ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Magnitude of Vacuolating Cytotoxin A (VACA) in *Helicobacter pylori* stool antigen and blood antibody-positive asymptomatic young children in Ziway, Ethiopia

By: Negusu Dawit

Advisors: Kassu Desta (MSc, PhD candidate)

Aster Tsegaye (MSc, PhD)

Bineyam Taye (MPH, PhD)

A research Thesis submitted to the Department of Medical Laboratory Sciences, College of Health Science, Addis Ababa University, in partial fulfillment of Master of Science Degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology)

December 2019

Addis Ababa, Ethiopia

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Negusu Dawit, entitled: **Magnitude of Vacuolating Cytotoxin A (VACA) In *H. pylori* stool antigen and blood antibody-positive asymptomatic young children in Ziway, Ethiopia**, and submitted in partial fulfillment of the requirements for Master of Science Degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

External Examiner

_____ Signature _____ Date _____

Internal Examiner

_____ Signature _____ Date _____

Advisors

_____ Signature _____ Date _____

_____ Signature _____ Date _____

_____ Signature _____ Date _____

Chairman of the Department or Graduate Program Coordinator

_____ Signature _____ Date _____

Acknowledgment

First of all, I would like to thank Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University (AAU) for giving me the opportunity to undertake this study. My gratitude also goes to my respectful advisors Dr. Aster Tsegaye, Mr Kassu Desta for their unreserved guidance, helpful advice, and encouragement for the development of the thesis. My sincere thanks go to Colgate University, USA and Dr Bineyam for the VACA test kit.

The study participants whose data is used in this study are gratefully acknowledged. I am also indebted to Mihret Tesfaye and Sosina Walelign for the *H pylori* data and other socio-demographic information of the children.

I would like to thank my family and friends for their support and for raising my morale throughout this work.

Table of content

Contents

List of table

List of figure

List of Abbreviation

Ab	antibody
Ag	antigen
ALA	alanine
ATP	adenine triphosphate
Bcl-2	B-cell lymphoma 2
CagA	cytotoxin-associated gene A
DUPA	duodenal ulcer promoting gene
EDTA	ethylene diamine tetraacetic acid,
ELISA	enzyme-linked immunosorbent assay
GEC	gingival epithelial cells
GLY	*Glycine*
GPI	glycol phosphatidylinositol
LE	late endoplasm
LFA-1	Lymphocyte function-associated antigen *1*
MCH	major histocompatibility complex
NFAT	nuclear factor of activated T-cells
OD	optical density
PUD	peptic ulcer disease
Taco	tryptophan aspartate coat
UBT	urea breath test
VacA	Vacuolating Cytotoxin A
VAL	valine
VDCA	voltage-dependent anion channel

Abstract

Background: Vacuolating Cytotoxin A (VacA) is the major *H.pylori* toxin that affects multiple cellular activities including cell membrane channel formation, disruption of endosomal /lysosomal function, and detachment of epithelial cells from the basement membrane. The toxin also, interferes with the process of antigen presentation, apoptosis and inhibition of activation-induced proliferation of T lymphocytes. In Ethiopia, information regarding VacA antigen is insufficient.

Objective: To determine the magnitude of VacA toxin production in *H.pylori* positive asymptomatic children at Ziway, Oromia Region, Ethiopia

Methods: A cross-sectional study was conducted in young children from selected schools and health facility in Ziway town. A convenient sampling technique was employed to include study participants who met the inclusion criteria. A structured questionnaire was used to collect socio-demographic and health status data of the study participants. Concentration of circulating VacA was determined by using the BG competitive ELISA antigen detection test kit. Information from the laboratory analysis and questionnaires were entered and analyzed using SPSS version 20. Categorical variables were compared using the chi-square test method. Pearson correlation was made between VacA positivity and test method, gender and age of the children. $P < 0.05$ was considering being statistically significant.

Result: A total of 21 *H. pylori* stool antigen (Ag) positive and 42 antibody (Ab) positive children aged 4-14 years were recruited (33 male, 30 female).The overall prevalence of VacA was 44.4% (28/63); 18 (64.28%) were male and 10 (35.7%) were female, 60.7% (17/28)of them were in the age group of 9-14 years. VacA positivity rate from stool antigen positives was 10/21 (47.6%) and from blood antibody positives 18/42 (42.9%).In this study there was no statistically significance between VacA prevalence and test method (stool Ag or serum Ab detection), age and sex.

Conclusion: The magnitude of H. pylori VacA toxin among asymptomatic children in Ziway, Ethiopia is high and there was no significant association between VacA positivity and age, sex and laboratory test method. Further large scale longitudinal studies should be conducted.

Key words: Helicobacter pylori,VAcA, young children, Ziway Ethiopia.

1. Introduction

1.1. Background

Helicobacter pylori (*H. pylori*) formerly known as Campylobacter pylorid, and then Campylobacter pylori, is a spiral or curved fastidious Gram-negative slow-growing micro-aerophilic and flagellated bacillus, (1-4). It was established in 1982 by Robin Warren and Barry Marshall as the causative agent of gastritis and peptic ulcer(3). The *H.pylori* genome (1.65 million bp) codes for about 1500 proteins (5).

Helicobacter pylori inflammation has been recognized as one of the most common chronic bacterial infections in humans (6) that affects about half of the world's population and is usually acquired in childhood (7).The prevalence among middle-aged adults is over 80 percent in many developing countries, and 20 to 50 percent in industrialized countries (8). The prevalence in children is 10% in developed countries but can be as high as 30%-40% in children from lower socioeconomic classes (9).

Most of the *H.pylori* infections usually acquired during early childhood and (10,11) persistent for lifelong unless eradicated by treatment. The majority of infected individuals remain free from symptom throughout their lifetime, only a small number develop a disease (12). The clinical presentation of *H.pylori* infection is a result of interaction between bacterial virulence (e.g.CagA, VacA, BabA), host factor including genetic polymorphisms,(e.g. IL-1β, IL-10, TNF-α influence the inflammatory response and the exasperation of mucosal damage), and environmental factors (e.g. diet, smoking)(13-15).

Epidemiological studies and experiments using animal models have suggested that VacA is one of the most important virulence factors in the pathogenesis of peptic ulceration and gastric cancer (16). The name refers to the most prominent effect of VacA capability of the toxin to cause large vacuoles in epithelial cells (17). However, *H.pylori* toxin implicated in multiple cellular activities including cell membrane channel formation, disruption endosomal /lysosomal function, detachment of epithelial cells from the basement membrane, interference with the process of antigen presentation, apoptosis and inhibition of activation-induced proliferation of T

lymphocytes. Many of these effects are dependent on the capacity of VacA to form anion-selective membrane channels (16,18-21).

The 3,864-bp VacA gene presents in all strains but has different allelic combinations and is expressed in only 50% of *H.pylori* isolates (22)due to sequence heterogeneity within the VacA gene at the (M) middle and (signal [s] region). This region encodes part of the cytotoxin's signal peptide and N-terminus, while the middle (m) region encodes part of the 55-kDa C-terminal sub unit. Two versions of the s-region (s1 and s2) and m-region (m1 and m2) exist, and this causes differences in the Vacuolating activities among individual *H.pylori* strains (14, 21-23).

The VacA s1 and m1 types can be further subdivided into s1a, s1b and s1c, and m1a, m1b, and m1c, respectively. The s2 type encodes a VacA protein with an additional N-terminal hydrophilic amino acid segment, which the s1 type lacks. The presence of this extra segment prevents the s2 type toxin from inducing vacuolation. On the other hand, disease with VacA s1 strains has been connected to gastric irritation and duodenal ulceration with enhanced Cytotoxin movement. Also the VacA s1m1 strains produce a massive quantity of toxin with high Vacuolating movement in gastric epithelial cells. Whereas s1m2 strains produce moderate measures of toxin, and s2m2 strains produce little or no toxin.The700-bp mid-region plays a role in host cell binding, and m1 forms are able to bind a wider range of cell types than m2.The m1 and m2 regions are <60% identical in amino acid sequence, while the other regions of the mature protein show >90% identity across a variety of strains(18,22,24).

The natural history of VacA positive *H.pylori* infection in children has not yet been extensively studied. The majority of infected children remain asymptomatic, but the inflammatory response may result in an ulcerogenic process. There are several reports which indicated that the clinical outcome of *H.pylori* infection is highly related to age of infection and virulence factors. The 95-kDa Vacuolating Cytotoxin (VacA) is one of *H.pylori* virulence factor are likely to play a crucial role in the most severe forms of gastric pathologies including peptic ulcer, gastritis and gastro esophageal reflux disease in children. The observed correlation between these proteins and the severity of *H. pylori*-induced gastric pathology has generated a lot of interest in these proteins (23, 25). There is paucity of data in Ethiopia about VACA in children; particularly no study is available that detects the toxin using ELISA.

1.2. Statement of the problem

The overall prevalence of *H.pylori* in developing countries is more than 80% and usually acquired in childhood (9). Among children, the prevalence of *H.pylori* infection is not more than 10% in developed countries, it may reach up to 75-80% in developing countries. It has been reported that the variation in the prevalence of *H.pylori* infection in different populations might be associated with socioeconomic factors and some habitual living and housing conditions (11,26).

Even though most *H.pylori* infections are clinically silent, the organism is associated with substantial morbidity and mortality (27).*H.pylori* is associated with different digestive diseases, such as gastritis, gastric and duodenal ulcer, and mucosa-associated lymphoid tissue lymphoma, and it considered to be a risk factor for the development of gastric cancer (28).

Epidemiological and basic studies have provided evidence that infection with *H.pylori* carrying specific virulence factors can lead to more severe outcome (14). VacA is one of the major type of toxin produced by *H.pylori* that induced cytoplasmic vacuolation in a variety of mammalian cell lines *in vitro* and produced epithelial cell damage and mucosal ulceration when administered interagastrical to mice (30). Over 90% of patients with duodenal ulcer disease harbored VacA s1a strains (29).

The prevalence of *H.pylori* in developing countries is high but there is a little data known about the geographic distribution of specific *H.pylori* virulence factor VacA, especially in children (28). Also, there is little data in developed and developing country's concerning the prevalence of VacA antigen in apparently healthy children. Most of the studies focus on antibody detection.

To the best of my knowledge, there is no published study conducted in this area in particular and in Ethiopia in general, about the prevalence *H. pylori* VacA antigen in young children; hence conducting this study may contribute to fill the existing gap.

1.3 Significance of the study

- ✓ This study provides new data in the prevalence of VacA antigen in *H. pylori* infected asymptomatic children.
- ✓ Determination of VacA prevalence is important to predict the clinical outcome of *H. pylori* infection; hence understanding the magnitude of the toxin will help clinicians to pay attention to *H pylori* positive individuals.
- ✓ The study provides a baseline data for the upcoming researchers in this area.

2. Literature review

2.1 Prevalence of Helicobacter pylori VacA, with gastro duodenal disease

The data reviewed by Sugimoto M *et.al*, 2009 in Latin American and African populations showed that the prevalence of VacA is associated with H. pylori-related disease development. Meta analysis was done on data from 2612 patients from Latin America (2285 strains) and 520 patients from Africa (434 strains). The frequencies of vacA s and m genotypes differed between strains from Latin America (77.2% for s1 and 68.1% for m1) and Africa (83.9% for s1 and 56.7% for m1). Latin American strains with s1 and m1 genotypes increased the risk of gastric cancer (OR 4.17, 95% CI 2.49–6.98 for s1, and 3.59, 2.27–5.68 for m1) and peptic ulcers (e.g. 1.73, 1.37–2.20 for s1). African strains with the s1 or m1 genotypes also increased the risk of peptic ulcers (8.69, 1.16–64.75 for s1) and gastric cancer (10.18, 2.36–43.84 for m1).(14)

The study conducted by Biernat M, M *et al*, in Poland from 130 gastro duodenal diseased children and adolescents (4-18 age) show that The vacAs1/m1 genotype was more frequent in children with ulcers than in other groups, whereas the vacAs2/m2 genotype was more frequent in patients with gastritis and gastroesophageal reflux disease(31).

Andreson H *et,al*, in Estonia conducted a study to determine the association of gastric disease with CagA and VacA *H. Pylori* antigen from 156 adults. The study shows that the prevalence of VacAs1/m1 and m2 gene associated with chronic gastritis, peptic ulcer disease, and perforated peptic ulcer in82.85%,93.93%, and 82%, respectively (32)

A total of 33 children,17 boys and 16 girls with an age range of 3 –15 years and have *H.pylori* gastritis were included in a study conducted by Ko JS et.al . The findings revealed that the prevalence of VacAs1 antigen was 100%. Among these, 24 (72%) were s1c and nine (27%) weres1a. But neutrophil activity, chronic inflammation, and *H. pylori* density were independent of VacA status(33)

The study conducted in Slovenia by Homan M et.al investigated 165 *H. pylori* positive children. The participants were 58 boys and 107 girls (age range 4– 18 years)who had recurrent abdominal pain. The prevalence of VacA gene was 90.9%(150 of 165 isolates). From those, thes1m1 genotype was the most frequent (63/150, 42%), followed by s1m2(42/150, 28%) and s2m2(36/150, 24%) (34).

Data from adults also support a high prevalence of VacA. The study conducted in Thailand by Chomvarin C et.al from 112 dyspeptic adults (34 patients with peptic ulcer disease,62 with non-ulcer dyspepsia or gastritis and 16 with gastric cancer) showed that the prevalence of VacA was 100% all of them were s1 signal sequence allele(35).

De Gusma V.Ret.al in Minas Gerais Brazil at University Hospital, Federal University of Minas Gerais, Belo Horizonte, investigated 24 patients (8 girls and 16 boys; mean age, 11.7 years; age range, 1 to 17 years) with an endoscopically documented duodena lulcer. As comparative group,41 patients(25girlsand16boys;meanage,9.3years; age range, 1 to 15 years) without a gastric or duodenal ulcer at endoscopy were included. Among the 55 patients infected with *H. pylori* strains with non-mixed VacA allelic types, the s1 signal sequence was found in strains isolated from all 19 patients with a duodenal ulcer and from 21 (58.3%) children without an ulcer. The s2 allele was found only in strains isolated from patients without an ulcer (n = 15; 41.7%). There was a strong correlation between the presence of the s1 allele and the presence of a duodenal ulcer (P =0.003)(27).

No published study is found related to VacA in children in Ethiopia. Nonetheless, the study conducted Asrat.D *et.al*, at Tikur Anbassa University Hospital, Addis Ababa, Ethiopia, investigated a total of 300 gastric biopsy samples and 50 *Helicobacter pylori* isolates from adult dyspeptic patients. The result showed that the prevalence of VacA genes was 79% and 87% in biopsy samples and clinical isolates, respectively (36).

2.2Epidemiology of VacA in asymptomatic subject

The prevalence of antibodies against the major *Helicobacter pylori* virulence markers Vacuolating Cytotoxin gene (VacA)was studied among One hundred and eighty-two*H. Pylori* positive asymptomatic young children (90 male and 92 female with an age range from 18–60 months) in a peri-urban community of Dhaka Bangladesh. This study by SarkerS showed that the prevalence of VacA antibodies from 145 urea breath test (UBT) positive children was 94%(25)

The study conducted in Virginia America by Elitsur Y et.al to determine the prevalence of CagA, VacA antibodies in symptomatic and asymptomatic children with *Helicobacter pylori*

infection recruited a total of 155 (23 symptomatic and 132 asymptomatic)infected children. The results indicated that the prevalence of CagA and VacA antibodies was 69% and 35% in symptomatic children and 54% and 52% in asymptomatic children, respectively. Regression analysis showed no significant difference between the presence of CagA or VacA antibodies and factors like age, gender, or community location (37).

The study conducted at King Abdul-Aziz University Maternity and Children's Hospitals, Jeddah, KSA by Jaber M.S in 215 asymptomatic sero-positive west Saudi- Arabia children(mean age 9.3±3.9 years; range, 1-14 years) indicated that the prevalence of VacA was 60% (129/215). The prevalence of VacA Ab is higher in males 91(42.3%)than females 38(17.7%) but there was no difference in the prevalence of VacA between Saudi and non-Saudi children (38).

Abasiyanik F.M et.al in Istanbul turkey investigated 30 symptomatic patients with dyspepsia and abdominal pain and 40 asymptomatic individuals 20 to 65 years of age (average age 37 years), with no history of abdominal pain. Their finding indicated that the prevalence of VacA in symptomatic and asymptomatic study participants was 21/30(70 %) and 26/40(65%),respectively (39).

A study conducted by Sicinschi L.A *et, al* in the rural villages of Narin ˜o or Genoy, in the State (‘‘departamento’’) of Narin ˜o, located at a high altitude in the Andes Mountains of southwestern Colombia involved 86 asymptomatic children (41 boys and 45 girls) for the determination of *H.pylori* virulence factor. Their age ranges from 4.1–8.7 years. The result demonstrated that the prevalence of VacA is 84.6%.The predominant vacA genotype was s1b, which was found in samples from 49 children (81.7%). The other genotypes detected were as follows: s1a for three children (5.0%), s2 for five children (8.3%), and s1 and s2 at different time points in samples from three children (5.0%)(40).

A total of 182 asymptomatic infants of whom 63 were females and 126 were males with an. age ranging from6-24 months (16.1m ± 5.6 m) were included in a study conducted by Hefzy E,M *et al* in Egypt. The overall prevalence of *H. pylori* in this study was 88.9% (168/189). From those *H. pylori* infected infants in 98.8% (166/168) of them VacA gene was identified. The most virulence VacAs1 gene was predominantly identified in 79.5% of positive cases, whereas 20.5% of isolates had the VacAS2 genotype. The middle (m2) region of VacA gene was predominant in

positive samples (68.7%),while m1genotype were 31.3%. On the other hand,S1m2 genotype was the most common combination of VacA(41).

The study conducted by Vivatvakin B, in 22 Thai native children aged 0-15 year without associated abdominal pain from different provinces in 4 parts of the Kingdom of Thailand show the prevalence of VacA genotype is 27/28 (96.43%)(42).

As reviewed above the magnitude of *H. pylori* VacA antibody is very high though the majority of the studies focus on genotyping, which has significance for disease pathogenesis. Considering the paucity of data related to VacA antigen detection globally and lack of studies in children in Ethiopia, this study will try to shade some light.

3. Objectives:

3.1. General objective:

To determine the magnitude of VacA toxin production in *H. Pylori* stool antigen and blood antibody positive asymptomatic children at Ziway, Oromia, Ethiopia from May to October 2019

3.2. Specific objectives

- To determine the magnitudes of VacA in young *H. pylori* positive children
- To compare the prevalence of VacA toxin in stool antigen positive and blood Ab positive children
- To compare the prevalence of VacA toxin by sex and age group

4. Hypothesis:

The prevalence of VacA in asymptomatic young children is low and does not differ by age and sex

5. Materials and methods

5.1. Study area

The samples used for this study were collected form Batu (Ziway) town, which is located in Oromia National Regional State, in East Shoa zone, Adami Tulu Jiddo KombolishaWoreda, at a distance of 160 Km from Addis Ababa. Its astronomical location is 7º 56‟ North Latitude and 38º 43‟ East Longitude with an elevation of 1643 meters above sea level. Batu is one of the reform towns in the region and has a town administration, municipality and two kebelles. The town also has governmental and private health facilities; from them, Batu hospital and the only private Sher hospital were selected conveniently. The average number of patient flow under 15 years of age per day in Sher and Batu hospital was 35 and 23 respectively. The city also has both government and private schools of which 14 are primary schools from them Sher (private) and Batu (public) primary schools were included (43).

5.2. Study design

This study is a sub study of a previous study on *H pylori* and allergy.(44) It employed both retrospective (demographic data, *H pylori* stool antigen and serum antibody positivity) and prospective design to measure VacA. A cross-sectional study design was used for the previous study involving one public and one private health facilities and schools. The current study utilized all *H pylori* stool Ag positives and age as well as facility matched serum Antibody positives from the previous study.

5.3. Study period

The current study was conducted from May to October 2019.

5.4. Population

5.4.1. Source population

All young children who were, below 15 years attending the selected schools and health facilities were used as source population for the previous study. Thus, all serum samples collected from these children were the source samples for the current study.

5.4.2. Study population

Serum samples from young children (who are below 15 years) who fulfilled the selection criteria were used as study samples to measure Vacuolated Cytotoxin antigen (VacA).

5.5. Eligibility

5.5.1. Inclusion criteria

- young children who were, below 15 years
- *H. pylori* antigen test or antibody test positive

5.5.2. Exclusion criteria

- The initial study excluded children who were taking antibiotics (like Pepto-Bismol and proton pump inhibitors Nexium or Prilosec) in the previous one month.
- Children with any peptic ulcer disease (PUD) and non ulcer dyspepsia asymptom(Heartburn or acid reflux) , Indigestion (dyspepsia), Nausea, vomiting , Non-cardiac chest pain, and feeling of fullness, bloating or belching) were also excluded

For the current study the following were used as criteria to exclude samples:

- Grossly hemolysed sample
- Lipemic sample
- Sample containing visible precipitant

5.6. Study variables

5.6.1. Dependent variables

- prevalence of VacA

5.6.2. Independent variable

- Socio demographic characteristics (sex, age)
- The test method used for the identification of *H. pylori* infection (stool Ag detection versus serum Ab detection)

5.7. Sample size calculation and sampling technique

5.7.1. Sample size calculation

Since this study was a sub-study of the "Association of Helicobacter pylori infection with atopy and allergic disorders in Ziway, Central Ethiopia", (44). I taken all stool antigen-positive samples and matched (with age and facility) blood antibody-positive samples were utilized for the purpose of this study. Accordingly, a total of 63 eligible samples were taken (21 stool antigen-positive samples, and 42 serum Antibody positives).

5.7.2. Sampling technique

A convenient sampling technique was used

5.8. Data collection and processing

5.8.1 Questionnaire based data collection:

The current study extracted only information required for its purpose from the original questionnaire data collected in the previous study Participant's information like demographic data (age and sex) and health status (if the children had suffered any relevant peptic ulcer disease (PUD) or gastritis disease and any antibiotic or other drugs taken) were extracted.

5.8.2 Laboratory data collection

H. pylori status of the participants by both stool antigen and serum antibody was already characterized and leftover serum samples stored at -80^{O}C in a previous study. For the identification of VacA concentration in the sample, BG competitive ELISA method was employed. Data collection procedure is summarized in Figure 1 below

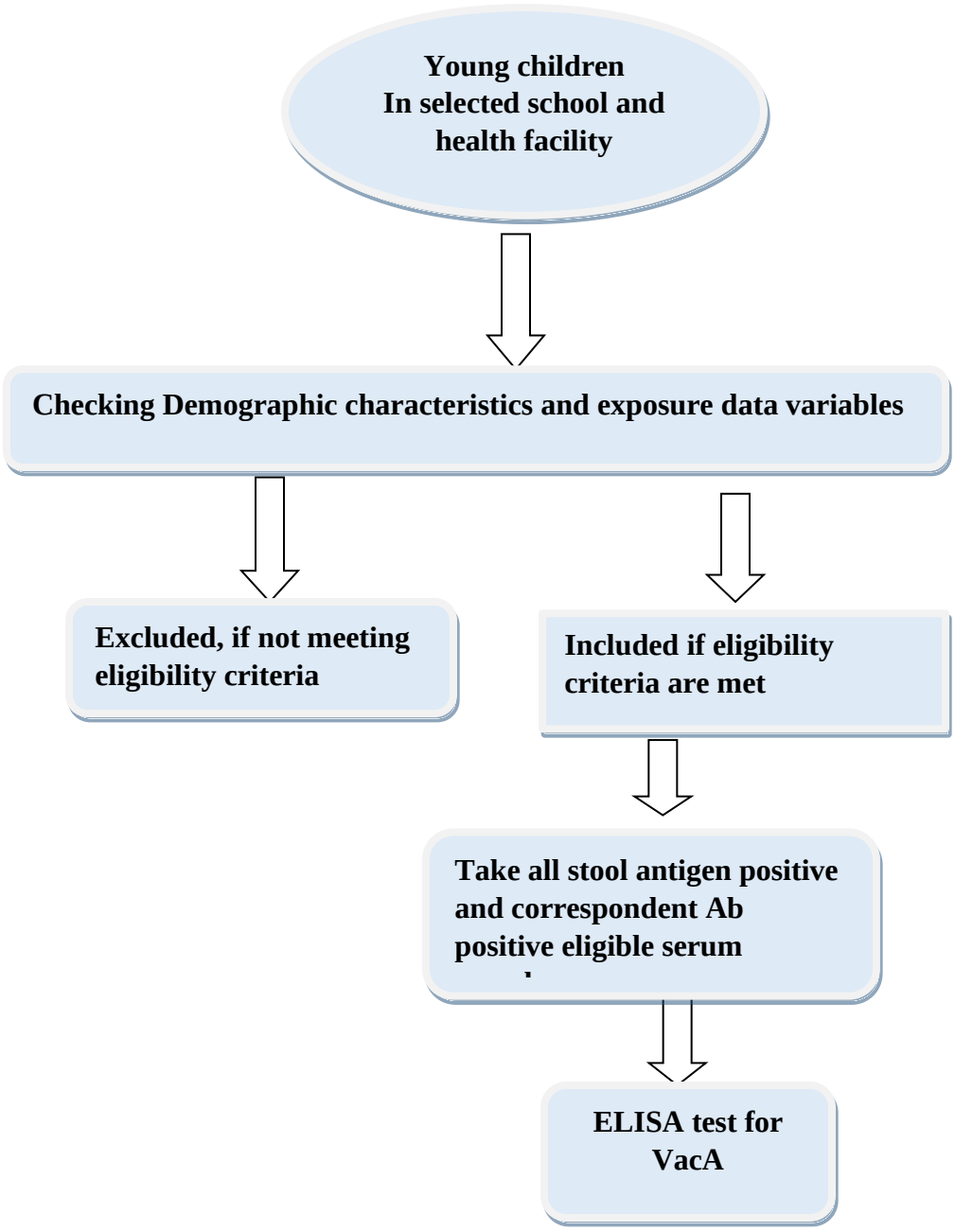

Figure:-1 Data collection and analysis work flow

5.8.3 ELISA VacA test kit

The serum VacA level was measured by a commercially available BG ELISA kit. This ELISA kit applies competitive enzyme immune assay techniques by utilizing an anti-VacA antibody and VacA-HPR conjugate. The assay sample and buffer was incubated together with VacA-HPR conjugate in a pre-coated plate for one hour. After the incubation period, the well is decanted and washed five times. The wells were then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue color complex. Finally, a stop solution was added to stop the reaction, which was then turning the solution to yellow. The intensity of the color was measured spectrophotometrically at 450nm in a microplate reader. The intensity of the color is inversely proportional to VacA concentration. A standard curve shown below was plotted relating the intensity of the color (OD) to the concentration of standard (Figure 2). The VacA concentration in each sample was interpolated from this standard curve. The test was done by following the manufacturer instruction as detailed in Annex: I

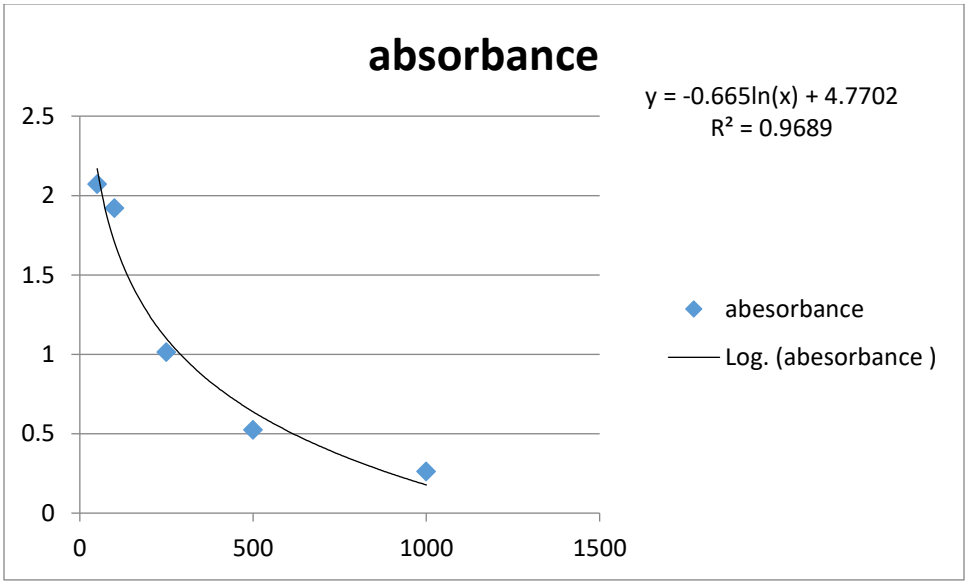

Figure 2.A standard curve plotted relating absorbance (y-axis) (OD) to the concentration of standard (x axis)

5.9 Data Quality Assurance:

To ensure data quality, Standard operating procedure(SOPs) was strictly followed and internal quality control materials was included from the test kits. Test was performed based on manufacturer's instructions.

5.9.1. Pre-analytical: serum samples with adequate volume and without any hemolysis or precipitant for analysis were used to produce reliable and valid data. Serum samples were stored at -80^{O}C until analysis.

5.9.2. Analytical phases: the tests were done under the supervision of senior and experienced laboratory personnel according to standard operating procedures of each test method. The reagents, kits and the methods were assessed with known positive and negative control materials, well-trained and experienced laboratory professionals participated in the laboratory analysis procedure. Finally, the results were checked by supervisors.

5.9.3. Post-analytical phases: the results were recorded by unique identification numbers, repeatedly checked before recording in result log and also raptly checking the result from the result login to instrument to avoid a transcriptional error.

The previous data in the original study also followed all the three phases to ensure data quality. In addition, they used a pretested questionnaire to collect socio-demographic and clinical data.

5.10 Operational Definition

Asymptomatic:-*H. pylori* positive participant may be defined as individuals who are positive for *H. pylori* (as determined by stool antigen test or blood Ab test) but do not have signs or symptoms for *H. pylori* infection (signs and symptoms of gastric or duodenal ulcer, non ulcer dyspepsia).

5.11 Data analysis and interpretation

Data was entered and analyzed using SPSS version 20 statistical software. All of the data was expressed as number and percentage. Comparisons of percentages were assessed using the chi-square test method. Pearson correlation was made between VacA positivity and gender, test method and age of the children. $P < 0.05$ was considered being statistically significant. Figures and tables were used for data presentation

5.12 Ethical considerations

Ethical clearance was obtained from the Departmental Research and Ethics Review Committee of the Department of Medical Laboratory Science of College of Health Sciences, Addis Ababa University. Results and any information regarding patients were kept confidential during and after the completion of the research project by password-protected electronic and locked hard copy files. No additional samples were collected from participants for the purpose of this study which is a continuation of a previous project. *H.pylori* antigen test-positive children have already been linked to health facilities.

5.13 Dissemination and utilization of results

The result of the study is submitted to the Department of Medical Laboratory Sciences (DMLS), Addis Ababa University (AAU). An oral presentation of the thesis will be made. The result will also be disseminated through publication in peer reviewed local and international journals and through presenting it in relevant workshops and seminars

6. Results

6.1 Baseline characteristics of the study participants

A total of 63 eligible study participants were recruited from four sites: Batu Hospital 3.2% (2/63), Sher Hospital 38.1% (24/63), Sher Elementary School 11.1% (7/63) and Batu Elementary School 47.6% (30/63). Just over half were male 52.4% (33/63). The mean +/-SD age was 8.58 +/-2.736 (the age range being between 4 and 14 years) and 65 % (41/65) were in the age group between 9-14 years. Out of the 63 participants, 66.6% (42/63) were *H.pylori* serum Antibody positive and 33.33% (21/63) were stool antigen positive (Table 1).

Table1: Baseline characteristics of study participants, Ziway, Ethiopia (n=63)

Variables		**Number**	**Percent**
Sex	Male	33	52.4
	Female	30	47.6
Age (years)	4-8	27	42.9
	9-14	36	57.1
Sample collection site	Batu Hospital	2	3.2
	Sher Hospital	24	38.1
	Sher Elementary School	7	11.1
	Batu Elementary School	30	47.6
Test method	Stool Antigen	21	33.3
	Blood Antibody	42	67.7

6.2 Prevalence of *H. pylori* Vacuolating Cytotoxin A

For the determination of the prevalence of VacA in stool Antigen and serum antibody *H. pylori* positive participants, competitive ELISA method was utilized. The overall positive rate of VacA, as shown in Table 2, was 44.4% (28/63); from those 18/28(64.28%) were male and 10/28 (35.7%) were female. About 60.7% (17/28) of them were in the age group of 9-14 years. VacA positivity rate from stool antigen positive individuals was 10/21(47.6%) and from blood antibody positive it was 18/42(42.8%). High prevalence of VacA was demonstrated in children from Share school 5/7(71.4%), Batu school 15/30 (50.0 %),Share hospital 8/24 (33.33%), and Batu hospital 0/2(0%)(Table 3)

Table 2.Frequency of VacA in H. pylori infected young children, Ziway

VacA	Frequency	Percent	Valid Percent	Cumulative Percent
Negative	35	55.6	55.6	55.6
Positive	28	44.4	44.4	100.0
Total	63	100.0	100.0	

Table 3, Frequency of VacA by sex, age, site and test method among *H. pylori* positive children, Ziway (n=63

Variable		Total	VacA Pos	Percentage
Age (Years)	4-8	27	11	40.7
	9-14	36	17	47.2
Sex	Male	33	18	54.5
	Female	30	10	33.3
Facility	Batu hospital	2	0	0
	Sher hospital	24	8	33.3
	Sher school	7	5	71.4
	Batuschool	30	15	50.0
Test method	Stool Antigen	21	10	47.6
	Blood Antibody	42	18	42.8

6.3 Concentration of VacA in stool antigen and serum antibody *H.Pylori* positive children

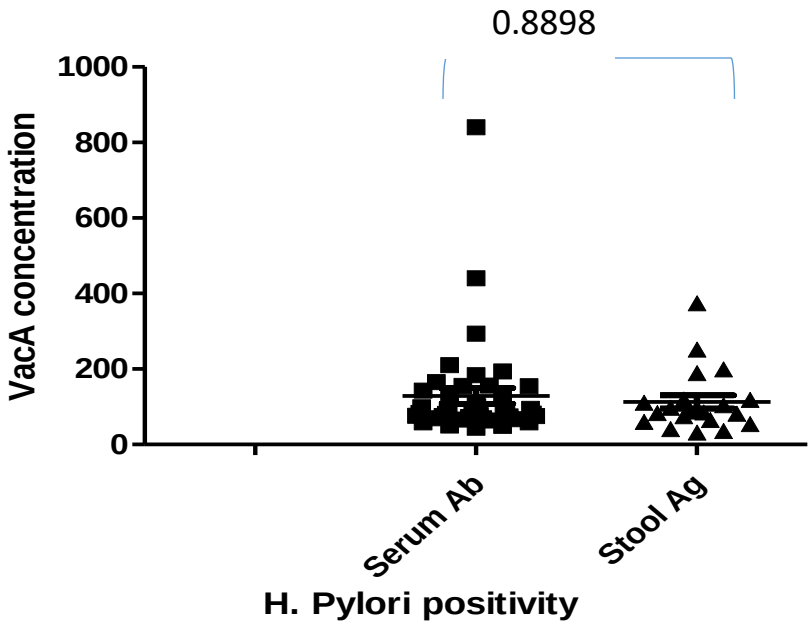

Figure 3: VACA concentration distribution by *H pylori* stool antigen and serum Antibody positivity among the study participant in Ziway (n=63)

The Distribution of VacA concentration among stool antigen and serum antibody *H.pylori* positives groups was assessed by using the Mann-Whitney U test. But it did not show statistically significant difference between those who are stool antigen and serum antibody positives as shown in Figure 3 below (P=0.8898).

6.3 Association of demographic factors of school children and test method with prevalence of VacA

From a total of 63 study participant *H.pylori* positive school children, 28 of them were VacA toxin positive. Chi-square statistical analysis showed that sex , test method and Age were not significantly associated with the prevalence of VacA as shown in Table 4.

Table 4:-Associations between prevalence of VacA with sex, age and test method among H, pylori positive young children, Ziway, Ethiopia

		ELISA VacA test		**P-value**
		Negative	Positive	
Sex	Male	15	18	0.091
	Female	20	10	
Laboratory method	Stool Antigen	11	10	0.720
	Blood Antibody	24	18	
Age	4-8	16	11	0.608
	9-14	19	17	

7. Discussion

H pylori is associated with several gastroduodenal diseases such as gastritis, gastric ulcer, duodenal ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (2,26,27). VacA is one of the major type of *H.pylori* virulence factor that has been associated with the development of Gastric mucosa injury, peptic ulcer disease and gastric cancer (19). It is present in nearly all strains but expressed in only 50% of the isolates (18).According of WHO(2010) the prevalence of *H.pylori* in Ethiopian children was about 80%(45) but to the best of my knowledge, information concerning prevalence of VacA among asymptomatic *Helicobacter pylori* positive young children in particular is not available in Ethiopia. Also determination of VacA in asymptomatic subject by the detection of Cytotoxin activity in vitro or by competitive ELISA, was not observed by others.

In this study using competitive ELISA analysis, the prevalence rate of VacA toxin was 44.4% and the prevalence of VacA toxin according to test method shows 10/21(47.6%) in stool antigen positives and 18/42 (42.8%) in serum antibody positive children. There was no significant difference in the prevalence and concentration of VacA toxin with test method. This shows that most of serum antibody positive children still have active *H.pylori* infection or the capability of toxin can survive more than a month in the circulation, suggesting careful follow up may be needed for individuals who are stool antigen negative in the presence of serum antibody positivity.

The current study also demonstrated no statistically significant association between the prevalence of VacA toxin by sex and age of children. This finding is different from study conducted in Jeddah, Saudi Arabia by Jaber SM *et al*,2005. According to their study, the prevalence of VacA was 60%. They also found that VacA antigen was significantly elevated in male compared to female and children ≥10 years compared to those in age group of 1-5 years and 6-9 year; (39). In the current study as well though statistically not significant, with increasing age of children, the percentage of VacA toxin positive against 89 kDa was elevated in children age group between 9-14 compared to those 4-8 years. This may be because of the increasing prevalence of *H.pylori* with age and the difference in prevalence might be due to difference in sample size, socio demography, and especially laboratory methods they used *H.pylori* infection identification tests and the later used VacA antigen identification.

The study conducted in West Virginia America by Elitsur.Y et al,1999 (37) demonstrated a 52%VacA antibody prevalence while another study conducted in Colombia by Sicinschi A,L et.al 2012 (40) showed a prevalence rate of 84.6%; in both studies there was no significant relationship between VacA antibody prevalence and age (37, 40). This finding was the same in the current study which demonstrated absence of statistically significant relationship between VacA and age, though antigen detection of the toxin was made as opposed the above two studies. This difference might be the sample size, heterogeneity of VacA gene and laboratory method, they used UBT and ELISA for the identification of H.pylori infection and , PCR Amplifications and Western blot for VACA antigen detection, respectively.

Other authors also found a high prevalence of infection with VacA positive strains in asymptomatic children. A study conducted in Samatya Hospital, Istanbul, Turkey by Abasiyanik MS at et.2002 (39), and in Bangladeshi by Sarker SA et,al(25) that reported prevalence of 65 %, and 82%, respectively. The former used /detection by ELISA and UBT tests and the later used VacA identification immunobloting and western blotting, which might partly explain for the differences amongst each other as well as with the present study.

Moreover, there was very high prevalence of VacA has been reported in the study conducted in Egypt by Hefzy EM *et.al*, 2014 (41) and by Vivatvakin B *et.al* 2004 in Thailand Thai (42) from asymptomatic children who reported prevalence rates of 98.8% and 96.43% respectively. Both studies used molecular techniques as opposed to the current study.

Though the current study was limited by the sample size, VacA toxin was determined in *H pylori* positive asymptomatic children for the first time in Ethiopia. Hence the implication of the finding are paramount as VacA positive *H.pylori* have been found to increase the risk of peptic ulcer disease and are associated with increased gastric epithelial damage (12). The study conducted in Poland by Biernat MM *etal*, shows the prevalence of VacA is associated with degree of bacterial density, as well as of chronic inflammation (31). This observation has been supported by other genotyping studies. Although a study conducted in Slovania children by Homan M*et al*,2009 m1 and s1 alleles of the vacA gene were more common in specimens with a higher bacterial density score ($P<0.01$, $P<0.01$, respectively), and type s1 strains were strong associated with a higher degree of chronic inflammation and duodenal ulcer (34).

This result agree with the study conducted in brazil children by DEGUSMA.V.R, et.al (2000),another study conducted in Poland children and adult by Biernat.M.M, et.al, showThe prevalence of vacAs1/m1 genotype was more frequent in children with ulcers than in other groups, whereas the vacAs2/m2 genotype was more frequent in patients with gastritis and GERD. While in Greek and Korea Children, the prevalence of disease do not significantly associated with severity of the disease. The difference might be due to difference in sample size, socio demography, and especially laboratory methods they used

Another study shows that an increase in the prevalence VacA antigen has been reported in patients with peptic ulcer disease and those with gastric cancer (24). A review conducted in Latin American and African populations by Sugimoto M& Yamaoka Y. 2009 show that s1 and m1 genotypes increased the risk of gastric cancer and peptic ulcers (14).As previously described the association between positivity of VacA and increased severity of gastritis in children was suggested by others. If only VacA strains are indeed associated with duodenal ulcer and gastric cancers, then the finding of a very high prevalence of such strains in the children of the current study in our setting is alarming. However, the prevalence of gastric cancer in Ethiopian population in2015 has been reported to be very low1.65/100,000 population (46). The current study cannot explain this disparity. These data in asymptomatic children may suggest that either the concentration VacA toxin is not enough to cause inflammation or other factors are involved in the development of clinical illnesses and in the determination of their clinical outcome.

8. Strength and limitation of the study

8.1 Strength of the study

- This study attempts to indicate the prevalence of *H.pylori* VacA antigen for the first time in asymptomatic children.
- Direct detection of VacA toxin as opposed to the antibody by using competitive ELISA method

8.2 Limitation of the study

- There might be some bias because of the convenient sampling technique that is used in this study.
- Small sample size
- Do not including another socio-demographic factors
- Other markers like pepsinogen ll for checking current progression of the infection(Type b gastritis) were not measured
- The study did not used advanced molecular technique for the identification of *H.pylori* infection VacA antigen and also mixed infection

9. Conclusion and recommendation

9.1 Conclusion

This study for the first time described the prevalence of *H. pylori* VacA toxin in asymptomatic children. The prevalence of *H. pylori* VacA toxin among asymptomatic children in Zeway, Ethiopia high and there no significant association between age, sex and laboratory test method.

9.2 Recommendation

- Since there is no study that have been done in Ethiopia on the prevalence of VacA in *H.pylori* positive asymptomatic young children, further Large scale study is recommended to supplement the results of this study to be used for future guideline in the development of national policy for *H.pylori* treatment.
- Further studies are required in symptomatic and asymptomatic study participants in this study area using different advanced diagnostic method to explain the actual role of VacA antigen in development and progression of *H.pylori* infection.

10. Reference

1. Khalifa MM, Sharaf RR&aziz R K. Helicobacter pylori: a poor man's gut pathogen? *Gut Pathog*2010;. 2**:** 2.https://doi.org/10.1186/1757-4749-2-2
2. Ali AM, Elkhatib W F. Potential Complications of Helicobacter pylori Infection in Children of a Non-Urban Community. *Arch Pediatr Infect Dis*2015; 3**:** e23510.DOI: 10.5812/pedinfect.23510
3. Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev*. 2006;19(3):449–490.
4. Rajindrajith S, Devanarayana NM, de Silva HJ. Helicobacter pylori infection in children. *Saudi J Gastroenterol*. 2009;15(2):86–94
5. Delahay RM &Rugge M .Pathogenesis of Helicobacter pylori Infection.2012 ; 9:15 https://doi.org/10.1111/j.1523-5378.2012.00976
6. Mera RM, Bravo LE, Goodman KJ, Yepez MC, Correa P. Long-term effects of clearing Helicobacter pylori on growth in school-age children. *Pediatr Infect Dis* 2012; 31**:** 263-6.
7. Tadesse E, Daka D, Yemane D, Shimelis T. Seroprevalence of Helicobacter pylori infection and its related risk factors in symptomatic patients in southern Ethiopia. BMC Research Notes. 2014;7(1):834.
8. Suerbaum S, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; 347**:** 1175-86.
9. Hunt R.H Helicobacter Pylori in Developing Countries. World Gastroenterology Organisation Global Guideline 2011;45/5: 383-388
10. Benenson S, Halle D, Rudensky B, Faber J, Schlesinger Y, Branski D, et al. Helicobacter pylori Genotypes in Israeli Children: The Significance of Geography. Journal of pediatric gastroenterology and nutrition. 2002;35:680-4.
11. Vandenplas Y, Blecker U. Helicobacter pylori infection in children. *Acta Paediatr*1998;87**:** 1105-12.
12. VandenplasY. Helicobacter pylori infection.World J Gastroenterol 2000;6: 20-31

13. Hagymasi K,Tulassay Z. Helicobacter pylori infection: new pathogenetic and clinical aspects. *World J Gastroenterol*2014; 20**:** 6386-99.
14. Sugimoto M,Yamaoka Y. The association of vacA genotype and Helicobacter pylori-related disease in Latin American and African populations. *Clin Microbiol Infect* 2009;15**:** 835-42.
15. Atherton JC. *H.pylori*virulence factors. *Br Med Bull* 1998; 54**:** 105-20.
16. Sundrud MS, Torres VJ, Unutmaz D, Cover TL. Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci U S A* 2004; 101**:** 7727-32.
17. Rassow J. Helicobacter pylori vacuolating toxin A and apoptosis. *Cell Commun Signal*. 2011;9:26. doi:10.1186/1478-811X-9-26
18. White JR, Winter JA, Robinson K. Differential inflammatory response to Helicobacter pylori infection: etiology and clinical outcomes. *J Inflamm Res* 2015; 8**:** 137-47.
19. Figueiredo C, Machado JC, Yamaoka Y. Pathogenesis of Helicobacter pylori Infection. *Helicobacter*,2005;10 Suppl **1:** 14-20.
20. Torres V J, Ivie SE, Mcclain MS, Cover TL. Functional properties of the p33 and p55 domains of the Helicobacter pylori vacuolating cytotoxin. *J Biol Chem*2005; 280**:** 21107-14.
21. Isomoto H, Moss J, Hirayama T. Pleiotropic actions of Helicobacter pylori vacuolating cytotoxin, VacA. *Tohoku J Exp Med.*2010; 220**:** 3-14.
22. Ghose C, Perez-Perez GI, Torres VJ, Crosatti M, Nomura A, Peek RM, et al. Serological assays for identification of human gastric colonization by Helicobacter pylori strains expressing VacA m1 or m2. Clinical and Vaccine Immunology. 2007;14(4):442-50.
23. Chang WL, Yeh YC, Sheu BS. The impacts of *H.pylori*virulence factors on the development of gastroduodenal diseases. *J Biomed Sci*. 2018;25(1):68.(20000)
24. Miernyk K, Morris J, Bruden D, McMahon B, Hurlburt D, Sacco F, et al. Characterization of Helicobacter pylori cagA and vacA genotypes among Alaskans and their correlation with clinical disease.Journal of clinical microbiology. 2011;49(9):3114-21.

25. Sarker S, Nahar S, Rahman M, Bardhan P, Gb N, Beglinger C, et al. High prevalence of cagA and vacA seropositivity in asymptomatic Bangladeshi children with Helicobacter pylori infection. Acta paediatrica. 2004;93:1432-6.

26. Ertem D, Harmanci H, Pehlivanoglu E. Helicobacter pylori infection in Turkish preschool and school children: role of socioeconomic factors and breast feeding. *Turk J Pediatr* 2003; 45**:** 114-22.

27. De Gusmão VR, Nogueira Mendes E, De Magalhães Queiroz DM, Aguiar Rocha G, Camargos Rocha AM, Ramadan Ashour AA, et al. vacA genotypes in Helicobacter pylori strains isolated from children with and without duodenal ulcer in Brazil. *Journal of clinical microbiology*,2000;38(8):2853-7.

28. Alarcón T, Martínez MJ, Urruzuno P, Cilleruelo ML, Madruga D, Sebastian M, et al. Prevalence of CagA and VacA antibodies in children with Helicobacter pylori-associated peptic ulcer compared to prevalence in pediatric patients with active or nonactive chronic gastritis. *Clin Diagn Lab Immunol.*2000;7(5):842-4.

29. Ahmed KS, Madompoyil B, Ghebremedhin AA, Issac J, Ahi JD, Khan AA, et.al. Studying the Importance of VacA Gene of Helicobacter pylori in Identifying the Pathogenicity of Strains by Comparing It with the Disease Status of the Subjects. Journal of Cancer Therapy. 2013;4:68-74.

30. Suzuki H, Masaoka T, Hosoda H, Ota T, Minegishi Y, Nomura S, et.al. Helicobacter pylori infection modifies gastric and plasma ghrelin dynamics in Mongolian gerbils. Gut. 2004 Feb 1;53(2):187-94

31. Biernat MM, Gosciniak G, Iwanczak B. Prevalence of Helicobacter pylori cagA, vacA, iceA, babA2 genotypes in Polish children and adolescents with gastroduodenal disease. Postepy higieny i medycyny doswiadczalnej. 2014;68:1015-21.

32. Andreson H, Loivukene K, Sillakivi T, Maaroos HI, Ustav M, Peetsalu A, et al. Association of cagA and vacA genotypes of Helicobacter pylori with gastric diseases in Estonia. Journal of Clinical Microbiology. 2002;40(1):298-300.

33. Ko JS, Kim KM, Oh YL, Seo JK. cagA, vacA, and iceA genotypes of Helicobacter pylori in Korean children. Pediatrics International 2008;50(5):628-31.

34. Homan M, Luzar B, Kocjan BJ, Orel R, Mocilnik T, Shrestha M, et al. Prevalence and clinical relevance of cagA, vacA, and iceA genotypes of Helicobacter pylori isolated from Slovenian children. Journal Of Pediatric Gastroenterology And Nutrition. 2009;49(3):289-96.

35. Chomvarin C, Namwat W, Chaicumpar K, Mairiang P, Sangchan A, Sripa B, et al. Prevalence of Helicobacter pylori vacA, cagA, cagE, iceA and babA2 genotypes in Thai dyspeptic patients.International Journal Of Infectious Diseases 2008;12(1):30-6.

36. Asrat D, Nilsson I, Mengistu Y, Kassa E, Ashenafi S, Ayenew K, et al. Prevalence of Helicobacter pylori vacA and cagA genotypes in Ethiopian dyspeptic patients. Journal Of Clinical Microbiology. 2004;42(6):2682-4.

37. Elitsur Y, Neace C, Werthammer MC, Triest WE. Prevalence of CagA, VacA antibodies in symptomatic and asymptomatic children with Helicobacter pylori infection. *Helicobacter.*1999 ;4**:** 100-5.

38. Jaber SM. The pattern of CagA and VacA proteins in Helicobacter pylori seropositive asymptomatic children in western Saudi Arabia. *Saudi Med J* 2005;26**:** 1372-7.

39. Abasiyanik MF, Sander E , Salih BA. Helicobacter pylori anti-CagA antibodies: prevalence in symptomatic and asymptomatic subjects in Turkey. *Can J Gastroenterol.*2002;16**:** 527-32.

40. Sicinschi LA, Correa P, Bravo LE, Peek RM, Jr.Wilson KT, Loh JT, et al. Non-invasive genotyping of Helicobacter pylori cagA, vacA, and hopQ from asymptomatic children. Helicobacter. 2012;17(2):96-106.

41. Hefzy E, Alkassem A, Ashraf S. Non-invasive detection of Helicobacter pylori virulence genotypes ureA, vacA, cagA and babA2 among asymptomatic Egyptian infants. African Journal of Microbiology Research. 2014;8:3276-83.

42. Vivatvakin B, Theamboonlers A, Semakachorn N, Wongsawadi L. Prevalence of CagA and VacA genotype of Helicobacter pylori in Thai Children. Journal of the Medical Association of Thailand = Chotmaihet thangphaet. 2004;87:1327-31.

43. *2007 Population and Housing Census of Ethiopia: Results for Oromia Region*, Vol. 1

44. Unpublished study was conducted by Tesfaye M. Association of Helicobacter pylori infection with atopy and allergic disorders in Ziway, Central Ethiopia. for the fulfillment of the requirements for master's degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology specialty track) in Addis Abeba university http://localhost:80/xmlui/handle/123456789/5268.2017-05

45. World Gastroenterology Organisation Global Guideline: Helicobacter pylori in developing countries. Journal of clinical gastroenterology. 2011;45(5):383-8.

46. Memirie ST, Habtemariam MK, Asefa M, Deressa BT, Abayneh G, Tsegaye B, et al. Estimates of Cancer Incidence in Ethiopia in 2015 Using Population-Based Registry Data. Journal of Global Oncology. 2018;4:1-11.

I: competitive ELISA Protocol for VacA

Principle of the Assay

VacA ELISA kit applies the competitive enzyme immunoassay techniques Utilization an anti-VacA antibody and anVacA-HRP conjugate. The assay sample and buffer are incubated together with VacA-HRP conjugated in pre-coated plate for one hour .after the incubation period; the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme –substrate reaction forms blue color complex. Finally a stoop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450 nm in a micro plate reader. The intensity of the color is inversely proportional to the VacA concentration since VacA from samples and VacA-HRP conjugate compete for the anti-VacA antibody binding site. Since the number of site is limited as more sites are occupied by VacA from the sample, fewer sites are left to bind VacA-HRP conjugate. a standard curve is plotted relating the intensity of the color(o.d) to the concentration of standards. The VacA concentration in each sample is interpolated from this standard curve.

Materials

All reagent provide are stored at 2-8^{o}c

Materials	Specification	Quantity
Microtiter plate	96 wells	
Enzyme conjugate	6.0 ml	
Standard A	0pg/ml	
Standard B	50 0pg/ml	
Standard C	100 0pg/ml	
Standard D	250 0pg/ml	
Standard E	500 0pg/ml	
StandardF	1000 0pg/ml	
Substrate A	6 ml	

Substrate B	6 ml	
Stop solution	6 ml	
Washing Solution (100X)	10ml	
Balance Solution	3 ml	

Note:- balance solution is used when the sample is cell culture supernatant

Specimen collection and storage

Plasma_ collect plasma using EDTA or heparin .assay as an anticoagulant centrifuge sample for 15 minute at 1000 x g(3000 rpm) at 2-8 ^{o}c within 30 minutes of collection. Assay immediately or aliquot and store sample at -20^{o}c or 80^{o}c

Note

1. Samplesshould be liquated and must be stored at -20^{o}c(less than 3 months)or -80^{o}c (less than 6 months) to avoid loss of bio activity and contamination. if sample are to be run within 24 hours,they may be stored 2-8^{o}c.avoid repeated freeze-thaw cycles. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples

2. Tissue or cell extraction samples prepared by chemical lysise buffer may cause an expected ELISA results due to the impact of certain chemicals.

3. Samples containing a visible precipitate must be clarified prior to use in the assay. care should be taken to minimize hemolysis.do not use grossly hemolysed or lipemic specimens.

4. do not use heat- treated specimens.

Materials and equipment required but not supplied

1. Precissionpipettes and disposables tips to deliver 10-1000µl.a multi-channel pipette desirable for large assays.

2. 100ml and 1 liter graduated cylinders.

3. Distilled or demonized water

4. Tubes or prepared sample dilutions.

5. Absorbent papers

6. Micro plateredder capable of measuring absorbance at 450nm.\

7. Centirfuge capable of 3000 xg

8. Microplatewasher or washingbottle

9. Incubater (37^{o}c)

10. Data analysis and graphing soft ware.

Reagent preparation

1. Bring all kit component and samples to room temperature (20-25^{o}c) before use.

2. Samples:- please predict the concentration before assaying. if concentration are unknown or not within the detection range, a preliminary experiment is recommended to determine the optimal dilution.PBS (ph 7.0-7.2)or 0.9% normal saline can be used as dilution buffer.

3. wash solution-dilute 10ml of wash solution concentrate (100x)with 990ml of demonized or distilled water to prepare 1000ml of wash solution(1x).if crystals have formed in the concentrate ,warm to room temperature and mix gently until the crystals have completely dissolved. The 1x wash solution is stable for to weak at 2-8^{o}c

4. Do not dilute the other components which are ready –to-use.

Assay procedure

Please read reagents preparation before starting assay procedure. It is recommended that all standards and samples be assayed in duplicate. It is strongly recommended to do a preliminary experiment before measuring all sample.

1. Secure the desired numbers of coated wells in the holder then add 100 µl of standards (shake the bottle of each standards gently by hand and pipette up and down the solution of standards for 3 times before adding) or samples to the appropriate well. add 100 µl of PBS (PH 7.0-7.2) in the blank control well.

2. Add 50 µl of conjugate to each well (NOT blank control well).mix well. Mixing well in this step is important. cover and incubate the plate for 1 hour at 37^{o}c
3. Wash the micro titer plate using one of the specified methods indicated below:
    a) Manual washing: remove incubation mixture by aspirating contents of the plate in to the sink or proper wastecontainer. Fill in each well completely with 1x wash solution, and then aspirate contents of the plate in to the sink or proper waste container. Repeat this procedure five times for a total of FIVEwashes. Afterwashing, invert plate and blot dry by hitting the plate on to absorbent paper or pear towels until no moisture appears. Note: hold the sides of the plates frame firmly when washing the plate to assure that all stripes remain securely in frames.complete removal of liquid at each steps is essential to good performance.
    b) Automatedwashing:-wash plate FIVE times with diluted wash solution (350-400 µl /well/wash) using an auto washer. Afterwashing, dray the plat as above. it is recommended that the washer be set for a soaking time of 10 seconds and shaking time of 5 seconds between each wash.
4. Add 50 µl substrate A and 50 µlsubstrate B to each well including blank control well,subsequently.cover and incubate for 15-20 mints at 37^{o}c.(avoid sun light in the colure is not dark, please prolong the incubation time, but the longest times is 30minut)
5. Add 50 µl of stop solution to each well including blank controlwell.mix well.
6. Determine the optical density (OD) at 450 nm using a micro plate reader immediately.

Calculating of result

1. The standard cureve is used to determine the amount of samples.
2. Firstaverage the duplication reading for each standard and samples. All O>D values are subtracted by the mean value of blank control before result interpretation. DO NOT subtract the OD of standard zero.
3. Construct a standard curve byplotting the average O.D. for each standard on the horizontal(X) axis against the concentration on the vertical(Y) axis, and draw a best fit curve using graph paper statistical software generate to a four parameter logistic (4-PL) curve fit or logit-log linear regression curve. An axis for the optical density and a y-axis for the concentration is also a choice. The data may be linearized by plotting the log of

the concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis.

4. Calculate the concentration of samples corresponding to the mean absorbance from the standard curve.
5. Standard curve for demonstration only.

Note:

1) Any variation in operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in result. Each user should obtain their own standard curve.
2) If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
3) If specimens generate values higher than the highest standard, dilute the specimens and repeat the assay.

CERTIFICATE OF ANALYSIS

1) In the same lot CV%:4.2,5.9
2) Different lot CV%:6.6,7.9
3) Spike recovery:92-104%
4) Linearity:
5) Sensitivity: The sensitivity in this assay is 1.0 pg/mL
6) Specificity: This assay has high sensitivity and excellent specificity for detection of VACA. No significant cross-reactivity or interference between VACA and analogues was observed. NOTE: limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between VACA and all the analogues, therefore, cross reaction may still exist in some cases.

SAFETY NOTES

1) This kit contains small amount of 3,3',5,5'-Tetramethylbenzidine (TMB) in substrate B. TMB is non-carcinogenic but it is hazardous in case of skin contact,eye contact, ingestion and inhalation. In case of contact, rinse affected area with plenty of water.
2) The stop solution provided with this kit is an acid solution. Wear protective gloves, clothing, and face protection.

3) Care should also be taken when handling the standard because of the known and unknown effects of it.
4) Care should also be taken to avoid contact of skin or eyes with other kit reagents or specimens. In the case of contact, wash immediately with water.
5) Do not pipette by mouth.
6) Avoid generation of aerosols.
7) Waste must be disposed of in accordance with federal, state and local environmental control regulations.
8) All blood components and biological materials should be handled as potentially hazardous. Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour a121.5^{0}c.

QUALITY CONTROL

1) It is recommended that all standards, controls and samples be run in duplicate. Standards and samples must be assayed at the same time.
2) The coefficient of determination of the standard curve should be $\geq$0.95.
3) Cover or cap all kit components and store at 2-8^{0}c when not in use.
4) Microtiter plates should be allowed to come to room temperature before opening the foil bags. Once the desired number of strips has been removed, immediately reseal the bag with desiccants and store at 2-8^{0}c to maintain plate integrity.
5) Sample should be collected in pyrogen/endotoxin-free tubes.
6) Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
7) When possible, avoid use of badly hemolyzedorlipemic serum .if large amounts of particulate matter are present, centrifuge or filter prior to analysis.
8) When pipetting reagents, maintain a consistent order of addition from well –to-well. This ensures equal incubation times for all wells.

9 .Don’t mix or interchange different reagent lots from various kit lots.

10. Do not use reagent after the kit expiration date.

11. Read absorbance immediately after adding the stop solution.

12. Incomplete washing will adversely affect the test outcome. All washing must be performed with wash solution provided. All residuals wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the well

13. Because TMB is light sensitive, avoid prolonged exposure to light. Also avoid contact between TMB and metal, otherwise color may develop.

Declaration

I the undersigned, declare that this MSc thesis is my original work and has not been presented for a degree in this or any other university and all sources of materials used for this thesis have been duly acknowledged.

Name: NegusuDawit (BSc)

Signature _____

Place _____

Date of submission _____

This thesis has been submitted with our approval as AU based advisors.

Aster Tsegaye (MSc, PhD, Associate Professor)

Department of Medical Laboratory Sciences

Addis Ababa University, Addis Ababa, Ethiopia

Signature _____

Place _____

Date _____

KassuDesta (MSc, PhD fellow) Assistant Professor

Department of Medical Laboratory Sciences

Addis Ababa University, Addis Ababa, Ethiopia

Signature _____

Place _____

Date _____