Immuno-diagnosis for human infected with *Helicobacter pylori* using C3 and C4

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Abstract: *Helicobacter pylori* (*H pylori*) is a micro-aerophilic, gram-negative, spiral shaped and flagellated organism present in almost half of the world population. Host genetics and host immune responses, as well as bacterial genotype and virulence factors contribute to the multifactorial nature of disease progression and outcome. Various tests have been developed to diagnose the infection but all have limitations. **Objective:** The current study aimed to determine levels of serum complement in patient infected with *H. pylori* and to compare different techniques used for diagnosis of the organism so that we may evaluate the role of C3 and C4 in diagnosis and pathogenesis of the disease. **Methods:** This study included 100 serum and 100 stool specimens, collected during November 2015 to December 2016, from subjects suffering from gastro-intestinal troubles. These studied subjects were 54 males and 46 females, aged from 1.5 to 70 years. The stool samples were analyzed for *H. pylori* antigen using: Enzyme linked Immune Sorbent Assay/antigen **(**ELISA/Ag**)** and Rapid antigen *H. pylori* test (Rapid/Ag). The serum was analyzed for *H. pylori* antibodies by Rapid antibody. *pylori* test (Rapid/Ab). Levels of complement C3 and C4 were measured in 46 blood serum (samples that were positive to *H. pylori*), using Radial Immunodiffusion (RID) plates. **Results:** out of 100 stool samples: 50%were positive to *H. pylori* using ELISA/Ag test (T1) and 89% were positive to *H. pylori* using Rapid/Ag test (T2). Out of 100 serum samples: 64% were positive to *H. pylori* using Rapid/Ab test (T3). All positive samples using T1were also positive using T2and all negative samples using T2were also negative using T1. Only 22% of negative samples using T1 were also negative using T2. Only 82% of positive samples using T1 were also positive using T3. Only 54% of negative sample using (T1) were negative using T3. Male patients had a slightly higher infection rate (28% and 47%) than female patients (22% and 42%) using T1 and T2 respectively, while female patients had a slightly higher rate (33 %) of infection than male patients (31%) using T3. The highest positive result (39%, 61% and 42%) was found in the Middle age group (21-40) years using T1, T2and T3, respectively. While the lowest positive result (03% and 9%) was found in the Young age group (1-20) years using T1 and T3 respectively and (13 %) was found in the Old age group (41-70) years using T2. C3 and C4 were measured in 46 patients; 41 (samples that were positive to *H. pylori*;56% male and 44% female) and 5 non-infected with *H. pylori*. Out of these positive samples40% had abnormal level of C3, 68% had abnormal level of C4. All 5 non-infected samples had normal level of C3and only20 % had abnormal level of C4. **Conclusion:** No single test can be considered as the master for the diagnosis of *H. pylori* infection. The detection of *H. pylori* infection using Antigen tests or Antibody test can be supported with test for C3 and C4. Bacteria immediately activate C3 by Antigen through alternative pathway and delayed activation of C4through classical pathway occurs by secreted specific antibodies. This correlation suggests possible use of changed levels of C3 and C4 as biomarker for infection with *H. pylori*.

1. Introduction:

Helicobacter pylori (H. pylori) is a micro-aerophilic, gram-negative, spiral shaped and flagellated organism. It is the most common chronic bacterial infection of humans, present in almost half of the world population. It has been shown to be a causative agent of disease of varying degrees of severity. Host genetics and host immune responses, as well as bacterial genotype and virulence factors contribute to the multifactorial nature of disease progression and outcome (**Marshall** and **Warren**, **1984**; **Prenck** and **Clemens**, **2003**).

There are several popular methods for detecting the presence of *H. pylori* infection: Enzyme-linked immunosorbent assay that detects *H. pylori* antigen (ELISA/Ag) in stool specimens. It has produced promising results for the detection of *H. pylori* in faecal samples (**Vaira *et al.,* 2000**). Antibody-based tests (Rapid/Ab or Rapid/Ag) that has significant advantages, as it is quicker, uses a smaller volume of serum, and has a lower threshold of detection (**Stege *et al.,* 2010**).

Each test type has its own advantages, disadvantages and limitations. Guidelines indicated that no single test can be considered as the

goldstandard for the diagnosis of *H. pylori* infection and that one should consider the method's advantages and disadvantages (**Mehmood** *et al.,* **2010** and **Miftahussurur** and **Yamaoka 2016**).

The majority of infected people remain asymptomatic, and only small portions develop illness, usually in adulthood (**Risch** *et al.,* **2010**). Some infected persons develop acute gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma (MALT), or gastric adenocarcinoma (**Suerbaum** and **Michetti**, **2002**). This bacterium is seemingly involved in the pathogenesis of several extra gastric diseases, such as increased risk of diarrhea, (**Passaro** *et al.,* **2001**). Also gastro esophageal reflux disease (**Rothenbacher** and **Brenner**, **2003**), iron deficiency anemia, skin disease (**Wang** *et al.,* **2012**), and rheumatologic conditions (**Shaweno** and **Daka, 2013**). Also a growing number of new data suggest the role of *H. pylori* in the development of systemic diseases including coronary heart disease (**Baudron** *et al.*, **2013**), diabetes (**Kuo** *et al.*, **2014**), obesity or anemia and growth disorders in children (**Chmiela** *et al.*, **2015**).

The host immune response is an important determinant of the outcome of the infection, but the mechanisms by which the immune response can eradicate gastric Helicobacter infection are unknown. Emerging data have indicated that *H. pylori* has multiple mechanisms to both evade and manipulate the immune response (**Ismail** *et al.,***2003**).

The complement system (C) plays an important defensive rule within human body. The rule of C is involved in both innate (natural, non-specific) and acquired (specific) immunity. While Complement component C4 plays a central role in classical and lectin pathways of complement. C3 is a key protein of the complement system, activation of C3 results in a variety of immunologic reactions such as immune adherence, phagocytosis, antibody response, cytolysis, inflammation, and killing of pathogenic microorganisms (**Shaldoum** *et al.,* **2012**).

Several lines of evidence suggest that the complement system may have an important role in *H. pylori*-induced gastritis, *H. pylori* can activate complement in vitro (**Ismail** *et al***., 2003**).

The current study aimed to determine the activity of complement in patient with *H. pylori* and to compare obtained data with ELISA/Ag, Rapid/Ag and Rapid/Ab diagnostic techniques for *H. pylori* so that we may evaluate the role of C3 and C4 in diagnosis and pathogenesis of this disease.

2. Subjects and Methods
Ethical consideration:
An approval was obtained from the hospitals in Alexandria before the commencement of the study**.**

Written informed consents were obtained from all volunteers after clear explanation for the objectives of the study. Patients received no anti-helminthic, antibiotic or proton pump inhibitor treatment, at least in the last two months before the study.
Subjects:
A total of 100 patients 54 males and 46 females, aged between 1.5 to70 years old, exhibiting symptoms related to gastro-intestinal troubles participated in this study at abdominal clinics of Alexandria University Hospitals.

Age group were divided into three groups: Young group from (1-20); Middle group from (21-40) and Old group from (41-70), years.

The occurrence of *H. pylori* infection was tested using different methods: ELISA/Ag and Rapid/Ag in stool; Rapid/Ab and Complement C3, C4 in blood serum.
ELISA/Ag (T1):
Stool samples were analyzed using ELISA stool antigen test (Accu Diag™ *H. pylori* Antigen ELISA Kit, California 91367, USA), according to manufacturer's instructions. Briefly, diluted fecal samples and peroxidase-conjugated polyclonal antibodies were added to the wells. After 90 minutes of incubation at room temperature, sample wells were washed to remove unbound samples and enzyme-labeled antibodies. The cut-off control corresponds to calibrator one. If the absorbance of the sample was higher than that of the cut-off, the sample was positive for the presence of specific Ag. The ratio between optical density (OD) value of the sample and that of the cut-off was calculated. Cut off: Negative, less than 15; Gray zone, 15–19.9 and positive more than 20, (**Choi** *et al.,* **2011**).
Rapid/Ag (T2):
Stool test was performed in the immune parasitology laboratory, department of Zoology, Central laboratories, Faculty of Science, Al-Azhar University using one Step *H. pylori* Card test (ABON Biopharm Co 2015). In this method, 2gm of each stool sample have been emulsified in 5ml of normal saline in a test tube, the emulsified stool samples were allowed to sediment, a stool test strip was dropped into the mixture and allowed to absorb it. Two red lines at the middle of the strip indicate a positive result, while negative result is an indication of only one red line (**Ahmed** *et al.,* **2016**).
Blood collection:
Serum samples were obtained in an empty vacutainer tube for the preparation of serum. The serum was obtained by allowing the blood to clot at room temperature for two hours and the tube was then centrifuged. Then serum was removed and stored at -80 °C till all samples were collected.

Rapid/Ab (T3): One step *H. pylori* Card test according to the protocol was performed in the immunoparasitlogy laboratory, department of Zoology, Central laboratories, Faculty of Science, Al-Azhar University, Egypt. In this method, one drop of serum was taken and applied to the sample well of strip in the kit. Result of the test was read after 15-20 minutes, two red lines at the middle of the strip indicate a positive result, while negative result is an indication of only one red line (**Al-Jumaily *et al.,* 2015**).

Levels of C3 and C4:

Levels of C3 and C4 were measured according to the standard procedure provided with the kits supplied from Biocientifica S.A. Argentina, Radial Immunodiffusion (RID) plates for determination of Immunoglobulin and other proteins in biological fluids. The procedure consists of an immuno precipitation in agarose between an antigen and its homologous antibody. It is performed by incorporating one of the two immune reactants (antibody) into wells duly punched in the gel. Antibody diffuses radially out of the well into the surrounding gel-antigen mixture, and a visible ring of precipitation forms where the antigen and antibody reacted, Ring diameters are measured by hand lens (0.1mm precision) then concentration were determined from the tables (**Shaldoum *et al.,* 2012**).

3. Results

The current study showed that, from the total 100 patients; 50% were positive using T1, 89% were positive using T2 and 64% were positive using T3, to *H. pylori* (**table 1 and fig. 1**).

From the total of 100 patients included in this study, male patients had a slightly higher of infection rate (28% and 47%) than female (22% and 42%) using T1 and T2 respectively, while female patients had a slightly higher rate (33%) of infection than male (31%) using T3. The differences observed in the gender were not statistically significant (**table 1 and fig.1**).

Table 1: The frequency of *Helicobacter pylori* distribution detected by ELIZA/Ag, Rapid antigen test and Rapid antibody test regarding sex and age groups (n=100).

Variable		ELISA/Ag test %		Rapid/Ag test %		Rapid/Ab test %	
		Positive	Negative	Positive	Negative	Positive	Negative
		50	50	89	11	64	36
Age	**Young** (19)	03	16	15	04	09	10
	Middle (65)	39	26	61	04	42	23
	Old (16)	08	08	13	03	13	03
P		0.17		0.005*		0.24	
Sex	**Male** (54)	28	26	47	07	31	23
	Female (46)	22	24	42	04	33	13
P		0.9		0.34		0.12	

Age groups: Young from 0-20 years, Middle from 21-40 years and Old from 41-70 years. P≤0.05 is significant

The highest positive result to infection with *H. pylori* (39%, 61% and 42%) was found in the Middle age group using T1, T2 and T3, respectively. While the lowest positive result (03% and 9%) was found in the Young age group using T1 and T3 respectively and (13 %) was found in the Old age group using T2. The differences observed in the age groups using T1 and T3 were not significant while those observed using T2 were extremely significant (**table 1 and fig.1**).

T1: 50% of all 100 patients were positive (<20) to *H. pylori* using T1, 34% were in crazy zone (15-19.9) and 16%of patients were negative (>15).

T2: 89% of patients were positive to *H. pylori* using T2 and 11% were negative. All positive samples using T1 were also positive (agree) using T2 and all negative samples using T2 were also negative (agree) using T1. Only 22% of negative samples using T1 were also negative (agree) using T2. About 78% of positive samples using T2 were negative samples (disagree) using T1 test (**table 2 and fig.2**).

T3: 64% of patients were positive to *H. pylori* using T3 and36% were negative. Out of 50 patients that were positive using T1 test, 41 samples were also positive using T3, only 82% of positive samples using T1 (agree) were also positive using T3. Also, only 54% of negative sample using T1 (agree) were negative using T3. About 46% of positive samples using T3 test were negative (disagree) using T1 test. Only 18% of negative sample using T3 test were positive sample (disagree) using T1 test (**table 3 and fig.3**).

Complement C3 and C4: C3 and C4 were measured in 46 patients: 41 samples that were positive to *H. pylori* (56% male and 44% female) that were diagnosed by T1, T2 and T3 and 5 cases that were not infected (3 males and 2 females), **table 3 and fig. 3**.

Male patients had 20% of abnormal complement C3 and 36% of normal complement C3. Male patients had 39% of abnormal C4 and 17% of normal C4. Female patients had also 20% of abnormal C3 and 24% of normal C3. Female patients had 29% of

abnormal C4 and had 15% of normal C4 (**table 4 and fig. 4**).

In the Young age groups; 4.9% of patient had abnormal C3, 2.4% of patients had normal of C3 and 2.4% of patients had abnormal C4, 4.9% of patients had normal C4. In the Middle age group; 29% of this patients had abnormal C3 and 46% of patients had normal of C3 and 54% of patients had abnormal C4, 22% of patients had normal C4. In the Old age group; 4.9% of patients had abnormal C3, 12% of patients had normal of C3 and 12% of patients had abnormal of C4, 4.9% of patients had normal of C4 (**table 4 and fig. 4**).

Comparison between normal and abnormal levels of C3 and C4 in patients infected with *H. pylori* revealed that; 16 (40%) had abnormal level of C3, and 28 (68%) had abnormal level of C4 (**table 5 and fig.5**).

The mean of complement components C3 inpatients infected with *H. pylori* was 85.7 mg/dl and in subjects without infection was also 110 mg/dl (where the normal range for C3 is 80-160 mg/dl). The mean of complement component C4 inpatients infected with *H. pylori* was 17.6 mg/dl lower than normal range while in subjects without infection was normal 23.4 mg/dl (where the normal range for C4 is 20–40 mg/dl) **table 5, fig. 6.**
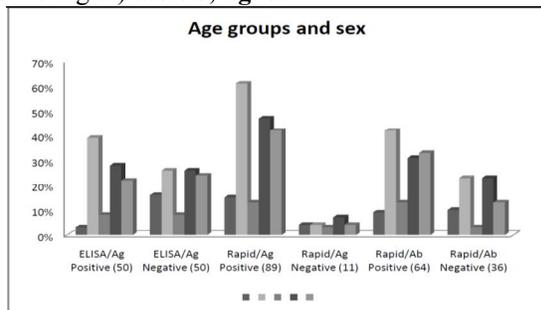

Figure 1: The frequency of *Helicobacter pylori* distribution detected by ELIZA/Ag, Rapid antigen test and Rapid antibody test regarding age groups and sex (n=100)

Table 2: Agreement and disagreement between ELISA/Ag and Rapid/Ag test

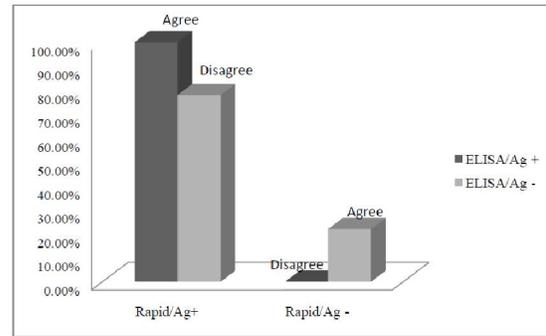| Status	Rapid/Ag + (89)	Rapid/Ag –(11)
ELISA/Ag+ (50)	Agree 50 (100%)	Disagree 0 (0%)
ELISA/Ag – (50)	Disagree 39 (78%)	Agree 11 (22%)

Figure 2: Agreement and disagreement between ELISA/Ag and Rapid/Ag test

Table 3: Agreement and disagreement between ELISA/Ag and Rapid/Abtest

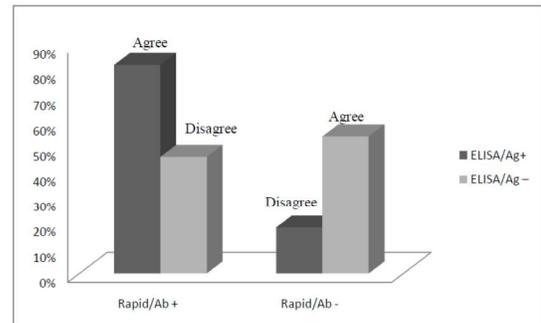| Status	Rapid/Ab + (64)	Rapid/Ab - (36)
ELISA/Ag+ (50)	Agree 41 (82%)	Disagree 09 (18%)
ELISA/Ag – (50)	Disagree 23 (46%)	Agree 27 (54%)

Figure 3: Agreement and disagreement between ELISA/Ag and Rapid/Abtest
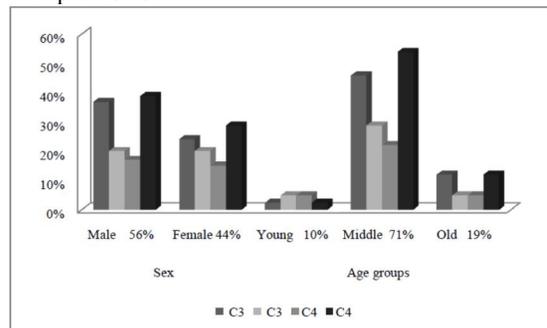

Figure 4: The normal and abnormal C3 and C4 in patients infected with *H. pylori* regarding gender and age groups

Table (**4**): The frequency of *Helicobacter pylori* determined by C3 and C4 regarding sex and age groups (n=41).

Variable (41)		Disagree	Complement C3		Complement C4	
			Normal	Abnormal	Normal	Abnormal
Sex	**Male** 23 (56%)		15 (36%)	08 (20%)	07 (17%)	16 (39%)
	Female 18 (44%)		10 (24%)	08 (20%)	06 (15%)	12 (29%)
Age groups (Mean = 31.5 years)	**Young** 03(10%)		01 (2.4%)	02 (4.9%)	02 (4.9%)	01 (2.4%)
	Middle 22(71%)		19 (46%)	12 (29%)	09 (22%)	22 (54%)
	Old 06(19%)		05 (12%)	02 (4.9%)	02 (4.9%)	05 (12%)

Age groups: Young from 0-20 years. Middle from 21-40 years. Old from 41-70 years

Table 5: The normal and abnormal C3 and C4 in patients infected with *H. pylori* and cases without infection

Variable	Grades	Infected with *H. pylori* No (%)	Without infection (%)	p value
C3	Abnormal	16 (40%)	00 (00.0%)	0.214
	Normal	25 (60%)	05 (100%)	
	Mean ± SE	85.7±4.6	110±3.8	
C4	Abnormal	28 (68%)	01 (020%)	0.0001***
	Normal	13 (32%)	04 (080%)	
	Mean ± SE	17.64±1.2	23.4±2.9	
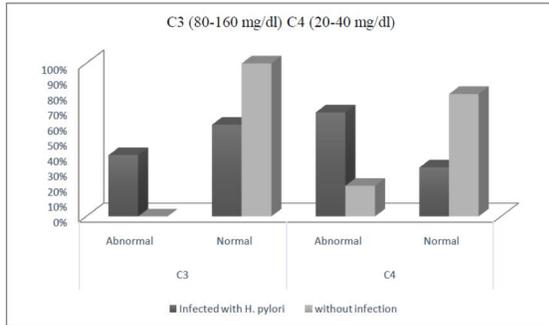
Figure 5: Normal and Abnormal of C3 and C4 inpatients infected with *H. Pylori* and cases without infection
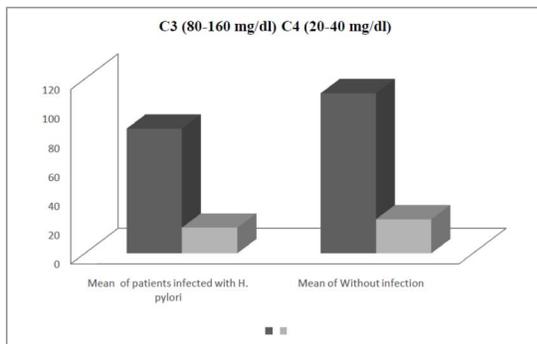
Figure 6: Mean of C3 and C4 in patients infected with *H. Pylori* and cases without infection

4. Discussion

Many diagnostic tests for *H. pylori* infection may have false negative results and using of multiple tests may help to provide a more accurate diagnosis of *H. pylori* infection (**Miftahussurur** and **Yamaoka 2016**). Detection of *H. pylori* antigen in stool, and antibodies in serum, has been strongly recommended as it is less expensive and more patient-friendly (**Awuku *et al.,* 2017**).

The prevalence of *H. pylori* was associated with age **(Zhang *et al.,* 2009)**. The current study showed that the prevalence of *H. pylori* infection was lowest in the Young age and increased in the middle age. These current results agree with **Shu *et al.* (2017,** in China); **Us and Hasçelik** (**1998,** in Turkey**)** and **Rashid *et al.* (2017,** in Pakistan) who observed that a significant increase in rate of infection was observed with increasing age. This may be due to the growing of age, expanding range of activity, collective living and meal

in high decades lead to the increase of exposure to *H. pylori* infection. Controversy to the current study, **Nasserolahei and Khalilian (2004,** in Iran*)* showed high prevalence of *H. pylori* infection in the first decades of life (10 to 19 years) and in the old life (60 years). The differences between the results may due to some factors as crowd, education, smoking, daily activity of the patients rather than age in infections or due to the environment and problems in social behavior**.**

In the present study, high level of *H. pylori* infection was observed among male patients. This finding agrees with **Talley (1992,** in the North-Central part of Nigeria**)** who found that most (60%) of the infected subjects were males. In contrasted to the current results, **Naji et *al.,* (2014,** in Yemen**)** found that the most of patients infected with *H. pylori* were females as compared with males and **AL-Segar (2007,** in Iraq) showed equality between the ratios of males to females**.** The variation among different studies can be explained that the gender is not considered as a risk factor in *H. pylori* infection (**Tadesse *et al.,* 2014** and **Rashid *et al.,* 2017**).

In the present study, detection of *H. pylori* has been preferred using ELISA/Ag (T1) or Rapid/Ag (T2) from stool. It is particularly appropriate for children as stool can be obtained from them without their active collaboration. Also, **Pourakbari *et al.* (2013**) and **Garza-González *et al.* (2014**) documented that *H. pylori* stool antigen test perform well in children. Another published study preferred analysis of H. pylori using ELISA/Ag tests than other tests (**Krogfelt *et al.,* 2005; Razaghi *et al.,* 2010).**

The prevalence of *H. pylori* using T2, in the present study, was positive at rate of 89%. This result is comparable with **Douraghi *et al.* (2013)** who recorded that the prevalence of *H. pylori* was 93.1%. Several other studies showed low prevalence of H. pylori using T2: **Jafar *et al.,* (**64.2%, **2013**); **Gulcanem *et al.,* (**46.3%, **2005**); **Issa *et al.,* (**45.7%, **2014); Kato *et al.,* (**30%, **2003**). The variation among different studies can be explained by insufficient amount of antigen in the stools, the sample size, the studied population, social economic, population density, geographical characteristics and may be quality of kit.

Using T3 in the present study, the prevalence of *H. pylori* was positive at rate of 64%. This result is concordance agrees with several other studies showed low prevalence of H. pylori using T3: **Luthra** (63%, **1998**); **Diab** *et al.,* (71.7%, **2009**); **Naji et al.** (72%, **2014**). Opposite to these findings, another study by **El Dine** *et al.,* (2008 in Egypt) recorded that the overall seropositive rate of *H. pylori* was 91.7%. IgG antibodies can be detected approximately 3 weeks after *H. pylori* infection. Therefore, the latent period between *H. pylori* infection and antibody production may be a source of false negative. Antibodies titers remain high for months after elimination of infection which may be a source of false positive (**Choi** *et al.,* **2011**). Regarding positive data all T1, T2 and T3 are in high agreement while low agreement between them is noticed in case of negative results. Hence negative test results should probably be confirmed with another test before eradication therapy is prescribed.

The complement system is one of the natural defense mechanisms that protect the human body from infections and perhaps tumors. Quantitative measurement of complement components may enable diagnosis of immunologic disorders, especially those associated with deficiencies of complement components (**Warner**, **1998**). The present work showed abnormal level of C4 in 68% and of C3 in 40% of patients. Activation of C4 is frequently found to be a more sensitive measure of classical pathway of activation due to bacteria activate complement system through delayed inducement of antibody to immune system. **Desar** *et al.* **(2009)** agreed with this point of view and observed that *H. pylori* is complement-sensitive and activates the classical pathway. Bacteria immediately activate C3 by Antigen through alternative pathway. However, **Hussain** *et al,* **(2008)** suggested that low C4 levels may falsely be regarded as classical pathway activation (reduced levels of total C4, reduced synthesis or increased catabolism of C4 without corresponding complement activation) and that several other factors may explain low C4 level. In the current work, the low level of C4 was associated with the development and exacerbation of *H. pylori.*

Conclusion:

No single test can be considered as the master test for diagnosis of *H. pylori* infection. The detection of *H. pylori* infection using antigens or antibodies test can be supported with test for C3 and C4. Bacteria immediately activate complement system by Antigen through alternative pathway and delayed activation of complement system through classical pathway occurs by secreted specific antibodies. The association between low levels of C3 and C4 with the development and exacerbation of *H. pylori* suggests possible use of changed levels of C3 and C4 as a biomarker for *H.pylori* infections.

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