Sudan University of Science and Technology

College of Graduate Studies

Relationship between *Helicobacter pylori* Seropositive Patients and Throat Infection

العلاقة بين الاصابة بالبكتيريا الحلزونية البوابية (***Helicobacter pylori***) في المرضى موجبي الفحص المصلي والعدوي البكتيرية في الحلق

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الملخص

معظم الشكاوي وسط مرضي العيادات الخارجيه تكون حول آلام الحلق والتشخيص السائد لها غالبا ما يكون التهاب البلعوم المزمن غير المحدد الذي لايزال بدوره تشخيص تختلف حوله الآراء. بجانب كون البكتيريا الحلزونية البوابية مسبب رئيس لإلتهاب المعدة المزمن، يبدو انها كذلك تلعب دورا هاما في آلام الحلق.
هدف هذا البحث لدراسة العلاقة بين الإصابة بالبكتريا البوابية الحلزونية والعدوي البكتيرية في الحلق لتحديد دور بكتريا البوابية الحلزونية في فسيولوجيا الإصابة بأمراض إلتهاب البلعوم و لوزة الحلق. تم التحقق من هذا بمعرفة معيار الاجسام المضادة عند المرضي الذين يعانون من من الاصابه بجرثومة المعدة، ليس هذا وحسب، بل تم ايضا التحقق من تواجد البكتريا السبحية التقيّحية من خلال التزريع بالإضافة لتقييم العلاقه بين الإصابه بالبكتريا الحلزونية البوابية وتدهور حالات إصابات آلام الحلق.

تم جمع عدد ثلاثمائة وسبعة إستفتاءات مصاحبة لعينات من مسحات الحلق من منطقة لوزة الحلق والبلعوم من المشاركين, جمعت من اشخاص موجبين ملصياً واخرين سليمين إكلينيكيا للمقارنة. تم عزل البكتريا الممرضه المسببة للحلق المتقرح من 74% من عينات مسحات لوزة الحلق و 76.3% من عينات مسحات البلعوم. نسبة الاشخاص اللذين عزلت منهم البكتريا الممرضه في المنطقتين معاً (لوزة الحلق والبلعوم) كانت 78.2%, لكن الأعراض الإكلينيكية شُخصت فقط فيما نسبتة 9.7% من مجموع المتطوعين في هذه الدراسة. بين كل البكتريات الممرضة المسببة لقرح الحلق كانت البكتريا السبحية التقيّحية هي السائدة عند العزل, بعد التعرف عليها بالإختبارات الكيميائية الحيوية وأجري تفاعل البلمرة المتسلسل للتأكد منها جميعا بواسطة البادئ spy 1258 الذي تفاعل إيجابيا مع 17.8% من عينات الحمض النووي من بكتريا السبحية التقيّحية.

أيضا تم جمع عينات دم من نفس الأشخاص المشاركين في الدراسة لتحديد مستوي الأجسام المضادة للبكتريا البوابية الحلزونيه في مصلهم, تحديدا الجلوبيولين المناعي النوع أ والنوع ج. بعد التحليل الإحصائي وجد أن معيار الجلوبيولين المناعي النوع ج مرتبط إرتباطاً معنوياً مع نسبة الجلوبيولين المناعي النوع أ (بمدى الثقة 99%). ووجد إرتباط معنوي بين الإصابة ببكتريا البوابية الحلزونية وأعراض إعتلال الجهاز الهضمي العلوي بالأخص إرتجاع القصبة الهوائية البلعومي (بمدي الثقة 99%). كما وجد إرتباط معنوي ووثيق بين أعراض إعتلال الجهاز الهضمي العلوي المصاحبة للعدوي بالبكتريا البوابية الحلزونية مع الإصابة بالحلق المتقرح (بمدى الثقة 95%).

كما وجدنا إرتباطاً بين الإصابة ببكتريا البوابية الحلزونية والعدوي البكتيرية في الحلق المسببة للحلق المتقرح, يفسر هذا الإرتباط نظريا كنتيجة لإرتجاع القصبة الهوائية البلعومي الذي يؤدي بدورة لتقليل الأس الهيدروجيني في الحلق مما يهيئ الظروف الملائمة للإصابة بالبكتريا السبحية المقيّحة المتحملة للأس الهيدروجيني الحمضي.
أظهرت الدراسة ان إنتشار الإصابة بالبكتريا البوابية الحلزونية مرتفع جداً في مجموعة المشاركين (88.2%) هذا يستنتج ارتفاع معدل الإصابة بهذه الجرثومة خلال السنوات المنصرمة في السودان ويتوقع إرتفاعه اكثر في الفترة القادمة مالم تجري دراسات أخري وحلول تراجع أساليب العلاج والوقاية منها. كما وجد ان البكتريا السبحية التقيّحية هي أكثر مسبب بكتيري للحلق المتقيح إنتشاراً في السودان.

بالرغم من أن إختبار كروماتوغرافيا المناعة المستخدم لتشخيص الإصابة ببكتريا البوابية الحلزونية هو أكثر الطرق شيوعاً لتشخيص هذا المرض في المعامل والمستشفيات السودانية, إلا ان الدراسة أظهرت انة ليس موثوقا به كثيرا كوسيلة تشخيصية مؤكدة للمرض.

ننصح بإجراء دراسة مماثلة يبحث العلاقة بين الإصابة بالبكتريا البوابية الحلزونية والحلق المتقرح الناتج عن المسببات الفيروسية لكثرة إنتشارها. كما ننصح بإختبار اهداف هذه الدراسة بإستعمال طرق تشخيصية أخرى للإصابة بالبكتريا البوابية الحلزونية غير الإختبارات المناعية. كما يجب تجنب إعادة إستعمال نفس المضادات الحيوية لعلاج الإصابة ببكتريا البوابية الحلزونية عندما تتكرر لتجنب المقاومة للمضادات الحيوية المتزايدة كثيرا مؤخراً. كما يفضل إجراء مزيد من الأبحاث عن المناعة الخلوية ضد البكتريا البوابية الحلزونية. كما ننصح بالإستعاضة عن إختبار كروماتوغرافيا المناعة لتشخيص الإصابة ببكتريا البوابية الحلزونية بإختبارات أخري أعلي موثوقية. من الضرورة بمكان إيجاد بادئ جديد لتفاعل البلمرة المتسلسل لتشخيص البكتريا السبحية المقيّحة غير البادئ spy 1258.

ABSTRACT

The most common complaint among patients in the out-patient clinics are sore throat, and the most frequent diagnosis is chronic non-specific pharyngitis. Chronic pharyngitis is still controversial. *Helicobacter pylori,* besides being the major cause of chronic gastritis, might also be involved in playing role in the upper respiratory tract infection.

This study aimed to determine the relationship between *H. pylori* infection and throat infection and its possible role in the etiology and pathogenesis of bacterial pharyngitis and tonsillitis. This was achieved by determination of antibody titer in patients with *H. pylori* infection. Not only that but also the presence of *Streptococcus pyogenes,* the main pathogen involved in throat infection, was investigated via culturing, and the assessment of the relationship between *H. pylori* infection and the aggravation of the sore throat infection.

From volunteers questionnaire data and three hundred and seven samples of throat swabs from tonsils and aurophaynx were collected and cultured from *H. pylori* seropositive and apparently healthy people. Conventional bacteriological methods were applied for isolation of bacteria among which is *Streptococcus pyogenes*.

Pathogenic sore throat causing bacteria were isolated from 74% tonsils and 76.3% pharynx of whole samples. 78.2% individuals had pathogenic bacteria in tonsil and auropharynx, but clinical respiratory signs were observed only in 9.7% of the whole individuals. *Streptococcus pyogenes* was the most predominant bacteria, PCR was conducted to confirm their identity, and dependingly 17.8% of them were detected by PCR using spy1258 primer.

Three hundred and seven blood samples were also collected from the same participants correspondingly for the determination of Anti-*Helicobacter pylori* IgG and IgA measurement. So that, IgG titer was found to be significantly correlated

with IgA ratio (at 99% confident interval, 2 tailed). In this study *H. pylori* positive cases were found to be significantly correlated with upper digestive tract signs (at 99% confident interval, 2 tailed). Besides the upper digestive tract illness signs were correlated with sore throat (at 95% confident interval, 2 tailed).

This study revealed a significant correlation between *H. pylori* infection and sore throat, theoretically due to the accompanied laryngeopharyngeal reflux which in turn reduces the throat pH enhancing the condition for *S. pyogenes* and other acidic pH tolerant pathogens. The results showed that the prevalence of *H. pylori* infection in our population is quite high (88.2%), this infer that the infection is elevated in Sudan during last years and expected to keep increasing unless further studies and solutions applied. *S. pyogenes* has been found to be the most causative agent of bacterial sore throat in Sudan whether it is related to *H. pylori* infection or not. In spite of commonly using ICT rapid test for *H. pylori* diagnosis in Sudanese labs and Hospitals, our study revealed the ICT antibody-based is not very reliable.

It is recommended to search the correlation between *H. pylori* positive infection and viral throat infection because it is also frequent than bacterial throat infection. Testing this research hypothesis should be followed depending on isolation of *H. pylori*, instead of conducting serological tests, with the isolation of *S. pyogenes*. The reuse of same antibiotics against *H. pylori* must be eschewed to avoid bacterial resistance. Such resistance is common in patients who had previous antibiotic treatment. Humoral immunity does not protect against *H. pylori* cellular does protect (Kuster *et al*, 2009). Further research in cellular immunity of *H. pylori* is needed. It is essential to develop a new PCR primer, specific for *Streptococcus pyogenes* and sensitive for its all strains to replace spy1258 primer. ICT method of *H .pylori* infection diagnosis should be avoided and replaced by more trusted methods.

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CHAPTER ONE

Introduction

In all otorhinolaryngology outpatient clinics the most common complaint among patients is sore throat, and the most frequent diagnosis is chronic nonspecific pharyngitis.

Chronic pharyngitis, a chronic inflammation of the pharyngeal mucosa and underlying etiopathogenesis, is still controversial. In general, chronic nonspecific pharyngitis is related to different processes, such as nasal obstruction and mouth breathing, laryngopharyngeal reflux, and acute or chronic upper respiratory tract infection (Aladag *et al.*, 2008). These patients present with symptoms such as chronic throat irritation, sore throat, chronic cough, foreign body and globus sensation in the throat, cervical dysphagia, and intermittent hoarseness persisting for three months or more (Monsur *et al.*, 2004). Treatment is usually difficult and based on reducing the symptoms by medical or behavioral methods (Emir *et al.*, 2006).

Helicobacter pylori (*H. pylori*) is a major cause of chronic gastritis and gastric ulcers and associated with gastric malignancy in addition to various other conditions including pulmonary, vascular and autoimmune disorders. Gastric juice infected with *H.pylori* might play an important role in upper respiratory tract infection. Although direct and/or indirect mechanisms might be involved in the association between *H. pylori* and upper respiratory tract diseases, the etiological role of *H. pylori* in upper respiratory tract disorders has not yet been fully elucidated. Although various studies over the past two decades have suggested a relationship between *H. pylori* and upper respiratory tract diseases, the findings are inconsistent (Kariya *et al.*, 2014). There are some studies that report extragastric *H. pylori* presence in salivary secretions (Botha *et al.*, 1998), tonsil and adenoids (Coskuner *et al.*, 2001), oral cavity (Gall-Troselj *et al.*, 1998), nasal and sinus mucosa (Bayiz *et al.*, 2003), middle ear (Bulut *et al.*, 2005) and dental plaques (Botha *et al*, 1998). A definitive relationship between *H. pylori* and upper respiratory

tract disorders has not been established and further studies are required (Kariya *et al*, 2014).

1.1 Rationale

Epidemiological studies have shown that the prevalence of carrying *H. pylori* ranges from 10%-20% to 80%-90% in developed and developing countries, respectively, and most carriers are asymptomatic (Sugimoto *et al*, 2009 and Malaty, 2007). The findings of published studies on the impact of *H. pylori* on the upper respiratory tract are inconsistent. Whether or not *H. pylori* is located in the upper respiratory tract and whether or not it plays a role in the pathogenesis of upper respiratory tract diseases remain unresolved, so that, more studies are required (Kariya *et al*, 2014).

1.2 Objectives

General objectives:

This study aimed to determine the relationship between *H. pylori* infection and throat infection to clarify whether it may play role in the etiology and pathogenesis of bacterial pharyngitis and tonsillitis.

Specific objectives:

1. To determine the *H. pylori* antibody titer in patients obtained from clinical labs information with the sore throat.
2. To investigate the presence of *Streptococcus pyogenes* as a common causative agents of the bacterial sore throat and its relationship with *H. pylori*.
3. To clarify whether there is other types of bacteria suspected to cause throat infections.
4. To clarify whether the *H. pylori* has ability to increase the sore throat after treatment of *H. pylori* or not.

CHAPTER TWO

Literature Review

2.1 *Helicobacter pylori:*

Helicobacter pylori was first observed by J Robin Warren, an Australian pathologist in 1979. The organism was determined to be Campylobacter-like because of similarity with said organism. It was first isolated and classified as *Helicobacter pylori* in 1993 by warren and Barry J Marshall (Copeland and Stahlfeld, 2012) and (Pajares and Gisbert, 2008).

Genus *Helicobacter* belongs to the subdivision of the *Proteobacteria* phylum, order *Campylobacterales*, family *Helicobacteraceae*. The genus *Helicobacter* consists of over 40 recognized species. *Helicobacter* species can be subdivided in two major lineages according to the colonization location, the gastric *Helicobacters* and the enterohepatic (non-gastric) *Helicobacters*. Both groups demonstrate a high level of organ specificity, such that gastric *Helicobacter* species in general are unable to colonize the intestine or liver, and vice-versa (Kuster *et al*., 2006).

Helicobacter pylori in vivo and under optimum *in vitro* conditions presents a spiral form with 2 to 4 μm long and 0.5 to 1 μm wide and have 2 to 6 unipolar sheathed flagella of approximately 3 μm in length, which often carry a distinctive bulb at the end (O'Toole *et al*., 2000). This bacterium converts to alternative coccoid form when exposed to inconvenient environment, passing through a U-shape during the conversion from one to another (Andersen, 2001).

H. pylori is microaerophilic, with optimal growth at O2 levels of 2 to 5% and the additional need of 5 to 10% CO2 and high humidity, growth occurs with an optimum 37°C. Although it is natural habitat is the acidic gastric mucosa, *H. pylori* is considered to be a neutralophilic. (Scott *et al.,* 2002) and (Stingl *et al.*, 2002). *H. pylori* is a fastidious microorganism and requires complex growth media.

2.2 Routes of Transmission

Numerous epidemiological studies have been conducted to identify the factors influencing transmission of this pathogen. Socioeconomic status is clearly the most important determinant for the development of *H. pylori* infection, with lower social classes exhibiting much higher prevalence (Mitchel, 2001). Factor encompasses conditions such as levels of hygiene, sanitation and educational opportunities, which have all been individually identified as markers of the bacterium presence.

Largely based on epidemiological and microbiological evidence, several routes of transmission have been conjectured. The most relevant pathways of person-to-person transmission encompass the gastro-oral, oral-oral and faecal-oral routes. Breastfeeding and iatrogenic transmission are also included as alternative ways for the dissemination of the pathogen. (Perez-Perez *et al.*, 2004).

2.2.1 Gastro-oral transmission

It has been suggested that exposure to microscopic droplets of gastric juice during endoscope manipulation could explain a higher prevalence of infection in gastrointestinal endoscopists (Hildebrand, 2000), but the gastro-oral transmission has been postulated mainly for young children, among whom vomiting and gastro-oesophageal reflux are common. In a recent epidemiological study, exposure to an infected household member with gastroenteritis and vomiting episodes was associated with a 6.3 fold increased risk of new infection (Perry, 2006).

2.2.2 Oral-oral transmission

The oral cavity has been considered to be a suitable reservoir for *H. pylori* subsistence, and oral-oral transmission has therefore been suggested to occur with contact with infected saliva. Identical strains of the pathogen have been detected by polymerase chain reaction (PCR) in the mouth and stomach of symptomatic infected individuals (Khandaker *et al.*, 1993), and in these populations detection of *H. pylori* in the oral cavity by PCR is in fact very common (Dowsett and Kowolik, 2003).

2.2.3 Faecal-oral transmission

It has been suggested that the faecal-oral route for *H. pylori* transmission is very unlikely due to the contact with human bile, to which it is very sensitive, during the passage through the intestine (Mitchell *et al.,* 1993). Well-established detection methods based on PCR or enzyme-linked immunoassays systematically identify the presence of the microorganism in the human faeces (Ndip *et al.*, 2004 -Queralt *et al.*, 2005).

2.2.4 Breastfeeding

The detection by PCR of *H. pylori* in breast milk has also raised the possibility of breastfeeding as a route of transmission (Kitagawa *et al.,* 2001), even though earlier studies stated that infants born from *H. pylori*-positive women are not more likely to acquire the infection (Blecker, 1994). The contamination of milk could be possible if the bacterium survived in nipples or fingers. However, most epidemiological studies appear not to find any correlation between breastfeeding and *H. pylori* acquisition (Malaty *et al*, 2001-Poms and Tatini, 2001).

2.2.5 Iatrogenic transmission

Acquisition of *H. pylori* by patients submitted to upper endoscopy, i.e. iatrogenic transmission, is supported by three out of four epidemiological studies (Goh *et al.*, 1996-Noone *et al.*, 2006). *H. pylori* has been consistently detected by culture in endoscopes after their use in infected patients (Nurnberg *et al.*, 2003-Katoh *et al.*, 1993), but adequate disinfection procedures are thought to greatly reduce or even eliminate the transmission risk for this microorganism (Cronmiller, 1999).

2.2.6 Zoonotic transmission

Including contact with animals as a possible transmission mode is an obvious reasoning, as zoonotic transmission represents one of the leading causes of illness and death from

infectious disease worldwide. Most epidemiological studies appear to support the role of animals in the acquisition of *H. pylori*, but the extent of this support depends on the animals under study. Considered vectors include cows (Fujimura, 2002), sheep (Dore, 2001), cockroaches (Imamura *et al.*, 2003), houseflies (Osato *et al.*, 1998) and domestic pets (Boomken, 2004).

2.2.7 Water ingestion

A large number of epidemiological studies have investigated drinking water, or drinking water-related conditions, as a risk factor for *H. pylori* infection (Falush, 2003, Adams *et al.*, 2003, Lage *et al.*, 1995, Jimenez-Guerra *et al.*, 2000, Lindo *et al.*, 1999, Goodman, 1996-Konishi *et al.*, 2007).

2.2.8 Food ingestion

Several epidemiological studies have found a positive relationship between the consumption of uncooked vegetables and *H. pylori* transmission (Goodman, 1996-Konishi *et al.*, 2007). Raw vegetables are suspected to be vulnerable to *H. pylori* colonization when contaminated water is used for washing or irrigation. It is important to bear in mind that this route assumes that *H. pylori* is also able to survive in water and has therefore all the problems associated with this possible transmission route(Gomes and De Martinis, 2004).

2.3 Epidemiology

Significant differences in prevalence across the world have been found within and between countries (Figure1) (Tan *et al.*, 2009). In undeveloped countries, most of the infections seem to be acquired during childhood while in developed countries the incidence increases gradually with age (Perez-Perez *et al.*, 2004).

The prevalence of *H. pylori* infection differs among countries and population groups. For middle-aged adults it varies in developed countries between 20 to 50% and up to 80% in many developing countries (Dunn *et al.*, 1997; Crew and Neugut, 2006;

Suerbaum and Michetti, 2002). The incidence, in developed countries, is about 1% per year until the age of 50 to 60 years (Bani-Hani *et al*., 2006). Epidemiological studies have shown that, in general, the high incidence of *H. pylori* is correlated with a deprivation in sanitation, hygiene and educational habits. (Axon, 2006; Bani-Hani *et al*., 2006; Dunn *et al*., 1997; Suerbaum and Michetti, 2002).

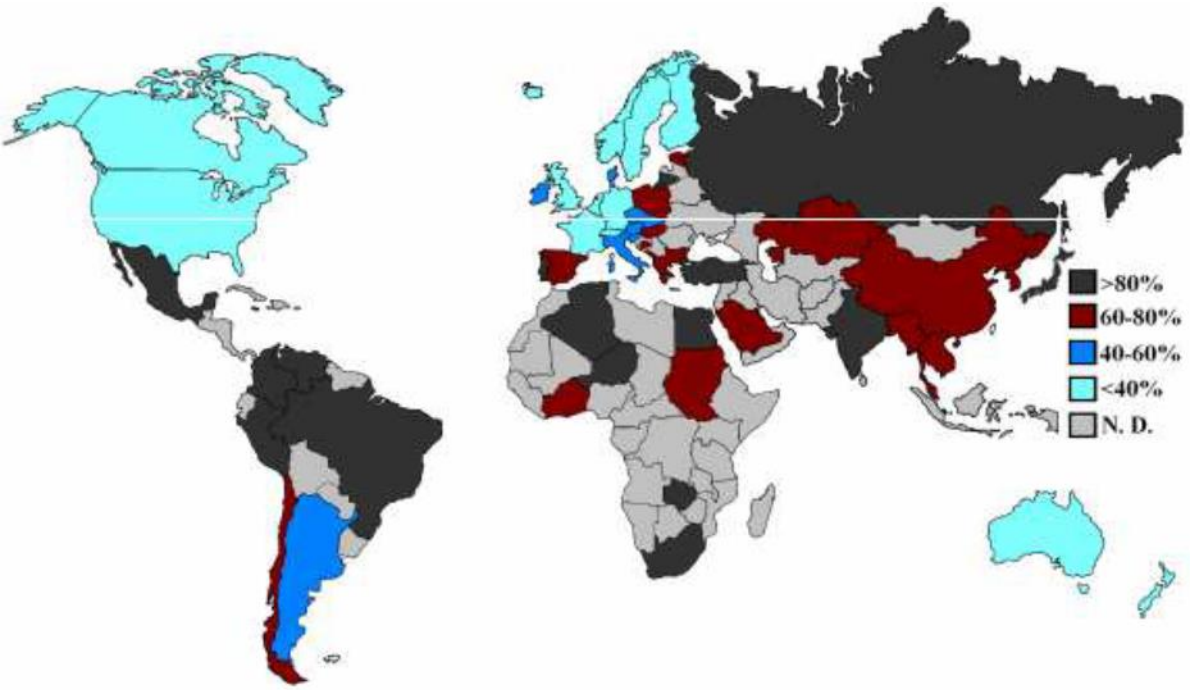


Figure 1: Worldwide prevalence of *H. pylori* after Tan *et al*, (2009); according to the latest.

2.3.1. The situation in Sudan:

Long time ago *H. pylori* was found in 80%of patients with gastritis, 56% of patients with duodenal ulcer, 60% of patients with duodenitis, 50% of patients with oesophagitis and 16% of normal control subjects, (Mirghani *et al.*, 1994), many years latter the seroprevalence among patients with dyspepsia was 65.8% in eastern Sudan (Abdallah *et al.*, 2014), in the same year Elhag and Omer published that the seropositive prevalence among Patients with Gastrointestinal Symptoms Attending Khartoum Teaching Hospital- Sudan was 21.1% positive for IgM antibody, 63.3% for IgG , while 16.7% were positive for both.

More recently, salih and his collague found that, the prevalence of HP is 56.3% among Sudanese children, although Generally HP is asymptomatic in children. The Prevalence of HP increased with age and was directly related to mother and father level of education, socioeconomic status and positive family history (Salih *et al.*, 2017)

2.4 Pathogenesis

H. pylori causes acute and chronic inflammation in the stomach, although the magnitude of inflammation varies from strain to strain and from host to host. In the majority of infected humans, there are no clinical consequences to *H. pylori* gastritis. In 20 to 30%, however, the end result of infection can be life-threatening. Four diseases are now widely acknowledged to be caused by *H. pylori*: duodenal ulcer, gastric ulcers, adenocarcinoma of the distal stomach (antrum and fundus), and gastric mucosa associated lymphoid tissue (MALT) lymphoma. Gastric adenocarcinoma is the 14th leading cause of death in the world (Covacci *et al.*, 1999).

2.4.1 Acute and chronic gastritis

Colonization with *H. pylori* virtually leads to infiltration of the gastric mucosa in both antrum and corpus with neutrophilic and mononuclear cells.

This chronic active gastritis is the primary condition related to *H. pylori* colonization, and other *H. pylori*-associated disorders in particular result from this chronic inflammatory process (Fig. 2) (Kuster *et al.*, 2006).

The acute phase of colonization with *H. pylori* may be associated with transient nonspecific dyspeptic symptoms, such as fullness, nausea, and vomiting, and with considerable inflammation of both the proximal and distal stomach mucosa, and pangastritis. This phase is often associated with hypochlorhydria, which can last for months (Perez-Perez *et al.*, 2003).

When colonization does become persistent, a close correlation exists between the level of acid secretion and the distribution of gastritis (Fig. 3). This correlation results from the counteractive effects of acid on bacterial growth versus those of bacterial growth and associated mucosal inflammation on acid secretion and regulation. In subjects with intact acid secretion, *H. pylori* in particular colonizes the gastric antrum, where few acid-secretory parietal cells are present. This colonization pattern is associated with an antrum-predominant gastritis. Histological evaluation of gastric corpus specimens in these cases reveals limited chronic inactive inflammation and low numbers of superficially colonizing *H. pylori* bacteria. Subjects in whom acid secretion is impaired, due to whatever mechanism, have a more even distribution of bacteria in antrum and corpus, and bacteria in the corpus are in closer contact with the mucosa, leading to a corpus-predominant pangastritis (Bloemena *et al.*, 1995).

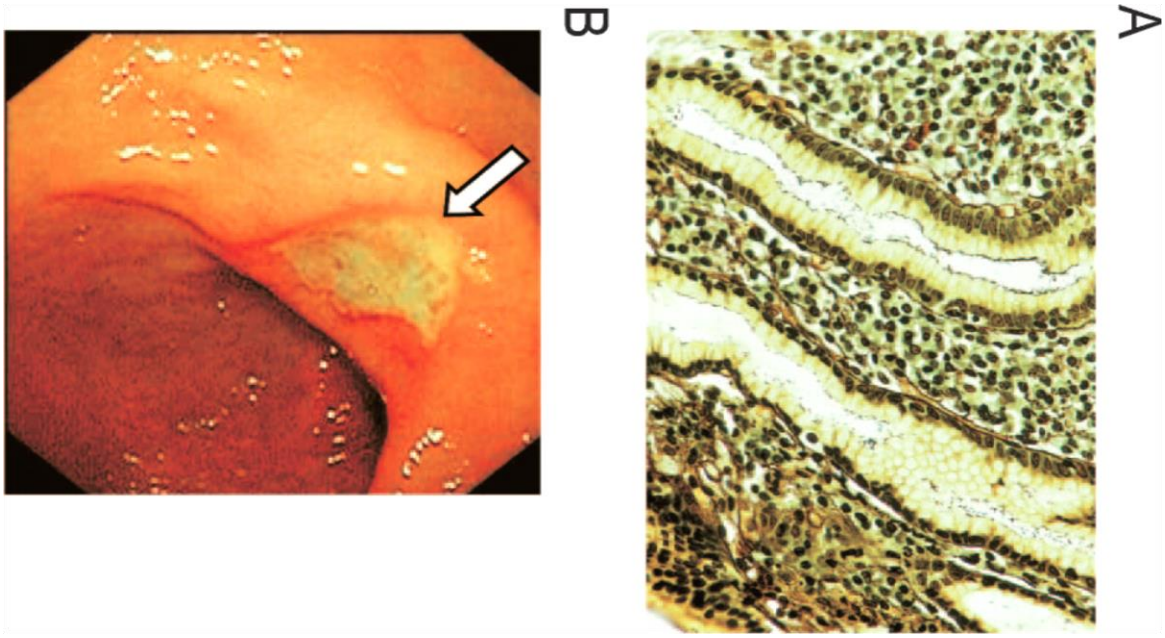


Figure 2: (A) Gastric glands abundantly colonized with *Helicobacter pylori*, shown as dark, curved bacilli closely aligning with the mucosal surface. (B) Endoscopic view of a gastric ulcer, with a clean base at the angulus. After Kuster *et al.*, (2006).

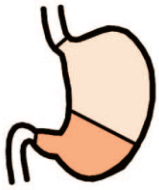
Pattern of gastritis	***Gastric histology***	***Duodenal histology***	***Acid secretion***	***Clinical condition***
 Pan-gastritis	• Chronic inflammation • Atrophy • Intestinal metaplasia	• Normal	• Reduced	• Gastric ulcer • Gastric cancer
 Antral-predominant	• Chronic inflammation • Polymorph activity	• Gastric metaplasia • Active chronic inflammation	• Increased	• Duodenal ulcer

Figure 3. Acid secretion and the associated pattern of gastritis play an important role in disease outcome in *H. pylori* infection. The figure displays the correlations between the pattern of *H. pylori* colonization, inflammation, acid secretion, gastric and duodenal histology, and clinical outcome.

The reduction in acid secretion can be due to a loss of parietal cells as a result of atrophic gastritis, but it can also occur when acid secretory capacity is intact but parietal cell function is inhibited by vagotomy or acid-suppressive drugs, in particular, proton pump inhibitors (PPIs) (Bloemena *et al.*, 1995). The resulting active inflammation of the corpus mucosa further augments hypochlorhydria, paralleling the acute phase of infection, as local inflammatory factors such as cytokines, including interleukin-1β (IL-1β), have a strong suppressive effect on parietal cell function, this is illustrated by various observations (El-Omer *et al.*, 1997) and (Correa *et al.*, 1996).

2.4.2 Peptic ulcer disease

Gastric or duodenal ulcers (commonly referred to as peptic ulcers) (Veldhuyzen *et al.*, 1999). In patients with duodenal ulcers, the inflammation of the gastric mucosa induced by the infection is most pronounced in the non–acid-secreting antral region of the stomach and stimulates the increased release of gastrin (El-Omer *et al.*, 1995). The increased gastrin levels in turn stimulate excess acid secretion from the more proximal acid-secreting fundic mucosa, which is relatively free of inflammation (El-Omer *et al.*, 1995). The increased duodenal acid load damages the duodenal mucosa, causing ulceration and gastric metaplasia. The metaplastic mucosa can then become colonized by *H. pylori*, which may contribute to the ulcerative process. Eradication of the infection provides a long-term cure of duodenal ulcers in more than 80% of patients whose ulcers are not associated with the use of NSAIDs (non-steroidal anti-inflammatory drugs).7 NSAIDs are the main cause of *H. pylori*–negative ulcers (Axon *et al.*, 1997).

2.4.3 Gastric Cancer

Extensive epidemiologic data suggest strong associations between *H. pylori* infection and noncardia gastric cancers (i.e., those distal to the gastroesophageal junction) (Aase *et al.*, 1999). The infection is classified as a human carcinogen by the World Health Organization (IARC, 1994). The risk of cancer is highest among patients in whom the infection induces inflammation of both the antral and fundic mucosa and causes

mucosal atrophy and intestinal metaplasia (Okamoto *et al.*, 2001). Eradication of *H. pylori* infection reduces the progression of atrophic gastritis, but there is little evidence of reversal of atrophy or intestinal metaplasia, (Ching *et al.*, 2004) and it remains unclear whether eradication reduces the risk of gastric cancer (Malfertheiner *et al.*, 2005).

2.4.4 Gastric MALT Lymphoma

Epidemiologic studies have also shown strong associations between *H. pylori* infection and the presence of gastric MALT lymphomas (Hansen *et al.*, 1994). Furthermore, eradication of the infection causes regression of most localized gastric MALT lymphomas (Dragosics *et al.*, 2004).

2.5 *H. pylori* and other conditions (extragastric manifestation):

- Cardiovascular diseases: several studies published on correlation between heart attack and *H. pylori* infection. Hughe et al showed the occurrence of concomitant decline in prevalence of heart attack and duodenal ulcer (which strongly related to *H. pylori* infection) due to eradication (Hughe, 2014).

  Another study reported that the high level of circulating IL-6 is abiomarker of heart failure in patients infected with CagA-positive strains (Figura *et al.*, 2014).
- Diabetes mellitus: a study reported that chronic *H. pylori* infection is significantly associated with high level of glycated haemoglobin A1c and type 2 DM. also there is possible role of *H. pylori* in complication of DM showing the risk of nephropathy and neuropathy.(Hsieh *et al.*, 2013) and (Wang *et al.*, 2013).
- Neurological diseases: there is significant positive predict value of antibodies level against and stroke (Sealy-Jefferson *et al.*, 2013). Other studies also evaluated the role of *H. pylori* on demedtia (Huang *et al.*, 2014).
- Haematological diseases: 75% of patients reported complete disappearance of iron deficiency anemia after *H. pylori* eradication (Hershko *et al.*, 2014).

Another report show the role of *H. pylori* on idiopathic thrompocytopenia purpure via modulation of Fcγ receptor balance of monocytes and macrophage or molecular mmimicry mechanisms between platelets and *H. pylori* peptides (Kuwana, 2014).

- Autoimmune diseases: a study report higher serum levels of rheumatoid factor and antinuclear antibodies in *H. pylori* infected patients with peptic ulcers. Thus, proposing that there is contribution of occurrence or worsening of some autoimmune diseases (Jafazaheh *et al.*, 2013).
- Hepatobiliary diseases: A report show a higher occurrence of cirrhotic nodules and liver fibrosis in patients coninfected with *H. pylori* and HCV. Besides *H. pylori* was identified in liver tissue from patient with hepatocellular carcinoma (Rabelo *et al.*, 2014).
- Respiratory diseases: *Helicobacter pylori* has been recently identified in the tracheobronchial aspirates, thus indicate the possibility that *H pylori* infection might cause ventilator-associated pneumonia has been raised (Mitz and Farber, 1993). Intense infiltration of the airway or gastric mucosa with neutrophils and T lymphocytes, (mediated by cytokines, especially IL-8, TNF-α, and IL-1b) (Tsang *et al.*, 1998). Occurs in both conditions. The increase in *H. pylori* seroprevalence appeared to correlate with disease activity, because the *H. pylori* immunoglobulin (Ig) G antibody level correlated with sputum production by the patients with bronchiectasis. The similarities between the disease process in bronchiectasis and ulceration might be due to a chronic inflammatory disease or bacteria induced tissue damage. It is also possible that aspiration of *H pylori* or its toxins into the respiratory tract could occur, particularly given the prevalence of gastrooesophageal reflux in patients with bronchiectasis (Tsang *et al.*, 1997).
- Metabolic change: *H. pylori* infection increases inflammatory factors, such as interleukin 1 (IL-1), interleukin 8 (IL-8), and tumor necrosis factor-alpha (TNF-α). These inflammatory factors may result in metabolic changes and systemic immune responses (Kim *et al.*, 2017) and (Roesler *et al.*, 2014).

2.1.5 Immune Response

Regarding the role of antibodies in protective immunity, Subsequent experiments have indicated that the relevance of the humoral system for protective immunity is only marginal. Antibodies can effectively prevent infection and reduce colonization in animal models (Marnila *et al.*, 2003), (Nomura *et al.*, 1994). *H. pylori* infection results in an induction of a Th1-polarized response that does not result, however, in clearance of the infection (Lindholm *et al.*, 1998), (Mohammadi *et al.*, 1996), (Smythies *et al.*, 2000). This is striking, as it is the cellular rather than the humoral immunity that has been reported to play the principal role in sterilizing immunity (Eaton *et al.*, 2001), (Castriotta *et al*., 1999), (Kosaka *et al*., 2000). *H. pylori* is thought to downregulate inflammation and control the host's immune response through a wide range of virulence factors that are involved in both provoking and maintaining a proinflammatory immune response (Eaton *et al.*, 2001).

2.1.6 Gastroeosophageal reflux disease (GERD)

GERD is a common condition result from the reflux of material (gastric acid, bile, pepsin, and duodenal contents overwhelm normal esophageal protective antireflux barriers,) through the lower eosophageal sphincter into eosophagus or oropharynx causing symptoms and/or injury to the eosophageal tissue (Spechler, 1992). Most people experience normal reflux which are not associate with pathogenic signs and may occur after meal (Szarka and Locke, 1999).

Pathologic reflux can results from variety of clinical presentations that lead to chronic symptoms, inflammation or eosophageal mucosal damage, whenever, GERD is more frequent and has longer duration. The lower eosophageal sphinicter relaxation is the key of etiologic factors (Storr *et al*, 2000).

The pathgenesis of this disorder (GERD) involves an imbalance between acid secretion and gastric mucosal defense. Important eosophageal symptoms include laryngitis and pharyngitis due reflux into the throat (Kahrilas, 2003).

Pathogenesis of GERD is similar to that of other secretory diseases such as duodenal ulcer disease and gastric ulcer disease. *H. pylori* infection is the factor in 85% to 100% of duodenal ulcers and 70% to 90% of gastric ulcers. Eradication of this organism result in a considerable decrease in recurrent ulcer (Sanders, 1996).

2.1.7 Diagnosis of *H. pylori* infection

Various tests have been developed for the detection of *H. pylori*, each with their specific advantages and disadvantages.The available tests are generally divided into invasive tests (endoscopic), based on gastric specimens, and noninvasive tests (nonendoscopic), based on peripheral samples for detection of antibodies, bacterial antigens, or urease activity (Logan, 1998).

Nonendoscopic Tests*:*

Serologic testing for IgG antibodies to *H. pylori* is often used to detect infection. However, a metaanalysis of studies of several commercially available quantitative serologic assays showed an overall sensitivity and specificity of only 85% and 79%, respectively (Loy *et al.*, 1996). The appropriate cutoff values vary among populations, and the test results are often reported as positive, negative, or equivocal. Also, this test has little value in confirming eradication of the infection, because the antibodies persist for many months, if not longer, after eradication.

Immunochromatography Test (ICT):

All invasive tests are limited by the high cost of endoscopy and sampling errors due to nonuniform distribution of *H. pylori* in the stomach (Greenberg *et al*, 1996). Noninvasive techniques to detect bacterial infection include urea breath tests (UBT) and anti-*H. pylori* antibody detection by serologic methods. The presence of antibodies to *H. pylori* strongly correlates with histologic evidence of infection in untreated patients (Talley *et al*., 1991).

The immunochromatographic test (ICT) method of anti-*H. pylori* antibody detection is based on the principle of reverse-flow immunochromatography, it includes the high-molecular-mass cell-associated protein (HMCAP), an antigen highly specific to *H. pylori* (Evans *et al.*, 1989), as the target antigen to detect *H. pylori*-specific IgG antibodies in human serum. It is preponderated in developing countrties laboratories because it is inexpensive, easy to perform, accurate, and produce results rapidly, i.e., during the encounter with the patient.

Enzyme-linked immunosorbent assay ELISA:

Enzyme-linked immunosorbent assay (ELISA) methodology is considered the optimal approach to serologic testing for *H. pylori* antibodies because such tests are noninvasive, simple to perform, rapid, and cost-effective compared to endoscopic biopsy. Further, serologic testing allows "global" sampling of gastric mucosal infection, whereas biopsy-based assays allow only localized sampling of the gastric mucosa. The choice of antigen is critical for the success of the ELISA. In general, four types of antigen have been used for detection of *H. pylori*-specific antibodies (Hoang *et al.*, 2004). These preparations include crude antigens, such as whole cells and whole-cell sonicates, cell fractions, such as glycine extracts and heat-stable antigens, component-enriched antigenic fractions, such as urease and the 120-kDa protein associated with vacuolating cytotoxic activity, and specific recombinant antigens, such as CagA. The sensitivities and specificities of several of the enriched antigen preparations typically exceed 95%. While analysis of whole-cell preparations of *H. pylori* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals a large number of polypeptides, generally no more than about 25 protein antigens are recognized by immunoblotting using sera from infected individuals. There is significant heterogeneity in the specificities of these antibodies. As shown by a variety of techniques, very few, if any, antigens are recognized by all positive sera. Among the most commonly recognized antigens are the 62- and 30-kDa subunits of urease and the 58-kDa homolog of the Cpn60 family of heat shock proteins (HspB). Considering the genetic heterogeneity of

H. pylori strains, it is generally thought that use of pooled extracts from multiple and genetically diverse strains as antigen preparations for detection of antibody will improve diagnostic performance. A significant fraction of individuals infected with *H. pylori* also produce serum and local antibodies directed against the 120-kDa CagA protein and the 89-kDa VacA protein. Antibodies directed against CagA and/or VacA protein antigen have a strong association with the severity of gastritis and with gastric cancer (Krah *et al.*, 2004).

The urea breath test:

The urea breath test involves drinking ^{13}C-labeled or ^{14}C-labeled urea, which is converted to labeled carbon dioxide by the urease in *H. pylori*. The labeled gas is measured in a breath sample. The test has a sensitivity and a specificity of 95% (Vaira and Vakil, 2001).

The fecal antigen test:

The infection can also be detected by identifying *H. pylori*–specific antigens in a stool sample with the use of polyclonal or monoclonal antibodies (the fecal antigen test). The monoclonal-antibody test is more accurate (Gisbert and Pajares, 2004). For both the breath test and the fecal antigen test, the patient should stop taking proton-pump inhibitors PPI 2 weeks before testing, should stop taking H2 receptor antagonists for 24 hours before testing, and should avoid taking antimicrobial agents for 4 weeks before testing, since these medications may suppress the infection and reduce the sensitivity of testing. (McColl and Kenneth, 2010).

EndoscopicTests:

H. pylori infection can be detected on endoscopic biopsy of the gastric mucosa, by means of several techniques. The biopsy specimens are usually taken from the prepyloric region, but an additional biopsy specimen obtained from the fundic mucosa may increase the test's sensitivity, especially if the patient has recently been treated with a proton-pump inhibitor. The urease-based method involves placement of the

endoscopic biopsy specimen in a solution of urea and pH-sensitive dye. If *H. pylori* is present, its urease converts the urea to ammonia, increasing the pH and changing the color of the dye. Recommendations for avoiding proton-pump inhibitors, H2 receptor antagonists, and antimicrobial therapy before testing apply to this test as well, to minimize the chance of false negative results (Midolo and Marshall, 2000) The test has a sensitivity of more than 90% and a specificity of more than 95% (Vaira and Vakil, 2001).

Another means of diagnosis involves routine histologic testing of a biopsy specimen; if there is *H. pylori* infection, the organism and associated gastritis are apparent on sections stained with hematoxylin and eosin or Giemsa. Although culturing of the organism is also possible and permits testing for sensitivity to antimicrobial agents, facilities for the culture of *H. pylori* are not widely available and the method is relatively insensitive. (McColl and Kenneth, 2010).

Additionally there are several molecular methods for detecting *H. pylori* include polymerase chain reaction (PCR), real- time PCR, stool PCR, multiplex PCR and fluorescent hybridization to identify *H. pylori* infection. There are several tests available to identify *H. pylori* infection, but none is considered a gold standard. (Malfertheiner *et al.*, 2012).

2.1.8 Infection recurrency of *H. pylori*

Although *H. pylori* is sensitive to a wide range of antibiotics in vitro, they all fail as monotherapy in vivo (Megraud, 1995). The lack of efficacy of monotherapy is related to the niche of *H. pylori*, residing at lower pH in a viscous mucus layer. Dual therapies, combining twice-daily-dosed PPI with, in particular, amoxicillin, are still in use in some countries, but dual therapies have mostly been replaced by triple therapies. These combine two antibiotics with either a bismuth compound or a PPI. A further alternative is provided by quadruple therapies, which combine the bismuth compound and PPI with two antibiotics. (Lambert and Midolo, 1997) Tetracycline,

amoxicillin, imidazoles, and a few selected macrolides (in particular clarithromycin, sometimes azithromycin) Are probably the drugs most widely used for *H. pylori* eradication therapy (Megraud and Lamouliatte, 2003). The use of these drugs has resulted in effective therapies against *H. pylori*, with consistent eradication rates over 80%. Failures are in particular related to insufficient therapy adherence, often because of side effects, and to the presence of antimicrobial resistance. Such resistance is common in patients who have had previous antibiotic treatment, including failed eradication therapies (Megraud, 2004). Therapeutic vaccination may already be beneficial, as it reduces the numbers of bacteria exposed to antibiotics and thus decreases the possibility of inducing antibiotic-resistant *H. pylori*. Much effort has been devoted to produce preliminary human vaccine trials (Voland *et al*., 2006).

Apparently Vaccines and antibiotics are not the only means for prevention and cure of *H. pylori*-associated disease. Poor socioeconomic status, living conditions, and hygiene have been repeatedly demonstrated to be major risk factors for *H. pylori* infection and *H. pylori*-associated disease (Kuster *et al*., 2006).

2.1.9 Previous studies about the role of *H. pylori* in throat infection:

Previous studies made by (Asefzadeh *et al.*, 2012, Gandomi *et al.*, 2007 and Aslan *et al*., 2007) showed that *H. pylori* has been identified in tonsillar tissue. Another Study suggested that, the rate of *H. pylori* infection was significantly higher in tonsillar tissues from a group of patients with tonsillitis compared with a group who had sleep-related breathing disorders (48% *vs* 24%) (Chang *et al.*, 2010). In addition to multiple regression analysis of confounding variables in patients with *H. pylori* gastric infection revealed that a history of tonsillectomy is associated with a decreased prevalence of gastric *H. pylori* (Minocha *et al*., 1997).

In contrast, another study found no significance difference between the incidence of *H. pylori*-positive tonsillar samples from patients with chronic tonsillitis and those with

obstructive sleep apnea syndrome (80% *vs* 83%) (Astl *et al*., 2013). Another report indicated that tonsillar tissue does not seem to be a reservoir for *H. pylori* infection and thattonsillectomy does not significantly affect gastric *H. pylori* eradication (Erzin *et al*., 2013). Nevertheless, *H. pylori* can survive for a certain period in gastric juice in the esophagus (Marshall, 2002). Gastric juices infected with *H. pylori* and systemic immune responses to gastric *H. pylori* infection might play a causative role in upper respiratory diseases (Paterson, 1997).

Gastropharyngeal reflux is considered an important factor in the pharyngeal disorders (Gross *et al*., 2002, and Biskin *et al*., 2010), and *H. pylori* residing in the pharynx might play a role in the development of pharyngitis. Some studies have tested this hypothesis by examining an association between *H. pylori* and pharyngitis (Kariya *et al.*, 2014). Moreover, it can be determined if a history of certain gastric diseases may contribute to pharyngitis infection. As a result it can be said that *H. pylori* is not detected in the pharynx of healthy people, chronic nonspecific pharyngitis is significantly related to *H. pylori* infection, and a stomach ailment history is associated with a higher rate of *H. pylori* infection of the pharynx (Emir *et al.*, 2006). The infection rate with *H. pylori* in the pharynx is higher in patients with stomach ailment histories than in patients without stomach ailment histories, suggesting that chronic pharyngitis may be related to stomach ailment history (Peng *et al.*, 2005).

2.2 Microbial agents of throat infection

Throat infection includes tonsillitis and pharyngitis may be caused by a wide variety of microbial agents, but the most common bacterial cause is group A beta-hemolytic streptococci. Group A Streptococcal pharyngitis is responsible for about 5% to 17% of sore throats in adults and 20-30% in children. However, the causative microorganisms in many cases remain unclear without critical diagnosis (Danis *et al.*, 2004), (Ebell *et al.*, 2000).

There is broad overlap between the signs and symptoms of streptococcal and nonstreptococcal (usually viral) pharyngitis, and the ability to identify streptococcal pharyngitis accurately on the basis of clinical grounds alone is insufficient (Mclsaac *et al.*, 2004). Therefore, a laboratory test should be performed to determine whether GAS is present in the pharynx (Bisno, 1996), (Choby, 2009).

Patients with GAS pharyngitis commonly present with sore throat (generally of sudden onset), pain on swallowing, and fever. Headache, nausea, vomiting, and abdominal pain may also be present, especially in children. On examination, patients have tonsillopharyngeal erythema, with or without exudates, often with tender, enlarged anterior cervical lymph nodes (lymphadenitis). Other findings may include a beefy, red, swollen uvula; petechiae on the palate; excoriated nares (especially in infants); and a scarlatiniform rash. However, none of these findings are specific for GAS pharyngitis. Conversely, the absence of fever or the presence of clinical features such as conjunctivitis, cough, hoarseness, coryza, anterior stomatitis, discrete intra-oral ulcerative lesions, viral exanthema, and diarrhea strongly suggest a viral rather than a streptococcal etiology (Shulman *et al.*, 2012).

Other bacteria can also cause pharyngitis *Arcanobacterium haemolyticum* is a rare cause of pharyngitis and associated with a rash, particularly in teenagers and young adults (Nyman *et al.*, 1997). *Neisseria gonorrhoeae* can occasionally cause acute pharyngitis in sexually active persons.

Other bacteria, such as *Francisella tularensis* and *Yersinia enterocolitica*, and mixed infections with anaerobic bacteria (eg, Vincent's angina) are rare causes of acute pharyngitis. Other pathogens, such as *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae,* are uncommon causes of acute pharyngitis. Other bacterial causes of acute pharyngitis include groups C and G β-hemolytic streptococci and *Corynbacterium diphtheria* (Gerber *et al.*, 1991). Group C streptococcus (GCS) is a relatively common cause of acute pharyngitis among college students and adults (Turner *et al.*, 1997). In addition to endemic pharyngitis, GCS can cause epidemic food-borne pharyngitis

after ingestion of contaminated products, such as unpasteurized cow's milk. Family and school outbreaks of GCS pharyngitis have also been described. Even though there are several well-documented food-borne outbreaksnof group G streptococcal (GGS) pharyngitis, as well as a community-wide respiratory outbreak of GGS pharyngitis in children (Gerber *et al.*, 1991).

Several recent reports have documented the isolation of *Fusobacterium necrophorum* from throat swabs of adolescents and young adults with nonstreptococcal pharyngitis (Amess *et al.*, 2007).

2.2.1 Diagnosis of throat infection:

2.2.1.1 Throat culture:

Culture of a throat swab obtained from the surface of both tonsils and the posterior pharyngeal wall on a sheep blood agar plate has been the standard for the documentation of the presence of GAS pharyngitis in the upper respiratory tract and for the confirmation of the clinical diagnosis of acute streptococcal pharyngitis (Snow *et al*, 2001). So that, culture of a single throat swab on a blood agar plate is 90%– 95% sensitive for detection of GAS pharyngitis (Gerber, 1984). However, false-negative results if the patient has received an antibiotic shortly before the throat swab is obtained. A major disadvantage of throat cultures is the delay (overnight or longer) in obtaining results.

2.2.1.2 Rapid antigen detection test (RADT):

RADT has been developed for identification of GAS pharyngitis directly from throat swabs. The first RADTs used latex agglutination methods, were relatively insensitive, and had unclear end points. Newer tests based on enzyme immunoassay techniques offer increased sensitivity and a more sharply defined end point (Gerber and Shulman, 2004). More recently, RADTs that use chemiluminescent DNA probes or optical

immunoassay have been developed. A variety of RADTs are available, and they have different performance characteristics (Tanz *et al.*, 2009). RADTs are highly specific (approximately 95%) when compared with blood agar plate cultures (Tanz *et al.*, 2009). False-positive test results are highly unusual, and therefore therapeutic decisions can be made with confidence on the basis of a positive test result (Johnson and Kaplan, 2001). The sensitivity of most of these tests is 70%–90%, compared with blood agar plate culture (Gerber and Shulman, 2004).

Neither conventional throat culture nor RADTs accurately differentiate acutely infected persons from asymptomatic streptococcal carriers with intercurrent viral pharyngitis. Nevertheless, they allow physicians to withhold antibiotics from the great majority of patients with sore throats for whom results of culture or RADT are negative. This is of extreme importance, because nationally up to 70% of patients with sore throats seen in primary care settings receive prescriptions for antimicrobials (Nyquist *et al.*, 1998), while only 20%–30% are likely to have GAS pharyngitis (Ebell *et al.*, 2000).

2.2.1.3 Measurement of anti-streptococcal antibody titers:

This test often useful for diagnosis of the nonsuppurative sequelae of GAS pharyngitis. However, such testing is not useful in the diagnosis of acute pharyngitis because antibody titers of the 2 most commonly used tests, antistreptolysin O (ASO) and AntiDNase B, may not reach maximum levels until 3–8 weeks after acute GAS pharyngeal infection and may remain elevated for months even without active GAS infection (Johnson *et al.*, 2010).

2.2.1.4 Molecular detection and characterization of GAS:

Polymerase chain reaction (PCR) methods have the necessary means to detect small quantities of pathogen (even dead ones). There are various schemes to PCR amplification of bacterial DNA in clinical samples. The first depending on usage of species-specific primers. This method misses the ability to detect bacterial infection

definitely. The second approach involves amplification of sequences persist in all bacteria based on universal sequences common in bacteria. A number of primer systems for 16S bacterial rRNA detection have been accomplished but they differ in focus to examinant clinical material and pathogens. Dnase B gene seems to be both existing in all *S.pyogenes* and also unique to this organism (Slinger *et al*., 2011). Liu *et al*. (2005) described Spy 1258 gene that is uniquely lay out in the *S.pyogenes*. Application of PCR primer formed from this gene facilitated amplification of DNA fragment from S.pyogenes only, but not from other species of the genus Streptococcus and common bacteria. Amplification and sequence analysis of the 16S rRNA gene can be applied to determine and identify bacteria in clinical samples. The discovery of PCR and DNA sequencing, comparisons of the gene sequences of bacterial species have revealed that the 16S rRNA gene is highly conserved within a species and among species of the same genus and thus can be used as the new "gold standard" for species-level identification of bacteria (woo *et al.*, 2001). rRNA gene RFLP analysis, or ribotyping, has been expandly used to characterize bacteria for epidemiologic and taxonomic purposes. The rRNA gene Restraction Fragments Length Polymorphisam (RFLP) analysis, or ribotyping, has been expandly used to qualify bacteria for epidemiologic and taxonomic determination. Numerous studies revealed that ribotyping was a stable, reproducible, and widely applicable typing system for determining the molecular epidemiology of genetically diverse bacteria (Maslow *et al.*, 1993). The main goal of this study to compare between different methods for detection and identification of *S.pyogenes* from (ENT) patients.

2.2.2 Treatment of throat infection:

A number of antibiotics are believed to be effective in treating GAS pharyngitis. These include penicillin and its congeners (eg, ampicillin and amoxicillin), as well as numerous cephalosporins, macrolides, and clindamycin. Penicillin, however, remains the treatment of choice because of its proven efficacy and safety, its narrow spectrum,

and its low cost [Report of the Committee on Infectious Disease 2012]. Penicillin-resistant GAS has never been documented.

GAS carriers do not generally require antimicrobial therapy because GAS carriers are unlikely to spread GAS pharyngitis to their close contacts and are at little or no risk for developing suppurative or nonsuppurative complications (eg, acute rheumatic fever) (Shulman *et al.*, 2012).

2.12 Microbial flora of throat:

S. salivarius is considered a dominant population in the human oral cavity and the upper airways, throughout the life of its human carrier (Nakajima *et al.*, 2013); (Tappuni *et al.*, 1993). And it plays a positive role in oral ecology (Jensen *et al.*, 2013). However *S. salivarius* displays only weakly virulent as an opportunistic infections, it can cause invasive disease in immunocompromised patients (Corredoira *et al.*, 2005) and (Han *et al.*, 2006).

Streptococcus morbillorum or *Gemella morbillorum* usually behaves as a commensal organism of the mucous membranes and is part of the normal flora of several surfaces, including that of the oropharynx and the gastrointestinal. However, *Gemella species* are able to cause severe localized and generalized infections as opportunistic pathogens (Benedetti *et al.*, 2009). It was eventually transferred from the genus *Streptococcus* to the genus Gemella in 1988 on the basis of its biomolecular features and physiological properties (Kilpper-Balz and Schleifer, 1988). Gemella species are important reservoirs of genes encoding resistance antibiotics such as macrolides. Consequently this resistance can be easily transferred to other pathogens that share their habitat, such as *Streptococcus pneumoniae* and *Streptococcus pyogenes*, then can affect the therapeutic issues (Cerda ` Zolezzi *et al.*, 2004; Asensi *et al.*, 1996)

Branhamella catarhalis: The only species of *Branhamella (Branhamella catarrhalis*) is reclassified to *Moraxella catarhalis* based on molecular analysis It was considered to be a harmless commensal of the upper respiratory tract, eventually it recognized was as

an important cause of respiratory tract infections in the elderly and in those with pre-existing respiratory disease as a common cause of otitis media in children (Enright *et al.*, 1997). And it had been also described by (Gordon, 1921) as a saprophyte of negligible virulence found in the throat of healthy adults.

Neisseria elongata: is a constituent of the commensal bacterial flora in the pharynx (Bøvre *et al.*, 1970). It was previously thought to be non-pathogenic, but has relatively recently been found to cause systemic infections such as endocarditis (Grant *et al.*, 1990), (Garner *et al.*, 1986) and (Yeon *et al.*, 2014).

Neisseria lactamica: This bacterium lives in a commensal relationship with humans, and frequently isolated from the upper respiratory tract (Kremastinou *et al.*, 2003 and Saez-Nieto *et al.*, 1985) and nasopharynx of children. It is rarely associated with invasive disease. (Changal *et al.*, 2016 and Everts *et al.*, 2010).

CHAPTER THREE

Materials and Methods

3.1 Materials

Disposable Betri-dishes

Test tubes

Flasks

Beakers, Cylinders

Micropipettes

Micropipette tips

McCartney bottles

200 ml Glass bottles

1.80 ml Cryo tubes

1.5 ml and 2ml Eppendorf tubes

Plastic pipette droppers

Racks

Oxidase discs bottles (HIMEDIA)

Bacitracin discs bottles (HIMEDIA)

Optichin discs bottles (HIMEDIA)

Novobiocin discs vial (HIMEDIA)

H. pylori rapid Ab detection strips

H. pylori IgG, IgA ELIZA kits (EUROIMUM)

Bacterial DNA extraction kit (Vivatis)

Lysozyme (thermo fisher)

Proteinase K (sigma)

PCR mastermix streps (ENTRON)

DNA ladder 100bp vial (ENTRON)

Agarose (thermo fisher)

3.2 Methodology

3.2.1 Study design:

Case control hospital based study.

3.2.2 Study area:

This study was performed in the state of Khartoum with the collaboration of the private clinical labs and hospitals in Khartoum around Ibrahim Malik Hospital.

3.2.3 Study population:

The study subjects were selected from patients who are atteniding the Gastroenterology clinics with abdominal complaints; patients who have *H. pylori* positive results were considered, and patients who have *H. pylori* negative results were considered as control. Subjects out of the clinics were also collected from heathy participants.

3.2.4 Inclusion criteria:

- Patients with *H. pylori* positive blood (serum) tests results.
- Patients with *H. pylori* negative blood (serum) tests results.

3.2.5 Exclusion criteria:

- Patients with epiglottis inflammation.
- Patients who are subjected to antibiotics will be excluded from this study.
- Patients with breathing labor such as apnea syndrome, asthma … etc.

3.2.6 Sampling:

Sample size: 307 throat swabs samples (from tonsils and posterior auropharynx) and 307 blood samples were obtained and calculated according to the following formula.

Sample size:

Calculation:

$$n = 1.96 \times \frac{Pexp^2(1 - Pexp)^2}{d^2}$$

n: required sample size

P_{exp}: expected prevalence

d: desired absolute precision = 0.05

Expected prevalence is 70% as recorded by using confidence level 95%.

3.2.7 Data collection tools:

Questionnaire form is included. Patient's demographic data should be retrieved from medical records on enrollment, including: Age, Gender, Smoking history, Body mass index. Hypertension, Diabetes mellitus. Questionnaires about general manifestations and symptoms.

3.2.8 Sample taking procedure:

Throat swabs: Sampling was obtained by taking throat swabs from tonsil and posterior auropharynx by the clinical lab's qualified experienced employees.

Blood: Sampling was obtained by taking 5 ml of venal blood by the clinical lab's qualified experienced practitioner.

3.2.9 Data analysis:

A analysis of the obtained results was performed by SPSS software.

3.2.10 Ethical consideration:

- Approval from University.
- Approval from Khartoum Ministry of Health Research Department.

- Approval from hospital.
- Research purpose and objectives were explained to participant in clear simple words.
- Participants has right voluntary informed consent.
- Participant has right to withdraw at any time, right to benefit (patient's phone number was taken to confirm him the positive result) , to no harm (samples were taken by qualified experienced person and swabs were never be reused), to privacy and confidentiality.

3.2.11 Sterilization and disinfection

3.2.11.1 Autoclaving:

Media, normal saline, micropipette tips, eppendorf tubes were sterilized by autoclaving at15 lbs (121°C) for 15 minutes.

3.2.11.2 Dry Heat:

Hot oven: Glass ware and equipment were sterilized in hot air oven at temperature 150-170°C for one hour. While test tubes and flasks or other glass wares containing media were sterilized by autoclaving.

Ultra-violet: UV lamp radiation was used for sterilization of sub-media room for 20 minutes.

3.2.11.3 Sanitation: Platinium loop and forceps were sterilized by direct flame.

3.2.11.4 Disinfection: Dettol solution and alcohol 70% were used for disinfecting benches and discards.

3.2 12 Preparation of media

The media were prepared according to Barrow and Feltham (2003).

3.2.12.1 Blood agar

Defibrinated blood 50 ml

Nutrient agar 950 ml

Nutrient agar was melted and sterilized by autoclaving, then it was cooled to 50ºC, the blood was added aseptically and mixed well then distributed in plates.

3.2.12.2 Blood agar slope

Defibrinated blood 50 ml

Nutrient agar 950 ml

Blood was taken from the jugular vein of sheep aseptically by syringe containing anticoagulant (citrate) with shaking 3-4 times to prevent coagulation.

Nutrient agar was melted and sterilized by autoclaving, then it was cooled to 50ºC, the blood was added aseptically and mixed well then it was distributed in screw capped bottles and allowed to set as slopes.

3.2.12.3 Serum broth

Sterile serum 50 ml

Nutrient broth 950 ml

Serum was prepared from blood which was collected without anticoagulant, by removal of serum that separates when clot contracts.

Nutrient broth was sterilized by autoclaving, it was cooled to 50ºC and serum was added aseptically. Then it was mixed and distributed in screw capped bottles.

3.2.12.4 Nutrient broth

Beef extract 10g

Peptone 10g

NaCl 5g

Distilled water 1000g

The ingredients were dissolved by heat in water and PH was adjusted to 7.2-7.4 and then sterilized at 121ºC for 15 minutes.

3.2.12.5 Nutrient agar

Beef extract	10g
Peptone	10g
NaCl	5g
Agar	28.09g
Distilled water	1000g

The ingredients were dissolved by heat in water and PH was adjusted to 7.2-7.4 and then sterilized at 121ºC for 15 minutes. After cooling at 45-50ºC, the medium was poured in sterile Petri-dishes about 15 ml in each, they were stored at 4ºC after they were solidified.

3.2.12.6 Voges Proskauer (VP) test media

Glucose-peptone medium:

Peptone	10g
Glucose	5g
Distilled water	1000ml

Solids were dissolved by heating in water, PH was adjusted to 7.6 and then the medium was sterilized at 121ºC for 5 minutes.

3.2.12.7 Broth sugars

Peptone	10g
Meat extract	3g
NaCl	5g

Distilled water 1000g

Andrate's indicator 10g

Solids were dissolved in water, the indicator was added and PH was adjusted to 7.1 to 7.2, then the broth sugars were sterilized at 121ºC for 15 minutes. The appropriate carbohydrates 0.5-1% were added aseptically, they were mixed, and distributed into sterile tubes.

3.2.12.8 Bile esculin agar

The medium was prepared according to (Himedia). It consist of:

Animal tissue 5g

Beef extract 3g

Ox gall 40g

Escullin 1g

Ferric citrate 0.5g

Agar 15g

64.5 grams were suspended in 1000 ml distilled water, the suspension was heated to boiling for dissolving the medium completely. It was then sterilized by autoclaving at 121ºC for 15 minutes. The medium was dispensed in screw capped bottles to form slants.

3.2.12.9 Arginine broth

Peptone (tryptone) 5g

Yeast extract 5g

K_2HPO_4 2g

Glucose 0.5g

Arginine monohydrochloride 3g

Distilled water 1000 ml

Ingredients were dissolves by heating and the PH was adjusted to 7.0, then it was sterilized by autoclaving at 121ºC for 15 minutes.

3.2.12.10 Diluted plasma

Rabbit plasma was obtained by removing the supernatant of the blood clott (EDTA) after the red cells had settled. The rabbit plasma was then diluted by addition of 0.8 ml of nutrient broth to 0.2 of rabbit plasma in each tube.

3.2.12.11 Nitrate broth:

KNO_3 1g

Nutrient broth 1000 ml

KNO3 was dissolved in the broth and distributed into containing inverted inner (Durham) tubes and then sterilized at 121ºC for 15 minutes.

3.2.12.12 Urea media

Peptone 1g

NaCl 5g

KH_2PO_4 2g

Agar 20g

Distilled water 1000g

The ingredients were dissolved by heating, the PH was adjusted to 6.8 and sterilized at 121ºC for 15 minutes.

Glucose 1g

Phenol red, 0.2% aq. soln 6ml

Were added to the molten base, the mixture was steamed for 1 hour and cooled at 50-55ºC.

Urea, 20% aq. soln 100 ml

Was sterilized by filtration and added aseptically to the base and cooled at 50-55ºC. The medium was distributed aseptically into sterile containers and were allowed to cool as slopes.

3.2.12.13 Oxidation fermentation media

Ingredients Grams/Litre

Casein peptone (pancreatic) 2.0g

Sodium chloride 5.0g

Dipotassium hydrogen phosphate 0.3g

Bromothymol blue 0.08g

Agar 2.5g

Final pH 7.1 +/- 0.2 at 25°C

Prepared media were stored below 8°C, and protected from direct light. The prepared culture medium is dark-green to blue-green in color and clear.

9.88 g in was dissolved in 1 litre distilled water. Then the suspension was sterilized by autoclaving at 121°C for 15 minutes. And Left to cool to approxmately 50°C and 100 ml of sterile filtered 10% D(+)-glucose, lactose, sucrose were added. They were mixed well and dispensed in 5 ml amounts in sterile tubes in duplicate for aerobic and anaerobic fermentation.

3.2.12.14 Brain hear infusion broth

Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5

pH 7.4 ± 0.2 @ 25°C

37g was dissolved in 1 litre of distilled water. Then suspention was mixed well and distributed into final containers. The media were sterilized by autoclaving at 121°C for 15 minutes.

3.2.12.15 Triple sugar iron

Meat extract	3	g
Yeast extract	3	g
Peptone	20	g
Glucose	1	g
Lactose	10	g
Sucrose	10	g
FeSO4-7H2O	0.2	g
Distilled water	1000	ml

Phenol red, 0.2% aq. soln 12 ml

The solids were heated to dissolve in the water, the indicator solution was added and mixed and dispensed into tubes. Then sterilized at 115 °C for 20 min and cooled to form slopes with deep butts about 3cm long.

3.2.12.16 Citrate media

Sodium citrate	3 g
Glucose	0.2 g
Yeast extract	0.5 g
L-cysteine hydrochloride	0.1 g
Ferric ammonium citrate	0.4 g
KH_2PO_4	1g
NaCl	5g
$Na_2S_2O_3$	0.08g
Agar	20g
Phenol red, 0.2% aq. Soln	6g
Distilled water	1000ml

Solids were dissolve in the water by heating; pH adjusted to 6.8-6.9, the indicator was added, and sterilized at 115 °C for 20 min.

3.2.12.17 Gelatin media

Gelatin	4 g
Nutrient Agar	50 ml
Distilled water	1000 ml

Soak the gelatin was soaked in the water and, when thoroughly softened, the was added to the melted Nutrient Agar. They mixed well, and sterilized at 115 °C for 10 min, and distribute into McCartney tubes.

3.2.12.18 Casein Agar (Milk Agar):

Milk, skim	500 ml
Nutrient Agar	500 ml

Prepare the skim-milk was prepared and sterilized by heating at 115 °C for 10 min. after cooling to about 50 °C and added to nutrient Agar after melting and cooling to 50-55 °C. The components were mixed well and distributed in Petri dishes.

3.2.12.19 Ammonium salt sugar

(NH4)2HPO4	1g
KC1	0.2g
MgSO4-7H2O	0.2g
Yeast extract	0.2g
Agar	20 g
Distilled water	1000 ml
Bromcresol purple, 0.2% aq. Soln	4 ml

Solids were added to the water and dissolved by steaming. Then the indicator was added and sterilized at 115 °C for 20 min. Then the basal medium was left to cool to about 60 °C and the appropriate carbohydrate as a sterile solution was added to give a final concentration of 0.5-1%. The solution was mixed and distributed aseptically into sterile tubes which are inclined so that the medium set as slopes

3.2.12.20 Motility medium

Peptone	10g
Meat extract	3 g
NaCl	5 g
Agar	4 g
Gelatin	80 g
Distilled water	1000 ml

The gelatin was soaked in the water for 30 min, then added the other ingredients, and heated to dissolve, and sterilized at 115 °C for 20 min.

3.2.12.21 Peptone water

Peptone	10g
NaCl	5g
Water	1000 ml

Solids were dissolved by heating in the water. Adjust to pH adjusted to 8.0-8.4 and boilled for 10 min. and sterilized at 115 °C for 20 min.

3.2.13 Reagent preparation:

3.2.13.1 Nessler´s reagent:

5g of potassium iodide was dissolved in fresh distilled water. Then Cold saturated mercuric chloride solution was added until a slight precipitation remains permanently after through shaking. Then 40 ml of 9N NaOH was added and all content was diluted to 100 ml with distilled water. The solution was allowed to stand for 24 hours.

3.2.13.2 α- naphthol solution

5% a-naphthol in ethanol (not 95% ethanol)
The solution should not be darker than straw colour; if necessary the a-naphthol should be redistilled.

3.2.13.3 KOH 40% (Oxoid limited)

3.2.13.4 H_2O_2 (sigma-Aldrich)

3.2.13.5 Kovacs reagent

7-dimethylaminobenzaldehyde	5 g
Amyl alcohol	75 ml
Cone. HC1	25 ml

Dissolve the aldehyde was dissolved in the alcohol by gently warming in a water bath (about 50-55 °C), after cooling the acid added with care. Then the soln was protected from light and stored at 4 °C.

3.2.13.6 Oxidase and Antibiotic discs (HIMEDIA Co.)

Bacterial isolates were preserved in 1:1 absolute glycerol and brain heart infusion broth.

3.2.14 Transportation control of collected samples

Throat swab specimens were obtained from the surface of both tonsils and the posterior pharyngeal wall. The swabs were coated with serum broth aseptically at the clinical lab and then preserved by using ice bags to prevent the multiplication of commensals; and transferred to the research lab.

3.2.15 Isolation and identification by conventional methods:

3.2.15.1 Throat swabs culture:

The specimens were inoculated on sheep blood agar and was preferred to be incubated anaerobically in carbon dioxide enriched atmosphere at 37°C for 24 hours. In case the swabs were dry they were moisten first with nutrient broth and then inoculated on the plates.

3.2.15.2 Microscopic examination

Smears were prepared from growth cultures and spread on slides for Gram stain technique. The smears were allowed to air-dry in a safe place and fixed by heating. Crystal-violet was applied for 30 seconds and then washed in water. Lugol's iodine solution was added for 30 seconds, the iodine was drained off without washing and then decolourized with few drops of acetone, and washed thoroughly in water. Counterstain with diluted carbol fuchsin was added for 30 second. Slides were washed and stand on end to dry.

3.2.15.3 Catalase test

The organisms were cultured overnight in serum broth, 1 ml of 3% of H_2O_2 was added for examination of immediate evolution of gas as positive reaction.

3.2.15.4 Oxidase

A loop full test culture was smeared on oxidase test discs with a platinum loop. The appearance of dark purple colour on the disc was donated as positive reaction.

3.2.15.5 Coagulase

0.5 ml of undiluted plasma was added to equal volume of 24 hours broth culture and incubated at 37ºC for 4 hours, the coagulation negative tubes were left at room temperature overnight and then were re-examined.

3.2.15.6 Antibiotic sensitivity

Streptococci were cultured on blood agar and incubated at 37ºC overnight. The organisms were tested for Bacitracin, Optichin and novobocin sensitivity by placing each antimicrobial disc on a quarter of cultured plate per an organism.

3.2.15.7 Acid from carbohydrates

About 200 µl of broth culture was added to sugar broth and incubated aerobically at 37ºC for 1-7 days. The tests were examined daily for 7 days for acid production. Reversion to alkalinity was also noted.

3.2.15.8 Aesculin hydrolysis

Aesculin agar was inoculated with the test organism, and incubated at 37ºC overnight to 5 days and then was examined daily for 5 days for blackening around bacterial growth.

3.2.15.9 Arginine hydrolysis

5 ml of arginine broth were inoculated with the organism and after incubation for 24 hours, 0.25 ml of Nessler's reagent was added to the culture, arginine hydrolysis was indicated by development of brown colour.

3.2.15.10 Voges- Proskauer test reaction

0.6 ml 5% α-naphthol solution and 0.2 ml 40% KOH aqueous solution were added to 48 hours incubated broth culture; the tubes were shaken and sloped open to increase the area of the air-liquid interface, and examined after 30 min. A positive reaction is indicated by a strong red colour.

3.2.15.11 Oxidation fermentation test

Duplicate tubes of OF semisolid medium were stab-inoculated with a straight wire. A layer of melted soft paraffin oil was added to one of the tubes to a depth of about 2 cm above the medium to seal it from air. The tubes were examined daily after incubation for up to 14 days. The oxidation of sugar was indicated by turning the green colour medium to yellow in the open tube, and the fermentative consumption indicated by yellow colour in the paraffin oil sealed tube.

3.2.15.12 Urease

A slope of Christensen's Urea medium was inoculated heavily, then the growth was examined after incubation for four hours and daily for 5 days. The red colour indicates that the test is positive.

3.2.15.13 Brain heart infusion broth

Brain heart infusion broth was inculated with loopful bacterial isolate and incubated overnight in 10% CO2.

3.2.15.14 Citrate

A slope of Simmons' citrate medium was inoculated on the surface, and examined daily - after incubation for up to 7 days - for growth and colour change. Blue colour and streak of growth indicated citrate utilization original green colour means citrate not utilized.

3.2.15.15 Ammonium salt sugar

Slopes of ammonium salt agar were inoculated heavily with bacterial isolate and incubated overnight in 37 °C up to five days.

3.2.15.17 Casein digestion

Plates of casein agar were inoculated with bacterial isolates and incubated overnight in 37 °C up to five days.

3.2.15.18 Gelatin liquefaction

Nutrient Gelatin was inoculated with the test organism and incubated at 37 °C for up to 14 days; every 2-3 days, the culture refrigerated for 2 h and then examined for liquefaction.

3.2.15.19 Motility test

The tubes of motility medium were Stab-inoculated in the cragie tubes inside the test tubes. They were incubated at 37°C. Motile organisms migrate out of the cragie tubes in the medium, which becomes turbid; while the growth of non-motile organisms is confined to the stab inoculum.

3.2.15.20 Indole production

Peptone Water broth media was inoculated with the test organism and incubated for 48 h. 0.5 ml of Kovacs' reagent was added, shaken well, and examined after about 1 min. A red colour in the reagent layer indicates indole production.

3.2.15.21 Hydrogen Sulphide production

Tubes of Triple Sugar Iron Agar were inoculated by stabbing the butt and streaking the slope; the growth was observed daily for up to 7 days. The blackening of the butt due to H2S production, and the dark pigment on the slope is a positive result.

3.3 Molecular biological identification

3.3.1 DNA extraction

In our preliminary experiments we estimated that the manual protocol modified from "plasmid DNA extraction" yields high concentration of extracted DNA compared with bacterial Kit DNA extraction protocol, using nanodrop apparatus (Thermofischer) (figure 5). However, the boiling method produced moderate extracted DNA concentration compared with both procedures mentioned above.

DNA extraction kit	32T: 63 ηg/µl
Bioiling method	115P: 32 ηg/µl
Plasmid DNA extraction	117P: 315 ηg/µl

Figure 4:Thermofischer Nanodrop

3.3.1.1 DNA extraction kit (GF-1 BA-100)

Kit components:

- GF-1 Columns
- Collection tubes
- Resuspension puffer 1
- Resuspension buffer 2
- Bacctreial genomic binding buffer
- Washing buffer
- Elution buffer

- Protienase K
- Lysozyme (50mg/ml)
- Absolute ethanol

Procedure:

1. Centrifugation: pellets of 3ml bacterial culture were centrifuged at 6000 xg for 2min. at room temperature. The supernatant was decanted completely.
2. Resuspend the pellets: 100µl of buffer1 was added to the pellet to resuspend the cells completely by pippeting up and down.
3. Lysozyme treatment: 20µl of lysozyme (50mg/ml)was added into the cell suspension. Then the suspension was mixed thoroughly and incubated at 37^{o}C for 20min.
4. Centrifugation: The digested cells were centrifuged at 10000xg for 3min. , the supernatant was decanted completely.
5. Protein denaturation: The pellet was resuspended in 180µl of buffer2 and 20µl of proteinase K eas also added. Then the suspension was mixed thoroughly and incubated at 65^{o}C in hotbath for 20min. with occasional mixing every 5min.
6. Homogenization: 400µl of binding buffer was added and the suspension was mixed thoroughly several times until a homogenous solution is obtained, the solution was then incubated for 10min. at 65^{o}C.
7. Addition of ethanol: 200µl of absolute ethanol was added and the solution mixed immediately and thoroughly.
8. Loading to columns: The samples of 650µl was transferred in columns and centrifuged at 10000xg for 1min. and their supernatants were discarded.
9. Columns washing: The columns were washed by adding 650µl of washing buffer and then centrifuged at10000xg for 1min. then were discarded flow thoroughly.
10. Columns drying: Additional centrifugation (10000xg) was performed to remove the residual ethanol.

11. DNA elution:The columns were replaced into clean eppendorf tubes, and 100 of preheated elution buffer directly into columns and stood for 2min. then centrifuged at 10000xg for 1 min to elute the DNA.
    The extracted DNAs were stored in -20°C.

3.3.1.2 Manual DNA extraction modified from plasmid DNA extraction protocol:

Solutions preparation:
Solution I:- consists of 50mM glucose, 25mM Tris- HCL PH 8.0 and 10mM EDTA PH 8.0. Sol.I was stored at 4°C.

Solution II:- consists of 1% SDS 50ml, 0.2N NaOH. Sol.II was stored at room temperature.

Solution III:- consists of 300ml of 5M potassium acetate, and 57.5 ml glacial acetic acid. Sol. III was prepared freshly.

In addition to isopropanol was used to separate the DNA.

Procedure:

1. 1.4 ml of saturated bacterial culture were spun in centrifuge machine for 1 min. at 6000xg. The supernatant was discarded and drain briefly.
2. 0.2 ml of ice- cold sol. I was added to the cell pellet and the cells were suspended by pipetting up and down.
3. 0.4ml of sol.II was added and mixed by inverting the tubes five times and left to sit at room temperature for 5 min.
4. 0.3 of ice-cold sol.III was the added, and mixed by inverting the tubes five times and incubated on ice for ten min. and then centifugated at 10000xg for 5min. supernatant was transferred into fresh tubes by pipette. Then the tubes was filled with isopropanol and kept at room temp. For 2min., then centrifugated for 5 min at 14000xg.
5. The supernatant was poured off and the tubes was drained.

6. One ml of ice cold 70% ethanol was added, and mixed by inversion several times and the tubes were spun at 10000xg for 1min.
7. Supernatant was poured off and the tubes was drained to dry for 5 min. and 50µl of double distilled water was added and kept at -20°C.

3.3.1.3 DNA extraction by boiling manual method (central lab protocol):

Two stricks of bacterial bacterial plate culture were optained in eppendorf tubes, and treated by10 µl of lysozyme (50mg/ml). 100µl of distilled water was added. Tubes were incubated at 37°C for 30min. then samples were treated by incudation in water bath at 100°C for 10min. after that samples were treated by freezing in -80°C for 2min. and treated by boiling again for 10min. and finally cetrifugated at 12000xg for 3 min. the supernatant containing DNA was obtained. And kept at -20°C.

3.3.2 Polymerase Chain Reaction (PCR):

iTaq DNA polymerase kits (intron) containing mastermix pre-mix tubes were used for DNA amplification.

Kit contents**:**

- iTaq DNA polymerase (5U/µl)
- 10x PCR buffer (100Mm Tris-HCL (PH8.3); 500mM KCl; 20mM $MgCl_2$; Enhancer solution)
- 10x $MgCl_2$ free buffer
- 10mM dNTPs (2.5mM/each)
- 25mM $MgCl_2$

Template preparation for PCR amplification:

1. -20°C stored DNA extracted samples were stirred on a vortex (IKA VORTEX) for complete thawing.
2. 13µl of dd.w; 5µl of sample templates and 2µl of primer were added in each mastermix pre-mix tube.

3. Tubes containing mixed contents were spun slightly for 2 sec. in a microcentrifuge (NEUATION) for mixing.
4. Tubes were put in an automated thermal cycler apparatus (AerisESCO) to perform amplification.

   Two primer (ordered from Macrogen Co.) were used in two different programs:

a. d-primer 480bp was used for the detection of *Streptococcus sp.*

   Sequence: F: 5¯-CCITAYICITAYGAYGCIYTIGARCC-3¯,

   R: 5¯-ARRTARTAIGCRTGYTCCCAIACRTC-3¯

   Program:

cycle		Program temperature	Time
Initial denatutation		95°C	3min.
35 cycle	denaturation	95°C	30sec.
	annealing	37°C	90sec.
	Extension	72°C	90sec.
Final extension		72°C	10min.

b. spy1258 primer 407bp., sequence: F:5¯-AAAGACCGCCTTAACCACCT-3¯; R: 5¯-TGGCAAGGTAAACTTCTAAAGCA-3¯

   Program:

cycle		Program temperature	Time
Initial denatutation		95°C	5min.
35 cycle	denaturation	95°C	30sec.
	annealing	64°C	30sec.
	Extension	72°C	45sec.
Final extension		72°C	2min.

3.3.3 Gel documentation system:

PCR products were resolved by electrophoresis in 1.5% agarose gel stained by ethedium bromide. 5x TBE (Tris-borate EDTA) was used as running buffer and diluted to 1x by adding1ml of 5x TBE to 99ml d.w.

Addition of 2µl of ethedium bromide resin each 100ml of TBE was recommended for clear band results.

After removing of comp from agarose gel, the tray was placed in electrophoresis apparatus (Biometra GmbH), the agarose gel soaked in TBE containing ethedium bromide. 5µ of each PCR products were pippeted in each well tactfully; in addition to 2µ of 100bp ladder; positive control and negative control. The electrophoresis apperatus powered on 120volt for 40min. and viewed by gel documentation system.

Figure 5: Gel documentation system (UV SoloTS Biometra)

3.4 Serologic tests

3.4.1 Screening of seroprevelance of *H. pylori* positive patients by using *H.pylori* Antibody Rapid test cassette (ALL TEST Co.):

Principle: the *H. pylori* rapid test cassette (serum/plasma) is a qualitative membrane based immunoassay for the detection of H. pylori antibodies in serum or plasma. In this test procedure anti-human IgG is immobilized with in the test line region of the test. Specimens react with *H. pylori* antigen coated particles. The mixure migrates chromatographically along the length of the test and interact with the immobilized anti-human IgG. If the specimen contains *H. pylori* antibodies a coloured line will appear in the test line region indicating positive result, if not no coloured line will appear in the test region.

Storage: packages of test strips were kept at room temperature as indicated in the brochure.

Procedure: three drops of serum were transferred to specimen well of the test cassette and left in clean area, the test read after 10 min. two red lines indicate positive result.

Sensitivity and specificity:

Table 1: Proposed sensitivity, specificity and accuracy of ICT according to manufacturer.

Method		ELISA IgG		Total result
H. pylori antibody rapid test Cassette (serum/plasms)	result	Positive	Negative	
	Positive	171	8	179
	Negative	0	102	102
Total result		171	110	281

Relative sensitivity: >99.9 % (95%CI*:98.3%-100%) *Confidence Interval

Relative specificity: 92.7 % (95%CI*:86.2-96.8%)

Accuracy: 97.2 % (95%CI*:94.5%-98.8%)

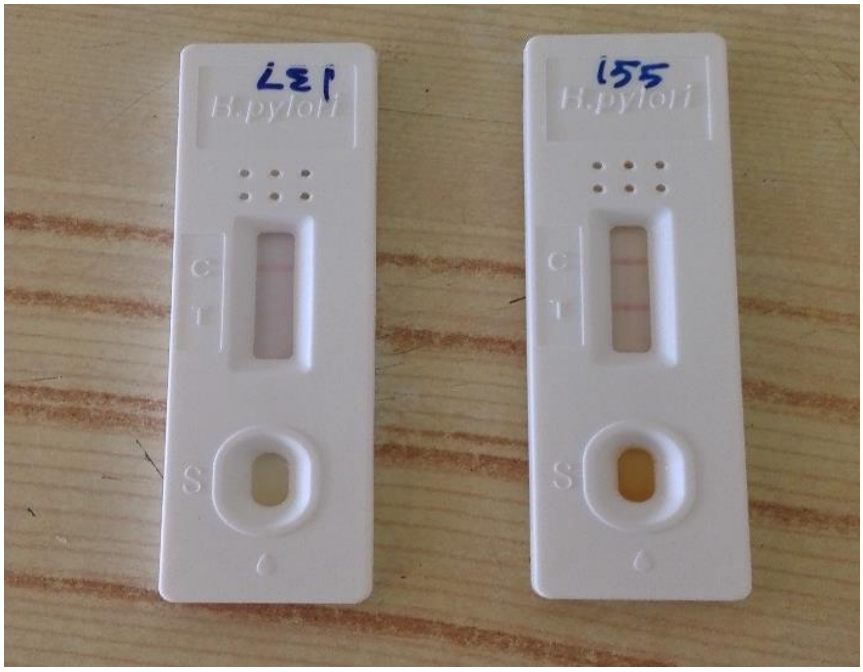


Figure 6: Immunochromatographic test (ICT)

3.4.2 Enzyme-linked immunoassay (ELISA)

3.4.2.1 Determination of Anti-*Helicobacter pylori* (IgG) titer

Anti-*Helicobacter pylori* ELISA (IgG) test kits (EUROIMUM) were used for measurement of a Anti-*Helicobacter pylori* IgG titer in patients serum.

Principle: The ELISA test kit provided a quantitative or semi quantitative in vitro assay for human antibodies of the IgG class against *Helicobacter pylori* in serum or plasma. The test kit contained microtiter strips each with 8 break-off reagent wells coated with *Helicobacter pylori* antigens. In the first reaction strip, diluted patients sample were incubated in the wells. In case of positive sample specific IgG antibodies will bind to antigens. To detect bound antibodies a second incubation carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalyzing a colour reaction.

Kit components:

Microplate wells coated with antigens	Positive control (IgG, human)
Calibrator1 200RU/ml (IgG, human)	Negative control (IgG, human)
Calibrator2 20RU/ml (IgG, human)	Sample buffer.
Calibrator3 2RU/ml (IgG, human)	Wash buffer 10x concentrate
Chromogen/ substrate solution TMB/H_2O_2	Stop solution.
Enzyme conjugate: peroxidase-labelled anti-human IgG (rabbit)	

The kits had been stored at 4^{o}C.

Procedure:

Samples preparation: patients' serums were diluted 1:101 in sample buffer and mixed wel by vortex (cyclone NICKEL ELECTRO)

Samples incubation: 100µl of diluted samples, three calibrators, positive and negative controls were pipetted into individual microplate wells. Then microplate's wells were incubated at room temperature for 30 min.

Washing: wells were empted and washed subsequently three times by using 300µl of washing buffer (1x) for each wash. The washing buffer left for 30 sec. before empting each time of washing.

Conjugate incubation: 100µl of enzyme conjugate (peroxidase-labelled antihuman IgG) was pipetted into each microplate well and incubated for 30min. at room temperature.

Washing: washing was done as described above.

Substrate incubation: 100µl of chromoogen/substrate was pipette into each of microplates' well and incubated for 15 min. at room temperature.

(Substrate had been protected from sunlight).

Stopping the reaction: 100µl of stop solution was pipette into each of microplates' well in the same order at the same speed as the substrate was introduced.

Measurement: photometric measurement of colour intensity was made in ELISA reader machine (Stat Fax 4200) at a wavelength of 450nm and the reference wavelength between 620 and 650nm, within 30min. of adding the stop solution. Prior to measurement a microplate was shaken slightly to ensure a homogenous distribution of the solution.

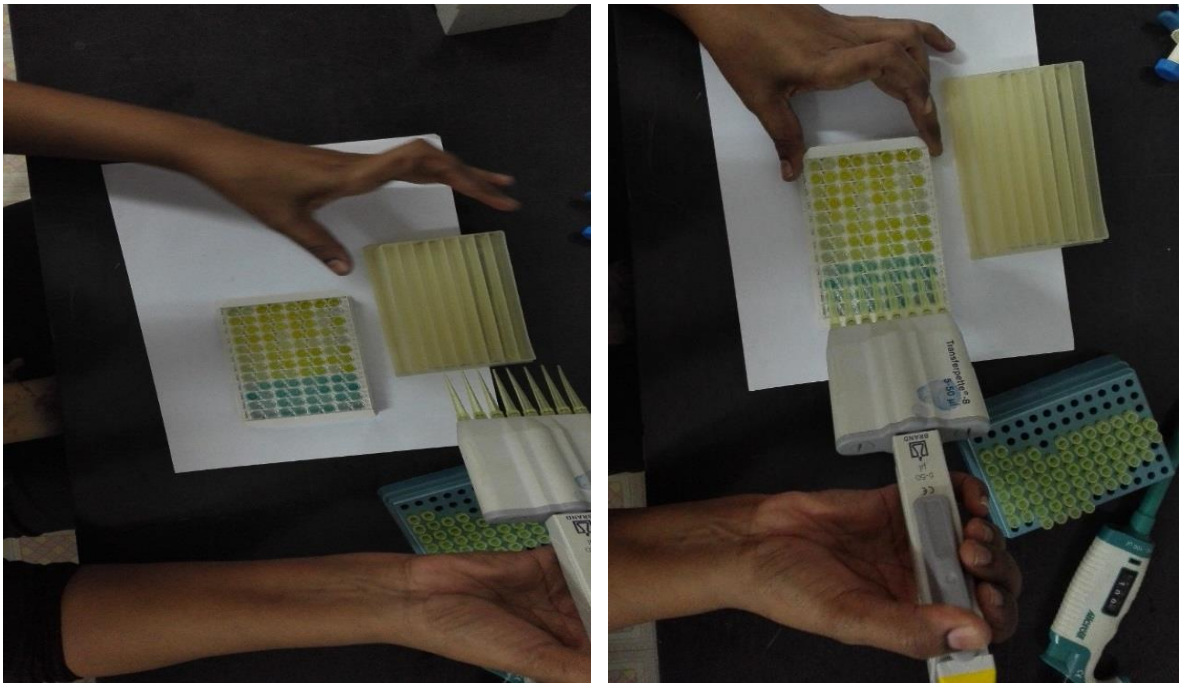


Figure 7: Addition of stop solution using multichannel pipette, color changed between wells of ELISA plate

Calculation:

Quantitative: results were evaluated quantitavely, the concentration of antibodies was obtained by point-to-point plotting of the extinction values measured for three calibration sera against the corresponding units (linear/linear). Point –to-point plotting for calculation of the standard curve by computer.

3.4.2.2 Detection of anti-helicobacter pylori (IgA):

Anti-*Helicobacter pylori* ELISA (IgA) test kits (EUROIMUM) were used**.**

Principle: The ELISA test kit provided a semi quantitative in vitro assay for human antibodies of the IgA class against *Helicobacter pylori* in serum or plasma. The test kit contained microtiter strips each with 8 break-off reagent wells coated with *Helicobacter pylori* antigens. In the first reaction strip, diluted patients sample are incubated in the wells. In case of positive sample specific IgA antibodies were bind to antigens. To detect bound antibodies a second incubation carried out using an enzyme-labelled anti-human IgA (enzyme conjugate) catalyzing a colour reaction.

Kit component:

Microplate wells coated with antigens	Sample buffer
Calibrator	Wash buffer 10x concentrate
Positive control (IgA, human)	Chromogen/ substrate solution TMB/H_2O_2
Negative control (IgA, human)	Stop solution.
Enzyme conjugate: peroxidase-labelled anti-human IgA (rabbit)	

The kits had been stored at 4^{o}C.

Procedure:

Samples preparation: patients' serums were diluted 1:101 in sample buffer and mixed wel by vortex (cyclone).

Samples incubation: 100µl of diluted samples, the calibrator, positive and negative controls were pipetted into individual microplate wells. Then microplate's wells were incubated at room temperature for 30 min.

Washing: wells were empted and washed subsequently three times by using 300µl of washing buffer (1x) for each wash. The washing buffer left for 30 sec. before empting each time of washing.

Conjugate incubation: 100µl of enzyme conjugate (peroxidase-labelled antihuman IgG) was pipetted into each microplate well and incubated for 30min. at room temperature.

Washing: washing was done as described above.

Substrate incubation: 100µl of chromoogen/substrate was pipette into each of microplates' well and incubated for 15 min. at room temperature.

(Substrate had been protected from sunlight).

Stopping the reaction: 100µl of stop solution was pipette into each of microplates' well in the same order at the same speed as the substrate was introduced.

Measurement: photometric measurement of colour intensity was made in ELISA reader machine (Stat Fax 4200) at a wavelength of 450nm and the reference wavelength between 620 and 650nm, within 30min. of adding the stop solution. Prior to measurement a microplate was shaken slightly to ensure a homogenous distribution of the solution.

Calculation: Semiquantitave: results were evaluated semiquantitatively by calculating a ratio of the extinction value of the control and patient sample over the extinction value of calibrator.

$$\textbf{Ratio} = \frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator}}$$

CHAPTER FOUR

RESULTS

4.1. Bactreial identification

Various types of gram positive and gram negative bacteria were isolated from 556 individual throat swabs. The results of the conventional method of identification was shown in table 2 to 7.

The frequency of *Streptococcus pyogenes* and others *Steptococccus sp.* isolates as they were dominant among our isolated bacteria was illustrated in chart 1 and 2.

4.1.1 Streptococcus sp. (Table2)

1. ***Streptococcus pyogenes**:* **396 isolates (71.2%)**:

- Number of confirmed *S.pyogenes* with ideal biochemical result was **27** isolates (6.8%).
- Number of confirmed *S.pyogenes* with uncommon biochemical results was **45** isolates (11.3%).
- Number of unconfirmed *S.pyogenes* with ideal biochemical result was **146** isolates (37%).
- Number of unconfirmed *S.pyogenes* with uncommon biochemical results was **178** isolates (44%).

2. Other *Streptococcus sp.* (Table2)*:*

Among isolated microorganism there were 4.8% isolates of *Streptococci* group C (*Streptococcus equi and S. disagalactiae)*, 2.3 % of all isolated bacteria were *Streptococcus spp*. Group L, 1.9% among isolated bacteria were *Streptococcus suis*, 1.1% *Streptococcus pneumonia*.

3. Commensals of *Streptococci* (Table2):

There were 8% *Streptococcus salivarius, 1.1% Streptococcus sanguinis* (one isolate), *6% Streptococcus sp.* (34 isolates), and *0.5% Streptococcus morbillorum* (3 isolates) among isolated microorganism.

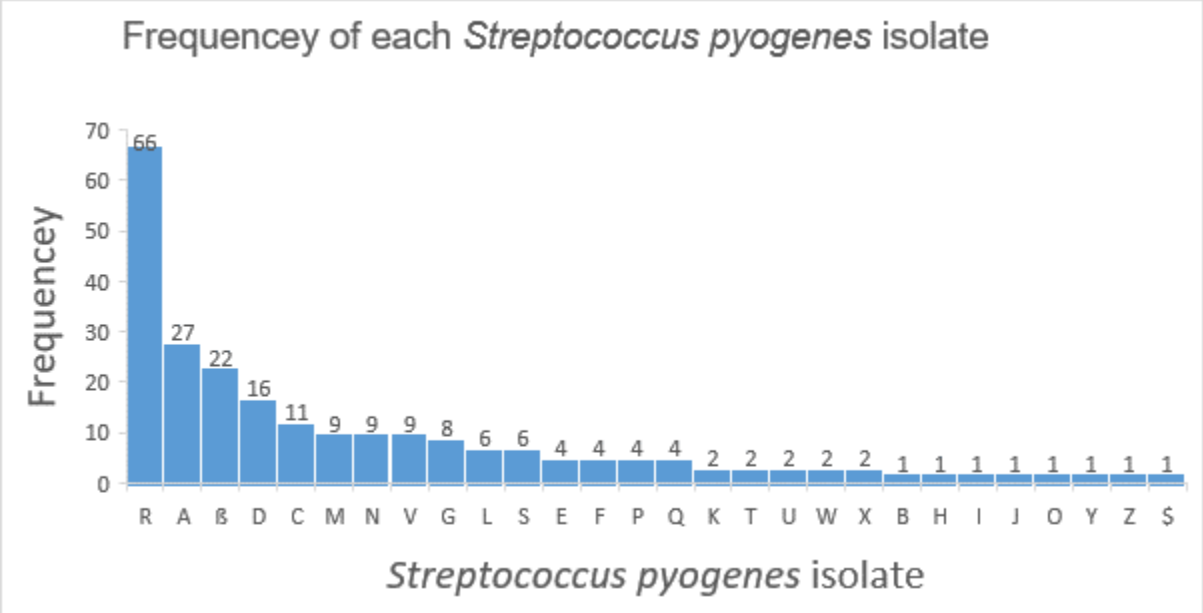


Chart1: The frequency of *Streptococcus pyogenes.*

Key chart for the frequency figure above:

c	Confirmed *S.pyogenes* with biochemical tests and PCR
B	Starch –ve *Trhalose –ve* PCR-*confirmed S. pyogenes*
C	Arginine –ve PCR-confirmed *S. pyogenes*
D	Arginine –ve PCR-unconfirmed *S. pyogenes*
E	*Arginin –ve* Starch –ve PCR-unconfirmed *S. pyogenes*
F	*Arginin –ve* Starch –ve PCR-confirmed *S. pyogenes*
G	*arginine –ve sorbitol* + PCR-unconfirmed *S. pyogenes*
H	Arginine and lactose –ve PCR-unconfirmed *S. pyogenes*
I	Arginine and lactose –ve PCR-confirmed *S. pyogenes*
J	Arginine –ve ribose + Confirmed *S. pyogenes*
K	Arginine and trehalose –ve Confirmed *S. pyogenes*
L	trehalose –ve PCR-Confirmed *S. pyogenes*
M	trehalose –ve PCR-unConfirmed *S. pyogenes*
N	Lactose –ve PCR-unconfirmed *S.pyogenes*
O	Ripose +ve lactose –ve confirmed *S. pyogenes*
P	*Sorbitol + trehalose –ve* PCR-unconfirmed *S. pyogenes*
Q	*Starch –ve and sorbitol* + PCR-unconfirmed *S. pyogenes*
R	Sorbitol + PCR-unconfirmed *S. pyogenes*
S	Sorbitol + PCR-Confirmed S. pyogenes
T	Sorbitol+, ribose + PCR-Confirmed *S. pyogenes*
U	*Starch & lactose –ve* PCR-unconfirmed *S. pyogenes*

v Starch and trehalose –ve PCR-unconfirmed *S. pyogenes*

w vp+ Confirmed *S. pyogenes*

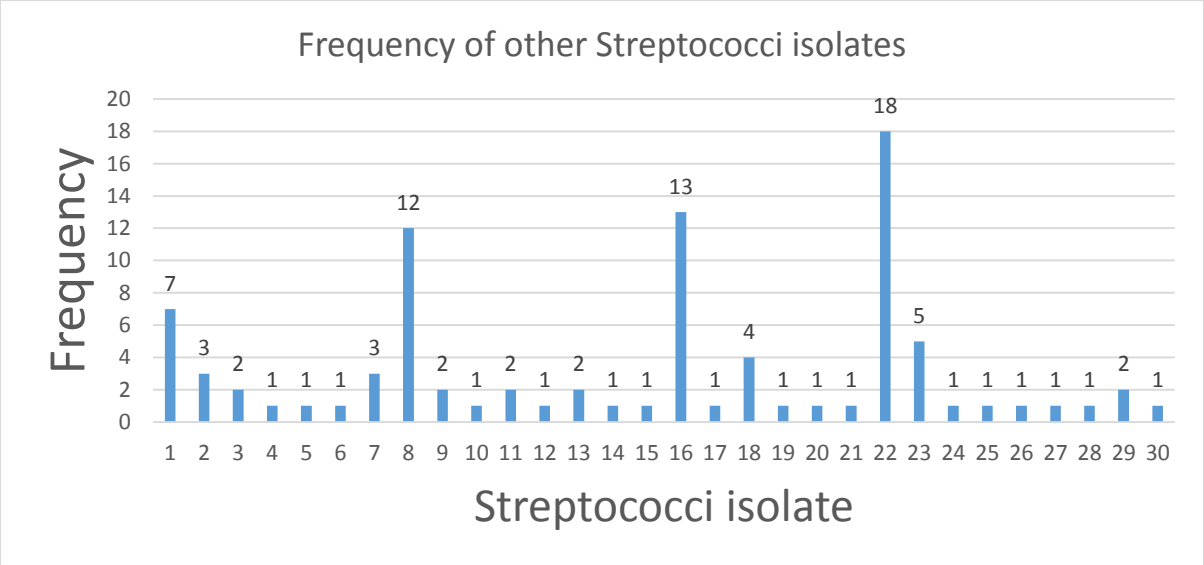
x Vp+ and ribose+ PCR-Confirmed *S.pyogenes*

y Trehalose –ve and vp+ PCR-unconfirmed *S.pyogenes*

z Lactose –ve and vp+ PCR-unconfirmed *S.pyogenes*

ß *Starch –ve* PCR-unconfirmed *S. pyogenes*

$ *Starch –ve* PCR-confirmed *S. pyogenes*



Chatr2: The frequency of other *Streptococcus sp.*

Key chart for the frequency figure above:

1 *Starch –ve strepto. Spp group L*

2 *Streptococcus. Spp group L*

3 *Mannitol + strepto. Spp group L*

4 *Starch & lactose –ve strepto. Spp group L*

5 *lactose –ve strepto. Spp group L*

6 *Starch -ve trehalose –ve S. spp. Group L*

7 *S. morbillorum*

8 *S. salivarius*

9 Trehalose –ve *S. pnemoniae*

10 Lactose –ve *S. pnemoniae*

11 *S.pnemonae*

12 *Mannitol +S. sanguis*

13 Ripose –ve mannitol +ve *s. dysagalactia*

14 Ripose –ve and lactose –ve *s. dysagalactia*

15 Ripose –ve trhalose –ve *s. dysagalactia*
16 Ripose –ve *s. dysagalactia*
17 Starch –ve *S. equi*
18 *S. equi* (group c)
19 *Mannitol + S. equi* (group c)
20 *Arginine –ve S. equi* (group c)
21 *S. zooepidemicus*
22 *Streptococcus sp.*
23 *S. suis*
24 *Arginine –ve S.suis*
25 *Mannitol + S. suis*
26 Sorbitol + *S. suis*
27 Starch –ve mannitol + *S. suis*
28 mannitol + *S. suis*
29 Trehalose – ve and arginine –ve *S. suis*
30 Starch and arginine –ve *S. suis*

4.1.2 Gram Negative Cocci (Table 3):

Gram negative cocci were also detected such as *Branhamella catarhalis (*4 isolates), *Neisseria elongate* (3 isolates), *Neisseria lactamica* (2 isolates) and *Neisseria sp.* (3 isolates)

4.1.3 *Staphylococcus sp.* (Table 4)*:*

0.4% of isolates were coagulase negative *Staphylococcus aureus* and 0.9 % were coagulase positive *Staphylococcus aureus. There were also 0.5% Staphylococcus chromogenes* (two isolates).

Other *Staphylococcus sp.* (Table 4):

One isolate of *Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus capitis, Staphylococcus hyicus, Staphylococcus simulans, Staphylococcus delphini, Staphylococcus scuiri* were found. Two isolates of *Staphylococcus lentus*, three isolates of *Staphylococcus intermedius*, four isolates of *Staphylococcus caseolyticus*, and 11 isolates of *Staphylococcus auricularis* were found among ll isolated bacteria.

4.1.4 Gram positive rods (Table 5):

Bacillus mycoides: 0.5%, *Bacillus licheniformis* 0.2%, *Bacillus pantothenicus* 0.4% and 0.2% *Clostridium sp.*

4.1.5 Gram Positive polymorphs (Table 6):

Corynbacteriium sp., Corynbacteriium kutscheri, Corynbacteriium amycolatum, Corynbacteriium bovis (2 isolates).

4.1.6 Gram negative rods (Table 7):

There were 0.4% *Shwanella putrefaction* among isolated microorganisms and 0.2% *Pseudomonas alkaligenes*.

4.1 Bacterial isolation and identification by conventional methods:

Varietis of gram positive and gram negative cocci, rods and polymorphs of isolated bacteria were displayed in the following tables with their primary and secondary tests resuls.

Table 2: The identification of streptoccoci obtained from each patient sample (either tonsils or pharynx swabs) using biochemical tests profile according to Barrow and Feltham (2003):

Tests / **Samples**	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
A t	β	_	R	R	NP	NP	NP	NP	NP	NP	NP	NP	NP	*Streptococcus sp.*
A p	β	_	S	R	NP	NP	NP	NP	NP	NP	NP	NP	NP	*Streptococcus sp.*
B t	β	_	R	R	NP	NP	NP	NP	NP	NP	NP	NP	NP	*Streptococcus sp*
B p	β	_	S	R	NP	NP	NP	NP	NP	NP	NP	NP	NP	*Streptococcus sp.*
C p	β	_	S	R	NP	NP	NP	NP	NP	NP	NP	NP	NP	Confirmed *S.pyogenes*
D t	β	_	S	R	NP	NP	NP	NP	NP	NP	NP	NP	NP	*Streptococcus sp.*
D p	β	_	R	R	NP	NP	NP	NP	NP	NP	NP	NP	NP	*Streptococcus sp.*
E t	β	_	S	R	_	+	_	_	_	_	+	+	_	Arginine –ve *S. pyogenes*
E phar	β	_	S	R	_	+	_	_	_	_	+	+	_	Arginine –ve *S. pyogenes*
F ton	β	_	S	R	+	w	_	_	w	_	w	+	_	Arginine *-ve S. pyogenes*
F phar	β	_	R	R	_	w	_	_	_	_	_	w	_	*S. morbillorum*
G ton	β	_	R	R	+s	w	_	_	+s	w	+s	+s	+s	*S. salivarius*
G phar	β	_	R	R	+s	w	_	_	+s	w	+s	+s	+s	*S. salivarius*
P phar	β	_	S	R	_	w	_	_	_	_	+	+	_	Arginine *–ve S.pyogenes*
R ton	β	_	S	R	_	+	+	_	_	_	+	+	W	*S.pyogenes*
R phar	β	_	S	R	_	+	+	_	_	_	+	+	_	Confirmed *S.pyogenes*
S ton	β	_	S	R	_	+	+	_	_	_	+	+	_	Confirmed *S.pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..														
Tests / **Samples**	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
S phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
T ton	β	_	S	R	_	+	+	_	_	_	+	–	+	Trehalose –ve vp+ *S.pyogenes*
T phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S. pyogenes*
U ton	β	_	S	R	_	+	+	_	_	_	+	+	_	Confirmed *S.pyogenes*
U phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
V ton	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
V phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
W ton	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
W phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyo*
X ton	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
X phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
29 ton	β	_	S	R	_	+	+	_	_	_	+	+	_	Confirmed *S.pyogenes*
29 phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
30 ton	β	_	S	R	_	+	+	_	_	_	+	+	_	*S.pyogenes*
30 phar	β	_	S	R	+s	+	+	_	_	_	+	+	_	Confirmed *S.pyogenes*
31 ton	β	_	S	R	_	+	+	_	_	+	+	+	–	Sorbitol + Confirmed *S.pyogenes*
31 phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S. pyogenes*
32 ton	β	_	S	R	_	+	+	_	_	_	+	+	_	*S. pyogenes*
33 ton	β	_	S	R	+s	+	+	_	_	_	+w	+	_	*S. pyogenes*
33 phar	β	_	S	R	_	+	+	_	_	_	+	+	_	Confirmed *S.pyogenes*
34 ton	β	_	S	R	_	+	+	_	_	_	+	+	_	Confirmed *S.pyogenes*
34 phar	β	_	S	R	_	w	+	_	_	_	+	+	_	Confirmed *S.pyogenes*
35 ton	β	_	S	R	_	+	_	_	_	_	+	+	_	Arginine –ve *S. pyogenes*
35 phar	β	_	S	R	_	w	_	_	_	_	+	+	_	Arginine –ve *S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
36 ton	β	_	S	R	_	+	+	_	_	_	+	+	_	*S. pyogenes*
36 phar	β	_	S	R	_	_	+	_	w	_	+	+	_	Starch –ve Confirmed *S.pyogenes*
37 ton	β	_	R	R	_	+ late	+	_	_	_	+	+	_	*S.suis*
37 phar	β	_	S	R	_	+	+	_	_	_	+	+	_	*S. pyogenes*
38 phar	β	_	S	R	_	+	+	_	+s	_	+	+	_	Confirmed *S. pyogenes*
39 ton	β	_	S	R	_	+	+	_	_	_	+	+	_	*S. pyogenes*
39 phar	β	_	S	R	_	w	+	_	_	_	+	+	_	*S. pyogenes*
40 ton	β	_	S	R	_	+	+	_	_	w	+	+	_	Confirmed *S. pyogenes week reaction with sorbitol*
40 phar	β	_	S	R	_	_	+	_	_	_	w	+	_	Starch –ve *S. pyogenes*
41 ton	β	_	S	R	_	+	w	_	+	+	+	+	_	Sorbitol + *S.pyogenes*
41 phar	β	_	S	R	+s	+	_	_	_	w	+	+	_	*Arginine –ve and w sorbitol S. pyogenes*
42 ton	β	_	S	R	+s		_	_	_	w	+	+	_	*Arginine –ve and w sorbitol S. pyogenes*
42 phar	β	_	S	R	_	w	+	_	_	_	+	+	_	Confirmed *S. pyogenes*
43 ton	β	_	S	R	+s	+s	+s	_	_	_	+	+	_	*S. pyogenes*
43 phar	β	_	S	R	+s	_	_	_	_	_	+	+	_	Starch and arginine –ve *S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / **Samples**	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
44 ton	β	_	S	R	_	w	_	_	_	_	_	+	_	Arginine and lactose –ve *S. pyogenes*
44 phar	α	+	S	S	_	_	_	_	_	_	+	+	_	*S. pnemoniae*
45 ton	β	_	S	R	_	_	_	_	_	_	+	+	–	Starch and arginine –ve Confirmed *S. pyogenes*
45 phar	β	_	S	R	_	w	w	_	_	_	_	+	w	Lactose –ve and w vp *S.pyogenes*
46 ton	β	_	S	R	+s	_	_	_	_	_	+	+	_	Starch and arginine –ve *S. pyogenes*
46 phar	β	_	S	R	_	w	_	_	_	w	w	w	_	*Arginine –ve and w sorbitol S. pyogenes*
47 ton	β	_	S	R	+s	+s	w	_	_	_	_	+	+	Lactose –ve *S.pyogenes*
47 phar	β	_	S	R	+s	+s	w	_	_	_	+	+	_	*S. pyogenes*
48 ton	β	_	S	R			_						_	*Streptococcus sp.*
48 phar	α	+	S	S	_	+	_	_	_	_	+	+	_	Trehalose –ve *S. pnemoniae*
49 ton	α	+	S	S	_	w	_	_	_	_	_	+	_	Lactose –ve *S. pnemoniae*
49 phar	β	_	S	R	_	+s	+s	_	_	_	+	+	_	*S. pyogenes*
50 ton	β	_	S	R	_	w	+	_	_	_	+	+	+s	vp+ Confirmed *S. pyogenes*
50 phar	β	_	S	R	_	+	+	_	_	_	+	+	+s	vp+ Confirmed *S. pyogenes*
51 ton	β	_	S	R	_	+	+	_	_	+	+	+	_	Sorbitol + *S. pyogenes*
51 phar	β	_	S	R	+s	+	+	w	+	+	+	+	_	Sorbitol + *S. suis*
52 ton	β	_	S	R	+s	+s	+	_	+	_	+	+	_	*S. pyogenes*
52 phar	β	_	S	R	+s	+	+	w	+s	+	+	+	–	Sorbitol+, ribose w Confirmed *S. pyogenes*
53 ton	β	_	S	R	+s	+	+	w	+s	+	+	+	–	Sorbitol+, ribose w Confirmed *S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
53 phar	β	_	S	R	+s	+	+	_	w	_	+	+	_	Confirmed *S. pyogenes*
54 ton	β	_	S	R	_	w	_	_	_	_	_	+	_	Arginine and lactose –ve Confirmed *S. pyogenes*
54 phar	β	_	S	R	_	+	_	_	_	_	+	_	_	Arginine and trehalose –ve Confirmed *S. pyogenes*
55 ton	β	–	S	R	–	+s	+	–	+s	+	+s	+	–	Sorbitol + *S.pyogenes*
55 phar	β	–	S	R	+s	+	w	_	+	+	+	+	_	Sorbitol + *S.pyogenes*
56 ton	β	_	S	R	_	+ late	_	_	_	w	+	+	_	*Arginine –ve and w sorbitol S. pyogenes*
56 P	β	–	S	R	+s	w	+	w	+	+	+	+	+	*Streptococcus sp.*
57 P	β	–	S	R	–	w	–	–	+	–	+	+	–	Arginine –ve *S.pyogenes*
58 ton	β	_	S	R	+s	w	+	_	w	_	+	w	+s	Vp+ and ribose w Confirmed *S.pyogenes*
58 P	β	_	S	R	+s	w	w	_	w	_	+ late	w	w	Vp and ribose week reaction Confirmed *S.pyogenes*
59 ton	β	_	S	R	_	w	+	_	w	_	+	+	–	*S. pyogenes*
59 P	β	_	S	R	+s	+	_	_	_	_	w	+	_	Arginine –ve *S. pyogenes*
60 ton	β	_	S	R	_	+	+s	–	+	_	+	+	_	*S. pyogenes*
61 ton	β	_	S	R	_	+	_	_			+	+	_	Arginine –ve Confirmed *S. pyogenes*
61 phar	β	_	S	R	+s	+	+s	_	_	_	w	+		Arginine –ve Confirmed *S. pyogenes*
62 ton	β	–	S	R	+s	+	+s	–	–	–	+	+	–	*S. pyogenes*
62 phar	β	_	S	R	+s	+	+	_	_	_	+	+	_	*S. pyogenes*
63 ton	β	_	S	R				–				+		*Streptococcus sp.*
63 P	β	_	S	R	+s	+	_	_	_	_	+	+	_	*S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
64 ton	β	_	S	R	_	+	_	_	_	_	_	+	_	Arginine –ve Confirmed *S. pyogenes*
64 phar	β	_	S	R	_	+	_	_	_	_	_	+	_	Arginine –ve Confirmed *S. pyogenes*
65 ton	α	+	S	R	+s	+	_	_	_	_	+	+S	_	Arginine –ve Confirmed *S. pyogenes*
65 phar	β	_	S	R	+s	+S	_	_	+	_	+S	+S	_	Arginine –ve Confirmed *S. pyogenes*
66 ton	β	_	S	R	+s	+	+	_	_	+	+	+	_	Sorbitol + Confirmed *S. pyogenes*
66 phar	β	_	S	R	+s	_	+	_	+	_	+S	+S	_	Starch –ve *S.pyogenes*
67 ton	β	_	S	R	+s	+	+	–	–	–	+S	+S	–	Confirmed *S. pyogenes*
67 P	β	_	S	R	+s	+S	_	_	_	_	+S	+S	_	Arginine –ve Confirmed *S. pyogenes*
68 ton	β	_	S	R	+s	+S	_	_	_	_	+S	+S	_	Arginine –ve Confirmed *S. pyogenes*
68phar	β	_	S	R	+s	+S	_	_	_	_	+S	+S	_	Arginine –ve Confirmed *S. pyogenes*
69 ton	β	_	S	R	+s	w	_	_	_	_	+S	+S	_	Arginine –ve *S. pyogenes*
69 phar	β	_	S	R	+s	+	_	w	w	_	+S	+S	_	Arginine –ve ribose w Confirmed *S. pyogenes*
70 ton	β	_	S	R	+s	_	_	_	w	_	+w	+S	_	Starch and arginine –ve Confirmed *S. pyogenes*
70 phar	β	_	S	R	+s	+	_	_	_		+S	+S	_	Arginine –ve Confirmed *S. pyogene*
71 ton	α	+	S	S	_	_	_	_	_	_	+	_	_	Trehalose –ve *S.pnemoniae*
71 P	β	_	S	R	_	_	_	_	_	_	+	+	_	Starch and arginine –ve *S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
72 ton	β	_	S	R	+s	+	_	_	_	_	+	+	_	Arginine –ve Confirmed *S. pyogenes*
72 phar	β	_	S	R	+s	w	w	_	_	+	+	+	_	Sorbitol+ Confirmed *S. pyogenes*
73 ton	β	_	S	R	+s	–	w	–	–	–	+	+	_	Starch –ve *S. pyogenes*
73 phar	β	_	S	R	+s	_	+	_	w	_	w	+	_	Starch and arginine –ve Confirmed *S. pyogenes*
74 T	β	_	S	R	+s	+	w	–	w	–	+	+	–	*S. pyogenes*
74 P	β	_	S	R	+s	+	w	–	w	–	+	+	–	*S. pyogenes*
75 ton	β	_	S	R	_	_	_	_	w	_	+	+	_	Starch and arginine –ve Confirmed *S. pyogenes*
75 phar	β	_	S	R	+s	_	_	_	_	_	+	+	_	Starch and arginine –ve *S. suis*
76 ton	β	_	R	R	+s	_	w	–	–	–	+	+	+	*S. salivarius*
76 P	β	_	S	R	+s	+	+	–	+w	_	+	+	–	Confirmed *S. pyogenes*
77 ton	β	_	S	R	_	w	+	–	–	–	+	+	–	Confirmed *S. pyogenes*
77 P	β	_	R	R	+S	+	+	–	–	–	+	+	–	*S.suis*
78 ton	β	_	R	R	+S	w	_	_	_	_	+	_	_	Arginine & trehalose –ve S. suis
78 P	β	_	R	R	+S	+	+	–	–	–	+	w	–	*S.suis*
79 T	β	_	S	R	+S	+	+	–	–		–	–	–	*S. equi* (group C)
79 P	β	_	R	R	_	+	w	_	_		+	+	_	*Streptococcus spp. Group* L
80 t	β	_	S	R	+S	+	w	_	_		_	+	_	Lactose –ve *S. pyogenes*
80 p	β	_	S	R	+S	w	w	_	w		+	+	_	Confirmed *S. pyogenes*
81 t	β	_	R	R	+S	w	w	_	_		+	+	_	*S.suis*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
81p	β	_	S	R	+S	+	+S	_	w		+	+	+	*S. salivarius*
82 t	β	_	R	R	+S	+	+S	_	+		+	+	w	*S. salivarius*
82 p	β	_	R	R	+S	w	+S	_	w		+	+	+	*S. salivarius*
83t	β	_	R	R	+S	_	+S	_	w		w	+	w	*S. salivarius*
83p	β	_	R	R	+	+	+S	_	_		+	+	+	S. zooepidemicus
84 t	β	_	R	R	+S	w	+S	_	+		w	+	+	*S. salivarius*
84 P	β	_	R	R	+S	w	+S	–	+		+	+	+	*S. salivarius*
85 T	β	_	R	R	+S	_	+	–	+	–	w	+	–	*Mannitol + S. suis*
85 P	β	_	R	R	+S	w		–	+		+	+	+	*S. salivarius*
86 T	β	_	R	R	+	w	–	–	w	–	+	–	–	*S. morbillorum*
86 P	β	_	R	R	+	+	–	–	–	–	–	+	_	*S. morbillorum*
87 T	β	_	R	R	+S	_	+	_	+	_	+	+	_	Starch –ve mannitol + *S. suis*
87 P	β	_	S	R	_	+	–	–	+	_	+	+	_	Arginine –ve *S.pyogenes*
88 T	β	_	S	R	+S	+	+	_	w	+	+	+	–	Sorbitol + Confirmes *S. pyogenes*
88 P	β	_	S	R	_	w	+	–	–	–	–	+	_	Lactose –ve *S. pyogenes*
89T	β	_	S	R	+S	–	+	w	+	+	+	+	_	*Streptococcus spp.*
89 P	β	_	S	R	+S	+	+	–	–	–	+	+	–	*S. pyogenes*
90 T	β	_	S	R	+S	_	+	w	+	+	+	+	–	*Streptococcus spp.*
90 P	β	_	S	R	_	w	+	–	–	–	+	+	–	*S. pyogenes*
91 T	β	_	S	R	+S	+	w	_	+	+	+	+	–	Sorbitol + *S. pyogenes*
91 P	β	_	S	R	_	+	_	_	_	_	+	+	–	Arginine –ve *S.pyogenes*
92 T	β	_	S	R	+S	+	+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
92 P	β	_	S	R	+S	+	+	_	+	+	+	+	–	Sorbitol + *S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..														
Tests / **Samples**	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
93 P	β	_	S	R	+S	_	+	_	_	_	_	+	_	Starch and lactose –ve *S. pyogenes*
94 T	β	_	S	R	+S	+	+	–	w	–	–	+	–	lactose –ve *S. pyogenes*
94P	β	_	S	R	+S	+	+	_	_	+	+	+	–	Sorbitol + Confirmed *S. pyogenes*
95 T	β	_	S	R	+S	w	+	_	w	–	+	+	–	*S. pyogenes*
95 P	β	_	S	R	+	w	+	_	_	_	+	_	_	Trehalose –ve Confirmed *S. pyogenes*
96T	β	_	S	R	+S	+	+	–	–	–	+	+	–	*S. pyogenes*
96 P	β	_	S	R	+S	+	+	–	–	–	+	+	–	*S. pyogenes*
97T	β	_	S	R	+S	+	+	–	–	–	+	+	–	*S. pyogenes*
97 P	β	_	S	R	+S	w	+	_	_	+	+	+	–	*Sorbitol + S. pyogenes*
98 T	β	+	R	R	+S	w	_	–	–	–	+	+	–	*Arginine –ve S.suis*
98 P	β	_	S	R	+S	+	+S	–	–	–	+	+	–	*S. pyogenes*
99 T	β	_	S	R	+S	+	+	–	w	w	w	w	–	*S.pyogenes*
99 P	β	_	R	R	+		w	–	–	–	+	–	–	Grroup G or *S. suis*
100T	β	_	S	R	+S	+	+	–	+	w	+	+	_	*S. pyogenes*
100P	β	_	S	R	+	w	+	–	–	–	+	+	_	*S. pyogenes*
101T	β	_	R	R	+S	w	_	_	_	_	+	_	_	*Arginine and trehalose –ve S.suis*
101P	β	_	S	R	+S	+	+	–	w	+	+	+	_	*S. pyogenes*
102T	β	_	R	R	w	+	_	_	_	_	+	+	–	Streptococcus spp. Pyridoxal or cysteine dependent

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
102P	β	_	R	R	+	w	_	_	_	_	+	_	_	Streptococcus spp. Pyridoxal or cysteine dependent
104T	β	_	S	R	w	w	+	–	–	–	+	+	–	*S. pyogenes*
104P	α	+	S	S	w	_	_	_	_	_	+	_	_	Trehalose –ve *S. pneumonae*
105P	β	_	S	R	+S	w	+	_	_	w	+	+	_	*Trehalose –ve S. pyogenes*
106T	β	_	S	R	+S	+	+	+	w	w	_	w	w	Ripose +ve lactose –ve confirmed *S. pyogenes*
106P	β	_	R	R	+	_	w	_	_	_	+	+	_	*Streptococcus spp. Group L*
107T	β	_	S	R	_	w	_	_	_	_	+	_	_	*Arginine and trehalose –ve S. pyogenes*
107P	β	_	S	R	+	+	+	–	w	+	–	+	–	lactose –ve *S. pyogenes*
108T	β	_	S	R	w	w	+	_	_	_	+	_	_	*Trehalose –ve S. pyogenes*
108P	β	_	S	R	_	w	+	_	_	_	+	_	_	*Trehalose –ve S. pyogenes*
109T	β	–	R	R	–	+	+	w	_	w	+	+	_	confirmed *S. pyogenes*
109P	β	–	R	R	–	+	+	w	_	+	+	+	+	*S. pyogenes*
110T	β	–	R	R	–	+	+	–	w	w	+	+	w	*S. pyogenes*
110P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
111T	β	–	S	R	–	w	–	–	–	–	–	–	–	*Streptococcus sp.*
111P	β	–	S	R	–	+	+	–	w	–	w	+	–	*S. pyogenes*
112T	β	_	S	R	_	w	+	_	_	_	+	_	_	*Trehalose –ve confirmed S. pyogenes*
112P	β	_	S	R	_	w	+	_	_	_	+	_	_	*Trehalose –ve S. pyogenes*
113T	β	_	S	R	_	w	+	_	_	_	w	_	_	*Trehalose –ve confirmed S. pyogenes*
113P	β	–	S	R	–	+	+	w	+	w	+	+	–	*S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
114T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
114P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
115P	β	–	S	R	–	+	+	_	_	_	+	+	_	*S. pyogenes*
116T	β	_	S	R	_	w	+	_	_	_	+	_	_	confirmed trehalose –ve *S. pyogenes*
116P	β	–	S	R	–	+	+	–	_	_	+	+	–	*S. pyogenes*
117T	β	–	S	R	–	+	+	–	w	_	+	w	–	confirmed *S. pyogenes*
117P	β	–	S	R	–	w	w	–	_	_	+	+	–	confirmed *S. pyogenes*
118T	β	_	S	R	_	w	w	_	_	_	+	_	_	confirmed trehalose –ve *S. pyogenes*
118P	β	–	S	R	–	+	+	–	–	–	–	–	–	s. equi (group c)
119T	β	–	S	R	–	+	–	–	w		w	+	–	confirmed *S. pyogenes*
119P	β	–	S	R										*Streptococcus sp.*
120T	β	–	S	R	–	+	–	w	+	+	+	+	–	confirmed *S. pyogenes*
120P	β	–	S	R	–	+	–	w	+	+	+	+	–	confirmed *S. pyogenes*
121T	β	–	S	R	–	+	–	–	+	+	+	+	–	*S. pyogenes*
121P	β	_	R	R	_	+	_	_	_	_	+	_	_	Trehalose –ve confirmed *S. pyogenes*
122T	β	_	S	R	_	+	_	w	w	w	w	w	_	*S. pyogenes*
122P	β	–	S	R	–	–	–	–	–	w	+	–	–	*Streptococcus sp.*
123T	β	–	S	R	–	+	–	–	+	+	+	+	–	*S. pyogenes*
123P	β	–	S	R	–	w	–	–	–	–	+	+	–	confirmed *S. pyogenes*
124T	β	–	S	R	–	+	+	–	+	+	+	+	–	Sorbitol + *S.pyogenes*
124P	β	–	S	R	–	+	w	–	+	–	+	+	–	*S.pyogenes*
125T	α	–	S	S	–	w	–	–	–	–	+	_	–	confirmed *S. pyogenes*
125P	β	–	S	R	–	w	–	–	–	–	+	+	–	confirmed *S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table 2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
126T	β	–	S	R	–	+	–	–	–	–	+	+	–	*S. pyogenes*
126P	β	–	S	R	–	w	–	–	–	–	+	+	–	*S. pyogenes*
127T	β	–	S	R	–	w	–	–	w	w	+	+	–	confirmed *S. pyogenes*
127P	β	–	S	R	–	w	–	–	–	–	+	+	–	*S. pyogenes*
128T	β	–	S	R	–	w	–	w	w	–	+	+	–	*S. pyogenes*
128P	β	_	S	R	_	w	_	_	_	_	_	_	_	*Trehalose –ve S. pyogenes*
129T	β	–	S	R	–	w	–	w	+	+	+	+	–	*S. pyogenes*
129P	β	–	S	R	–	+	–	–	–	+	+	+	–	*S. pyogenes*
130T	β	–	S	R	–	w	–	–	–	–	+	+	–	*S. pyogenes*
130P	β	–	S	R	–	+	–	–	–	–	+	+	–	*S. pyogenes*
131P	β	–	S	R	–	w	–	–	w	–	+	w	–	*S. pyogenes*
132P	β	–	S	R	–	w	–	–	–	–	+	+	–	*S. pyogenes*
136T	β	–	S	R	–	w	–	–	–	–	+	+	–	*S. pyogenes*
136P	β	–	S	R	–	w	–	–	w	–	+	_	–	*Trhalose –ve S. pyogenes*
137T	β	–	S	R	–	–	–	–	–	–	+	+	–	Starch –ve *S. pyogenes*
137P	β	–	S	R	–	–	–	–	–	–	+	+	–	Starch –ve *S. pyogenes*
138P	β	_	S	R	_	+	_	_	_	_	+	+	_	*S. pyogenes*
139T	β	–	S	R	–	+	–	–	–	–	+	+	–	*S. pyogenes*
139P	β	–	S	R	–	+	–	–	–	–	+	+	–	*S. pyogenes*
140T	β	–	S	R	–	+	–	–	w	–	+	+	–	*S. pyogenes*
140P	β	–	S	R	–	–	–	–	–	–	+	+	–	*Streptococcus sp.*
141T	β	–	S	R	–	–	–	–	+	–	+	+	–	Starch –ve *S. pyogenes*
141P	β	–	S	R	–	–	–	–	w	–	+	+	–	*S. pyogenes*
145P	β	–	S	R	–	–	–	–	–	–	–	–	–	*Streptococcus sp.*
147P	β	–	S	R	–	+	–	–	–	–	–	w	–	*Lactose –ve S. pyogenes*
148P	β	–	S	R	–	+	–	–	w	_	+	+	–	*S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table 2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
150T	β	_	S	R	_	w	_	_	_	_	+	_	_	*Trhalose –ve S. pyogenes*
151T	α	_	S	S	_	_	_	_	_	_	+	_	_	*Trehalose –ve S. pneumonae*
151P	β	–	S	R	–	+	–	–	–	–	+	+	–	*S. pyogenes*
152T	β	_	S	R	_	_	_	_	_	_	+	–	–	Starch –ve *Trhalose –ve confirmed S. pyogenes*
152P	β	–	S	R	–	+	–	w	–	–	+	+	–	*confirmed S. pyogenes*
154T	β	_	S	R	_	_	_	_	_	_	+	_	_	*Trhalose –ve* Starch –ve *S. pyogenes*
154P	β	_	S	R	_	w	_	_	_	_	+	_	_	*Trhalose –ve S. pyogenes*
155T	β	_	S	R	_	+	–	–	–	–	+	+	–	S. pyogenes
155P	β	_	S	R	_	+	_	_	+	_	+	_	_	*Trhalose –ve S. pyogenes*
156T	β	–	S	R	–	w	–	–	w	–	–	w	–	*Lactose –ve S. pyogenes*
156P	β	_	S	R	_	+	_	_	+	+	+	+	_	Sorbitol +ve *S. pyogenes*
158T	β	–	S	R	–	+	–	–	w	w	+	+	_	*S. pyogenes*
158P	β	–	S	R	–	–	–	–	–	–	–	–	–	*Streptococcus sp.*
159T	β	_	S	R	_	+	_	_	+	+	+	+	_	Sorbitol +ve *S. pyogenes*
159P	β	–	S	R	_	w	_	_	_	w	+	+	_	*S. pyogenes*
160T	γ	_	S	R	_	+	_	_	w	+	+	+	_	Sorbitol +ve *S. pyogenes*
160P	β	+	S	R	–	+	–	–	–	–	–	–	–	*S. equi (group c)*
161T	γ	_	S	R	–	+	–	–	–	–	+	+	–	*S. pyogenes*
161P	β	_	S	R	_	_	+	_	_	_	+	–	–	Starch –ve *Trhalose –ve confirmed S. pyogenes*
162T	β	_	S	R	_	+	+	_	+	+	+	+	_	Sorbitol +ve *S. pyogenes*
162P	γ	_	S	R	_	+	_	_	+	+	+	+	_	Sorbitol +ve *S. pyogenes*
163P	γ	–	S	R	–	+	+	–	+	–	+	+	–	*S. pyogenes*
164P	γ	–	S	R	–	+	+	–	w	w	+	+	–	*S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table 2: Continued..														
Tests / **Samples**	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
165T	γ	_	S	R	_	+	+	_	_	+	+	+	_	Sorbitol +ve *S. pyogenes*
165P	β	_	S	R	_	w	+	_	w	+	+	_	_	Sorbitol +ve *Trhalose –ve S. pyogenes*
166T	β	_	S	R	_	w	+	_	+	+	+	+	_	Sorbitol +ve *S. pyogenes*
166P	β	_	S	R	_	_	+	_	+	_	+	+		Starch –ve *S. pyogenes*
167T	α	_	S	R	_	_	_	_	_	_	+	w	_	Ripose –ve *s. dysagalactia*
167P	α	_	S	R	_	w	w	_	w	_	+	_	_	Ripose –ve trhalose –ve *s. dysagalactia*
168T	α	_	S	R	_	w	w	_	_	_	+	w	_	Ripose –ve *s. dysagalactia*
168P	α	_	S	R	_	w	w	_	_	_	+	+	_	Ripose –ve *s. dysagalactia*
169P	α	–	S	R	–	+	w	–	w	–	+	w	–	*Streptococcus sp.*
170T	α	_	S	R	_	+	w	_	w	_	+	+	_	Ripose –ve *s. dysagalactia*
170P	α	_	S	R	_	+	w	_	w	w	+	+	_	Ripose –ve *s. dysagalactia*
171T	α	_	S	R	_	+	w	_	w	_	+	+	_	Ripose –ve *s. dysagalactia*
171P	α	–	S	R	–	w	w	–	–	–	–	–	–	*Streptococcus sp.*
172T	α	_	S	R	_	+	w	_	_	_	+	w	_	Ripose –ve *s. dysagalactia*
172P	β	–	S	R	–	+	w	–	w	w	+	+	–	*S. pyogenes*
173P	α	–	S	R	–	w	+	–	–	–	–	–	–	*Streptococcus sp.*
175P	α	_	S	R	_	w	_	_	w	_	_	w	_	Ripose –ve and lactose –ve *s. dysagalactia*
176P	β	–	S	R	–	w	+	w	+	w	+	+	–	*S. pyogenes*
178P	α	_	S	R	_	+	_	_	_	_	+	+	_	Ripose –ve *s. dysagalactia*
180T	α	_	S	R	_	+	_	_	+	+	_	+	_	Strepto spp (either *s. dysagalactia or s. uberis)*
180P	α	_	S	R	_	+	_	_	_	_	+	w	_	Ripose –ve *s. dysagalactia*
181T	α	_	S	R	_	w	_	_	w	w	+	+	_	Ripose –ve *s. dysagalactia*
184P	β	_	S	R	_	+	+	_	_	_	+	_	_	*Trhalose –ve S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
185T ®	β	_	S	R	_	+	w	_	w	_	+	+	_	*S. pyogenes*
185P®	β	_	S	R	_	+	+	_	_	_	+	+	_	*S. pyogenes*
188T	β	_	S	R	_	+	_	_	_	_	+	_	_	*Arginin –ve Trhalose –ve S. pyogenes*
189T	β	–	S	R	–	+	–	–	–	–	+	w	–	*Arginin –ve S. pyogenes*
189P	β	–	S	R	–	+	+	–	–	w	+	+	–	*S. pyogenes*
189Pα	α	_	S	R	_	w	+	_	_	_	+	_	_	Ripose –ve *s. dysagalactia*
190T	β	–	S	R	–	+	+	–	+	w	+	+	–	*S. pyogenes*
190P	β	–	S	R	–	+	+	–	+	w	+	+	–	*S. pyogenes*
191T	β	–	S	R	–	+	+	–	+	w	+	+	–	*S. pyogenes*
191P	β	–	S	R	–	+	+	–	+	w	+	+	–	*S. pyogenes*
192T	β	–	S	R	–	+	+	–	+	w	+	+	–	*S. pyogenes*
192P	β	–	S	R	–	+	+	–	+	–	+	+	–	*S. pyogenes*
193T	β	–	S	R	–	w	+	–	+	w	+	+	–	*S. pyogenes*
193P	β	–	S	R	–	w	+	–	–	–	+	w	–	*S. pyogenes*
194T	β	_	S	R	_	_	+	_	+	w	+	+	–	Starch –ve *S. pyogenes*
194P	β	_	S	R	_	+	_	_	w	+	+	_	_	*Arginin –ve Trhalose –ve S. pyogenes*
195T	β	–	S	R	–	+	+	–	w	+	+	+	–	*S. pyogenes*
195P	β	–	S	R	–	+	+	–	_	_	+	+	–	*S. pyogenes*
196T	β	–	S	R	–	+	+	–	+	w	+	+	–	*S. pyogenes*
196P	β	–	S	R	–	+	+	–	w	+	+	+	–	*S. pyogenes*
197T	β	–	S	R	–	w	+	–	+	w	+	+	–	*S. pyogenes*
197P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
198T	β	–	S	R	–	+	+	–	–	+	+	+	–	*S. pyogenes*
198P	β	–	S	R	–	+	–	–	+	+	+	+	–	*Streptococcus sp.*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
199T	β	–	S	R	–	+	+	–	+	–	+	+	–	*S. pyogenes*
199P	β	_	S	R	_	+	+	_	_	_	+	_	_	*Trehalose –ve S. pyogenes*
200T	β	–	S	R	–	w	+	–	+	w	+	+	–	*S. pyogenes*
200P	β	–	S	R	–	+	+	–	+	+	+	+	–	*S. pyogenes*
201T	α	_	S	R	_	+	w	_	_	w	+	+	_	Ripose –ve *s. dysagalactia*
201P	α	_	S	R	_	+	w	_	w	w	+	+	_	Ripose –ve *s. dysagalactia*
202T	α	_	S	R	_	+	w	_	+	+	+	+	_	Ripose –ve mannitol +ve *s. dysagalactia*
202P	α	_	S	R	_	+	w	_	+	w	+	+	_	Ripose –ve mannitol +ve *s. dysagalactia*
203T	β	–	S	R	–	+	w	–	–	+	+	+	–	*S. pyogenes*
203P	β	–	S	R	–	+	w	–	–	–	+	+	–	*S. pyogenes*
204T	β	_	S	R	_	+	+	_	_	_	+	+	_	*Trhalose –ve S. pyogenes*
204P	β	_	S	R	_	+	+	_	_	_	+	+	_	*Trhalose –ve S. pyogenes*
205T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
205P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
206T	β	–	S	R	–	w	+	–	–	–	+	+	–	*S. pyogenes*
206P	β	–	S	R	–	w	+	–	–	–	+	+	–	*S. pyogenes*
207T	β	–	S	R	–	+	+	–	–	–	+	+	–	lactose –ve *S. pyogenes*
207P	β	–	S	R	–	+	–	–	–	w	+	+	–	Starch –ve *S. pyogenes*
208T	β	–	S	R	–	+	+	–	–	w	+	+	–	*S. pyogenes*
209T	β	_	S	R	_	_	_	_	+	_	+	+	_	*Arginin –ve* Starch –ve *S. pyogenes*
209P	β	_	S	R	_	w	+	_	_	_	+	+	_	*Trhalose –ve S. pyogenes*
210T	β	_	S	R	_	_	w	_	_	_	+	+	_	Starch –ve *Trhalose –ve S. pyogenes*
210P	β	–	S	R	–	w	w	–	–	–	+	+	–	*S. pyogenes*
211P	β	_	S	R	_	w	+	_	_	_	+	+	_	*Trehalose –ve S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..														
Tests / **Samples**	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
212T	β	_	S	R	_	w	w	_	_	_	+	_	_	*Trehalose –ve S. pyogenes*
212P	β	–	S	R	–	+	w	–	–	–	+	+	–	*S. pyogenes*
213T	β	_	S	R	_	w	+	_	_	_	+	_	_	*Trehalose –ve S. pyogenes*
213P	β	–	S	R	–	w	+	_	+	w	+	+	–	*Sorbitol (w) S. pyogenes*
214T	β	_	S	R	_	+	+	_	_	_	+	_	_	*Trehalose –ve S. pyogenes*
214P	β	_	S	R	_	+	_	_	+	+	+	+	_	*Arginin –ve* sorbitol +ve *S. pyogenes*
215T	β	–	S	R	–	w	–	–	–	w	+	+	–	*Sorbitol (w) S. pyogenes*
215P	β	_	S	R	_	+	_	_	_	_	+	_	_	*Arginin –ve Trhalose –ve S. pyogenes*
215T-4	β	_	S	R	_	w	_	_	_	_	_	_	_	*S. equi (group c)*
216T	β	–	S	R	–	–	–	–	–	–	–	–	–	*Streptococcus sp.*
216P	β	_	S	R	_	+	_	_	_	_	+	_	_	*Arginin –ve Trhalose –ve S. pyogenes*
216T-1	β	_	S	R	_	_	w	_	_	_	+	+	_	*Starch –ve S. pyogenes*
217T	β	–	S	R	–	–	–	–	–	–	–	–	–	*Streptococcus sp.*
217P	β	–	S	R	–	–	–	–	–	–	–	–	–	*Streptococcus sp.*
218T	β	_	S	R	_	w	+	_	_	_	+	_	_	*Trhalose –ve S. pyogenes*
218P	β	_	S	R	_	w	+	_	_	_	+	_	_	*Trhalose –ve S. pyogenes*
218T-6	β	_	S	R	_	+	w	_	+	_	w	+	_	*Sorbitol +S. pyogenes*
219T	β	_	S	R	_	+	_	_	_	_	+	_	_	*Arginin –ve Trhalose –ve S. pyogenes*
219P	β	–	S	R	–	+	–	–	–	–	–	–	–	*Arginin –ve S. equi*
220T	β	–	S	R	–	+	+	–	–	–	+	–	–	*S. pyogenes*
220P	β	_	S	R	_	+	_	_	_	_	+	w	_	*Trehalose –ve S. pyogenes*
221T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table 2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
221P	β	_	S	R	_	+	+	_	_	+	+	_	_	*Trehalose –ve S. pyogenes*
222T	β	_	S	R	_	–	+	_	_	+	+	+	_	*Starch –ve, sorbitol + S. pyogenes*
222p	β	–	S	R	–	+	+	–	–	w	+	+	–	*S. pyogenes*
223T	β	–	S	R	–	–	+	–	–	–	+	+	–	*Starch –ve S. pyogenes*
223P	β	–	S	R	–	–	+	–	–	–	+	–	–	*Starch –ve S. pyogenes*
224T	β	–	S	R	–	–	+	–	–	–	+	–	–	*Starch –ve S. pyogenes*
224P	β	–	S	R	–	w	+	–	–	–	+	+	–	*S. pyogenes*
225T	β	–	S	R	–	+	+	–	w	w	+	+	–	*S. pyogenes*
225P	β	–	S	R	–	+	+	–	+	_	_	+	–	*S. pyogenes*
226P	β	–	S	R	–	w	+	–	–	–	+	+	–	*S. pyogenes*
227T	β	–	S	R	–	+	+	–	–	+	+	+	–	*S. pyogenes*
227P	β	+	S	R	–	–	+	–	+	–	+	+	–	*Mannitol +S. sanguis*
228T	β	–	S	R	–	+	+	–	+	+	+	+	–	*Sorbitol +S. pyogenes*
228P	β	–	S	R	–	+	+	–	+	+	+	+	–	*Sorbitol +S. pyogenes*
229T	β	–	S	R	–	+	+	–	–	+	+	+	–	*Sorbitol +S. pyogenes*
229P	β	–	S	R	–	+	w	–	+	+	+	+	–	*Sorbitol +S. pyogenes*
230T	β	_	S	R	_	+	w	_	+	+	+	_	_	*Sorbitol + trehalose –ve S. pyogenes*
230P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
231T	β	–	S	R	–	+	+	–	+	+	+	+	–	*Sorbitol +S. pyogenes*
231P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
232T	β	_	S	R	_	+	_	_	_	+	+	+	_	Arginine –ve sorbitol + *S.pyogenes*
232P	β	_	S	R	_	+	_	_	_	+	+	+	_	*Manitol + S. equi (group c)*
233T	β	–	S	R	–	+	+	–	+	–	–	–	–	*Streptococcus sp.*
233P	β	–	S	R	–	+	+	–	–	+	+	+	–	*Sorbitol +S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Tests / Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
234T	β	_	S	R	_	+	+	_	_	+	+	_	_	*Sorbitol + trehalose –ve S. pyogenes*
234P	β	_	S	R	_	+	+	_	_	_	+	_	_	*trehalose –ve S. pyogenes*
235T	β	–	S	R	–	+	+	–	–	+	+	+	–	*Sorbitol +S. pyogenes*
235P	β	_	S	R	_	_	+	w	_	_	+	_	_	*Starch -ve trehalose –ve S. spp. Group L*
236T	β	–	S	R	–	+	+	–	–	+	+	+	–	*Sorbitol +S. pyogenes*
236P	β	_	S	R	_	+	+	_	_	+	+	_	_	*Sorbitol + trehalose –ve S. pyogenes*
237T	β	_	S	R	_	_	+	_	_	_	+	+	_	Starch –ve *S. pyogenes*
237P	β	_	S	R	_	_	+	_	_	_	+	_	_	Starch & trehalose –ve *S. pyogenes*
238T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
238P	β	_	S	R	_	+	_	_	_	+	+	+	_	Arginine –ve sorbitol + *S. pyogenes*
239T	β	–	S	R	–	+	+	–	–	+	+	+	–	*Sorbitol +S. pyogenes*
239P	β	_	S	R	_	+	+	_	_	_	+	_	_	*trehalose –ve S. pyogenes*
240T	β	–	S	R	–	+	+	–	+	–	+	+	–	*S. pyogenes*
240P	β	–	S	R	–	+	+	–	+	+	+	+	–	*Sorbitol +S. pyogenes*
241T	β	–	S	R	–	+	+	–	w	+	+	+	–	*Sorbitol +S. pyogenes*
241P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
242T	β	_	S	R	_	+	_	_	_	_	+	+	_	Arginine –ve *S. pyogenes*
242P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
243T	β	–	S	R	–	+	+	–	–	+	+	+	–	*S. pyogenes*
243P	β	–	S	R	–	+	+	–	_	+	+	+	–	*Sorbitol + S. pyogenes*
244T	β	–	S	R	–	+	+	–	+	–	+	+	–	*S. pyogenes*
244P	β	–	S	R	–	+	+	–	_	+	+	+	–	*Sorbitol +S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..														
Tests / **Samples**	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
245T	β	–	S	R	–	+	+	–	+	+	+	+	–	*Sorbitol + S. pyogenes*
245P	β	–	S	R	–	+	+	–	+	+	+	+	–	*Sorbitol + S. pyogenes*
246T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
246P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
247T	β	–	S	R	–	+	+	–	–	+	+	+	–	*S. pyogenes*
247P	β	–	S	R	–	+	+	–	–	w	+	+	–	*S. pyogenes*
248P	β	_	S	R	_	+	_	_	_	_	+	+	_	*Arginine –ve S. pyogenes*
249T	β	–	S	R	–	+	+	–	–	+	+	+	–	*Sorbitol + S. pyogenes*
249P	β	–	S	R	–	+	+	–	–	+	+	+	–	*Sorbitol + S. pyogenes*
250T	β	_	S	R	_	_	w	_	_	_	+	+	_	*Arginine –ve S. pyogenes*
250P	β	–	S	R	–	_	+	–	+	+	+	+	_	*arginine –ve sorbitol + S. pyogenes*
251T	β	–	S	R	–	+	+	–	+	+	+	+	–	*sorbitol + S. pyogenes*
251P	β	–	S	R	–	+	w	—	+	–	+	+	–	*sorbitol + S. pyogenes*
252T	β	_	S	R	_	_	+	_	_	_	+	+	_	*arginine –ve S. pyogenes*
252P	β	_	S	R	_	_	+	_	+	+	+	+	_	*arginine –ve S. pyogenes*
253T	β	–	S	R	–	–	–	–	–		–	–	–	*Streptococcus sp.*
253P	β	–	S	R	–	+	–	–	–	–	+	+	–	*Starch –ve S. pyogenes*
254T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
254P	β	–	S	R	–	+	+	–	+	–	+	+	–	*S. pyogenes*
255T	β	–	S	R	–	–	–	–	–	–	–	–	–	*Streptococcus sp.*
255P	β	–	S	R	–	–	–	–	–	–	–	–	–	*Streptococcus sp.*
256T	β	_	S	R	_	+	_	_	_	_	_	_	_	Starch –ve *S. equi* (group c)
256P	β	–	S	R	–	+	+	–	+	–	+	+	–	*Sorbitol + S. pyogenes*
257T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
257P	β	–	S	R	–	+	+	–	+	–	+	+	–	Sorbitol + *S. pyogenes*
258T	β	–	S	R	–	+	+	–	+	–	+	+	–	*S. pyogenes*
258P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..														
Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Result**
259T	β	–	S	R	–	–	+	–	–	_	+	+	_	*Arginine –ve S. pyogenes*
259P	β	–	S	R	–	–	–	–	–	–	–	w	–	*Streptococcus sp.*
260T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
260P	β	–	S	R	–	+	w	–	–	_	+	+	_	*S. pyogenes*
261T	β	–	S	R	–	+	w	–	w	–	+	+	–	*Sorbitol + S. pyogenes*
261P	β	–	S	R	–	+	+	–	w	–	+	w	–	*Sorbitol + S. pyogenes*
262T	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
262P	β	–	S	R	–	+	+	–	+	–	+	+	–	*Sorbitol + S. pyogenes*
263T	β	–	S	R	–	+	+	–	+	–	+	+	–	*Sorbitol + S. pyogenes*
263P	β	–	S	R	–	_	_	+	_	_	+	_	_	Arginine &trehalose –ve *S. pyogenes*
264T	β	–	S	R	–	–	–	–	–	–	–	–	–	*Streptococcus sp.*
264P	β	–	S	R	–	+	w	–	+	_	+	+	–	Confirmed *S. pyogenes*
265T	β	–	S	R	–	+	+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
265P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
266T	β	–	S	R	–	+	w	–	–	–	+	+	–	*S. pyogenes*
266P	β	–	S	R	–	+	+	–	–	–	+	+	–	*S. pyogenes*
268T	β	–	S	R	–	_	_	–	+	_	+	+	-	*Starch –ve Sorbitol + S. pyogenes*
268P	β	–	S	R	–	+	+	–	+	+		+	–	*Sorbitol + S. pyogenes*
269T	β	–	S	R	_	+	+	_	+	_	+	+	_	*Sorbitol + S. pyogenes*
269P	β	–	S	R	–	–	–	–	w	–	–	–	–	*Streptococcus sp.*
270T	β	–	S	R	–	–	–	–	_	–	–	–	–	*Streptococcus sp.*
271T	β	–	S	R	–	w	+	w	+	–	+	+	–	*Sorbitol + S. pyogenes*
271P	β	–	S	R	–	w	+	–	+	+	+	+	–	*Sorbitol + S. pyogenes*
272T	β	–	S	R	–	+	+	–	–	+	+	+	–	*S. pyogenes*
272P	β	–	S	R	–	+	–	w	–	–	+	+	–	Starch –ve *S. pyogenes*
273T	β	–	S	R	–	+	+	–	+	w	+	+	–	Sorbitol + *S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..														
Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Results**
274T	β	–	S	S	–	+	–	–	–	w	–	w	–	Starch –ve *S. pyogenes*
274P	β	–	S	R	–		+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
275T	β	–	S	R	–		+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
275P	β	–	S	R	–	+	+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
276T	β	–	S	R	–	+	+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
276P	β	–	S	R	–	+	_	w	+	–	+	+	–	Sorbitol + *S. pyogenes*
277T	β	–	S	R	–		+	–	–	–	+	+	–	*S. pyogenes*
277P	β	–	S	R	–		+	–	–	–	+	+	–	*S. pyogenes*
278T	β	–	S	R	–		w	–	–	–	+	+	–	*S. pyogenes*
279T	β	–	S	R	–		+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
279P	β	–	S	R	–		+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
280T	β	–	S	R	–		+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
280P	β	–	S	R	–		W	–	–	–	+	+	w	*S. pyogenes*
281T	β	–	S	R	–		+	–	+	W	+	+	–	Sorbitol + *S. pyogenes*
281P	β	–	S	R	–		+	–	+	W	+	+	–	Sorbitol + *S. pyogenes*
282P	β	–	S	R	–		+	–	+	+	+	+ late	–	Sorbitol + *S. pyogenes*
283T	β	–	S	R	–		+	–	W	W	+	+	–	Sorbitol w *S. pyogenes*
284T	β	–	S	R	–		+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
284P	β	–	S	R	–		+	–	W	W	–	+	–	Sorbitol w *S. pyogenes*
285T	β	–	S	R	–		+	w	W	W	+	+	–	Sorbitol w *S. pyogenes*
285P	β	–	S	R	–		+	–	–	W	+	+	–	*S. pyogenes*
294P	ɤ	–	S	R	–	+	–	–	–	+	+	–	–	*Streptococcus sp.*
295T	β	–	R	R	–	+	–	–	–	_	+	+	–	*Starch –ve S. pyogenes*
295P	β	–	R	R	–	+	–	–	–	+	+	_	–	*Starch & trehalose –ve S. pyogenes*
296P	β	–	S	R	–	+	–	–	–	–	w	+	–	*Starch –ve S. pyogenes*
297T	β	_	S	R	_	+	_	w	_	_	+	+	_	*Starch –ve strepto. Spp group L*
Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.														

Table2: Continued..														
Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Results**
297P	β	–	S	R	–	+	+	w	–	+	+	+	–	*Mannitol + strepto. Spp group L*
298T	β	–	S	R	–	+	+	w	+	+	+	+	–	*Mannitol + strepto. Spp group L*
298P	β	–	S	R	–	w	–	–	–	–	+	–	–	Starch and trehalose –ve *S. pyogenes*
299T	β	–	S	R	–	w	–	w	–	–	+	+	–	*Starch –ve strepto. Spp group L*
299P	β	–	S	R	–	+	–	w	–	–	–	+	–	*Starch & lactose –ve strepto. Spp group L*
300T	β	–	S	S	–	+	w	–	+	–	+	+	–	*S.pnemonae*
300P	β	–	S	R	–	+	+	–	+	+	+	+	–	Sorbitol + *S. pyogenes*
301T	β	–	S	R	–	w	–	–	–	–	+	+	–	*Starch –ve S. pyogenes*
302T	β	_	R	R	_	+	w	w	_	_	+	+	_	*Streptococcus. Spp group L*
302P	β	_	S	R	_	+	w	_	_	w	+	_	_	*Trehalose –ve S. pyogenes*
303T	β	–	S	R	–	+	–	w	–	–	+	+	–	*Starch –ve strepto. Spp group L*
303P	β	–	S	R	–	+	–	–	–	–	+	–	–	Starch and trehalose –ve *S. pyogenes*
304T	α	–	S	R	–	+	–	–	–	+	–	+	–	*Streptococcus sp.*
304P	β	–	S	R	–	+		w	w	+	–	+	–	*lactose –ve strepto. Spp group L*
305T	β	–	S	R	–	+	–	–	–	–	–	+	–	*Starch & lactose –ve S. pyogenes*
305P	β	–	S	R	–	+	–	–	+	+	+	+	–	*Starch –ve sorbitol + S. pyogenes*
306T	β	–	S	R	–	+	–	w	–	–	+	+	–	*Starch –ve strepto. Spp group L*
306P	β	–	S	R	–	+	–	–	–	–	+	+	–	*Starch –ve S. pyogenes*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table2: Continued..

Samples	H	Cat	Bac	opt	Es	Star	arg	Rib	Man	Sor	Lact	Treh	VP	**Results**
307T	β	–	S	R	–	+	–	w	–	–	+	+	–	*Starch –ve strepto. Spp group L*
307P	β	–	S	R	–	+	–	w	–	–	+	+	–	*Starch –ve strepto. Spp group L*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer. NP: not performed.

Table 3: The dentification of Gram negative cocci obtained from each patient sample (either tonsils or pharynx swabs) using biochemical tests profile according to Barrow and Feltham (2003):

Samples	H	Catalase	Oxidase	O/F	Maltose	glucose	sucrose	Lactose	Final result
59P-1	β	w	+	F	+	_	+	+	*Branhamella catarhalis*
65T-5	β	w	+	F	+	+	+	+	*Branhamella catarhalis*
167-1	Non	–	+	–	+	+	+	–	*Neisseria elongata*
203T-1	Non	_	w	_	+	_	+	_	*Neisseria elongata*
204T-2	Non	_	_	_	_	_	+	+	*Neisseria elongata*
206T-4	β	+	+	F	+	+	+	+	*Branhamella catarhalis.*
248P-19	Non	+	+	F	+	+	+	+	*Branhamella catarhalis.*
249P-20	Non	+	+	_	+	+	+	+	*Neisseria sp.*
250P-22	Non	+	+	O	+	+	+	+	*Neisseria lactamica*
252P-25	Non	+	+	_	+	+	+	+	*Neisseria sp.*
252P-30	Non	w	+	_	+	+	+	+	*Neisseria sp.*
252T-24	Non	+	_	O	+	+	+	+	*Neisseria lactamica*
Keys: H: hemolysis on sheep blood agar, O/F: oxidation fermentation test.									

Table 4: The dentification of Gram positive cocci obtained from each patient sample (either tonsils or pharynx swabs) using biochemical tests profile according to Barrow and Feltham (2003):

Sampl.	H	Cat	Ox	O/F	Arg	ure	glu	Tre	Lac	suc	man	raf	fruc	mann	mal	xyl	VP	Nov	Cog	Final result
60P-2	No	+	_	F	_	_	_	_	_	NP	_	_	_	_	NP	NP	+	S	+	*S.aureus*
63P-3	No	+	_	O	+	+	+	+	+	NP	+	_	+	+	NP	NP	+	S	NP	*S. epidermidis*
168P-2	No	+	_	O	_	_	+	_	+	+	_	NP	+	+	+	_	_	NP	+	*S. delphini*
170P-4	No	+	+	F	_	+	_	+	+	+	+	+	+	+	+	_	+	NP	_	*S.lentus*
172P-6	No	+	+	O		_	+	_	_	+	_	_	+	_	+	_	_	NP	_	*S.sciuri*
183T-7	β	+	_	O	+	+	+	+	_	+	+	+	+	+	+	_	+	S	+	*S.aureus*
196P-10	No	+	_	_	+	+	+	+	+	+	+	+	+	+	+	+	_	S	NP	*S. chromogenes*
194T-11	No	+	_	_	+	+	+	+	+	+	+	+	+	+	+	+	_	S	NP	*S. chromogenes*
205T-3	β	+	_	F			+	+	_	+	_		+	+	+	_	_	NP	NP	*S. auricularis*
206T-5	β	+	+s	_	_		+			+			+				_			*Micococcus lylae*
208T-1	No	+	+	_	_		+			–			+				_			*Micococcus sp.*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer, ure: urease, raf: raffinose, fruc: fructose, Mann: mannose, malt: maltose, suc: sucrose, glu: glucose, xyl: xylose, Nov: novobiocon, Cog: coagulase NP: not performed.

Table 4: Continued..																			
Sampl.	**H**	**Cat**	**Ox**	**O/F**	**Arg**	**ure**	**glu**	**Tre**	**Lac**	**suc**	**man**	**raf**	**fruc**	**mann**	**mal**	**xyl**	**VP**	**Nov**	**Cog**	**Final result**
209T-2	No	+	+	_	_		+			+			+				_			*Micococcus lylae*
212T-1	No	+	+	F	_	_	+	+	+	+	_		+	+	+	_	_			*S. caseolyticus*
213T-3	No	+	+	F	_	+	+	+	+	+	w		+	+	+	_	_			*S. caseolyticus*
219P-1	No	+	_	F				+	+	+	_	+	+	+	+	_	+			*S. hominis*
222T-1	No	+	+	F	+	+		w		+	_		_	_		_	_	S	_	*Near to S. caseolyticus*
225T-3	β	+	_	_	+	+		+		+	+		+	+		_	_	S	_	*S. chromogenes*
185T R-4	No	+	+	_	+	+		+		+	+		+	+		_	_	S	_	*S. caseolyticus*
228T-3	No	+		F	+	_		+	+	+	+		+	+	+	_	_		+	*S. intermedius (maltose +ve)*
229P-5	No	+	_	F	+	+		+	+	+	+		+	+	+	_	+		+	*S. aureus*
230T-6	No	+	_	F	_	_		+	_	+	_		+	_	+	_	_		_	*S. auricularis*
230P-7	No	+	_	F	+	+		_	_	+	_		+	+	+	_	_		_	*S. capitis*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer, ure: urease, raf: raffinose, fruc: fructose, Mann: mannose, malt: maltose, suc: sucrose, glu: glucose, xyl: xylose, Nov: novobiocon, Cog: coagulase NP: not performed.

Table 4: Continued..

Sampl.	H	Cat	Ox	O/F	Arg	ure	glu	Tre	Lac	suc	man	raf	fruc	mann	mal	xyl	VP	Nov	Cog	Final result
234T-12	No	+	–	F	+	+		+	+	+	+		+	+	+	–	–		+	*S.intermedius (maltose +ve)*
234T-13	No	+	+	F	–	–		–	+	+	–		+	+	+	–	–		NP	*S. caseolyticus*
235T-14	No	+	–	F	–	+		–	+	+	+		+	+	+	–	–		–	*S. lentus*
236T-16	No	+	–	F	+	+		+	+	+	+		+	+	+	–	–		+	*S.intermedius (maltose +ve*
235T-15	No	+	–	F	+	+		–	+	+	–		+	+	+	–	–		+	*S. hyicus (maltose +ve)*
238T-1	No	+	–	F		–		–	+	–	–		–	–	–			S	–	*S. simulans*
238P-2	No	+	–	F		–		–	–	–	–		–	–	–			S	–	*S. auricularis*
239T-3	No	+	–	F		–		+	–	+	–		–	–	–			S	–	*S. auricularis*
239P-4	No	+	–	F		+		w	+	+	–		–	–	–			S	–	*S. auricularis*
240T-5	No	+	–	F		+		–	–	–	–		–	–	–			S	–	*S. auricularis*
240P-7	No	+	–	F		–		–	–	–	–		–	–	–			S	+	*S. aureus*

Keys: H: hemolysis on sheep blood agar, Cat: catalase, Bac: bacitracin, opt: optichin, Es: bile esculine, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, sor: sorbitol, lact: lactose, Tre: trehalose, VP: voges proskauer, ure: urease, raf: raffinose, fruc: fructose, Mann: mannose, malt: maltose, suc: sucrose, glu: glucose, xyl: xylose, Nov: novobiocon, Cog: coagulase NP: not performed.

Table4: Continued..																			
Sampl.	**H**	**Cat**	**Ox**	**O/F**	**Arg**	**ure**	**glu**	**Tre**	**Lac**	**suc**	**man**	**raf**	**fruc**	**mann**	**mal**	**xyl**	**V P**	**Nov**	**Cog**	**Final result**
242T-9	β	+	_	_		+		+	+	+	+		+	+	+			S	_	*S. felis*
243T-10	No	+	_	F		_		_		_	_		w	_	+			S	_	*S. auricularis*
243T-11	No	+	_	F		_		_		_	_		_	_	_			S	_	*S.auricularis*
244T-12	No	+	_	F		_		_		_	_		_	_	_			S	_	*S. auricularis*
245T-13	No	+	_	F		_		_		_	_		_	_	_			S	_	*S.auricularis*
245P-14	β double zone	+	_	F		+		+		+	+		+	+	+			S	–	*S. aureus*
247T-17	β	+	_	F		_		+		+	+		+	+	+			S	_	*S.auricularis*
251T-23	No	+		F		+		+		+	+		+	+	_			S	_	*S.hyicus*
253P-27	β	+	_	F	+	+		+		_	+		+	+	+			S	+	*S. aureus*
246T-29	β	+	_	F	+	+		+		_	+		+	+	+			S	+	*S.aureus*

Keys: H: hemolysis on sheep blood agar, Cat: catalase,OX: oxidase, O/F: oxidation fermentation test, Arg: arginine, ,Man: mannitol, , lact: lactose, Tre: trehalose, VP: voges proskauer, ure: urease, raf: raffinose, fruc: fructose, Mann: mannose, malt: maltose, suc: sucrose, glu: glucose, xyl: xylose, Nov: novobiocon, Cog: coagulase NP: not performed.

Table 5: The dentification of Gram positive rods obtained from each patient sample (either tonsils or pharynx swabs) using biochemical tests profile according to Barrow and Feltham (2003):

Samples	H	Endo	ox	M	Sp	glu	gal	Mann	raf	xyl	cit	ure	Ind	VP	cas	Final resul
78T-4	non	+		_	TX	+	_	_	_	_	+	_	NP	_	+	*Bacillus mycoides*
103T-14	non	+		_	VX	_	_	_	_	_	_	_	NP	_	NP	*Bacillus mycoides*
109T-17	+	+	_	+	TX	+	+	+	+	+	_	+	NP	_	NP	*Bacillus licheniformis*
110T-18	non	+		_	TX	+	_	_	_	_	+	_	NP	_	NP	*Bacillus. mycoides*
246P-15	Non	+		+		+		+		_	_	_	_		+	*Bacillus pantothenticus*
246T-16	Non	+		+		+		+		_	_	+	_		+	*Bacillus pantothenticus*

Keys: H: hemolysis on sheep blood agar, endo: presence of endosposes, ox: oxidase, M: motility, sp: sporse position, glu: glucose, gal: galactose, mann: mannose, raf: raffinose, xyl: xylose, cit: citrate, ure: urease, Ind: indole, VP: voges proskauer, cas: casein. T: spores terminal, X: sporse oval, V: variable in position. NP: not performed.

Sample	H	Gram	Sp	M	glu	lac	suc	Indol	casinase	urease	Lecithinase C	Final result
240P-8	No	Gram variable rods	Drum stick terminal	_	_	_	_	_	+	_	_	*Clostridium sp.*

Keys: H: hemolysis on sheep blood agar, Sp: sporse position and shape, M: moltility, glu: glucose, lac: lactose, suc: sucrose.

Table 6: The dentification of Positive polymorphic shape obtained from each patient sample (either tonsils or pharynx swabs) using biochemical tests profile according to Barrow and Feltham (2003):

Sampl.	H	O/F	cat	M	Es	Gel	glu	lac	Malt	Man	tre	xyl	star	suc	ure	Arg	VP	Final result
104T-11	non	_			_	+	+	_	+	w	+	_	_	+	_	_		*Corynbacterium sp.*
208T-0	Non	_	+	_	_	_	+	_	+	_	+	_	+	+	+	w	_	*C. kutscheri*
213T-2	Non	F	+	_	_	NP	+	_	_	_	_	_	_	_	_	_	+	*C. amycolatum*
233P-10	Non	O		_	_	NP	+	+	+	+	+	_	+	+	_	+	_	*C.bovis*
247P-18	non	O		_	_	NP	+	+	+	+	_	_	_	_	_	+	_	*C.bovis*

Keys: H: hemolysis on sheep blood agar, o/f: oxidation fermentation test, Es: esculin hydrolysis, Cat: catalase, M: motility, Star: starch, Arg: arginine, Rib: ribose,Man: mannitol, lact: lactose, Tre: trehalose, VP: voges proskauer, ure: urease, raf: raffinose, malt: maltose, suc: sucrose, glu: glucose, xyl: xylose, Gel> gelatin liquifaction, NP: not performed.

Table 7: The dentification of Gram negative rods:obtained from each patient sample (either tonsils or pharynx swabs) using biochemical tests profile according to Barrow and Feltham (2003):

					Ammonium salt agar								
Sampl.	M	O/F	cat	ox	Glu	Ara	fruc	malt	man	suc	xyl	ure	Final result
135T	+	_	+	+	+	+	+	+	_	+	+	+	*Shwanella putrefaction*
135P	+	_	+	+	+	+	+	+	_	+	+	+	*Shwanella putrefaction*
Sample		M	cat	ox	Glu	Gal	raf	man	xyl	TSI			Final result
114P-22		+	_	+	+	_	_	_	_	_			*Pseudomonas alkaligenes*

Keys: M: moltility, O/F: oxidation fermintaion test, cat: catalase, Ox: oxidase, glu: glucose, Ara: arabinose, Fruc: fructose, malt: maltose, Man: mannitol, suc: sucrose, xyl: xylose, ure: urease, Gal: galactose, Raf: raffinose, TSI: trible sugar iron.

4.2 PCR RESULTS

4.2.1 Detection of *S. pyogenous:*

Seventy two samples of 556 samples (13%) were detected by PCR and confirned as *Streptococcus pyogenes* on the size 407bp of spy 1258 primer. Which represent 6.8% of the isolated *Streptococcus pyogenes*.

Plate 1: shows PCR run using spy 1258 primer (407bp in length) sample no. 45T is positive.

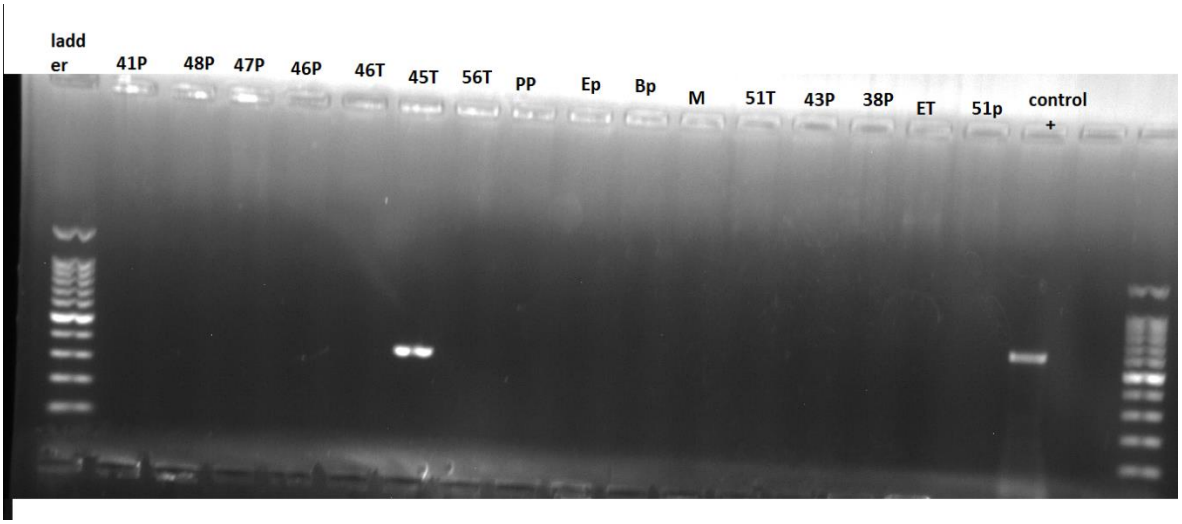


Plate 2: Shows PCR run using spy 1258 primer (407bp in length) samples no. 42P, ST, 74P, RP, 34P, 34T, 36P are apparently positive. Sample no. 60T shows multiple bands and samples no. 33P, 67P and 38T show positive faint bands.

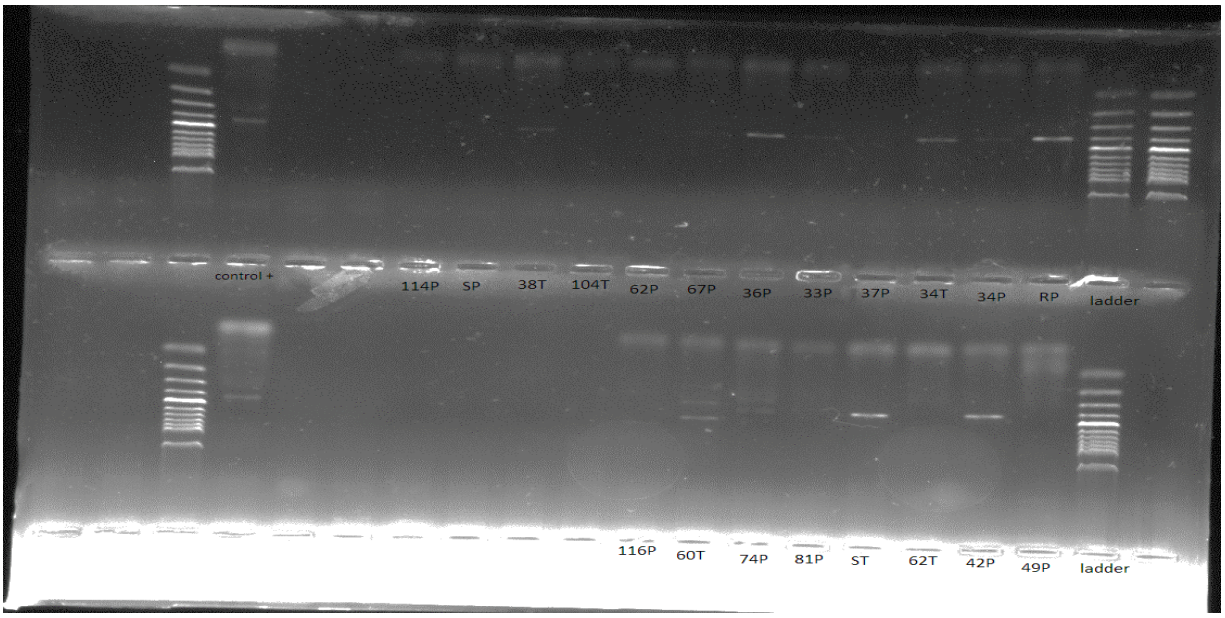


Plate 3: shows PCR run using spy 1258 primer (407bp in length) samples no. 54T, 77T, 68T, 85P, 52P, 80P and 64P are positive with faint bands. 76P and 58P show very faint bands. Sample no 66T is positive with multiple bands. The lower part of plate shows multiple bands on 73Pand 65T. There are many other positive faint bands.

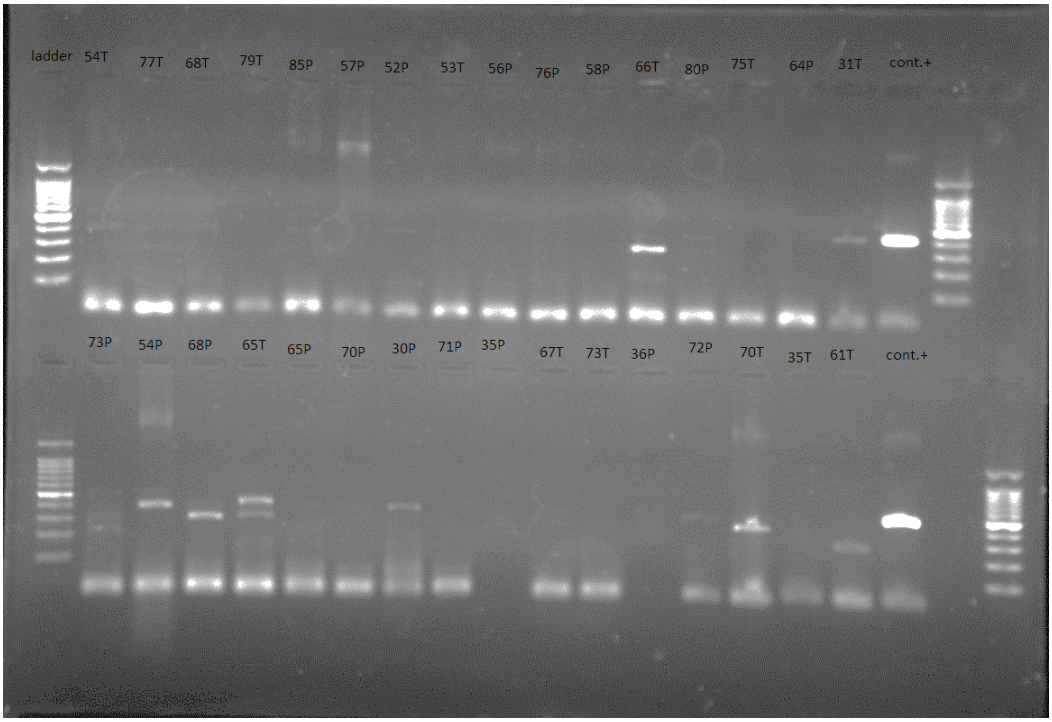


Plate 4: shows PCR run using spy 1258 primer (407bp in length) there are many positive bright and faint bands.samples no. 123P and 118T show concentrated DNA in spite they are positive. There are other negative samples with concentrated DNA.

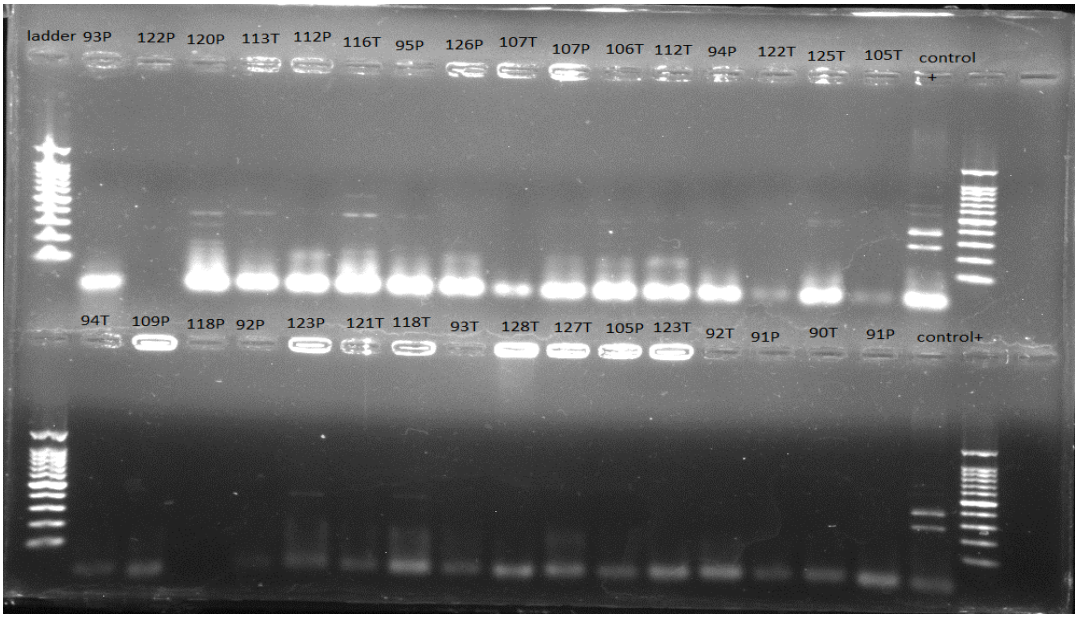


Plate 5: shows PCR run using spy 1258 primer (407bp in length) samples no. 64T, 72Tand 69P result multiple positive bands. 32P, 58T, 88T and 127T are apparently positive. 121P, 109T, 119T, 120T and 125P show positive faint bands.

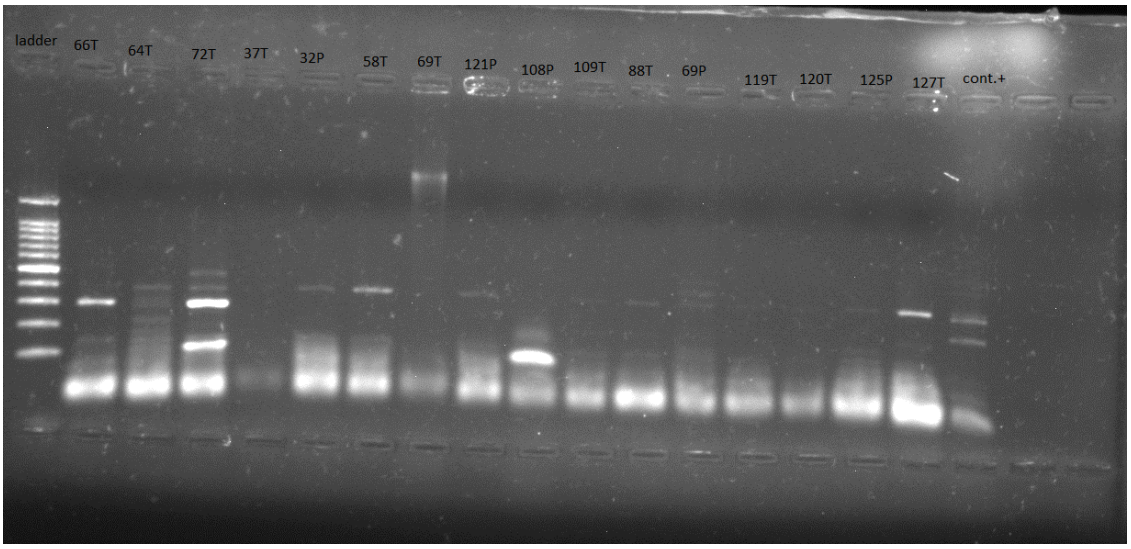
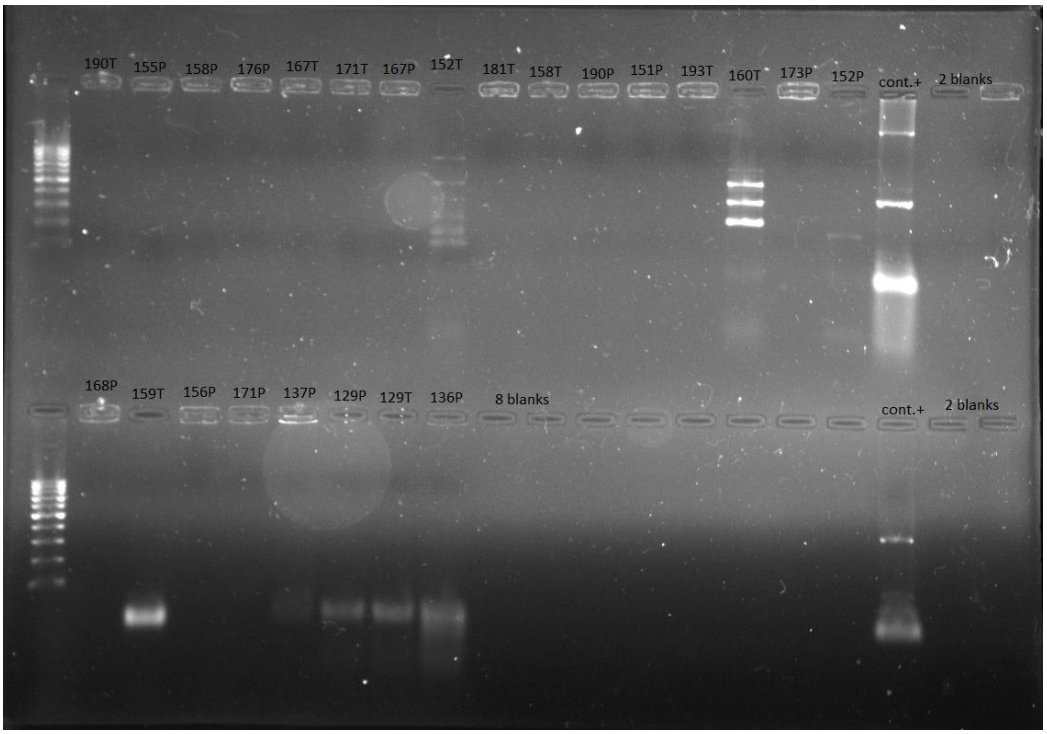


Plate 6: shows PCR run using spy 1258 primer (407bp in length) shows only three samples positive sample no. 152T,160P and 152P with multiple strong bands.



4.2.2 Detection of *Streptococcus sp.*:

Eigty five samples of 556 samples (15.3%) were detected by PCR and confirned as *Streptococci* on the size 480bp of the Dprimer, which represent 15.3% of the isolated *Streptococci*.

Plate 7: shows PCR run using D primer (480bp in length) samples no.81P, 93T, XT, 55P, 116T, 36T,49T, 74T, 53T,263P,118P, 48P, 43T are clearly positive. 112P, 92Tand 37T are positive with faint bands.

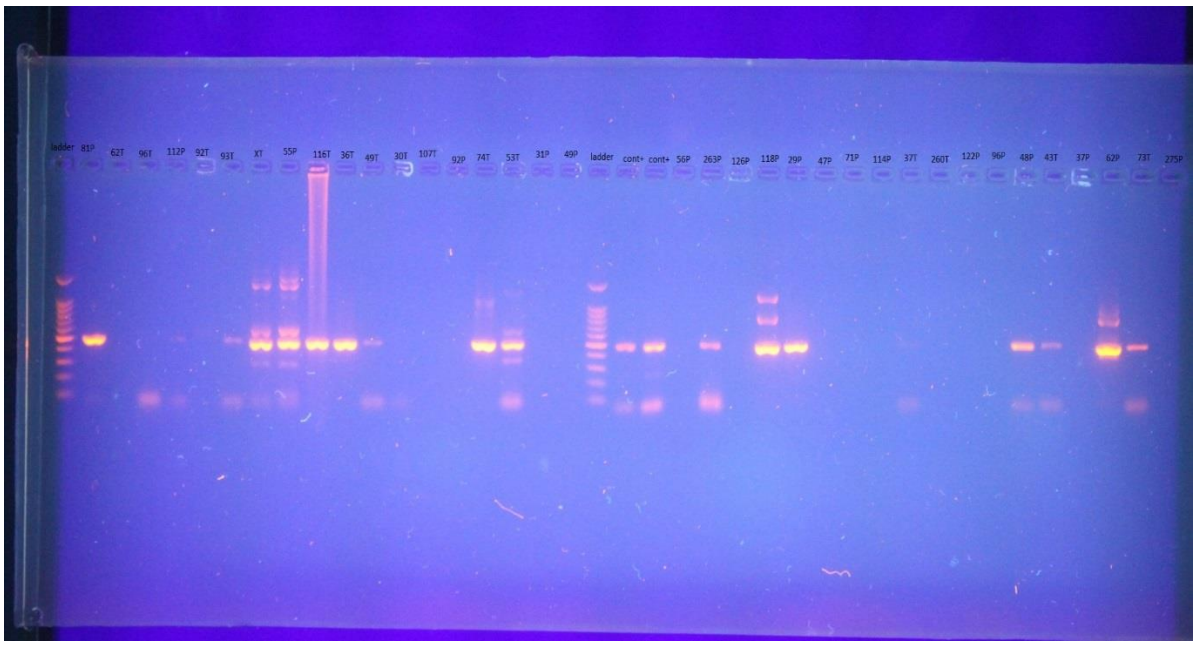


Plate 8: shows PCR run using D primer (480bp in length) three positive bands for samples no. 139T, 154T and 141P.

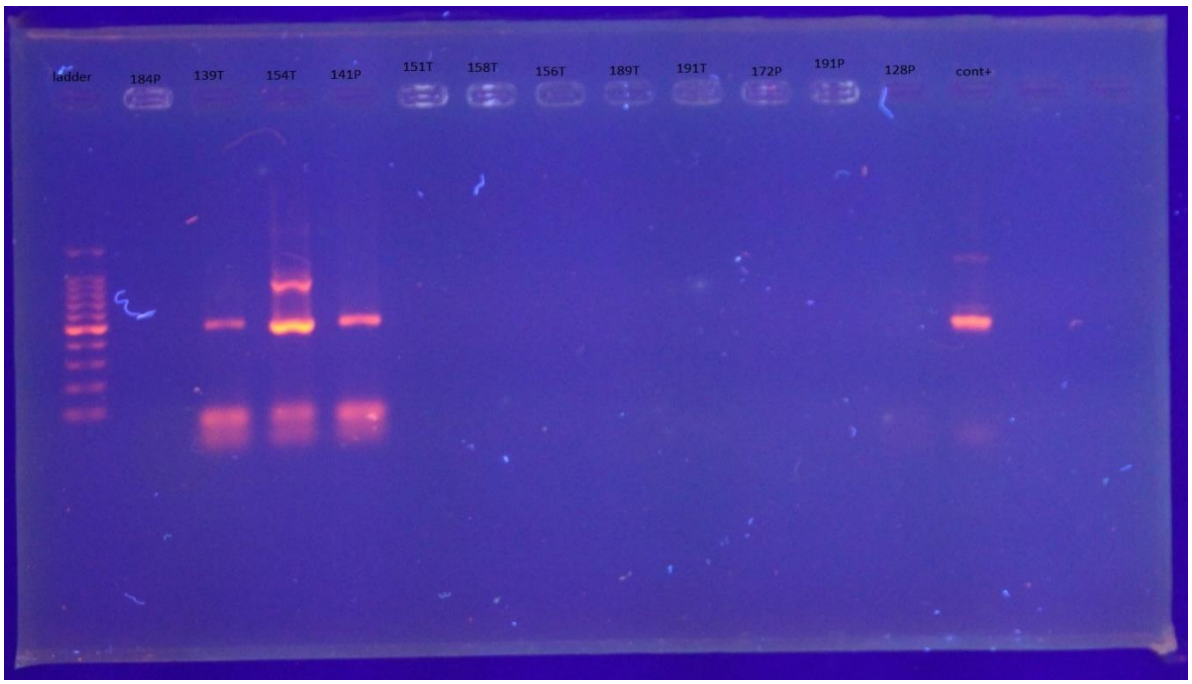


Plate 9: shows PCR run using D primer (480bp in length) samples no. 159T, 138P, 141T are positive and 136T positive with faint band.

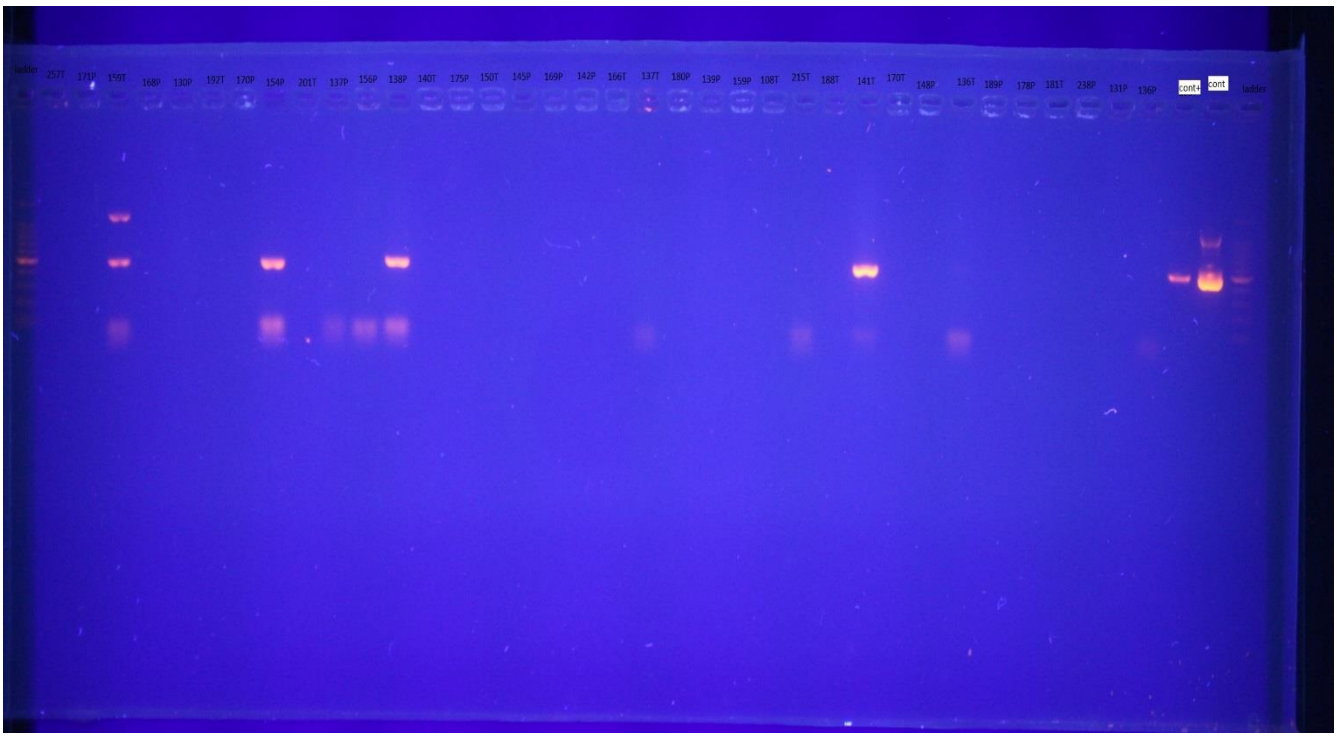


Plate 10: shows PCR run using D primer (480bp in length) samples no. 127P, 87P, DT, 126T and 105P are apparently positive.

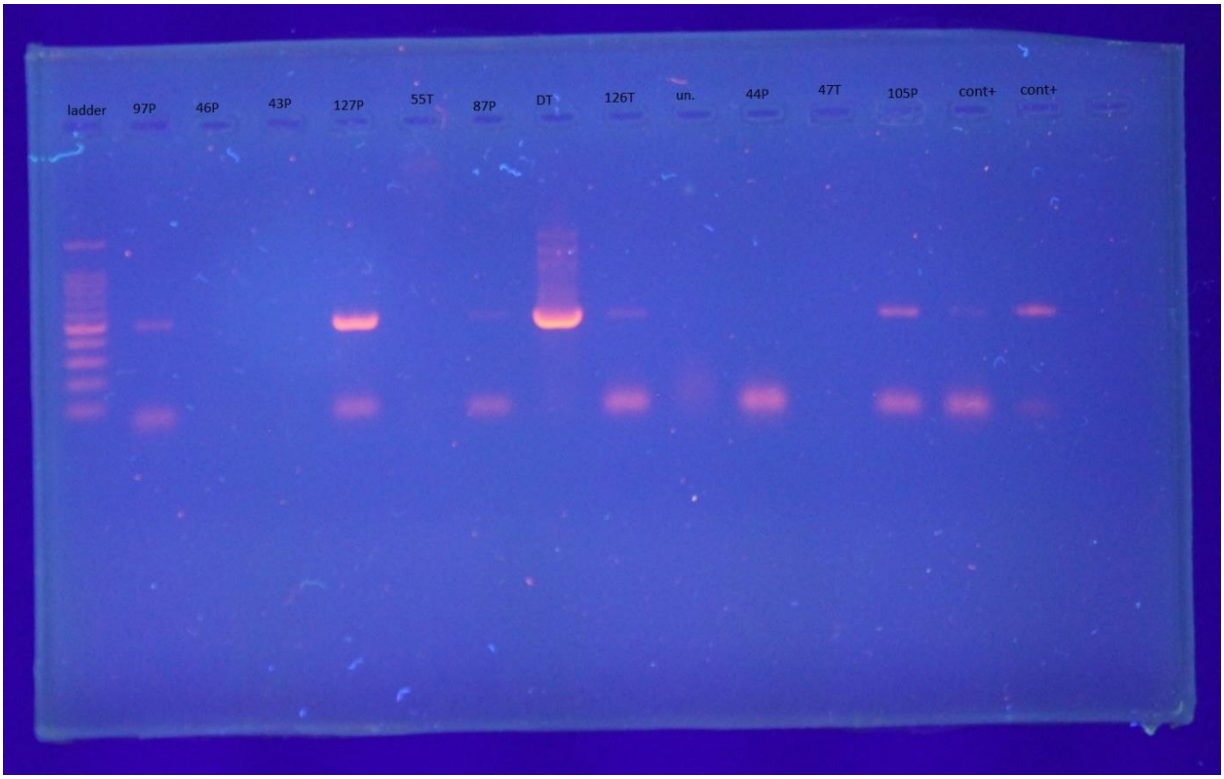


Plate 11: shows PCR run using D primer (480bp in length) samples no. 261P, 202T and 218P only are positive.

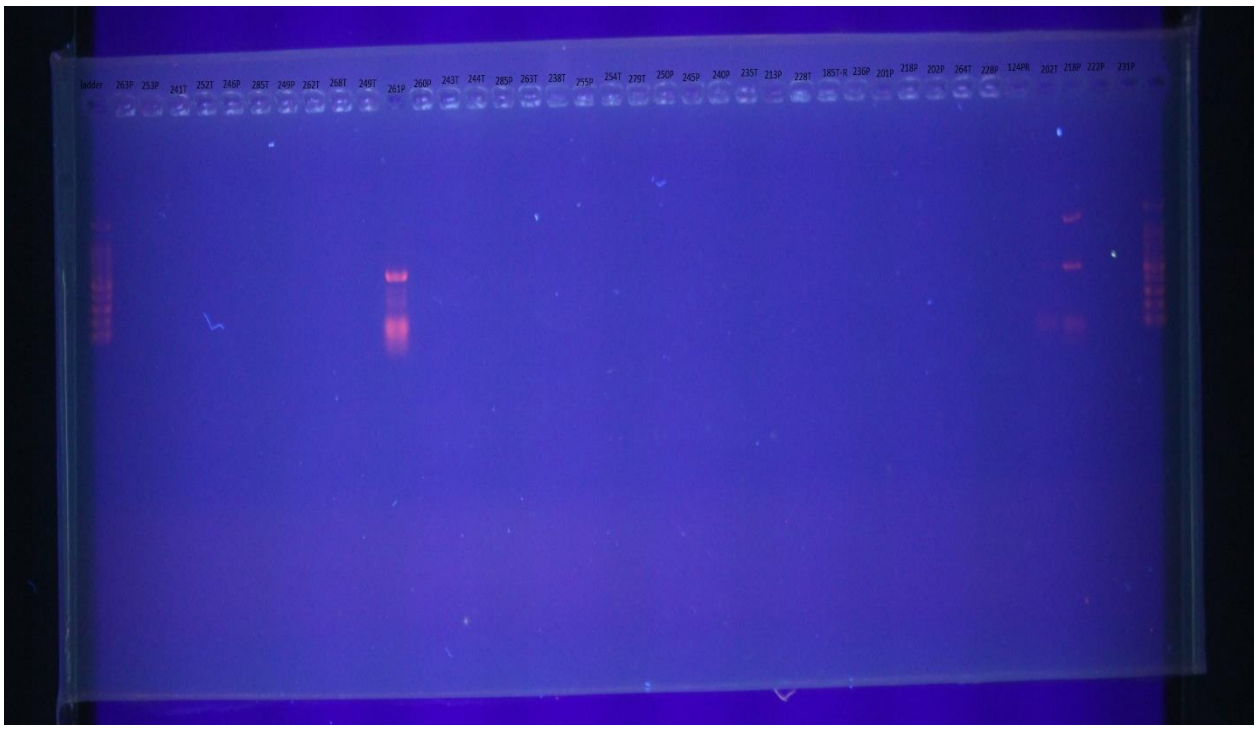


Plate 12: shows PCR run using D primer (480bp in length) samples no. 121T,111T,49T,51P,BP,EP,110T, AP, 111P, ET, MP, 100T, 52T, FT, 110P, PP, 51T,49P, 106P, 115P, 44T,56T and 128T. Sample no. 122T show faint positive band.

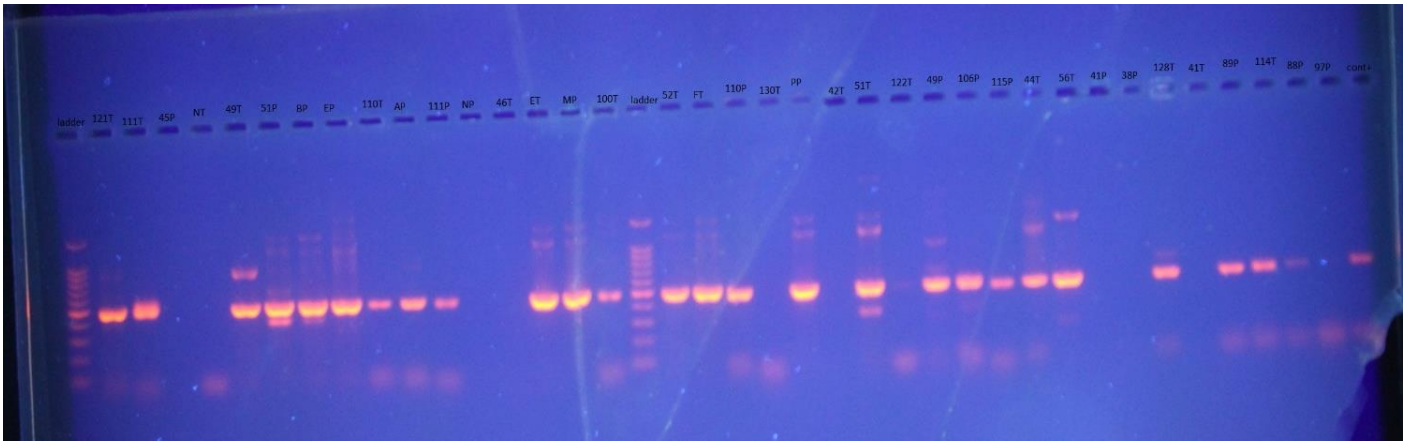
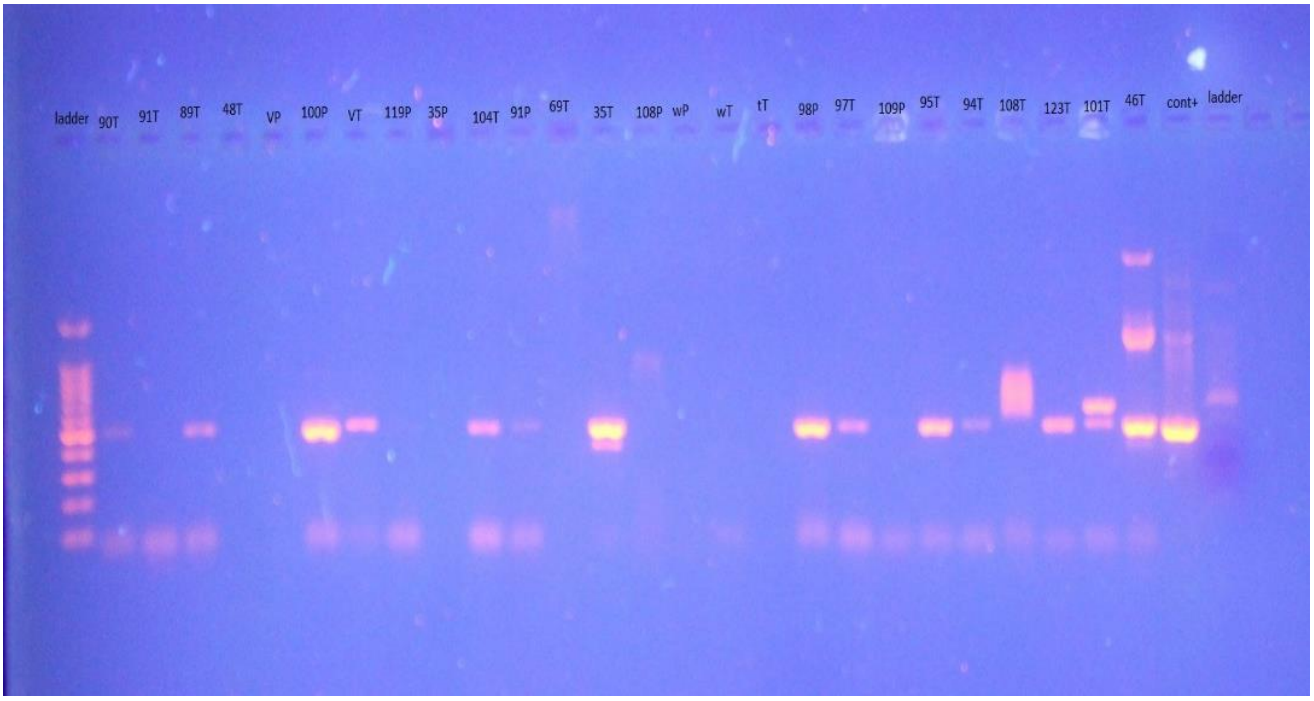


Plate 13: shows PCR run using D primer (480bp in length) samples no. 90T, 89T, 100P, VT, 104T, 91P, 97 T, 95T, 123T, 46T are positive with strong bands. 91T, 119P and 109 P are positive with faint bands. 35T and 101P show multiple bands.



4.3 Serological tests result:

The following table (Table 8) exhibit the IgG antibody titre for each sample and the IgA ratio of the mucisal immunity as it is semiquantitative measurement. Besides the ICT result of the preliminary screening as shown in the table it is qualitative test only.

The distribution of IgG Conc. RU/ml and IgA positive samples were displayed in chert 3 and 4 with the linear best fit line calculation for the IgG and IgA quantities.

Table 8: the results of over all ELIZA IgG, IgA and ICT.

All IgG				All IgA			ICT
Sample	Conc.RU/ml	Extinction	Result	Extinction	Ratio	Result	N/A
C1	200	3.16	N/A	N/A	N/A	N/A	N/A
C2	20	0.68	N/A	N/A	N/A	N/A	N/A
C3	2	0.31	N/A	0.68	1	N/A	N/A
pos	114.05	1.98	N/A	1.15	1.69	pos	N/A
neg	0	0.2	N/A	0.03	0.04	neg	N/A
A	205.05	3.23	pos	0.27	0.4	neg	+
B	68.7	1.35	pos	0.25	0.37	neg	+
C	144.1	2.39	pos	0.15	0.22	neg	+
D	218.5	3.42	pos	0.27	0.4	neg	+
E	47.7	1.06	pos	0.28	0.41	neg	+
N	35.9	0.9	pos	0.37	0.54	neg	+
P	16.8	0.61	BL	0.15	0.22	neg	+
Q	116.3	2	pos	0.28	0.41	neg	+
U	205.1	3.23	pos	0.49	0.72	neg	+
V	174.3	2.81	pos	0.76	1.12	pos	+
W	140.5	2.34	pos	0.48	0.71	neg	+
X	157.6	2.58	pos	0.17	0.25	neg	+
30	109.9	1.92	pos	0.65	0.96	BL	+
31	40.3	0.96	pos	0.11	0.16	neg	+
32	66.2	1.32	pos	0.3	0.44	neg	+
33	70.05	1.37	pos	0.25	0.37	neg	+
34	5.88	0.39	neg	0.41	0.6	neg	+
35	14.5	0.57	neg	0.12	0.18	neg	+
36	60.5	1.24	pos	0.19	0.28	neg	+
37	55.3	1.16	pos	0.43	0.63	neg	+
38	35.06	0.89	pos	0.2	0.29	neg	+
40	5.25	0.38	neg	0.1	0.15	neg	+
41	107.25	1.88	pos	0.61	0.9	BL	+
42	6.65	0.4	neg	0.17	0.25	neg	+
45	200.5	3.17	pos	0.41	0.6	neg	+
48	111.1	1.94	pos	0.74	1.09	BL	+

All IgG				All IgA			ICT
Sample	Conc.RU/ml	Extinction	Result	Extinction	Ratio	Result	N/A
49	218.5	3.42	pos	2.13	3.13	pos	+
51	25.5	0.75	pos	0.3	0.44	neg	+
54	25.7	0.76	pos	0.53	0.78	neg	+
55	47.6	1.06	pos	0.12	0.18	neg	+
58	101.6	1.8	pos	0.4	0.59	neg	+
59	70.6	1.38	pos	0.36	0.53	neg	+
60	18.7	0.65	BL	0.56	0.82	BL	+
61	116.1	2	pos	0.78	1.15	pos	+
63	84.8	1.57	pos	0.67	0.99	BL	+
64	11.6	0.51	neg	0.28	0.41	neg	+
65	22.5	0.71	pos	0.17	0.25	neg	+
67	41.6	0.98	pos	0.16	0.24	neg	+
68	31.3	0.83	pos	0.9	1.32	pos	+
69	105.1	1.85	pos	0.51	0.75	neg	+
70	83.6	1.56	pos	0.51	0.75	neg	+
71	83.1	1.55	pos	0.75	1.1	pos	+
73	81.8	1.53	pos	0.6	0.88	BL	+
75	68.6	1.35	pos	0.3	0.44	neg	+
77	71.8	1.39	pos	0.35	0.51	neg	+
78	35.9	0.9	pos	0.2	0.29	neg	+
79	78.8	1.49	pos	0.74	1.09	BL	+
81	47.07	1.05	pos	0.13	0.19	neg	+
82	71.07	1.38	pos	0.33	0.49	neg	+
83	72.6	1.4	pos	0.56	0.82	BL	+
86	69.08	1.36	pos	0.12	0.18	neg	+
88	140.9	2.35	pos	0.95	1.4	pos	+
89	101.5	1.8	pos	0.29	0.43	neg	+
90	87.7	1.61	pos	0.24	0.35	neg	+
91	119.2	2.05	pos	0.29	0.43	neg	+
92	3.5	0.35	neg	0.29	0.43	neg	+
93	8.8	0.45	neg	0.21	0.31	neg	+
96	169.7	2.75	pos	0.56	0.82	BL	+
97	109.7	1.92	pos	0.39	0.57	neg	+
98	64.7	1.29	pos	0.33	0.49	neg	+
103	42.1	0.98	pos	0.17	0.25	neg	+
104	32.9	0.85	pos	0.29	0.43	neg	+
105	91.8	1.67	pos	0.16	0.24	neg	+
106	14.01	0.56	pos	0.13	0.19	neg	+
107	75.09	1.44	pos	0.68	1	BL	+
108	23	0.72	pos	0.5	0.74	neg	+
109	27.09	0.78	pos	0.33	0.49	neg	+

All IgG				All IgA			ICT
Sample	Conc.RU/ml	Extinction	Result	Extinction	Ratio	Result	N/A
110	9.7	0.47	neg	0.38	0.56	neg	+
111	151.2	2.49	pos	0.15	0.22	neg	+
113	71.6	1.39	pos	0.51	0.75	neg	+
114	89.9	1.64	pos	1.74	2.56	pos	+
115	41.9	0.98	pos	0.27	0.4	neg	+
116	106.2	1.87	pos	0.21	0.31	neg	+
117	83.6	1.56	pos	0.29	0.43	neg	+
118	44.4	1.01	pos	0.67	0.99	BL	+
121	0	0.2	neg	0.25	0.37	neg	+
122	15.7	0.59	neg	0.49	0.72	neg	+
123	22.5	0.71	pos	0.41	0.6	neg	+
124	15.2	0.58	pos	0.46	0.68	neg	+
125	46.6	1.04	pos	0.4	0.59	neg	+
126	130.8	2.21	pos	0.47	0.69	neg	+
127	22.8	0.72	pos	0.38	0.56	neg	+
129	93.5	1.69	pos	0.26	0.38	neg	+
130	0	0.2	neg	0.12	0.18	neg	+
131	23.5	0.73	pos	1.79	2.63	pos	+
132	71.9	1.39	pos	1.05	1.54	pos	+
C1	200	1.217	N/A	N/A	N/A	N/A	N/A
C2	20	0.188	N/A	N/A	N/A	N/A	N/A
C3	2	0.035	N/A	0.973	2.8	N/A	N/A
Con posit	123.9	0.782	N/A	0.042	0.1	N/A	N/A
Cont neg	1.99	0.035	N/A	0.347	1.0	N/A	N/A
204-	N/A	N/A	N/A	0.163	0.5	neg	-
213-	N/A	N/A	N/A	0.295	0.9	BL	-
95	179.5	1.1	pos	0.735	2.1	pos	+
87	72.71	0.489	pos	0.216	0.6	neg	+
136	255.4	1.534	pos	0.723	2.1	pos	+
137	82.53	0.546	pos	0.4	1.2	pos	+
84	274.9	1.645	pos	0.524	1.5	pos	+
94	94.66	0.615	pos	0.61	1.8	pos	+
148	30.15	0.246	pos	0.16	0.5	neg	+
152	278.8	1.667	pos	0.387	1.1	pos	+
145	20.79	0.193	BL	0.476	1.4	pos	+
138	7.64	0.083	neg	0.237	0.7	neg	+
139	54.58	0.386	pos	0.409	1.2	pos	+
162	16.76	0.161	BL	0.116	0.3	neg	+
150	37.3	0.287	pos	0.164	0.5	neg	+
158	155	0.96	pos	0.908	2.6	pos	+
156	7.81	0.084	neg	0.273	0.8	BL	+

All IgG				All IgA			ICT
Sample	Conc.RU/ml	Extinction	Result	Extinction	Ratio	Result	N/A
168	158.9	0.982	pos	0.479	1.4	pos	+
169	109.8	0.702	pos	0.461	1.3	pos	+
170	137.4	0.859	pos	0.448	1.3	pos	+
159	18.73	0.177	BL	0.186	0.5	neg	+
172	299.2	1.784	pos	0.479	1.4	pos	+
167	147.9	0.919	pos	0.348	1.0	BL	+
173	105.1	0.675	pos	0.267	0.8	BL	+
171	128.6	0.809	pos	0.309	0.9	BL	+
174	14.88	0.145	neg	0.164	0.5	neg	+
179	210.4	1.277	pos	0.386	1.1	pos	+
178	245.3	1.476	pos	0.73	2.1	pos	+
176	129.5	0.814	pos	0.824	2.4	pos	+
180	138.8	0.867	pos	0.861	2.5	pos	+
181	198.1	1.206	pos	0.88	2.5	pos	+
183	34.08	0.269	pos	0.177	0.5	neg	+
190	132	0.829	pos	0.298	0.9	BL	+
189	82.14	0.543	pos	0.415	1.2	pos	+
187	188.7	1.153	pos	0.896	2.6	pos	+
185	146.9	0.914	pos	0.427	1.2	pos	+
188	240.6	1.449	pos	0.646	1.9	pos	+
182	32.63	0.26	pos	0.329	0.9	BL	+
191	178.2	1.092	pos	0.338	1.0	BL	+
192	145.1	0.903	pos	0.371	1.1	pos	+
193	122.9	0.777	pos	0.361	1.0	BL	+
194	83.74	0.552	pos	0.067	0.2	neg	+
196	88.71	0.581	pos	0.078	0.2	neg	+
197	98.79	0.638	pos	0.079	0.2	neg	+
175	93.35	0.607	pos	0.36	1.0	BL	+
155	217.1	1.315	pos	0.243	0.7	neg	+
198	112.1	0.715	pos	0.35	1.0	BL	+
199	76.41	0.511	pos	0.511	1.5	pos	+
200	12.71	0.126	neg	0.235	0.7	neg	+
184	22.11	0.2	pos	0.27	0.8	BL	+
177	77.47	0.517	pos	0.172	0.5	neg	+
142	184.3	1.128	pos	1.022	2.9	pos	+
134	26.74	0.227	pos	0.118	0.3	neg	+
133	103.4	0.665	pos	0.177	0.5	neg	+
144	98.34	0.636	pos	0.57	1.6	pos	+
149	69.94	0.474	pos	0.219	0.6	neg	+
225	68.38	0.467	pos	0.439	1.3	pos	+
224	285.4	1.705	pos	0.775	2.2	pos	+

All IgG				All IgA			ICT
Sample	Conc.RU/ml	Extinction	Result	Extinction	Ratio	Result	N/A
124 R	218.6	1.323	pos	1.563	4.5	pos	+
185 R	120.5	0.763	pos	0.31	0.9	BL	+
157	198.6	1.209	pos	0.344	1.0	BL	+
147	69.78	0.473	pos	0.245	0.7	neg	+
221	129.6	0.815	pos	0.426	1.2	pos	+
220	125.1	0.789	pos	0.188	0.5	neg	+
218	72.85	0.49	pos	0.312	0.9	BL	+
219	121.4	0.768	pos	0.434	1.3	pos	+
214	148.8	0.925	pos	1.439	4.1	pos	+
216	134.3	0.841	pos	0.648	1.9	pos	+
215	93.44	0.608	pos	0.691	2.0	pos	+
212	15.49	0.15	neg	0.159	0.5	neg	+
210	114.6	0.729	pos	0.274	0.8	BL	+
211	137.9	0.862	pos	0.245	0.7	neg	+
207	278.3	1.667	pos	0.86	2.5	pos	+
205	73.53	0.494	pos	0.18	0.5	neg	+
117	19.64	0.185	BL	0.112	0.3	neg	+
143 -	29.08	0.24	pos	0.266	0.8	BL	-
146 -	18.84	0.178	BL	0.203	0.6	neg	-
m-	120.5	0.763	pos	0.332	1.0	BL	-
203	44.23	0.327	pos	0.242	0.7	neg	+
195 -	15.57	0.15	neg	0.441	1.3	pos	-
163-	18.02	0.171	BL	0.352	1.0	BL	-
202 -	33.72	0.267	pos	0.162	0.5	neg	-
161 -	143.9	0.896	pos	0.159	0.5	neg	-
186 -	32.08	0.257	pos	0.197	0.6	neg	-
201 -	78.05	0.52	pos	0.277	0.8	BL	-
164 -	25.05	0.217	pos	1.24	3.6	pos	-
165 -	30.08	0.246	pos	0.197	0.6	neg	-
135 -	21.32	0.196	BL	0.695	2.0	pos	-
166 -	20.1	0.189	BL	0.247	0.7	neg	-
154 -	8.43	0.089	neg	0.246	0.7	neg	-
153 -	251.4	1.511	pos	0.956	2.8	pos	-
226 -	42.24	0.315	pos	1.091	3.1	pos	-
222 -	17.76	0.169	BL	0.384	1.1	pos	-

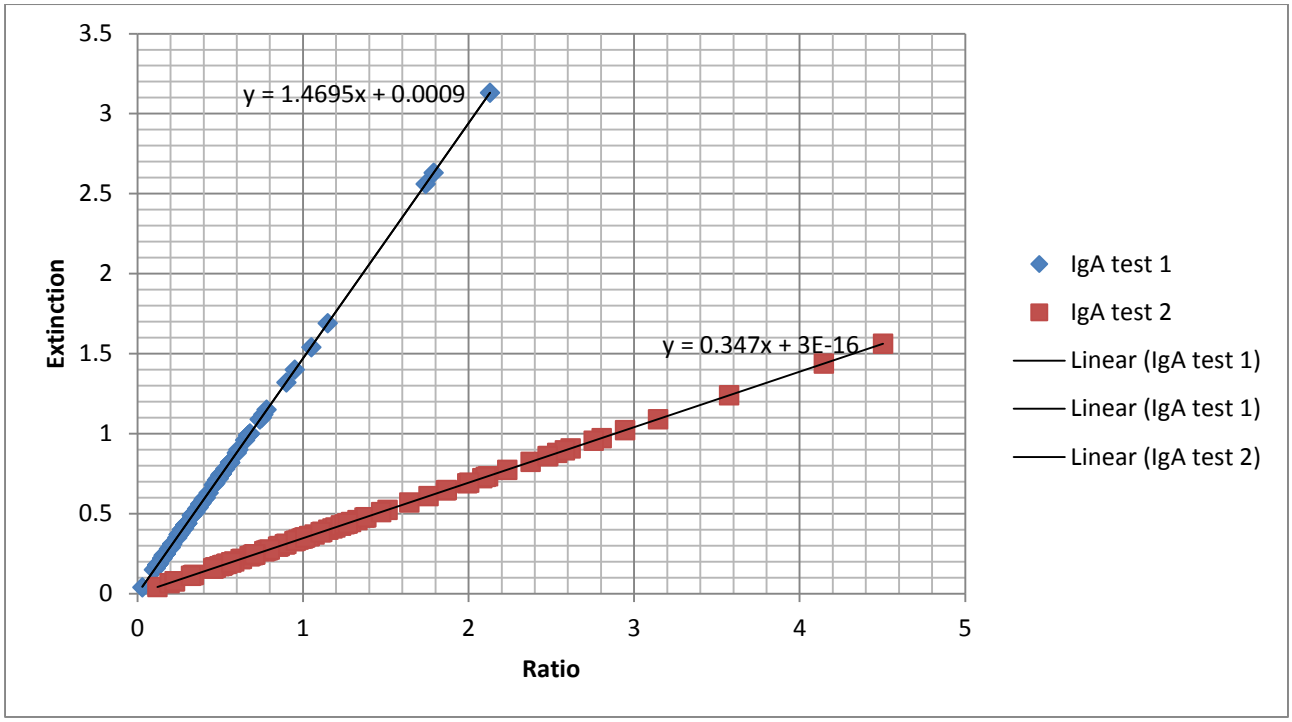


Chart3: The linear regression equation for the IgA ratio with the obtained extinction value.

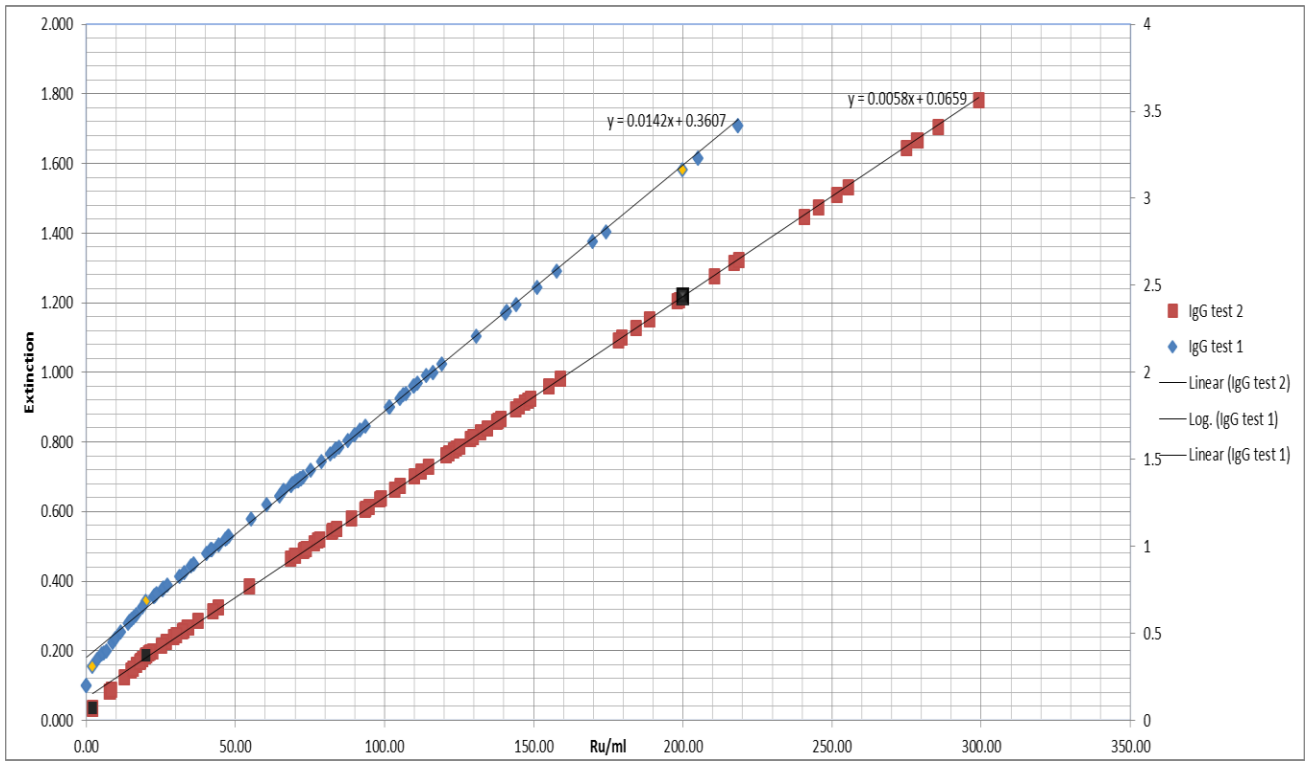


Chart4: the standard curve of the IgG titer calculation with the obtained extinction value.

4.4 Questionnare data:

Table 9 include the questionnaire assumed from respodents before collecting samples; this tool was complemented with the overall results of parameters in this study.

Key descriptions for the report table below:

- Gender: 0=male / 1=female.

- H. pylori: result by ELISA 0= Negative / 1= doubtful / 2= Positive

-H. pylori by ICT: 0= Negative/ 1= Positive

- History of H. pylori: 0= had been infected at least once before / 1=has never been infected before.

- Treatment if previously infected: 0= treatment completed / 1=interrupted.

- Age: 0= (1-14 years children) / 1= (14-40 years youth) / 2=(40-60 years middle ages) / 3=(over 60 years old).

- Chronic Health issues and symptoms: 0= absent / 1= present.

Symptoms:

- **Headache and dizziness:**
- **General body fatigue: Exhaustion, tiredness, insomnia and lack of appetite.**
- **Fever: generalized fever feeling or localized hot foot and balms.**
- **Lower Digestive tract illness: nervous colon, indigestion, gases, diarrhea and constipation.**
- **Respiratory tract: sinusitis, sore throat, sneezing, nausea, nostril congestion and cough.**
- **Upper digestive tract illness: stomach reflux, feel acid burn in chest, pharynx and mouth**

Bacterial isolates:

- Identified Bacterial isolate: 0=commensal / 1=pathogenic / 2=*S.pyogenes*.

Table9: The questionnare report:

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
A T/P	1	205.05	-	1	1	0	0	0	1	1	0	1	1`	1	0	0	2 2
B T/P	1	68.7		1	1	0	0	0	0	0	0	0	0	1	0	0	2 2
C P	1	144.1	-	1	1	0	0	0	1	1	0	1	1	1	0	0	2
D T/P	1	218.5	-	1	1	0	0	0	1	1	0	1	1	1	0	0	2 2
E T/P	1	47.7		0	2	0	0	0	1	1	0	0	0	0	0	0	2 2
P T/P	1	16.8	-	1	1	0	0	0	1	1	0	0	0	1	1	1	 2
Q T/P	1	116.3	-	1	1	0	0	0	1	1	0	0	0	1	1	1	
U T/P	1	205.1	-	0	1	0	0	0	1	1	0	0	0	1	1	1	2 2
V T/P	1	174.3	Pos.	0	1	0	0	0	1	1	0	0	0	1	1	1	2 2
W T/P	1	140.5	-	1	3	0	1	1	0	0	0	0	0	1	1	1	2 2
X T/P	1	157.6	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2 2
30 T/P	1	109.9	BL	0	1	0	0	0	1	1	0	0	0	1	1	1	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
																	2
31 T/P	1	40.3	-	1	1	0	0	0	1	1	0	0	0	1	1	1	2
																	2
32 T/P	1	66.2	-	1	1	0	0	0	1	1	0	0	0	1	1	1	2
																	2
33 T/P	1	70.05	-	0	1	0	0	0	1	1	0	0	0	1	1	1	2
																	2
34 T/P	1	-	-	1	3	0	1	1	1	1		0	0	1	1	1	2
																	2
35 T/P	1	-	-	1	1	0	0	0	0	1		0	0	1	1	1	2
																	2
36 T/P	1	60.5	-	0	1	0	0	0	0	1	0	0	0	1	1	1	2
																	2
37 T/P	1	55.3	-	0	1	1	0	0	0	1		0	0	1	1	1	1
																	2
38P	1	35.06	-	1	1	0	0	0	1	1		0	0	1	1	1	2
40 T/P	1	-	-	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
41 T/P	1	107.25	BL	1	0	0	0	0	1	1	0	0	0	1	1	1	2
																	2
42 T/P	1	-	-	1	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
45 T/P	1	200.5	-	0	3	0	1	0	1	1	0	0	0	1	1	1	2
																	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
48 T/P	1	111.1	BL	1	1	0	0	0	0	1	0	0	0	1	1	1	0 1
49 T/P	1	218.5	Pos.	0	1	0	0	0	0	1	0	0	0	1	1	1	1 1
51 T/P	1	25.5	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2 1
54 T/P	1	25.7	-	0	3	0	0	0	0	0	0	0	0	1	1	1	2 2
55 T/P	1	47.6	-	1	1	0	0	0	0	0	0	0	0	1	1	1	2 2
58 T/P	1	101.6	-	0	2	0	0	0	0	0	0	0	0	1	1	1	2 2
59T/P	1	70.6	-	0	2	0	0	0	0	0	0	0	0	1	1	1	2 2
60T/P	1	BL	BL	0	1	1	0	0	0	0	0	0	0	1	1	1	2
61T/P	1	116.1	Pos.	0	1	0	0	0	0	0	0	0	0	1	1	1	2 2
63T/P	1	84.8	BL	0	0	0	0	0	0	0	0	0	0	1	1	1	1 2
64T/P	1	-	-	0	2	0	0	0	0	0	0	0	0	1	1	1	2 2
65T/P	1	22.5	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
																	2
67T/P	1	41.6	-	0	2	0	0	0	0	0	0	0	0	1	1	1	2
																	2
68T/P	1	31.3	Pos.	0	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
69T/P	1	105.1	-	0	1	0	0	0	0	0	0	0	0		1	1	2
														1			2
70T/P	1	83.6	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
71T/P	1	83.1	Pos.	0	1	1	0	0	0	0	0	0	0	1	1	1	2
																	2
73T/P	1	81.8	BL	0	1	0	0	0	0	0	0	0	0	1	0	0	2
																	2
75	1	68.6	-	0	2	0	0	0	0	0	0	0	0	1	1	1	2
																	2
77	1	71.6	-	1	2	0	1	0	0	0	0	0	0	1	1	1	1
																	1
78	1	35.9	-	1	2	0	0	0	0	0	1	0	1	1	1	1	1
																	1
79	1	78.8	BL	0	1	0	0	0	0	0	0	0	0	1	1	1	1
																	1
81	1	47.07	#NAME?	1	1	0	0	0	0	0	0	0	0	1	1	1	1
																	0

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
84	1	274.9	Pos.	0	2	1	0	0	0	0	0	0	0	1	1	1	0 0
87	1	72.71	-	0	2	0	0	0	0	0	0	0	0	1	1	1	1 2
88	1	140.9	Pos.	1	2	0	0	0	0	0	0	0	0	1	1	1	2 2
89	1	101.5	-	1	1	0	0	0	0	0	0	0	0	1	1	1	 2
90	1	87.7	-	1	1	0	0	0	0	0	0	0	0	1	1	1	 2
92T/P	1	-	-	1	3	0	0	0	0	0	0	0	0	1	1	1	2 2
93T/P	1	-	-	1	2	0	0	1	0	0	0	0	0	1	1	1	2 2 2
94T/P	1	94.66	Pos.	1	3	0	0	0	0	0	0	0	0	1	1	1	2 2
95T/P	1	179.5	Pos.	0	1	0	0	0	0	0	0	0	0	1	1	1	2 2
96T/P	1	244.9	BL	1	2	0	0	0	0	0	0	0	0	1	1	1	2 2
97T/P	1	192.78	Pos.	1	1	0	0	0	0	0	0	0	0	1	1	1	2 2
98T/P	1	205.2	BL	0	1	0	0	0	0	0	0	0	0	1	1	1	1 2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
103	1	148.19	-	1	1	0	1	0	0	0	0	1	0	1	1	1	
104	1	122		0	1	0	0	0	1	0	0	0	0	0	1	1	2 1
105	1	91.8	-	0	1	0	0	0	1	1	0	0	0	0	1	1	2 2
106	1	-	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2 1
107	1	75.09	BL	0	1	0	0	0	0	1	0	0	0	1	1	1	2 2
108	1	23	-	1	1	0	0	1	0	0	0	0	0	1	1	1	2 2
109	1	124.6		1	1	0	0	0	0	0	0	0	0	1	1	1	2 2
110	1	-	-	0	1	0	0	0	0	0	1	1	0	1	1	1	2 2
111	1	151.2	-	0	1	0	0	0	0	0			0	1	1	1	2 2
113	1	71.6		0	1	0	0	0	0	1	1	0	0	1	1	1	2 2
114	1	89.9	Pos.	0	1	0	0	0	0	0	0	0	0	1	1	1	2 2
116	1	106.2	-	0	3	0	0	0	0	0	0	0	0	0	0	0	2 2
117	1	83.6	-	1	3	0	0	0	0	0	0	0	0	1	1	1	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
																	2
118	1	44.4	BL	0	2	0	0	0	0	0	0	1	0	1	0	0	2
																	1
121	1	-	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2
																	0
122	1	-	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
124	1	-	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2
																	0
125	1	46.6	-	1	1	0	0	0	0	1	0	0	0	1	1	1	2
																	2
126	1	130.8	-	0	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
127	1	22.8	-	0	2	0	0	0	0	1	1	0	1	0	1	1	2
																	2
129	1	93.5	-	1	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
130	1	0	-	1	1	0	0	0	0	0	0	0	1	1	1	1	2
																	2
131	1	23.5	Pos.	0	0	0	0	0	0	1	1	0	0	1	1	1	2
132	1	71.9	Pos.	0	1	0	0	0	1			0	0		1	1	2
136	1	255.4	Pos.	1	1	0	0	0	0	1		0	0	1	1	1	2
																	2
137	1	82.53		1	0	1	0	0	0	1		0	0	0	1	1	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
			Pos.														2
138	1	-			2	0	0	0	0	0	0	0	0	1	1	1	
																	2
139	0	54.58	Pos.			0	0	0	0	0	0	0	0	1	1	1	2
																	2
																	2
144	1	98.34	Pos.	1	3	0	0	0	0	0	0	0	1	0	1	1	2
																	2
145	1	BL	Pos.	1	1	0	0	1		1	0	0	0	1	1	1	
																	0
148	1	30.15	-	0	1	0	0	0	0	0	0	0	0	1	1	1	
																	2
150	0	37.3	-	0	1	0	0	0	0	0	0	0	1	1		1	2
152	1	278.8	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
153	0	251.4	Pos.		2	0	1	0	0	0	0	0	0	1	1	1	2
155	1	217.1		0	1	0	0	0	0	0	0	0	1	0	1	1	2
																	2
156	0	-	-	1	1	0	0	0	0	0	0	0	0	1	1	1	0
																	0
158	1	155	Pos.	1	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
161	0	143.9	-	0	2	0	1	0	0	0	0	0	0	1	1	1	2

Sample	*H. pylori* ICT	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
																	2
162	1	16.76	-	0	1	0	0	0	0	0	0	0	0		1	1	2
																	2
164	0	25.05	Pos.	0		0	0	0	0	0	0	0	0	1	1	1	2
																	2
166	0	20.1	-	1	1	0	0	0	0	0	0	0	1	1	1	1	2
																	2
167	1	147.9	BL	0	1	0	0	0	0	0	0	1	1	1	1	1	1
																	1
168	1	158.9	Pos.	0	2	0	0	0		1			0	1	1	1	1
																	1
169	1	109.8	Pos.	1	2	0	0	0	0	0	0	1	0	1	1	1	1
																	1
170	1	137.4	Pos.	1	1	0	0	0	0	1	0	0	0	0	1	1	1
																	1
171	1	128.6	BL	0	2	0	0	0	0	0	0	0	1	1	1	1	1
																	1
172	1	*299.2	Pos.	0		0	0	0	0	0	0	0	0	0	1	1	1
																	2
173	1	105.1	BL	0		0	0	0	0	0	0	0	0	0	1	1	
																	2
174	1	-	-	0		0	0	0		1	1	1	0	1	1	1	0
																	0
176	1	129.5	Pos.	0		0	0	0	0	0	1	0	0	1	1	1	
																	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
178	1	245.3	Pos.	0	1	0	0	0	1	0	0	0	0	1	1	1	1
179	1	210.4	Pos.	0	1	0	0	0	0	0	0	0	0	1	1	1	
185	1	146.9	Pos.	0	1	0	0	0	1	1	0		0		1	1	2
188	1	240.6	Pos.	0	2	0	0	0	0	1	0	0	0	1	1	1	2
189	1	82.14	Pos.	0	2	0	0	0	0	0	0	0	0	1	1	1	2
190	1	132		0	2	0	0	0	0	0	0	0	0	1	0	0	1
192	1	145.1	Pos.	1	1	0	0	0	1				0	1	1	1	2
194	1	83.74		1	0	0	0	0	0	0	0	0	0	1	0	1	2
195	0	-	Pos.	0	1	0	0	0	0	0	0	0	0	1	1	1	2
197	1	98.79		0	0	0	0	0	0	0	0	0	1	0	1	1	2
199	1	76.41	Pos.	1	1	0	0	0	0	0	0	0	1	0	1	1	2
205	1	73.53		1	1	0	0	0	0	1	0	0	0		0	0	2
207	1	278.3		1	1	0	0	0	0	0	0	0	0	1	0	0	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
			Pos.														2
211	1	137.9		1	1	0	0	0	0	0	0	0	0	1	0	0	2
																	2
212	1	15.49	Pos.	0	0	0	0	0	0	0	0	0		1	1	1	2
																	2
214	1	148.8	Pos.	1	0	0	0	0	0	0	0	0	0	0	1	1	2
																	2
215	1	93.44	Pos.	1	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
216	1	134.3	Pos.	1	2	0	0	0	0	0	0	0	0	1	1	1	0
																	0
218		72.85		0	1	0	0	0	0	0	0	1	0	0	1	1	0
																	0
219	1	121.4	Pos.	1	0	0	0	0	0	0	0	0	0	1	1	1	2
																	2
221	1	129.6	Pos.	0	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
222	0		Pos.	1	2	0	0	0	0	0	0	0	0	1	1	1	2
																	2
224	1	285.4	Pos.	0	1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
225	1	68.38	Pos.		1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
226	0	42.24		0	2	0	0	0	0	0	0	0	0	1	1	1	

Sample	*H. pylori* ICT	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
			Pos.														2
228	1	171.7	Pos.	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
229	1	227.8	Pos.	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
230	1	210.6	Pos.	0	1	0	0	0	0	0	0	0	0	0	0	0	2
																	2
231	1	38.94		0	1	0	0	0	0	0	0	0	0	0	0	0	2
																	2
232	1	152.7463		0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	1
233	1	158.78467	BL	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
234	0	77.220226		0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
235	0	19.530568		0	1	0	0	0	0	0	0	0	0	0	1	1	1
																	2
236	1	19.530568	BL	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
237	1	188.97	BL	0	1	0	0	0	0	0	0	0	0	0	0	0	2
																	2
238	1	205.60526		0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
239	0			1	1	0	0	0	0	0	0	0	0	0	1	1	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
		39.782332															2
240	1	158.50598	BL	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
241	0	95.7	BL	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
242	1	218.14	Pos.	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
243	1	83.16	Pos.	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
244	0	19.251874	Pos.	1	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
245	1	82.4		1	1	0	0	0	0	0	0	0	1	0	0	0	2
																	2
246	0	73.225612		1	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
247	0	73.225612		1	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
248	1	197.2	Pos.	1	1	0	0	0	0	0	0	0	1	0	1	1	
																	2
249	1	211.45784		1	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
250	0	56.03	Pos.	1	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
251	0	24.2	-	1	1	0	0	0	0	0	0	0	1	0	1	1	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
																	2
252	0	143.4565	-	1	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
253	0	67.5	-	1	1	0	0	0	0	0	0	0	1	0	1	1	0
																	2
254	0	98.772562	-	0	1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
255	1	205.69816	BL		1	0	0	0	0	0	0	0	0	1	1	1	1
																	1
256	1	167.42	Pos.		1	0	0	0	0	0	0	0	0	1	1	1	1
																	2
257	1	119.12	Pos.		1	0	0	1	0	0	0	0	0	1	1	1	2
																	2
258	0	119.21012	BL		1	0	0	0	0	0	0	0	0	0		1	2
																	2
259	1	116.42318	-		1	0	0	0	0	0	0	0	0	1	1	1	2
																	1
260	1	207.64	Pos.		1	0	0	0	0	0	0	0	0	1	1	1	2
																	2
261	0	89.48	Pos.		1	0	0	0	0	0	0	0	0	0		1	2
																	2
262	1	132.68	-		1	0	0	0	0	0	0	0	0	1	0	0	2
																	2
263	0	213.63	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2
																	2

Sample	*H. pylori* ICT	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
264	1	162.68	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2 2
265		79.63	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2 2
266	1	82.9	-	1	1	0	0	0	0	1	0	0	1	0	0	0	2 2
267	1	202.4	BL	1	1	0	0	0	0	1	0	0	1	0	1	1	1 1
268	1	59.198014	-		1	0	0	0	0	0	0	0	0	0	1	1	2 2
269	1	132.86	Pos.	0	2	0	0	0	0	0	0	0	0	1	1	1	2 1
270	1	113.6	-	1	1	0	0	0	0	0	0	0	1	0	1	1	1
271	1	86,69	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2 2
272	0	-	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2 2
273	1	63	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2 2
274	0	-	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2 2
275	1	193.15	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2 2
276	0				1	0	0	0	0	0	0	0	0	0	1	1	2

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
		102.76718															2
277	1	139.18	Pos.		1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
278	1	140.29797	BL		1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
279	0	117.16			1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
280	1	154.88295			1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
281	1	156.92671	BL		1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
282	0	56.039482			1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
283	1	161.9432			1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
284	0	113.54334			1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
285	1	114.65812			1	0	0	0	0	0	0	0	0	0	1	1	2
																	2
286	0	5.317174		0	0	0	0	0	0	0	0	0	0	0	1	1	1
																	1
295	1	86.04		0	1	0	0	0	0	0	0	0	1	0	1	1	2
																	2
296	1	166.49		0	1	0	0	0	0	0	0	1	0	1	1	1	

Sample	*H. pylori* *ICT*	H. pylori ELISA IgG Titre	IgA	Gendr	Age	Chronic Health issues			Current Symptoms						History of H. pylori	Treatment	*Isolated bacteria*
						Smoking	Hyper-tenion	Diabetes	Headache and dizziness	body fatigue	Fever	LDT illness	URT signs	UDT illness			
			Pos.														2
297	0	90.690436		0	1	0	0	0	0	0	0	0			1	1	2 1
298	1	9.12		0	1	0	0	0	0	0	0	1	1	1	0	0	1 2
299	1	133.79		0	1	0	0	0	0	0	0	0	0	1	0	0	1 1
300	1	114.75102			1	0	0	0	0	0	0	0			1	1	 2
301	1	44.52	BL	1	1	0	0	0	0	0	0	0	1	1	0	0	2
302	1	120.88	Pos.	1	1	0	0	0	0	0	0	0	1	0	0	0	1 2
303	0	98.77	BL	0	1	0	0	0	0	0	0	0	1	0	1	1	1 2
304	0	1.69		1	0	0	0	0	0	0	0	1	0	0	1	1	0 1
305	1	119.67	Pos.	1	0	0	0	0	0	0	0	0	1	0	1	1	2 2
306	1	107.5		0	0	0	0	0	0	0	0	0	1	1	0	0	1 2
307	0	119.95		1	0	0	0	0	0	0	0	0	1	1	1	1	1 1

4.5 Statistical Analysis

- Sore throat cases were found to be significantly (at 95% confident interval, 2 tailed) correlated with ICT results of this study, and also significantly correlated with symptoms of general body fatigue and respiratory signs.

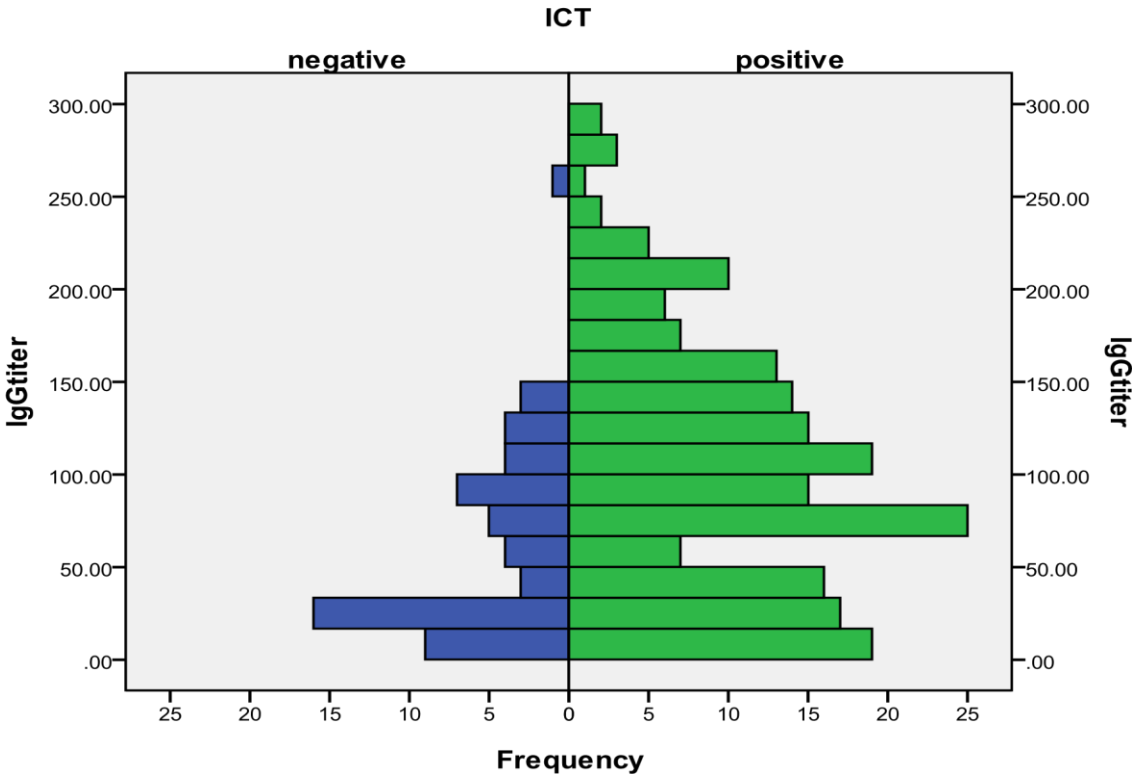


Chart 5: The difference in accurecy between two diagnostic tools for *Helicobacter pylori* infection (IgG ELISA and ICT)

- IgG Antibody titer was found to be significantly (at 99% confident interval, 2 tailed) correlated with ICT (chart 5) and IgA ratio (chart 7).
- *H. pylori* positive (by ELISA or ICT) cases were found to be significantly (at 99% confident interval, 2 tailed) correlated with upper digestive tract signs (chart 6).
- In this study Diabetes was found to be significantly (at 95% confident interval, 2 tailed) more prevalent in females.
- In the present study hypertension and upper GIT signs were found to be significantly (at 99% confident interval, 2 tailed) more related with Age and more prevalent in

elderly. Also in elderly sore tonsils were related significantly (at 99% confident interval, 2 tailed) with Age.

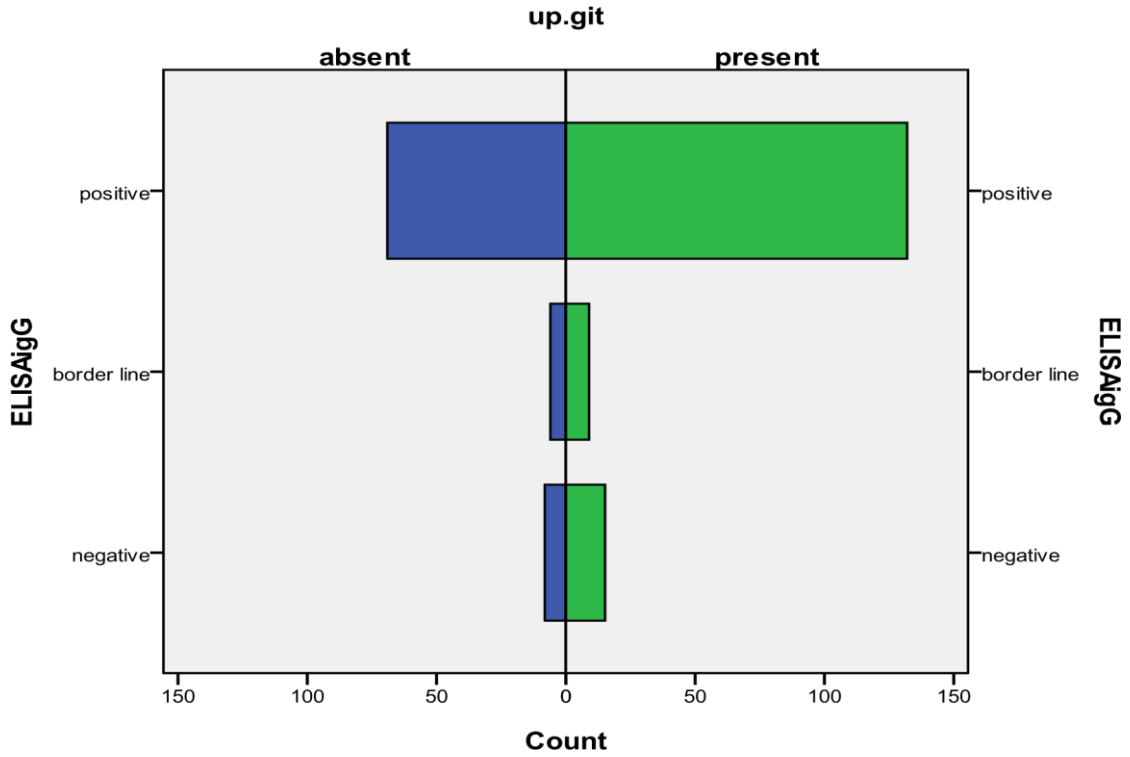


Chart 6: The increase of IgG conc. correlating with upper digestive tract signs.

- Negative correlation was found between IgG titer and fever (at 95% confident interval, 2 tailed). But fever has positive correlation with presence of respiratory symptoms and lower digestive tract illness.
- Most of the sore throat cases were found to be subclinical with neither respiratory symptoms nor air way signs.
- Contrary to upper digestive tract illnesses signs, the lower digestive tract signs have negative correlation with sore throat and *H. pylori* infection (at 95% confident interval, 2 tailed).
- Presence of previous *H. pylori* infection history was found to be significantly correlated with isolation of pathogenic bacteria particularly from tonsils. Tonsils can be a reservoir organ for throat pathogenic bacteria (i.e *Streptococcus pyogenes*).

- A significant correlation was found between ICT method of diagnosis and IgG titer detected by quantitative ELISA.
- Among collected samples 11.3% individuals were complaining from respiratory signs. And 11% had been infected at least once before
- IgA ELISA result had detect 53.2% of individual samples were negative, where as 16.8% were border line and 30% were positive (Table 12).
- 28.1% were detected negative by ICT and 71.9% were positive (Table 11).

Table10: The sensitivity and specificity calculating methods.

	Disease present	Disease absent
Test positive	a (TP)	b (FP)
Test negative	c (FN)	d (TN)
	Sensitivity: a/ (a+c)	Specificity: d/ (b+d)

TP: True positive, FP: False positive, FN: False negative, TN: True negative

Table11: The sensitivity, specificity and accuracy of ICT in this study.

Method		IgG ELISA*		Total results
H. pylori rapid test cassette (serum/ plasma) ICT	result	+	-	
	+	149 a	12 b	161
	-	48 c	15 d	63
Total results		197	27	224

- * 11 cases were in border line has been excluded.

Accuracy of ICT = $\frac{N\ true\ positive\ (a)+N\ True\ negative(d)}{N\ true\ positive\ (a)+N\ true\ negative(d)+\ N\ false\ positive(b)+N\ false\ negative(c)}$

Accuracy = 74.6% (95% CI*) * Confidence Interval

Sensitivity = $\frac{N\ true\ positive}{N\ true\ positive+N\ false\ negative}$

Sensitivity = 75.6% (95% CI*)

Specificity = $\frac{N\ True\ negative}{N\ true\ negative + N\ false\ positive}$

Specificity = 88.2% (95% CI*)

- By IgG ELISA 84% were Positive, 11.4% were Negative and 4.6% were border line.
- IgA ELISA result had detect 53.2% of individual samples were negative, where as 16.8% were border line and 30% were positive (Table 12).

Table12: The sensitivity, specificity and accuracy of IgA ELISA in this study

Method		IgG ELISA*		Total results
IgA ELISA	result	+	-	
	+	68 a	3 b	71
	-	106 c	21 d	127
Total results		174	24	198

Accuracy of IgA ELISA =

$$\frac{N\ true\ positive\ (a) + N\ True\ negative(d)}{N\ true\ positive\ (a) + N\ true\ negative(d) +\ N\ false\ positive(b) + N\ false\ negative(c)}$$

Accuracy = 45% (95% CI*) * Confidence Interval

Sensitivity = $\frac{N\ true\ positive}{N\ true\ positive + N\ false\ negative}$

Sensitivity = 39.1% (95% CI*)

Specificity = $\frac{N\ True\ negative}{N\ true\ negative + N\ false\ positive}$

Specificity = 87.5% (95% CI*)

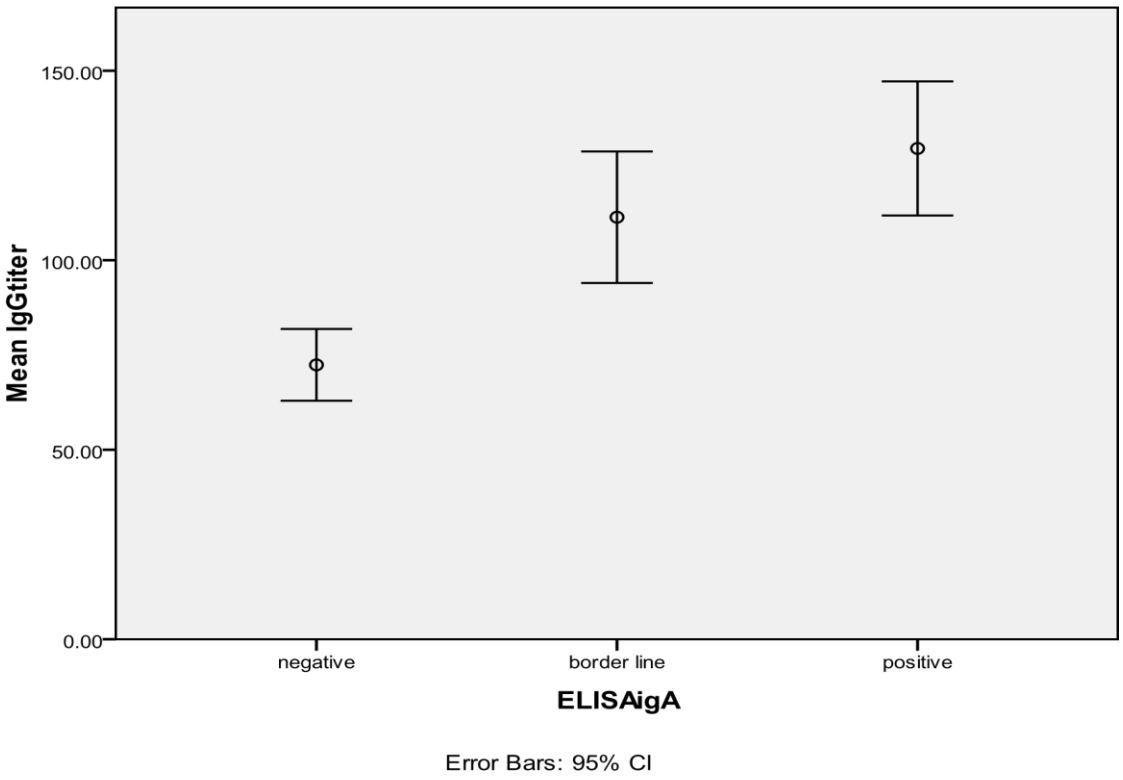


Chart 7: The mean of Anti-*Helicobacter pylori* IgG for each categori of Anti-*Helicobacter pylori* IgA (Positive, border line and Negative cases), this graph showed that even the Positive cases of IgA have low IgG titer; the maximum IgG titer among samples were about 299 Ru/ml.

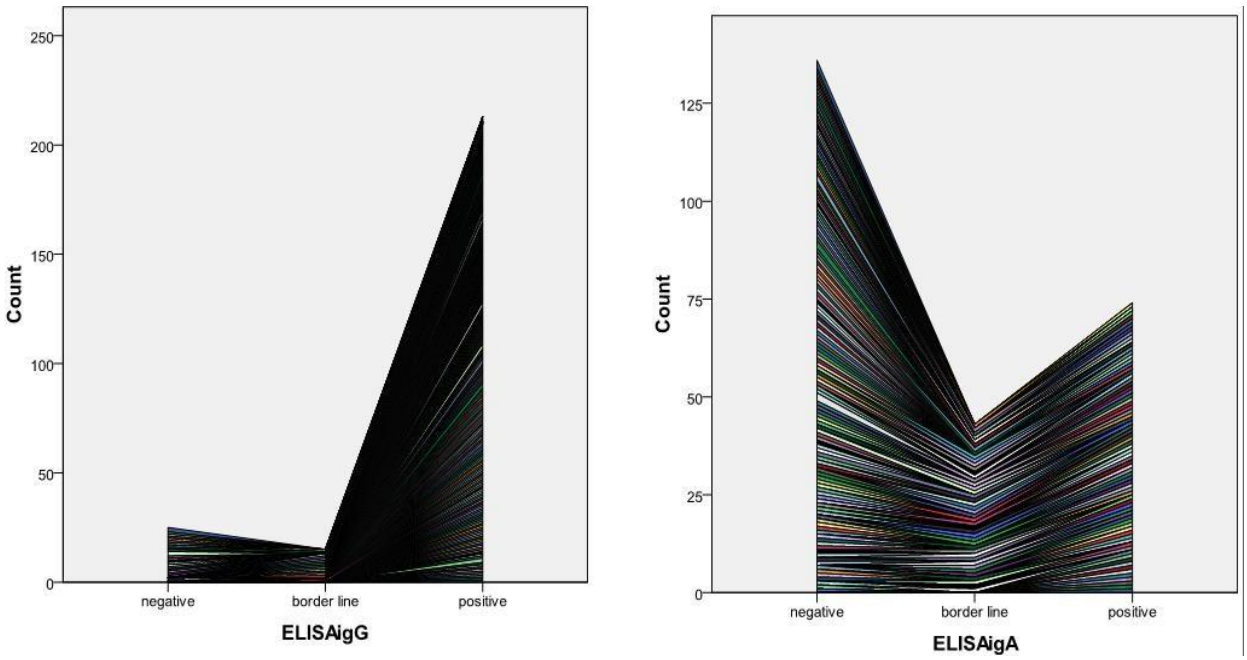


Chart 8: The big difference in patient frequencies between Anti-*Helicobacter pylori* IgG and IgA categories

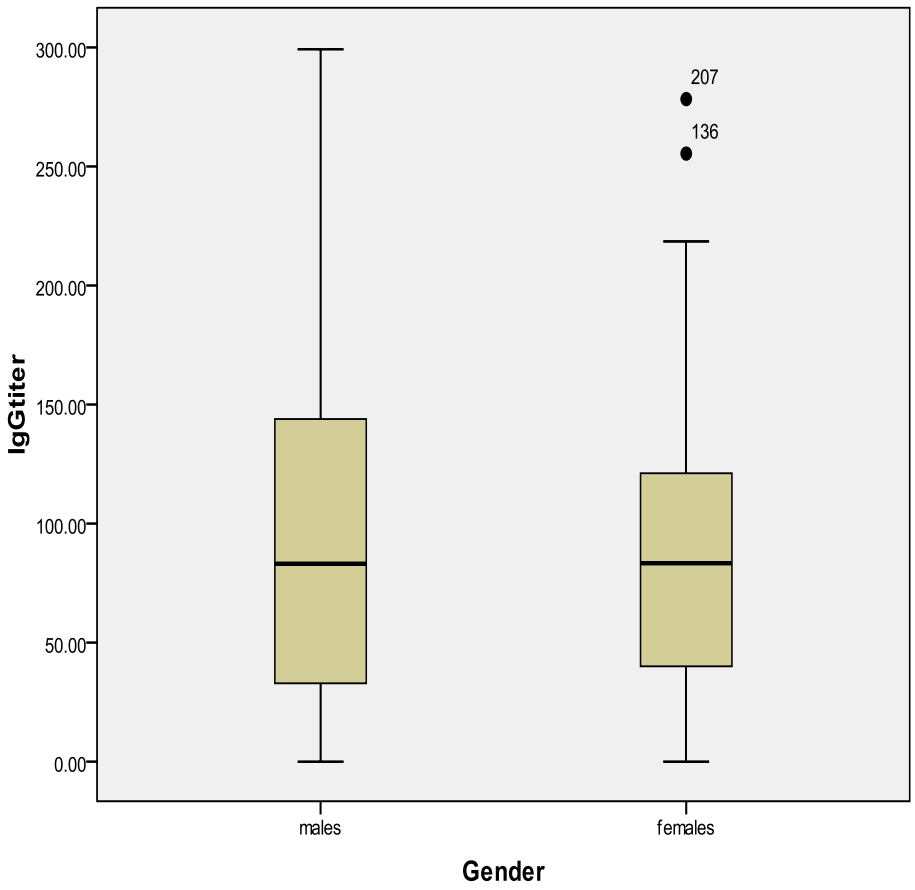


Chart 9: The difference in anti-Helicobacter IgG titer between males and female, as it is clear males were more affected than females (among IgG positive patients there were 76.6% males and 38.3% females).

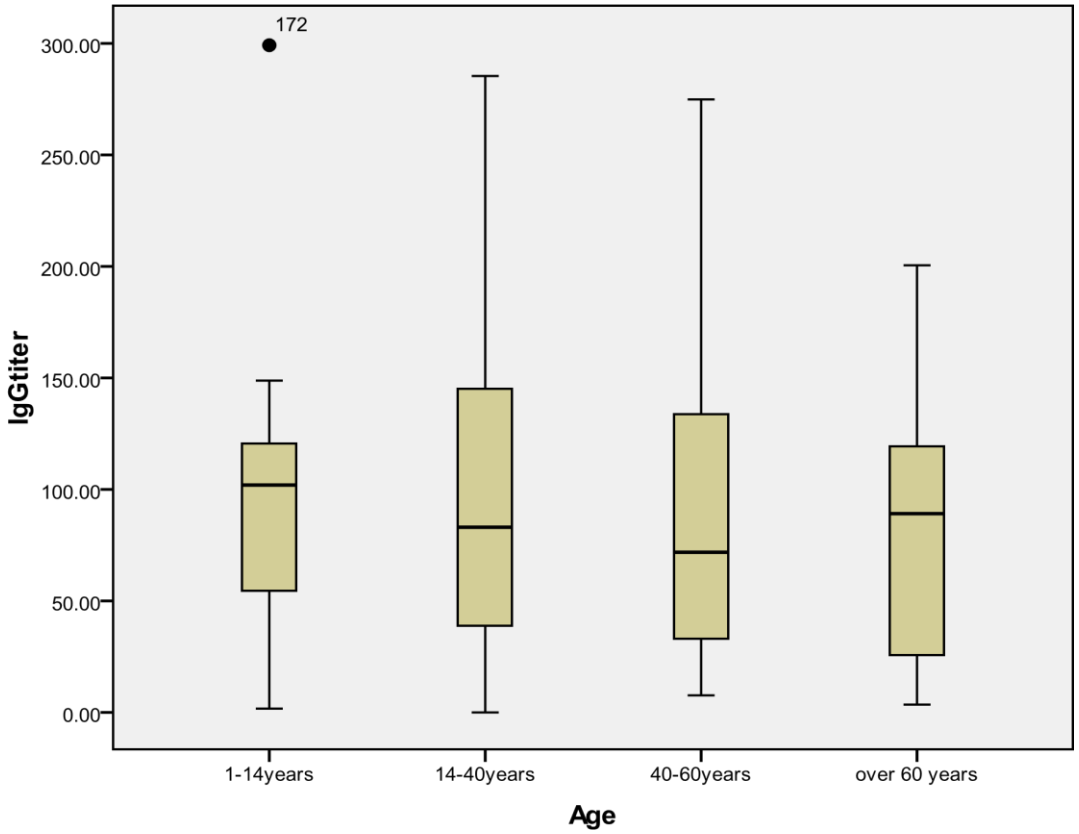


Chart 10: Differences in Anti-*Helicobacter pylori* IgG titer between Age groups. Please notice that the youth group between 14 and 40 years old has highest Anti-*Helicobacter pylori* IgG titer.

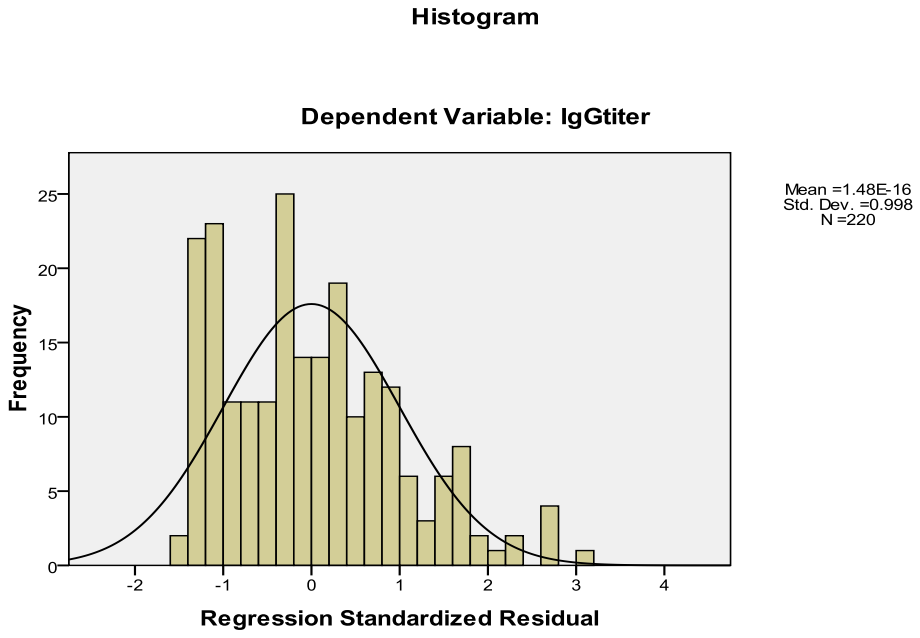


Chart 11: The normal distribution of IgG titer.

The linear Regression equasion for the sore throat has been found to be:

Y= 93.248 + 4.127 X

This means presence of sore throat increase the serum anti-*Helicobacter pylori* IgG 4 times more than normal throat individuals, as it is shown as well in the chart below.

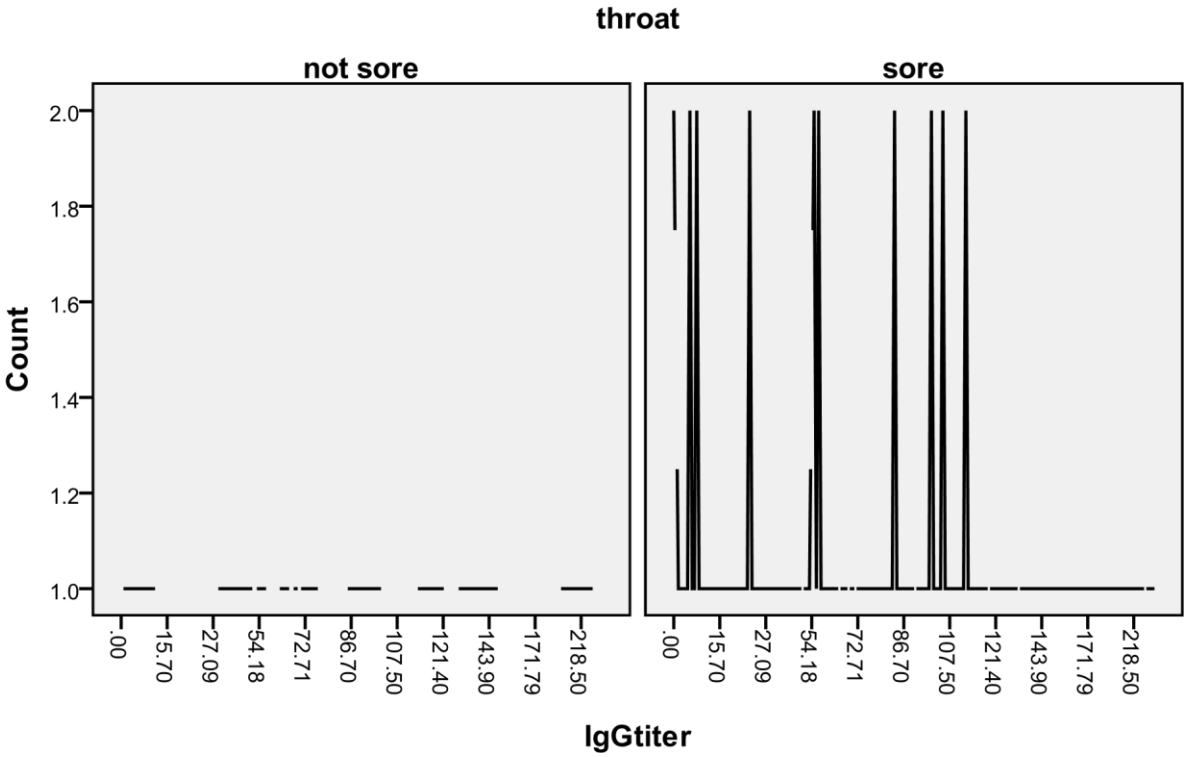


Chart 12: This chart compare the difference in Anti-*Helicobacter pylori* IgG titer between patients with sore throat and those with no sore throat

Sore throat:

- Pathogenic sore throat causing bacteria were isolated from **tonsils** of 228 individual (74%) from whole samples.
- Pathogenic sore throat causing bacteria were isolated from **pharynx** of 235 individual (76.3%) from whole samples.

- Among the 308 collected samples 4.1% had normal bacterial biofilm in **tonsils and pharynx**, 17.7% had a pathogenic bacteria either only in the **tonsils or** in the **pharynx**, while 78.2% had pathogenic bacteria simultaneously in the **tonsils and pharynx.**

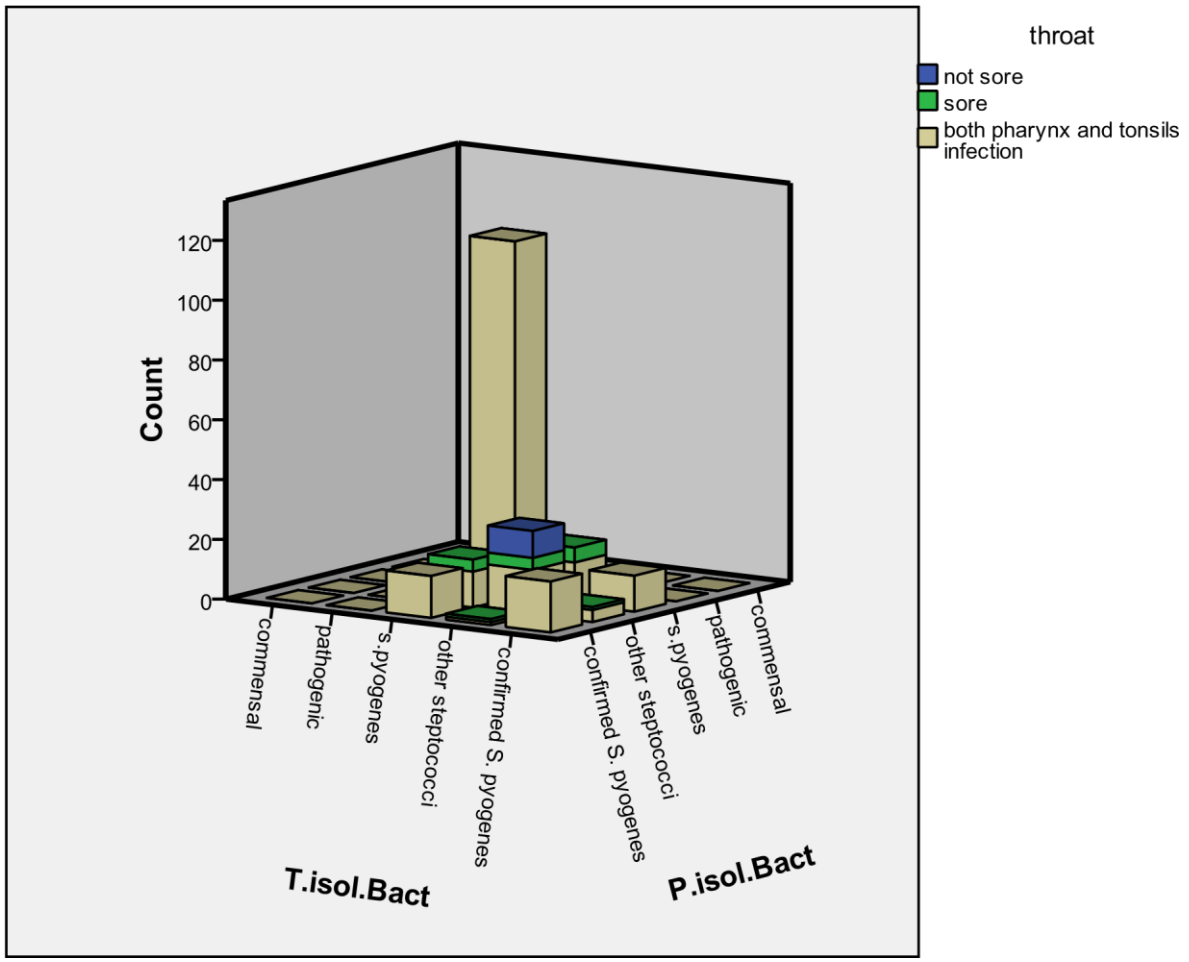


Chart 13: The high frequency of isolation *Streptococcus pyogenes* among all other isolated bacteria. *S. pyogenes* represent about 71.2% among about 520 bacterial isolate, obtains and identified from throat in this study.

- Although sore throat has been observed to be very prevalent, but clinical respiratory symptoms were observed only in 9.7% of participants in this study.
- Among those isolated bacteria *Streptococcus pyogenes* was the most predominant bacteria 71.2% among other isolates (were detected by Microscopic features along with cultural characteristics and biochemical properties in 65.1% among whole samples) and were detected by PCR using D primer and SPY 1258 jointly in 13.3% among whole samples.

- Among all individuals complaining from respiratory signs, 89% of them were confirmed with *H. pylori* infection.

Table13: The sensitivity, specificity and accuracy of the conventional methods used for the isolation of *Streptococcus pyogenes* in this study.

Method		*Streptococcus pyogenes* by Conventional bacteriological methods		Total results
Streptococcus pyogenes by PCR	result	+	-	
	+	41 a	0 b	41
	-	159 c	108 d	267
Total results		200	108	308

Accuracy of PCR method = $\frac{N\ true\ positive + N\ True\ negative}{N\ true\ positive + N\ true\ negative + N\ false\ positive + N\ false\ negative}$

Accuracy = 48.4% (95% CI*) * Confidence Interval

Sensitivity = $\frac{N\ true\ positive}{N\ true\ positive + N\ false\ negative}$

Sensitivity of PCR = 20.5% (95% CI*)

Specificity = $\frac{N\ True\ negative}{N\ true\ negative + N\ false\ positive}$

Specificity of PCR ≥ 99.9% (95% CI*)

***H. pylori* :**

- 10.1% of whole collected samples were from individuals with history of previous *H. pylori* infection.

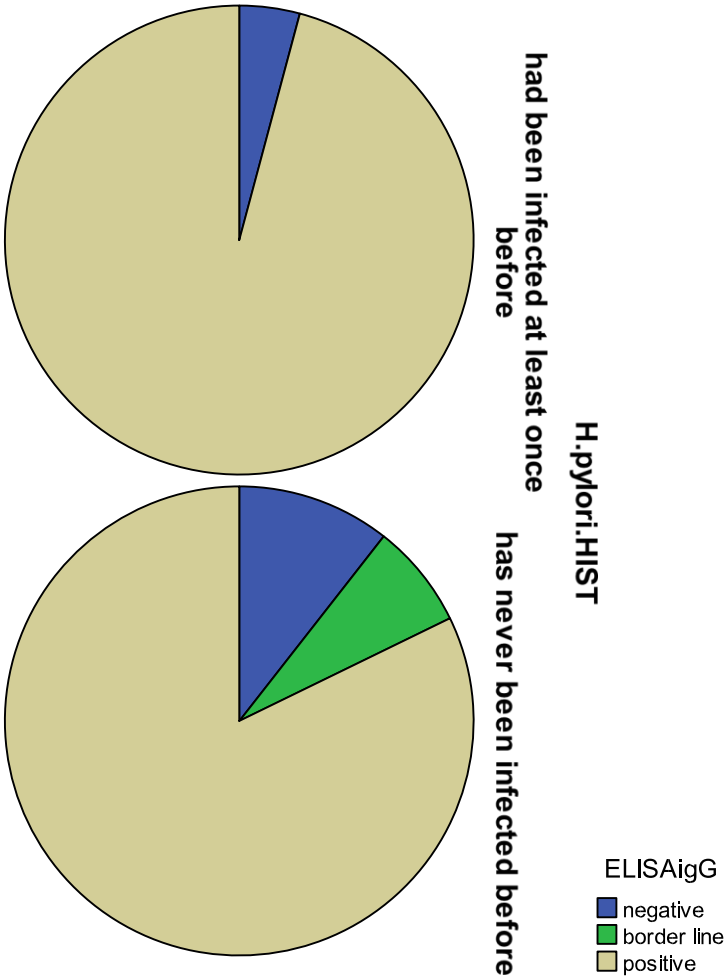


Chart 14: The difference between patients with *H. pylori* history and those with absolutely no previous *H. pylori* infection in IgG categories (Positive, Border line or Positive). Please notice that the majority of patients with previous *H. pylori* history were positive for Anti-*Helicobacter pylori* IgG.

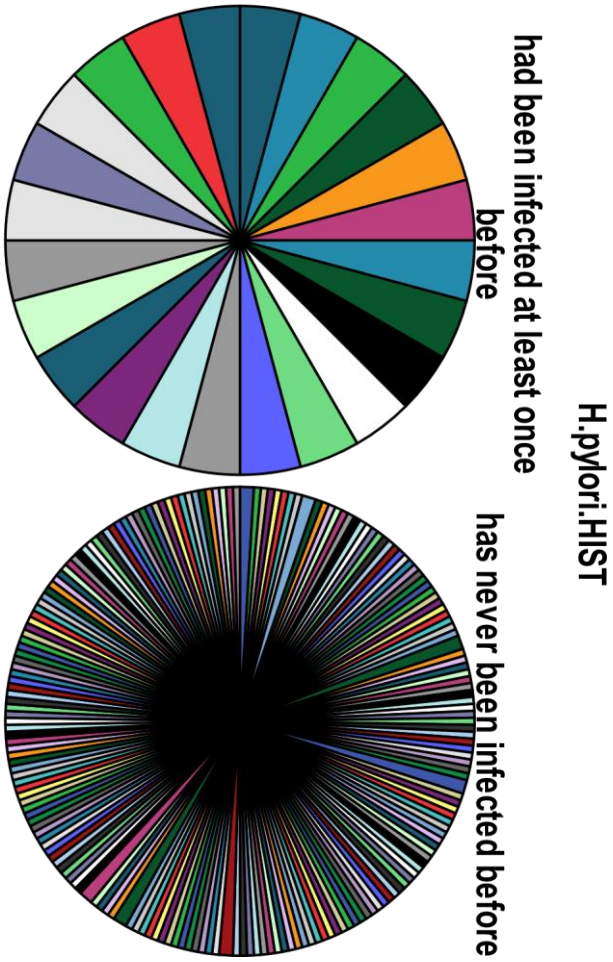


Figure 15: This Pie Chart reflect the huge difference between patients with *H. pylori* infection history and those with no previous *H. plori* history in Anti-*Helicobacter pylori* IgG titer (each color represent individual Anti-*Helicobacter pylori* IgG titer)

- Among those with previous infection, about 96% were Positive by IgG ELISA, 80% were positive by ICT and only 8.3% were positive by IgA ELISA. In other word The majority of cases that had at least one *H. pylori* infection before had very low concentration of anti- *H. pylori* IgA in their body and most of them were diagnosed infected with *H. pylori* in this study; this suggest that developing high level of anti- *H. pylori* IgA is qite essential to protect previously *H. pylori* infected people from being infected again. Also those parients severing from recurrent *H. pylori* infection are basically have an issue with developing high anti- *H. pylori* IgA titer in their blood and this in turn makes them vulnerable for further *H. pylori* infections in the future.

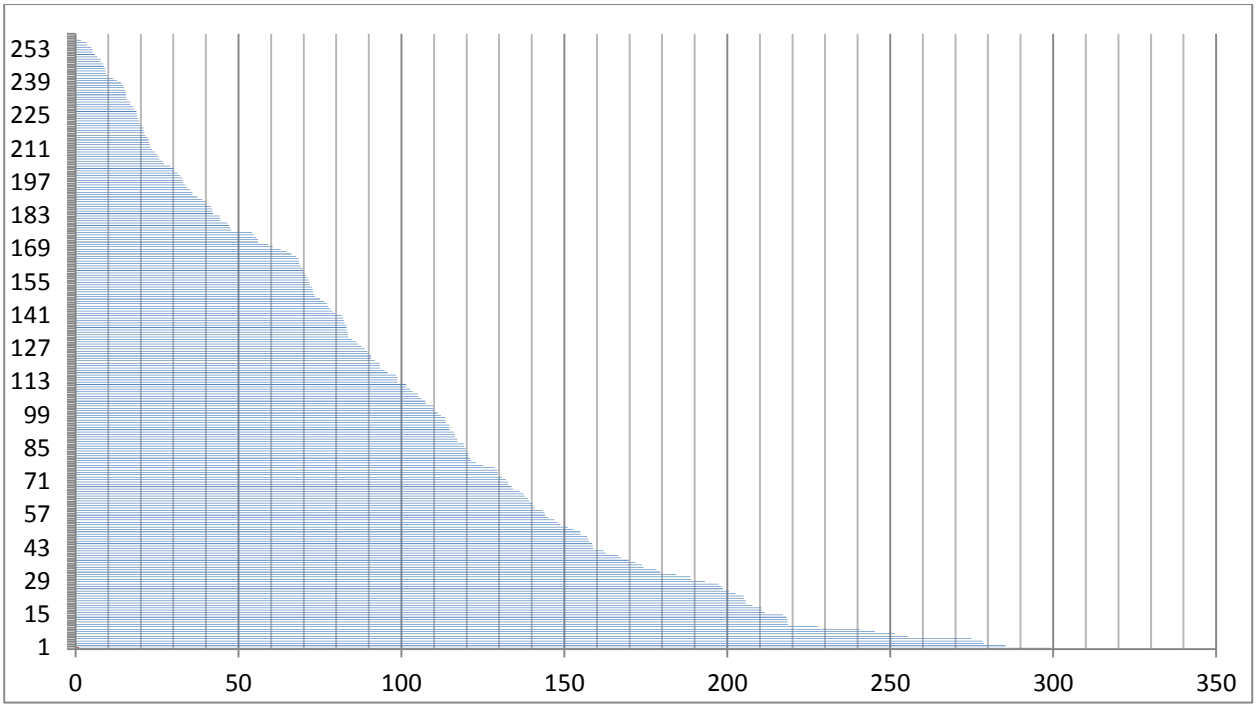


Chart 16: IgG Cons. (Ru/ml) among patients

This histogram shows that there is very high significant correlation between pathogenic bacteria (*Strepto. pyogenes*) and *H. pylori* infection.

CHAPTER FIVE

DISCUSSION

Previous studies showed that *S.equi* is associated with a wide variety of diseases in horses and other animals including humans. *S.equi* shares over 80 % DNA sequence identity with the important human pathogen *Streptococcus pyogenes* (Holden *et al*., 2009). However, *S.equi* rarely cause human infections, several patients with *S. equi* have animal contacts (Kristina *et al,* 2016). Thus this study goes in line with Kristina *et al*. (2016). and Holden *et al.* (2006). Clinical presentations are variable, and may include mild upper respiratory tract signs, and pneumonia (Downar *et al*., 2001).

The present findings are in accordance with the findings reported by Brandt *et al.*, (2009) and Jensen and Kilian (2011). They found that *S. dysgalactiae* colonizes the human upper respiratory, gastrointestinal, and female genital tracts. That was previously considered as nonpathogenic (Brandt and Spellerberge, 2009). A full list of *Streptococcus dysgalactiae* strains have been provided by Jensen and Kilian (2011) from human throat as an original habitat.

The original strains of group L streptococci were isolated by Hare and Fry (1938) from dogs and pigs (Laughton, 1948). Most of the group L streptococci isolates from blood and cerebrospinal fluid were believed by Broome *et al.* (1976) to be nonpathogenic, and there have been very few reports of infection in man (Barnham and Neilson, 1987).
β hemolytic Group L Streptococci were first found in the human throat (White *et al*., 1939) in patients both with and without signs of respiratory infection (Nordlander *et al*., 1975 ; Olsen, 1957).
In our knowledge since 1987 no incident of infection by group L or even colonization in man was reported.

Our result ties well with Lun *et al.* (2007) and Ishigaki *et al.* (2009); they reported that *Streptococcus suis* is a pathogen in pigs that can cause severe systemic infection in humans, infections can be complicated by acute respiratory distress. The number of

human *S. suis* cases reported in the literature has increased significantly (Lun *et al*, 2007). Human infection was also associated with cattle (Ishigaki *et al*., 2009).

A similar conclusion that *S. pneumoniae* colonizes the upper respiratory tract was reached by several authors (Gwaltney *et al*. 1975; Bogaert *et al*. 2004 and Simell *et al*. 2012). They also have shown that pneumococcal carriage at the individual level plays an important role in pneumococcal disease dissemination in communities.

Some recent studies have identified the oropharynx as a potential site of *Staphylococcus aureu*s colonization (Bignardi and Lowes, 2009 and Marshall and Spelman, 2007). Results by Caroline *et al*. (2011) that showed higher throat than nasal carriage of *S. aureus* also confirm earlier observations that the oropharynx is an important reservoir for *S. aureus* (Marshall and Spelman, 2007 ; Nilsson and Ripa, 2006 ; Hamdan-Partida *et al.*, 2010).

The present findings on 0.6% *S. aureus* at least hint the presence of coagulase positive and negative *S. aureus* in human throat.

Many healthy people may carry *S. aureus* as a part of their normal microflora in the nose, throat, perineum or skin (Narmeen *et al*., 2009). The coagulase test usually correlates well with staphylococci pathogenicity (Markey *et al*., 2013). A study showed that staphylococci are widely spread among humans and the most common isolates were coagulase-negative staphylococci (Sleiniute and Siugzdaite, 2015).

The present results strongly agree with Mackay *et al.* (1993) in that the misidentification of atypical coagulase-negative *S. aureus* strains from clinical specimens could be dangerous and lead to failure of treatment.

To our knowledge no study has yielded in isolation of *Staphylococcus chromogenes* from human. Therefore, as far as we know, no previous research has investigated the isolation of *S. chromogenes* in human throat. Our findings on 0.5% *S. chromogenes* of our isolates at least hint that this bacteria could cololnise human throat.

- The present study involved different age groups each had identified prevalence rate (PR) 1-14years (PR= 11.9%), 14-40years (PR= 69%), 40-60years (PR= 13.5%) and Over 60 years (PR= 5.6%).
- The mean of IgG antibody titer was 95.21 RU/ml and the maximum was 299.20 RU/ml while every sample above 22 RU/ml was considered positive according to manufacturer. The positive samples for IgG was quite high (88.2%) while for IgA the positives was 30% and the positive cases by ICT were 71.9%. IgA ELISA showed that, 28.7% of males were Positive, while 25.8% of females were affected. On the other hand IgG ELISA showed 84% of males were positive and 81.8% of females were Positive. 83% of children between 1 to 14 years were positive, 85% of youth between 14 to 40 years, 80% of older people between 40 and 60 years were positive and 85% of very old people over 60 years old were positive by IgG ELISA.
- Sore throat with pathogenic bacteria in both pharynx and tonsils in Children between 1 to 14 years was 74%, youth between 14 to 40 years was 77%, older people between 40 and 60 years was 80% and very old people over 60 years old was 84%.
- Significant correlation has been noticed between smoking and sore throat as 87.5% of smokers had sore throat caused by pathogenic bacteria.
- The result showed that 88.2% of our population were positive for *H. pylori* IgG, only 29.5% were positive for IgA, while 26.7% were positive for both. In contrast a previous study published that 63.3% of samples in Khartoum, Sudan, were positive for IgG (Elhag and Omer, 2014), this may suggest that the prevalence of *H. pylori* infection is increasing. In Kenya Jontathan and his colleagues (2003), found 70.2% of patients were seropositive for IgG. While in Saudi Arabia only 22% were seropositive for IgG (Mubashir and Hani, 2007).
- In the present study males were more affected than females; among positive IgG samples76.6 % were males and 38.3% were females which is contrary to Elhag and Omer study in 2014 who found that females were more affected than males.
- The present study results showed insignificant correlation between age and *H. pylori* antibodies, this was similar to study done in Iran by Alavi *et al.* (2010) and also same

as what found 5 years age in Sudan by Elhag and Omer (2014), however this differs from the findings of Forman and Burley (2006) and Kabir (2007) who reported that the prevalence of *H. pylori* infection increases with age.

- In this study the higher percentage of infection was observed among age group 14-40 years (70%). This high percent may be due to the vast majority of individuals acquiring this infection during childhood (Cherian *et al*., 2008).
- Although our results differ from Elhag and Omer (2014); because we found that males were more affected and had higher IgG and IgA antibodies than females but we still agree with them along with Mirghani and his colleagues (Mirghani *et al*., 2002), as there was insignificant correlation between *H. pylori* antibodies and gender (P >0.05), also it was in agreement with that obtained in Egypt by Manal *et al*. (2007), in addition to other studies by Huang *et al*. (2004) in Malaysia, Kikuchi and Dore (2005) in Iran, and Mukherjee *et al*. (2005) in Netherlands. On the other hand, Leandro *et al*. (2005) found that the prevalence was significantly higher in boys, also Versalovic and Fox (2003) reported that *H. pylori* is more prevalent among the elderly and more frequent in males than females.
- Anti *Helicobacter pylori* IgG antibody is mostly affected by presence or absence of the pathogen and it is the more trustful than IgA, although there were significant positive (p= 0.05) correlation between IgG and IgA levels, but high number of positive IgG were negative by IgA
- On the other hand IgA has been found to be more effective in protection and immunogenicity against *H. pylori* infection and its absence or low level may be the cause of active recurrent *H. pylori* infection.
- So we suggest that, failure of *H. pylori* infection treatment could be, beside antibiotic resistance (particularly clarithromycin), attributed to failure of immune system to raise effective IgA level. These reasons justify the necessity of a novel vaccine to prevent *H. pylori* infection.
- Among all individuals complaining from respiratory signs, 89% of them had been confirmed with *H. pylori* infection. Some authors identified *H. pylori* VacA toxin in

human lungs, possibly influencing the course of some respiratory diseases by promoting inflammation (Nakashima *et al.*, 2015).

- Among our volunteers (n=308) almost half (n=159) had signs of laryngeopharyngeal reflux (LPR) with acid burn in chest, pharynx and/or mouth, about 95% of those with LPR had a sore throat due to pathogenic bacteria; this copes with what was found previously by Koufman *et al.* (2002).
- Serological methods in diagnosis of *H. pylori* become increasingly important in the last year's and we are persuaded that it will be in the future even more trustworthy corner stone for diagnosis of *H. pylori* infection specially in developing countries like Sudan; because of the growing rates of disease prevelance and also due to its efficiency, availability and simplicity. More than twenty years ago Cutler *et al.* (1995) have speculated that serology probably will be the test of choice for patients not previously treated for *H. pylori.* Serological testing may be accomplished either by enzyme-linked immunosorbent assay performed in a reference laboratory (IgG or IgA serology) or by one of the newly available in-office immunoassay kits (i.e. ICT). For all the above mentioned reasons we used these three serological method and measure their specificity, sensitivity and accuracy in Sudanese patients in order to report the pros and cons of each method and hence help the researchers and physicians to appoint the suitable diagnostic tool for each case accordingly.
- IgA ELISA has low specificity and sensitivity For *H. pylori* infection compared to IgG ELISA; this finding has many agreements since Cutler *et al.* (1995).
- On the other hand, among all individuals with upper digestive tract illness signs (signs of laryngeopharyngeal reflux) (LPR), 85% of them had been confirmed to be infected with *H. pylori*.
- Among Diapetic patients 83.3% of them were infected with *Helicobacter pylori*, while the majority of them were negative for Anti- *H. pylori* IgA. Other previous studies found a significant association between Diabetes Mellitus (DM) and *H. pylori* infection (Hsieh *et al.*, 2013 and Yang *et al.*, 2014). We propose that this finding could be attributed to the Immune system debilitation accompanied with DM.

- One of the most terrific conditions associated with *H. pylori* is the Cardiac Syndrome X, the pathogenesis of this condition remained unexplained with so many theories for years by Rasmi *et al.* (2012) had proved that this association is mainly due to the inflammatory cytokines triggered by *H. pylori* infection (mainly tumor necrosis factor alpha and interleukin-6) by contributing to endothelial dysfunction, dependently we strongly think this manifestation could be worsen even more due to the subsequent *Streptococcus pyogenes* secondary infection caused by *H. pylori*; because *S. pyogenes* itself trigger even more inflammatory cytokines specially tumor necrosis factor alpha, interleukin IL-6 and IL-10 (Müller-Alouf *et al.*, 1994)
- Although sore throat has been very prevalent, yet clinical respiratory symptoms were observed only in 9.7%. So most of the patients with *H. pylori* infection were having subclinical *Strep. pyogenes* infection.
- Among those isolated bacteria *Streptococcus pyogenes* was the most predominant bacteria 71.2% among other isolates (were detected by Microscopic features along with cultural characteristics and biochemical properties in 65.1% among whole samples) and were detected by PCR using D primer and SPY 1258 jointly in 13.3% among whole samples. Therefore, we belive that the molecular detection of *Strep. pyogenes* strains infection by PCR in Sudan and surroundings need to develop a new specific primer.
- Our results indicates that PCR accuracy was 48.4%, PCR sensitivity was 20.5% and PCR specificity was ≥ 99.9%. These results agree with each of Liu *et al*. (2005), Dunne *et al.* (2013), Schabauer *et al*. (2014) and Al-Saadi *et al.* (2015). All of these studies showed the gene Spy 1258, was specific for *S.pyogenes* only, but not for other species of the genus *Streptococcus* and common bacteria.
- Although Spy1258 gene was very common gene among *S.pyogenes* strains and detected in slight number of bacterial sequences availavbe at the GenBank, the Spy1258 gene sequence was clearly absent in other bacterial genomes available at GenBank.
- The very low sensitivity of Spy1258 PCR assay had been noticed by Dunne *et al*. (2013), but the reasons still remain unclear.

- Although the degenerative primer d1 and d2 provide a PCR product that represents approximately 85% of the *sodA* gene encoding a manganese-dependent enzyme (Mn-SOD) 29 type strains of streptococci (*S. acidominimus*, *S. agalactiae*, *S. alactolyticus*, *S. anginosus*, *S. bovis*, *S. canis*, *S. constellatus*, *S. cricetus*, *S. downei*, *S. dysgalactiae*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, *S. equinus*, *S. gordonii*, *S. iniae*, *S. intermedius*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguis*, *S. pneumoniae*, *S. porcinus*, *S. pyogenes*, *S. salivarius*, *S. sanguis*, *S. sobrinus*, *S. suis*, *S. thermophilus*, and *S. vestibularis*), (Poyart *et al*, 1998); this means it has very low specificity for *S.pyogenes*, but we found that it has more sensitivity than Spy 1258 primer. Therefore for molecular diagnosis of *S.pyogenes* we recommend using both of them (d1 and d2 primers at first for scanning followed by Spy 1258 primer for confirmation). But the development of new primers especially for North Africa strains still necessary.
- Sore throat cases have been found to be significantly (at 95% confident interval, 2 tailed) correlated with ICT results, and also significantly correlated with symptoms of general body fatigue and respiratory signs.
- IgG Antibody titer has been found to be significantly (at 99% confident interval, 2 tailed) correlated with ICT and IgA ratio.
- After data analysis we found that ICT accuracy was 74.6%, while ICT sensitivity was 75.6% and ICT specificity was 88.2%, this was different from ICT manufacturer provided data, which mention that sensitivity is greater than 99.9%, relative specicificity = 92.7% and accuracy is 97.2%. We speculated that the difference could be attributed to the use of fresh samples in most of our samples, the reduction in accuracy, specificity and sensitivity rates while using fresh samples instead of stored samples had been mentioned before by Sharma *et al.* (1997). Another possible justification is the use of high-molecular-mass cell-associated protein (HMCAP), an antigen highly specific to *H. pylori*, as the only Ag in ICT kit (Sharma *et al.*, 1997 ; Evans *et al*., 1989). On the other hand, the antigen used in IgG ELISA was the whole bacterial cell lysate derived from *H. pylori* strain "ATCC43504".

- Although the spy 1258 is the most widely common primer used for molecular detection of *S. pyogenes* (Liu *et al.*, 2005), it can only detect 13 strains out of more than 200 strains and we found so many isolates which are typical for *S. pyogenes* by microscopic characteristics, colony features and biochemical tests results, but couldn't be detected by spy 1258 primer.
- Colman and Ball, (1984) reported that fermentation of lactose was present in 72% and 89% of isolates cultured in Hartley-digest horse and Columbia horse blood agar respectively, also hydrolysis of arginine was present in 62% and 60% of isolates cultured in Hartley-digest horse and Columbia horse blood agar blood agar respectively. In contrast all our isolates were cultivated in Sheep blood Agar and 76.4% had arginine hydrolysis activity while 90.3% of them could ferment lactose.
- We had 1 isolate which was frankly fermenting ribose and other 13 isolates tested were weakly positive to ribose; 8 of which were confirmed as *S. pyogenes* by PCR, that was unlike Colman and Ball (1984) and Barrow and Feltham (2005).
- Occasionally nonhaemolytic strains of Group A streptococci are isolated (James and McFarland, 1971), but all the isolates of *S. pyogenes* in this study were haemolytic.
- M-type 6 and M-type 55 are known to ferment mannitol, and we had many as 38 isolates fermented mannitol weakly (7 of them were confirmed by PCR) and another 64 isolates were strongly positive for Mannitol fermentation test (only 3 were confirmed by PCR).
- Data analysis of this study showed very significant correlation between isolation of Group A Streptococci (*S. pyogenes*) and signs of upper digestive tract. According to Koufman *et al* (2002) sore throat is a symptomatic feature of extraeosaphageal reflux (laryngeopharyngeal reflux LPR) and the golden and dominant feature of LPR is the elevated acidic pH of throat, which is the most common upper digestive tract signs we found. So we concluded that this is mainly because of *Streptococcus pyogenes* (71.2% among others), because this bacteria can tolerate and resist acidic conditions up to 4 pH. This remarkable properties of *S. pyogenes* is linked to possesion of a virulent factor known as Arginine deiminase ADI system (Cotter and Hill, 2003). We think that the

laryngeopharyngeal reflux favors the conditions for secondary Group A streptococci pharyngitis.

- There are two problems with the PCR primers for group A Streptococci GAS, the first one is that, now a days there is more than 60 different types of strains of (GAS), http://lab.rockefeller.edu/fischetti/mstocks , but the most known ubiquitous PCR primer available can only anneal for about 13 strains according to MFEprimer-2.0 Report , the second proplems is the variability of GAS genome, along with rareness of sequenced genomes from developing countries in Africa. This necessitates developing new primer, possible by sequencing new isolates from Africa including Sudan.

CHAPTER SIX

6.1 Conclusion:

- There is a significant correlation between *H. pylori* infection and sore throat, due to the accompanied laryngeopharyngeal reflux which inturn reduce the throat pH enhancing the condition for *S. pyogenes* and other acidic pH tolerant pathogens.
- The prevelance of *H.pylori* infection in our study population is quite high (88.2%) this indicated the infection is expected to keep increasing unless furthers studies and solutions applied.
- *Streptococcus pyogenes* constituted 71%of isolated bacteria from throat swabs; and it is the most causative agent of bacterial sore thoat in Sudan whether it is related to *H.pylori* infection or not.
- ICT ethod of *H.pylori* infection diagnosis is less reliable compared to ELISA.

6.2 Recommendation:

1- Searching the correlation between *H.pylori* positive infection and viral throat infection because as it is also frequent than bacterial throat infection.

2- More research on the isolation of *H.pylori* is needed, instead of conducting serological tests, with the isolation of *S. pyogenes*

3- Physicians should avoid prescriping the same antibiotics against *H. pylori* to avoid bacterial resistance. Such resistance is common in patients who had previous antibiotic treatment.

4- Further research in cellular immunity of *H. pylori* is needed. Because humoral immunity does not protect against *H. pylori* like cellular immunity (Kuster *et al*, 2006).

5- It is essential to develop a new PCR primer, specific for *Streptococcus pyogenes* and sensitive for its all strains to replace spy1258 primer.

6- ICT method of *H.pylori* infection diagnosis should be avoided and replaced by more trusted methods.

7- Further reseach for the investigation of the low level IgA response.

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Appendix

Questionnaire

Personal information: -

- Patient's code:……..phone no.:……
- Age: ……. - Gender: ……..
- Body Mass: ……....
- Diabetes: yes ( ) no ( )
- Hypertension: yes ( ) no ( )
- Smoking History: ……...…….
- Symptoms Present: ……...
  ……...……..
- Pain of Throat: yes ( ) no ( )
  If yes the level of pain:

- Pain of Abdomen: yes ( ) no ( )
  If yes the level of pain:

- History of H. pylori:

Others

……

……

وزارة الصحة ولاية الخرطوم

الادارة العامة للتخطيط الاستراتيجي

ادارة البحوث

التاريخ: ٨ /٨ /٢٠١٦م

السيد مدير ادارة المؤسسات العلاجية الخاصة.

الرجاء السماح بتنفيذ بحث The Correlation between Helicobacter Pylori Seroprevalent Positive and Throat Infections in Private Hospitals in Khartoum 2016.

والذي يقوم به الباحث صلاح البيت أرجو محمد

ومده بما يحتاج اليه من معلومات

وجزاكم الله خيرا

د/منى فتح الرحمن

مدير ادارة الدراسات و البحوث

بسم الله الرحمن الرحيم

وزارة الصحة - ولاية الخرطوم

إدارة المؤسسات العلاجية الخاصة

النمرة: وص وع/ م ح ح/44/2 | التاريخ: ١٥/٨/٢٠١٦م

السيد / مدير طبي مستشفى ....[illegible]........

المحترم

السلام عليكم ورحمة الله تعالى وبركاته

الموضوع/ تنفيذ بحث

بالإشارة للموضوع أعلاه نفيدكم بأنه قد تمت الموافقة على تنفيذ بحث بعنوان :-

(The correlation between helicobacter pylori positive and threat infections in private hospitals in Khartoum 2016)

والذي تنفذه الباحثة :-

أ/ هبة صلاح الدين .

من الجامعة الوطنية - كلية الطب .

الرجاء تسهيل مهمة جمع البيانات ومساعدة الباحثات .

وجزاكم الله خيراً.....

د/ محمد عباس أحمد الحوراوي

مدير إدارة المؤسسات العلاجية الخاصة