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Immunological and molecular detection of *Helicobacter pylori* in patients clinically diagnosed with chronic urticarial and atopic dermatitis

Tasneem Z. Abd-Alrahman[1], Rana S. Aboud[1] and Saife D. Al-Ahmer[2,*]

[1] Department of Biology, College of Science, Baghdad University, Iraq.
[2] Institute of Genetic Engineering and Biotechnology for Post Graduate Studies,Baghdad University, Iraq.

* Corresponding author: Saife D. Al-Ahmer; e-mail: saifealahmer@yahoo.com

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ABSTRACT

To determine the relationship between *Helicobacter pylori* infection and skin disorders, sixty six patients who suffering from skin diseases include chronic urticarial (CU) and atopic dermatitis (AD) who attended at Dermatological Clinic/ Al-Numan Teaching Hospital from the beginning of October 2015 to the end of January 2016 with age (6-62) have been investigated and compared to twenty two samples of apparently healthy individuals were studied as control group. All the studied groups were subjected to measurement of anti-*Helicobacter pylori* IgG antibodies by enzyme linked immuno sorbent assay (ELISA) and detection of *16S rRNA* and *CagA* genes by using singleplex and multiplex PCR methods. The results of current study revealed that there was a highly significant elevation (P<0.01) in concentration of *H. pylori* IgG antibodies in sera of the CU and AD patients compared with control group, also the results revealed that there was a significant elevation (P<0.05) in concentration of *H. pylori* IgG antibodies in sera of the CU patients compared with control group, and significant elevation (P<0.05) in concentration of *H. pylori* IgG antibodies in sera of AD patients compared with control group. The results of present study indicated that 26(66.67%) patients out of 39 CU patients were positive for both of *16S rRNA* and *CagA* genes, while 13(33.33%) patients out of 39 CU patients were negative for those genes. Also, the results revealed that 19(70.37%) patients out of 27 AD patients were positive for both of *16S rRNA* and *CagA* genes, whereas only 8(29.62%) were negative for both those genes comparing with control group which showed 1(4.54%) individuals out of 22 apparently healthy individuals were positive for both *16S rRNA* and *CagA* genes, the statistical analysis was highly significant (P<0.01).

Keywords: Chronic urticarial, atopic dermatitis, *Anti-Helicobacter pylori* Abs, *16S rRNA* gene, *CagA* gene.

1. INTRODUCTION

Helicobacter pylori (H. pylori), a microaerophilic, flagellated, curved or spiral, Gram-negative bacterium, colonizes the human stomach. Its infection is widespread throughout the world, and is present in about 50% of the global human population; with 80% in developing countries and 20-50% in industrialized countries [1, 2]. Infection in the stomach with *H. pylori* causes inflammation of the gastric mucosa, which lead to gastritis, duodenal or gastric ulcer and even in rare cases to gastric carcinoma or mucosa associated lymphoid tissue (MALT) lymphoma [3].

There are two types of *Helicobacter pylori* strains type I and II. Type I strains express cytotoxin associated antigen (CagA) and vacuolating cytotoxin antigen (Vac A), while type II strains do not express any antigens [4]. Translocation of CagA into the epithelial cells of gastric mucosa leads to increased levels of pro-inflammatory cytokines such as tumour necrosis factor–α, interleukin (IL)-6, IL-10, and IL-8. VacA protein interacts with macrophages, B- and T-lymphocytes and causes reduced IL-2 production with suppression of IL-2-mediated T lymphocyte proliferation. The interaction between *H. pylori* and B-lymphocytes results in

uncontrolled growth and proliferation of predominantly CD5+ B-cells that produce polyreactive and autoreactive immunoglobulin M (IgM) and immunoglobulin G3 (IgG3) antibodies. The antibodies produced do not result in clearance of the HP and may result in production of autoreactive antibodies, such as anti-H/KATPase antibodies [5].

In the past 10 years, an increasing number of research concerning the association between *H. pylori* infection and extra digestive conditions such as haematological, cardiovascular, neurological, metabolic, autoimmune, and skin diseases [6]. *Helicobacter pylori* infection triggers a marked local inflammatory response and a chronic systemic immune response. It is possible that inflammatory mediators released during the immune response to *H. pylori* infection play a role in the pathogenesis of skin diseases [7]. There are several diagnostic assays for *H. pylori* include bacterial culture, urease test, urea broth test, histology, serology, stool antigen test and the molecular methods such as polymerase chain reaction (PCR), the potential to accurately determine both the presence of infection and the genotype of bacteria, and have marked sensitivity and specificity. The PCR technique has been used to detect the *H. pylori* DNA in human blood and bacterial colonies by amplifying the *16S rRNA* gene [8, 9]. This study aims to evaluate the association of *H. pylori* infections with persistence of chronic urticarial and atopic dermatitis skin diseases in human.

2. MATERIALS AND METHODS

2.1 Patients and methods

The present study included sixty six patients who are suffering from skin diseases (chronic urticarial and atopic dermatitis) attended at Dermatological Clinic/ Al-Numan Teaching Hospital. The samples were collected from the beginning of October 2015 to the end of January 2016. The ages of the total patients ranged from 6-62 years. Twenty two samples of apparently healthy individuals (control group) including16 males and 6 females were studied as control group. All the samples were marked by number of samples, name of patient and the day of sample collection.

2.2 Blood samples collection

Five milliliters of blood sample was collected from patients clinically diagnosed with chronic urticarial and atopic dermatitis and healthy individuals, and then the blood sample was divided for 2 ml into EDTA tube for DNA extraction and 3 ml into plain tube to collect the serum for immunological tests. All the serum samples were stored at -20ºC until use.

2.3 Immunological detection of anti-*Helicobacter pyloi* IgG by ELISA method

All the studied groups include the chronic urticarial and atopic dermatitis patients and apparently healthy individuals (control group) were submitted to estimate the anti-*Helicobacter pyloi* IgG antibodies level by using ELISA test (Germany) according to a protocol of the kit according to the method described in [10].

2.4 Molecular detection of *Helicobacter pylori* by singleplex and multiplex PCR methods

a) DNA extraction: DNA was extracted from blood samples that were collected from the patients clinically diagnosed with chronic urticarial and atopic dermatitis and from the healthy individuals using a commercial gSYNCDNA extraction kit and according to the manufacturer's instructions. All the extracted DNA samples were stored at -20˚C until use.

b) DNA quantification: The extracted DNA from blood samples that collected from the patients clinically diagnosed with urticarial and atopic dermatitis and from the healthy individuals was quantified by spectrophotometer at O.D. 260/280 nm.

c) Primers selection: The primers for *16S rRNA* and *CagA* genes of *H. pylori* as the target genes for this study were selected according to [11] and [12], respectively. These sets of primers were designed based on the conserved regions in *H. pylori*, primers were synthesized by Alpha DNA, Kanda. The name, sequence and expected product size of these primers are shown in table [1].

Table 1: Primer name, sequence and expected product size of *16S rRNA* and *CagA* genes of *H. pylori*.

Primer name	Primer sequence (5'→3')	Expected product size (bp)
16Sr-F	GCTAAGAGATCAGCCTAT	~522
16Sr-R	TGGCAATCAGCGTCAGG	
CagA-F	GATAACAGGCAAGCTTTT	~394
CagA-R	CTGCAAAAGATTGTTTGG	

d) Singleplex PCR Master Mix of *16S rRNA* and *CagA* genes: The singleplex PCR master mix for detection of *16S rRNA* and *CagA* genes of *H. pylori* was performed individually for each one of those genes in 20 µl volumes containing 4 µl of nuclease free water, 5 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 3 µl of 10 pmol of forward primers (16Sr-F, CagA-F) and 3 µl of

10 pmol reverse primers (16Sr-R, CagA-R), and 5 µl of DNA sample.

e) Singleplex PCR program of *16S rRNA* and *CagA* genes: The singleplex PCR program was performed individually for each one of those genes and carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by [12] with some modifications. Briefly, the Amplification of *16S rRNA* and *CagA* genes of *H. pylori* was carried out with initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds (for primers of *16S rRNA* gene) and annealing at 54°C for 30 seconds (for primers of *CagA* gene), extension at 72°C for 30 seconds, and then the thermal cycles were terminated by a final extension for 7 minutes at 72°C.

f) Multiplex PCR Master Mix of *16S rRNA* and *CagA* genes: The multiplex PCR master mix for detection of both *16S rRNA* and *CagA* genes of *H. pylori* in one reaction was performed in 50 µl volumes containing 12 µl of nuclease free water, 15 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 3 µl of 10 pmol of each forward primers (16Sr-F, CagA-F) and 3 µl of 10 pmol of each reverse primers (16Sr-R, CagA-R), and 5 µl of DNA sample.

g) Multiplex PCR program of *16S rRNA* and *CagA* genes: The multiplex PCR program for detection of both *16S rRNA* and *CagA* genes of *H. pylori* in one program was carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by [12] with some modifications. Briefly, the Amplification of *16S rRNA* and *CagA* genes of *H. pylori* was carried out with initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds (for primers of both *16S rRNA* and *CagA* genes), extension at 72°C for 30 seconds, and then the thermal cycles were terminated by a final extension for 7 minutes at 72°C.

2.5 Statistical Analysis

The Statistical Analysis System (SAS) [13] program was used to study the effect of difference factors in study parameters. T-test and Chi-square test data was reported.

3. RESULTS AND DISCUSSION

The results of current study revealed that there was a highly significant elevation (P<0.01) in the concentration of *H. pylori* IgG antibodies in sera of patients with chronic urticarial (CU) and atopic dermatitis (AD) (29.24± 1.54) U/ ml compared to control group (21.79± 3.03) U/ml, as shown in table [2]:

Table 2: Mean level of anti *H. pylori* IgG antibodies U/ml in sera of chronic urticarial (CU) and atopic dermatitis patients (AD) and control group.

Group	No.	Mean ± SE of IgG concentration
Patient's groups (CU, AD)	49	29.24 ± 1.54
Control group	22	21.79 ± 3.03
LSD value	-	6.136 **
P-value	-	0.0112
** (P<0.01)		

Table 3: Mean level *H. pylori* IgG antibodies U/ml in sera of the chronic urticarial patients (CU) and control group.

Group	No.	Mean ± SE of IgG concentration
Chronic urticarial patients (CU)	29	28.41 ± 2.12
Control group	22	21.79 ± 3.03
LSD value	-	6.232 *
P-value	-	0.0524
* (P<0.05)		

Also, the results of this study revealed that there was a significant elevation (P<0.05) in the concentration of *H. pylori* IgG antibodies in sera of the chronic urticarial patients (CU) (28.41 ±2.12) U/ml compared to the control group (21.79± 3.03) U/ml, as shown in table [3]. Also, the present study revealed that there was a significant elevation (P<0.05) in the concentration of *H. pylori* IgG antibodies in sera of the atopic dermatitis patients (AD) (30.46±2.25) U/ml compared to the control group (21.79± 3.03) U/ml, as shown in table [4]:

Table 4: Mean level of *H. pylori* IgG antibodies in sera of atopic dermatitis patients (AD) and control group.

Group	No	Mean ± SE of IgG concentration
Atopic dermatitis patients (AD)	20	30.46 ± 2.25
Control group	22	21.79 ± 3.03
LSD value	-	7.767 *
P-value	-	0.0297
* (P<0.05)		

While there were significant differences (P<0.05) in the concentration of *H. pylori* IgG antibodies in sera of the atopic dermatitis patients (AD) according to the ages. The total concentration of IgG antibodies were (26.12±23.7)U/ml in sera of the atopic dermatitis patients (AD) within age group less than 40 years compared to (24.96±2.49) U/ml in sera of the atopic dermatitis patients (AD) within age group more than 40 years as shown in table [5].

Table 5: Effect of gender and age in Level *H. pylori* IgG antibodies with atopic dermatitis disease (AD).

The factors	No.	Mean ± SE of IgG concentration
Gender		
Male	8	30.78 ± 4.59
Female	12	30.25 ± 2.36
LSD value	-	9.336 NS
P-value	-	0.906
Age group		
Less than 40	13	26.12 ± 237
More than 40	7	24.96 ± 2.49
LSD value	-	7.588 *
P-value	-	0.0509
NS: Non-significant, * (P<0.05)		

The results of present study indicated that 26(66.67%) patients out of 39(100%) chronic urticarial patients (CU) were positive to *16S rRNA* and *CagA* genes, while 13(33.33%) patients out of 39(100%) chronic urticarial patients (CU) were negative to both of these genes. Also, the results of current study revealed that 19(70.37%) patients out of 27 (100%) atopic dermatitis patients (AD) were positive to *16S rRNA* and *CagA* genes, whereas only 8(29.62%) out of 27 (100%) atopic dermatitis patients (AD) were negative to both of these genes comparing with control group which showed 21(95.45%) out of 22(100%) healthy individuals (control group) were negative for *16S rRNA* and *CagA* genes and only 1(4.54%) person out of 22(100%) healthy individuals was positive to both of these genes, the statistical analysis showed highly significant different (P<0.01) as shown in table [6], figure [1], figure [2] and figure [3].

Table 6: Distribution of chronic urticarial patients (CU), atopic dermatitis patients (AD) and control group according to positive result for *16S rRAN* and *CagA* genes.

Type of group	Total No.	Positive No. (%)	Negative No. (%)	Chi-square
Chronic urticarial patients (CU)	39	26 (66.67%)	13 (33.33%)	9.721 **
Atopic dermatitis patients (AD)	27	19 (70.37%)	8 (29.62%)	11.856 **I
Control group	22	1 (4.54%)	21 (95.45%)	14.476 **
Chi-square	-	12.405 **	12.369	-
** (P<0.01).				

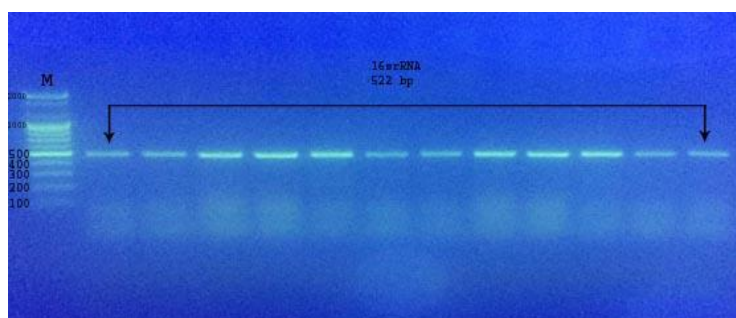

Figure 1: Gel electrophoresis of singleplex PCR products of *16S rRNA* gene of *Helicobacter pylori* isolates using 1.5% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-13: Singleplex PCR products of *16S rRNA* gene.

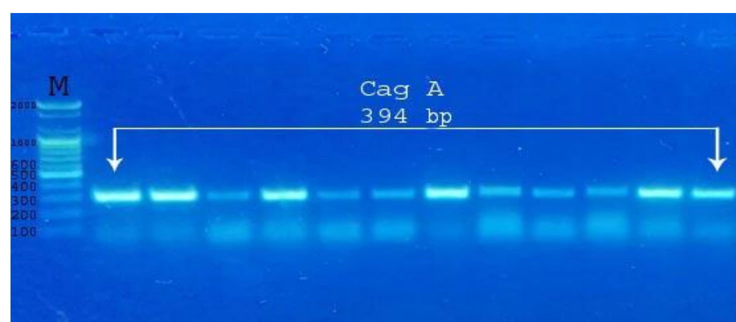

Figure 2: Gel electrophoresis of singleplex PCR products of *CagA* gene of *Helicobacter pylori* isolates using 1.5% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-13: Singleplex PCR products of *CagA* gene.

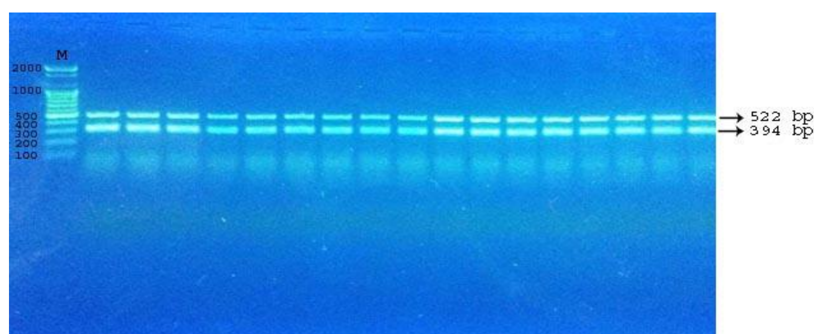

Figure 3: Gel electrophoresis of multiplex PCR products of *16S rRNA* and *CagA* genes of *Helicobacter pylori* isolates using 1.5% agarose gel at 7volt /cm for 1 hour. Lane 1: 100 bp DNA ladder, lane 2-18: Multiplex PCR products of *16S rRNA* and *CagA* genes.

The results of the present study were in agreement with other studies. One study showed the prevalence rate of *H. pylori* infection was 49, 74% in urticarial group compared to control group [14]. While [15] showed that *H. pylori* infection identified in 43(43%) of 100 patients with chronic urticaria. Other study assessed 21 patients with chronic urticaria and *H. pylori* infection [16]. While [17] showed that the prevalence of infection was found to be higher in adults than in children, and higher prevalence in men than in women. Another study in Italy reported an increase in anti *H. pylori* IgG antibody in children with AD compared to control group [18]. Also, the result of present study agrees with other study done by [8] who showed that 20(100) % of cases positive result from human blood by PCR. While [19] indicated that 18% from serum sample contain CagA DNA.

Several factors lead to translocation of *H. pylori* across the gastrointestinal barrier and lead blood stream include disruption of the ecology of micoflora lead to overgrowth of bacteria, impaired host immunity and physical disruption of intestinal barrier by endotoxin. Also, infection with *H. pylori* decrease synthesis of gastric mucin producing inflammatory response and induce changes in the endothelial lining of the vessels [20].

The postulate role of *H. pylori* in the pathogenesis of extra-intestinal disorders is based on the fact that local inflammation has systemic effects. *H. pylori* colonize gastric mucosa and induce strong inflammatory responses with release of various bacterial and host-dependent cytotoxic substances. Gastric *H. pylori* infection is a chronic process lasting for decades, and persistent infection induces a chronic inflammatory and immune response able to induce lesions both locally and remote to the primary site of infection. The systemic effects may involve increased mucosal permeability to alimentary antigens, immunomodulation, an autoimmune mechanism or the impairment of vascular integrity. The various immunopathogenesis during the *H. pylori* –caused disease might be due to an unbalanced TH1 or TH2 mediated response post infection [21, 22].

Several theories have been proposed to explain this finding, first, as full antigens; *H. pylori* is able to cause allergy and immune responses. Chronic infection with *H. pylori* cause production of specific antibodies such as IgG and IgA antibodies to 19-kDa *H. pylori*-associated lipoprotein were found to play a role in the pathogenesis of CU. When these antibodies and IgE-mediated immune response against *H. pylori* antigens, some bacterial immunoresponsive proteins were identified in cases of CU. Second, the cagA protein of the virulent strain stimulates the gastric epithelium to secrete inflammatory cytokines such as IL-1, TNF-, LTC4 and PAF, may play a role in the pathogenesis of urticarial lesions. Third the infection process development impairs the barrier function of the alimentary tract, and this creates conditions for allergic food particles to enter bloodstream. Fourth, *H. pylori* may up regulate the cytotoxic eosinophilic cationic protein secreted by activated eosinophiles, which contributes to the etiopathogenesis of chronic urticaria. Also, infection with *H. pylori* might be a source of circulating immune complexes and these immune complexes may trigger urticaria [14, 16, 23].

Regard with ELISA technique, fast, easy, and relatively in expensive could identify patients who have been infected with the organism. This method is not useful in confirming eradication of *H. pylori* [24]. Molecular methods such as PCR have the potential to accurately determine both the presence of infection by amplifying *16S rRNA* gene. The 16S rRNA gene is one of the specific targets to confirm *H. pylori* infection and positive amplification of *H. pylori* specific DNA may be considered as a direct evidence for the presence of the pathogen [25, 26].

4. CONCLUSION

Helicobacter pylori may have a role in the pathogenesis of chronic urticarial and atopic dermatitis, and the results of present study suggest that *H. pylori* infection should be included in diagnostic workup of patients with skin disorders. In addition, several evidences may revealed that *H. pylori* is based upon inaccurate isolate identification, resulting from inadequate identification methods, on the other hand, *16S rRNA* and *CagA* genes, appeared to be useful molecular tool for detection of *H. pylori* isolates in chronic urticarial and atopic dermatitis patients and, they could represented a useful molecular method for detection of *H. pylori* isolates in other human diseases.

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