PREVALENCE OF *HELICOBACTER PYLORI* GENOTYPES AND THEIR ASSOCIATION WITH INTERLEUKIN-1- BETA IN PATIENTS WITH GASTRITIS FROM AIZAWL, MIZORAM

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Submitted

In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Biotechnology of Mizoram University, Aizawl

CERTIFICATE

This is to certify that **Mr. Subhajit Mukherjee**, Ph.D. Scholar, Registration No. MZU/Ph.D./751 of 22.05.2015 has work on the Thesis entitled **"Prevalence of *Helicobacter pylori* genotypes and their association with Interleukin-1- Beta in patients with gastritis from Aizawl, Mizoram".** He has fulfilled all criteria prescribed by the UGC (Minimum Standard and Procedure governing Ph.D. Regulations). He has fulfilled the mandatory publication (Publication enclosed) and completed Ph.D. course work.

This also certifies that the scholar has been admitted in the Department through an entrance test, followed by an interview as per clause 9 (i) and (ii) of the UGC Regulation 2009.

Dated:

Prof. N. Senthil Kumar

<p align="center">**DECLARATION**
Mizoram University
October, 2021</p>

I, **Subhajit Mukherjee,** hereby declare that the subject matter of this thesis entitled **"Prevalence of *Helicobacter pylori* genotypes and their association with Interleukin-1- Beta in patients with gastritis from Aizawl, Mizoram"** is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Biotechnology.

Dated:

Candidate

Head   Prof. N. Senthil Kumar
                                                                              Supervisor

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Dated: Subhajit Mukherjee

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Chapter I

Introduction and Review of literature

Helicobacter pylori (H. pylori) area major bacterial pathogen in humans causing active chronic gastritis, peptic ulcer, acute erosive gastritis and as well as a major risk factor for gastric carcinoma and MALT lymphoma (Dorjiet al.,2013). The International Agency for Research on Cancer (IARC) in 1994 reported that there is a correlation between *H. pylori* contamination and stomach cancer. The World Health Organization (WHO) reported the incidence of gastric carcinoma to be very high in Mizoram among all the other states in India (Phukanet al., 2006).

Helicobacter pylori used to colonize in the human stomach and create a long-term infection of the gastric mucosa (Dunne et al., 2014). The formation of a reservoir of bacteria in gastric mucus helps *H. pylori* to escape from mucus flow and gastric acids. This infection causes chronic non-atrophic gastritis, which further develop into chronic atrophic gastritis, intestinal metaplasia, and dysplasia toward gastric carcinoma (Park et al., 2015). However, this development occurs only in some people and is dependent on several factors, like bacterial and patients genetic factors(Kusters et al., 2006; Ailloud et al., 2019).There are some particular *H. pylori* genotypes (*cagA, vacA*) found which are observed as more virulent strains because these strains are associated with moderate gastroduodenal infections in humans (Yamaoka et al., 1999).In earlier studies it was observed that *H. pylori* involves different virulence factors including cytotoxin associated gene (cagA) and vacuolating cytotoxin A (vacA), and these two are linked with increase in the chances of disease development (Kusters et al., 2006).In some countries, reports explained that most strains of the bacteria produce CagA and VacA and that proteins expressed by these virulence factors have different disease outcomes (Smith et al., 2001; Harrison et al., 2017; Seriki et al., 2017).

In 2005, scientists Warren and Marshal won the Nobel Prize for physiologyor medicine for their discovery those *Helicobacter pylori* (Niyaz, 2005). This discovery has changed the concept that peptic ulcer as long term acute illness of unspecified reasons into major infection as it may be resolved by different antibiotics and acid secretion inhibitors (Basset et al., 2002). Currently, evidences show that *H. pylori* is a most common threat with the aim of adenocarcinoma of the stomach and MALT lymphoma (Amieva and EL-Omar 2008; Kuipers 1999; Malfertheiner et al., 2002).

The reasons for this higher incidence of gastric carcinoma have not been understood fully. Further, it is difficult to understand the epidemiology of the infection and *H. pylori* related diseases including gastric cancer. *H. pylori* infection varies based on the type of virulence factor (*cagA*, *vacA* genotype) present in different strain of *H. pylori* (Amieva and EL-Omar, 2008). *CagA*is the member of a large pathogenicity island (cag PAI) and is present in the more virulent *H. pylori* strains, having the ability to produce cytotoxicity in the host cell. The acuteness of cytotoxicity depends on the genotype of *cagA* present in *H. pylori*. Another important cytotoxin present in *H. pylori* which affects the host by formation of pores in patients' epithelial cells of the stomach is known as *VacA*, vacuolating cytotoxin gene A. Some other threat factors for highoccurrence of gastric carcinoma are the abundance of antibiotic resistance in *H. pylori* and isbecause of the existence of SNPs in 16S rRNA, 23s rRNA and GyrA genes.

Inflammation of the gastric mucosa leading to gastritis occurs due the *H. pylori* infection. Gastritis is found in the antral region and is correlated with excessive acid production and a high probability of duodenal ulcer disease. Gastritis leads to hypochlorhydria, increased gastric atrophy and a high probability of gastric carcinoma (EL-Omar et al., 2000). The occurrence of ulcer, gastric carcinoma and other gastric related diseases, not only depends on bacteria and environmental factors but also it depends on the polymorphic status of genes which play an active role in colonization of bacteria. *Interleukin-1-Beta* (*IL-1B*) polymorphism revealed the abundance of *H. pylori* colonization (Amieva and EL-Omar, 2008). The polymorphism of the gene influences the successive development of *H. pylori* infection.

Significant geographic diversity in the occurrence of *H. pylori* virulence factors has been reported worldwide (Dorjiet al., 2013). *H. pylori* exhibits genetic diversity as evidenced by an apparently unlimited number of unique strains that differ in genome size, gene order and genetic content (Kuipers, 1999). *H. pylori* showed more frequent recombination events with heterologous strains than any other known bacterial species (Seckaet al., 2011). Different reports of European countries observed that mostly every category of *H. pylori* isolated from patients show homogeneous DNA profiles, while population from Mexico and China suffer from the heterogeneous strains (Patraet al., 2012). In India, the occurrence of *H. pylori* infection is at high risk than the other western civilizations and most of the cases were reported that to carry multiple strains of *H. pylori* (Vatsalaet al., 2014) and the probability of the diseases due to *H. Pylori* strains in a particular patient is mostly increased than the Western countries (Megraud, 2004). The present thesis work aims to determine the genotypic and antibiotic resistance status of *H. pylori* in relation to the demographic factors and host interleukin gene from Mizo tribal population, Northeast India.

The correlation of *H. pylori* genotype with clinical diseases remains contentious, but studies on gene variations of *H. pylori* are not only essential for predicting the clinical outcome, but also for understanding their distribution and evolutionary processes. The patients who have *H. pylori* infection have at least two fold higher probability for the growth gastric carcinoma compared with uninfected persons (Uemuraet al., 2001). According to a study, it is assessed that patients suffering with this bacterial infection have a 10 to 20% of threat for growth of gastric ulcer disease and a 1 to 2% life threatening for growth of gastric carcinoma (Kusterset al., 2006). Depending on age, race, and social status the *H. pylori* have a world-wide distribution and its prevalence in healthy asymptomatic persons is 15 to 70% (Kusterset al., 2006). Epidemiological research works have reported a correlation between colonization and age, low socio-economic status, and overcrowding (Yamaoka, 2012).

Different studies reported that the *vacA* s1a or s1b genotypes were most important among strains from Western populations, and on the other hand, s1c is much

frequent from East Asian countries (Cover et al.,1994; Atherton, 1995). In the western population, the *vacA* m1a and m2a genotypes were much frequent, and in South Asia the m1c genotype was frequently available strain, and the m1b and m2b genotypes were more abundant from East Asia (Saribasaket al., 2004). Overall, different categories from Western populations have *cagA* type 2a; *vacA* s1a, s1b, or s2/m1a; or m2a genotypes. Strains from South Asia mostly have *cagA* type 2a and *vacA* s1a/m1c genotypes, and bacteria from East Asia mainly acquire *cagA* type 1a, *vacA* s1c/m1b, or m2b genotypes (Martínezet al., 2001; Jang *et al.,* 2010). These deviations among the world wide allocations of the *cagA* and *vacA* genotypes may consider the contrast of reports correlating with the *cagA* and *vacA* genotypes with the medicinal outcome from different geographic regions (Pernget al., 2003; Cover, and Blanke, 2005).

According to the other study reports, frequency of antibiotic resistance strain of *H. pylori* affects the eradication process and indirectly increases the incidence of GC worldwide (Nishizawa, and Suzuki, 2014). *H. pylori* suppression treatment was carried out for many stomach diseases, like gastric ulcer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric carcinoma at the first stage of cancer where it could beresectedby endoscopy(Uemuraet al., 2001). The current trend forthe eradication of *H. pylori* infection contains a proton pump inhibitor and any two antimicrobial agents, such as amoxicillin, clarithromycin, and metronidazole. Some studies say a variety of such triple regimens, triple therapies with a proton pump inhibitor (PPI; i.e. omeprazole, rabeprazole and lansorprazole), clarithromycin and amoxicillin are representative standard regimens in Japan (Asakaet al., 2001). Triple therapies with a PPI or ranitidine bismuth citrate, fused with clarithromycin and amoxicillin or metronidazole are effective regimes in Europe (Malfertheineret al., 2002). Therefore, clarithromycin is very effective antibiotics in *H. pylori* mitigation treatment. Reduction percentages of bacteria by triple therapies involving clarithromycin count on the sensitivity of bacteria to clarithromycin (Furutaet al., 2001). The number of clarithromycin-resistant strains of *H. pylori* seems to vary worldwide (12.2% in USA, 1.7% in the Netherlands, 4.4 % in the UK and 12.9 % in Japan) (Murakami et al., 2002). Clarithromycin-resistant strain of *H. pylori* is due to mutation from adenine (A) to guanine (G) at position 2142 or 2143 of the 23S rRNA

gene (Furutaet al., 2007). The *Interleukin-1* (*IL-1*) gene cluster on chromosome 2q contains 3 related genes within a 430-kilobase (kb) region, *IL-1A*, *IL-1B* and *IL-1RN*, which encrypt the pro-inflammatory cytokines IL-1α and IL-1β and their endogenous receptor antagonist IL-1ra, respectively. IL-1βis up regulated in the appearance of *H. pylori* and is essential in instigating and expanding the agitation to this infection (Dinarello, 1996).

In the present study, demographic risk factors were evaluated on the basis of *H. pylori* presence or absence as well as list of risk factors predominant in the virulent genotypes of the bacteria. Furthermore, clarithromycin resistant status of virulent strain of *H. pylori* were measured and analyzed depending on associated risk factors. The host *Interleukin- 1-β* genotypes were examined and correlated with the presence of *H. pylori* virulent strains.

Chapter II

Aim and Objectives

The following aims are set forth to accomplish the proposed work in this study:

- To identify the *H. pylori* genotypes (16S rRNA, *vacA*, *CagA*, *Urec*) in relation to the demographic factors.

- To detect the clarithromycin antibiotic resistance status of *H. pylori* by PCR.

- To determine the polymorphism in *Interleukin 1 Beta* gene in patient's genome.

Chapter III

To identify the *H. pylori* genotypes (16S rRNA, vacA, CagA, Urec) in relation to the demographic factors

1. Introduction

Mizoram has high incidence of *H. pylori* infection and stomach adenocarcinoma in India (Ghatak et al., 2016). Although reasons are not clear for increased rate of *H. pylori* infection in this region, the standard of living is not so high among the people and there may be chances of easy spreading among the communities. The frequency of *H. pylori* infection varies based on the host and is also associated with lifestyle habits, living conditions, cleanliness and sewage (Ahmed et al., 2007). The acuteness of infection which is especially high among those living in developing countries(Dorji et al., 2013; Jang et al., 2010), is multifactorial including interaction with potentially environmental contaminants such as potable water and the ingestion of faecal contaminated vegetables (Kusters et al., 2006).

Additionally, the lifestyle is unique with a high consumption of meat and fermented pork fat (sa-um) (Ghatak et al., 2016; Lalrohlui et al., 2021). They also use a tobacco smoke–infused aqueous solution called, "Tuibur" - a small amount of Tuibur is kept in the mouth for few minutes, and is spit out and very rarely is swallowed (Madathil et al., 2018).The unregulated makeshift industry of tuibur production usually occurs in unhygienic conditions using unfiltered water, increasing the potential to harbor *H. pylori* bacterium (Madathil et al., 2018). Furthermore, tuibur could modify the pH of saliva by making it more alkaline, thus it could increase the acuteness of *H. pylori* contamination in the buccal cavity and could set out as a reservoir for the germs (Lalmuanpuii et al., 2016). Presence of some carcinogenic factors like TSNAs (tobacco-specific nitrosamines), NNN (N-nitrosonornicotine), NNK [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone] in Tuibur can be a probablethreat for gastric cancer (Muthukumaran, 2016).

While evidence suggest that this specific bacteria and tuibur are deeply correlated with the consequences of gastric carcinoma (Ghatak et al., 2016), no in-

depth investigation was conducted to find the correlation of tuibur with *H. pylori*c ontamination. Additionally, no investigation reported the association of environmental and life style factors with *H. pylori* in the tribal people staying in mountain region with distinctive life style habits. The exact threat for this particular infection is still unrevealed.

Helicobacter pylori is a primary major risk factors for gastric carcinoma development, through the development of atrophy in stomach (Wroblewski et al., 2010; Cheung et al., 2018). *H. pylori* is genetically evolved and diverse bacteria and various genotypes have been correlated with virulence and gastric disease risk (Blaser et al., 2001; Šterbencet al., 2019). *H. pylori* strains contain the vacA gene that translates a vacuolating cytotoxin. High inflammation and epithelial destruction in the gastric mucosa is strongly correlated to the vacA gene (Palframan et al., 2012; Winter et al., 2014). According to the previous report, the cagA gene of *H. pylori* is a marker for the existence of the cag pathogenicity island (PAI) (Kauser et al., 2004). The CagA protein is translocated by a type IV secretion system (encoded by the cag PAI) into gastric epithelial cells, where it provides changes in the tyrosine phosphorylation states of different cellular proteins (Li et al., 1999). Various genes of the cag PAI translate proteins which help in over expression of the pro-inflammatory interleukin 8 (IL-8) by the gastric epithelium (Li et al., 1999). Some studies denoted that there is an association present between infection with cagA-positive strains and gastric ulcer (Bulent et al., 2003), and for development of atrophic gastritis and carcinoma of the stomach (Cheung et al., 2017).

2. Materials and methods

2.1. Sample collection

A total of 863 patients with gastritis were recruited from February 2014 to August 2016 at the gastroenterology clinic Trinity diagnostic Centre in Aizawl, Mizoram Northeast India. The consent for the study was approved by the ethical committee of Civil Hospital, Aizawl (No. B.12018/1/13-CH(A)/IEC/36) as well as the Mizoram University ethical committee.

Inclusion Criteria

Mizo ethnic patients who had dyspeptic indications or gastro intestinal irritations requiring upper GI endoscopy.

Exclusion Criteria

Patients who had already used antibiotics for gastrointestinal irritations for past two weeks, active GI bleeding; pregnancy and the record of gastrectomy are excluded.

2.2. Data collection

A structured interview was done by trained nurse and the data was collected on the basis of few factors including age, educational qualification, profession, domestic location, marital status, number of children, medical background, health condition, family record of *H. pylori* infection and daily habits (e.g., tobacco consumption and alcohol intake, diet) by using a bilingual verified and standardized questionnaire (Mizo and English language).
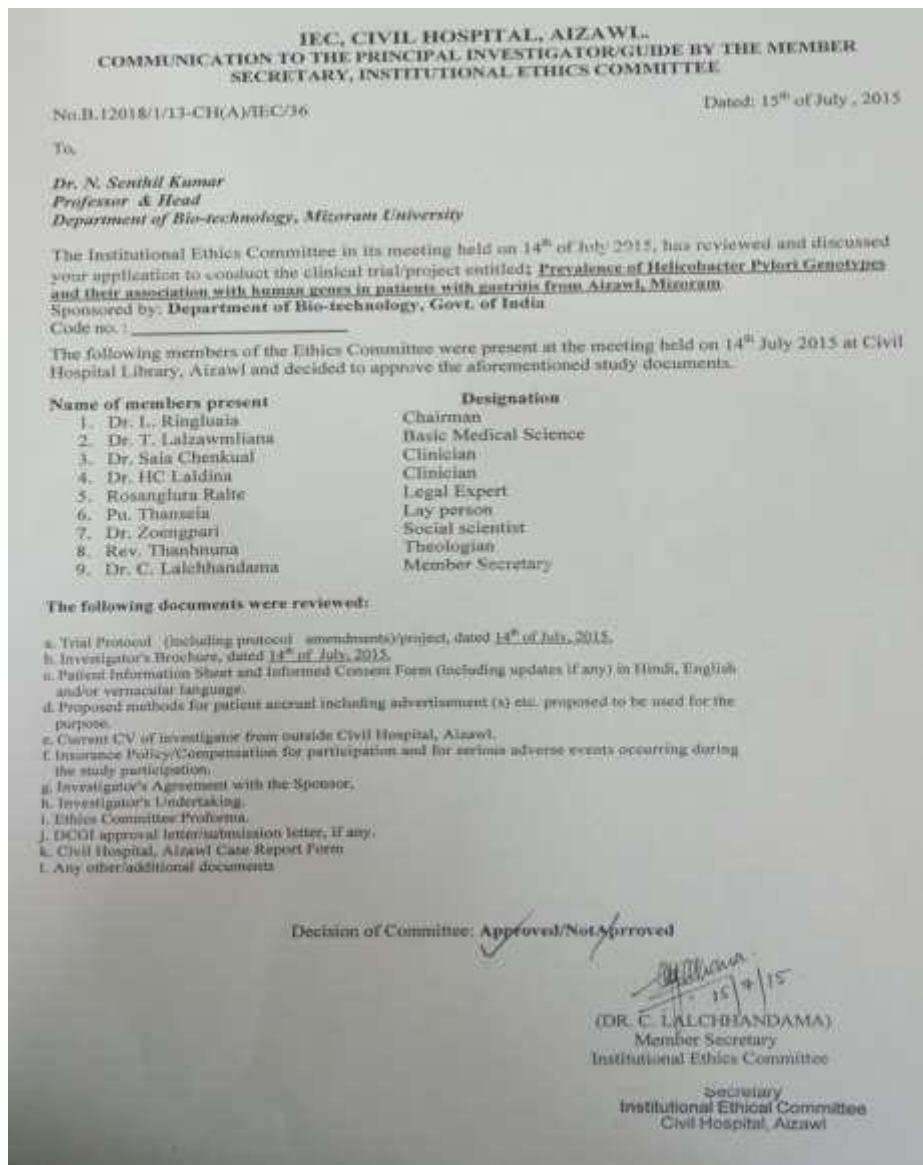
Figure: 3.1. Ethical approval by the IHE Committee of Civil Hospital Aizawl, Mizoram

On the basis of self-reported measures (regular, occasional, former, or never consumers), the following information were collected:

- Tobacco smoking and alcohol consumption
- Consumption of various smokeless tobacco products like Tuibur, betel nut, pan, zarda
- Food habits like completely vegetarian diet, raw vegetables intake, fermented pig fat (sa-um), intake of salt and pickle

- Sanitation (commode, and in field)
- Potable water sources (e.g., unfiltered, filtered [using ceramic, active carbon/charcoal, UV sterilization and reverse osmosis] and boiled).

PATIENT'S DETAIL

Damlo hming (Patient's name) :
....

Damlo No. (Patient's No.) :..

Gender : Mipa(Male) [ ]   Hmeichhia(Female) [ ]

Kum(Age) :   Pianni leh Thla (D.O.B.) :

Hnam (Nationality) :
Nupui/ Pasal inei tawh em (Marital status): Nei(Married): [ ]   Neilo(Unmarried): [ ]

Mobile No:
Hnam hming(Ethnicity):   Tawng hman (Language Used):
District: Khua/Veng(Town/Village):
Hnathawh(Occupation):
Thiamna/Zirsan zawng(Edu. Qualification):
Address :

Fa I nei em? (Do you have a children ?) Nei (Yes) [ ]   Neilo (No) [ ]
I neih chuan engzat nge? (If Yes, how many children do you have?) [ ]
Mipa engzat nge? (No. of male child) [ ]   Hmeichhia engzat nge? (No. of female child) [ ]
(Tuna piangtur te pawh huamin, boral tawh tiamlo in) (Please include stillbirths; it is not necessary to include miscarriages)
In Inah engzah nge cheng (Family size living in the same household):

HELICOBACTER PYLORI **STATUS** :   Positive [ ]   Negative [ ]

Figure: 3.2. Sample questionnaire and consent form to conduct the study.

(contd.)

QUESTIONNAIRE

1) Zu i in em? (Do you consume alcohol?)   In (Yes) [   ]   In lo(No) [   ]
   In chuan, in reng (regularly) [   ]   Chang chag in (Occasionally) [   ]
   Nghei tawh chuan, engtik atngin nge ? (If quit already, Since when?) :

2) Mei i zu em? (Do you Smoke?) Zu(Yes) [   ] Zu lo (No) [   ]
   I zuk chuan, Zu reng (regularly) [   ]   Chang chang in (Occasionally) [   ]
   Nghei tawh chuan, engtik atngin nge ? (If quit already, Since when?) :

3) Zuk leh hmuam dang tih I nei em? (Do you consume other tobacco product?)
   Nei(Yes) [   ]   Nei lo (No) [   ]
   Neih chuan, Ei reng (Regularly) [   ]   Chang chang in (Occasionally) [   ]
   Eng ber nge? Supari [   ] ; Beetle Nut [   ] ; Pan /Zarda pan [   ] ; Gutkha [   ] ; Sahdah [
   ];Khaini [   ] ; Tiranga [   ] ; Others _____

4) Ei leh in (Diet)
   Thlai nilo I ei em ? (Do you consume non- Veg?) Ei (Yes) [   ]   Ei lo (No) [   ]
   Heng- Sa- Bawngsa(Beef) [   ]; Vawksa(Pork) [   ]; Kelsa(Mutton) [   ]; Arsa(Chicken) [   ];
   Sangha(Fish) [   ]{chhum(boiled) [   ]; Kan(fried) [   ]}; Artui(egg) [   ] {chhum(boiled) [   ];
   Kan(fried) [   ]};
   Ei vek(all) {chhum(boiled) [   ]; Kan(fried) [   ]};
   Thlai hel/ chhumloh in I ei ngai em?
   (Do you normally consume raw/ uncooked fruits/ vegetables) ; ei(Yes) [   ];   Ei lo (No)
   [   ]
   Ei chuan(if yes); ei reng (Regularly) [   ]   chang chang in (Occasionally) [   ]
   Saum I ei ngai em?(Do you consumed fermented pig fat); ei(Yes) [   ];   Ei lo (No) [   ]
   Ei chuan, Heh(Heavy) [   ]; Pangai(Average) [   ]; Hehlo (Little) [   ]
   Chi I hmeh ngai em? (Do you consume salt during taking your food?)
   Ei chuan, Heh(Heavy) [   ]; Pangai(Average) [   ]; Hehlo (Little) [   ]
   Pickle/ Chutney I ei ngai em?(Do you consumed pickles, chutneys?)
   Ei chuan, Heh(Heavy) [   ]; Pangai(Average) [   ]; Hehlo (Little) [   ]; ei ngailo(none) [   ];

5) Tui in tur khawi tanga hmuh nge(Source of drinking water):
   a)Public point atang in (Public water distribution system)
   b)Tuikhur/ lui tui atang in (Stream, Lake or river)
   c)Diltui/ tuichhunchhuah,(Ponds, ditches, wells)

6) Engtiangin nge I in thin(types of drinking water consumed):
   thlitfim loh(unfiltered) [   ]; thlitfim(filtered) [   ], Chhuan so(boiled) [   ]
   Thlitfim chuan, engtia thlitfim nge,(if filtered,) Ceramic filter [   ]; Active carbon/ charcoal
   filter [   ]; UV sterilization [   ]; Reverse Osmosis [   ]

7) Eng ekin nge I hman thin (Type of Sanitation use): Commode [   ],   In field [   ].

8) In Inah Positive **H.Pylori** pakhat tal I chenpui chuan, akimchang han sawi teh (If any one
   of your house having **H. pylori** positive, please give details).

Figure: 3.2. Sample questionnaire and consent form to conduct the study.

Clinical Details:

6) (*H.pylori* **neih tan kum)Year of** *H.pylori* **infection detected** ?
   Endoscopy result : Positive [   ];   Negative [ ]

7) **Dyspepsia** : Duration: Chang changin(Intermittent) [ ]; Contineous [ ]; Nocturnal [ ];
   Nalo(Mild) [ ] ; Na deuh(moderate) [ ]; Na lutuk (severe) [ ]

8) **Medication** : Antacids [ ] H2 antagonists [ ] Proton-pump inhibitors [ ]
   Taking aspirin or other NSAID [ ]   Reason :

9) **What are the indications for testing** *H.pylori*? (More than one can betrue)

   - Dyspepsia   Yes(   ) ; No(   )
   - Gastric MALToma   Yes(   ) ; No(   )
   - Gastro oesophageal reflex disease   Yes(   ) ; No(   )
   - Family history of gastric cancer   Yes(   ) ; No(   )
   - On patient's request   Yes(   ) ; No(   )
   - Gastritis   Yes(   ) ; No(   )
   - Duodenal Ulcer   Yes(   ) ; No(   )
   - Gastric Ulcer   Yes(   ) ; No(   )
   - Patient's on long term PPI   Yes(   ) ; No(   )
   - Idiopathic thrombocytopenic purpura   Yes(   ) ; No(   )
   - Unexplained iron deficiency anemia   Yes(   ) ; No(   )

10) **Treatment taken?**
   - Proton pump inhibitors + Clarithromycin + Amoxicillin   ( )
   - Proton pump inhibitors + Clarithromycin + Metronidazole   ( )
   - Bismuth citrate + Clarithromycin + Furazolidone   ( )
   - Furazolidone + Amoxicillin + PPI + Bismuth citrate   ( )
   - Sequential therapy (5days PPI + Amoxillin followed by 5 days
     PPI + Clarithromycin + Tinidazole   ( )

Remtihna (Consent) :
Heng achunga thu te hi ka hriatpui a , Ka biological sample hi zirchian atan pek ka remti thlap e.

The information provided above was given with my full consent and I do not have any objection with providing my biological sample for research purposes. I have read and understood the consent information.

Place: **Signature:**

Date: **Name:**

THANK YOU VERY MUCH FOR YOUR HELP
(Collector information)

Name :   Signature :

Date and Time :

Figure: 3.2. Sample questionnaire and consent form to conduct the study.

2.3. Endoscopy and biopsy sampling

The endoscopy tested samples were collected from antrum portion of participant's stomach on the basis of rapid urease test kit. Endoscopy was done by using Fujinon VP-4450 HD Series on patients after an over-night fasting. Two biopsy specimens were collected through endoscopy from each patient's mucosa of the gastric antrum and placed in a small screw capped bottle containing 0.2ml sterile normal saline to maintain humidity. The biopsy samples were used for Rapid urease test.

2.4. Rapid urease test (RUT) for *H. pylori* status

The rapid urease kit (RUT DRY Test kit, Gastro Cure System, WB, India) available in market were used for the screening of endoscopic sample (Brown et al., 2002). The samples of endoscopic test were collected into the well of kit. *H. pylori* produces urease enzyme which hydrolyses urea producing ammonia. A pH based color indicator detected the rise in the pH of the medium by ammonium ions. In the case of positive *H. pylori* infection, there was an immediate change in color from yellow to pink. Samples that remained yellow, without any color change were inferred as *H. pylori*-negative. This test was read after 2 hours for positive and negative results.

2.5. Statistical analysis

The demographic factors were discovered using descriptive statistics. The correlation of demographic factors among case–control subjects was examined for Hardy–Weinberg equilibrium by a chi-square test with one degree of freedom (df). Unconditional logistic regression was used for the reorganization of different factors correlated with *H. pylori* infection. A hierarchical modeling strategy was performed to assess the variation in strength of this correlation when modified for possible confounder. Four sets of confounders were examined: (i) Model 1: tobacco and alcohol factors (smoked tobacco, smokeless tobacco, and alcohol consumption); (ii) Model 2: demographic factors (age, sex, marital status); (iii) Model 3: socioeconomic position (no. of persons in household, drinking water sources, and sanitation); (iv) Model 4: dietary factors (raw food, salt, sa-um, pickles). The association of epidemiological factors with *H. pylori* prevalence was estimated by means of odds ratios (ORs) and 95% confidence intervals (CIs) using conditional and unconditional multiple logistic

regression. For all tests, a two-sided p-value <0.05 was observed statistically remarkable. All analysis of statistics were carried out using of SPSS 20.0 program (SPSS, Spain) and confirmed by R statistical package ver3.3.0 (The R Foundation for Statistical Computing).
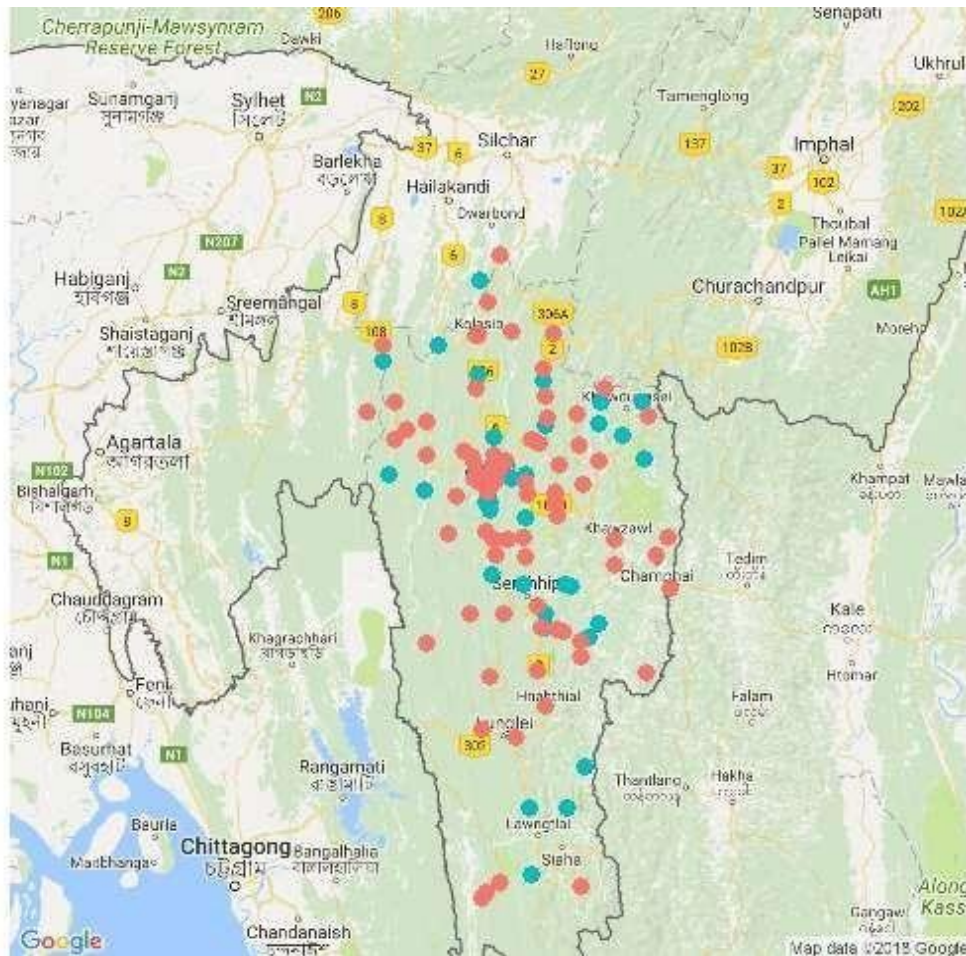
Figure 3.3. Sample collection area plotted using R. Studio. 3.2.

2.6. DNA Isolation

Endoscopic sample tissues were kept in formalin solution. Formalin-fixed tissues were subjected to glycine–Tris–ethylene-di-amine tetra acetic acid buffer for removal of formalin. The condition applied for digestion of pulverized endoscopic tissue samples are with 100 mg/ml proteinase K in cell lysis buffer for 2-3 hours at 56°C. The DNA was pull out from gastric antral biopsy specimens using modified precipitation method of Sambrook and Russell (2001). The process of homogenization

of biopsy specimens was done with a sterile micro pestle. DNA was collected based on phenol, chloroform, and isoamyl alcohol-based separation method precipitated with 2 propanols and 3 M sodium acetate 10:1 ratio. After these processes it washed with 70% ethanol and eluted in 50 µL of nuclease-free water (pH 8.3). Extracted genomic DNA was observed by gel electrophoresis technique (Figure: 3.4)
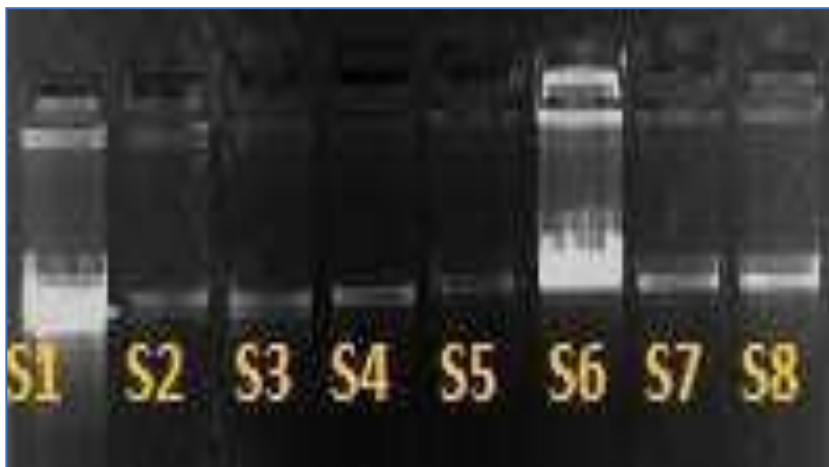
Figure: 3.4. Genomic DNA isolated from the endoscopy samples (0.8% agarose gel) S1 to S8: Representative Samples

2.7. PCR amplification of the *H. pylori* specific gene regions

RUT results were again verified by Polymerase chain reaction (PCR) using *H. pylori* specific 16S rRNA primers, Thermal condition was maintained by initial denaturation at 95^0C for 7 minutes followed by cycle denaturation at 95^0Cfor 1 minute, annealing at 62^0Cfor 30 seconds followed by cyclic extension 72^0Cfor 40 seconds for 35 cycles and final extension at 72^0Cfor 5 minutes followed by 4^0C to stop the reaction. Thermal amplifications were done by using Eppendorf (vapoprotect) instrument and subjected to 2% agarose gel electrophoresis for the presence or absence of the partial16S rRNA region. Based on 16S rRNA positive samples, PCR amplification of cagA1, cagA2, vacA, ureC was performed (Figures: 3.5- 3.9).
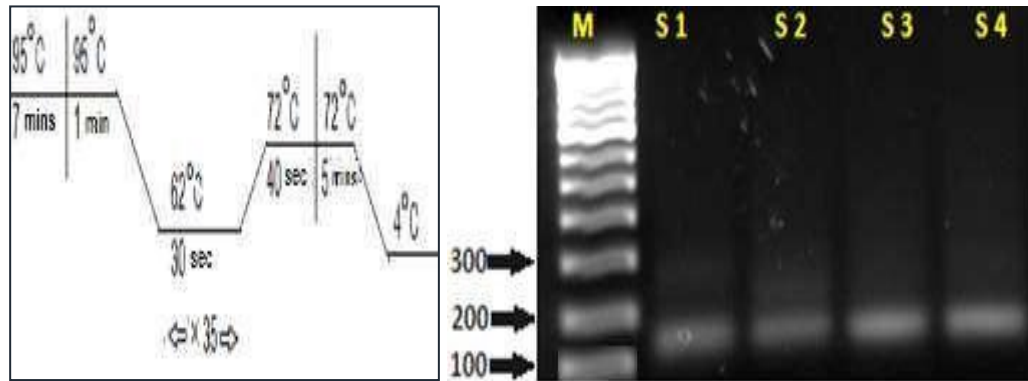
Figure: 3.5. Partial amplification of 16S gene of 109 bp size (2% agarose gel)

M: 100 bp Marker; S1 to S4: Representative Samples;
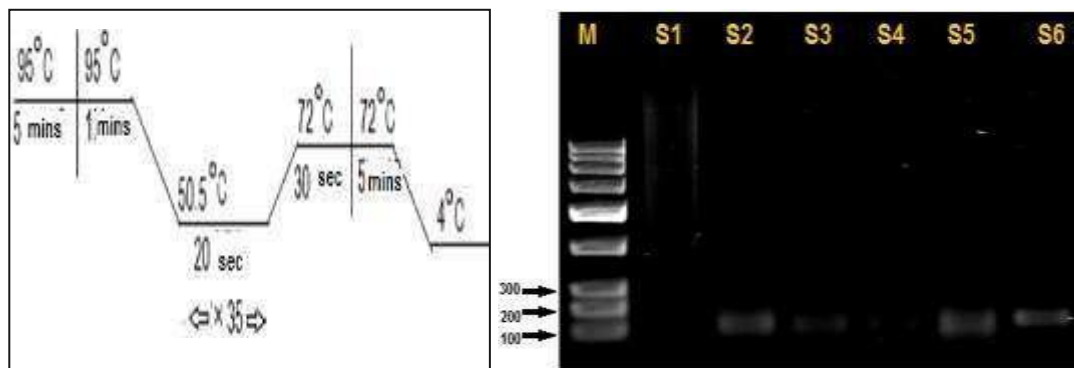
Figure: 3.6. Partial amplification of cagA1 gene of 100bp size (2%agarose gel)

M: 100 bp Marker; S1 to S6: Representative Samples
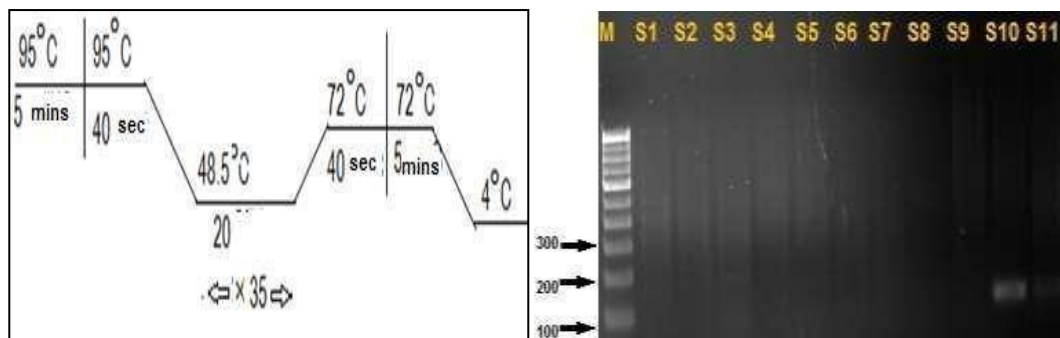
Figure: 3.7. Partial amplification of cagA2 gene of 220 bp size (2%agarose gel)

M: 100 bp Marker; S1 to S11: Representative Samples
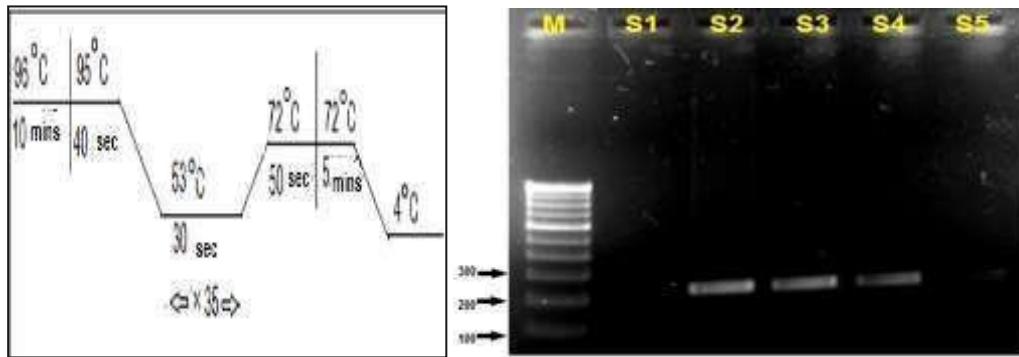
Figure: 3.8.Partial amplification of vacA gene of 229 bp size (2%agarose gel)

M: 100 bp Marker; S1 to S5: Representative Samples
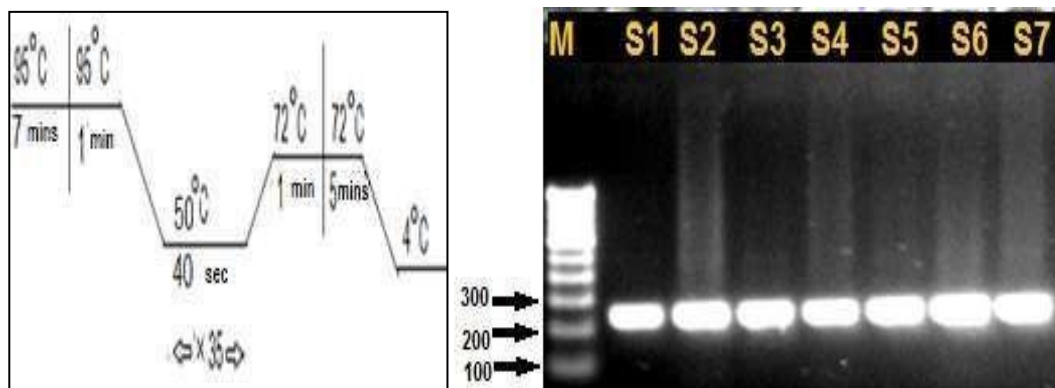
Figure: 3.9. Partial amplification of Urec gene of 294 bp size (2%agarose gel)

M: 100 bp Marker; S1 to S7: Representative Samples

Gene Name	Primer name	Primer Sequences	product length (bp)	Genotypes	References
16S rRNA	Hp1 Hp2	5'-CTGGAGAGACTAAGCCCTCC-3' 5'-ATTACTGACGCTGATTGTGC-3'	109	– H. pylori absence + H. pylori presence	Thomson et al. (2011)
cagA1 (Asian population based)	CagA1-F CagA1-R	5'-TGGCTCAAGCTCGTAAT-3' 5'- TGGAAAACTTGAACGAATCAGA-3'	120	–CagA 1 absence + CagA 1 presence	
cagA2 (European population based)	CagA2-F CagA2-R	5'-TCAGTTAGCCCTGAACC-3' 5'-GCCCTACCTTACTGAGAT-3'	100	–CagA 1 absence + CagA 1 presence	
vacA	VacA-F VacA-R	5' -GAGCGAGCTATGGTTATGAC- 3' 5' -ACTCCAGCATTCATATAGA- 3'	229	–VacA absence + VacA presence	Johannessen et al. (2013)
ureC	Urec-F Urec-R	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' 5' -AAGCTTACTTTCTAACACTAACGC-3'	294	–Urec absence + Urec presence	Johannesse net al. (2013)

Table 3.1. List of primers used to amplify bacterial specific gene regions

2.8. *H. pylori* vacA and cagA Genotyping

DNA that was extracted from endoscopic samples was further used for partial amplification of vacA gene. Thermal condition was maintained by initial denaturation at 96^0C for 10 minutes followed by cycle denaturation at 95^0C for 40 seconds, annealing at 53^0C for 30 seconds followed by cyclic extension 72^0C for 50 seconds for 35 cycles and final extension at 72^0C for 5 minutes followed by 4^0C to stop the reaction and cagA genotyping was performed by amplification of partial cagA gene for asian lineage. PCR initial denaturation at 95^0C for 5 minutes followed by cycle denaturation at 95^0C for 1 minute, annealing at 50.5^0C for 20 seconds followed by cyclic extension 72^0Cfor 30 seconds for 35 cycles and final extension at 72^0C for 5 minutes followed

by 4^0C. Similarly for European lineage, PCR condition was initial denaturation at 95^0C for 5 minutes followed by cycle denaturation at 95^0C for 40 seconds, annealing at 48.5^0C for 20 seconds followed by cyclic extension 72^0C for 40 seconds for 35 cycles and final extension at 72^0C for 5 minutes followed by 4^0C. Genotyping was done by polymerase chain reaction (PCR) followed by 1.5% agarose gel electrophoresis. The gold standard, di-deoxy chain termination method was performed forcagA lineage identification and for the sequencing of vacA partial gene using Automated Sanger sequencing (ABI 3500 Genetic analyzer).

3. Results

3.1. *Helicobacter pylori* infection in relation to the demographic factors

Out of 943 patients, 863 expressed their willingness to participate in this research work and the rate of feedback is 91.5 %. The percentage of refusal was high among male participants (75% of the total refusal). Out of all samples, 475 (55%) were positive for this specific infectious disease. The participants were from the age between 8 to 92 years and were categorized as *H. pylori* positive or negative groups. The prevalence was almost identical among middle age groups: 4.6% at 10–20, 13% at 21–30, 23.1% at 31–40, 24% at 41–50, 20% at 51–60, 8.6% at 61 -70, 4.2 at 71-80 and 2.5 at >80 years (Table 3.1). However, the *H. pylori* urease test positive was not so remarkably different between male and female subjects (p = 0.56). The percentage of males (58.5%), married persons (83.4%), those with lesser than matriculation educational level (52%) are slightly higher in *H. pylori*-positive than the *H. pylori* negative persons.

Among the participants, access to a common drinking water distribution system was *H. pylori*-positive (81.1%) and negative (83.8%), respectively. However, a very less percentage of people with *H. Pylori* positive consumed filtered drinking water. The food and life style habits of the *H. pylor*i-positive and -negative groups are listed in Table 3.2.

	H. pylori Negative	H. pylori Positive
	n=388	**n=475**
	n (%)	**n (%)**
Age (Mean SD)	44.36 (15.59)	45.22 (15.30)
Sex		
Female	173 (44.6)	197 (41.5)
Male	215 (55.4)	278 (58.5)
Marital status		
Unmarried	78 (20.1)	79 (16.6)
Married	310 (79.9)	396 (83.4)
Location		
North Aizawl	111 (28.6)	139 (29.3)
East Aizawl	55 (14.2)	54 (11.4)
South Aizawl	56 (14.4)	77 (16.2)
West Aizawl	78 (20.1)	94 (19.8)
Other	88 (22.7)	111 (23.4)
Occupation		
House wife/No Job	103 (26.5)	120 (25.3)
Student	120 (30.9)	131 (27.3)
Government Employee	95 (24.5)	115 (24.2)
Self employed	39 (10.1)	35 (7.4)
Farmer	31 (8.0)	74 (15.6)
Education qualification		
Above matriculation	93 (24.0)	98 (20.6)
Matriculation	112 (28.9)	130 (27.4)
Below matriculation	183 (47.2)	247 (52.0)
No. of persons living in same household (Mean SD)	5.82 (2.19)	6.58 (2.36)
Source of drinking water		
Public water distribution system	325 (83.8)	385 (81.1)
Stream / Lake / River	58 (14.9)	81 (17.1)
Ponds / Ditches / Wells	5 (1.3)	9 (1.9)

Water purification method used		
Filtered (Ceramic/Charcoal/UV/Reverse osmosis)	284 (73.2)	324 (68.2)
Boiled	88 (22.7)	140 (29.5)
Un purified	16 (4.1)	11 (2.3)
Sanitation		
Commode		
Open field	228 (58.8)	264 (55.6)
Saum (Fermented pig fat) consumption		
Never	63 (16.2)	62 (13.1)
Mild consumption	106 (27.3)	117 (24.6)
Moderate consumption	191 (49.2)	264 (55.6)
Heavy consumption	28 (7.2)	32 (6.7)
Raw or uncooked vegetables consumption		
Never	26 (6.7)	25 (5.3)
Occasionally	150 (38.7)	186 (39.2)
Regularly	212 (54.6)	264 (55.6)
Salt consumption		
Never / mild consumption	155 (39.9)	207 (43.6)
Moderate consumption	184 (47.4)	218 (45.9)
Heavy consumption	49 (12.6)	50 (10.5)
Pickle consumption		
Never	72 (18.6)	96 (20.2)
Mild consumption	193 (49.7)	235 (49.5)
Moderate consumption	98 (25.3)	122 (25.7)
Heavy consumption	25 (6.4)	22 (4.6)
Tobacco smoking		
Never	228 (58.8)	203 (42.7)
Former user	22 (5.7)	10 (2.1)
Current user	138 (35.6)	262 (55.2)
Alcohol consumption		
Never	262 (67.5)	248 (52.2)

Former user	15 (3.9)	21 (4.4)
Current user	111 (35.6)	206 (43.4)
Chewed Tobacco product consumption		
Never		
Ever	248 (63.9)	352 (74.1)
Tuibur consumption		
Never	365 (94.1)	403 (84.8)
Ever	23 (5.9)	75 (15.2)

Table 3.2. Distribution of socio-demographic and lifestyle factors with *H. pylori* infection.

Among the patients with *H. pylori*, almost 47.4% were living with 5 to 7 family members, and almost greater than quarter (26.9%) were living with 8 to 10 family members (Table 3.2). The number of person in same household was a significant factor for *H. pylori* infection in univariate and multivariate analysis, when the family members are 5 to 7, 8 to 10 and more than 10 persons. Occupation as farmer was also a significant factor with OR: 1.94; 95% CI: 1.09 – 3.50 for *H. pylori* infection in the Mizo population in multivariate analysis. But there is no statistical significance for the qualification and occupation, because majority of the patients and family members are educated, having a formal education (Table 3.2).

The maximum numbers of patients infected with *H. pylori* were smokers (55.2%) and consumption of local alcoholic beverages was found in 43.3%. Statistical association between locally processed alcohol made from rice and *H. pylori* status was detected with high OR and 95% CI in regular consumers (at least thrice a week) by univariate analysis. Consumption of betel leaf with areca nut and tuibur (tobacco infused water) were significant risk factors for the *H. pylori* infection in univariate analysis, but in the multivariate analysis only regular tuibur consumption was significant factor. Consumption of raw uncooked meat/vegetables at least twice a week

was by 94.6% of the patients, but the factor is not statistically significant for *H. pylori* infection (Table 3.2).

The consumption of fermented pork fat and extra salt with *H. pylori* infection was not significant in Mizo population. Prevalence of Intermittent, nocturnal and continuous dyspeptic symptoms (upper abdominal pain or discomfort) was of 60.2%, 34% and 5.6%, respectively. Maximum patients had pursued medication due to stomach problems. Family history of different gastrointestinal diseases and gastro-oesophageal reflux were reported by 31.7%, 51.4% and 13.4%, respectively out of the 462 participants who were aware (at the time of the interview) of the medical diagnosis to their symptoms disease history. The existence of dyspeptic symptoms was remarkably correlated with the occurrence of *H. pylori* infection with symptomatic individuals having a prevalence of 21% higher than among the non-symptomatic individuals. No association between gastro-oesophageal reflux disease and bacterial contamination was detected. There is a strong association and statistical significance observed for ulcer and familial history of gastric cancer with OR: 22.13; 95% CI: 12.60 – 40.93, OR: 10.03; 95% CI: 5.74 – 18.43, respectively.

Majority of the infected patients (81%) were using public welfare department (PWD) water supply for house-hold use, but very less number of patients (17.1%) were using stream, lake or river water. Consequently, most of the infected patients were using ceramic (59.1%) and boiled (29.5%) water as a drinking water, but there are no statistical significance for water resource and drinking water quality for the *H. pylori* infection (Table 3.3).

Factor	Category	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Gender	Female	Reference	
	Male	1.13 (0.86 – 1.48)	1.13 (0.86 – 1.50)
Age group (years)	10-20	Reference	
	21-30	1.38 (0.69 – 2.74)	1.32 (0.64 – 2.70)
	31-40	1.34 (0.71 – 2.55)	1.12 (0.51 – 2.42)

	41-50	1.47 (0.77 – 2.80)	1.11 (0.50 – 2.47)
	51-60	1.38 (0.72 – 2.65)	1.04 (0.46 – 2.33)
	61-70	1.72 (0.81 – 3.68)	1.18 (0.48 – 2.92)
	71 - 80	1.26 (0.53 – 2.99)	0.80 (0.29 – 2.18)
	Above 80	1.56 (0.53 – 4.71)	1.01 (0.30 – 3.43)
Marital status	Unmarried	Reference	
	Married	1.26 (0.89 – 1.78)	1.19 (0.72 – 1.97)
No. of person in same house hold	1 - 4	Reference	
	5 - 7	1.47 (1.05 – 2.06)*	1.47 (1.04 – 2.07)*
	8 - 10	2.45 (1.62 – 3.71)***	2.51 (1.65 – 3.83)***
	> 10	4.21 (2.08 – 9.12)***	4.24 (2.05 – 9.35)***
Occupation	Govt. employee	Reference	
	Self employed	0.93 (0.65 – 1.34)	0.89 (0.59 – 1.35)
	House wife	1.03 (0.71 – 1.51)	0.98 (0.61 – 1.58)
	Student	0.77 (0.45 – 1.30)	0.75 (0.44 – 1.28)
	Farmer	2.04 (1.25 – 3.39)**	1.94 (1.09 – 3.50)*
Qualification	Higher Education	Reference	
	Matric pass	1.10 (0.75 – 1.61)	1.10(0.73 – 1.67)
	Under matriculation	1.28 (0.90 – 1.80)	1.10 (0.70 – 1.73)
Locally processed alcohol made from rice fermentation	Non alcoholic	Reference	
	Quit	1.47 (0.75 – 2.98)	1.59 (0.44 – 3.47)
	Occasionally	1.99 (1.41 – 2.83)***	1.43 (0.96 – 2.13)
	Regularly	1.90 (1.28 – 2.86)**	1.28 (0.81 – 2.05)
Smokers	Non smokers	Reference	

	Quit	0.51 (0.22 – 1.07)	0.45 (0.19 – 0.99)
	Occasionally	1.86 (1.31 – 2.67)***	1.61 (1.09 – 2.39)*
	Regularly	2.39 (1.70 – 3.37)***	1.98 (1.32 – 2.98)***
Betel leaf with areca nut consumer	Non consumers	Reference	
	consumers	1.44 (1.10 – 1.89)**	0.94 (0.63 – 1.39)
Tuibur consumer	Non consumers	Reference	
	Occasionally	0.57 (0.37 – 0.86)**	1.47 (0.94 – 2.29)
	Regularly	2.47 (1.77 – 3.45)***	4.02 (1.68 – 4.61)***
Raw uncooked Meat/vegetables	Non consumers	Reference	
	Occasionally	1.28 (0.71 – 2.33)	1.19 (0.64 – 2.21)
	Regularly	1.29 (0.72 – 2.31)	1.17 (0.64 – 2.15)
Fermented pig fat (Sa-um)	Non consumers	Reference	
	Little	1.21 (0.72 – 1.73)	1.10 (0.69 – 1.74)
	Average	1.40 (0.94 – 2.09)	1.42 (0.93 – 2.17)
	Heavy	1.16 (0.62 – 2.15)	1.22 (0.64 – 2.34)
Extra salt	Non consumers	Reference	
	Little	2.08 (0.84 – 5.45)	1.68 (0.65 – 4.53)
	Average	1.77 (0.71 – 4.62)	1.37 (0.53 – 3.67)
	Heavy	1.53 (0.58 – 4.21)	1.28 (0.46 – 3.68)
Pickles	Non consumers	Reference	
	Little	0.91 (0.63 – 1.30)	0.89 (0.60 – 1.30)
	Average	0.93 (0.62 – 1.39)	0.90 (0.58 – 1.39)
	Heavy	0.66 (0.34 – 1.26)	0.70 (0.35 – 1.40)

Household Water Resources	PWD	Reference	
	Stream, lake or river	1.17 (0.81 – 1.70)	1.15 (0.79 – 1.68)
	Pond, ditch or well	1.51 (0.51 – 4.98)	2.26 (0.70 – 8.27)
Drinking water	UV/RO	Reference	
	Active carbon	0.37 (0.14 – 0.92)	0.31 (0.11 – 0.81)
	Ceramic	0.98 (0.57 – 1.66)	0.96 (0.56 – 1.64)
	Boiled	1.31 (0.73 – 2.30)	1.25 (0.70 – 2.22)
	Unfiltered	0.56 (0.22 – 1.40)	0.52 (0.20 – 1.32)

Table 3.3. Association of risk factors *H. pylori* positive cases in Mizo population

About 74.1% of the individuals in the *H. pylori*-positive group were ever users of chewed tobacco, and tobacco smoking (57.3%) compared to 63.9% and 35.6% among the *H. pylori*-negative people, respectively. The tuibur users are much higher in *H. pylori*-positive (15.2%) than the *H. pylori*-negative group (5.9%).

Results of logistic regression showed that ever-users of Tuibur had increased odds of *H. pylori* positive endoscopy by 3.32 (OR=3.32; 95%CI=1.95 – 5.83) times compared to never users. This association was not altered even after considering other known confounding risk factors of this bacterial contamination. In addition, chewed tobacco users had 1.49 (OR=1.49; 95%CI=1.06 – 2.09) higher odds of having *H. pylori* positive endoscopy result (Table 3.4).

	Odds Ratio (95% CI)			
	Model 1[#]	**Model 2[$]**	**Model 3[¥]**	**Model 4[£]**
Tuibur consumers				
Never	1	1	1	1
Ever	3.23 (1.98 – 5.44)	2.94 (1.78 – 5.00)	3.32 (1.96 – 5.80)	3.32 (1.95 – 5.83)

Tobacco Chewers				
Never	1	1	1	1
Ever	1.51 (1.11 – 2.06)	1.51 (1.10 – 2.08)	1.49 (1.07 – 2.08)	1.49 (1.06 – 2.09)
Tobacco Smokers				
Non smokers	1	1	1	1
Former smoker	0.45 (0.19 – 0.99)	0.46 (0.19 – 1.01)	0.36 (0.14 – 0.83)	0.36 (0.14 – 0.86)
Current smoker	1.78 (1.28 – 2.50)	1.89 (1.34 – 2.67)	1.82 (1.27 – 2.60)	1.81 (1.26 – 2.61)
Alcohol consumers				
Never	1	1	1	1
Former user	1.57 (0.74 – 3.45)	1.71 (0.80 – 3.78)	1.77 (0.80 – 4.02)	1.74 (0.78 – 3.98)
Current user	1.37 (0.97 – 1.94)	1.68 (1.13 – 2.50)	1.84 (1.22 – 2.80)	1.81 (1.19 – 2.76)

Table 3.4.Association between tobacco and alcohol habits and *H.pylori* infection

 # Model 1 – mutually adjusted;**$ Model 2** – further adjusted for age, sex, marital status;**¥ Model 3** – further adjusted for occupation, education, no. of persons in household, drinking water sources, and sanitation;**£ Model 4** – further adjusted for dietary factors (raw food, salt, sa-um, pickles).

3.2. Distribution of cagA and vacA genotypes in *H. pylori*-positive samples

A total number of 475 *H. pylori*-positive (55.04%)endoscopic specimenswere collected from 863 collected samples from Trinity Diagonostic Centre, Aizawl, Mizoram. All the positive samples were confirmed by rapid urease test, 16S and Urec gene PCR test followed by cagA and vacA virulence genes identification. The vacA genotype was identified in 31 (6.5%) and the cagA genotype was identified in 23 (4.8%) *H. pylori*-positive samples. Out of cagA positive samples, a total of 18 (78.26%) and 5 (21.74%) samples were represented as cagA lineage 1 (Asian specific lineage) and cagA lineage 2 (European specific lineage), respectively. Whereas 7 samples show combinations of vacA and cagA genotypes: cagA lineage1/vacA (10.6%), cagA lineage2/vacA (4.32%) (Table 3.5.).

Categories	No. of cases (%)
Genotype	
vacA (+ve)/ *cagA* (-ve)	24 (51.1)
vacA (-ve)/ *cagA1*(+ve)	13 (27.7)
vacA (-ve)/ *cagA2*(+ve)	3 (6.43)
vacA (+ve)/ *cagA1*(+ve)	5 (10.6)
vacA (+ve)/ *cagA2*(+ve)	2 (4.32)
Gender	
Female	14 (29.85)
Male	33 (70.23)
No. of Person in a Family	
1-4	8 (17.02)
5-7	12 (25.58)
8-10	27 (57.44)
Alcohol consumer	
Non-alcoholic	25 (53.19%)
Occasionally	13 (27.7%)
Regularly	9 (19.1%)
Smoker	
Non-Smoker	17 (36.2%)
Occasionally	11 (23.4%)
Regularly	19 (40.4%)

Table 3.5. Demographic factors *and the H. pylori* genotype in gastritis patients

3.3. Association between *H. pylori* genotype and demographic factors

Male gastritis patients (70.23%) were more affected with *H. pylori* compared to the female patients. Large family size was marked as the high-risk factor for *H. pylori* infection in this population, because 57.44% of *H. pylori*-infected gastritis patients have more than 8 people in the family. Almost , 50% of *H. pylori*-infected gastritis patients were alcohol consumers and 63.8% of *H. pylori*-infected gastritis patients were regular or occasionally smokers in study cohort (Table 3.5.).

Some more significant factors for the existence of cagA genotypes were alcohol, non-veg, and extra pickle consumption in the univariate analysis among all studied lifestyle factors. Alcohol consumption (p = 0.03) and extra pickle intake (p = 0.03) were the significant factors in multivariate analysis for cagA genotype patients' group (Table 3.6). The values of probability are significantly higher for the cagA positive group of people with the alcohol and extra pickle consumer group. The multivariate model got a high region under the curve values (AUC = 0.75, p = 0.001)

for the correlation among alcohol and extra pickle consumption and the presence of cagA genotype (Figures: 3.10A and 3.11A). Whereas, Asian lineage cagA-linage1 remarkbly correlated with alcohol consumer (OR = 0.63; 95% linage cagA-linage1 were remarkbly correlated with alcohol consumption (OR = 0.63; 95% CI = 0.38 - 1.04, p = 0.05) and non-veg consumption (OR = 0.26; 95% CI = 0.08 – 0.85, p = 0.02) in univariate analysis. While the independent threat for the existance of Asian lineage of cagA in multivariate analysis was non-veg consumption for this population (AUC=0.72, p=0.001) (Figures: 3.10B and 3.11B). European lineage of cagA also showed high curve valuewhen correlated with food habits (AUC=0.83, p=0.001) (Figures: 3.10C and 3.11C).Interestingly, none of the demographic factors (food and lifestyle habits) were significant with the existance of vacA genotypes in this population (Table 4.3.).

	Odd ratio	95% CI	P value
cagA	**Univariate**		
Marital status	0.46	0.11 - 1.84	0.27
Alcohol	**0.63**	**0.39 - 1.01**	**0.05**
Smoking	0.85	0.55 - 1.32	0.47
Tobacco consumption	0.86	0.51 - 1.44	0.56
Nonveg consumption	**0.34**	**0.11 - 1.06**	**0.05**
Raw food consumption	1.01	0.39 - 2.61	0.97
Sa-um consumption	0.59	0.29 - 1.19	0.14
Extra salt intake	0.48	0.17 - 1.37	0.17
Extra pickle intake	**0.31**	**0.10 - 0.92**	**0.03**
	Multivariate		
Alcohol consumption	**0.54**	**0.31 - 0.95**	**0.03**
Nonveg consumption	0.37	0.10 -1.39	0.14
Extra pickle intake	**0.54**	**0.31 - 0.95**	**0.03**
cagA-lineage1	**Univariate**		

Marital status	0.41	0.10 - 1.64	0.21
Alcohol	**0.63**	**0.38 - 1.04**	**0.05**
Smoking	0.82	0.52 - 1.28	0.38
Tobacco consumption	0.78	0.45 - 1.33	0.36
Nonveg consumption	**0.26**	**0.08 - 0.85**	**0.02**
Raw food consumption	1.53	0.56 - 4.12	0.43
Sa-um consumption	0.97	0.49 - 1.93	0.94
Extra salt intake	0.49	0.16 - 1.50	0.21
Extra pickle intake	0.47	0.17 - 1.37	0.42
	Multivariate		
Alcohol consumption	0.62	0.35 - 1.09	0.09
Nonveg consumption	**0.23**	**0.06 - 0.87**	**0.03**
vacA	**Univariate**		
Marital status	1.14	0.28 - 4.68	0.85
Alcohol	1.15	0.71 - 1.86	0.56
Smoking	0.99	0.63 - 1.56	0.97
Tobacco consumption	0.73	0.41 - 1.35	0.29
Nonveg consumption	1.3	0.67 - 2.56	0.43
Raw food consumption	0.59	0.21 - 1.66	0.32
Sa-um consumption	1.25	0.61 - 2.57	0.53
Extra salt intake	1.15	0.42 - 3.31	0.79
Extra pickle intake	2.28	0.79 - 6.55	0.12

Table 3.6. Univariate and multivariate analysis of the risk factors compared between negative and positive samples for cagA, cagA-lineage 1 and vacA genotypes in gastritis patients.
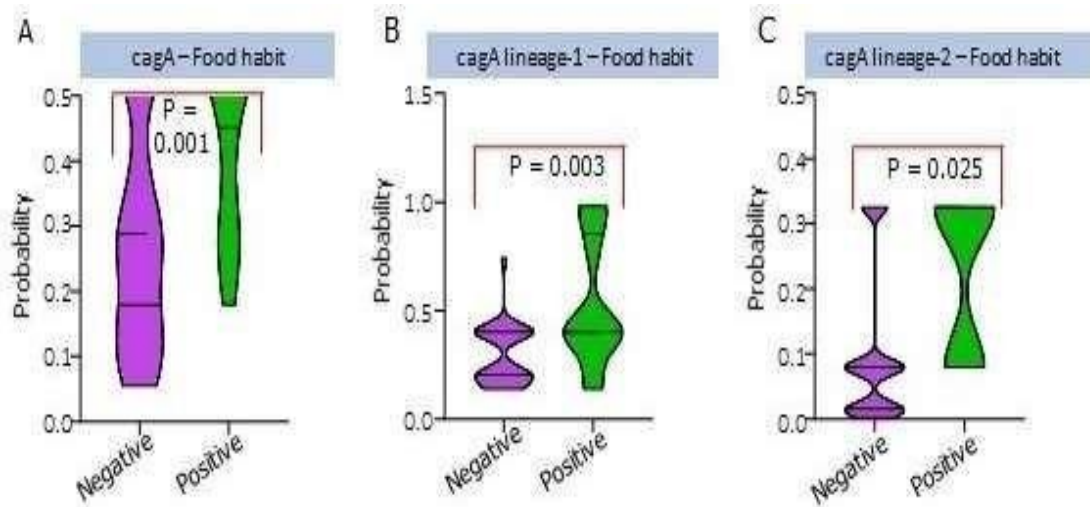
Figure: 3.10. Association plot for *H. pylori* cagA genotype and food habits
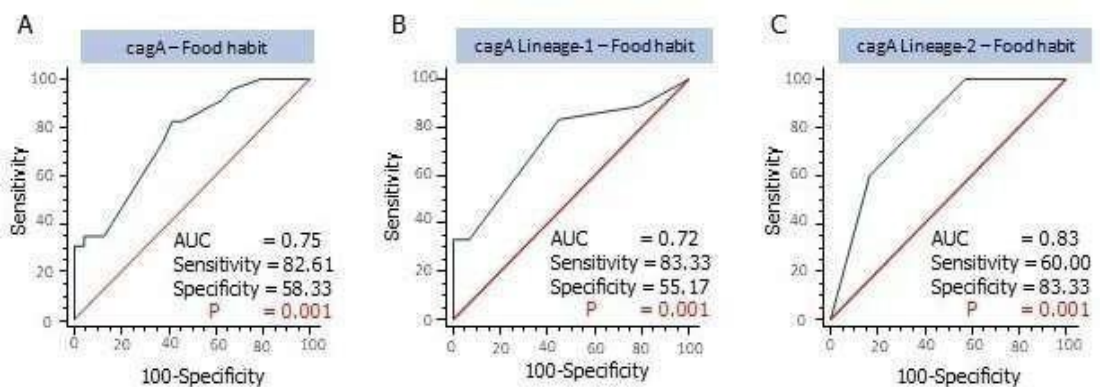
Figure: 3.11. Receiver operating curve (ROC) for *H. pylori* cagA genotype and food habits

4. Discussion

This study is the first and foremost large scale estimate of the abundance of *H. pylori* infection in gastric cancer prone state of Mizoram, Northeast India. The population of India is relatively heterogeneous and prevalence of gastritis is geographically divergent. In the present study, no statistical correlation was found between patients of both genders. However, since the refusal percentage to participate in the present work was much high among men, it is possible that certain cases might

have been excluded that were with greater chances of infection from demographic factors. Some other study reported excessive occurrence of infection in men (Franceschi et al.,1998). This study also correlates the socio-demographic factors connected with status of infection. The percentage of occurrence of gastrointestinal diseases reported variations according to the region and the life style among the different states of north eastern zone of India (Phukan et al., 2006). Mizoram is remarkably on top in gastric cancer incidence, as in Aizawl the age-adjusted incidence and death rate of gastric carcinoma per 100,000 individuals in 2003-04 was 57.3 and 25.3 for male and 33.6 and 18.0 for females, respectively which is well above the national average (Dikshit et al., 2011).

Factors linked to present family size were most relevant determinants of *H. pylori* infection in this adult population because of the habit of sharing food from the same bowl, which is a common practice for the society. Similar, finding was remarked in crowded families in developed and developing countries (Brown, 2000). In mostof these studies, a clear correlation was noticed between socio-economic status and overcrowding in childhood, but not with present household conditions (Kuipers,1999; Yamaoka, 2012; Goh et al., 2011). This study evaluated the socioeconomic status and overcrowding of the subjects, a similar study on the age and the number of individuals present per room were observed to be remarkable threat for *H. pylori* infection (Brown et al., 2002; Alizadeh et al., 2009). Basically, it is found that the domestic congestion is a major transmission factor for spreading infection inside the families (Windsor et al., 2005; Ozaydin et al., 2013). An observation reported on Mexican patients showed high *H. pylori* sero-prevalence with low educational levels of the patients (Zeidel et al., 2002; Eusebi et al., 2014). But in the present study, no correlation was found between *H. pylori* infection and educational level. There was an association with farmer as an occupation, working in the agriculture field with many people and consuming unhygienic food and water may be the major causes of this risk factor.

In another study, the correlation between intake of Tuibur and threat of gastric carcinoma was reported (Ghatak et al., 2016), but it is the foremost research to investigate the correlation between use of Tuibur and *H. pylori* infection. Moreover,

this association was not attenuated when we included important risk factors (e.g., indicators of socioeconomic position) in the models (Table 3.4).

Previous study reported the unhygienic conditions in which Tuibur is produced (Madathil et al., 2018) and furthermore, viability of *H. pylori* bacterium may facilitate by the alkaline pH of Tuibur. Tuibur also contains some carcinogenic agents like TSNAs, NNN, NNK which are responsible for different health hazards along with cancer and other diseases. These molecular particles can have association with increased rate of *H. pylori* infection by changes in pH level of buccal cavity as well as in abdomen and jointly influenced the disease rate (Muthukumaran, 2016; Lalmuanpuii et al., 2016).

Regarding behavioural factors, the recent reports did not find tobacco use or alcohol consumption to be a threat for *H. pylori* infection (Darnindro et al., 2015; Santos et al., 2005). Another research work designed to evaluate the smoking or alcohol habits in southwest England reported that the habit of smoking was not linked to *H. pylori* infection and drinking of alcohol is correlated, but not statistically remarkable (Rodrigues et al., 2005). In a EUROGAST Study, a univariate analysis reported that drinking alcohol was correlated with a less occurrence of *H. pylori*, but this consequence fully departed after alteration in the multivariate analysis (Mendoza et al., 2013; Nell et al., 2013; Eusebi et al., 2014). No correlation was observed between *H. pylori* and drinking alcohol in other studies (Darnindro et al., 2015; Goh et al., 2011; Ghatak et al., 2016). In the present study, no strong correlation was observed between the intake of local alcohol and *H. pylori* infection, but occasional and regular habits of smoking were significant factors with *H. pylori* infection, because of the high usage of local cigarette (zozial). The higher occurrence of *H. pylori* among smokers might be because of the sharing of the cigarette within the people assembled together at the time of smoking and also because of the weakening of the systemic and local immunity (Zeidel et al., 2002).

The correlation between smoking and drinking used and the infection of *H. pylori* is inconclusive (Brown et al., 2002; Vatsala et al., 2014). Studies from European countries have reported positive or negative (Sharma et al., 2016; Ahmed et al., 2006)

or inconclusive relationship between tobacco, alcohol behaviours and *H. pylori* infection (Sharma et al., 2016; Ahmed et al., 2006; Abebaw et al., 2006; Brown 2000). In this study, tobacco smokers and alcohol consumers were correlated with higher odds of *H. pylori* infection (Table 3.4). A negative correlation was found between earlier smokers of tobacco and *H. pylori* infection. In fact, changed habits of smoking and alcohol were found in 43.4% and 55.2% in the dyspepsia patients, respectively.

In the present research work, the consumption of betel quid (a chewed tobacco product) was correlated with high odds for *H. pylori* infection. The regular cleaning of betel vine leaves and areca nut for the betel quid, under dirty and unclean water can be the cause of the outcome (Abebaw et al., 2014). Interestingly, the report was not totally reduced even after altering for food habits, sources of water and socio-economic variable.

It is believed that the occurrence of *H. pylori* changes with resources of potable water. In the present study, the occurrence of *H. pylori* was increased in patients who used unprotected surface water than the people who drank PWD supplied water, but there was none the statistical significance observed. This is similar to a research work done in China which observed that having river water as potable water had much risk than the having tap water. In India, also the occurrence of *H. pylori* was much higher among peoples who used to have well-water than piped tap water (Ahmed et al., 2007). Conversely, a research work reported that in Ethiopia, the occurrence of *H. pylori* was much more among the people who used to have piped water than the well water (Ahmed et al., 2006).

Regarding gastrointestinal complaints, only nocturnal dyspeptic symptoms was correlated to the infection. Meta-analyses of trials which have been done in individuals with functional (that is, investigated) dyspepsia have shown no advantages from removal of *H. pylori* (Abebaw et al., 2014). Since patients sorted as presenting of dyspepsia, in the present study work it was not examined for identification of the reasons behind their symptoms.

The present work has few hindrances like; firstly, the study sample only included patients of Mizo origin. However, high incidence on gastric carcinoma among the people of this region suited aim of this work ideally. Second, recruitment of participants from patients reporting with gastritis at a gastroenterology clinic in Aizawl has done due to the feasibility. Although this strategy allowed focusing in a group with high probability of *H. pylori* infection, it could bias the results. Like, if use of Tuibur can influence the gastric disease, the present work may have stratification bias as it deals only with gastritis patients (Cole et al., 2010). Although, a correlation between Tuibur and gastritis existed (not-interposed through *H. pylori* infection) has not been reported in literature. However, the present study has primary evidence for a correlation and replication of the findings can be done by prospective and mechanistic studies.

The infection of *H. pylori* is dependent on some factors like food and lifestyle habits for a population and the genetics of the host, and the reasons of infections are also dependent on the virulent genotypes of *H. pylori* (Oforiet al., 2019). There are various strong evidences that the existence of CagA genotype in stomach epithelial cells had the effect of cytoskeletal rearrangements, alterations in membrane dynamics, disruption of cell junctions, and promotion of mitogenic, pro-inflammatory, and anti-apoptotic genes transcription (Hatakeyama et al., 2017). The changes in the cytoskeleton included because of the phosphorylation of the CagA gene in the cytoplasm of the epithelial cell give a result in morphological changes known as the "hummingbird" phenotype (Takahashiet al., 2020). In this study, it was observed that this phenomenon was accompanied by an increase in the production of interleukin in response to the infection. Therefore, its induction is counted a possible event in tumour initiation. Hence, the strains of *H. pylori* which used to express CagA are related with an increased risk of peptic ulcer and resulting gastric adenocarcinoma (Zhang et al., 2017). In different studies, it has been shown that cagA+ genotype has a higher risk of gastritis in the Indian population (Ghatak et al., 2016; Salih et al., 2013).

Furthermore, the absence of correlation of vacA maybe because the outcome of cancer or ulcer disease is a complex process that also involves factors other than the

vacA, such as cagA. Unlike this study, other studies have also identified that the presence of vacA is related with peptic ulcer diseases (Fakhre et al., 2014; Keikha et al., 2020; Faundez et al., 2002). The results of the study also had shown a not significant difference in vacA genotype distribution in gastritis patients. However, different disease results were encountered in subjects infected with *H. pylori* strains sharing the multiple virulence genes cagA and vacA genotype (14.92%). The various outcomes of *H. pylori* infection could be dependent on different pathological as well as various genetic factors of the host. Considering host genetic factors, a genome-wide screen analysis to detect the genetic factor(s) defining susceptibility to *H. pylori* infection recommended the presence of a possible linkage with chromosome4 (Miftahussurur et al., 2015).

Some important factors for the existence of cagA genotypes were alcohol consumption and extra pickle intake, whereas alcohol consumptions and non-veg intake were the significant factors for the existence of cagA – Asian lineage 1 for this population. But this study did not find any link between the different lifestyle habits and the existence of vacA genotype in gastritis patients. It was observed that in this population most of the occasional or regular smokers got infected by *H. pylori*. The higher percentage of male gastritis patients were *H. pylori*-infected than female patients. In this study it was observed that Betel leaf and nut (pan consumption) consumers were highly infected with *H. pylori* strain.

In conclusion, Alcohol consumption might be the vital threat for the existence of cagA genotypes or *H. pylori* infection among this population. CagA-lineage1 (Asian specific lineage) is wide spread in this population than cagA -lineage2 (European specific lineage).

Chapter IV

PCR based detection of Clarithromycin antibiotic resistance status of *H. pylori*

1. Introduction

Prevalence of antibiotic resistance strain of *H. pylori* affects the suppression process and enhances the occurrence of gastric cancer (GC) worldwide (Nishizawa, and Suzuki, 2014). *H. pylori* reduction treatment was executed for many different gastrointestinal disorders, such as peptic ulcer diseases, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer at a very early stage when the cancer has been endoscopically exterminated (Uemura et al., 2001). Many studies show that the increase in the antibiotic resistance in past few years has reduced the potentiality of treatment regimens (Jina et al., 2020). The occurrence of antibiotic resistance in *H. pylori* is due to the existence of SNPs in 16S rRNA, 23S rRNA and GyrA genes which can be another risk factor for high incidence of gastric cancer.

The recent way for the eradication of *H. pylori* infection comprises of a proton pump inhibitor (PPI) and some antimicrobial agents, such as amoxicillin, clarithromycin, and metronidazole. Few varieties of such triple regimens, triple therapies with a proton pump inhibitor (PPI; i.e., omeprazole, rabeprazole and lansorprazole), clarithromycin and amoxicillin are standard drugs in Japan (Asaka et al., 2001). Triple therapies with a PPI or ranitidine bismuth citrate, combined with clarithromycin and amoxicillin or metronidazole are standardized regimes in Europe (Malfertheiner et al., 2002). However, different studies say that the global recovery rate is 80% after using standard triple therapy. (Graham et al 2007; Megraud, 2004). Therefore, clarithromycin is most prime antibiotics in *H. pylori* reduction therapy. Reduction frequency by triple therapies involving clarithromycin, depend on bacterial sensitivity to clarithromycin (Furuta et al., 2001). The World Health Organization announced that clarithromycin-resistant *H. pylori* are of importance as a pathogen (Kuo et al., 2017). The prevalence of Clarithromycin-resistant strains of *H. pylori* varies in different parts of the world (12.2% in USA, 1.7% in the Netherlands, 4.4 % in the UK and 12.9 % in Japan) (Murakami et al., 2002). In a study it was found that

H.pylori strain resistance to clarithromycin is also correlated with point mutations in the 23S rRNA gene (Megraud, 2004). According to the studies Clarithromycin-resistant strain of *H. pylori* is possible due to mutation from adenine (A) to guanine (G) at position 2142 or 2143 of the 23S rRNA gene (Furuta et al., 2007).

2. Materials and methods

2.1. Multiplex PCR amplification of the 23S rRNA gene

The 23S rRNA gene different region with SNP was amplified by polymerase chain reaction (PCR) using two different primer pairs (Table 4.1). PCR was done in 25 µl total reaction volumes each containing 100 ng of template DNA, 0.2 pM of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2mMdNTPs, and 1 unit of Taq DNA polymerase (Fermentas, Germany). The reaction mixture was heated to 95^0 C for 10min, followed by 30 cycles each consisting of 15 sec denaturation at 94^0 C, 20sec annealing at 60.5^0 C temperature, 30 sec of extension at 72^0 C and a final 5 min extension at 72^0 C. The PCR amplification products (3 µl) were subjected to electrophoresis in a 2 % agarose gel in 1X TBE buffer at 80 V for 30 min, stained with Ethidium Bromide and images were obtained in GBOX gel documentation systems (UK). (Figure: 4.1)

Primer name	Primer Sequences	Expected product size (bp)	Genotypes	References
FP-1	5′ -TCGAAGGTTAAGAGGATGCGTCAGTC- 3′	320	320= wild type	(Li et al., 1999)
RP-1	5′ -GACTCCATAAGAGCCAAAGCCCTTAC- 3′			
RP2142G	5′ -AGTAAAGGTCCACGGGGTATTCC- 3′	238	238= A-G Mutant	
FP2143G	5′ -CCGCGGCAAGACAGAGA- 3′	118	118= A-G Mutant	

Table 4.1. List of 23S rRNA primers used to identify susceptible or resistant clarithromycin *H. pylori* virulent strains
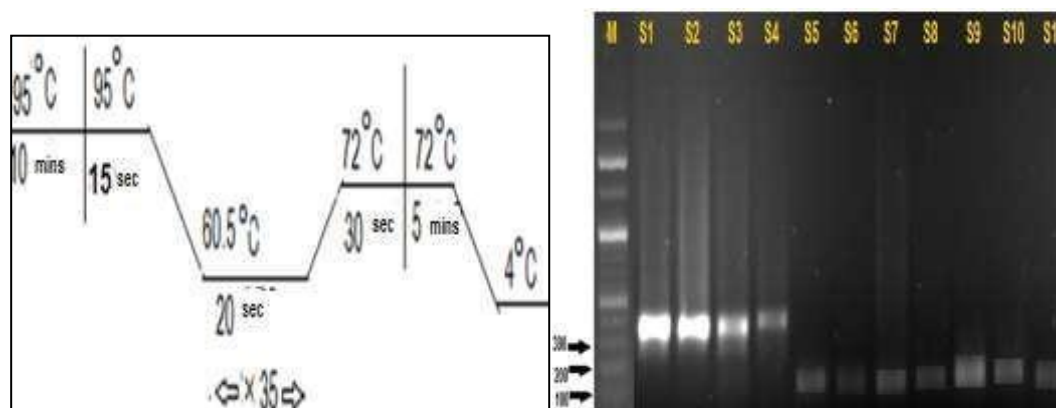
**Figure: 4.1. Partial amplification of 23S rRNA gene of 320 bp size (wild type)
and 118 bp (mutant type) (2% agarose gel)
M: 1 kbp Marker; S1 to S11: Representative Samples**

2.3. Statistical Analysis

Single Nucleotide Polymorphisms from 23S rRNA gene and virulent genes were tested for Hardy–Weinberg equilibrium by a chi-square (v2) test with one degree of freedom (df). The distribution of susceptibility or resistance to clarithromycin was compared with demographic factors and estimated using odds ratios (ORs), and 95% confidence intervals (CIs). A logistic regression analysis was done to assess the potential confounder's influence of both genetic and environmental factors for the status of clarithromycin resistance. Then, the independent effect of risk factors was analysed in a multivariate model (introducing all variables and terms of interactions) keeping only those statistically significant or showing a confounding effect on the studied factors. For all tests, a two-sided p-value<0.05 was considered statistically significant. All statistical analyses were achieved using SPSS 20.0 program (SPSS, Madrid, Spain).

3. Results

Clarithromycin is utmost frequently used antibiotics for the eradication process of *H. pylori*. Presence of clarithromycin resistant *H. pylori* strains causes adverse effect on eradication process and affect the host with a higher degree of medical complications. This study assists to find out the demographic, as well as socio-

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economic factors, relate to the existence of clarithromycin-resistant strains in this particular population.

In this study, males were prone to carry clarithromycin-resistant *H. pylori* strains with 70.2% of the total study population were males. Similarly, an ascending order was found with the number of persons in the same household with 1-4, 5-7, 8-10 and above 10 persons contributed to the population 17%, 25.5%, 40.4% respectively except above 10 persons 17%, due to less availability in this population. This population are mostly non-veg consumers and the way of consuming differs with 87.2% of the population were consuming boiled. About 63.8% and 66% of the total population consuming betel leaf and ceramic filtered water, respectively (Table 4.2).

Factors	No of participant (%)
Clarithromycin	
Susceptible	17(36.2%)
Resistance	30(63.8%)
Gender	
Female	14(29.8%)
Male	33(70.2%)
No. of Person in a Family	
1-4	8(17.0%)
5-7	12(25.5%)
8-10	19(40.4%)
Above 10	8(17.0%)
Non-Veg	
Non-Consumer	3(6.4%)
Less than 3	1(2.1%)
Fried	2(4.3%)
Boiled	41(87.2)
Pan	
Non-consumer	17(36.2%)
Consumer	30(63.8%)
Drinking Water	
UV-RO	2(4.3%)
Active Carbon	1(2.1%)
Ceramic	31(66.0%)
Boiled	13(27.7%)

Table 4.2. Distribution of risk factors among virulent *H. pylori* infected patients

Base on statistical analysis, the risk factors were included for further studies. Logistic regression was done, univariates analysis results showed the existence of clarithromycin-resistant *H. pylori* strain was higher among the male of the population with an odd ratio of 1.35 and 95%CI (1.01-1.69) for a p-value of 0.02. Similarly, ceramic filtrate water consumers showed a high odd ratio of 2.28 and 95% CI (6.02-8.62) for a p-value of 0.001 (Table 4.3). Gender (male) adjusted odd ratio of fermented pig fat consumers showed odd ratio of 2.53 with 95%CI 1.01-6.31 for a p-value of 0.04 (Table 4.4).

Categories	Odd ratio	Confidence interval	P value
Gender			
Female	Reference		
Male	1.35	1.01 – 1.698	0.02
Non vegetarian			
Non consumers	Reference		
fried	0.5	0.13 – 19.56	0.71
boiled	0.86	0.72 – 10.38	0.91
Fermented pig fat			
Non consumers	Reference		
Little	0.85	0.16 – 4.55	0.85
Average	0.9	0.15 – 5.25	0.9
Heavy	0.75	0.78 – 7.21	0.8
Drinking water			
UV_RO	Reference		
Ceramic	2.28	6.02 – 8.62	0.001
Pan			
Non consumers	Reference		
Consumers	0.62	0.17 – 2.23	0.46

Table 4.3. Unadjusted odd ratio and confidence interval of risk factors with respect to clarithromycin resistance

	Odd ratio	Confidence interval	P value
Fermented pig fat			
Non-consumers	Reference		
Consumers	2.53	1.01 – 6.31	0.04

Table 4.4. Gender (male) adjusted odds ratio and confidence interval of risk factor concerning Clarithromycin resistance

4. Discussion

The triple therapy, a combination of one proton pump inhibitor with two antibiotics has been the first line of drugs used preferentially to eradicate the *H. pylori* infection (Thung et al., 2016; Malfertheiner et al., 2017). Some factors that can reduce the eradication rate are the drug combination, the time of treatment, the adhesion to the therapy, and the resistance to clarithromycin in the population (Chey et al., 2017). In this study, results were considered with life style determinants associated with clarithromycin resistance which might be responsible for eradication failures.

Clarithromycin resistance was found to be high among men with an odds ratio of 1.35 and 95% CI (1.01-1.69), conflicting with the study based on Korea where women patients were in majority. Gender can be a factor associated with clarithromycin resistance (Chang et al., 2019). Mizoram is a hilly state with rain water harvest as the main source of stagnant drinking water and can easily contaminate with *H. pylori*. Earlier studies revealed potable water source related to clarithromycin resistance and in the present study, ceramic filtrate water consumers were at a high risk of *H. pylori,* which might be a source of clarithromycin resistant strain of *H. pylori* (Hollander et al., 2013; Mentis et al., 2018).

As the present study showed that men were more prone to clarithromycin resistant strain of *H. pylori* infection. Hence, men were considered for further investigation and the report found that food habits as a causative factor for clarithromycin resistance. The Mizo populations are mostly non-veg consumers and fermented pig fat has been preferred as an additive in the boiled soup with monosodium glutamate (MSG), which is a toxic agent, may affect the stomach by changing the pH level. In the study, consumption of fermented pig fat was another important factor associated with male predominance in clarithromycin resistance.

Chapter V

Interleukin -1 -Beta gene polymorphism and its association with *H. pylori* virulence.

1. Introduction

H. pylori are genetically diverse and various genotypes have been associated with virulence and gastric disease risk. Human genetic polymorphisms also play a significant role in the disease sensitivity of the host. In a recent study, it is found that the polymorphism of the interleukin 1 beta (*IL-1B*) gene and the IL-1 receptor antagonist gene (IL-1RN) have been correlated with ahigh chances of both hypochlorhydia and gastritis (Ruzzo et al., 2005). *IL-1B* encodes IL-1, a potent pro-inflammatory cytokine and powerful inhibitor of gastric acid secretion that plays an important role in initiating and increasing the agitating reaction to *H. pylori* contamination (Figueiredo et al., 2014). A polymorphic allele with a T instead of a C at position –511 of the regulatory region of theIL-1B gene (*IL-1B*-511*T) is associated with increased IL-1 production (Kimang'a, 2012). IL-1RN encodes the IL-1 receptor antagonist (IL-1ra), an anti-inflammatory cytokine that ambitiously attach to IL-1 receptors, and thereby regulates the potentially destructive effects of IL-1(Volarevic et al., 2010). The IL-1RN gene has a variable number of tandem repeats in intron 2, resulting in a short allele (IL-1RN*2, with two repeats) or long alleles (IL-1RN*L, with three to six repeats). The IL-1RN*2 alleles are corelated with increased IL-1b production (Santtila et al 1998). *H. pylori* infection in patients with the presence of these alleles may result in development of gastric.IL-1b, driven to major and constant inflammation, gastric atrophy, and hypochlorhydia, and finally to the occurrence of gastritis (Wroblewski et al., 2010).

In this study, the investigation deals with whether there are combinations of bacterial and host genotypes that are highly correlated to the occurrence of gastritis. This study is about the correlation of *H. pylori* vacA and cagA virulence-associated genes and human IL-1B and IL-1RN susceptibility polymorphisms with the demographic features of gastritis.

2. Materials and methods

2.1. PCR amplification of *Interleukin -1 -Beta* gene

The Interleukin -1 -Beta gene different VNTR region and SNPs were amplified by polymerase chain reaction (PCR) using three different primers pair (Table 5.1). PCR was performed in 25 µl total reaction volumes each containing 100 ng of template DNA, 0.2 pM of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2mMdNTPs, and 1 unit of Taq DNA polymerase (Fermentas, Germany). The reaction mixture for IL-1β-511 primer was heated to 95^0 C for 5 min, followed by 35 cycles each consisting of 40-sec denaturation at 95^0 C, 30-sec annealing at 61^0 C temperature, 1min of extension at 72^0 C, and a final 5 min extension at 72^0 C (Figure: 5.1). Similarly, the reaction mixture for IL-1β-31 primer was heated to 95^0 C for 5 min, followed by 35 cycles each consisting of 40-sec denaturation at 95^0 C, 40-sec annealing at 53^0 C temperature, 1min of extension at 72^0 C, and a final 7 min extension at 72^0 C (Figure: 5.2). The reaction mixture for IL-1RN primer was heated to 95^0 C for 5 min, followed by 35 cycles each consisting of 40-sec denaturation at 95^0 C, 40-sec annealing at 61^0 C temperature, 1min of extension at 72^0 C, and a final 5 min extension at 72^0 C (Figure: 5.3).The PCR amplification products (3 µl) were subjected to electrophoresis in a 2 % agarose gel in 1X TAE buffer at 80 Vh for 30 min, stained with Ethidium Bromide, and images were obtained in GBOX gel documentation systems (UK) (Figures: 5.1 - 5.3).

Primer name	Primer Sequences	Product length (bp)	Genotypes	References
IL-1β-511 F IL-1β-511 R	5'-GCCTGAACCCTGCATACCGT- 3' 5'-GCCAATAGCCCTCCCTGTCT- 3'	155	Sanger sequencing	(Morshedy et al., 2017)
IL-1β-31 F IL-1β-31 R	5'-AGAAGCTTCCACCAATACTC- 3' 5' -AGCACCTAGTTGTAAGGAA- 3'	239	Presence of T or C or T/C	
IL-1RNF IL-1RNR	5' -CCCCTCAGCAACACTCC- 3' 5' -GGTCAGAAGGGCAGAGA- 3'	Variable tendan repeat	allele 1= 4 repeats (wild type), allele 2= 2 repeats (270 bp), allele 3= 5 repeats, allele 4= 3 repeats, allele 5= 6 repeats	(Rudijono et al. 2018)

Table 5.1. List of primers to identify VNTR and SNPsin IL-1β**in** *H. pylori* **positive virulent patient samples**
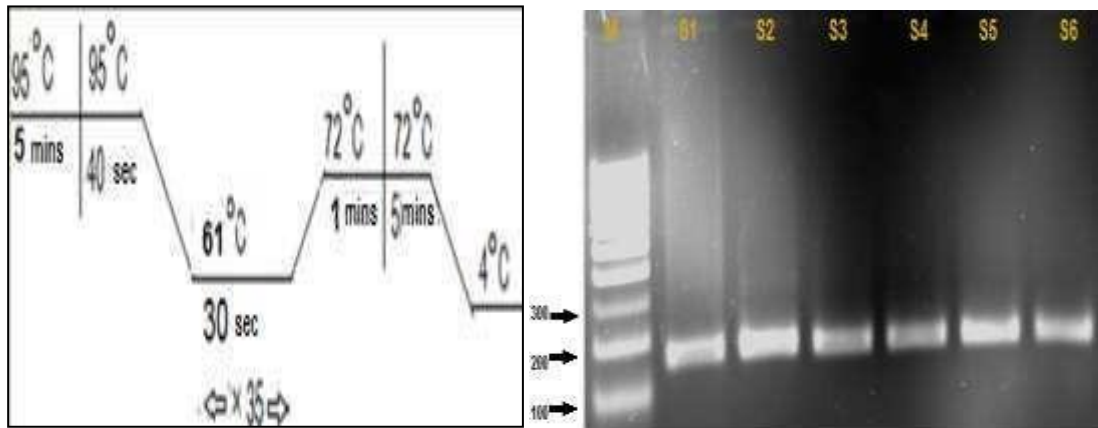
Figure: 5.1. Partial amplification of IL β 511 gene of 155bp size

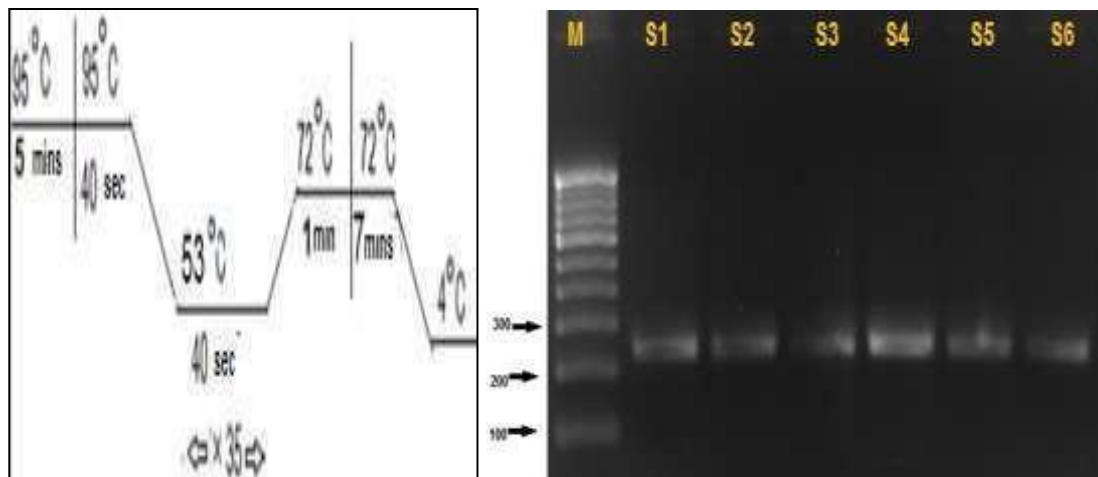**M: 100 bp Marker; S1 to S6: Representative Samples**

Figure: 5.2. Partial amplification of IL 1β -31 gene of 239bp size
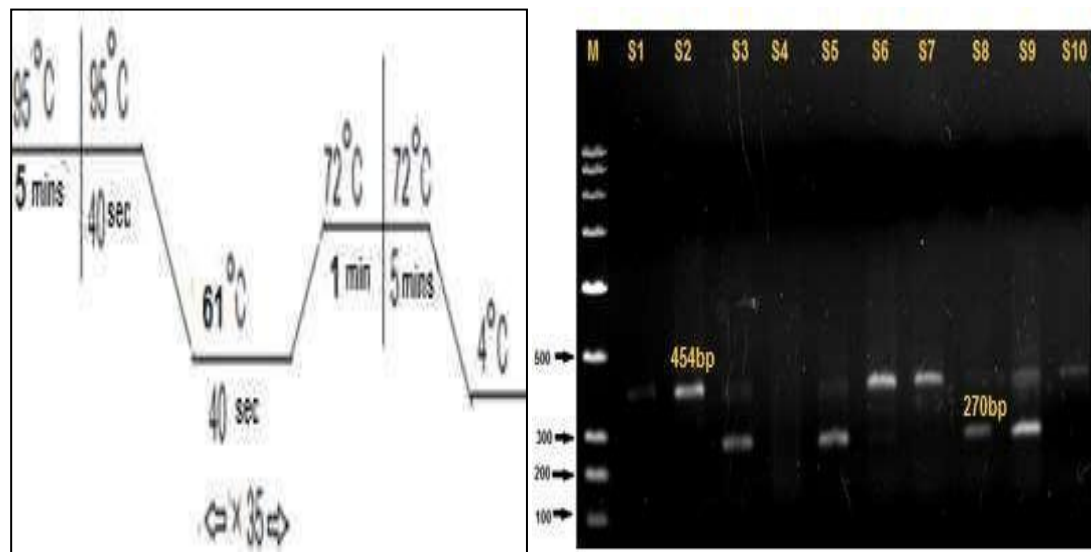
M: 100 bp Marker; S1 to S6: Representative Samples

Figure: 5.3. Partial amplification of IL 1-RN gene

M: 1 kbp Marker; S1 to S10: Representative Samples

2.2. Statistical Analysis

Differences in the combined score and gastric bacterial different genotypes were assessed with the Mann–Whitney test. Correlations between genotypes and the existence of host genotypes were assessed. Only the matter containing single *H. pylori* genotypes were inserted in the analyses of the histopathologic features of gastritis. Associations between bacterial genotypes and host genotypes and association of genotype frequencies between gastritis were measured by the logistic regression.

Comparisons of the genotype prevalence among group of people determineby age, sex, food, and lifestyle habits were done by the logistic regression. Odds ratios (ORs) with 95% confidence intervals (CIs) and unconditional logistic regression models were calculated with SPSS software (version 20; SPSS Science, Chicago, IL). Area under the curve (AUC) values was calculated through the Receiver operating curve (ROC) analysis using MedCalc software (version 14.8; MedCalc, Belgium). Differences were measured statistically significant when P<.05. All statistical tests were two-sided.

3. RESULTS

***Association between H. pylori* and host IL-1B/IL-1RN Genotypes**

Among all the *H. pylori* participants' group, a total number of 42.63% of patients carried genotype C and 57.42% of patients carried genotype T for IL-511. Whereas 38.35%, 19.16%, and 42.63% patients' group carried allele 1/3/4/5, allele 2, allele 2/4 for IL-RN tandem repeats, respectively (Table 5.2).

Interleukin-RN tandem repeats allele 2 was statistically significantly connected with cagA positive genotypes of *H. pylori* (Table 5.3.; Figures: 5.4.A and 5.5.A). The probability values were much higher for the cagA positive samples than negative samples. Interestingly, IL-RN tandem repeats allele 2 was remarkably correlated with the existence of cagA lineage 1 and 2 for this population (Table 5.3., Figures: 5.4.B and 5.4.C). The model reached a high AUC score for the association between cagA lineage 1 (AUC = 0.71) and 2 (AUC = 0.69) with, IL-RN tandem repeats allele 2 (Figures: 5.5.B and 5.5C).

In the cagA positive gastritis patients, IL-1B-511*T carriers represented an OR of 1.07 (95% CI 0.31 – 3.64) of the case subjects, although it was not statistically remarkable (Table 5.3). Whereas there is no significant association between vacA genotypes and host IL-1B/IL-1RN Genotypes.

Categories	No. of cases(%)
Genotype	
vacA (+ve)/*cagA*(-ve)	24(51.1)
vacA (-ve)/*cagA1*(+ve)	13(27.7)
vacA (-ve)/*cagA2*(+ve)	3 (6.43)
vacA (+ve)/*cagA1*(+ve)	5(10.6)
vacA (+ve)/*cagA2*(+ve)	2(4.32)
***IL-RN* Alleles**	
Allele 1/3/4/5	18(38.35)
Allele 2	9(19.16)
Allele 2 and Allele 4 (Heterozygous)	20(42.63)
IL-511	
Genotype C	20(42.63)
Genotype T	27(57.42)

Table 5.2. Demographic factors and the host *interleukin β*alleles in gastritis patients.

	Odd ratio	95% CI	P value
IL allele		**cagA**	
1	3.6	0.39 - 33.87	0.25
2	**0.17**	**0.03 - 0.89**	**0.04**
4	0.72	0.23 - 2.59	0.61
511	1.07	0.31 - 3.64	0.92
IL allele		**CagA-lineage1**	
1	0.13	0.01 - 1.24	0.07
2	**7.88**	**1.84 - 33.76**	**0.005**
4	1.63	0.51 - 5.43	0.42
			0.92
511	0.93	0.29 - 2.95	

IL allele	cagA-lineage2		
1	-	-	-
2	**7.47**	**1.45 - 38.51**	**0.01**
4	1.22	0.35 - 4.19	0.75
511	0.88	0.27 - 2.92	0.83
IL allele	**vacA**		
1	1.5	0.14 - 15.81	0.73
2	2.22	0.23 - 21.73	0.49
4	2.46	0.25 - 24.02	0.44
511	1.125	0.17 - 7.457	0.91

Table 5.3. Association between IL-β alleles with cagA, cagA-lineage 1 and 2 and vacA genotypes in gastritis patients using logistic regression analysis.
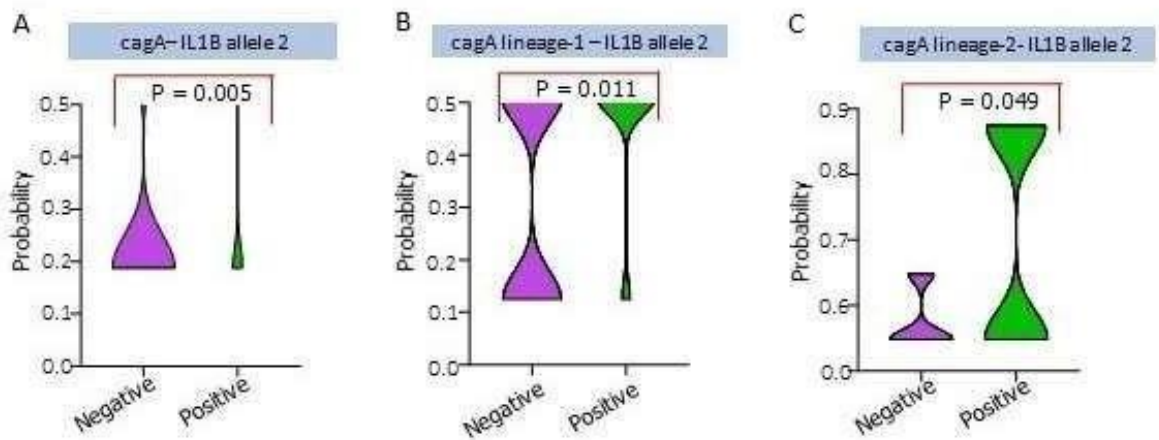

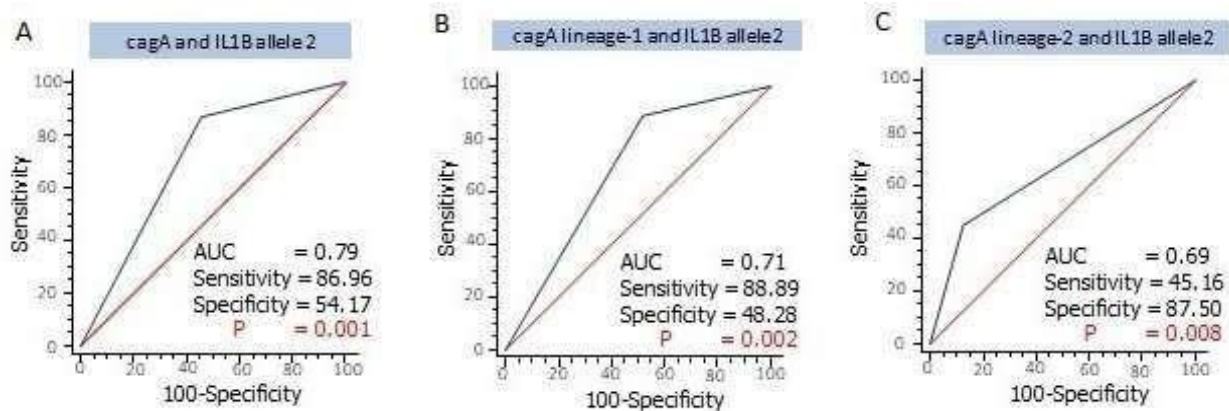**Figure: 5.4. Association plot for *H. pylori* and host genotypes**

Figure: 5.5. Receiver operating curve (ROC) for *H. pylori* and host genotypes

4. DISCUSSION

The various results of *H. pylori* infection may depend not only on other bacterial factors but also on the unique genetic factors of the host. Regarding host genetic factors, a genome-wide screen analysis to recognize the genetic factor(s) defining sensitivity to *H. pylori* occurrence suggested the existence of a possible linkage with chromosome4 (Miftahussurur et al., 2015). Considering the allocation of the human IL-1 gene on chromosome 4 (4q13-q21), the outcome of different study may support the hypothesis that the IL-1 gene polymorphism is possibly connected with *H. pylori*-induced gastrointestinal diseases. Impressively, the inheritance of the IL-1 -511T allele was correlated with progression of gastric atrophy in patients with *H. pylori* infection and raised the chances of gastric carcinoma in Japanese and Chinese people (Long et al., 2015), which gave a strong support to this study. Results of this study also recommend that gastritis is significantly correlated with the functional polymorphism in the promoter region of the IL-1 gene. The study found that there was higher risk of gastric carcinoma in high IL-1 producers, in agreement with the concept that IL-1 may increase the risk of development of gastric carcinoma by altering the quality and vigor of inflammatory responses developed by the host after exposure to *H. pylori*. Other than IL-1 stimulated neutrophils to synthesize active radicals such as nitric oxide (Soufli et al., 2016). These radicals by their mutagenic potential could

cause mutations in gastric epithelial cells (Smith et al., 2006). Other than IL-1 by inducing angiogenesis would be other significant factors in gastric carcinogenesis. Thus, the inheritance of the high producer allele of IL-1 (carriers of -511T and IL-RN tandem repeats allele) may produce chronic gastritis, which may then be followed by the occurrence of gastric carcinoma through the existence of the cagA genotype.

The IL-1B-511*T carrier genotype was correlated with the hypocaloric condition in the stomach, whereas the IL-1RN*2/*2 genotypes were connected with this parameter in both areas of the abdomen. Patients who carried IL-1B-511*T and homozygous for IL-1RN*2 had more chances of corpus gastritis and a higher corpus *H. pylori* frequency than IL-1B-511*C homozygotes/IL- 1RN*L carriers (Table 5.2). IL-1B-511*T carriers/ IL-1RN*2 homozygotes had a much higher occurrence of epithelial destruction. These patients also had a higher threat of glandular atrophyand intestinal metaplasia. There was some evidence that the risks correlated to the IL-1B-511*T and IL-1RN*2 alleles act independently (Table 5.4), with the risk in combined *T carrier/*2 homozygotes being greater than the risk correlated with each allele individually (OR - 3.3, 95% CI - 1.3 to 8.2). However, this outcome could be treated with some precaution due to the individual ORs were not statistically remarkably different.

Furthermore, this study provides more evidences that attachment of genetic polymorphism of the IL-8 gene in the host, genetically determined differences in IL-8 production via promoter polymorphisms could contribute to patients susceptibility to gastric cancer development after *H. pylori* infection in Indian population.

Chapter VI

Summary and conclusion

Mizoram, the state of Northeast India has reported a high incidence of gastric cancer among other states which instigate to conduct the study to find out the prevalence of *H. pylori* infection in this region.

The unique traditional habits were considered to evaluate risk factors associated with disease occurrence.

The Mizo population has a homemade preparation of pickle as a food habit, study indicated pickle consumption as a confounding factor for the presence of virulent strain of *H. pylori*.

Crowding was found as a risk factor associated with *H. pylori* infection as reported in an earlier study with a different population.

Smoking habit was found to be a risk factor for *H. pylori* infection as smoking can weaken immunity.

The use of tuibur showed the direct connection to the *H. pylori* infection by altering the stomach pH and creating a beneficial situation for *H. pylori* to sustain inside the patients.

In this study, it was also found that *H. pylori* infection was insignificant with drinking alcohol but the occurrence of cagA virulent gene among *H. pylori*-infected patients is high among alcohol consumers.

The commonly use triple therapy medication for *H. pylori* eradication included Clarithromycin antibiotic. In this study Clarithromycin resistant status of *H. pylori*-positive cases were screened and risk factors predominant with clarithromycin resistance were measured.

Ceramic filtrate drinking water is one of the reasons for the occurrence of clarithromycin resistance among *H. pylori*-infected patients.

The study showed that the Mizo population has a unique habit of having fermented pig fat which is also called "sa-um" in the local language, intake of "sa-um" makes the high rise graph of the incidence rate of clarithromycin resistance.

The role of host genotype concerning *H. pylori* infection was considered and Interleukin-RN tandem repeats allele 2 was found statistically remarkably correlation to cagA Asian and European lineage for this population.

Additionally, the study also informed that there is no valuable correlation between vacA genotypes and host IL-1B/IL-1RN Genotypes. Furthermore, this study has given more evidence of the IL-8 gene in the host, genetically determined differences in IL-8 production via promoter polymorphisms could contribute to patients' sensitivity to the occurrence of gastric carcinoma after *H. pylori* infection in the Mizo population.

Finally, this study gave an idea about initial measurements of development of *H. pylori* infection among adenocarcinoma prone Mizo population and found the threats as crowding, smoking habit, tuibur addition and alcohol consumption among *H. pylori* cagA positive patients and confounding factor as pickle intake correlated to high incidence, and can help to take a prior measurement. This study also provided clarithromycin resistant status which is one of the commonly use antibiotic for *H. pylori* suppression, so that the various medicinal therapy can be plan. In this study, clarithromycin-resistant was high among men and the potable water source was a risk factor for clarithromycin resistance. Further consideration of men for clarithromycin resistance showed consumption of fermented pig fat as a risk factor for clarithromycin resistance. Host genotypic status enhances the *H. pylori* infection, the study showed an association between Interleukin-RN tandem repeats allele 2 and occurrence of cagA genotype, which can also help physicians to improve the medication for these individuals.

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Abbreviation

H. pylori-*Helicobacter pylori*

MALT – Mucosa Associated Lymphatic Tissue

IARC - International Agency for Research on Cancer

WHO - World Health Organization

cag PAI – cag Pathogenicity Island

cagA – Cytotoxin associated gene A

VacA - Vacuolatingcytotoxin gene A

ureC – Urease Subunit Alpha

GyrA – Gyrase gene A

IL-1 - Interleukin-1

IL-1B - Interleukin-1-Beta

IL-1RN - Interleukin-1Receptor Antagonist gene

IL-1β 31- Interleukin-1-Beta 31 region

IL-1β 511- Interleukin-1-Beta 511 region

SNP – Single Nucleotide Polymorphisim

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

rRNA – Ribosomal Ribonucleic Acid

GC – Gastric Cancer

GI–Gastro Intestinal

PPI - Proton Pump Inhibitor

Kb – Kilobase

Bp- Base Pair

TSNAs - Tobacco-Specific Nitrosamines

NNN - N-nitrosonornicotine

NNK – Nicotine –derived nitrosamine ketone

RUT - Rapid Urease Test

df – Degree of Freedom

OR – Odd Ratios

CI – Confidence Intervals

SD – Standard Deviation

SPSS – Statistical Package for the Social Sciences

PWD - Public Welfare Department

UV – Ultraviolet

RO – Reverse Osmosis

PCR- Polymerase Chain Reaction

ABI - Applied Biosystems Instruments.

AUC – Area Under the Curve

dNTP-Deoxynucleotide triphosphate

TBE – Tris-borate-EDTA

EDTA- Ethylene Diamine Tetra Acetic acid

VNTR– Variable Numbers of Tandem Repeats

ROC - Receiver operating curve

MSG – Monosodium Glutamate

<div align="center">**BRIEF BIODATA**</div>

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Career objective: Obtain the career in the field of gastritis and gastric carcinoma and become an integral part of the medical biotechnology.

Area of interest: Post genetic susceptibility to acquire pathogen related diseases and identification of pathogenicity directed degree of infection.

Academic Qualification:

Education	Institute	University/ Boad	Year of Passing	Division
M.Sc. Biotechnology	MITS School of Biotechnology	Utkal University	2012	First(1st)
B.Sc. Biotechnology	Majhighariani Institute of Technology and Science	Berhampur University	2010	First(1st)
Higher secondary(+2)	MCTI School, Hirapur	W.B.C.H.S.E.	2003	Second(2nd)
Secondary (1st)	SPBN School, Burnpur	W.B.B.S.E.	2001	First(1st)

Workshop Attended:

Institute	Name of Workshop	Sponsored	Duration
Mizoram University	National workshop on **"Structure Determination of Macromolecules"**	DBT-BIF Centre, India	March 26-28, 2013.
Mizoram University	International seminar on **"Structural Elucidation of Microbial Natural Products: Opportunities and Challenges"**	DST, Govt. of India, The Royal Society, London	January 14-16, 2014
Mizoram University	National workshop on **"Techniques in Molecular Biology and Bioinformatics"**	DBT, India	January 26-31, 2014
Mizoram University	National workshop on **"Advances in Cancer Genomics"**	DBT, India, IIT Guwahati	May 30-31, 2014
Mizoram University	National workshop on **"Biostatistics using Sigma plot"**	DBT-BIF Centre, India	June 27, 2014
Mizoram University	National workshop on **"Capacity Building in Effective Management of Intellectual Property Rights"**	BCIL, Govt. of India	Aug 27-28, 2014.
Assam University	National workshop on **"Human Genetics: Techniques and Statistical Analyses"**	ISI Kolkata	Nov 10-14, 2014
Mizoram University	National workshop on **"Cancer Mutation Detection and analysis"**	DBT, India	April 17-18, 2015
Mizoram University	National workshop on **"Browsing Genome with ENSEMBL"**	DBT, India	May 30, 2015
Mizoram University	National workshop on **"Gene ontology and Cytoscape"**	DBT, India	July 13-14, 2015
Mizoram University	National workshop on **"North-East Autumn School on Human Genetics: Techniques and Statistical Analyses"**	ISI Kolkata	September 08-11, 2015
Mizoram University	National workshop on **"Exploring the Cancer Genomics"**	DBT, India	February 22-27, 2016
Mizoram University	National workshop on **"Understanding Basic**	DBT, India	September 07-11, 2016

	Principles in Human Molecular Genetics"		
Mizoram University	International workshop on **"Cancer Epidemiology"**	DBT, India	November 29-30, 2016
Mizoram University	National Workshop on **"A Brief Introduction to Bioinformatics and System Biology"**	DBT-BIF Centre, India	December 13 -14, 2018
Mizoram University	4th Advanced Research Training Workshop on **"Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches"**	NIBMG, Kolkata	October 15- 23, 2019

National and Inter-National Conference Attended:

Institute	Title of Presentation	Sponsored	Duration
Mizoram University	Tobacco use and crowding are the major risk factors for *Helicobacter pylori* infection in gastritis patients.	ABAP and BEHIET 2018, India	November 12-14, 2018

Publications:

1. **Mukherjee S**, Madathil S, Ghatak S, Lalrintluanga J, Pautu J L, Zohmingthanga J, Pachuau L, Nicolau B, Senthil Kumar N. (2020); **Association of tobacco smoke-infused water (Tuibur) of Mizo people and risk of *Helicobacter pylori* infection.** Environmental Science and Pollution Research 27, 8580–8585 (Impact factor: 4.223)

2. Chakraborty P, Ghatak S, Yadav R, **Mukherjee S,** Chhakchhuak L, Chenkual S, Zomuana T, Lalruatfela S, Maitra A, Senthil Kumar N. (2020);Novel Somatic Mutations of the CDH1 Gene Associated with Gastric Cancer: Prediction of PathogenicityUsingComprehensiveInsilicoMethods.CurrentPharmacoge nomicsand Personalized Medicine 17(3): 1. DOI: 10.2174/1875692117999201109210911.

3. Sailo C V, Pandey P, **Mukherjee S**, Zami Z, Lalremruata R, Nemi L and Senthil Kumar N. (2019); Pathogenic microbes contaminating

mobile phones in hospital environment in Northeast India: incidence and antibiotic resistance. Tropical Medicine and Health 47:59

4. Ghatak S, Lallawmzuali D, **Mukherjee S**, Mawia L, Pautu JL, Senthil Kumar N. (2016); Polymorphism in mtDNA control region of Mizo-Mongloid Breast Cancer samples as revealed by PCR-RFLP analysis. Mitochondrial DNA Part A 27 (3), 2205-2208.

5. **Mukherjee S**, Ghatak S, Yadav RP, Zothansanga, Guru subramanian G. Senthil Kumar N.(2015); Advance in PCR based molecular marker and its application in biodiversity conservation. Biodiversity in Tropical Ecosystems 395-422.

6. **Mukherjee S**, Senthil Kumar N. (2014); Terminator gene technology– their mechanism and consequences. Science Vision 14, 51-58.

7. Zodinpuii D, Ghatak S, **Mukherjee S** and Senthil Kumar N. (2013); Genetic relatedness of genus Oryza from Eastern Himalayan region as revealed by chloroplast matK gene. Asian Journal of Conservation Biology2,2: 144-151.

Papers communicated:

Mukherjee S, Ghatak S, Lalrinpuia B, Jahau L, Pautu J L and Senthil Kumar N. (2021); *Helicobacter pylori* virulent genes and host Interleukin 1 are associated with demographic factors for an increased risk of Gastritis. Libyan journal of medicine (Impact factor: 1.29).

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PARTICULARS OF THE CANDIDATE

NAME OF CANDIDATE: SUBHAJIT MUKHERJEE

DEGREE: Ph. D.

DEPARTMENT: BIOTECHNOLOGY

TITLE OF THESIS: Prevalence of *Helicobacter pylori* genotypes and their association with Interleukin-1- Beta in patients with gastritis from Aizawl, Mizoram

DATE OF ADMISSION: 04-08-2014

APPROVAL OF RESEARCH PROPOSAL:

1. DRC: NA

2. BOS: 18.05.2015

3. SCHOOL BOARD: 22.05.2015

MZU REGISTRATION NO.: 6743 of 2014

Ph.D. REGISTRATION NO & DATE: MZU/Ph.D./751 of 22.05.2015

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Association of tobaccosmoke–infused water (tuibur) use by Mizo people and risk of Helicobacter pylori *infection*

Subhajit Mukherjee, Sreenath Arekurnnath Madathil, Souvik Ghatak, Lalrintluanga Jahau, Jeremy L. Pautu, John Zohmingthanga, et al.

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RESEARCH ARTICLE

Association of tobacco smoke–infused water (tuibur) use by Mizo people and risk of *Helicobacter pylori* infection

Subhajit Mukherjee [1] & Sreenath Arekurnnath Madathil [2] & Souvik Ghatak[1] & Lalrintluanga Jahau[3] & Jeremy L. Pautu[4] & John Zohmingthanga [5] & Lalawmpuii Pachuau [5] & Belinda Nicolau [2] & Nachimuthu Senthil Kumar[1]

Abstract

The study aims to understand the influence of environmental and lifestyle factors and more specifically the role of tobacco smoke–infused water (tuibur) on *Helicobacter pylori* infection. It was a cross-sectional study to measure the epidemiological risk factors associated with *H. pylori* infection among the tribal population in Northeast India. Endoscopic samples were collected from the antrum region of the stomach from 863 participants with gastritis. *H. pylori* infection was confirmed in 475 samples by the rapid urease test and PCR-based methods. Information on demographic and lifestyle factors was collected using a validated and standardized questionnaire. Logistic regression was used to estimate odds ratio (OR) and 95% confidence interval (CI) for the association between the various factors and *H. pylori*. The use of tuibur was associated with an increased OR of *H. pylori* infection (OR = 3.32, 95% Cl = 1.95–5.83). Tobacco chewers (OR = 1.49, 95% Cl = 1.06–2.09), smokers (OR = 1.81, 95% Cl = 1.26–2.61), and alcohol consumers (OR = 1.81, 95% Cl = 1.19–2.76) were also infected with *H. pylori*. The results were not attenuated after adjusting for major well-known risk factors of *H. pylori* infection. The habit of tuibur consumption may be a contributing factor to the high prevalence of *H. pylori* infection and in turn, may contribute to the high prevalence of gastritis among the Mizo population.

Keywords *Helicobacter pylori* · Tuibur · Tobacco chewer · Smoker · Alcohol consumer · Mizoram

Introduction

In 2005, Warren and Marshal received the Nobel prize for physiology or medicine for their discovery about *Helicobacter pylori* (*H. pylori*) being one of the common human bacterial pathogen that colonizes in the stomach causing gastroduodenal disorders (Niyaz 2005). This discovery

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changed the peptic ulcer disease paradigm from a chronic disease of unknown causes to an infectious disease that can be cured with the use of antibiotics and acid secretion inhibitors (Basset et al. 2002). Currently, evidence supports that *H. pylori* is the causative agent of several gastroduodenal disorders including active chronic gastritis, peptic ulcer, and acute erosive gastritis and is a major risk factor for gastric carcinoma and MALT lymphoma (Amieva and EL–Omar 2008; Kuipers 1999; Malfertheiner et al. 2002).

H. pylori infection is a very common and chronic infection around the globe and varies among and within populations, but it is almost universal in developing countries (Dorji et al. 2013; Jang et al. 2010). The risk of infection is multifactorial and is related to the standards of living, hygiene, and sanitation (Ahmed et al. 2007), including the contamination of drinking water and ingestion of fecal-contaminated vegetables as well as lifestyle habits (Kusters et al. 2006; Zhu et al. 2014).

Mizoram, a small state located in Northeastern India, is one of the hotspot regions for *H. pylori* infection and also the state that has the highest incidence of gastric cancer in India (Ghatak et al. 2016). Although the reasons for the high levels

of *H. pylori* infection in the region are not fully understood, the standard of living in the state is low with more than 20% of the population living under the poverty line (Phukan et al. 2006). In addition, Mizo people have a unique diet and lifestyle factor; they are predominantly non-vegetarians with high intake of animal fat in the form of pork meat and fermented pork fat (saum) that is used to prepare vegetable stew (bai) (Ghatak et al. 2016; Phukan et al. 2006). Also, this population has a peculiar habit of consumption of tobacco smoke–infused aqueous solution called "tuibur." About 5 ml of tuibur is kept in the mouth for few minutes and then is usually spit out; occasionally, some individuals may also swallow the product. We have previously reported the production and distribution of tuibur (Madathil et al. 2018). The unregulated makeshift industry of tuibur production uses unfiltered water and is usually located in unhygienic conditions. Presence of carcinogenic TSNAs (tobacco-specific nitrosamines), NNN (N'-nitrosonornicotine), and NNK [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone] in tuibur can be a potential risk factor for gastric carcinoma (Muthukumaran 2016). Furthermore, tuibur even if not swallowed may modify the saliva pH making it more alkaline, thus increasing the risk of *H. pylori* infection in the mouth which may serve as a reservoir of the bacteria (Lalmuanpuii and Muthukumaran 2016).

H. pylori and tuibur have been associated with risk of gastric cancer (Ghatak et al. 2016). Moreover, although there is evidence for some risk factors for *H. pylori* infection, the exact risk factors for the bacterial infection remains largely unknown. In addition, no studies have investigated the environmental and lifestyle factors associated with *H. pylori* in a tribal population living in a hilly mountainous region with unique lifestyle habits. Hence, the present study hypothesizes that tuibur use might be a major risk factor associated with *H. pylori* infection.

Methods

Study population

A total of 863 patients with gastritis who were scheduled for an endoscopy in Trinity Diagnostic Centre, Aizawl District, Mizoram, Northeast India were recruited during February 2014 to August 2016. All the participants included were Mizo and presented with dyspeptic symptoms or gastrological problems requiring upper GI endoscopy. Patients who had taken antibiotics for gastrological problems for 2 weeks, or with gastrointestinal bleeding, are pregnant, or with a history of gastrectomy were excluded from the study because of the high chances of false-negative result in urease test for *H. pylori*. We used a convenience sample to increase the probability to capture potential individuals with *H. pylori* infection. The study was

approved by the ethical committee of Civil Hospital, Aizawl (No. B.12018/1/13-CH(A)/IEC/36) as well as the Mizoram University ethical committee.

Data collection

An in-person interview using a validated and standardized questionnaire in both languages, Mizo and English, was used to collect information on array of exposures including socio-demographic characteristics (e.g., age, levels of education, occupation, residential location, marital status, number of children), medical history, health status, family history of *H. pylori* infection, and lifestyle factors (e.g., tuibur consumption, tobacco and alcohol use). Smoking tobacco and alcohol consumption were collected as self-reported measures (regular, occasional, former, or never consumers). In addition to the frequency of use, combinations of different smokeless tobacco products (e.g., tuibur, betel nut, pan, zarda) and dietary habits (e.g., strictly vegetarian diet, raw or uncooked vegetable consumption, fermented pig fat (saum), salt and pickle consumption) were also collected. Finally, we also recorded information on sanitation and drinking water sources (e.g., unfiltered, filtered [using ceramic, active carbon/charcoal, UV sterilization, and reverse osmosis] and boiled).

Endoscopy and biopsy sampling

Endoscopic samples collected from the antrum portion of the participant's stomach were used to detect *H. pylori* infection based on the rapid urease test kit. Endoscopy was carried out using Fujinon VP-4450 HD Series on patients after an overnight fast. Two biopsy specimens per patient (replicates) from the mucosa of the gastric antrum were obtained by endoscopy and were placed in a small screw-capped bottle containing 0.2-ml sterile normal saline to maintain humidity. From the biopsy specimens collected, one was used for the rapid urease test and the other for PCR amplification of urease C gene.

Rapid urease test for *H. pylori* status

The endoscopic sample was screened by a commercially available rapid urease kit (RUT DRY Test kit, Gastro Cure System, WB, India) (Brown et al. 2002). The endoscopic samples were directly placed into the well of the kit. The urease enzyme produced by *H. pylori* rapidly hydrolyzes urea in the well, producing ammonia. The rise in the pH of the medium by ammonium ions was detected with a pH-based color indicator. In the case of *H. pylori* infection in the samples, there will be an immediate change in color from yellow to pink (*H. pylori*-positive). Samples that remained yellow, that is, without color change were inferred as an absence for *H. pylori* infection (*H. pylori*-negative). This test was read

after 2 h for positive and negative results. Cross validation was performed by PCR amplification of urease C gene using forward primer 5′-AAGCTTTTAGGGGGTGTTAGGGGTTT-3′ and reverse primer 5′-AAGCTTACTTTCTAACACTAACGC-3′ with thermal condition of 95 °C for 7 min followed by 35 cycle of 95 °C for 1 min, 54 °C for 40 s, 72 °C for 1 min, and final extension of 72 °C for 5 min. PCR amplicons were visualized on a 1.5% agarose gel (Lu et al. 1999).

Statistical analysis

The characteristics of the population were explored using descriptive statistics. The association between tuibur and *H. pylori* infection was investigated using unconditional logistic regression. We performed a hierarchical modeling strategy to evaluate the change in the strength of this association when adjusted for potential confounder. Four sets of confounders were considered. (i) Model 1: tobacco and alcohol factors (smoked tobacco, smokeless tobacco, and alcohol consumption); (ii) model 2: demographic factors (age, sex, marital status); (iii) model 3: socioeconomic position (no. of persons in household, drinking water sources, and sanitation); and (iv) model 4: dietary factors (raw food, salt, saum, pickles). Odds ratios and 95% confidence intervals were estimated. All statistical analyses were performed using R statistical package ver 3.3.0 (The R Foundation for Statistical Computing).

Results

A total of 943 patients was invited to participate in the study, and 863 accepted the invitation to participate in the study (response rate of 91.5%). However, the percentage of men refusing to participate in the study was higher (75% of the total refusal). More than half of the samples (475 (55%)) were tested positive for *H. pylori* infection by both rapid urease test (RUT) as well as urease C gene PCR-based methods. The age of the participants ranged from 8 to 92 years, with comparable distribution between *H. pylori*-positive and -negative groups. The *H. pylori*-positive group had a slightly higher proportion of male (58.5%), married people (83.4%), those with below matriculation level of education (52%) compared with the *H. pylori*-negative group (55.4%, 79.9%, 47.2%, respectively). Most of the participants had access to a public water distribution system (81.1% and 83.8% *H. pylori*-positive and -negative, respectively). However, a lower proportion of participants in the *H. pylori*-positive group drank filtered water. Dietary factors showed a similar distribution between *H. pylori*-positive and -negative groups (Table 1).

The majority of the participants in the *H. pylori*-positive group were tobacco chewers (74.1%) and smoked forms of tobacco (57.3%) compared with 63.9% and 35.6% among the *H. pylori*-negative group, respectively. There was approximately three times higher proportion of tuibur users in the *H. pylori*-positive (15.2%) compared with the *H. pylori*-negative group (5.9%).

Results from the logistic regression analysis showed that tuibur users increased the odds of *H. pylori*-positive endoscopy by 3.32 (OR = 3.32; 95% CI = 1.95–5.83) times compared with never users. This association was not attenuated after adjusting for several known risk factors of *H. pylori* infection. In addition, chewed tobacco users had 1.49 (OR = 1.49; 95% CI = 1.06–2.09) higher odds of having *H. pylori*-positive endoscopy result (Table 2).

Discussion

Our results suggest an association between the use of tuibur and *H. pylori* infection among Mizo individuals with gastritis. Although one previous study has reported an association between tuibur consumption and the risk of gastric cancer (Ghatak et al. 2016), to our knowledge, this is the first study investigating the association between tuibur use and *H. pylori* infection. Moreover, this association was not attenuated when we included important risk factors (e.g., indicators of socioeconomic position) in the models (Table 2).

Although we have not tested the tuibur samples for the presence of *H. pylori* bacterium, our previous work reported the unhygienic conditions in which tuibur is produced (Madathil et al. 2018). A higher incidence of gastric cancer was reported for the consumers of smoked tobacco as well as tuibur in Northeast India (Misra et al. 2014), and in the present study, we found a strong association between tuibur and *H. pylori* presence. Furthermore, the average alkaline pH of tuibur is above 9 and may have an impact on the oral cavity and the stomach which may facilitate the viability of *H. pylori* bacterium (Lalruatfela et al. 2017). The prolonged exposure to smokeless tobacco extract affects the drug-metabolizing enzymes of the gastrointestinal tract, which may be an important factor that determines the susceptibility to different organisms leading to carcinogenesis (IARC Working Group 2007). Tuibur also contains carcinogenic agents like TSNAs, NNN, NNK, and others which is reported as health hazards as well as causes cancer and other diseases. These molecules may have a correlation with *H. pylori* proliferation by changing pH in the oral cavity as well as in the stomach and together influencing the disease outcome (Muthukumaran 2016; Lalmuanpuii and Muthukumaran 2016).

The association between tobacco and alcohol use and *H. pylori* infection is inconclusive (Brown et al. 2002; Misra

Table 1 Distribution of sociodemographic and lifestyle factors among *H. pylori*-positive and -negative participants

	H. pylori-negative n = 388 n (%)	*H. pylori*-positive n = 475 n (%)
Age (mean)	44.36	45.22
Sex		
Female	173 (44.6)	197 (41.5)
Male	215 (55.4)	278 (58.5)
Marital status		
Unmarried	78 (20.1)	79 (16.6)
Married	310 (79.9)	396 (83.4)
Location		
North Aizawl	111 (28.6)	139 (29.3)
East Aizawl	55 (14.2)	54 (11.4)
South Aizawl	56 (14.4)	77 (16.2)
West Aizawl	78 (20.1)	94 (19.8)
Other	88 (22.7)	111 (23.4)
Occupation		
House wife/no Job	103 (26.5)	120 (25.3)
Student	120 (30.9)	131 (27.3)
Government employee	95 (24.5)	115 (24.2)
Self-employed	39 (10.1)	35 (7.4)
Farmer	31 (8.0)	74 (15.6)
Education qualification		
Above matriculation	93 (24.0)	98 (20.6)
Matriculation	112 (28.9)	130 (27.4)
Below matriculation	183 (47.2)	247 (52.0)
No. of persons living in same household(mean SD)	5.82 (2.19)	6.58 (2.36)
Source of drinking water		
Public water distribution system	325 (83.8)	385 (81.1)
Stream/lake /river	58 (14.9)	81 (17.1)
Ponds/ditches/wells	5 (1.3)	9 (1.9)
Water purification method used		
Filtered (ceramic/charcoal/UV/ reverse osmosis)	284 (73.2)	324 (68.2)
Boiled	88 (22.7)	140 (29.5)
Unpurified	16 (4.1)	11 (2.3)
Sanitation		
Commode	160 (41.2)	229 (44.4)
Open field	228 (58.8)	264 (55.6)
Saum (fermented pig fat) consumption		
Never	63 (16.2)	62 (13.1)
Mild consumption	106 (27.3)	117 (24.6)
Moderate consumption	191 (49.2)	264 (55.6)
Heavy consumption	28 (7.2)	32 (6.7)
Raw or uncooked fruits and vegetables consumption		
Never	26 (6.7)	25 (5.3)
Occasionally	150 (38.7)	186 (39.2)

Table 1 (continued)

	H. pylori-negative n = 388 n (%)	*H. pylori*-positive n =475 n (%)
Regularly	212 (54.6)	264 (55.6)
Extra salt consumption		
Never/mild consumption	155 (39.9)	207 (43.6)
Moderate consumption	184 (47.4)	218 (45.9)
Heavy consumption	49 (12.6)	50 (10.5)
Pickle consumption		
Never	72 (18.6)	96 (20.2)
Mild consumption	193 (49.7)	235 (49.5)
Moderate consumption	98 (25.3)	122 (25.7)
Heavy consumption	25 (6.4)	22 (4.6)
Tobacco smoking		
Never	228 (58.8)	203 (42.7)
Former user	22 (5.7)	10 (2.1)
Current user	138 (35.6)	262 (55.2)
Alcohol consumption		
Never	262 (67.5)	248 (52.2)
Former user	15 (3.9)	21 (4.4)
Current user	111 (35.6)	206 (43.4)
Chewable Tobacco consumption		
Never	140 (36.1)	123 (25.9)
Ever	248 (63.9)	352 (74.1)
Tuibur consumption		
Never	365 (94.1)	403 (84.8)
Ever	23 (5.9)	75 (15.2)

et al. 2014). Studies from European countries have reported positive or negative (Sharma et al. 2016; Ahmed et al. 2006) or inconclusive relationship between tobacco, alcohol use, and *H. pylori* infection (Sharma et al. 2016; Ahmed et al. 2006; Abebaw et al. 2014; Brown 2000). In our study, tobacco smokers and alcohol consumption were associated with increased odds of *H. pylori* infection (Table 2). A negative association was observed between the past users of tobacco and *H. pylori* infection. In fact, changes in alcohol and smoking habits were reported by 43.4% and 55.2%, respectively, of the participants with dyspeptic symptoms.

The use of betel quid (a chewable tobacco product) was associated with increased odds for *H. pylori* infection in our study. The practice of washing raw leaves of betel vine and areca nut (used to prepare the betel quid) in unhygienic water may explain our results (Abebaw et al. 2014). Interestingly, the results were not grossly attenuated even after adjusting for dietary, water sources, and socioeconomic variables.

Our study has several limitations. First, the study sample only included participants of Mizo origin. However, a high incidence of gastric cancer among this population suited our

Table 2 Association between tobacco and alcohol habits and *H. pylori* infection

	Odds ratio (95% CI)			
	Model 1[#]	Model 2[$]	Model 3[¥]	Model 4[£]
Tuibur				
Never	1	1	1	1
Ever	3.23 (1.98–5.44)	2.94 (1.78–5.00)	3.32 (1.96–5.80)	3.32 (1.95–5.83)
Chewed tobacco				
Never	1	1	1	1
Ever	1.51 (1.11–2.06)	1.51 (1.10–2.08)	1.49 (1.07–2.08)	1.49 (1.06–2.09)
Smoker tobacco				
Non smokers	1	1	1	1
Former smoker	0.45 (0.19–0.99)	0.46 (0.19–1.01)	0.36 (0.14–0.83)	0.36 (0.14–0.86)
Current smoker	1.78 (1.28–2.50)	1.89 (1.34–2.67)	1.82 (1.27–2.60)	1.81 (1.26–2.61)
Alcohol consumption				
Never	1	1	1	1
Former user	1.57 (0.74–3.45)	1.71 (0.80–3.78)	1.77 (0.80–4.02)	1.74 (0.78–3.98)
Current user	1.37 (0.97–1.94)	1.68 (1.13–2.50)	1.84 (1.22–2.80)	1.81 (1.19–2.76)

[#] Mutually adjusted

[$] Further adjusted for age, sex, marital status

[¥] Further adjusted for occupation, education, no. of persons in household, drinking water sources, and sanitation

[£] Further adjusted for dietary factors (raw food, salt, saum, pickles)

aim ideally. Incidence rates of gastric ulcer, gastritis, and gastric carcinoma show variations in geographical location and lifestyle habits among Northeast Indian states (Jang et al. 2010; Aziz et al. 2015; Darnindro et al. 2015). Second, for feasibility reasons, we recruited our participants from patients reporting with gastritis at a gastroenterology clinic in Aizawl. Although this strategy allowed us to focus on a group with a high probability of *H. pylori* infection, it might have biased our results. For example, if tuibur use can induce gastritis regardless of *H. pylori* infection, our study may have succumbed to collider stratification bias (Cole et al. 2010). However, a direct link between tuibur use and gastritis, not-mediated through *H. pylori* infection, has not been established in the literature.

Unfortunately, due to the cross-sectional nature of our study, reverse causality bias cannot be ruled out. However, it is rather unlikely that anyone would start using tuibur due to gastritis induced by *H. pylori* infection. Moreover, our study provides initial evidence for an association and replication of our findings, though prospective and mechanistic studies are warranted. Isolation of *H. pylori* bacterium from samples of tuibur collected from point of sales and potentially from the oral cavity of regular tuibur users is the next essential step in the scientific inquiry.

In conclusion, we report a positive cross-sectional association between tuibur use and *H. pylori* infection. Future studies should consider the possibility of the oral cavity of regular tuibur user, being a reservoir for repeated *H. pylori* infections.

Compliance with ethical standards

Ethical approval The study was approved by the ethical committee of Civil Hospital, Aizawl (No. B.12018/1/13-CH(A)/IEC/36) as well as the Mizoram University ethical committee.

Conflict of interest The authors declare that they have no conflict of interest.

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ABSTRACT

PREVALENCE OF *HELICOBACTER PYLORI* GENOTYPES AND THEIR ASSOCIATION WITH INTERLEUKIN-1- BETA IN PATIENTS WITH GASTRITIS FROM AIZAWL, MIZORAM

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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1. Introduction

Helicobacter pylori is an important threat for gastric ulcer and gastric carcinoma. International Agency for Research on Cancer (IARC) in 1994 said that there is a correlation between *H. pylori* infection and gastric carcinoma (Ford et al., 2014). The occurrence of *H. pylori* infection depends on both among and within individuals and is inversely proportionate to standards of living and cleanliness and sanitation. Northeastern zones of our country have always been one of the active regions for *H. pylori* contaminations, and the major factor of this is genotoxic stress from tobacco exposure (Ghatak et al., 2016). Various environmental or food factors are also liable for the infection of *H. pylori*, including mainly living standards and reproductive factors, have been identified.

Mizoram is a small state located in the north eastern India, at the eastern end of the Himalayas, and it shares borders with Tripura Bangladesh, Myanmar, Assam and Manipur. Most of the Mizo people used to take non-veg meal, and intake of fat of animals is much higher in the form of pork meat and addition of fermented pork fat (saum) while preparing bai (vegetable stew), which is a common food habit in this population. Some studies have also informed that countries with high intake of uncooked meat or vegetables were observed to have higher occurrenceof *H. pylori* infection (Patra et al., 2012; Phukan et al., 2006). The habit of intake of "tuibur" (tobacco smoke–infused aqueous solution) has been reported in Mizoram. Therefore, association of tobacco consuming with the occurrence of *H. pylori* infection in Mizoram cannot be ruled out (Ghatak et al., 2016). The occurrence of *H. pylori* in Mizoram has not been interpreted. Therefore, the objective of the study was to detect the occurrence of *H. pylori* infection in relation to various demographic factors in a unique geographical region.

However, the chances of improvement is there only in few patients, which is dependent on various factors, like bacterial and host genetic factors (Kusters et al., 2006; Ailloud et al., 2019).There are few particular *H. pylori* genotypes (*cagA, vacA*)

found which are observed as higher virulent strains, because these strains are correlated with severe gastroduodenal diseases in humans (Yamaoka et al., 1999). In was observed that *H. pylori* involves different risk factors including cytotoxin associated gene (cagA) and vacuolatingcytotoxin A (vacA), and these two are correlated with higher threat for occurrence of diseases (Kusters et al., 2006). Highly agitation and epithelial reduction in the gastric mucosa is strongly linked to the vacA gene (Palframan et al., 2012; Winter et al., 2014). According to the study the cagA gene of *H. pylori* is a marker for the existence of the cag pathogenicity island (PAI) (Kauser et al., 2004).Many studies say that the arising of antibiotic resistance in past few years has decreased the effectiveness of treatment regimens (Jina et al., 2020). The ocurrence of antibiotic resistance in *H. pylori* is because of the existence of SNPs in 16S rRNA, 23S rRNA and GyrA gene that might be an acute risk factor for occurrence of gastric carcinoma.

The way for mitigation of *H. pylori* infection is the proton pump inhibitor (PPI) and some antimicrobial agents, such as amoxicillin, clarithromycin, and metronidazole. Few categories of such triple regimens, triple therapies with a proton pump inhibitor (PPI; i.e. omeprazole, rabeprazole and lansorprazole), clarithromycin and amoxicillin are standard controls in Japan (Asaka et al., 2001). Triple therapies with a PPI or ranitidine bismuth citrate compiled with clarithromycin and amoxicillin or metronidazole are standardized regimens in European countries (Malfertheiner et al., 2002). Some studies reported that Clarithromycin-resistant strain of *H. pylori* is possible because of the mutation from adenine (A) to guanine (G) at position 2142 or 2143 of the 23S rRNA gene (Furuta et al., 2007).

It is found that the polymorphisms of the interleukin 1 beta (*IL-1B*) gene and the IL-1 receptor antagonist gene (IL-1RN) to be correlated with a high occurrence of both hypochlorhydria and gastritis (Ruzzo et al., 2005). *IL-1B*encripts IL-1, a potent pro-inflammatory cytokine and powerful inhibitor of gastric acid secretion that has an important value in occurrence of agitation to *H. pylori* infection (Figueiredo et al., 2014).A polymorphic allele with a T instead of a C at position – 511 of the regulatory region of theIL-1B gene (*IL-1B*-511*T) is correlated with high

IL-1production (Kimang'a, 2012). IL-1RN encrypt the IL-1 receptor antagonist (IL-1ra), an anti-inflammatory cytokine that ambitiously attach to IL-1 receptors, and thereby controls the possibly destruction affects of IL-1 (Volarevic et al., 2010). The IL-1RN gene has a variable number of tandem repeats in intron 2, resulting in a short allele (IL-1RN*2, with two repeats) or long alleles (IL-1RN*L, with three to six repeats). The IL-1RN*2 alleles are correlated with increased IL-1b production (Santtila et al 1998). *H. pylori* infection in patients with these alleles may therefore result in increased production of gastric IL-1b, leading to major and sustained inflammation, gastric atrophy, and hypochlorhydria, and ultimately to the incidence of gastritis (Wroblewski et al., 2010).

In this study, there are combinations of bacterial and host genotypes that are much correlated to the occurrence of gastritis. This research work deals with the correlation of *H. pylori* vacA and cagA virulence-associated genes and human IL-1B and IL-1RN susceptibility polymorphisms with the demographic features of gastritis.

2. Aim and objectives

- To identify the *H. pylori* genotypes (16S rRNA, *vacA*, *CagA*, *Urec*) in relation to the demographic factors.
- To detect the clarithromycin antibiotic resistance status of *H. pylori* by PCR.
- To determine the polymorphism in *Interleukin 1 Beta* gene in patient's genome.

3. Materials and methods

3.1. Sample collection

About 863 patients with gastritis, scheduled for an endoscopy at the gastroenterology clinic Trinity diagnostic Centre in Aizawl District, Mizoram Northeast India were recruited. Patients who presented with dyspepsia or gastrological problem requiring upper GI endoscopy were included in the present study and the individuals who had taken antibiotics for gastrological problem for

past two weeks, active GI bleeding; pregnancy and history of gastrectomy were excluded for false *H. pylori* negative result in urease test.

A proper sample was utilized to make the higher rate of probability to collect data for possible *H. Pylori* infection. The study was approved by the ethical committee of Civil Hospital, Aizawl (No. B.12018/1/13-CH(A)/IEC/36) as well as the Mizoram University ethical committee.

3.2. Data collection

The demographic data was collected on the basis of few factors including age, educational qualification, profession, domestic location, marital status, number of children, medical background, health condition, family record of *H. pylori* infection and daily habits (e.g., tobacco consumption and alcohol intake, diet) by using a bilingual verified and standardized questionnaire (Mizo and English). On the basis of self-reported measures (regular, occasional, former, or never consumers),some information were collected about tobacco smoking and drinking of alcohol, consumption of various smokeless tobacco products like Tuibur, betel nut, pan, zarda, food habits like completely vegetarian diet, raw vegetables intake, fermented pig fat (sa-um), intake of salt and pickle, sanitation(commode, and in field), and drinking water sources (e.g., unfiltered, filtered [using ceramic, active carbon/charcoal, UV sterilization and reverse osmosis] and boiled).

3.3. Endoscopy and biopsy sampling

To detect *H. pylori* infection, endoscopy tested samples were accumulated from antrum portion of individual's stomach on the basis of rapid urease test kit. Endoscopy was done by using Fujinon VP-4450 HD Series on patients after an over night fasting. Two biopsy specimens were accumulated through endoscopy from each patient's mucosa of the gastric antrum and placed in a small screw capped bottle containing 0.2ml sterile normal saline to maintain humidity which. The biopsy samples were used for Rapid urease test.

3.4. Rapid urease test (RUT) for *H. pylori* status

The rapid urease kit (RUT DRY Test kit, Gastro Cure System, WB, India) available in market were used for the screening of endoscopic sample (Brown et al. 2002). The samples of endoscopic test were collected into the well of kit. A pH based colour indicator detected the rise in the pH of the medium by ammonium ions. In the positive case of *H. pylori* contamination among the samples, there was an immediate change in colour from yellow to pink.

3.5. DNA Isolation

Endoscopic sample tissues were placed in formalin solution. Formalin-fixed tissues were subjected to glycine–Tris–ethylene-di-amine tetra acetic acid buffer for removal of formalin. The DNA was extracted from gastric antral biopsy specimens. DNA was collected based on phenol, chloroform, and isoamyl alcohol-based separation method. After these processes sample washed and extracted genomic DNA was observed by gel electrophoresis technique.

3.6. PCR amplification of the *H. pylori* specific gene regions

RUT results were again verified by Polymerase chain reaction (PCR) using *H. pylori* specific 16S rRNA primers, Thermal amplifications were done by using Eppendorf (vapoprotect) instrument and subjected to gel electrophoresis for the presence or absence of the partial 16S rRNA region. Based on 16S rRNA positive samples, PCR amplification of cagA1, cagA2, vacA, ureC was performed.

3.7. *H. pylori* vacA and cagA Genotyping

DNA that was isolated from endoscopic samples was further used for partial amplification of vacA gene. cagA genotyping was performed by amplification of partial cagA gene for asian lineage. Genotyping was done by polymerase chain reaction (PCR) followed by 1.5% agarose gel electrophoresis cagA lineage identification and for the sequencing of vacA partial gene, gold standard di deoxy chain termination method was used with the help automated Sanger sequencing (ABI 3500 Genetic analyzer).

3.8. Multiplex PCR amplification of the 23S rRNA gene

The 23S rRNA gene different region with SNP was amplified by polymerase chain reaction (PCR) using two different pair of primers. PCR was done in 25 µl total reaction volumes each containing 100 ng of template DNA, 0.2 pM of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, and 1 unit of Taq DNA polymerase (Fermentas, Germany). The PCR amplification products (3 µl) were subjected to electrophoresis in a 2 % agarose gel and images were obtained in GBOX gel documentation systems (UK).

3.9. PCR amplification of *Interleukin -1 -Beta* gene

The Interleukin -1 -Beta gene different VNTR region and SNPs were amplified by polymerase chain reaction (PCR) using three different primers pair. PCR was performed in 25 µl total reaction volumes each containing 100 ng of template DNA, 0.2 pM of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, and 1 unit of Taq DNA polymerase (Fermentas, Germany). The PCR amplification products (3 µl) were subjected to electrophoresis in a 2 % agarose gel in 1X TBE buffer at 80 Vh for 30 min, stained with Ethidium Bromide, and images were obtained in GBOX gel documentation systems (UK).

3.10. Statistical analysis

The risk factors of the patients were discovered using descriptive statistics. The association of demographic factors among case–control subjects was examined for Hardy–Weinberg equilibrium by a chi-square test with one degree of freedom (df). Unconditional logistic regression was used for the reorganization of different factors correlated with *H. pylori* infection. A hierarchical modeling strategy was performed to assess the variation in strength of this correlation when modified for possible confounder. The associations of epidemiological factors with *H. pylori* prevalence were evaluated by means of odds ratios (ORs) and 95% confidence intervals (CIs) using conditional and unconditional multiple logistic regression.

23S rRNA gene SNPs among the *H. pylori* virulent genes samples were tested for Hardy–Weinberg equilibrium by a chi-square (v2) test with one degree of freedom (df). The distributions of susceptibility and resistance of clarithromycin among demographic factors were evaluated by using odds ratios (ORs), and 95% confidence intervals (CIs). With that, logistic regression analyses were done to assess the potential confounder's influence of both genetic and environmental factors for the status of clarithromycin. Then, the independent effect of risk factors was analyzed in a multivariate model (introducing all variables and terms of interactions). All statistical analyses were achieved by using SPSS 20.0 program (SPSS, Madrid, Spain).

Differences in the combined score and gastric bacterial different genotypes were assessed with the Mann–Whitney test. Correlations between genotypes and the existence of host genotypes were assessed. Only the cases containing single *H. pylori* genotypes were inserted in the analyses of the histopathologic features of gastritis. Associations between bacterial genotypes and host genotypes and association of genotype frequencies between gastritis were measured by the logistic regression. Comparability of genotype prevalence among group of people determines by age, sex, food, and lifestyle habits were done by the logistic regression. Odds ratios (ORs) with 95% confidence intervals (CIs) and unconditional logistic regression models were calculated with SPSS software (version 20; SPSS Science, Chicago, IL). Area under the curve (AUC) values was calculated through the Receiver operating curve (ROC) analysis using MedCalc software (version 14.8; MedCalc, Belgium). Differences were measured statistically significant when $P<.05$. All statistical tests were two-sided.

4. Results

Out of 943appoimtments, 863 patients participated in the study. However, the percentage of men refusing to participate in the study was higher. The participants were from the age between 8 to 92 years old, with comparative allocation between *H. pylori* positive and negative groups.However, the *H. pylori* urease test positive was not so remarkably different between male and female subjects. The proportions of

male, married people, those with below matriculation level of education are slightly higher *H. pylori*-positive group compared to the *H. pylori* negative group. However, a lower proportion of participants in *H. pylori*-positive group consumed filtered water. The same way dietary factors showed the distribution between *H. pylori*-positive and -negative groups.

Among the patients with *H. pylori*, almost half lived with 5 to 7 family members, and almost more than one fourth were lived with 8 to 10 family members. The No. of person in same house-hold was a significant factor for *H. pylori* infection in univariate and multivariate analysis.

In this study, it was found that more than half of the smokers were infected with *H. pylori*. Consumption of local alcoholic beverages was found in many patients. The statistical association between locally processed alcohol made from rice drinking and *H. pylori* status was detected with high OR and 95% CI, of the men and women were regular local alcohol drinkers (at least thrice a week) for univariate analysis. *H. pylori* status is statistically remarkable with occasionally and regularly smoking habit in univariate and multivariate analysis with high OR and 95% CI. Intake of betel leaf with areca nut and tuibur (tobacco infused water) were a major factor for the *H. pylori* infection in univariate analysis, but for the multivariate analysis only regular consumption of tuibur was remarkable factor. Most of the individuals had to consult for medical care because of the stomach pain. The presence of nocturnal dyspeptic symptoms was significantly correlated to the incidence of *H. pylori* infection, with symptomatic individuals having a higher occurrence than the observed among negative infection ones. Most of the affected patients were using public welfare department (PWD) water supply for house hold use, Consequently, most of the affected patients are using ceramic and boiled water as a potable water, but there are no statistical significant for water resource and potable water quality for the *H. pylori* infection.

Many of the participants in the *H. pylori*-positive group were ever users of chewed tobacco, which are belongs to the majority group and smoked forms of

tobacco. There was approximately three times higher proportion of Tuibur users between the *H. pylori*-positive compared to the *H. pylori*-negative group.

Results of logistic regression showed that being ever users of Tuibur increased the odds of *H. pylori* positive endoscopy compared to never users. This association was not weakened after adjusting for several known risk factors of *H. pylori* infection. In addition, chewed tobacco users had 1.49 higher odds of having *H. pylori* positive endoscopy result.

All the positive samples were tested by cagA and vacA virulence genes identification. Out of cagA positive samples, a total of 78.26% and 21.74% samples were represented as cagA lineage 1 (Asian specific lineage) and cagA lineage 2 (European specific lineage), respectively. Whereas 4.32% samples show combinations of vacA and cagA genotypes: cagA lineage1/vacA (10.6%), cagA lineage2/vacA.

Male patients of gastritis were more affected with *H. pylori* than the female patients. Large family size was marked as the increased-risk factor for *H. pylori* infection in this population, because 57.44% of *H. pylori*-infected gastritis patients have more than 8 people in the family. Almost , 50% of *H. pylori*-infected gastritis patients were alcohol consumer, and 63.8% of *H. pylori*-infected gastritis patients were regular or occasionally smokers in study cohort.Some more important factors for the presence of cagA genotypes were alcohol, non-veg, and extra pickle consumption in the univariate analysis among all studied lifestyle factors. Alcohol consumption and extra pickle intake were the significant factors in multivariate analysis for cagA genotype patients' group. The values of probability are remarkably higher for the cagA positive group of patients with the alcohol and extra pickle consumer group. The multivariate model got a high area under the curve values (AUC = 0.75, p = 0.001) for the correlation between alcohol and extra pickle consumption and the presence of cagA genotype. Whereas, Asian lineage cagA-linage1 remarkably correlatedto alcohol consumer (OR = 0.63; 95% linage cagA-linage1 were remarkably correlated todrinking alcohol (OR = 0.63; 95% CI = 0.38 -

1.04, p = 0.05) and non-veg consumption (OR = 0.26; 95% CI = 0.08 – 0.85, p = 0.02) in univariate analysis. While the independent risk factor for the existance of Asian lineage cagA-linage1 in multivariate analysis was non-veg consumption for this population.

Clarithromycin is one of the most common antibiotics used for *H. pylori*. Presence of clarithromycin resistant *H. pylori* strains causes complications. This study assists to find out the demographic, as well as socio-economic factors, relate to the presence of clarithromycin-resistant strains in this particular population. In this study, males were prone to carry clarithromycin-resistant *H. pylori* strains. Similarly, an ascending order was found with the number of patients in the same household with 1-4, 5-7, 8-10 and above 10 persons contributed to the population 17%, 25.5%, 40.4% respectively except above 10 persons (17%), due to less availability in this population. These populations mainly consume non-veg food items. Logistic regression was done, univariates analysis results showed the presence of clarithromycin resistant *H. pylori* strain was higher among the male of the population. Gender (male) adjusted odd ratio of ceramic filtrate water consumers and fermented pig fat consumers showed 13.14 and 2.53, respectively.

Among all the *H. pylori* participants' group, a total number of 42.63% of patients carried genotype C and 57.42% of patients carried genotype T for IL-511. Interleukin-RN tandem repeats allele 2 was statistically remarkably connected to cagA positive genotypes of *H. pylori*. The probability values were much higher for the cagA positive samples than negative samples. Interestingly, IL-RN tandem repeats allele 2 was significantly associated with the presence of cagA lineage 1 and 2 for this population. The model reached a high AUC score for the association between cagA lineage 1 (AUC = 0.71) and 2 (AUC = 0.69) with, IL-RN tandem repeats allele 2.

In the cagA positive gastritis patients, IL-1B-511*T carriers represented an OR of 1.07 (95% CI 0.31 – 3.64)of the case subjects, although it was not statistically remarkable. The study couldn't found any remarkable correlation between vacA genotypes and host IL-1B/IL-1RN Genotypes.

5. Conclusion

This study is the first and foremost large scale estimate of the abundance of *H. pylori* infection in gastric cancer prone state of Mizoram, Northeast India. The unique food habits and living standard were the causative factors for *H. pylori* infection among the mizo population. Among the various factors, number of individuals stay in same household showed gradual increase in the occurrence rate with increased number of persons. Farming as an occupation showed relation with *H. pylori* occurrence and it is may be due to lack of awareness of cleanliness. This study gave an idea that smokers are at a high threat of *H. pylori* infection as longer period habits of smoking alter the pH condition and indirectly preparing a preferable condition for the bacterium inside the abdomen. Intake of tuibur among Mizo population developed a direct relation with *H. pylori* infection by changing the stomach pH and making a favorable condition for *H. pylori* to sustain inside the host. This study reported the occurrence of cagA virulent gene among *H. pylori* infected patients is high among alcohol consumers and intake of alcohol for longer period of time may alter the pH of the stomach and indirectly help the bacteria to sustain in side host.

The study reports that the source of ceramic filtrate potable water is one of the reasons of occurrence of clarithromycin resistant among *H. pylori* infected patients. The study showed that the Mizo population has unique habit of consumption of fermented pig fat which is also called "sa-um" in local language, consumption of "sa-um" also make the graph high of the incidence rate of clarithromycin resistant.

In this study the Interleukin-RN tandem repeats allele 2 was statistically remarkably correlated with cagA Asian lineage and additionally, the study also showed that there is no remarkable correlation between vacA genotypes and host IL-1B/IL-1RN genotypes.

The study gave an idea about initial measurements of occurrence of *H. pylori* infection among gastric carcinoma prone Mizo population and examines the risk factors as well as confounding factor related with high incidence.

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