

Sequential Therapy versus Standard Triple Therapy in *Helicobacter pylori* Eradication

Abstract

Background: The belief that no organism can survive in the acidic environment of the stomach was shattered by Barry Marshall and Robin Warren in 1982 when they identified the organism *Helicobacter pylori*. *H. pylori* is the main cause of gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma. The aim of treatment of *H. pylori* infection in any clinical situation is eradication of bacterium from the foregut with armamentarium of antibiotic of choice.

Aim: to study whether sequential therapy is more effective than standard triple therapy in terms of eradication of *Helicobacter pylori*.

Material and methods: Our hospital based, prospective, randomized study entitled "Sequential therapy versus standard triple therapy in *Helicobacter pylori* eradication" was conducted and concluded in post graduate department of Medicine, tertiary care institute in 2012. Three hundred patients with documented *H. pylori* infection studied, were randomized into 3 groups to receive standard or sequential (clarithromycin or levofloxacin based) anti *H. pylori* therapy.

Results: Three hundred patients studied were randomized into 3 groups, one group received standard triple therapy for 10 days (Group A), second group clarithromycin based sequential therapy for 10 days (Group B) and third levofloxacin based sequential therapy for 10 days (Group C). Group A achieved eradication rate of 68% only. While sequential therapy group B and C showed a success of 81 and 86% respectively. In our study, Group B sequential therapy achieved 13% higher eradication as compared to standard triple therapy.

Conclusion: In conclusion, our large, prospective, hospital based study shows the superiority of sequential treatment for eradicating *H. pylori* infection compared with conventional triple therapy. The sequential regimen is less expensive and is more effective than conventional therapy for patients with clarithromycin-resistant organisms. Our data suggest that sequential therapy may have a role as a first line treatment for *H. pylori* infection.

Keywords: *Helicobacter pylori*; Standard therapy; Sequential therapy; Eradication

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Introduction

Helicobacter pylori was discovered by two Australian Nobel Prize winning scientists, Barry Marshall and Robin Warren in 1982 [1]. It plays a key role in the development of both stomach and intestinal ulcers. It is a spiral, highly mobile, microaerophilic gram negative bacterium with multiple unipolar sheathed flagella [2]. It mostly resides in the deeper mucus gel coating gastric mucosa and between the mucus layer and the gastric epithelium of antrum and proximal segments of the stomach. The genome in *H. pylori* encodes-1500 proteins [3].

Multiple virulence factors of *Helicobacter pylori* that promote colonization and induce tissue injury.

- Factors promoting colonization
- Flagella [4]
- Urease [5]
- Adherence Factors [6]
- Factors Inducing tissue injury
- Lipopolysaccharide [7]

- Leukocyte recruitment and activating factors [8]
- Vacuolating Cytotoxin (Vac A) [9]
- Cytotoxin-associated antigen (Cag A) [10,11]
- Outer membrane inflammatory protein (Oip A) [12]
- Heat shock protein (HSP A, HSP B)

The prevalence of *H. pylori* is strongly correlated with socioeconomic conditions with over 80% of the population in developing countries and 20%-50% in industrialized countries affected [13]. Infection is acquired by oral ingestion of the bacterium in vomitus, saliva or feces and is mainly transmitted within families in early childhood. There is frequent reinfection following eradication therapy in adults [14,15].

H. pylori infection has pathogenic role in majority of duodenal and gastric ulcers, and there is strong evidence that it also increases the risk of gastric cancer and gastric mucosa associated lymphoid tissue lymphomas [13]. *H. pylori* infection that involves the antrum predominantly, while relatively sparing the acid-secreting portion of the stomach, will predispose to duodenal ulceration whereas intense inflammation in the oxytic mucosa will result in gastric atrophy with a decreased acid output and a predisposition to gastric ulceration and cancer [16].

Non-gastrointestinal tract diseases [17] possibly associated with *H. pylori* infection has come up recently although the data supported these associations are weak.

The diseases include Iron deficiency anemia, Coronary artery disease, Cerebrovascular disease, Hypertension, Raynaud's phenomena, Migraine headaches, Vomiting of pregnancy, Immune thrombocytopenic purpura, Hyperammonemia, Sudden infant death syndrome, Growth retardation, Anorexia of aging, Rosacea and Chronic urticaria.

Diagnoses of *H. pylori* may be divided into that do (Biopsy based tests) and that do not require sampling of gastric mucosa (non-invasive tests). In biopsy based tests at least three samples (e.g. from the lesser curvature angularis, the greater curve pre-pyloric antrum, and the greater curve body) are taken. The standard hematoxylin and eosin (H&E) stain is excellent to determine histological chronic or chronic active inflammation (gastritis) and demonstrates *H. pylori* if large number of organisms are present. A special stain (e.g. silver stain) is better at detecting the organism if small numbers of bacteria are present. Attributes of both H&E and a special stain are found in the genta and El-Zimaity 'triple' stains, which combine the H&E stain, *H. pylori* selective stains, and alcian blue to detect intestinal metaplasia [18]. The alternative is to use two different stains, a combination of an H&E and a Diff-Quik stain is probably the best alternative [19]. Rapid urease test is a rapid test for diagnosis of *H. pylori*. The basis of the test is the ability of *H. pylori* to secrete the urease enzyme, which catalyzes the conversion of urea to ammonia and bicarbonate. The test is performed at the time of gastroscopy. A biopsy of mucosa is taken from the antrum of the stomach, and is placed into a medium containing urea and an indicator such as phenol red. The urease produced by *H. pylori* hydrolyzes urea to ammonia, which raises the pH of the medium, and changes the color of the specimen from yellow (negative) to red (positive).

There is evidence to suggest that *H. pylori* moves proximal in the stomach in patients on therapy with proton pump inhibitors, and, as such, samples from the fundus and antrum should be taken in these patients.

Non-invasive tests include serological tests, urea breath tests and stool antigen tests. Serological tests (IgG antibodies) are generally not useful to confirm cure after antimicrobial therapy, a fall in antibody titers of 20% or more 6 months after completion of therapy may be sensitive in confirming cure of infection [20]. Urea breath tests using urea labeled with either ¹³C or ¹⁴C that is ingested [21] and are preferred means of evaluating the success of antimicrobial therapy in clinical practice, but patient should be off PPIs for at least 7 days before the test can be done otherwise one third of patients will give false negative tests [22]. H2 receptor antagonists can be continued up to the day before urea breath testing and provide an alternative for the patient who derives continued antisecretory therapy. Another new, non-invasive diagnostic test is a stool antigen test based on detection of *H. pylori* antigens in stool. Overall, studies using pretreatment *H. pylori* stool antigen tests have shown that the sensitivity and specificity of the tests are comparable to histology or urea breath tests [23].

Cure of *H. pylori* infection is not easy and requires combination of antibiotics often with additional non-antibiotic adjunctive agents; single agents are ineffective. The finding that the elimination of *H. pylori* infection changes the natural history of peptic ulcer disease [24] and gastric mucosa associated lymphoid tissue lymphoma [25] has led to the development of successful strategies to clear the organisms from persons with these disorders, keeping in view the prevalence of peptic ulcer disease (gastric ulcer and duodenal ulcer) in the United States, with four million individuals affected per year. Lifetime prevalence of peptic ulcer disease in United States is ~12% in men and 10% in women [26].

Several combination therapies have been an effective standard of treatment, however resistance rates have been rising and eradication failures have increased to 1 in 5 patients [27]. Reported clarithromycin resistance is 10 to 12% in patients infected with *H. pylori* and that of metronidazole is 25.1% during the period from 1999 through 2002 [28].

Several therapies have come like dual therapy (PPI plus amoxicillin, PPI plus clarithromycin, ranitidine bismuth citrate (tritec) plus clarithromycin) is not recommended in view of eradication rates of <80-85%. At present the standard treatment for *H. pylori* infection that has been endorsed rely on clarithromycin or metronidazole in conjunction with other antibiotics and PPI [29,30] i.e. amoxicillin 1 g BD + clarithromycin 500 mg BD + PPI like Pantoprazole 40 mg BD for 10-14 days. But the rate of eradication with such a regimen has decreased (88% in 1996 and 69.4% in 1999) [31,32]. Novel first line anti *H. pylori* therapy in 2011 includes sequential therapy, concomitant quadruple therapy, hybrid (dual-concomitant) therapy and bismuth containing quadruple therapy. In bismuth containing Quadruple therapy we use (bismuth + metronidazole/clarithromycin + tetracycline + PPI) where clarithromycin is substituted for metronidazole (or vice versa) [33]. So it remains to be determined which therapy is best and cost effective in terms of *H. pylori* eradication.

Material and Method

The study was conducted in Postgraduate Department of Medicine tertiary care Hospital Srinagar. It included both inpatients and out-patients respectively. It was a hospital based prospective study and included subjects between 18 to 70 years who presented with symptoms of dyspepsia and GI bleed. The patients were endoscoped by expert gastroenterologists and rapid urease test was done on them, for which single biopsy sample was taken from the antrum and patients were asked to look for change in color of urea containing medium within 24 hours after putting biopsy specimen in rapid urease test kit (RUT kit), change to red color (phenol red used as indicator) indicated that test was positive for *H. pylori* and subject henceforth was included in study group. Rapid urease test kit was supplied to us by hospital (Allied Marketing Co.).

A total of 654 patients participated in our study out of which rapid urease test was positive in 463 and 163 subjects dropped out of study, the rest 300 were randomized into three groups (A, B and C) of 100 each, by using method of simple random sampling to avoid selection bias. Group A received standard triple therapy, Group B clarithromycin based sequential therapy and Group C levofloxacin based sequential therapy.

Exclusion criteria

- Patients less than 18 years or more than 70 years of age.
- Pregnant and lactating mother.
- Patients on prolonged PPI therapy, anticoagulants, steroids and/or NSAID.
- Malignancy of esophagus and stomach, chronic liver disease (CLD) patients.
- Comorbid medical conditions, severe or unsuitable cardiovascular, pulmonary or endocrine disease, clinically significant hepatic or renal disease or dysfunction.

Patients or their guardians signed consent before participation in the study. A detailed clinical history, relevant physical and abdominal examinations were carried out. Routine laboratory studies were performed and USG abdomen of subjects was also done.

Treatment Regimens used In Three Groups

Standard triple therapy (Group A)

Pantoprazole 40 mg (twice daily) + Clarithromycin 500 mg (twice daily) + Amoxicillin 1 g (twice daily)

Total duration of treatment (10 Days).

Sequential therapy

(I) Clarithromycin Based (Group B)

(DAY 1-5): 1. Pantoprazole (40 mg twice daily)

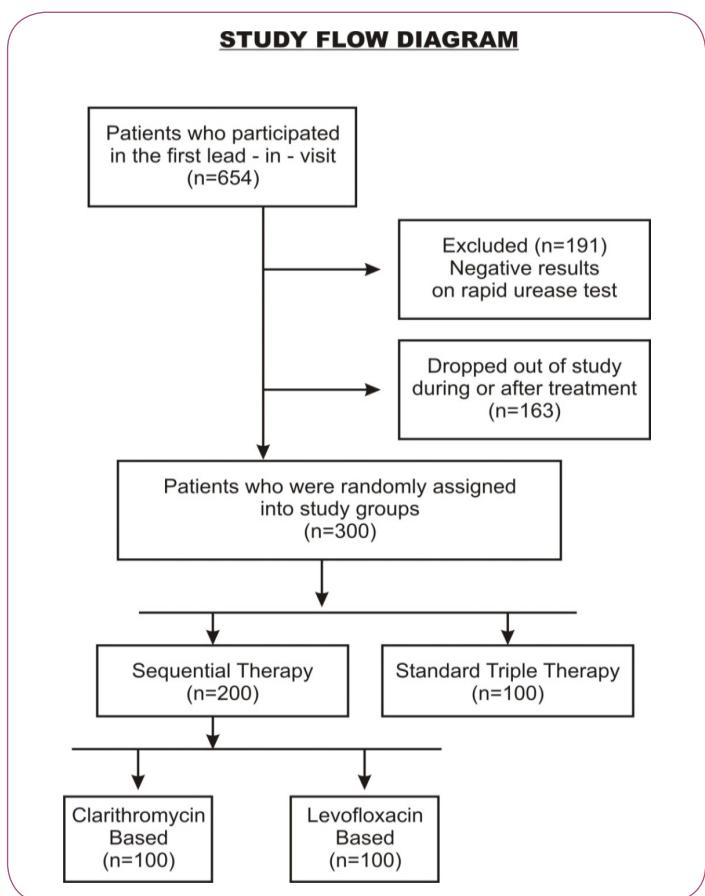
2. Amoxicillin (1 g twice daily)

(DAY 6-10): 1. Pantoprazole (40 mg twice daily)
 2. Clarithromycin (500 mg twice daily)
 3. Tinidazole (500 mg twice daily)

Levofloxacin based (Group C)

(DAY 1-5) 1. Pantoprazole (40 mg twice daily)
 2. Amoxicillin (1 g twice daily)
 (DAY 6-10): 1. Pantoprazole (40 mg twice daily)
 2. Levofloxacin (250 mg twice daily)
 3. Tinidazole (500 mg twice daily)

The aim of our study was eradication of *H. pylori* infection. All the participants in the study were re-endoscoped four weeks after completion of drug regimen for *H. pylori* eradication and test used was RUT.



Statistical Analysis

The difference between the proportions of eradicated infections for the three treatments was calculated by using the method recommended by Newcombe and Altman. The level of significance was assessed by using Mannwhitney 'U' test and Kruskal Wallis test for Non metric variables. Student's t test and ANOVA was used for metric Variables. Intergroup variance was checked at 95% CI. MS Excel, Minitab and SPSS software was used for data analysis.

The analysis of data enabled us to determine whether sequential treatment regimen is better than standard triple therapy and henceforth can be recommended as initial therapy for *H. pylori* eradication.

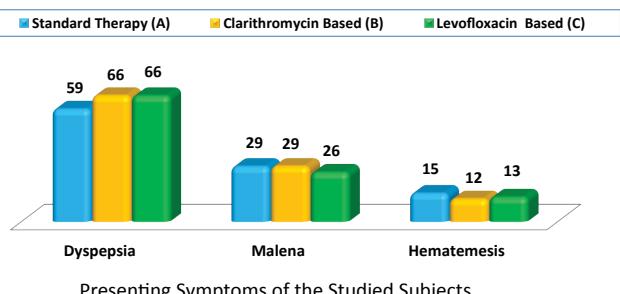
Results

The age of 300 patients in our study ranged from 18 to 70 (mean age 44.3 ± 15 yrs). Most of the patients in our study belonged to age group 41 to 50 and least between 61 to 70 yrs. Distribution of subjects across age and gender in our three study groups is as given in Graphs 1 and 2.

Most of patients in studied groups presented with dyspepsia, which was seen in 59% in Group A, 66% in Group B and 66% in Group C. Malena was seen in 29%, 29% and 26% subjects in different groups. Hematemesis was seen in 15, 12 and 13% patients in Group A Group B and Group C respectively. The intergroup difference was not statistically significant as depicted in Graph 3.

Esophagogastroduodenoscopy (EGD) was normal in 94 study subjects. Rest subjects have findings as depicted in Table 1.

Rapid urease test determined eradication of *H. pylori* in patients receiving levofloxacin based sequential therapy (Group C) was



Top of columns gives no's in each group

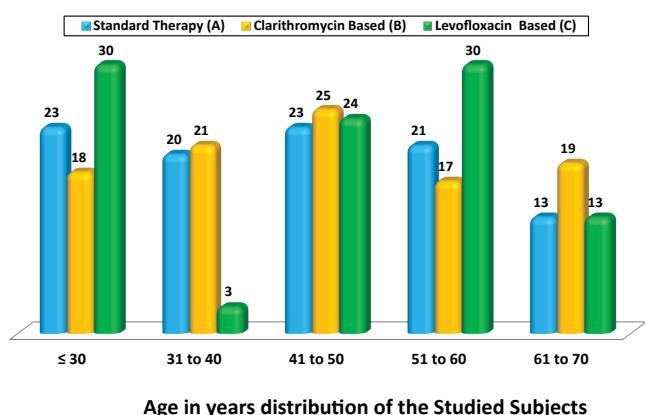
Graph 3 Presenting Symptoms of the Studied Subjects.

86% whereas the subsequent reduction in clarithromycin based sequential therapy (Group B) and standard triple therapy group (Group A) were 81% and 68%. The intergroup difference of A and C was significant ($p < 0.05$), besides A and B was also significant ($p < 0.05$) as depicted in Tables 2 and 3.

Discussion

The belief that no organism can survive in the acidic environment of the stomach was shattered by Barry Marshall and Robbin Warren in 1982 when they identified the organism *H. pylori*. The Maastricht III consensus report on *H. pylori* has recommended that a triple therapy consisting of a PPI, clarithromycin and amoxicillin for 7 days is the first choice treatment for *H. pylori* infection [34]. The eradication rate with standard triple therapy has declined to unacceptable levels (i.e. 80% or less) with some European countries reporting 25-60% success rate only [35,36]. As a general rule for the treatment of infectious diseases, clinicians should use regimens that have an eradication rate of $\geq 90\%$. Although the main reasons for failure of eradication of *H. pylori* infection includes antibiotic resistance, poor compliance and rapid metabolism of PPI [37] Failure of eradication is more likely in younger patients i.e. aged 50 years or less [38]. The other factor for failure is diarrhoea due to triple therapy. One study from Japan reports that the use of probiotic bacterium *Clostridium butyricum* MIYAIRI 588 strain reduced fluctuations in intestinal flora and decreased incidence of GI side effects [39]. PPI plays a major role in *H. pylori* eradication therapy by (a) increasing the intra gastric pH which improves antibiotic stability and bioavailability (b) increasing the intra gastric pH to 6 or more prompting *H. pylori* to replicate and thus become sensitive to antibiotics (c) suppressing acid secretion (d) direct anti *H. pylori* effect [40]. PPI are metabolized primarily by cytochrome P 450 (CYP) 2C19. The genetic polymorphism for CYP 2C19 are classified into 3 groups. Rapid metabolizers (RM) intermediate metabolizers (IM) and poor metabolizers (PM), maximum acid suppression is achieved with PM [40].

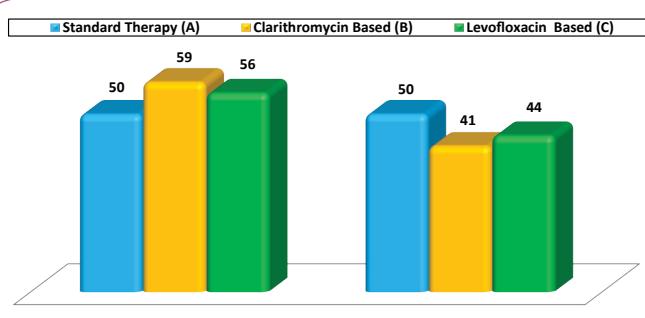
The aim of treatment of *H. pylori* infection in any clinical situation is eradication of bacterium from the foregut. Eradication is currently defined as negative test for *H. pylori* at least 28 days after the end of anti-microbial therapy. At present the standard treatment for *H. pylori* infection that has been endorsed by US and European authorities rely on clarithromycin or metronidazole in conjunction with other antibiotics and PPI [29,30]. Novel first



Age in years distribution of the Studied Subjects

Top of columns gives no's in each group

Graph 1 Age in years distribution of the Studied Subjects.



Gender distribution of the Studied Subjects

Top of columns gives no's in each group

Graph 2 Gender distribution of the Studied Subjects.

Table 1 Endoscopic Finding in the Studied Subjects.

Endoscopic Findings	Standard therapy (Group A)		Clarithromycin Based (Group B)		Levofloxacin Based (Group C)		p value			
	n	%	n	%	n	%				
Duodenal Ulcer	13	13	19	19	21	21	AB	AC	BC	Overall
Gastric Ulcer	6	6	11	11	4	4	0.456 (NS)	0.869 (NS)	0.656 (NS)	0.772 (NS)
Gastritis (Antral/Pan)	69	69	53	53	57	57				
Normal EGD	12	12	17	17	18	18				

Table 2 Rapid Urease test.

		Standard Therapy (Group A)		Sequential Therapy				Result			
				Clarithromycin Based (Group B)		Levofloxacin Based (Group C)					
		n	%	n	%	n	%	AB	AC	BC	Overall
Rapid Urease Test before treatment	+Ve	100	100	100	100	100	100	1.000 (NS)	1.000 (NS)	1.000 (NS)	1.000 (NS)
Rapid Urease Test after treatment	+Ve	32	32	19	19	14	14	0.035 (Sig)	0.003 (Sig)	0.342 (NS)	0.006 (Sig)
	-Ve	68	68	81	81	86	86				
Interpretation success		68	68	81	81	86	86	0.035 (Sig)	0.003 (Sig)	0.342 (NS)	0.006 (Sig)

Table 3 Data of *H. pylori* eradication rate in Indian patients are available from several clinical trials.

Author	Treatment regimen		No. of patients		Time of testing		Eradication rate	
Dayal (1997)	BC/T4/M		57		4 weeks		54%	
	LAS		21		6 & 12 weeks		86%	
Ahuja (1998)	LCS		18		6 & 12 weeks		83%	
	LPS		21		6 & 12 weeks		71%	
Bhasin (1999)	OC		22		4 weeks		68%	
	OAC		20		4 weeks		70%	
	BC/A/M		22		4 weeks		59%	
Bhasin (2000)	LAC 2wk		24		6 weeks		96%	
	LAC 2wk		22		6 weeks		54%	
Pari (2003)	LAC		35		4 weeks		82%	
	L/BC/T4/M		33		4 wks		72%	

L – Lansoperazole; A = Amoxicillin, S = Secnidazole; O = Omeperazole; C = Clarithromycin; BC = Bismuth Citrate; T4 = Tetracycline; M = Metronidazole

line anti *H. pylori* therapies in 2011 include sequential therapy, concomitant quadruple therapy [41], hybrid (dual-concomitant) therapy [42] and bismuth containing quadruple therapy [33]. Sequential therapy is a novel approach for *H. pylori* infection because of high eradication rates (93.5% eradication rate; 95% CI 92-95) [43,44]. The mechanism proposed for the success of the sequential therapy is that bacteria develop efflux channels for clarithromycin, which rapidly transfers the drug out of the bacterial cell, preventing the antibiotic from binding to the ribosome [45-62]. Because amoxicillin acts on the bacterial cell wall and weakens it, the initial phase of treatment prevents the development of efflux channels by weakening cell wall of bacterium [62].

Our hospital based, prospective, randomized study entitled “Sequential therapy versus standard triple therapy in *Helicobacter pylori* eradication” was conducted and concluded in post graduate department of medicine in 2012. Three hundred patients studied were randomized into 3 groups, one group received standard triple therapy for 10 days (Group A), second group clarithromycin based sequential therapy for 10 days

(Group B) and third levofloxacin based sequential therapy for 10 days (Group C). Group A achieved eradication rate of 68% only. While sequential therapy group B and C showed a success of 81 and 86% respectively.

To understand the relative efficacy of sequential therapy compared with standard triple therapy, we performed a systematic literature review and meta-analysis of randomized, controlled trials (RCTs) comparing these 2 treatment.

Patients taken in our study were between 18 and 70 years of age. The mean age in standard therapy group (Group A) was 43.3 ± 14.7 years, in clarithromycin based study group (Group B) mean age was 45.4 ± 14.4 years and in levofloxacin therapy based sequential therapy group (Group C) mean age was 44.1 ± 16.1 . In a Study by De Francesco et al. mean age of standard therapy was 46 and sequential was 44.2 years.

Dyspepsia is defined as pain or discomfort in the central upper abdomen which originates in the upper gastrointestinal tract. Most of patients in studied groups presented with dyspepsia, which was seen in 59% in Group A, 66% in Group B and 66% in

Group C. Malena was seen in 29, 29 and 26% subjects in different groups. Hematemesis was seen in 15, 12 and 13% patients in Group A Group B and Group C respectively. Endoscopically most subjects had gastritis (Antral/Pan) across all groups. It was 69% in Group A (standard therapy group), 53% in Group B and 57% in Group C. Duodenal ulcer was seen in 13, 19 and 21% of patients in Group A, Group B and Group C respectively. This was followed by normal EGD i.e. non-ulcer dyspepsia (12%, 17%, and 18%), least common finding seen endoscopically was gastric ulcer in studied subjects (6%, 11% and 4%). The pathophysiological mechanisms by which the infection may cause dyspepsia are unclear, but may include changes in acid secretion, abnormal motility, or altered visceral perception. Most researchers believe that there is a relation, although an imperfect one, between non-ulcer dyspepsia and infection with *H. pylori*. In our study we involved even non ulcer dyspepsia patients. Bruley Des Varannes et al. [45] showed that there are benefits for eradicating *H. pylori* in patients with non-ulcer dyspepsia, although in majority of patients relief of symptoms is less likely. Prevalence of peptic ulcer was quiet similar to study by MS Khuroo et al. in 1989 [46] showed the life time prevalence of peptic ulcer 11.2% with peak incidence in 5th decade of life. R A Moore MA DPhil in 1994 [47] showed peptic ulcers are found in 25% of dyspeptic patients whose blood tests positive for *H. pylori*, compared with only 3% of similar patients who test negative. Combining data from three separate studies shows that rates of gastric and duodenal erosions, and gastric cancer, are also higher in patients who test positive for *H. pylori*. This indicates that testing blood for *H. pylori* can be a useful way of determining which patients would benefit from conventional conservative therapy (acid-suppressing medicines) and those who would benefit from curing *H. pylori* infection.

The overall eradication rate with our standard triple therapy of 10 day duration (Group A) was 68%, with clarithromycin based sequential therapy (Group B) showed a success rate of 81% and levofloxacin based sequential therapy (Group C) 86% respectively. The results of this study show that sequential therapy is superior to triple therapy for the eradication of *H. Pylori* infection. The study also demonstrates that triple therapy, which is the current standard treatment, has low eradication rate. Our study supported most other studies on *H. Pylori* eradication that show higher eradication rates with sequential therapy than standard therapy. Choi WH et al. [48] conducted a study in Asia in 2008 showed eradication by 10 day sequential therapy by 77.9% and 71.6% by standard triple regimen, quiet similar to our findings. Nadim S Jafri et al. [49] showed the eradication rates by sequential therapy (clarithromycin based) by 93.4% and by standard triple therapy by 76.9%, which was an European study and showed higher eradication rates as compared to our study, reason may be that in it study 2747 patients were taken as compared to ours were only 300 participated in study and difference in *H. pylori* between east and west [50]. Sanchez-Delgado et al. in 2008 [51] showed eradication rates by sequential therapy(clarithromycin based) by 84.2% results quiet similar to our study in which eradication rate of *H. Pylori* was 81% respectively by clarithromycin based therapy. Uygun et al. [52] also conducted a study in which eradication by sequential therapy was 80.1% and 63% by standard triple

regimen again, slightly deviating from our study, causes may be it used tetracycline and eradication was confirmed 6 weeks after therapy as against ours where were endoscoped subjects after 4 weeks. Moayyedi P et al. [53] identified 12 trials with 3271 patients and concluded that sequential therapy achieved 12% better eradication rates. In our study sequential therapy was 13% superior to standard triple regimen. Romano M et al. [54] compared levofloxacin and clarithromycin based sequential therapy with eradication of 80.8% with clarithromycin sequential therapy, 96% with levofloxacin 250 mg sequential therapy and 96.8% with levofloxacin 500 mg sequential therapies. Our study also showed higher efficacy of levofloxacin based regimen as compared to clarithromycin based therapy. Recent several studies have shown that a novel 10 day sequential therapy can achieve success rate of 90-94% [55] Gatta L et al. [56] reported a systemic review of 13 trials (3271 patients) and showed that sequential therapy achieved 12% higher eradication rate than standard triple therapy. In our study, Group B i.e. clarithromycin based sequential therapy achieved 13% higher eradication as compared to standard triple therapy.

Data of *H. pylori* eradication rate in Indian patients are available from several clinical trials.

Our trial has limitations; the results may not be applicable to other countries and populations. It does not tell us about the percentage of subjects having clarithromycin resistance and our study design does not tell us whether the improved effect with sequential therapy is due to the sequential administration or to the additional antibiotic (tinidazole) that is not contained in the standard regimen. Although sequential therapy is an improvement over current therapies, it does not decrease the duration of therapy. In conclusion, our large, prospective, hospital based study shows the superiority of sequential treatment for eradicating *H. pylori* infection compared with conventional triple therapy. The sequential regimen is less expensive and is more effective than conventional therapy for patients with clarithromycin-resistant organisms. Side effects with both regimens were similar and consisted mostly of diarrhoea and abdominal discomfort. Our data suggest that sequential therapy may have a role as a first line treatment for *H. pylori* infection.

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Availability of Data and Materials

The data on which this study has been based are freely and publicly available from hospital record department.

Authors' Contributions

All authors contributed in every aspect of study. All authors read and approved the final manuscript.

Competing Interests

No competing interest.

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