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Molecular screening of *Helicobacter pylori* among Egyptian patients with decompensated liver cirrhosis

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Abstract

Background: Globally *Helicobacter Pylori (H. Pylori)* infection and liver diseases are renowned with their high incidence rate. Recent studies have observed the relation between *H. Pylori* infection and liver disease progression to cirrhosis. This study aimed to determine the prevalence of *H. pylori* in liver cirrhotic patients. **Patient and Methods:** This study was conducted on 100 liver cirrhotic patients to detect *H. Pylori* infection based on *UreA* gene detection in ascites using nested polymerase chain reaction (nPCR). Biochemical analysis was performed on ascitic fluid, serum, complete blood count (CBC), and polymorph nuclear leukocytes count (PMN). **Result:** *H. Pylori* infection was detected in 75% of patients with liver cirrhosis. In ascitic fluid, *H. pylori* infection showed a highly significant association with albumin and PMN (P=0.0001) and also with other biochemical analyses including serum ascites albumin gradient (SAAG), specific gravity, and glucose (P=0.005, 0.0031, and 0.02, respectively). In serum only, the infection showed significant association with alkaline phosphatase, albumin, and hemoglobin with P values of 0.008, 0.014, and 0.008, respectively. **Conclusion:** The current study results shed light on the significant role of *H. Pylori* infection in ascites of patients with decompensated end-stage liver cirrhosis suggesting a prospective relation between *H. pylori* infection and liver diseases progression due to hepatic infections especially HCV.

Key words: *H. pylori*, Nested PCR, *UreA* gene, Liver cirrhosis, HCV

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1. Introduction

Helicobacter pylori is a slow-growing, microaerophilic, highly motile, a gram-negative spiral bacterium. In developed countries, infection with this type of bacteria reached 50%, while in developing countries, infections may reach 90% **(Mitchell and Katelaris. 2016).** *H. Pylori* infection closely correlates with chronic gastritis, peptic ulcer, gastric carcinoma, and malignant lymphoma of gastric mucosa related lymphoid tissue (MALTOMA). In addition, it has been recognized as a class I carcinogenic factor by WHO. This type of bacteria may cause extragastric organ disturbances including aggravate cardiovascular diseases and metabolic diseases and affect the liver functions, especially in patients with liver cirrhosis (**Waluga *et al*., 2015).** Molecular methods are vastly used for diagnosing *H. pylori* infection for analyses of variance and studying the virulence and resistance patterns (**Cirak *et al*., 2007**). However, the high level of genomic plasticity between *H. pylori* different strains complicates the choice of target genes. Diversity of genes such as *cagA* , *ureC* (*glmM*) , *ureA* , 16S rRNA, and 26-kDa species-specific antigen (SSA) genes have been used as targets (**Lage *et al*., 1995**). *UreA* gene was used as a target in several studies due to its good sensitivity and specificity (**Uribe *et al*., 1998 and Kawamata *et al*., 1996)**.

Hepatitis C virus (HCV) is the most prevalent infectious agent of hepatic diseases. It has severe complications varying from chronic inflammatory diseases, hepatic cirrhosis, and end-stage liver diseases to hepatocellular carcinoma (HCC) (**Butel *et al*., 2007**). Egypt has a high prevalence of HCV where it is recognized to be the main cause of high morbidity and mortality caused by liver diseases (**El-Zanaty and Way 2008**). Cirrhosis is scarring of the liver where the normal liver tissue is damaged and replaced with regenerated hepatic nodules surrounded by fibrous tissue septa. Cirrhosis eventually develops in 0 to 50% of patients with chronic HCV and HBV infection; however, it is not clear why only a proportion of patients with these infections progress to end-stage disease or by what mechanisms HCV-related cirrhosis may ultimately lead to HCC. Moreover, cirrhosis is the final common stage in patients with progressive liver diseases of various causes. It is recognized as the leading cause of death in people between the ages of 25 and 64 years (**Kung *et al*., 2008**).

Bacterial infections in patients with end-stage liver diseases affect their candidacy for liver transplantation as up to one-third of all hospitalized patients with cirrhosis are infected with sepsis (**Fernandez *et al*., 2012 and Merli *et al*., 2010**) which increases their mortality rate to more than 50% (**Olson *et al*., 2011**). With regard to liver diseases, *H. pylori* infection has been reported in the liver tissue by detecting its DNA suggesting that these bacteria could be participating in the progression of the disease to cirrhosis and HCC (**Ponzetto *et al*., 2000**). The association between *H. pylori* and liver diseases has been observed but remains unknown (**Waluga *et al*., 2015)**. The incidence of *H. pylori* or *Helicobacter* species infection was reported in patients with various liver diseases (**Esmat *et al*., 2012 and Pirouz *et al*., 2009)**.

The main objective of the present study is to determine the prevalence of *H. pylori* infection among Egyptian patients with decompensated liver cirrhosis by using nested PCR targeting the *UreA* gene in ascites.

2. Patients & methods

2.1. Study design & individuals

This prospective cross-sectional study was conducted on Egyptian patients with end-stage chronic liver disease who were referred to the Hepatology and Tropical Medicine Research Institute, Al-Kasr Al-Ainy Faculty of Medicine, Cairo University for liver work-ups from March 2016 to May 2017. Clinical and laboratory parameters including etiology of cirrhosis, sex, age, liver function tests, and complete blood count were assessed at the baseline (date of paracentesis and/or endoscopy).

2.2. Sample Collection

Venous blood samples were collected by plastic disposable syringes. After centrifugation, the resulted sera stored immediately at -20°C until use. Ascites fluid was obtained by abdominal paracentesis in the first 24 hours after admission of patients using aseptic techniques according to **Runyon 1986.**

2.3. Biochemical and molecular analysis

The serological and biochemical analyses were conducted on both serum and ascites fluid in the Clinical Pathology Department, Al-Kasr Al-Ainy Faculty of Medicine, Cairo University, while the molecular analysis was performed at the Lab of Molecular Medical Parasitology (LMMP) at the same faculty. The Research Ethics Committee of the faculty approved the study and informed consent was obtained from the study participants. Sera samples of the study patients were analyzed for albumin, total protein, bilirubin, creatinine, glucose, alkaline phosphatase, ALT, and AST, and the whole blood samples were analyzed for CBC and PMN using dialysis kit (Diagnostic Systems international Mc15, Cat. No.1224, Germany) kinetic method according to the manufacturer's instructions. Ascites fluid samples were analyzed for albumin, protein, SAAG, glucose, and specific gravity**.** Albumin concentration in the serum and ascitic fluid was determined according to **Bakker and Mücke 2007**, albumin concentration in serum and fluid is determined according to these factors: Serum protein concentration**,** Differentiation between serum albumin and ascitic fluid albumin, Serum glucose concentration (**Bakker and Mücke 2007)** and specific gravity (**Light 1979**).

2.4. Molecular assay

2.4.1. Genomic DNA extraction

Genomic DNA was extracted from the ascitic fluid using Zymo research spin columns, isolation mini kit (cat. no D 3024) according to the manufacturer's instructions. The extracted DNA was stored at -20 °C until use.

2.4.2. Nested Polymerase Chain Reaction (nPCR) for H. Pylori

Nested PCR was performed with *Helicobacter* genus-specific *UreA* primers and it included a two-squinted PCR reaction. The first amplification was carried out in a final volume of 25 µl reaction mixture containing: DNA and the primers 2F2 5'ATATTATGGAAGAAGCGAGAGC-3' and 2R2 5'ATGGAAGTGTGAGCCGATTTG-3'. The second reaction amplified by internal sets 2F3 (IF) 5'CATGAAGTGGGTATTGAAGC-3 and 2R3 (IR) 5'AAGTGTGAGCCGATTTGAACCG-3'. The amplification conditions were as follows: 35 cycles of 1 min at 96°C (denaturation), 1 min at 57°C (annealing), 1.5 min at 72°C (extension). Amplification was obtained according to **Sasaki *et al.*, 1999.** The amplified products were analyzed by 1.5 % agarose gel electrophoresis with ethidium bromide at 100 V for 30 min. The band of gels was viewed under ultraviolet light.

2.5. Statistical analysis

All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows. Data were statistically described in terms of mean, standard deviation (SD), median and range, or frequencies (number of cases) and percentages when appropriate. A comparison of the numerical variables between the study groups was done using Student's *t*-test for independent samples in comparing normally distributed data and Mann Whitney *U* test for independent samples in comparing non-normal data. For comparing categorical data, Chi-square (^{2}) test was performed. The exact test was used instead when the expected frequency is less than 5. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Determination of infection with *H. pylori*

The demographic and laboratory data of the 100 patients included in this study were summarized in **Table 1**. The mean age of patients was 58 ±9. The majority of patients were males (68). *H. pylori* DNA was detected in 75% of the included patients using *Helicobacter* genus-specific *UreA* gene primers

(**Fig. 1)**. The infection incidence rate was higher among males than females (69.3%); however, this difference was not statistically significant.

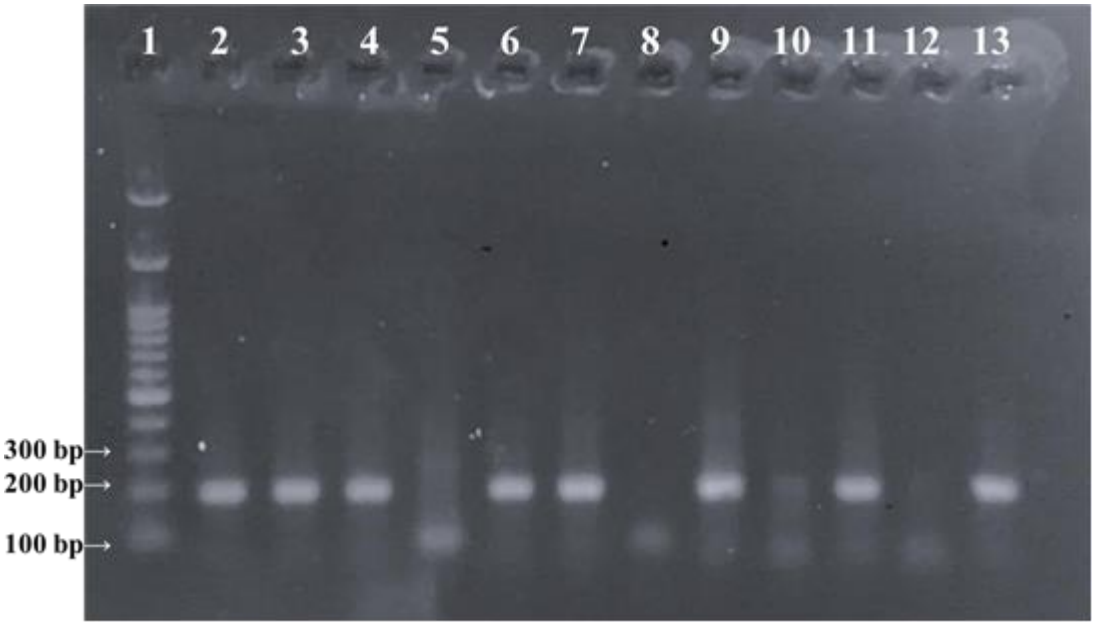

Figure1: Agarose gel electrophoresis for the products of the nPCR targeting *UreA* gene of *H.pylori*at (200bp). Lane 1: 100 bp "ladder". Lanes 2-4, 6, 7, 9 & 11: Positive samples. Lanes 5, 8 & 10: Negative samples. Lane 12: Negative control. Lane 13: Positive control.

Table 1: Demographic and clinical characteristics of tested sero-positive patients

	Tested group (no:100)	**P value**
	mean±SD	
Age (years)	58 ±9	**0.02***
sex		**0.794**
male	68 (68%)	
female	32(32%)	
HB (gms/dL)	9.7 ± 1.9	**0.284**
TLC (cells/mcL)	5.7 ± 2.1	**0.447**
Platlets (Platlets/ mcL)	106 ± 42.8	**0.934**
Bilirubin(mg/dl)	2 ± 1.7	**0.098**
ALT(U/L)	51 ± 34.9	**0.295**
AST(U/L)	59.5 ± 31.9	**0.459**
ALP(U/L)	162 ± 104	**0.23**
Albumin(gms/dl)	2.8 ± 0.5	**0.394**
Creatinin(mg/dl)	1.2 ± 0.6	**0.563**
B.Sugar(mg/dl)	146 ± 59	**0.782**

Data presented as mean± SD, with (*) *P* value < 0.05 is significant.

3.2. Comparison of liver functions between patients with and without H. Pylori infections

The comparisons between levels of liver enzymes in patients with and without *H. pylori* infection were performed in both sera and ascitic fluid and summarized in **Tables 2** and **3**. Among the results, albumin and PMN showed a highly significant association (P=0.0001) with H. pylori infection. Albumin was higher in *H. pylori*-negative cases than in positive cases (negative correlation) (**Fig. 2**), while PMN was higher in *H. pylori*-positive cases than in negative ones (positive correlation) (**Fig. 3)**. Moreover, other variables such as SAAG, specific gravity, glucose, and alkaline phosphatase showed a significant positive association with *H. pylori* infection, while hemoglobin showed a negative association with it (**Table 2 and 3).**

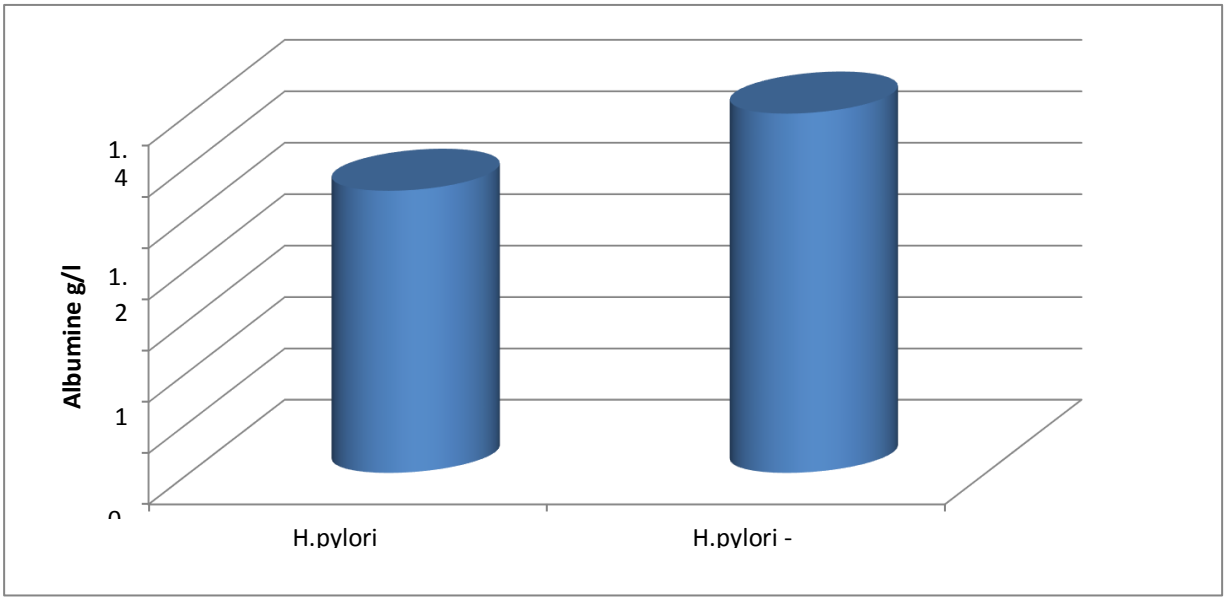

Figure (2): serum albumin (g/dl) between *H.pylori* +ve and *H.pylori* -ve groups

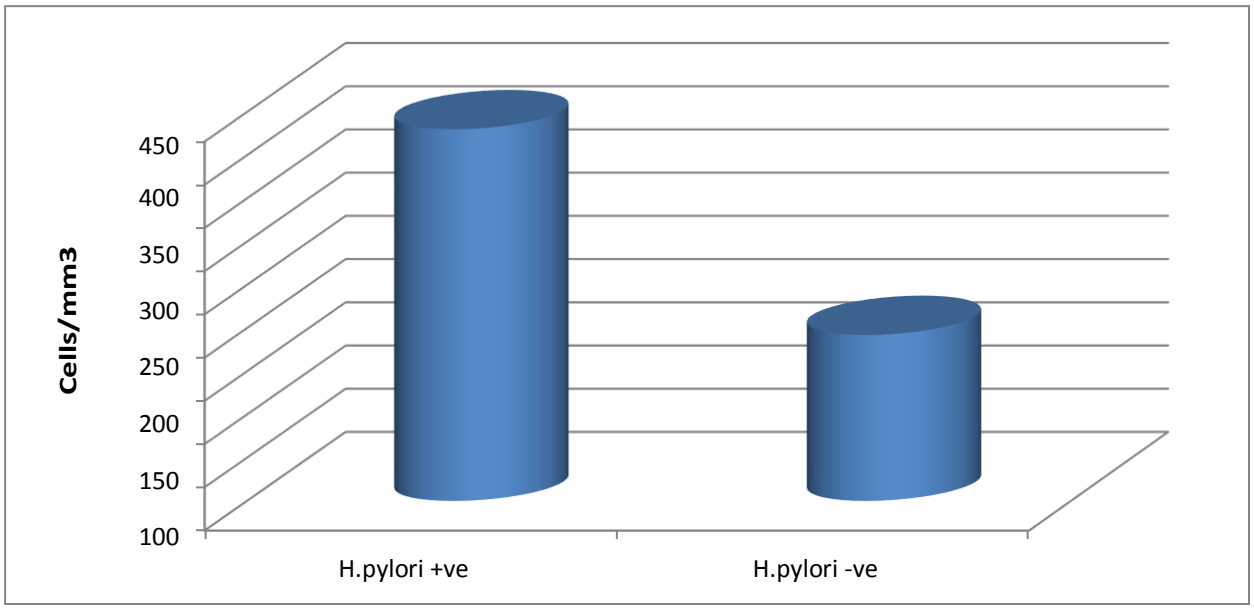

Figure (3): PMN (cells/mm^3) between *H.pylori* +ve and *H.pylori* -ve groups

Table 2: Serological and biochemical analyses association with *H. Pylori* infection

Variable	H.pylori +ve group (no:75)	H.pylori -ve group (no:25)	P value
	mean±SD	mean±SD	
HB (gms/dL)	9.9 ± 1.8	8.8 ± 1.9	**0.008***
TLC (cells/mcL)	5.7 ± 2.2	5.5 ± 2.1	**0.536**
Platlets (Platlets/ mcL)	106± 40.4	112 ± 50	**0.466**
Bilirubin(mg/dl)	1.93 ± 1.92	1.5 ± 1.2	**0.063**
ALT(U/L)	52 ± 36	47 ± 29	**0.333**
AST(U/L)	58± 31	53 ± 32	**0.39**
ALP(U/L)	157± 111	110 ± 57	**0.008***
Albumin(gms/dl)	2.8± 0.5	3± 0.4	**0.014***
Creatinin(mg/dl)	1.2 ± 0.7	1.1 ± 0.5	**0.31**
B.Sugar(mg/dl)	145± 58	141 ± 60	**0.655**

Data presented as mean± SD, with (*) *P* value < 0.05 is significant.

Table 3: Correlation between ascitic fluid analysis and *H. pylori* infection

Variable	All study population
	mean±SD
Albumin(gms/dl)	**2.8 ± 0.5**
SAAG	**1.5 ± 0.5**
S.gravity	**1.4 ± 0.7**
Total Protein(gms/dL)	**2.6 ± 0.8**
Sugar A.F(mg/dL)	**183 ± 54**
PMN(cells/mm3)	**371 ± 132**

4. Discussion

In Egypt, hepatic diseases, especially hepatitis C and B viruses, are currently the main causes of cirrhosis according to **Perz *et al*., 2006**. In the current study, percentages of HCV, HBV, and co-infected patients having cirrhosis are reported to be 90%, 2%, and 3%, respectively. These results are in agreement with those of **Perz *et al*., 2006, Dan and Longo 2012, and Friedman 2014**. In addition, our finding is consistent with **Edwards and Kushner 1993** who reported that 5% of patients with hereditary hemochromatosis have cirrhosis.

PCR tests and other molecular techniques for investigation have been recognized to be the most accurate methods for detecting *H. pylori* (**Belda *et al.*, 2012)**. PCR is a fast, cost-effective method that can be used to detect genotypes (**Garza-Gonzlez *et al*., 2014**). Moreover, it can be performed on tissue and stool specimens and is used to detect antibiotic resistance and virulence (**Guarner *et al.*, 2010**). *UreA* gene was used in this study as a PCR target. A PCR product with amplification size of 200 bp was obtained from 75% of the ascitic fluid samples of patients with *H. Pylori* infection; this is in agreement with **Hunt *et al*., 2010** who reported the *H. Pylori* to be an endemic infectious agent in Egypt with an incidence rate of 90%. The results of the current study are also in agreement with **He *et al*., (2002)** and **Vinette *et al*.**, **(2004)** results who reported the *ureA* gene is conserved and specific to *H. pylori*. On the

contrary, **Lu *et al*., (1999)** showed that *ureA* gene PCR has low sensitivity (75%).

Ebruasicioglu *et al*., (2010) reported that there may be an unusual presentation of peritonitis without any white cells in the peritoneal aspirate despite multiple positive peritoneal cultures. Furthermore, they stated that immune suppression is a possible mechanism for the lack of white cells in patients with peritonitis. Furthermore, the prompt treatment with subsequent improvement of survival, detection, and identification of *H. pylori* by culture is still tricky (**Runyon 2009**).

Cirrhotic patients are often screened for early signs of HCC according to **Singal *et al*., 2014.** Laboratory data of all the study populations showed an elevation of bilirubin with mean ±SD (2 ± 1.7) which agrees with the findings of **Friedman 2014.** Inconsistency with **Christou *et al*., 2007** study, sugar in the ascitic fluid was reported to be significantly associated with *H. pylori* infection (P = 0.0155) as the exudative ascitic fluid has lower glucose level than transudatives due to the destruction or glycolysis of glucose by the action of bacteria and cells. Ascitic fluid glucose can drop significantly in severe infections like secondary peritonitis or late stage of spontaneous bacterial peritonitis.

Koss 1997 reported that specific gravity is less than 1.05 in transudative ascitic fluid and more than 1.05 in exudative ascitic fluid; this agrees with our study results that showed a significant association between *H. pylori* infection and specific gravity (P=0.0033). In the present study, PMN showed a highly significant association with the incidence of *H. pylori* infection (P= 0.0001); this agrees with a previous study by **Dever and Sheikh 2015** which showed that the spontaneous bacterial peritonitis requires an ascitic fluid PMN count of at least 250 cells/mm^3**.** According to **Portillo *et al*., 2000**, ascites with high protein content were reported in up to 25% of patients with chronic liver diseases although it is a consistent finding in malignant ascitic fluid. Conversely, a relatively low ascites protein concentration may be found in patients with exudative ascites if there is hypoproteinemia. Spontaneous bacterial peritonitis (SBP) is often associated with low protein and high albumin gradient; this agrees with the present study data which showed a significant association of *H. pylori* infection with albumin level and SAAG (P = 0.0153, 0.0198, respectively).

Diagnostic paracentesis or ascitic fluid analysis should be done for all patients with new-onset of ascites as SBP is the most frequent and fatal complication of ascites. Late or misdiagnosed SBP can lead to increased mortality. SBP is estimated to affect 10-30% of cirrhotic patients hospitalized with ascites and the percentage of mortality in this group could approach 30% **(Grabau *et al*., 2004).** SBP diagnosis still relies on positive cultures as well as cell count of more 250 cell/mm^3 in the peritoneal aspirate (**Ebruasicioglu *et al*., 2010)**. However, a variant of peritonitis was characterized by negative ascites. This is attributed to the fact that general media is not suitable to culture bacteria of ascitic fluid and this may cause false-negative results (**Runyou, 2009**).

Conclusion

The current study showed an association between the incidences of *H. pylori* infection and decompensated liver cirrhosis which mainly resulted from HCV and HBV infections in Egyptian patients. The prognosis of liver diseases is appreciably affected by *H. pylori* infection. The incidence of *H. pylori* infection affected a number of biochemical parameters of the liver function. Further studies are recommended to emphasize the relation between *H. pylori* infection and prognosis of liver diseases.

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Compliance with ethical standards

Conflict of interest: The authors have declared that no competing interest exists**.**

Author Contributions Statement: **1: Mohamed Y. Nasr (**Participated in the study design, manuscript revision**) 2: Ehsan H. Hassan (**Participated in samples and data collection) 3: **Ghada Hassan (**Participated in the study design and lab work) 4**: Asmaa Ibrahim (**corresponding author**,** participated in lab work as well as manuscript writing and revision) 5**: Amal Abdel-Aziz** (provided technical help, manuscript revision)

Ethical approval: The ethical board of University of Sadat City, Genetic Engineering, and Biotechnology Research Institute, Egypt approved the study. Patients included in the study were verbally informed about its aims and the collection of specimens was done after their consent was obtained.

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