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A NOVEL LATERAL FLOW TEST STRIP FOR THE DETECTION OF CagA ANTIGEN OF *Helicobacter pylori* IN SERA OF INFECTED SUBJECTS

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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Date
June 2012

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MS Thesis - Biology
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Supervisor: Dr. Barık SALİH (Assoc. Prof.)

ABSTRACT

It is well known that half of the world populations are infected with *Helicobacter pylori*. The diagnosis of such infection exerts a big burden that is not easy to handle taking into considerations that millions of infected subjects suffer from severe pathological outcome. The CagA protein is considered to be one of the key virulence factors of *H. pylori*, since CagA-positive strains provoke severe mucosal damages and act as a risk factor for the development of peptic ulceration and gastric cancer. Our aim is to develop a lateral flow test strip based on double-antibody sandwich format using gold conjugated monoclonal antibody to detect the free CagA protein of *H. pylori* in sera of infected subjects and to correlate the presence of such protein with the severity of the disease and the clinical outcome. Two commercially available anti-CagA monoclonal antibodies were used. One was conjugated with a 20 nm gold nanoparticle to provide red color in positive reaction. These monoclonal antibodies were first evaluated in a dot blot test whether or not they will recognize the epitopes on our recombinant CagA protein then used in the test strip. The test strip was assembled from the following materials: sample pad, conjugate pad, membrane and absorbent pad. Optimization of the test was conducted to determine the concentration of monoclonal antibodies to be used for gold labeling, the concentration of the antigen and the type of the membrane to determine the speed of the reaction. The test strip was evaluated by testing serum samples supplemented with recombinant CagA protein. Several concentrations of the CagA protein were tested and the minimum concentration that gave a red color was 4 µg/ml. The control line always appeared red in color which confirms the proper working of the test strip. The test strip showed no cross-reactivity or interference with other bacterial antigens or serum proteins.

Keywords: *Helicobacter pylori*, Lateral flow test strip, CagA

ENFEKTE VAKALARIN SERUMLARINDA *Helicobacter pylori* CagA ANTİJENİNİN TESBİTİ İÇİN YENİ BİR LATERAL AKIM TEST ÇUBUĞU

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ÖZ

Dünya nüfusunun yarısının *Helicobacter pylori* ile enfekte olduğu çok iyi bilinmektedir. Bu enfeksiyonun teşhisi, enfekte vakaların milyonlarcasının şiddetli patolojik sonuçlardan muzdarip olduğu gerçeği kolaylıkla idrak edilemediğinden büyük bir güç sarfetmeyi gerektirir. CagA-pozitif suşları ciddi mukoza hasarına yol açıp mide ve duodenum ülserinde ve mide kanserinde risk faktörü olarak görev aldığından, CagA proteini *H. pylori*'nin önemli virülans faktörlerinden bir olarak kabul edilmektedir. Amacımız enfekte vakaların serumlarındaki serbest CagA proteininin kendisini belirlemek için altın bağlı monoklonal antikor kullanarak çift antikorlu sandiviç formatında bir lateral akım test çubuğu üretmek ve bu proteinin varlığıyla hastalığın derecesi ve klinik çıktısı arasındaki bağlantıyı bulmaya yardımcı olmak. Bunun için iki tane ticari mevcut anti-CagA monoklonal antikor kullanıldı. Bir tanesi positif reaksiyonda kırmızı rengi sağlamak için 20 nm altın nano parçacık ile konjuge edildi. Bu monoklonal antikorlar, bizim recombinant CagA proteinimiz üzerindeki epitopları tanıyıp tanımadığını tespit etmek için ilk olarak dot blot testinde değerlendirildi. Test çubuğu şu malzemelerden meydana gelmiştir: örnek pedi, konjugat pedi, membran ve emici ped. Testin optimizasyonu, altınla işaretlenebilmesi için monoklonal antikorların konsantrasyonlarını, antijenin konsantrasyonunu ve reaksiyonun hızını tespit etmek için membranın çeşidinin belirlenmesi ile sağlandı. Test çubuğu, rekombinant CagA proteini ilave edilmiş serum örnekleri test edilerek değerlendirildi. CagA proteinin birkaç konsantrasyonu test edildi ve kırmızı rengi veren minimum konsantrasyon 4 µg/ml olarak bulundu. Kontrol çizgisi test çubuğunun iyi bir şekilde çalıştığını doğrulayan kırmızı renk olarak her zaman göründü. Test çubuğu, diğer bakteri antijenleri ya da serum proteinleri ile çapraz reaksiyon veya engellenme göstermedi.

Anahtar Kelimeler: *Helicobacter pylori*, Lateral akım test çubuğu, CagA

DEDICATION

To my parents

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LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

BSA	Bovine serum albumin
cagA	Cytotoxin-associated gene A
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GTxMS	Goat anti-mouse
h	Hour
hCG	Human chorionic gonadotropin
HRP	Horseradish peroxidase
ICA	Immunochromatographic assay
IgG	Immunoglobulin G
LFIA	Lateral flow immunoassay
LFTS	Lateral flow test strip
McAb	Monoclonal antibody
min	Minute
PBS-T	Phosphate buffered saline containing Tween 20
PcAb	Polyclonal antibody
PCR	Polymerase chain reaction
PUD	Peptic ulcer diseases
rCagA	Recombinant CagA
RT	Room temperature
RUT	Rapid urease test
TMB	3,3’,5,5’-Tetramethylbenzidine
UBT	Urea breath test

CHAPTER 1

INTRODUCTION

Approximately 50 % of the world populations (over 3 billion) are known to be infected with *Helicobacter pylori* mainly in the developing countries (Kimmel et al., 2000). Eventhough the majority of subjects are asymptomatic, still few percentages (several millions) develop peptic ulceration that might progress to gastric cancer (Sipponen, 1998; Sipponen and Marshall, 2000). Thus, diagnosis of *H. pylori* infection is the most important step to be carried out for treatment and for prevention of the disease. Variations in the clinical outcome were found potentially due to high levels of genotypic diversity among *H. pylori* strains (Rudi et al., 1998; Saribasak et al., 2004). The CagA protein encoded by the cytotoxin-associated gene (cagA) is considered to be one of the key virulence factors of *H. pylori*, since *cagA*-positive strains provoke severe mucosal damages and act as a risk factor for the development of peptic ulceration and gastric cancer (Ong and Duggan, 2004). Unfortunately the cagA gene status can only be determined by molecular techniques (PCR) that requires the use of invasive method (endoscopy) for biopsy collection. It can however be evaluated indirectly by detecting serum anti-CagA antibodies by the ELISA test (a non-invasive approach) which indicates either current or previous infection with *H. pylori*. The lateral flow immunoassay (LFIA) is another alternative for the ELISA test that can be used in the detection of such antibodies in sera of infected subjects.

Our aim is to develop a novel lateral flow test strip using monoclonal antibodies to detect the free CagA protein of *H. pylori* rather than anti-CagA antibodies in sera of infected subjects. Such test will help a great deal to determine the active CagA status in *H. pylori* infected patients particularly those with gastritis since CagA protein plays an important role in the progression of the disease in such patients. In addition, it will help

to correlate the presence of such protein with the severity of the disease and the clinical outcome. It can also provide a predictive evaluation whether or not to treat such patients in order to block future progression to peptic ulceration and gastric cancer.

The LFIA strip that we will develop utilizes a sandwich-type format and will be validated by testing serum samples from infected subjects. No such test for the detection of *H. pylori* CagA antigen is being reported or commercially available. In addition and to the best of our knowledge this will be the first test worldwide that will detect the free CagA antigen in patient sera rather than its antibodies.

CHAPTER 2

LITERATURE REVIEW

2.1 *Helicobacter pylori* INFECTION

Infection with *Helicobacter pylori* generally leads to the development of chronic gastritis that progress over many years in a few percentages of infected patients to peptic ulcer diseases (PUD) and possibly to gastric cancer (Sipponen, 1998; Sipponen and Marshall, 2000). Several mechanisms are known to play a role in pathogenesis these include host immunological factors, environmental influences, and genetic variability of the bacterium (Fox and Wang, 2002). *H. pylori* is genetically diverse and certain strains are more virulent and cause more severe diseases than others which is also reflected on the clinical outcome. The cytotoxin-associated gene (*cagA*) encodes a highly immunodominant protein (CagA) of 120-140 kDa molecular weight that affects host cell physiology after being delivered to gastric epithelial cells (Yamaoka et al., 1998; Backert et al., 2000; Salih et al., 2007). The link between the expression of *cagA* and PUD in symptomatic subjects has been investigated. Several studies done on subjects from European countries (Portugal, Netherlands, Italy, Germany), USA and our study in Turkey showed that individuals with peptic ulceration are generally infected with *cagA*-positive *H. pylori* strains as compared to subjects with gastritis harboring *cagA*-negative strains (Navaglia et al., 1998; Rudi et al., 1998; Rudi et al., 2000; Fox and Wang, 2002; Troost et al., 2003; Saribasak et al., 2004). Thus, the CagA protein is considered to be one of the key virulence factors of *H. pylori*, since strains that are *cagA*-positive provoke the most severe mucosal lesions and also act as a risk factor for the development of peptic ulceration and gastric cancer (Ong and Duggan, 2004).

2.2 DIAGNOSIS OF *H. pylori* INFECTION

The diagnosis of *H. pylori* infection is an important approach for the selection of therapy and for the follow up of eradication success. *H. pylori* infection can be diagnosed by invasive and non-invasive techniques. The invasive technique (endoscopy) relies on the collection of gastric biopsy specimen that can be then tested by either rapid urease test, culture, PCR and/or histopathology (Ricci et al., 2007). This is a time consuming and expensive approach unlike the non-invasive tests such as ^{13}C-urea breath test (UBT), stool antigen test, and serology tests. The UBT and stool antigen tests detect the presence of *H. pylori* and are called active tests. Serology however (e.g. the ELISA test) detects anti-*H. pylori* antibodies which is an indicator of *H. pylori* exposure and is called passive test (Sciortino, 1993; Kimmel et al., 2000; Ricci et al., 2007). In addition all these tests are not informative since they do not provide a mean for the evaluation of the pathological potential of *H. pylori* and the CagA status (Sciortino, 1993; Kimmel et al., 2000). Therefore, searching for *cagA*-positive strains of *H. pylori* by more reliable methods will help identify populations at a greater risk for developing peptic ulceration and gastric cancer.

Increased prevalence of anti-CagA antibodies in both PUD and gastric cancer patients was reported (Navaglia et al., 1998; Kimmel et al., 2000). However there appears to be major geographic differences in the prevalence of cagA-positive strains. In Australia and in our study in Turkey, anti-CagA antibodies in duodenal ulcer group were of higher titers than those of the asymptomatic group, while in China these antibodies were prevalent in both asymptomatic and gastric cancer group (Mitchell et al., 1996; Abasiyanik et al., 2002). So there is a demand for the development of a test using monoclonal antibodies (McAbs) that specifically recognize the free CagA protein itself in sera of infected patients rather than its anti-CagA antibodies. Such test will provide a tool for the determination of pathological potential of *H. pylori* strains in symptomatic subjects, determine an active status of infection, provide a predictive evaluation for the progression of the disease and eliminate the need for additional endoscopies in the follow up of eradication therapy. Unlike the drop of anti-CagA antibody titers that occur slowly and may last for up to 2 years even after successful eradication, the drop of CagA antigen is expected to be much faster when the source of this antigen (*H. pylori* bacterium) will be eradicated.

The lateral flow test strip is another alternative for the ELISA test used in serological diagnosis of CagA antigen of *H. pylori*. We will develop our own test strip using monoclonal antibodies to detect the free CagA protein in sera of infected subjects.

2.3 LATERAL FLOW IMMUNOASSAY (LFIA) STRIPS

When antibodies are used as recognition elements, the lateral flow tests are called "lateral flow immunoassays"(LFIA) (Posthuma-Trumpie et al., 2009). The LFIA is an immunochromatographic assay (ICA) that is commercially available for the detection of a wide array of targets such as infectious agents (bacteria, viruses) (Nakasone et al., 2007; Cui et al., 2008; Kawatsu et al., 2008; Peng et al., 2008), hormones (Posthuma-Trumpie et al., 2008), drugs (Zhang et al., 2006; Zhu et al., 2008; Xie et al., 2009), pesticides (Zhou et al., 2004; Guo et al., 2009), mycotoxins (Kolosova et al., 2007; Wang et al., 2007b) etc.. It is intended particularly for a quick diagnosis and as a single use supporting test at a health care unit. A visual qualitative (on/off) signal is enough for most applications. The best-known and first developed (LFIA) was the pregnancy test for the detection of human chorionic gonadotropin (hCG) hormone in urine sample (Leuvering et al., 1980; van Amerongen et al., 1993). Several investigators have also developed their own lateral flow test strips for the detection of various target molecules (Tanaka et al., 2006; Wang et al., 2007a; Chiao et al., 2008; Zhao et al., 2008; Li et al., 2009; Xu et al., 2009; Omidfar et al., 2010; Wang et al., 2011; Yu et al., 2011). In general the lateral flow test strips have the advantages of being rapid (2-5 min), easy to use (self-performing), cost effective, portable, highly sensitive and specific. They do not require complicated equipment and technical expertise that are critical parameters for point-of-care, and have long shelf life at room temperature (12-24 months) (Posthuma-Trumpie et al., 2009; Ngom et al., 2010). In this section, it is purposed to review the components and the principles of lateral flow immunoassay (LFIA) devices and to compare the currently available rapid *H. pylori* commercial test devices.

2.3.1 Principles of the LFIA

The LFIA strips are designed to detect the presence of an analyte (antigen or antibody) by a specific labeled-antigen or labeled-antibody. The test strip consists of four sections; sample pad (cellulose), conjugate pad (glass fiber), membrane (nitrocellulose) and absorbent pad (cellulose) which are laminated onto a sheet of

plastic backing orderly to allow cutting and handling (Figure 2.1). The pads overlap the membrane to allow a continuous flow path for the sample. The sample pad allows the diffusion of the sample into the conjugate pad that is impregnated with detector reagent (labeled-antigen or labeled-antibody) depending on the application. If the sample contains an analyte, it will bind to the detector reagent and the complex will continue to flow and then irreversibly binds capture reagent (antigen or antibody) at the test line on the membrane and forms a colored line. A continuous flow of the sample through the strip toward the control line will form a second colored line that indicates a proper function of the test device. The absorbent pad at the end of the strip wicks the fluid through the membrane to ensure a continuous flow and thus maintains a clear background. Strips can be housed in a plastic holder (cassette), where only the sample application window and a reading window are exposed, for protection and easier handling (Millipore, 2008; Posthuma-Trumpie et al., 2009; Ngom et al., 2010).

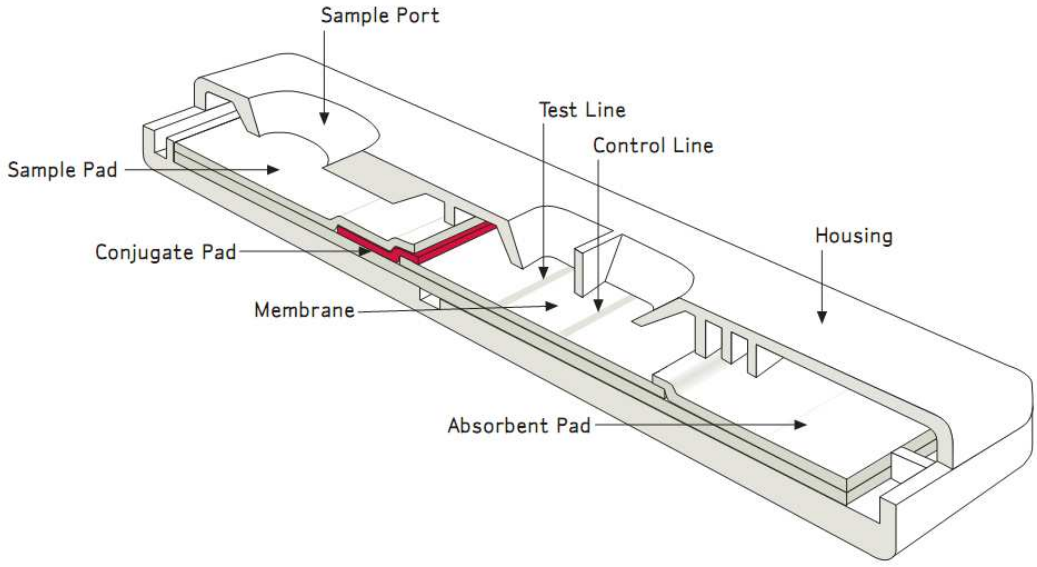


Figure 2.1 Schematic view of a lateral flow test strip (Millipore, 2008)

2.3.1.1 Antibodies

The affinity of the specific antibody mostly influences the sensitivity of the test. Both monoclonal and polyclonal antibodies have been used in these tests; however the type of such antibodies used in commercial LFIA strips were not usually referred to in the manufacturer data sheets. Several investigators used either monoclonal antibodies or

polyclonal antibodies both in the conjugate pad and on the test line (Shyu et al., 2002; Chiao et al., 2004; Kawatsu et al., 2006; Tanaka et al., 2006; Nakasone et al., 2007; Chiao et al., 2008; Kawatsu et al., 2008; Peng et al., 2008; Jiang et al., 2011; Yu et al., 2011). Others used both monoclonal and polyclonal antibodies on the same strip (Wang et al., 2011; Yang et al., 2011). The control line is coated with primary or secondary (anti-IgG) antibody depending on the application to capture excess detector reagents regardless of the presence or absence of target analyte (Posthuma-Trumpie et al., 2009; Ngom et al., 2010). The control line and the clear background that appear on the reading window are the indicators of an internal positive and internal negative procedural control.

2.3.1.2 Label Particles

The majority of the available commercial LFIA strips (around 94 %) use colloidal gold particles of red to pink in color for labeling, while the rest uses colored latex particles. According to the recent review articles many investigators also used colloidal gold particles for labeling (Posthuma-Trumpie et al., 2009; Ngom et al., 2010) while few others used colored latex particles (Gussenhoven et al., 1997; Greenwald et al., 2003). The size of the gold particles varies between 2-150 nm, but generally 15-25 nm particles were used (Zhou et al., 2004; Zhang et al., 2006; Nakasone et al., 2007; Zhao et al., 2008; Li et al., 2009; Xie et al., 2009; Omidfar et al., 2010). The advantages of gold particles are being stable, easy to use and have convenient surfaces to accelerate the antibody-antigen recognitions, which increases the immunoassay signals and allow a smooth flow through the membrane (DiScipio, 1996).

2.4 LFIA STRIPS FOR THE DIAGNOSIS OF *H. pylori*

Several LFIA strips are currently commercially available for the diagnosis of *H. pylori* infection. This is a qualitative test that is used either to detect anti-*H. pylori* antibodies in blood samples (whole blood, serum and/or plasma) or to detect *H. pylori* antigens in stool samples. Both are intended to aid in the diagnosis of *H. pylori* infection in adult patients with symptoms of gastrointestinal disorders and to monitor the success of eradication in treated patients. In this review 22 commercially available anti-*H. pylori* antibody test strips (Table 2.1 and Table 2.2) and 14 *H. pylori* antigen test strips (Table 2.3 and Table 2.4) were compared.

Table 2.1 Commercially available lateral flow test strips for the detection of antibodies to *H. pylori* in serum samples.

Commercial test	**Manufacturer**	**Country**	**Reference**
Rapid Response™ *H. pylori* Rapid Test	BTNX Inc.	Canada	http://www.btnx.com
CLIAwaived™ *H. pylori* Rapid Test	CLIAwaived ™, Inc.	USA	http://www.cliawaived.com
OneStep *H. pylori* RapiCard™ Serum InstaTest	Cortez Diagnostics, Inc.	USA	http://www.rapidtest.com
INSTANT-VIEW® *H. pylori* Rapid Test	American Screening Corporation	USA	http://www.americanscreeningcorp.com
Clarity *H. pylori* Rapid Test	Diagnostic Test Group, LLC.	USA	http://www.claritydiagnostics.com
BioStar® Acceava® *H. pylori* Test	Inverness Medical–BioStar Inc.	USA	http://www.invernessmedicalpd.com
ICON® HP-One-Step Anti-*H. pylori* Antibody Test	Beckman Coulter, Inc.	USA	https://www.beckmancoulter.com
BioSign® *H. pylori* WB	Princeton BioMeditech Corporation	USA	http://www.drugtesting.co.za
QuickView™ *H. Pylori* Antibody Test	LumiQuick Diagnostics, Inc.	USA	http://www.lumiquick.com
Immunospec *H. Pylori* Antibody Test	Immunospec Corporation	USA	http://www.immunospec.com
Clearview® *H. pylori* Test	Inverness Medical–BioStar Inc.	USA	http://www.invernessmedicalpd.com
iScreen™ *H. pylori* Rapid Test	Instant Technologies, Inc.	USA	http://www.cliawaived.com

Table 2.1 Continued.

Commercial test	**Manufacturer**	**Country**	**Reference**
H. pylori Rapid Test	Innovacon, Inc.	USA	http://www.mddoctorsdirect.com
ACON® *H. pylori* Rapid Test	ACON Laboratories, Inc.	USA	http://www.accessdata.fda.gov
QuickVue® One-Step *H. pylori* gII® Test	Quidel Corporation	USA	http://www.quidel.com
SureStep™ *H. pylori* test	Applied Biotech, Inc.	USA	http://www.cliawaived.com
LINK2™ *H. pylori* Rapid Test	Becton Dickinson	USA	http://www.bd.com
NOVAtest® One-Step *H. pylori* Test	Atlas Link Biotech. Co., Ltd	China	http://www.ivdpretest.com
Accu-Tell® *H. pylori* Test	AccuBioTech Co., Ltd.	China	http://www.accubiotech.com
Helicobacter pylori Serum Antikorları Kart Test	RTA Labs	Turkey	http://www.rtalabs.com.tr
RAPIRUN *H. pylori* Antibody Detection Kit	Otsuka Pharmaceutical Co., Ltd.	Japan	http://www.accessdata.fda.gov
ASSURE® *H. pylori* Rapid Test	MP Biomedicals Asia Pacific Pte Ltd.	Singapore	http://www.mpbio.com

Table 2.2 Principles and performances of commercially available lateral flow test strips for the detection of antibodies to *H. pylori* in serum samples.

Commercial test	**Assay Format**	**Label**	**Sensitivity**	**Specificity**
Rapid Response™ *H. pylori* Rapid Test	NA	NA	95.1 %	94.1 %
CLIAwaived™ *H. pylori* Rapid Test	NA	NA	95.1 %	94.1 %
OneStep *H. pylori* RapiCard™ Serum InstaTest	Sandwich	Colloidal gold	95.1 %	94.1 %
INSTANT-VIEW® *H. pylori* Rapid Test	Sandwich	Colloidal gold	95.1 %	94.1 %
Clarity *H. pylori* Rapid Test	Sandwich	Colloidal gold	95.9 %	89.1 %
BioStar® Acceava® *H. pylori* Test	Sandwich	Colloidal gold	95.9 %	89.1 %
ICON® HP-One-Step Anti-*H. pylori* Antibody Test	Sandwich	Colloidal gold	95.9 %	89.1 %
BioSign® *H. pylori* WB	Sandwich	Colloidal gold	95.9 %	89.1 %
QuickView™ *H. Pylori* Antibody Test	Sandwich	Colloidal gold	100 %	97 %
Immunospec *H. Pylori* Antibody Test	Sandwich	Colloidal gold	100 %	97 %
Clearview® *H. pylori* Test	Indirect	NA	89 %	89 %

NA: Not available

Table 2.2 Continued.

Commercial test	**Assay Format**	**Label**	**Sensitivity**	**Specificity**
iScreen™ *H. pylori* Rapid Test	Indirect	NA	89 %	89 %
H. pylori Rapid Test	Indirect	NA	89 %	89 %
ACON® *H. pylori* Rapid Test	NA	NA	89 %	89 %
QuickVue® One-Step *H. pylori* gII® Test	Indirect	NA	90 %	78 %
SureStep™ *H. pylori* test	Sandwich	Colloidal gold	92.6 %	82.4 %
LINK2™ *H. pylori* Rapid Test	Sandwich	Blue latex	88.6 %	85.1 %
NOVAtest® One-Step *H. pylori* Test	Sandwich	Colloidal gold	95.9 %	89.6 %
Accu-Tell® *H. pylori* Test	Sandwich	Colloidal gold	92 %	96.6 %
Helicobacter pylori Serum Antikorları Kart Test	Sandwich	Colloidal gold	92 %	96.6 %
RAPIRUN *H. pylori* Antibody Detection Kit	Indirect	Colloidal gold	84.71 %	89.32 %
ASSURE® *H. pylori* Rapid Test	Indirect	Colloidal gold	92 %	93 %

NA: Not available

Table 2.3 Commercially available lateral flow test strips for the detection of *H. pylori* antigens in stool samples.

Commercial test	**Manufacturer**	**Country**	**Reference**
ImmunoCard STAT!® HpSA	Meridian Bioscience Inc.	USA	http://www.meridianbioscience.com
QuickView™ *H. pylori* Antigen Test	LumiQuick Diagnostics, Inc.	USA	http://www.lumiquick.com
Immunospec *H. pylori* Antigen Test	Immunospec Corporation	USA	http://www.immunospec.com
OneStep *H. pylori* Antigen RapiCard™ InstaTest	Cortez Diagnostics, Inc.	USA	http://rapidtest.com
HELISTOOL *H. pylori* Gaita Ag Kart Test	RTA Labs	Turkey	http://www.rtalabs.com.tr
Rapid *H. pylori* Antigen Test	Boson Biotech Co., Ltd.	China	http://www.bosonbio.com
Helicobacter pylori Stool Antigen (HPSA) Rapid Test	Jei Daniel Biotech Corp.	China	http://jdbiotech.en.alibaba.com
StrongStep® *H. pylori* Antigen Rapid Test	Liming Bio-Products Co., Ltd.	China	http://www.limingbio.com
Accu-Tell® *H.pylori* Antigen Rapid Test	AccuBioTech Co., Ltd.	China	http://www.accubiotech.com
RAPID Hp StAR™	Oxoid (Ely) Ltd.	UK	http://www.oxoid.com
H. pylori Antigen Rapid Test	Globe Diagnostics S.r.l.	Italy	http://www.gdsrl.com

Table 2.3 Continued.

Commercial test	**Manufacturer**	**Country**	**Reference**
SD BIOLINE *H. pylori* Ag Rapid Test	Standard Diagnostics, Inc.	Korea	http://www.standardia.com
H. pylori Ag Card Test	AXIOM Diagnostics	Germany	http://www.axiom-mms.com
Rapidan Tester® *H. pylori* Antijen Test	Türklab	Turkey	http://www.turklab.com.tr

Table 2.4 Principles and performances of commercially available lateral flow test strips for the detection of *H. pylori* antigens in stool samples.

Commercial test	**Assay Format**	**Label**	**Sensitivity**	**Specificity**
ImmunoCard STAT!® HpSA	Sandwich	Red latex	90.6 %	91.5 %
QuickView™ *H. pylori* Antigen Test	Sandwich	Colloidal gold	94.0 %	96.7 %
Immunospec *H. pylori* Antigen Test	Sandwich	Colloidal gold	94.0 %	96.7 %
OneStep *H. pylori* Antigen RapiCard™ InstaTest	Sandwich	Colloidal gold	94.0 %	96.7 %
HELISTOOL *H. pylori* Gaita Ag Kart Test	Sandwich	Colloidal gold	94.0 %	96.7 %
Rapid *H. pylori* Antigen Test	Sandwich	Colloidal gold	94.0 %	96.7 %
Helicobacter pylori Stool Antigen (HPSA) Rapid Test	Sandwich	Colloidal gold	94 %	96.7 %
StrongStep® *H. pylori* Antigen Rapid Test	Sandwich	NA	98.5 %	98.1 %
Accu-Tell® *H.pylori* Antigen Rapid Test	Sandwich	Colloidal gold	99.9 %	99.9 %
RAPID Hp StAR™	Sandwich	Colloidal gold	91.2 %	82.4 %

NA: Not available

Table 2.4 Continued.

Commercial test	**Assay Format**	**Label**	**Sensitivity**	**Specificity**
H. pylori Antigen Rapid Test	Sandwich	Red polystyrene	4-8 ng/mL	95 %
SD BIOLINE *H. pylori* Ag Rapid Test	Sandwich	Colloidal gold	100 %	100 %
H. pylori Ag Card Test	Sandwich	Colloidal gold	94.0 %	98.6 %
Rapidan Tester® *H. pylori* Antijen Test	Sandwich	NA	99.9 %	99.9 %

NA: Not available

2.4.1 LFIA for the Detection of Anti-*H. pylori* Antibodies

The test strips are available in two formats:

2.4.1.1 Sandwich Format

In this format the *H. pylori* antigens coated on gold particles were placed into the conjugate pad, *H. pylori* unlabeled antigens were immobilized on the test line and similarly anti-*H. pylori* antibodies were immobilized on the control line (Figure 2.2a). The addition of a sample drop (Figure 2.2b) onto the sample pad leads to a lateral flow of the sample fluid containing anti-*H. pylori* antibodies toward the conjugate pad where it binds to the antigen coated on gold particles. The complex then flow to the test line where it binds to the immobilized antigen and result in a red color line. The flow of the sample fluid will continue toward the control line where the remaining antigen coated gold particles will bind to the immobilized anti-*H. pylori* antibodies and give a red color line.

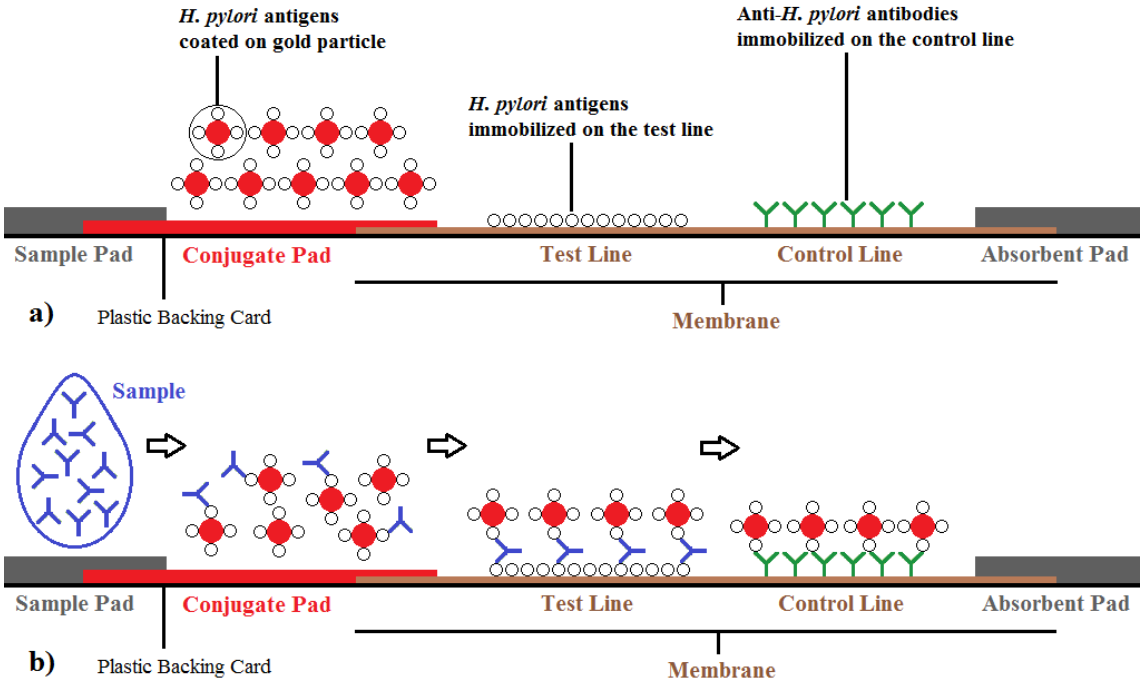


Figure 2.2 Schematic representation of a lateral flow test strip (sandwich format for the detection of anti-*H. pylori* antibodies) showing the constituents of the strip before (a) and after (b) the addition of the sample.

2.4.1.2 Indirect Format

In this format either *H. pylori* antigens (Figure 2.3) or antibodies (Figure 2.4) were immobilized on the test line. In the antigen coated indirect format (Figure 2.3), anti-human IgG antibodies coated on gold particles were placed into the conjugate pad, *H. pylori* unlabeled antigens were immobilized on the test line and similarly protein A were immobilized on the control line (Figure 2.3a). The addition of a sample drop (Figure 2.3b) onto the sample pad leads to a lateral flow of the sample fluid containing specific antibodies toward the conjugate pad where it binds to the antibody coated on gold particles. The complex then flow to the test line where it binds to the immobilized antigen and result in a red color line. The flow of the sample fluid will continue toward the control line where the remaining immune-complexes will bind to the immobilized protein A and give a red color line.

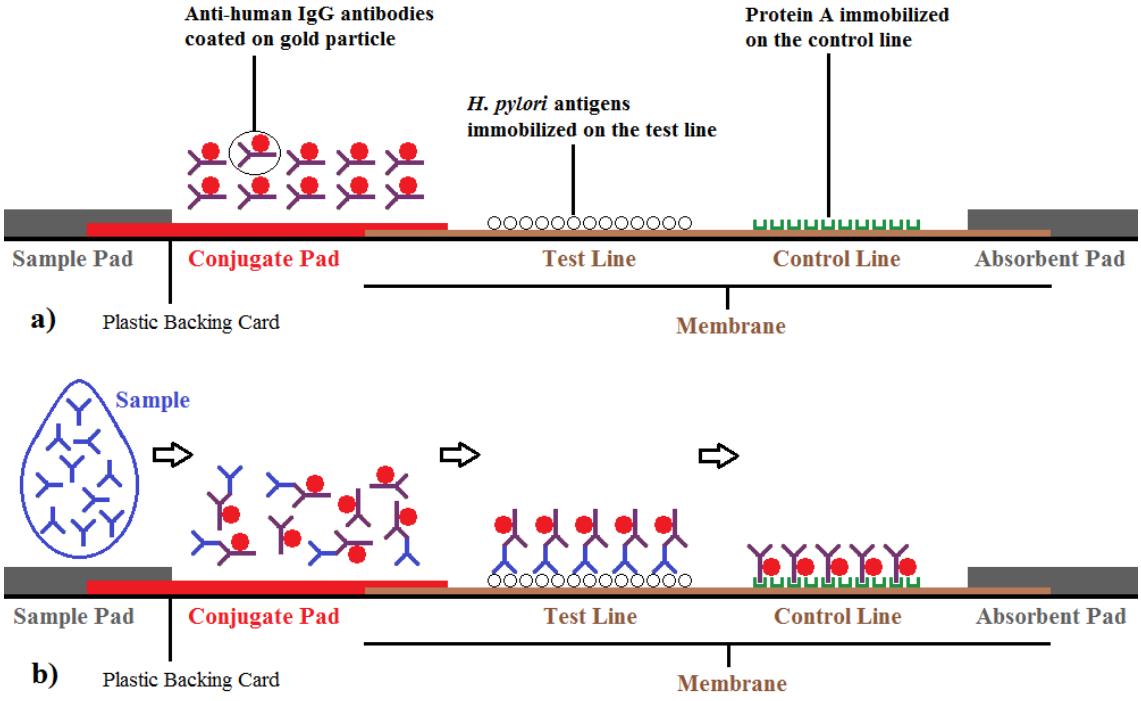


Figure 2.3 Schematic representation of a lateral flow test strip (indirect format using antigen immobilized on the test line for the detection of anti-*H. pylori* antibodies) showing the constituents of the strip before (a) and after (b) the addition of the sample.

In the antibody coated indirect format (Figure 2.4), *H. pylori* antigens coated on gold particles were placed into the conjugate pad, anti-human IgG antibodies were immobilized on the test line and similarly anti-*H. pylori* antibodies were immobilized on the control line (Figure 2.4a). The addition of a sample drop (Figure 2.4b) onto the sample pad leads to a lateral flow of the sample fluid containing specific antibodies toward the conjugate pad where it binds to the antigen coated on gold particles. The complex then flow to the test line where it binds to the immobilized antibody and result in a red color line. The flow of the sample fluid will continue toward the control line where the remaining antigen coated gold particles will bind to the immobilized anti-*H. pylori* antibodies and give a red color line.

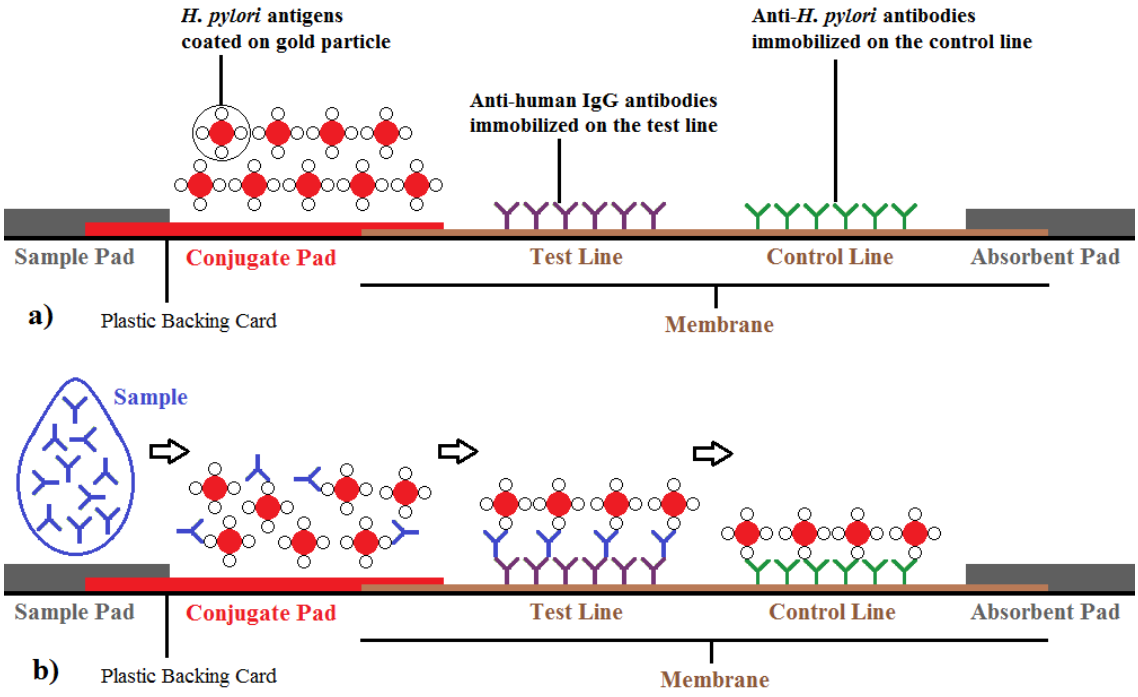


Figure 2.4 Schematic representation of a lateral flow test strip (indirect format using antibody immobilized on the test line for the detection of anti-*H. pylori* antibodies) showing the constituents of the strip before (a) and after (b) the addition of the sample.

The sensitivity and specificity of these test strips were compared with culture, histology, RUT, UBT and/or ELISA. According to the manufacturer data sheets the sensitivity of different commercially available tests were in the range of 85-100 % (average 93 %) while the specificity were in the range of 85-100 % (average 90 %). Of these 50 % showed a sensitivity of >95 %, while only 20 % had a specificity of >95 %.

The anti-*H. pylori* IgG used in these test strips showed no cross reactivity when reacted with closely related organisms such as *C. jejuni*, *C. fetus*, *C. coli*, and *E. coli*.

2.4.2 LFIA for the Detection of *H. pylori* Antigens

The test strips detecting *H. pylori* antigens were based on the sandwich format (Figure 2.5). On the test strip an anti-*H. pylori* antibodies coated on gold particles were placed into the conjugate pad, another anti-*H. pylori* antibodies were immobilized on the test line and similarly anti-IgG antibodies were immobilized on the control line (Figure 2.5a). The addition of a sample drop (Figure 2.5b) onto the sample pad leads to a lateral flow of the sample fluid containing antigens toward the conjugate pad where it binds to the antibody coated on gold particles. The complex then flow to the test line where it binds to the immobilized antibody and result in a red color line. The flow of the sample fluid will continue toward the control line where the remaining antibody coated gold particles will bind to the immobilized anti-IgG antibodies and give a red color line.

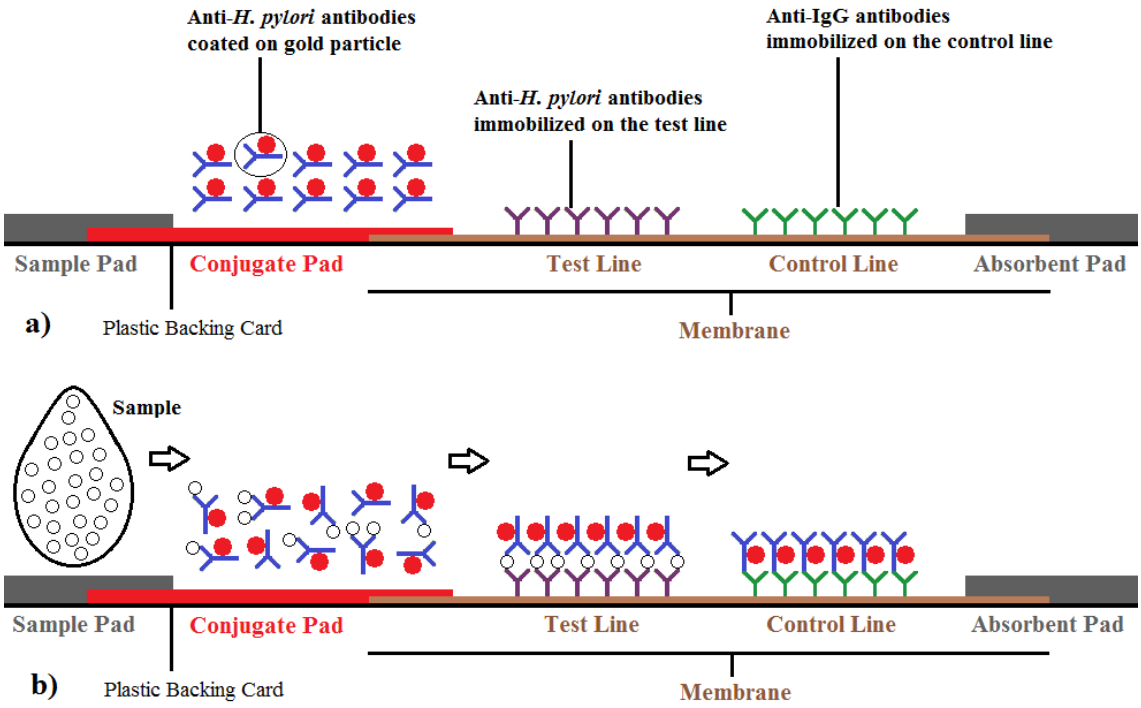


Figure 2.5 Schematic representation of a lateral flow test strip (sandwich format for the detection of *H. pylori* antigens) showing the constituents of the strip before (a) and after (b) the addition of the sample.

The performance characteristics of rapid *H. pylori* antigen test strips have been evaluated by comparison with culture, histology, RUT, UBT or ELISA. The sensitivity of the test strips were in the range of 90-100 % (average 95 %) while the specificity were in the range of 80-100 % (average 96 %). The test showed no cross reactivity when stools were spiked with a list of microorganisms (manufacturer data sheets).

CHAPTER 3

MATERIALS AND METHOD

3.1 MATERIALS

3.1.1 Immunoreagents

Monoclonal antibodies: Three commercially available antibodies were used.

1. Monoclonal antibody B237H (Abcam, Cambridge, England) that recognizes an epitope localized within the 580 amino acids of *H. pylori* CagA C-terminal end.
2. Monoclonal antibody B818M (Abcam, Cambridge, England) that recognizes an epitope localized between 562 and 795 amino acids of *H. pylori* CagA.
3. Monoclonal antibody A-10 (Santa Cruz Biotech., CA, USA) that recognizes an epitope localized within the 1-300 amino acids of *H. pylori* CagA.

Polyclonal antibodies: Two commercially available antibodies were purchased.

1. Polyclonal goat anti-mouse IgG (AP124) (Millipore, USA).
2. Polyclonal goat anti-mouse IgG-HRP (sc-2005) (Santa Cruz Biotech., CA, USA).

Antigen:

A recombinant CagA antigen (rCagA) prepared in our laboratory by cloning the *cagA* gene of *H. pylori* strain isolated from a Turkish patient (Z. Ulupınar, MS Thesis) was used in this study.

3.1.2 Chemicals

Colloidal gold (20nm) was obtained from Sigma–Aldrich (MO, USA) for labeling the antibodies. Sucrose, bovine serum albumin (BSA) for blocking of the antibody immobilized nitrocellulose membrane, NaCI, Tris, Tween 20, phosphate buffered saline (PBS) (pH 7,4), sodium azide (NaN_3) for preserving the proteins in blocking and diluting solutions, sodium carbonate, sodium borate, EDTA and Protein Quantification Kit-Rapid (Sigma–Aldrich, MO, USA); nutrient agar (Merck, Germany); brucella broth (Becton-Dickinson, USA); 5 % sheep blood agar (Salubris, Istanbul, Turkey); fetal bovine serum (FBS) and TMB substrate (Invitrogen, USA). Silica gel from the commercial products was used as desiccant.

3.1.3 Membranes

For the development of immunochromatographic assay the following materials were used:

1. Cellulose fiber sample and absorbent pad (Millipore, MA, USA)
2. Glass fiber conjugate pad (Millipore, MA, USA)
3. Hi-Flow Plus 240 Membrane Card (Millipore, MA, USA)
4. Hi-Flow Plus 90 Membrane Card (Millipore, MA, USA)

3.2 PREPARATION OF MONOCLONAL ANTIBODY-GOLD CONJUGATES

The anti-CagA monoclonal antibodies (B237H and B818M) were conjugated to 20 nm colloidal gold as described previously (Yokota and Fujimori, 1992). Briefly, 20 nm colloidal gold solution was adjusted to pH 9.0 with 0.2 mM sodium carbonate (Figure 3.1). The pH was measured with pH paper (Merck, Germany) in order not to damage colloidal gold with electrode of pH meter.

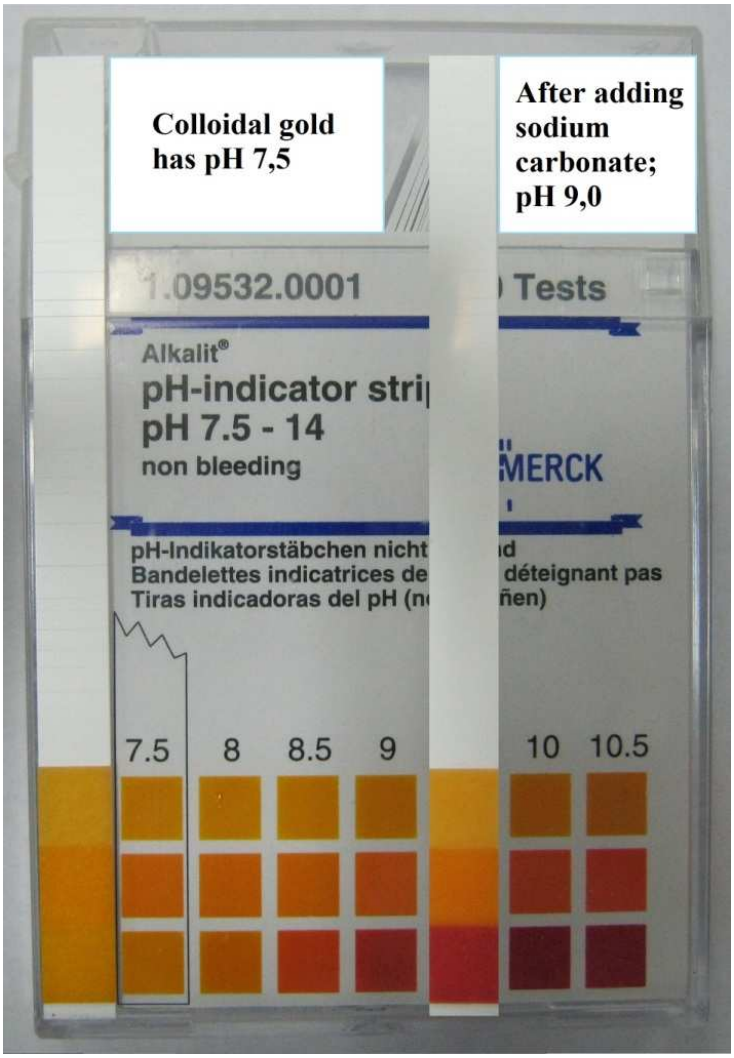


Figure 3.1 pH indicator strip for the adjustment of colloidal gold solution.

The optimum protein concentration for labeling was determined by the following steps: 25 µl of anti-CagA McAb solution was 2-fold diluted in double deionized water and then 25 µl of colloidal gold solution was added. Mixtures were incubated for 15 min at room temperature and then 100 µl of 10 % NaCl solution was added. The color of samples gradually changed from brilliant red to blue as the concentration of McAb decreased. The optimum concentration of McAb for colloidal gold labeling was the lowest concentration of McAb solution that did not change color.

200 µl of anti-CagA McAb solution at the optimum concentration was added drop by drop to 1 ml colloidal gold solution (pH 9.0) stirred vigorously on magnetic shaker and then incubated for 45 min at room temperature. After adding 100 µl of 10 % BSA solution in 20 mmol/l sodium borate (pH 9.0), the mixture was incubated for another 15

min at room temperature. Labeled McAb was washed two times with 20 mmol/l sodium borate (pH 9.0) containing 1 % BSA and 0.1 % sodium azide then centrifuged at 25000×g at 10 °C for 30 min with Beckman Coulter Allegra 64R benchtop centrifuge (USA). The precipitate was resuspended in 1 ml washing buffer and stored at 4 °C until used.

3.3 DOT BLOT ASSAYS

The dot blot assays were used to confirm that the 3 McAb will recognize our rCagA protein before using them in the LFTS. Three different dot blot formats were used for this purpose.

3.3.1 Indirect Assay

Anti-CagA monoclonal antibodies (B237H and B818M) were tested for immunoreactivity using the indirect dot blot assay. This was used to confirm that these antibodies recognize epitopes of our recombinant CagA protein (rCagA).

Principles

- Using narrow-mouth pipet tip, 2 µl of CagA-positive *H. pylori* whole-cell lysate, rCagA and anti-CagA McAb (diluted in PBS containing 0,01 % Tween 20) were spotted onto the nitrocellulose membrane (1 x 2,5 cm). The anti-CagA McAb was used as internal positive control.
- The membrane was let to dry for 30 min at RT.
- Non-specific sites were blocked by soaking into 5 % BSA in PBS-T (Phosphate buffered saline containing 0,05 % Tween 20) for 1 hr at RT.
- The membrane was then incubated with 1 µg/ml primary antibody (anti-CagA McAb) dissolved in PBS-T containing 0,1 % BSA for 30 min at RT with shaking at low speed.
- The membrane was washed three times with PBS-T (3 x 5 min).
- The membrane was incubated with 0,2 µg/ml secondary antibody (goat anti-mouse IgG-HRP) dissolved in PBS-T containing 0,1 % BSA for 30 min at RT with shaking at low speed.

- The membrane was washed three times with PBS-T (15 min, 2 x 5 min), then once with PBS (5 min) on the shaker.
- After that, the membrane was incubated with 50 µl TMB substrate for 5 min.
- Color change was an indicator for a positive reaction.
- In addition, 2 µl of rCagA was spotted onto a separate nitrocellulose membrane. After the blocking, the membrane was incubated with goat anti-mouse IgG-HRP and TMB substrate was added to act as a negative control for secondary antibody.

3.3.2 Direct Assay

Gold labeled anti-CagA McAb conjugates were tested for immunoreactivity using the direct dot blot assay.

Principles

- 2 µl of CagA-positive *H. pylori* whole-cell lysate, rCagA and GTxMS PcAb which was diluted in PBS containing 0,01 % Tween 20 and 0,1 % BSA were spotted onto the nitrocellulose membrane (1 x 2,5 cm). The GTxMS PcAb was used as internal positive control.
- The membrane was let to dry for 30 min at RT.
- Non-specific sites were blocked by soaking into 5 % BSA in PBS-T (Phosphate buffered saline containing 0,02 % Tween 20) for 0.5-1 hr at RT.
- Then, the membrane was incubated with colloidal gold conjugated anti-CagA McAb solution for overnight at RT with shaking at low speed.
- The membrane was washed three times with PBS-T (3 x 5 min), then once with PBS (5 min) on the shaker.
- The presence of red color is an indicator of positive reaction.

3.3.3 Sandwich Assay

Gold labeled anti-CagA McAb conjugates were tested by the sandwich assay for the detection of rCagA protein. The B237H McAb was used as the detection antibody, while the B818M McAb was used as capture antibody.

Principles

- 2 µl of diluted concentrations of the B818M McAb diluted in PBS containing 0,01 % Tween 20 and 0,1 % BSA were spotted onto the nitrocellulose membrane (1 x 2,5 cm). Also 2 µl of rCagA and GTxMS PcAb diluted in PBS containing 0,01 % Tween 20 and 0,1 % BSA were spotted onto the nitrocellulose membrane as internal positive controls.
- The membrane was let to dry for 30 min at RT.
- Non-specific sites were blocked by soaking into 5 % BSA in PBS-T (Phosphate buffered saline containing 0,05 % Tween 20) for 0.5-1 hr at RT.
- The membrane was incubated with 10 µg/ml rCagA antigen solution in PBS for 6 hour at RT with shaking at low speed.
- It was washed three times with PBS-T (3 x 5 min) on the shaker.
- Then, the membrane was incubated with colloidal gold conjugated anti-CagA B237H McAb solution for overnight at RT with shaking at low speed.
- The membrane was washed three times with PBS-T (3 x 5 min), then once with PBS (5 min) on the shaker.
- The presence of red color is an indicator of positive reaction.

3.4 INDIRECT DOT BLOT ASSAY TO TEST CROSS-REACTIVITY

Anti-CagA McAb B237H, B818M (Abcam) and McAb A-10 (Santa Cruz) were used to test cross-reactivity with closely related microorganisms by using the indirect dot blot assay.

Principles

- 2 µl of cell lysates of *E. coli*, *P. vulgaris*, *S. aureus*, CagA-positive *H. pylori* strain 722, CagA-positive *H. pylori* strain 13206 and rCagA were spotted onto the nitrocellulose membrane (1 x 2,5 cm). The rCagA was used as internal positive control.
- The membrane was let to dry for 30 min at RT.
- Non-specific sites were blocked by soaking into 5 % BSA in PBS-T (Phosphate buffered saline containing 0,05 % Tween 20) for 0.5-1 hr at RT.

- Then, the membrane was incubated with 1 µg/ml primary antibody (anti-CagA McAb) dissolved in PBS-T containing 0,1 % BSA for 30 min at RT with shaking at low speed.
- The membrane was washed three times with PBS-T (3 x 5 min).
- The membrane was incubated with 0,2 µg/ml secondary antibody (goat anti-mouse IgG-HRP) dissolved in PBS-T containing 0,1 % BSA for 30 min at RT with shaking at low speed.
- The membrane was washed three times with PBS-T (15 min, 2 x 5 min), then once with PBS (5 min) on the shaker.
- After that, the membrane was incubated with 50 µl TMB substrate for 5 min.
- Color change was an indicator for a positive reaction.

3.5 LATERAL FLOW TEST STRIP (LFTS) PREPARATION

The test strip consists of the following materials:

a. Membrane: Two types of nitrocellulose membranes with different flow time were used.

    1. Hi-Flow Plus 240 Membrane Card (Millipore, MA, USA): The membrane has a nominal capillary flow time of 240s/4cm and a nominal membrane thickness of 135 µm direct cast onto 50 µm polyester backing (~185 µm overall thickness). The card is supplied with an acrylic PSA adhesive with coated-paper release liners.
    2. Hi-Flow Plus 90 Membrane Card (Millipore, MA, USA): The membrane has a nominal capillary flow time of 90s/4cm and a nominal membrane thickness of 135 µm direct cast onto 50 µm polyester backing (~185 µm overall thickness). The card is supplied with an acrylic PSA adhesive with coated-paper release liners.

a. Conjugate pad: A 0,5 x 1 cm pad made of glass fiber that is laminated between the sample pad and the membrane. Labeled monoclonal antibodies are placed into this pad.
b. Sample pad: The sample pad is made of cellulose fiber and used to distribute the sample to the conjugate pad.

c. Absorbent pad: The absorbent pad is made of cellulose fiber and its function is to wick the sample fluid through the membrane by capillary force.

3.6 PREPARATION OF THE CONJUGATE PAD

The conjugate pad was impregnated with gold labeled anti-CagA McAb-B237H (Abcam, Cambridge, England).

3.7 IMMOBILIZATION OF CAPTURE REAGENTS

2 µl of anti-CagA McAb-B818M was dispensed on the Hi-Flow Plus 240 Membrane Card (test line). 2 µl of goat anti-mouse (GTxMS) PcAb was dispensed on the Hi-Flow Plus 240 Membrane Card (control line) (Figure 3.2). After drying for 1h at 37 °C with desiccant, non-specific sites on the membrane were blocked with 4 % BSA in PBS-T (0,04 % Tween 20) for 1h. The membrane was washed once with PBS-T (0,04 % Tween 20), dried and stored at RT with desiccant.

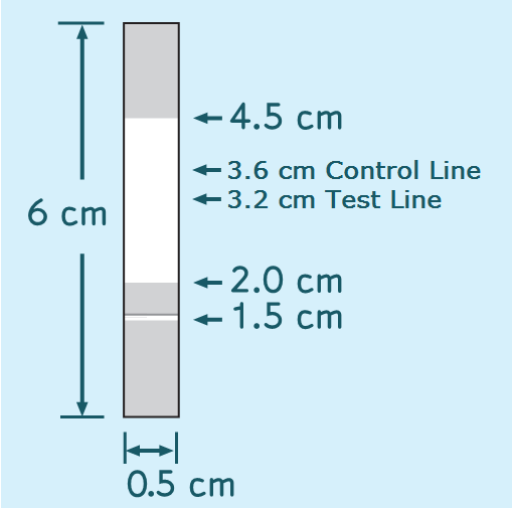


Figure 3.2 Hi-Flow Plus 240 Membrane Card dimensions.

3.8 TEST STRIP ASSEMBLY

The 3 pads were overlaid on the membrane to ensure a continuous flow and proper function of the strip as illustrated in Figure 3.3-1,2. The absorbent pad (5 x 20 mm), conjugate pad (5 x 10 mm) and sample pad (5 x 20 mm) were attached in proper alignment to the Hi-Flow Plus 240 or 90 Membrane Card (5 x 60 mm) and stored in the presence of desiccant gel at RT. The strip was housed in a plastic cassette, where only

the sample application window and a reading window were exposed, for protection and easier handling (Figure 3.3-3,4). The strip was ready to use for the detection of CagA antigen of *H. pylori* in serum samples.

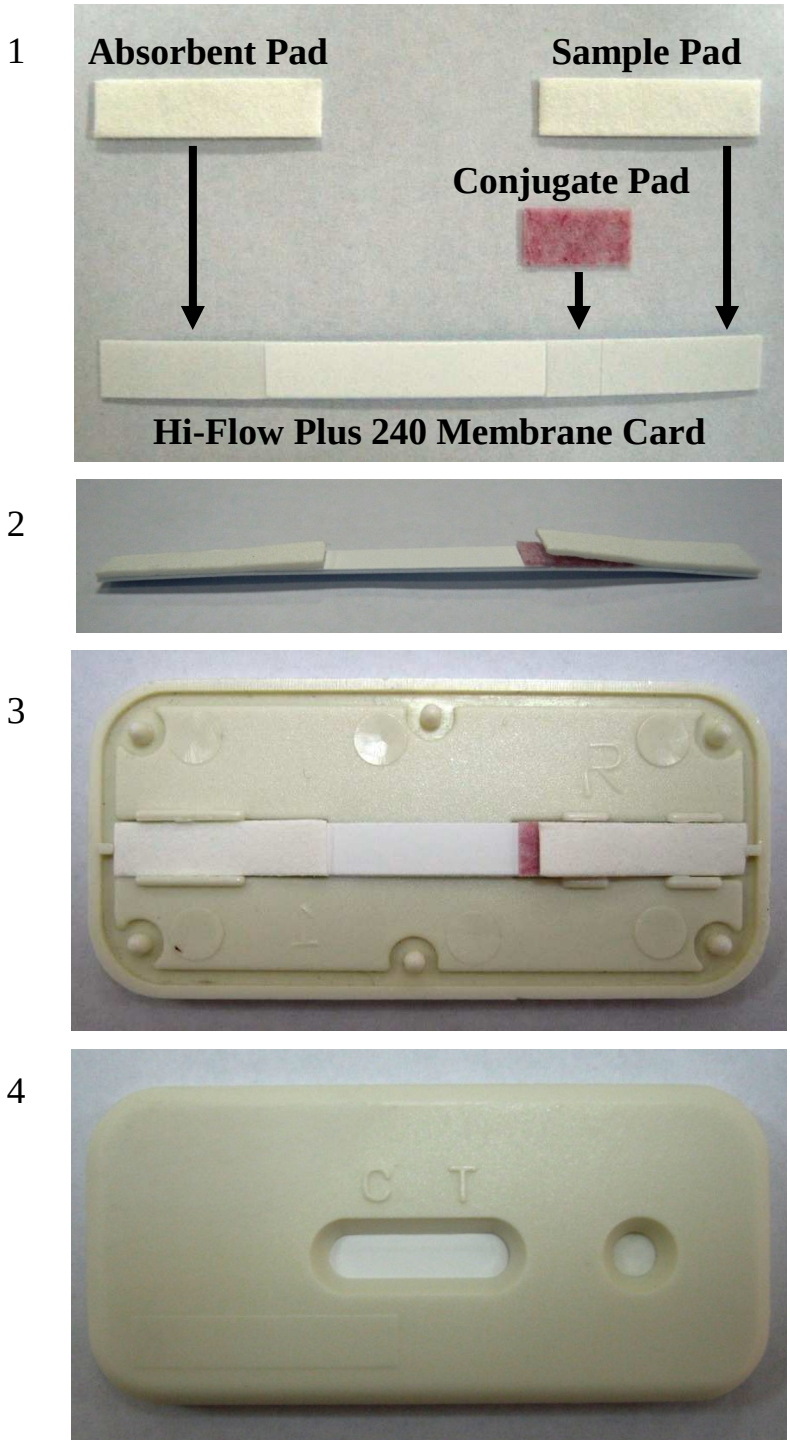


Figure 3.3 Test strip assembly. This figure shows the assembly of the conjugate pad, sample pad and absorbent pad on the membrane background (1). A side view of the assembled strip (2). Placement of the strip in a plastic cassette (3). Plastic cassette ready to use (4).

3.9 PREPARATION OF STANDARD SERUM SAMPLES

Serum sample from asymptomatic subject was tested for the absence of *H. pylori* by the commercial rapid *H. pylori* antigen test device (Rapidan Tester, Turklab, Izmir, Turkey). The 5 ml of venipuncture whole blood of subjects was collected in a tube containing no anticoagulant and centrifuged at 3000 rpm for 5 min at room temperature. After that, the 1.5-2.0 ml of serum was collected into a sterile 2.0 ml microtube. rCagA was diluted in this serum at 0, 2, 4, 8, 16, 32 and 64 µg/ml, and these were applied to our rapid CagA test strip to determine sensitivity of the test.

3.10 PREPARATION OF LYSATES OF *H. pylori* AND OTHER GASTROINTESTINAL BACTERIA

H. pylori cagA-positive strains (722 and 13206) which have been stored at -80 °C were incubated at 37 °C for 5 minutes and then inoculated onto colombia agar (5 % sheep red blood cell) and incubated under microaerophilic conditions in a CO_2 incubator at 37 °C for 5-7 days. The growth culture of H. pylori was identified by colony morphology, gram stain and positive reactions to catalase and urease activities (Figure 3.4). Cultures were aseptically transferred in separate sterile 25 cm² flasks containing Brucella broth with 5 % Fetal Bovine Serum (FBS) for 5 days.

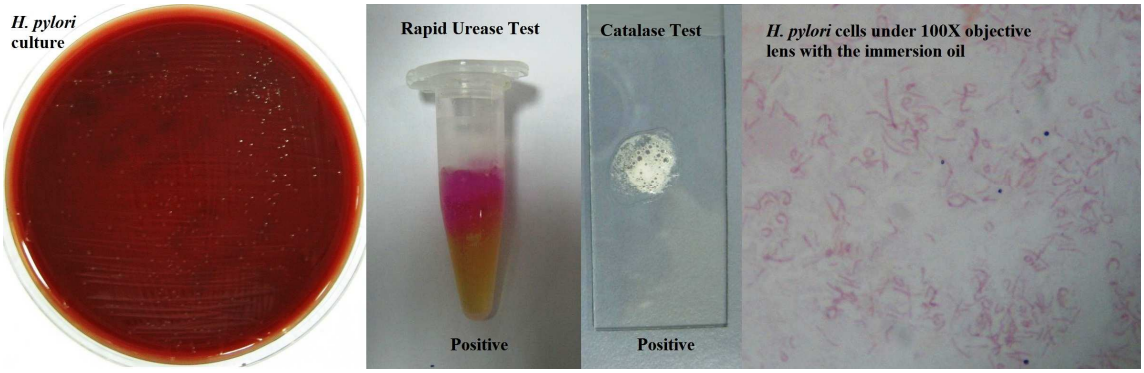


Figure 3.4 Identification the *H. pylori* by colony morphology, rapid urease test, catalase and gram staining.

Colonies of *Escherichia coli*, *Proteus vulgaris* and *Staphylococcus aureus* were transferred to separate nutrient broth media and incubated at 37 °C for 2 days. Bacterial cultures were centrifuged at 12000xg for 10 min and the pellet was resuspended in PBS (pH 7,4) and then disrupted by sonication at 20000 Hz for 45 s; this process was

performed five times. The supernatant was then collected (bacterium whole-cell lysate) (Shin et al., 2003). The concentrations of the lysates were measured with Bradford assay by using Protein Quantification Kit-Rapid.

3.11 PRINCIPLES OF THE LFTS

Our test strip prepared for detecting the CagA antigen of *H. pylori* used a sandwich-type immunochromatographic assay (Figure 3.5). The test strip and samples was brought to room temperature before testing. The test strip was labeled with sample identity. 80 µl of sample was added to the sample pad with a micropipette and left for 1 minute. Then, 50 µl of wash buffer (PBS, pH 7.4) was placed into the sample well. The addition of a sample drop onto the sample pad lead to a lateral flow of the sample fluid containing CagA antigens toward the conjugate pad where it bound to the McAb coated on gold particles. By capillary action, the complex then moved to the test line where it bound to the immobilized second McAb and resulted in a red color line. The flow of the sample fluid was continued toward the control line where the remaining antibody coated gold particles was bound to the immobilized goat anti-mouse PcAb and gave a red color line to indicate that the test has been correctly performed and the test device functioned properly. The more CagA protein present in the sample, the stronger color of the test line. If CagA antigen is lower than the detection limit of the test, then only the control line was visible. If the control line shows no color, then the test will be considered invalid, and similarly no results were interpreted after 30 minutes.

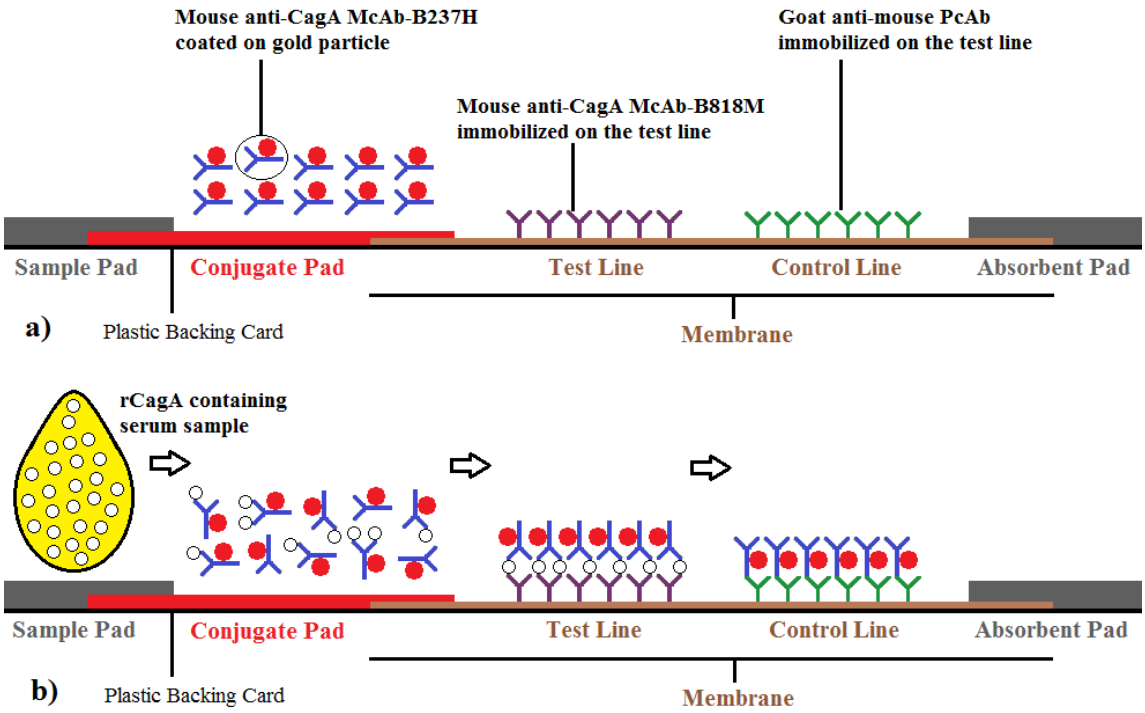


Figure 3.5 Schematic representation of lateral flow test strip in sandwich format for the detection of CagA showing the constituents of the strip before (a) and after (b) the addition of the sample.

Interpretation of the results (Figure 3.6):

Positive: Two red lines in the test region (T) and the control region (C) means that CagA antigen of *H. pylori* has been detected in the sample.

Negative: One red line in the control region (C) with no red line in the test region (T) means that CagA antigen of *H. pylori* has not been detected in the sample or is below the detection limit of the test.

Invalid: The test will be considered invalid if no color develops in the control line. The test will be repeated with a new strip.

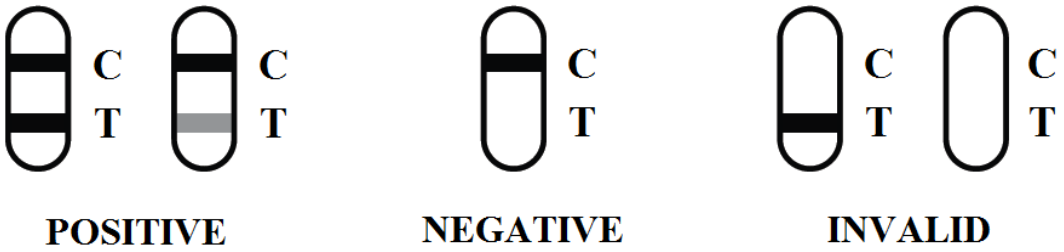


Figure 3.6 Interpretation of the results.

3.12 INVESTIGATION OF CROSS REACTIVITY AND INTERFERENCE

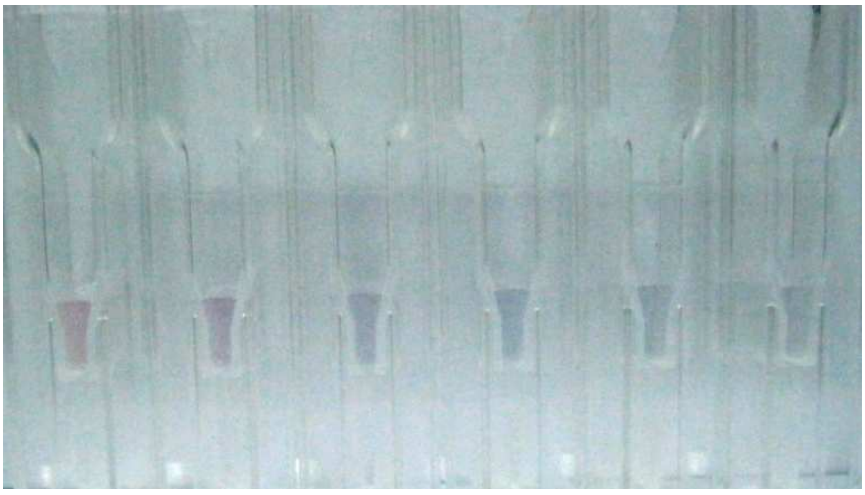
Some closely related microorganisms (*Escherichia coli*, *Proteus vulgaris* and *Staphylococcus aureus*) were used to test the specificity of rapid CagA test strip. Positive (64 µg/ml rCagA supplemented serum) and negative (only serum) samples were spiked with 800 µg/ml concentrated whole cell lysates of these bacteria and tested by our rapid CagA test strip separately. For this purpose, 50 µl of serum sample was mixed with 50 µl lysate of microorganism and then the test procedure explained before was repeated. (The final concentration of lysate in sample became 400 µg/ml.) Also, the test strip was evaluated with two CagA-positive *H. pylori* strains (722 and 13206).

CHAPTER 4

RESULTS

4.1 DETERMINATION OF THE OPTIMUM CONCENTRATIONS OF MONOCLONAL ANTIBODIES TO BE CONJUGATED TO COLLOIDAL GOLD NANOPARTICLES

A 2-fold dilutions of anti-CagA McAb B237H and B818M was done in double distelled water. 25 µl of gold nanoparticles was added to each tube and incubated for 15 min, then 100 µl of 10 % NaCl solution was added. The color of the dilutions gradually changed from brilliant red to blue as the concentration of McAb decreased. The optimum concentration of McAb for colloidal gold labeling was the lowest concentration of McAb solution that did not change color. We found that the optimum concentration for labeling of B237H and B818M monoclonal antibodies were 32 µg/ml (Figure 4.1) and 64 µg/ml respectively (Figure 4.2).



32 16 8 4 2 1

Dilutions of B237H anti-CagA McAb (µg/ml)

Figure 4.1 Optimum concentration of B237H anti-CagA monoclonal antibody for colloidal gold labeling.

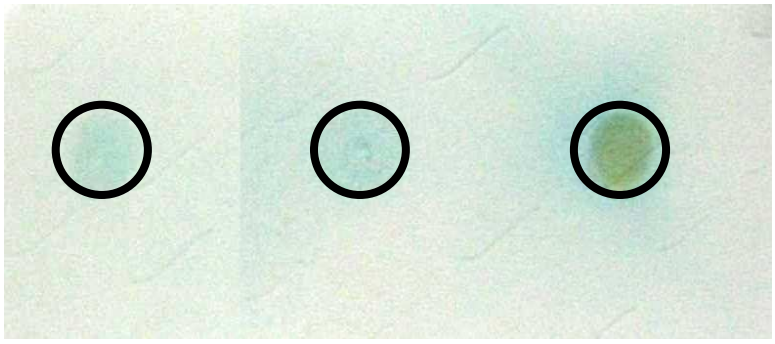
64 32 16 8 4 2

Dilutions of B818M anti-CagA McAb (µg/ml)

Figure 4.2 Optimum concentration of B818M anti-CagA monoclonal antibody for colloidal gold labeling.

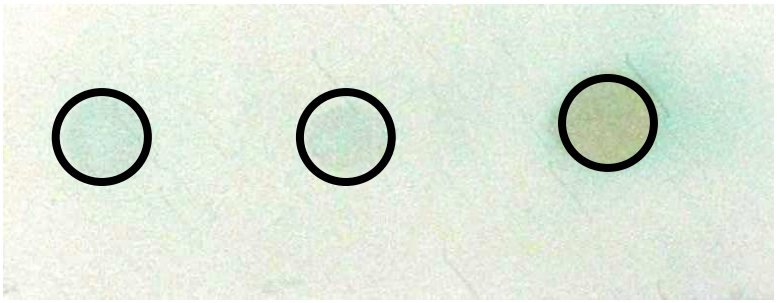
4.2 DOT BLOT ASSAYS TO TEST MONOCLONAL ANTIBODIES

The B237H anti-CagA McAb and the B818M anti-CagA McAb bound to both CagA-positive *H. pylori* whole cell lysate (800 µg/ml) and the rCagA (80 µg/ml) according to blue colored dots appeared on the membrane. Clear background on the membrane represented negative control and the colored dot on the areas of B237H anti-CagA McAb and the B818M anti-CagA McAb represented positive control (Figure 4.3 and 4.4). In addition, there was no blue colored dot on the membrane on which only the rCagA was spotted and then, incubated with the goat anti-mouse IgG-HRP (Figure is not shown). This shows that the secondary antibody did not bind to the rCagA confirming that the anti-CagA monoclonal antibodies bound to the rCagA on the membranes in Figure 4.3 and Figure 4.4 exactly.



CagA-positive *H. pylori* lysate (800 µg/ml)	rCagA (80 µg/ml)	B237H anti-CagA McAb (1500 µg/ml)

Figure 4.3 Indirect dot blot assay to test the reactivity of anti-CagA McAb B237H.

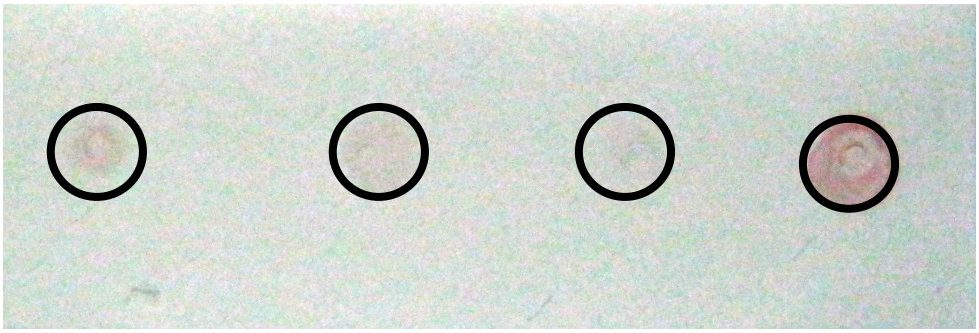


CagA-positive *H. pylori* lysate (800 µg/ml)	rCagA (80 µg/ml)	B818M anti-CagA McAb (1500 µg/ml)

Figure 4.4 Indirect dot blot assay to test the reactivity of anti-CagA McAb B818M.

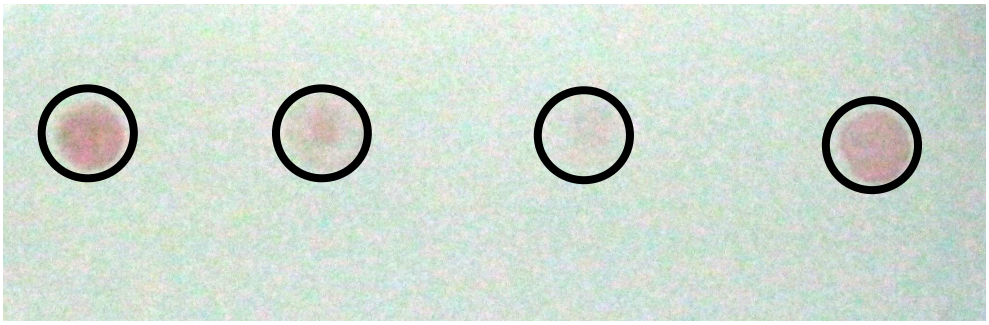
4.3 DOT BLOT ASSAYS TO TEST GOLD LABELED MONOCLONAL ANTIBODIES

The gold labeled B237H anti-CagA McAb and the gold labeled B818M anti-CagA McAb bound to the both CagA-positive *H. pylori* whole cell lysate (800 µg/ml) and the rCagA (80 µg/ml) while weakly red color occurred on the area of rCagA with low concentration (12 µg/ml rCagA). Clear background on the membrane represented negative control and the red colored dot on the area of GTxMS PcAb represented positive control (Figure 4.5 and 4.6).



CagA-positive *H. pylori* lysate (800 µg/ml)	rCagA (80 µg/ml)	rCagA (12 µg/ml)	GTxMS PcAb (500 µg/ml)

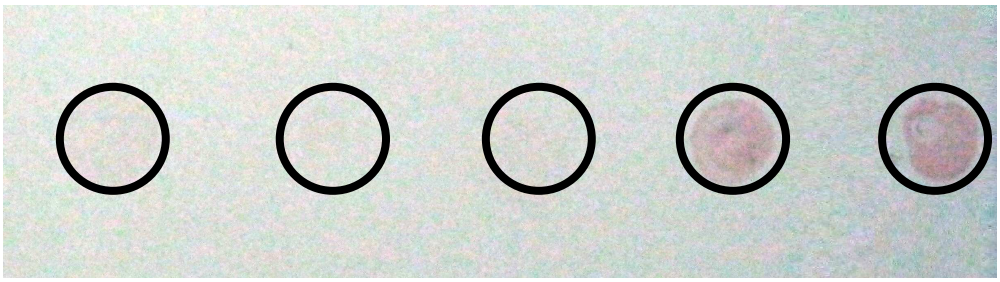
Figure 4.5 Direct dot blot assay to test the reactivity of gold labeled anti-CagA McAb B237H.



CagA-positive *H. pylori* lysate (800 µg/ml)	rCagA (80 µg/ml)	rCagA (12 µg/ml)	GTxMS PcAb (500 µg/ml)

Figure 4.6 Direct dot blot assay to test the reactivity of gold labeled anti-CagA McAb B818M.

The B818M anti-CagA McAb as a capture reagent and the gold labeled B237H anti-CagA McAb as a detector reagent were used to test sandwich assay. The red colored signal decreased by decreasing the concentration of capture reagent. Clear background on the membrane represented negative control and the red colored dots on the areas of rCagA and GTxMS PcAb represented positive controls (Figure 4.7).

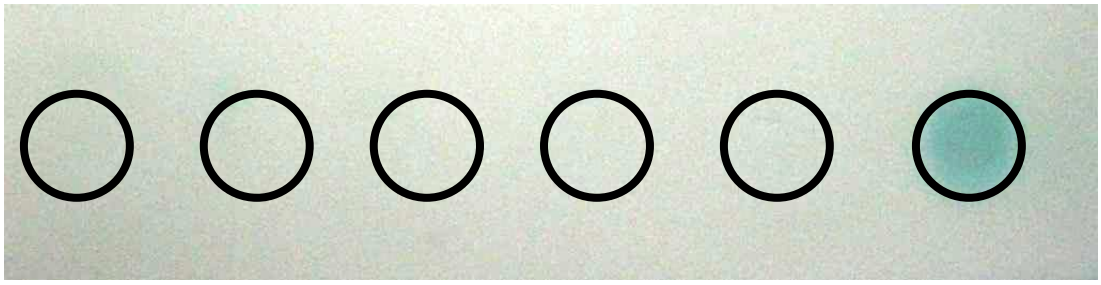


B818M McAb (1000 µg/ml) | B818M McAb (500 µg/ml) | B818M McAb (250 µg/ml) | rCagA (80 µg/ml) | GTxMS PcAb (500 µg/ml)

Figure 4.7 Sandwich dot blot assay using anti-CagA McAb (B818M) as a capture reagent and anti-CagA McAb (B237H) gold labeled as a detector reagent.

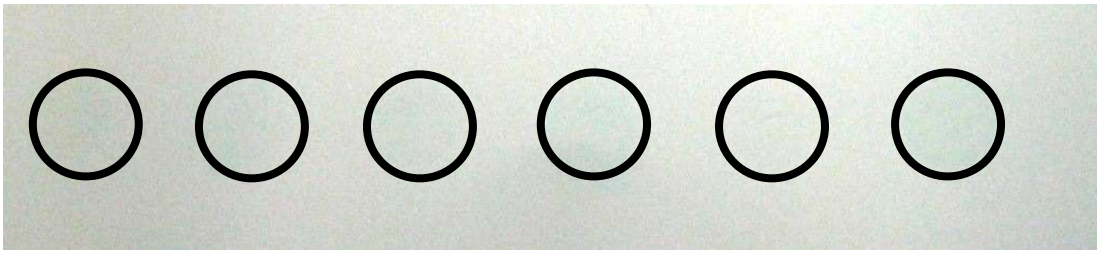
4.4 DOT BLOT ASSAY TO TEST CROSS-REACTIVITY FOR ANTI-CagA MONOCLONAL ANTIBODIES

Closely related microorganisms were evaluated for cross reactivity with A-10 (Santa Cruz), B237H (Abcam) and B818M (Abcam) anti-CagA monoclonal antibodies by using the indirect dot blot assay. This was one more time confirmed that these antibodies recognized rCagA protein efficiently (Figures below). Also, they recognized the CagA protein inside the CagA-positive *H. pylori* lysates (722 and 13206) as seen slightly blue color on membrane in figure. However, there were no blue colored dots on the areas of the other microorganisms. Clear background on the membrane represented negative control and the colored dot on the areas of rCagA represented positive control.



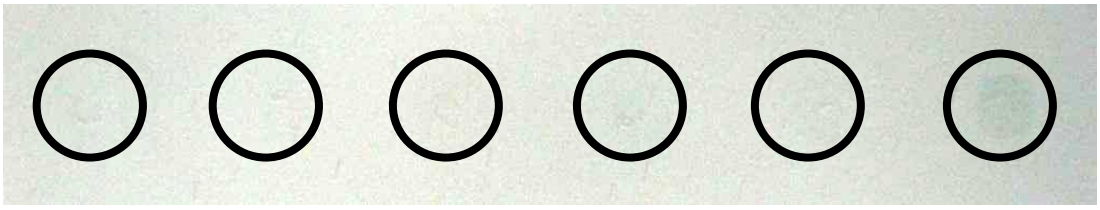
E. coli | *P. vulgaris* | *S. aureus* | *H. pylori* (722) | *H. pylori* (13206) | rCagA

Figure 4.8 Cross-reactivity test using anti-CagA McAb A-10 (Santa Cruz). An 80 µg/ml of the above listed bacterial lysates were used.



E. coli *P. vulgaris* *S. aureus* *H. pylori* (722) *H. pylori* (13206) rCagA

Figure 4.9 Cross-reactivity test using anti-CagA McAb B237H (Abcam). An 80 μg/ml of the above listed bacterial lysates were used.



E. coli *P. vulgaris* *S. aureus* *H. pylori* (722) *H. pylori* (13206) rCagA

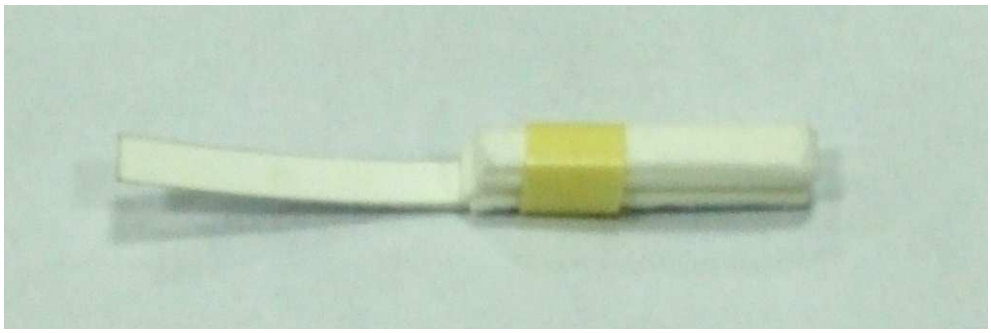
Figure 4.10 Cross-reactivity test using anti-CagA McAb B818M (Abcam). An 80 μg/ml of the above listed bacterial lysates were used.

4.5 LATERAL FLOW TEST STRIP

4.5.1 First Developed Test Strip

The first test strip was developed by assembling the nitrocellulose membrane with a few pieces of Whatman cellulose paper (Figure 4.11-1). *H. pylori* lysate was spotted on the membrane and then the strip was dipped into the gold labeled McAb. After completion of the flow through the absorbent cellulose paper, a red colored dot was appeared on the membrane. This indicated that the gold labeled McAb was bound to the CagA antigen inside the CagA-positive *H. pylori* whole cell lysate (Figure 4.11-2). However, the test strip did not provide a complete LFIA strip.

1



2

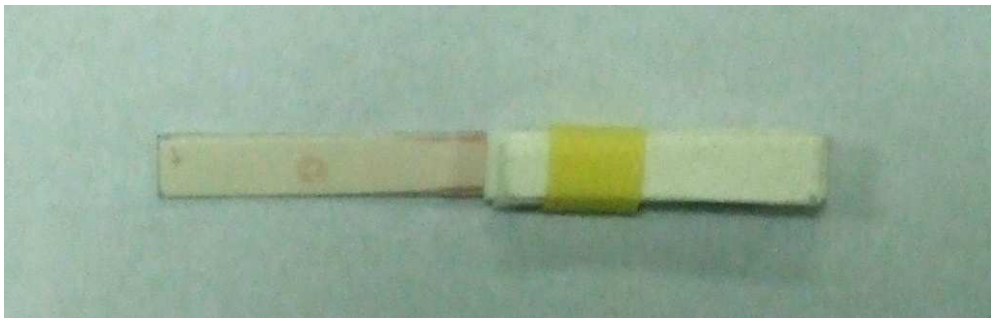
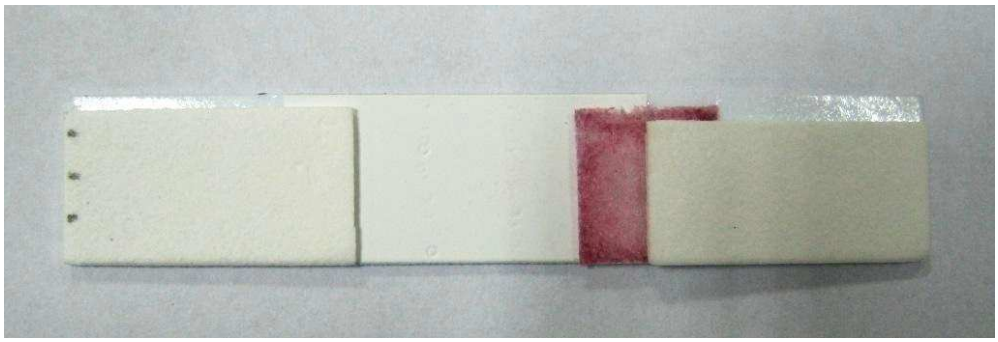


Figure 4.11 First developed test strip. The Whatman cellulose paper was attached on the membrane (1). After dipping the membrane into the gold labeled McAb vertically, the red color was appeared on the area of the immobilized CagA positive *H. pylori* lysate.

4.5.2 Cutting of the Test Strip

The Hi-Flow Plus 240 Membrane Card was then purchased and the test strip was cut into 3 x 60 mm with scissors (Figure 4.12-1, 2).

1



2

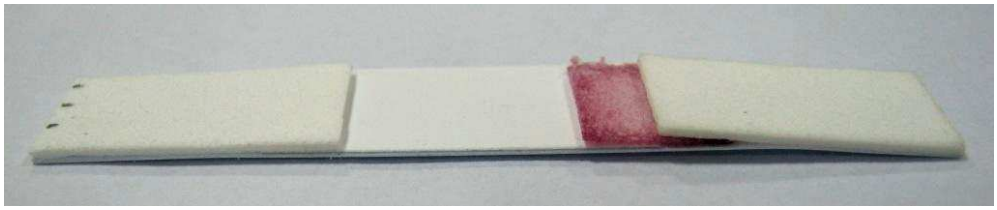


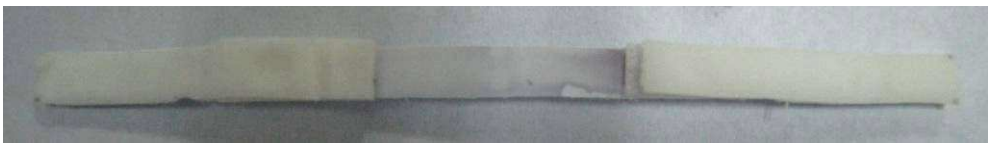
Figure 4.12 Preparation of the test strip by cutting 10 x 60 mm strip into 3 x 60 mm as marked on the absorbent pad (1). The side view of the test strip (2).

4.5.3 Strip Pads Assembly

Several attempts were made to assemble the different pads on the Hi-Flow Plus 240 Membrane Card but all configurations were unsuccessful. Because, gold labeled monoclonal antibodies did not reach to the absorbent pad completely (Figure 4.13-1, 2, 3).

1

2



3

Figure 4.13 Different placements of pads on the membrane (1, 2, 3) showing the lack of flow of gold labeled monoclonal antibodies through the membrane.

At the end, we were able to assemble the pads on membrane as shown in figure 4.14 and obtained a continuous flow.

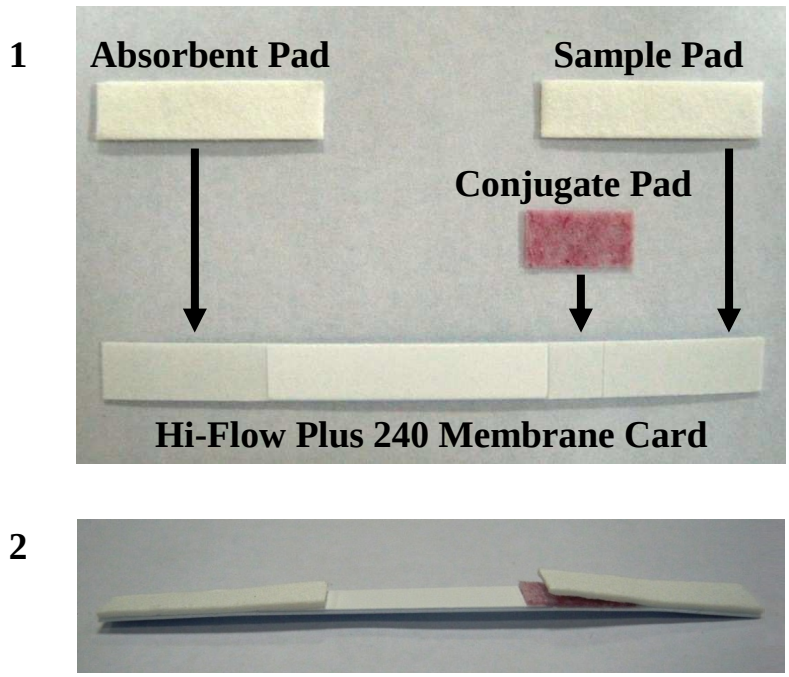
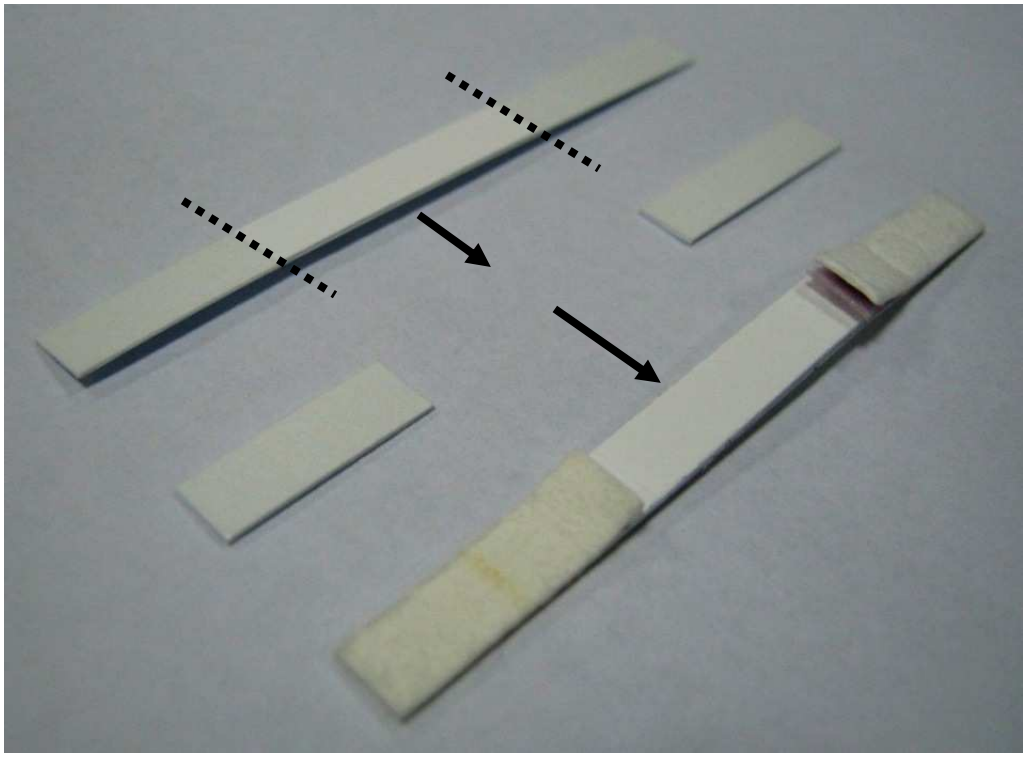


Figure 4.14 Test strip assembly showing the placement of the conjugate pad, sample pad and absorbent pad on the membrane (1). Side view of the assembled strip (2).

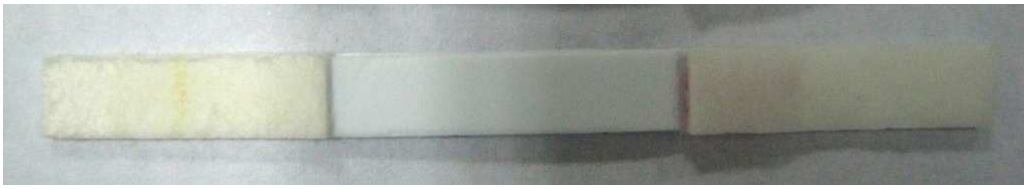
4.5.4 Comparing Membrane with a Commercial Membrane

To compare the flow properties of our membrane with a commercial one, the membranes were exchanged (Figure 4.15-1). The membrane of Hi-Flow Plus 240 Membrane Card was cut and then placed on the membrane area of a commercial test strip. A drop PBS was added on the sample pad but no migration of commercial gold labeled McAbs on our membrane (Figure 4.15-2). However, our gold labeled McAbs were migrated through the commercial membrane slightly (Figure 4.15-3).

1



2



3

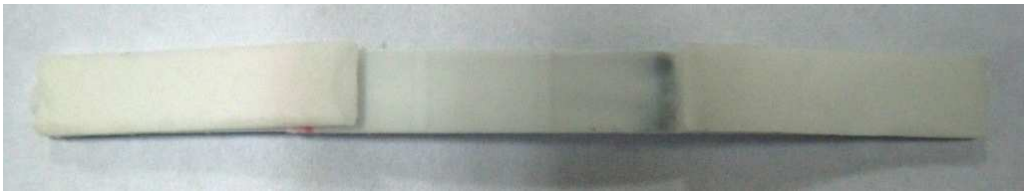


Figure 4.15 Comparing the flow properties of the membrane with the commercial one. The membrane of Hi-Flow Plus 240 Membrane Card was cut and then placed on the membrane area of a commercial test strip (1). There was no migration of commercial gold labeled McAbs on our membrane (2). However, our gold labeled McAbs were migrated through the commercial membrane slightly (3).

4.5.5 Flow of Gold Labeled Antibodies through the Strip

The flow of the gold labeled antibodies through the membrane was not possible without the addition of special conjugate buffer to the gold labeled antibodies suspension (Figure 4.16).

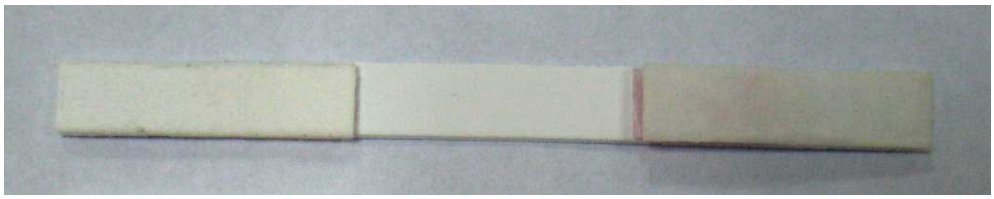


Figure 4.16 No flow of gold labeled antibodies through the membrane.

4.5.6 Preparation of the Conjugate Pad

Zhang et. al. (2006) recommended to dry the conjugate pad at 56 °C for 1 h but this resulted in the accumulation of conjugates through the edges of the pad. We were able to overcome this problem by drying the conjugate pad at 37 °C for 3 h (Figure 4.17).

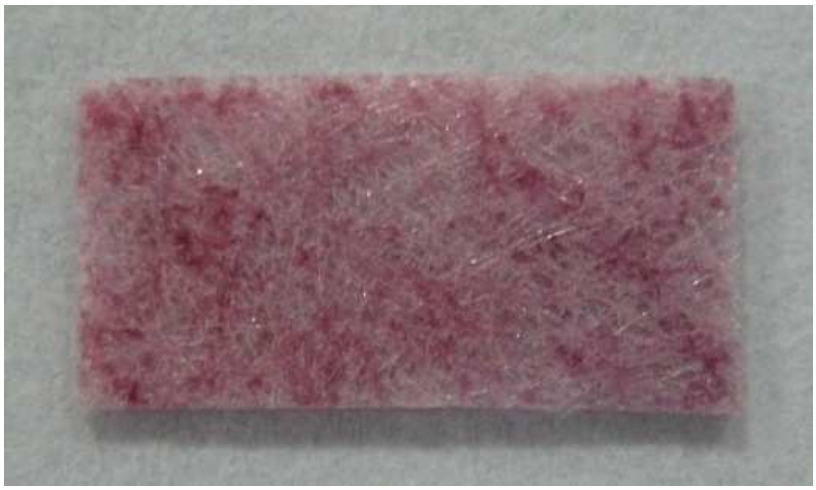


Figure 4.17 Conjugate pad showing even distribution of the gold labeled McAbs into the pad.

4.5.7 Selecting the Best Membrane

We have tried 2 types of membranes; the Hi-Flow Plus 240 Membrane and the Hi-Flow Plus 90 Membrane to test the flow of conjugates through the membrane. No good flow was obtained in both membranes (Figure 4.18 and Figure 4.19).

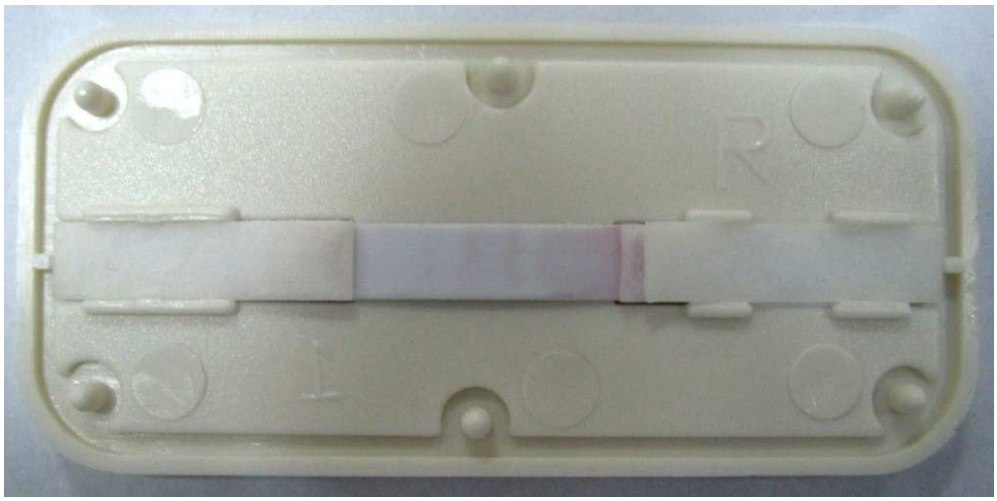


Figure 4.18 Hi-Flow Plus 240 Membrane showing no flow of conjugate.

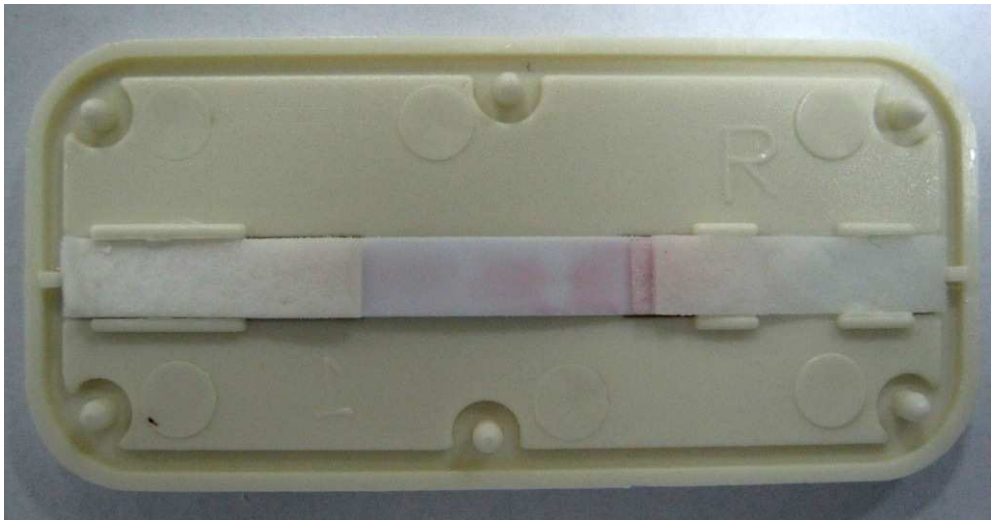


Figure 4.19 Hi-Flow Plus 90 Membrane showing no flow of conjugate.

To overcome this problem *H. plyori* lysate (800 µg/ml) was first spotted onto the Hi-Flow Plus 90 Membrane then the membrane was blocked with 5 % BSA in TBS-T. After that, 100 µl PBS was added onto the sample pad and we noticed the flow of conjugates through the membrane. The conjugates reached the absorbent pad and the background became clear. The CagA protein inside the lysate was detected as a red colored dot (Figure 4.20).

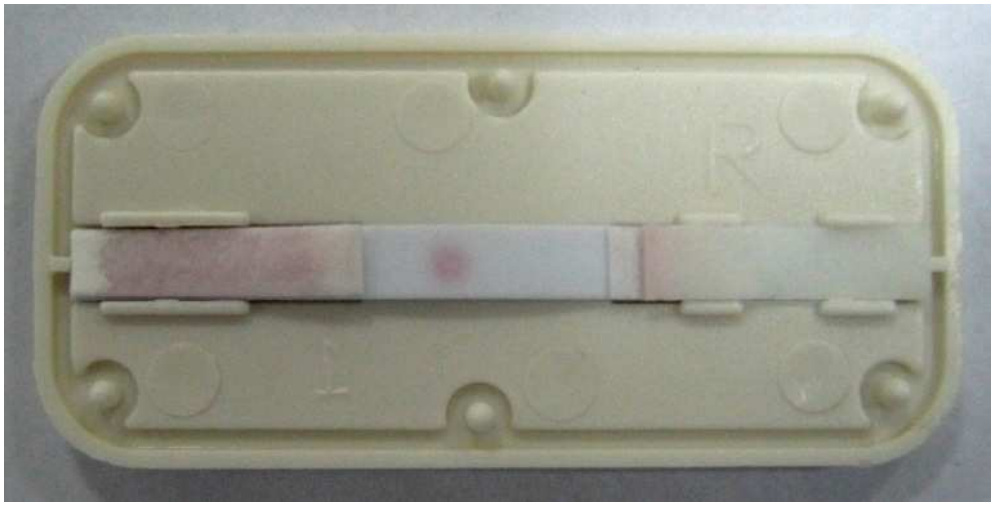


Figure 4.20 The CagA protein inside the *H. plyori* lysate was detected as a red colored dot.

The *H. plyori* lysate (800 μg/ml) was also spotted onto the Hi-Flow Plus 240 Membrane. Then, the membrane was blocked with 5 % BSA in TBS-T. After that, 100 μl PBS was added onto the sample pad. The conjugates reached the absorbent pad and the background remained clear. The CagA protein inside the lysate was detected as a red colored dot (Figure 4.21).

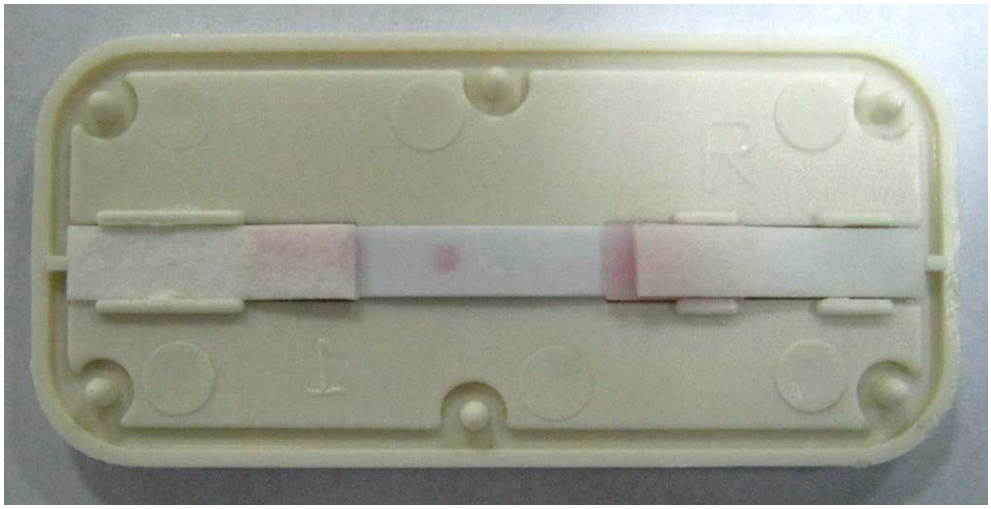


Figure 4.21 The CagA protein inside the *H. plyori* lysate was detected as a red colored dot.

The above approach was applied again using recombinant CagA protein (rCagA). The rCagA proteins were detected as a red colored dot (Figure 4.22).

Figure 4.22 The rCagA protein was detected as a red colored dot.

4.5.8 Test Line and Control Line Setup

A line was drawn on the membrane to immobilize the capture reagent as a straight line (Figure 4.23-1). rCagA protein was immobilized on this line. After adding a drop of PBS onto the sample pad, a red color line was noticed on the membrane (Figure 4.23-2).

1

2

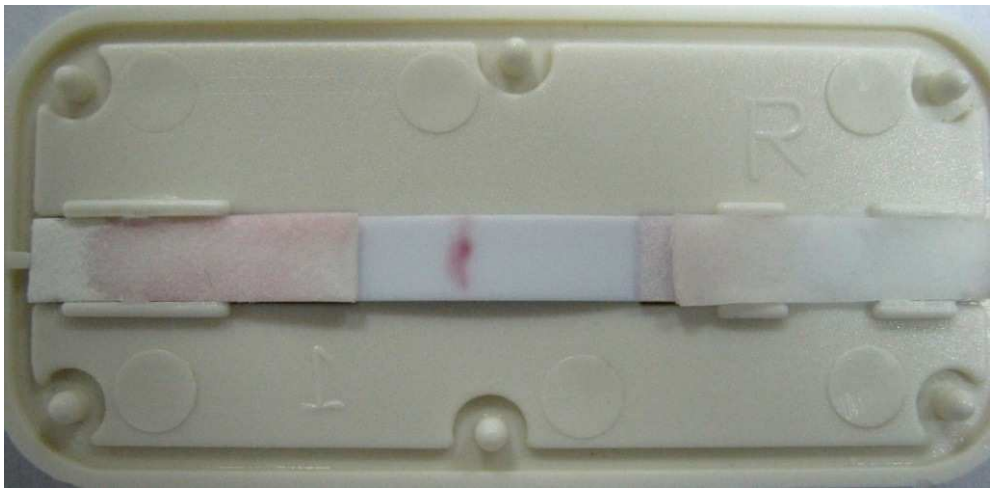


Figure 4.23 An indented line was made on the membrane (1). A red color was seen on this line where rCagA has been immobilized (2).

The above approach was also used to test the control line. The capture reagent (goat anti-mouse PcAb) was immobilized on this line (Figure 4.24-1). After applying sample, the control line was appeared as a straight red line (Figure 4.24-2).

1

2

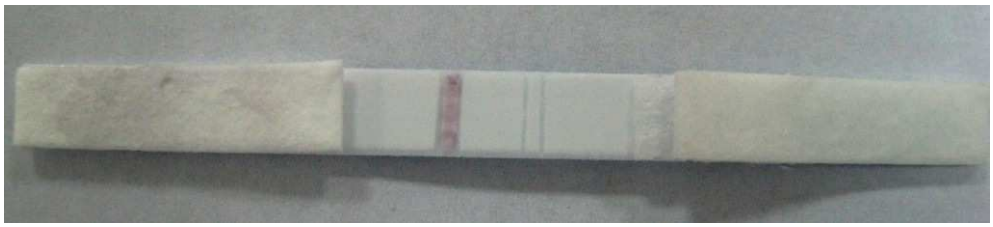


Figure 4.24 An indented line was made on the membrane (1). A red color was seen on this line where the capture reagent was immobilized (2).

4.5.9 Position of the Test Line and Control Line

The anti-CagA McAb (B818M, Abcam) was immobilized on the test line and the goat anti-mouse PcAb (Millipore) was immobilized on the control line onto the Hi-Flow Plus 90 Membrane. The test was run but no colored line appeared.

To overcome this problem, the Hi-Flow Plus 240 Membrane was used. But only the control line was seen (Figure 4.25).

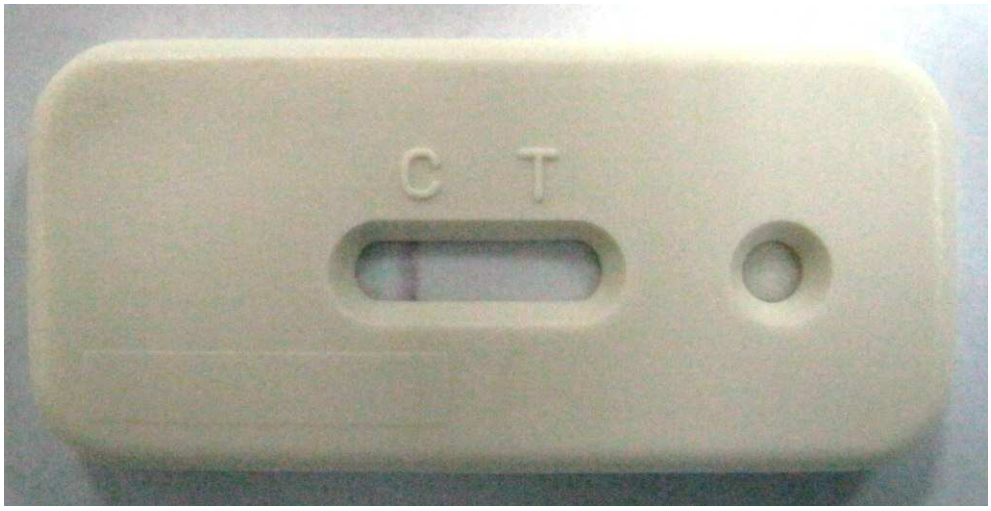


Figure 4.25 Red colored control line on the Hi-Flow Plus 240 Membrane after applying rCagA containing solution.

The locations of the lines were optimized as shown in figure below (Figure 4.26). 2 µl of anti-CagA McAb-B818M (test line) and 2 µl of goat anti-mouse (GTxMS) PcAb (control line) were dispensed on the Hi-Flow Plus 240 Membrane Card. 32 µg/ml rCagA in the serum sample was loaded onto the sample pad. After 15 min incubation at RT two visible lines were detected on the test strip (Figure 4.27).

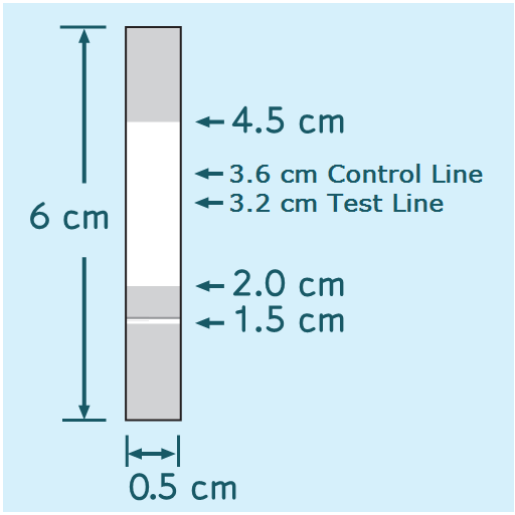


Figure 4.26 The locations of the test line and control line on the Hi-Flow Plus 240 Membrane Card.

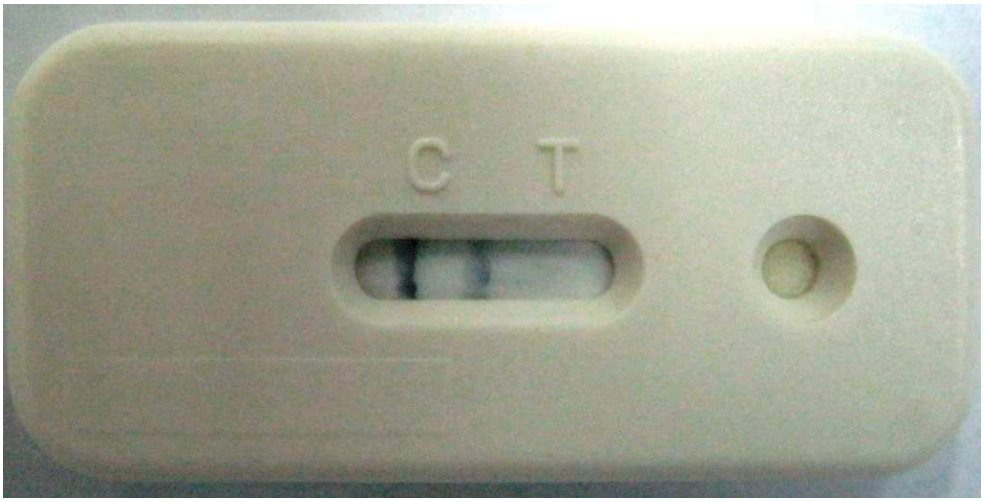


Figure 4.27 LFTS showing the test line and the control line.

4.6 SENSITIVITY OF THE TEST STRIP

The sensitivity of the test strip was determined by testing the serum standards supplemented with rCagA at 0, 2, 4, 8, 16, 32 and 64 µg/ml. In the present study the lower detection limit of the test strip by visual detection was 4 µg/ml (Figure 4.28).

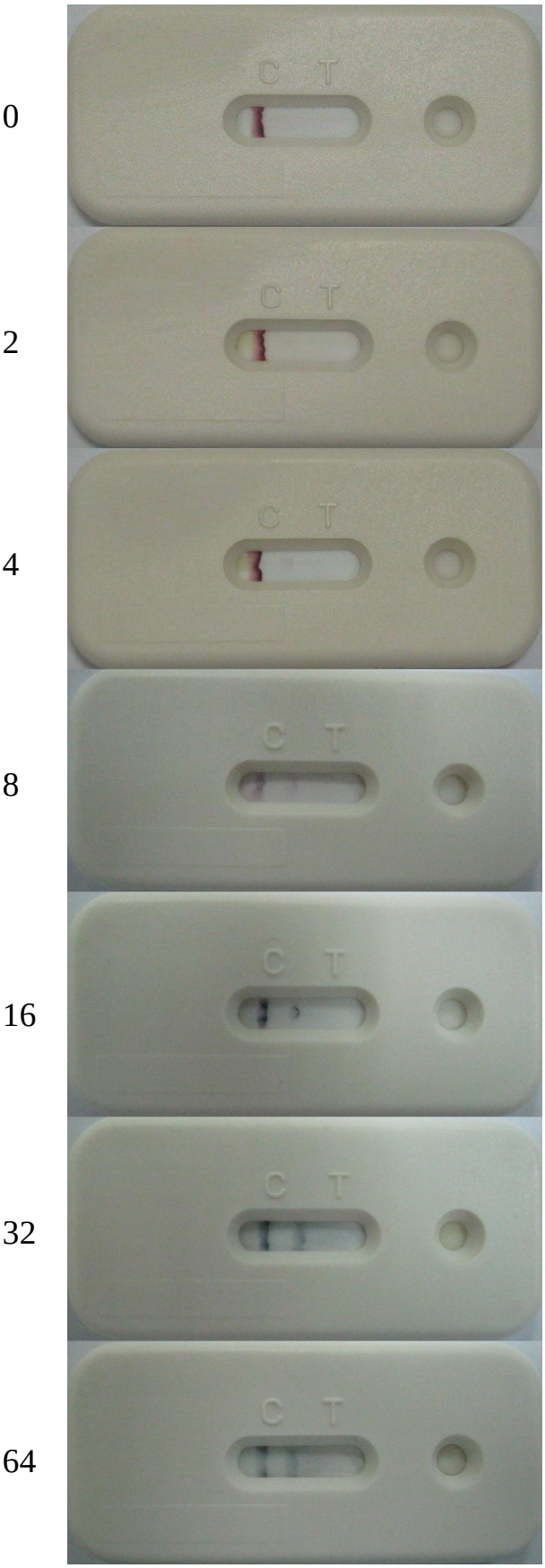


Figure 4.28 rCagA protein concentrations (µg/ml) added to serum sample was used to determine sensitivity of the test strip.

4.7 SPECIFICITY OF THE TEST STRIP

Closely related microorganisms were evaluated for cross reactivity with the test strip. Whole cell lysates of *E. coli, Proteus vulgaris* and *Staph. aureus* were spiked into the CagA positive and negative sera at high concentrations and tested separately. None of the microorganisms tested yielded a positive result in the negative serum or interfered with detection of the positive serum; CagA-positive serum remained positive and CagA-negative serum remained negative with the spiked organisms. Both the negative and positive sera were positive when spiked with CagA-positive *Helicobacter pylori* strains 722 and 13206. In addition, there was no interference with the serum proteins such as albumin, bilirubin, and hemoglobin according to results in the previous and current sections.

CHAPTER 5

DISCUSSIONS AND CONCLUSION

The lateral flow test strip is a promising biosensor where no equipment is required, easy to handle, rapid visual observation of the results and convenience in performance on the spot. The gold nanoparticles used in this test allow an easy and rapid procedure and eliminate the use of toxic reagents (Paek et al., 2000; Chiao et al., 2004; Sato et al., 2004; Xiulan et al., 2005). These gold nanoparticles are more stable than the conventional systems utilizing fluorescence or enzyme labels. In addition, the nanoscale surfaces of these particles are appropriate for accelerating the antibody–antigen recognitions, which enhances the immunoassay signals (DiScipio, 1996).

The basic principle of the test is as follow: In the sandwich-type assay the antigen binds to the antibody–gold nanoparticle impregnated in the conjugate pad and the flow of this immunocomplex to the test line will result in binding to the antibody immobilized on the membrane. A red color will be seen at the test line and its intensity will increase as the accumulation of gold nanoparticles increases with time and the results can be obtained in approximately 10 min (Dok An et al., 2001).

The worldwide prevalence of *H. pylori* infection and the high percentages of patients with peptic ulcer diseases necessitate the use of different diagnostic tests for the detection of *H. pylori.* The commercially available rapid LFIA strips is one of the non-invasive tests that provide good support and aid in the diagnosis of *H. pylori* infection rather than the expensive, time consuming and laborious invasive (endoscopy) approach. Around 44 % of the LFIA strips showed sensitivity and specificity of over 95 % as indicated in the manufacturer data sheets. The majority of the test strips detects anti-*H. pylori* antibodies in serum samples, of these 68 % utilize the sandwich format while 32 % the indirect format. However, the test strips detecting *H. pylori* antigens in

stool samples utilize only the sandwich format where the antigens are sandwiched between two different antibodies and these have higher sensitivities and specificities.

The CagA protein encoded by the cagA gene is considered to be one of the key virulence factors of *H. pylori*, since cagA-positive strains provoke severe mucosal damages and act as a risk factor for the development of peptic ulceration and gastric cancer (Ong and Duggan, 2004). Our developed LFIA strip uses monoclonal antibodies (McAbs) that specifically recognize the free CagA protein itself in sera of infected patients rather than its anti-CagA antibodies. This will provide a tool for the determination of pathological potential of *H. pylori* strains in symptomatic subjects, determine an active status of infection, provide a predictive evaluation for the progression of the disease and eliminate the need for additional endoscopies in the follow up of eradication therapy. But, it should be known that antibiotics, proton pump inhibitors and bismuth preparations are known to suppress growth of *H. pylori*. So serum samples must be collected not earlier than 2 weeks after termination of drug ingestion and 4 weeks after termination of antibiotics therapy.

The dot blot assay that we developed using 2 commercial McAbs recognized our rCagA protein. This shows that our rCagA possess two different epitopes; one epitope might be located on the C terminal 580 amino acids region and the other epitope might be localized in between the Asp562 and Gln795 amino acids region.

After adding a drop of serum sample on the sample pad of our test strip, the colloidal gold conjugated B237H McAb impregnated into the conjugate pad bound to one epitope on the rCagA and then by capillary flow of the complex through the membrane the second McAb B818M immobilized on the test line bind to the other epitope on the rCagA (sandwich format). So the B237H McAb as the detection antibody in the conjugate pad, the B818M McAb as capture antibody on the test line and the GTxMS IgG as control antibody on the control line yielded very good immunochromatographic assay that detect CagA antigen of *H. pylori.*

In conclusion, we were able to develop a novel lateral flow test strip successfully. We utilized CagA-specific monoclonal antibodies and were able to detect CagA antigen in serum samples. The test strip showed no cross-reactivity with closely related bacteria and no interference with other bacterial or serum proteins. This test provide a predictive evaluation whether or not to treat such patients in order to block future progression to peptic ulceration and gastric cancer, and eliminate the need for additional endoscopies in the follow up of eradication therapy. To the best of our knowledge this is the first LFTS developed in our country.

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