant

mismatches were noted for the mutation at A2143G, A2142G, and also mutation at A2143C nucleotide location[29]. However, in molecular management of the CAM-resistant infected patients, we focused on *infB* (G160A) and *rpl22* (deletion or insertion) the novel variants which might potentially be related to CAM-R strain formation. *infB* (translation initiation factor IF-2) which acts as the suppressor of the formyl methionyl transfer RNA from spontaneous hydrolysis, promotes its binding to 30S ribosomal subunits during the initiation of protein synthesis, and is involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex strongly related to synergic effect on Clarithromycin resistance mechanisms. According to Binh et al., construction of G160A mutant in 26695 wild-type strain of *H. pylori* indicated the increasement of the MIC, although it did not exhibit resistance breakpoint. Effect of *infB* G160A coupling with *rpl22*-9bp insertion exhibited increment of MIC to the level of ≥ 2 mg/L[10]. *rpl22* encode the accessory protein of the large ribosomal subunit (ribosomal protein L22) that interacts with all the domains of 23S rRNA [31]. According to the first report on transformation of 3bp deletion (strains without GTG nucleotides) or TTCCATGTA insertion in 2014 Binh et al., showed that the strains that contain *rpl22*-9bp insertion are potent to exhibit lower levels of Clarithromycin inhibition $MIC \geq 2$ mg/L, and the strains that contain *rpl22* GTG deletion correlated to higher level of Clarithromycin inhibition in concentration $MIC \geq 4$ mg/L. Based on the coupling of A2143G and *rpl22* 9bp insertion along with *rpl22* 3bp deletion through transformation demonstrated the highest level of clarithromycin concentration in bacterial inhibition, $MIC \geq 256$ mg/L[10]. Based on this experiment, six out of 34 infected patients characterized with *infB* G160A nucleotide mismatches were reported: 4/6 with $MIC \geq 4$, 8 mg/L, and the 2/6 were characterized among CAM-sensitive infected patients. In contrast to Binh et al., and near to Miftahussurur et al., our data propose that there is no accurate attribution between CAM-R *H. pylori*- positive infected patients screening and *infB* mutations with or without *rpl22* mutations [10, 11].

Accumulation of our knowledge demonstrated that among all CAM-R strains 16/16 (100%) *rpl22* TTCCATGTA insertion has been distributed with low or high minimal inhibitory concentration of 2,4,8mg/L and 13.3% of the strains considered with GTG deletion pattern with $MIC \geq 8$ mg/L. Based on the present findings the highest minimal inhibitory concentration in A2143C with *infB* G160A beside *rpl22* (GTG deletion and 9bp insertion) considered to be 8 mg/L, that looks different from the reports of Miftahussurur et al., in 2016, and Binh et al., in 2014, respectively [10, 11]. In line with the Indonesian study, *rpl22* mismatches could be remarkable target gene in rapid monitoring of CAM-R infected patients [11].

Conclusion

We are honored to introduce economical, non-invasive molecular tests that can be used as a routine/daily clinical practice. Based on our hands-on experience there is a highlighted, significant overlapping between the phenotypically separated Clarithromycin resistant strains and *rpl22* 9bp insertion, at the second step *rpl22* GTG deletion. These changes alone might be enough to express CAM resistance among *H. pylori*-positive infected patients. Based on the positive outcome of our study, the next step would be to produce such a study on a broader level with a larger number of subjects.

Declarations

Ethics Approval and consent to participate:

This survey was approved by the Ethics Committee of Tarbiat Modares University (IR-MODARES.REC.1398/019), Tehran, Iran; all the participants have accepted and signed the informed consent.

Consent for publication:

Written informed consent was obtained from the patient for publication of this cross-sectional study. A copy of the written consent is available for review by the Editor of this journal.

Availability of data and materials:

The data that support the findings of this study are available from the corresponding author upon reasonable request. Any accession

numbers/web links will be listed in the final form.

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Competing interests:

The authors declare that they have no competing interests.

Contributions:

AAM and AMM developed the idea, designed the study, AAM, and AY collected the samples, AAM, AMM, and MN analyzed the data and drafted the manuscript. AMM reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Abbreviations

H. pylori: *Helicobacter pylori*
 MALT: mucosal-associated lymphoid tissues
 STT: standard triple therapy
 PPIs: proton pump inhibitors
 AMX: amoxicillin
 CAM: Clarithromycin
 MTZ: metronidazole
 CAM-R: Clarithromycin resistance
 infB: translation initiation factor IF2
 rpl22: ribosomal protein L22
 MIC: minimal inhibitory concentration
 PCR: polymerase chain reaction
 ASP-PCR: Allele-specific polymerase chain reaction
 WHO: World Health Organization
 CYP2C19 *2, *3: p450 cyp 2c19 Pharmacogene Variation
 MFS : major facilitator superfamily
 RND : Resistance-nodulation-cell division
 min: minute
 s: second
 °C: centigrade degree
 l: litre
 mg: milligram
 µl: microlitre
 v: volume
 w: weight