Contents

Impact of *Helicobacter pylori* Eradication on Absolute Telomere Length in Gastric Mucosa

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Key words:
Helicobacter pylori,
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absolute telomere
length, gastric ulcer.

Background and study aim: This study proposed to assess the relation between absolute telomere lengths (TLs) in gastric mucosa and *Helicobacter pylori* (*H. pylori*) infection and study the impact of (*H. pylori*) eradication therapy on TLs.

Patients and Methods: This study was conducted on (42) subjects divided into two groups, group I: included (17) *H. pylori* negative cases served as control group, group II: included (25) *H. pylori* positive patients. Absolute TLs was measured in base pairs (bp) in gastric mucosa and analyzed by real time polymerase chain reaction (RT-PCR) in all groups and re-evaluated in *H. pylori* positive patients 4 weeks after eradication therapy.

Results: Prior to eradication therapy, there was highly significant shortening (P<0.001) in TLs (bp) in gastric mucosa

of *H. pylori* positive patients compared to *H. pylori* negative controls. While there was highly significant elongation was observed after *H. pylori* eradication therapy in H. pylori positive patients (P < 0.001). There was significant negative correlation between TLs and ages of patients (P<0.001) while there was no significant relation between TLs and sex (P= 0.5) before and after eradication therapy.

Conclusion: *H. pylori* positive patients had significantly shorter TLs than *H. pylori* negative controls. TLs were increased after *H. pylori* eradication therapy in H. pylori positive cases. This finding may indicate the importance of *H. pylori* eradication to avoid the development of gastric cancer by its effect on TLs.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram- negative, non-invasive, helix-shaped, microaerophilic agent. It is the most common chronic bacterial infection in humans. *H. pylori* infection is strongly related with socioeconomic status and its prevalence is over 80% in developing countries and 20-50% in developed countries [1]. *H. pylori* is the etiological agent of chronic gastritis, peptic ulcer, and has been documented to be linked with the development of gastric adeno-carcinoma and lymphoma [2]. Histological pre-neoplastic changes that might progress into gastric cancer are found in around 50% of people infected with *H. pylori*. Despite this fact, less than 2% of the patients develop gastric cancer [3]. Although many factors may be related to *H. pylori* associated gastric carcinogenesis, the underlying molecular mechanisms are still unknown. The complications of

chronic inflammation associated with *H. pylori* infection are believed to be related to the genetic features of the host, virulence factors of the bacteria strain, and environmental factors [4]. Telomerase, located at the distal end of human chromosomes, comprises simple, repetitive and G-rich hexameric sequences (TTAGGG), and is vital for chromosomal stability and replication [5]. Short telomeres are associated with cellular senescence and decreased tissue renewal capacity [6]. Longer telomere length (TL) appears to prevent genomic instability and development of cancer in human aged cells by limiting the number of cell divisions. However, shortened telomeres impair immune function that might also increase cancer susceptibility [7]. Telomere length has been analyzed in many human cancers, and it has been found to be shorter in some tumors

(e.g. colon cancer, gastric cancer and glioblastoma) and longer in others (e.g. chordoma) compared to normal tissue **[8,9].** In this study we aimed to assess the absolute (TLs) in gastric mucosa of H. pylori positive patients compared to H. pylori negative controls and to determine any changes in (TLs) by *H. pylori* eradication therapy.

PATIENTS AND METHODS

Patients:

This cohort prospective study was carried out in the Hepatology, Gastroenterology and Infectious Diseases and Medical Microbiology and Immunology Departments, Faculty of Medicine, Benha University from January 2016 to July 2016. The study was approved by the local ethics committee of Benha University Hospitals and written consent was taken from each participant. Forty two adult patients were enrolled in this study were complaining of dyspeptic symptoms (defined as abdominal pain related to the meals). According to the results of upper GIT endoscopy, biopsy urease test and microbiological culture, selected patients were divided into 2 groups. Group I: (17) *H. pylori* negative patients with functional dyspepsia (according to Rome II criteria) **[10]** and they were considered as a control group. Group II: (25) *H. pylori* positive patients. Patients with chronic disease as liver cirrhosis, chronic renal failure, diabetes, hypertension, obstructive airway diseases, patients with history of non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors (PPI) within previous month, heavy smokers and patients with cancers were excluded from the study.

All patients were subjected to full history taking, thorough clinical examination and routine laboratory investigation.

Upper GIT endoscopy: was done for all patients and controls by the same endoscopist after fasting at least 8 hours using video endoscope (OLYMPUS GIF TYPE Q180 2001629, Japan). Four quadrant biopsies were taken from antral mucosa within 5 cm of the pyloric opening for detection of *H. pylori* infection using urease test and microbiological culture and TL analysis. In positive *H. pylori* cases upper GIT endoscopy was repeated one month after *H. pylori* eradication therapy (a full dose PPI, Clarithromycin 500 mg and Metronidazole 400 mg, all twice daily for 2 weeks) and biopsy samples were again taken to determine the changes in telomere length after eradication therapy, gastric fragments were kept in thioglycolate broth (Difco Laboratories, Detroit, Mich.) at 4°C. Samples for TL analysis were sent to the laboratory in tubes with 0.9% NaCl. Then they were centrifuged at 3000g for 5 minutes, the supernatant was removed, and the tissue samples were stored at -80°C until TL was analyzed using (RT.PCR).

Identification of *H. pylori*

Urease test: Biopsies were placed in tubes containing christensen's 2% urea agar and examined within 24 h of incubation at 37℃ for urea hydrolysis.

Microbiological Culture: Fragments in thioglycolate broth (Difco Laboratories, Detroit, Mich.) were ground in a tissue homogenizer. Biopsies were rubbed onto Dent's agar plates using Columbia agar base supplemented with 7% human blood containing vancomycin, trimethoprim, cefsulodin and amphotercin B (Oxoid, Basingstoke, United Kingdom). Plates were incubated for 4-7 days in microaerophilic environment at 37°C. Bacteriological identification of *H. pylori* was done. Patients considered positive for *H. pylori* if direct urease and culture were positive or if culture alone was positive and considered negative if both tests were negative **[11].**

Genomic DNA extraction: DNA was extracted using QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer instructions. The extracted DNA concentration was confirmed through measurement by NanoDrop 2000c Spectrophotometer. Readings were taken at wave lengths of 260 and 280 nm. Concentration of DNA samples were measured = 30 ng /μL at wave lengths 260 and 280 nm **[12].**

Quantitative real time PCR: as described by Cawthon **[13].**

Telomere length assay

Telomere and human β- Globin (HBG) gene were analyzed on DNA samples in 36-well rotor (Rotor-Gene Q 5plex. Qiagen, Germany) using Rotor-Gene_2_3_1_software.

HBG was used as a single copy gene needed for normalization. Necessary standards for absolute quantitation of β-globin expression were prepared using QuantiTect SYBR Green PCR Kit (Roche, Germany).

The telomere length assay was performed according to manufacturer's instructions with a Telo TAGGG Telomere Length Assay (Roche, Germany) kit. The product derived from the

telomere PCR assay was normalized with the product obtained by the β-globin PCR assay and telomere length was measured in base pairs (bp). The primers used in amplifications were described in table1.

Cycling conditions (for both telomere and HBG amplicons) are: 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, followed by a meltingcurve analysis.

A plot of CT (the fractional cycle number at which the well's accumulating fluorescence crosses a set threshold that is several standard deviations above baseline fluorescence), versus log (amount of input target DNA) was generated by performing serial dilutions of the standards ranging between 10^{-1} to 10^{-6} dilution. This linear curve allows absolute quantitation of unknowns in the same PCR run.

Statistical analysis:
Collected data were analyzed using SPSS 16 (SpssInc, Chicago, ILL Company). Categorical data were presented as number and percentages while quantitative data were expressed as mean ± standard deviation, and range. Chi square test (X2) test was used to analyze categorical variables. Quantitative data were tested for normality using KolomogrovSmirnove test, using Wilcoxon test, Man Whitney U test and Spearman's correlation coefficient (rho) as proved to be non-parametric. P<0.05 was considered significant.

Table (1): Primer sequences in amplification reaction [13]

Gene	Primers	
	Forward	**Reverse**
THR[*]	5'GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGT GAGGGT-3'	5'TCCCGACTATCCCTATCCCTATCCCTATCCC TATCCCTA-3'
HBG[**]	5'-GCTTCTGACACAACTGTGTTCACTAGC-3'	5'-CACCAACTTCATCCACGTTCACC-3'

THR[*] telomere hexamer repeats.
HBG[**] human β-globin.

RESULTS

This cohort prospective study was conducted on 25 *H. pylori*-positive patients (16 males and 9 females) with mean age 39.4±8.12 years and 17 H. pylori- negative patients served as a control group (12 males and 5 females) with mean age 38.5±8.5 years. There was insignificant statistical difference between patients and controls as regard sex and age, (P value = 0.65 and 0.74 respectively) (Table 2).

The absolute TLs was shorter in *H. pylori* positive patients than control group (*H. pylori* negative patients) before treatment regimen (2326.4 ± 165.06 bp Vs 3046.7 ± 331.60 bp respectively) with highly statistically significant difference between both groups (P<0.001) (Table 3).

In Table (4) there was highly statistically significant difference between *H. pylori* positive patients before and after eradication therapy (P<0.001). The telomere length was significantly increased after eradication therapy (absolute TLs was 2326.4±165.06 bp before treatment Vs 2842.3 ± 296.04 bp after treatment).

There was insignificant difference in absolute TLs in *H. pylori* positive patients after eradication therapy compared to control group (P = 0.053) (Table 5).

There was significant negative correlation between ages of patients and TLs before and after treatment (before treatment, r= -0.713, P<0.001, after treatment r= -0.846, P<0.001) (Figs. 1,2 respectively). While as regard the relation between sex and TLs there was no significant relation (before treatment TLs in males was 2335.5 ± 166.97 bp, in females it was 2310.1 ± 170.28 bp and P value = 0.51 and after eradication therapy TLs was in males 2881.6 ± 305.00 bp and in females it was 2772.3 ± 282.65 bp with P value = 0.55) (Table 6).

Table (2): Comparison between studied groups regarding sex and age

Variable	Group I (N = 17) *H. pylori* negative cases	Group II (N = 25) *H. pylori* positive cases	Test	P value
Sex Females (%) Males (%)	 5 (29.4) 12 (70.6)	 9 (36.0) 16 (64.0)	X^2=198	0.65
Age (years) Mean ±SD; (range)	 38.5 ± 8.5 (25-52)	 39.4 ± 8.12 (25-52)	t= 0.32	0.74

Table (3): Comparison between *H. pylori* positive patients and control group as regard TLs before treatment

Study groups	TLs*in bp Mean ±SD
H. pylori **positive patients before treatment (No.=25)	2326.4±165.06
H. pylori **i negative control group (No.=17)	3046.7±331.60
Test	***MWU Z= 5.25
P value	**< 0.001**

***Telomere length, ****Helicobacter pylori*, ***MWU= Man Whitney U Test

Table (4): TLs in *H. pylori* positive patients before and after eradication therapy

| Study groups (No.=25)	TLs*in bp Mean ±SD
*H. pylori*** positive patients before eradication therapy	2326.4±165.06
*H. pylori*** positive patients after eradication therapy	2842.3±296.04
Test	Wilcoxon test Z= 4.4
P value	**< 0.001**

*Telomere length, **Helicobacter pylori.*

Table (5): Comparison between *H. pylori* positive patients and control group as regard TLs after eradication therapy

Study groups	TLs* Mean ±SD
H. pylori ** positive patients after eradication therapy (No.=25)	2842.4±296.04
*H. pylori*** negative control group (No.=17)	3046.7±331.60
test	MWU Z = 1.93
P value	0.053
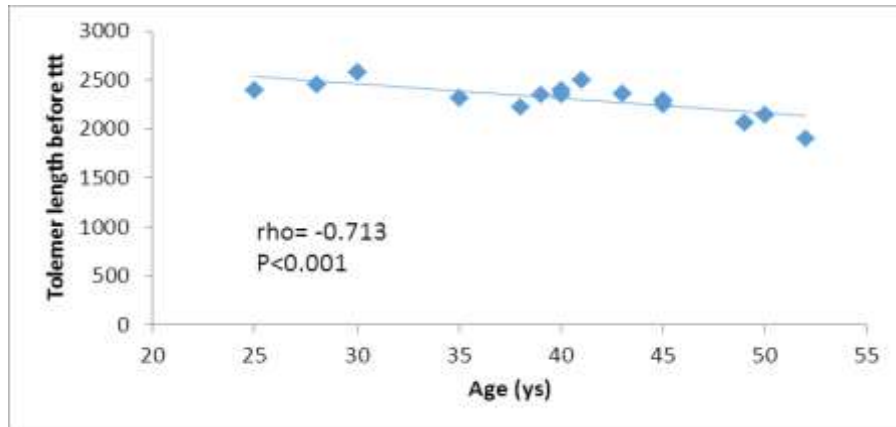
Figure (1): Correlation between age and telomere length before treatment
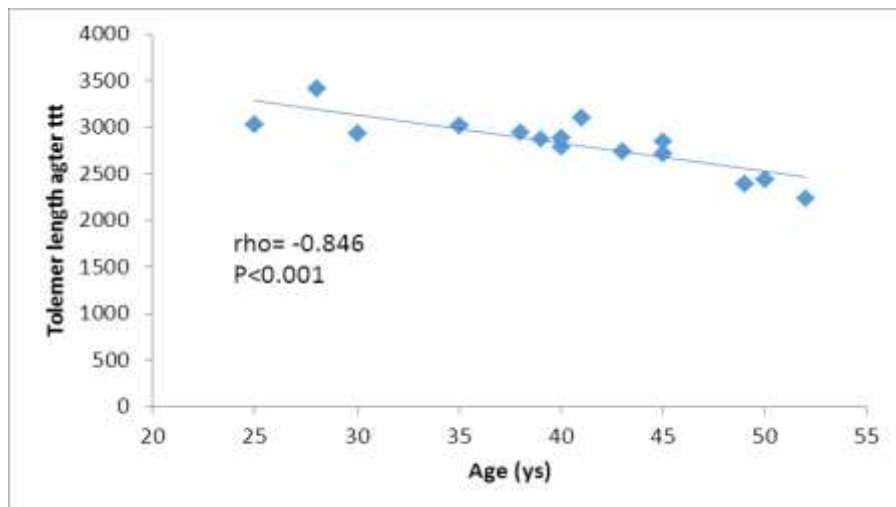
Figure (2): Correlation between age and telomere length after treatment

Table (6): Relation between sex and telomere length

Sex	Telomere length (bp) Mean ± SD	Test	P value
Before eradication therapy			
Male (N = 16)	2335.5 ± 166.97	MWU	0.51
Female (N = 9)	2310.1 ± 170.28	Z = 0.65	
After eradication therapy			
Male (N = 16)	2881.6 ± 305.00	MWU	0.55
Female (N = 9)	2772.3 ± 282.65	Z = 0.59	

DISCUSSION

Telomeres are special chromatin structures that protect the ends of chromosomes from degrading and restructuring activities [4]. They consist of repetitive nucleotide sequences and an associated terminal protein complex that is vital for chromosomal stability, replication and prevent loss of chromosomal integrity [5]. Telomere shortening results in the deterioration of the protective functions, fusion in chromosomes, breaking and bridging, and gene amplifications. All of which lead to genomic instability, the most significant feature of solid tumors [14]. Inflammation, oxidative stress and increased cell replication are major environmental factors associated with accelerated shortening of telomeres [15]. Meta-analyses suggest 1.4 to 3.0 fold increased risk of cancer for those with the shortest versus longest telomeres [16,5]. *H. pylori* infection in gastric mucosa leads to chronic inflammation and complications, such as ulcer, metaplasia and cancer. Even though the relation of *H. pylori* and the pathogenesis of gastric cancer is not completely known, it has been shown that a change in telomere length and telomerase activity in pre-neoplastic and cancerous tissue might arise. This change is considered a stage or cause in the development of cancer [17]. Pre-neoplastic changes that might progress into gastric cancer are found in around 50% of people infected with *H. pylori* and TL has been found to be shorter in some tumors a scolon cancer and gastric cancer compared to normal tissue [8,9]. This study was performed to assess the absolute TLs in gastric mucosa of *H. pylori* infected patients compared to *H. pylori* negative controls and determine the effect of eradication of *H. pylori* infection on TLs. In the present study we found highly significant shortening in TLs in *H. pylori* positive patients than control group (P<0.001), this finding was in agreement with Kuniyasu et al. [18]. Who reported that TL was significantly shorter in the group infected by *H. pylori* than in the uninfected group, in the same hand Aida et al. [19] demonstrated that *H. pylori*–positive gastric mucosa has been shown to have shorter TL than H. pylori negative mucosa. Similarly Aslan et al. [20] found that the telomere length was found to be 2958.9±1345.7 bp in the control group and 2481.2±1823bp in the study group prior to eradication; however, this difference was not significant (p=0.11). In the present work, after *H. pylori* eradication therapy, there was highly

statistically significant elongation in TLs in *H. pylori* positive cases (P<0.001), that in agreement with Aslan et al. [20] who found that The telomere length was found to be 3766.3± 1608.8 bp in the study group after eradication. In this group, the telomere length was significantly increased after eradication compared to the period before eradication (p=0.01), and considered this finding provides new approach for effectiveness of *H. pylori* eradication therapy in increasing the TLs and decrease incidence of gastric cancer, also Chung et al. [5] elucidated that peptic ulcer and intestinal metaplasia are closely related to *H. pylori* infection. Furthermore, chronic gastritis due to *H. pylori* infection may progress to intestinal metaplasia and even gastric cancer. This finding was considered to provide the effectiveness of *H. pylori* eradication therapy in increasing the TLs and decrease incidence of gastric cancer. Also in the present study we found significant negative correlation between age and absolute TLs in patients group before and after treatment (P<0.001), this result disagreement with Aslan et al. [20] who found negative correlation but insignificant (r = 0.172, p>0.05), while Hou et al. [21] reported that telomeres were significantly shorter in association with aging (P<0.001), and the extent of telomere shortening may vary considerably among individuals within age groups, suggesting that environmental and lifestyle factors could play critical roles in the rate of telomere attrition. As regard relation between TLs and sex the present work found insignificant relation between TLs and gender before eradication therapy in patients group (2335.5 ± 166.97 bp in males and 2310.1 ± 170.28 bp in female with P value 0.51) this in agreement with Aslan et al. [21] as they found TLs before eradication was found (2721.3 ± 2003.44 bp in males, 2161.1 ± 1608.6 bp in females, p>0.05), on the same hand Hou et al. [21] reported insignificant relation between TLs and sex (P value = 0.09), when studying TLs in gastric cancer related to *H. pylori* positivity.

CONCLUSION

H. pylori positive patients have significantly shorter absolute TLs in their gastric mucosa compared to *H. pylori* negative controls. *H. pylori* eradication therapy increases the absolute TL in *H. pylori* infected patients and that can open new approach for consider effectiveness of H. pylori eradication therapy in increasing the telomere length one of preventable methods for gastric cancer.

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Relationship between Plasma Concentrations of Interleukin-10 and Thrombocytopenia in Chickenpox Patients

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Background and study aim: Chickenpox is highly infectious, self-limiting disease, caused by varicella-zoster virus. Thrombocytopenia is considered a common hematological complication of chickenpox. This study was conducted to assess the correlation between serum interleukin 10 levels and thrombocytopenia in adult chickenpox patients.

Patients and Methods: Fifty patients of chickenpox complicated by thrombocytopenia were included in this study as a patient group. Fifty patients of chickenpox with normal platelet of comparable age, race and gender were identified as a control group. All patients were diagnosed by clinical picture of chickenpox and by presence of varicella - zoster virus immunoglobulin M (VZV IgM) in the patient blood. Estimation of complete blood count, liver function tests, kidney function tests, fasting blood sugar, and levels of cytokines IL-10 and TNF-α were done in both groups on the day of admission and day of discharge.

Results: At time of admission, IL-10 levels were significantly higher in patients group than in control group, while TNF-α level were not significantly different between the two groups. At time of discharge, we observed a significant decline in IL-10 levels in the patients group as compared with time of admission. A negative correlation between IL-10 levels and platelet count was observed while no correlation was found between TNF-α and platelet count.

Conclusion: IL-10 level could play an important role in development of thrombocytopenia in patients with chickenpox.

INTRODUCTION

Chickenpox is highly infectious, self-limiting disease, caused by varicella-zoster virus affecting all age groups typically associated with fever and characteristic exanthematous vesicular skin rash [1]. It can cause serious life-threatening complications particularly in elderly, pregnant women, smokers, or in immune-compromised patients.

However, reports have shown that the incidence of Chickenpox in adults has doubled in recent years and this has been paralleled with an increase in hospital admissions [2] and mortality [3]. The reason for this age shift is not known, though it may be the result of decreased exposure to Varicella-Zoster virus (VZV), increased virus virulence, the immigration of non immune adults from the tropics, increasing vaccine coverage and a decrease in transmission may result in accumulation of susceptible adults followed by a shift of incidence into the older age group [4]. Furthermore, as compared to children the clinical presentation in adults is severe and more commonly associated with complications [5].

Varicella is generally considered as a benign, self limiting disease; however, it may be associated with serious complications especially in adults and immunocompromised [6]. The complication rate has been reported variably; varicella pneumonia, skin infection, encephalitis, cerebellar ataxia and subclinical hepatitis are reported frequently, whereas, acute myocarditis, acute pancreatitis, acute liver failure, glomerulonephritis, disseminated intra-vascular coagulation (DIC) and rhabdomyolysis are among the rare complications [7].

Thrombocytopenia is considered a common hematological complication of chickenpox and it is four times more common in adults than children [8]. Varicella associated thrombocytopenia is well described in number of observational studies but mechanisms involved in platelet reduction are not well understood, The cytokines released during an acute inflammatory response to varicella contribute to the pathogenesis of thrombocytopenia. No previous studies have addressed the association of IL-10 and thrombo-cytopenia in chickenpox [8]. The aim of this study was to assess the potential correlation of serum interleukin 10 levels in the pathogenesis of thrombocytopenia in adult chickenpox patients admitted in the hospital.

PATIENTS AND METHODS

Study design and the participants:
This study was conducted between December 2014 and December 2015, at the Infectious Disease Hospital (IDH), Kuwait, which is a tertiary care hospital and accredited by the national Canadian accreditation program at 2014. A total of fifty patients of chickenpox complicated by thrombo-cytopenia were included in this study as patients group. Of the patients included in the study, 35 were Indian, 3 were Indonesian, 5 were Bangladeshi, 4 were Sri Lankan and 3 were Kuwaiti. In addition, fifty patients of chickenpox with normal platelet of comparable age, race and gender were identified as controls.

Diagnosis of chickenpox was based on the presence of a typical rash associated with fever and throat pain and serological confirmation was based on the presence of VZV IgM in the patient blood.

Exclusion criteria :
Patients with history of chronic liver disease, immunocompromised status (HIV/Drugs), blood disorders, pregnant, recent intake of drugs/ conditions which might cause thrombocytopenia and active alcohol consumers were excluded from the study.

Data collection :
Both groups were submitted to full history taking, comprehensive clinical examination, complete blood count, liver function test, kidney function test, fasting blood sugar, and levels of cytokines interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) (Biomedix medical group, Synlab, German).

According to the platelet count, thrombocytopenia was defined if count was less than 150×10^3/ul (Ref.range:$150-400 \times 10^3$/ul) [9]. Grading of thrombocytopenia was carried out according to National Cancer Institute (NCI) Criteria for Adverse Events Version 3 [10]. According to that patients with thrombocytopenia have been divided into following five grades :

Grade 0: Within normal limit, platelet count 150,000 or above.
Grade I: Platelet count between 75,000 and 150,000.
Grade II: Platelet count between 50,000 and 75,000.
Grade III: Platelet count between 25,000 and 50,000.
Grade IV: Platelet count less than 25,000.

Management was done as per standard guidelines for the treatment of chickenpox and its complications. All patients received acyclovir intravenously or orally in proper dose according to body weight for 5 to 7 days [11]. Platelet count was repeated on alternate days and patients were discharged from the hospital once the platelet count returned to the reference range and patients became asymptomatic.

Statistical analysis :
The data was analyzed using the statistical package for social sciences (SPSS) version 8.0 software. The significance of differences between mean values of the study variables was evaluated by using t-test. The significance of differences between proportions was performed using the Chi-square test. The P value less than 0.05 is considered significant.

RESULTS

In this study, 50 patients with chickenpox complicated by thrombocytopenia were included. The mean age ± SD of the patients under the study was 31.12±6.25 years and males out-numbered the females 40 (80%) vs. 10 (20%) with different grading of thrombocytopenia. There was no significant age difference among the two groups (Table 1). All the patients had fever (100%) at the time of presentation, followed by Pleomorphic itchy skin rash (100%), nausea (40%), vomiting (40%), anorexia (60%), diarrhea (05%), abdominal pain (10%), cough (14%) and breathlessness (08%).

Out of 50 cases in patients group, 26 (52%) cases had Grade I thrombocytopenia, 14 (28%) cases had Grade II thrombocytopenia, 8 (16%) cases had Grade III thrombocytopenia and 2 (4%) cases had Grade IV thrombocytopenia (Table 2). None of the patients with thrombocytopenia developed purpuric spot, ecchymosis or bleeding manifestation during the course of disease.

At time of admission, mean platelet count was 101.84±40.2 and there was significant difference between the studied groups. While Mean hemoglobin value was 12.3±1.8 and mean white blood cell count was 7.8±1.5 and there were not significantly different between the two groups (Table 1). IL-10 levels were significantly higher in patients group than in control group (mean serum IL-10 levels of 13.7±1.8 vs. 6.9±0.8, P<0.001). It has been further observed that there was no significant deference as regard pro-inflammatory cytokine TNF-α in patients group

when compared with controls. There were significant differences between the studied groups as regard liver enzymes alanine transaminase and aspartate transaminase (Table 1).

At time of discharge, we observed a significant decline in IL-10 levels in the patients group as compared with time of admission (mean serum IL-10 levels of 13.7±1.8 vs. 7.46 ± 1.02, P<0.001). Also, at time of discharge, there was significant increase in number of platelet in patients group as compared with time of admission (mean platelet count was 152.73±34.46 vs. 101.84 ± 40.2, P<0.05) and Platelet was still significant low in patients group as compared with controls (Table 3).

In this study, we observed a negative correlation between IL-10 levels and platelet count. While no correlation was found between pro-inflammatory cytokine TNF-α and platelet count (Table 4).

Table (1) : Comparison between studied groups at time of admission

	On admission		
	patients group	**control group**	**P-value**
Age	31.12±6.25	30.23±5.67	0.82
ALT	144.13±34.28 u/L	81.90±8.86 u/L	0.01
AST	113.0±24.61 u/L	67.8±4.47 u/L	0.01
IL-10	13.7±1.8	6.9±0.8	0.001
TNF-α	6.2±1.5	5.1±1.2	0.11
Platelet	101.84±40.2 10³/uL	211.85±45.5 10³/uL	0.001
WBCs	7.8±1.5 10³/uL	7.34±1.24 10³/uL	0.72
Hemoglobin	12.3±1.8 g/dL	13.1±2.2 g/dL	0.51
s. creatinine	99.57±13.02 μmol/L	91.45±13.23 μmol/L	0.21

TNF-α**:** tumor necrosis factor alpha; IL-10: interleukin-10; ALT: alanine transaminase
AST: aspartate transaminase; WBCs: White blood cells; SC: serum creatinine

Table (2) : Distribution of sex as regard grading of thrombocytopenia

	Male (%)	**Female (%)**	**Total**
Grade 0	0 (0.0)	0 (0.0)	0
Grade I	20 (76.9)	6 (23.1)	26
Grade II	11 (78.5)	3 (21.5)	14
Grade III	7 (87.5)	1 (12.5)	8
Grade IV	2 (100.0)	0 (0.0)	2
Total	40 (80.0)	10 (20.0)	50

Table (3): Comparison between studied groups at time of discharge

	On discharge		
	Patients group	**Control group**	**P-value**
ALT	50.35±10.03	49.71±9.26	0.62
AST	38.7±5.92	37.95±6.78	0.71
IL-10	7.46±1.02	4.14±0.9	0.018
TNF-α	5.9±0.9	6.01±1.01	0.94
Platelet	152.73±34.46	243.10±33.34	0.01
WBCs	8.42±1.63	8.39±1.67	0.95
Hemoglobin	13.5±2.1	13.9±1.9	0.91
S. creatinine	84.3±11.4	85.8±11.78	0.71

Table (4): Correlation between serum IL10 levels, TNFα , and other parameters in studied groups

	IL-10				TNF-α			
	Patient Group		**Control Group**		**Patient Group**		**Control Group**	
	R	**p**	**R**	**p**	**R**	**p**	**r**	**P**
Age	-0.02	0.944 NS	-0.01	0.96 NS	0.19	0.48 NS	0.06	0.81 NS
ALT	0.29	0.1 NS	0.05	0.24 NS	-0.27	0.25 NS	0.03	0.68 NS
AST	0.25	0.41 NS	0.44	0.09 NS	-0.23	0.404 NS	0.22	0.43 NS
Platelet	-0.52*	<0.05 S	0.43	0.104 NS	-0.32	0.25 NS	0.11	0.68 NS
IL-10	1.000	0	1.000	0	0.19	0.49 NS	0.31	0.52 NS
TNF-α	0.19	0.49 NS	0.31	0.52 NS	1.000	0	1.000	0

DISCUSSION

Thrombocytopenia is considered a common hematological complication of chickenpox infection; however, hemorrhagic manifestations are rare [12]. A number of observational studies have confirmed the association of thrombocytopenia to chickenpox [8]. Thrombocytopenia in varicella usually develops early in the disease process and the incidence has been reported variably. The frequency of thrombocytopenia in chickenpox patients has been reported as 1%, 22.5%, 30% and 45% in various studies [12,13,14,15]. The mechanisms involved in platelet reduction are not well understood [8], Two likely pathogenetic mechanisms are suggested: Both non-immunological as well as immunological destruction of platelets have been implicated in causing thrombocytopenia. Non immunological pathogenesis is the infectious one with thrombocytopenia during the period of viremia: the other immunological is post infectious with

thrombocytopenia continuing for weeks and months [16]. Cr15-labelled platelet studies suggest marked platelet destruction. IgG and IgM anti-platelet antibody on platelets has been demonstrated, suggesting involvement of immune mediated mechanisms [16]. Thrombocytopenia may be detectable before the characteristic rash appears, suggesting direct destruction of platelets. Immune thrombocytopenia (ITP) is a rare complication of chickenpox which appears as a delayed complication [17].

The cytokines released during an acute inflammatory response to varicella contribute to the pathogenesis of thrombocytopenia [18]. The present study revealed a significant higher levels of IL-10 in patients group at time of admission. The induction of VZV-specific T cells and IgG antibodies was accompanied by transient increases in IL-10 production has been demonstrated in a study by Jenkins et al. [19]. No previous studies have addressed the association of IL-10 and thrombocytopenia in chickenpox. Here, we

showed that thrombocytopenia in adult with chickenpox is negatively correlated with plasma concentrations of IL-10. There is evidence that IL-10 may directly induce thrombocytopenia. The administration of a low dose of recombinant human IL-10 (8 µg/kg/d) decreased platelet production in healthy adult volunteers [18]. In the same study, there was a corresponding reduction in splenic sequestration of platelets in the IL-10 treated group compared with the placebo treated subjects. In the IL-10 treated group, there was a trend toward lower numbers of megakaryocyte colony-forming units (CFU-MKs) compared with volunteers who received placebo [18]. This study suggests that IL-10 induced reduction in platelet count is caused, at least in part, by a reduction in platelet production.

Tumor necrosis factor-α (TNF-α) has been associated with platelet consumption in mice [20], but in this study, we could not demonstrate an association between thrombocytopenia and plasma concentration of TNF-α, suggesting but not unequivocally that TNF-α does not have a significant role in the development of thrombocytopenia in patients with chickenpox.

CONCLUSION

Thrombocytopenia is frequently associated with chickenpox. Furthermore, low platelet count usually does not cause bleeding tendency and count improves with the treatment of varicella. Plasma concentration of IL-10 was negatively correlated with platelet count. IL-10 induced reduction in platelet count is caused, at least in part, by a reduction in platelet production.

Conflicts of interest: None.
Ethical approval:Approved.

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Frequency of Cholelithiasis in Patients with Chronic Liver Disease: A Hospital-Based Study

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Background and study aim: Liver Cirrhosis is a strong and a common known risk factor for Cholelithiasis. Cholelithiasis is a multifactorial disease, based on a complex interaction of environmental and genetic factors. The primary aim of this study is to determine the frequency of cholelithiasis in chronic liver disease (CLD) patients admitted to Zagazig university hospitals. The secondary aim is to determine the risk factors and their association with the underlying etiology and severity of liver disease.

Patients and Methods: We conducted a hospital based study including 131 patients with chronic liver disease based on clinical, laboratory and Ultrasonographic findings. Demographic, clinical and etiological data were recorded, using a pre-coded questionnaire. A number of laboratory tests as fasting plasma glucose, total cholesterol, triglyceride, aspartate aminotransferase (AST), alanine amino-transferase (ALT), alkaline phosphatase (ALP), hepatitis B surface antigen (HBsAg), and antibody to hepatitis C virus (HCV-Ab) were analyzed.

Results: The number of registered cases was 131 with age (52.9±11.7).There were 55 (42%) males and 76 (58%) females. Hepatitis C (HCV) was present in 101 (77%) cases. The prevalence of cholelithiasis was 50.4%% (66 of 131 patients). Most of cholelithiasis patients presented with child C stage (68.2%), followed by child B (21.2%) and the least one was Child A. Hepatitis C (10.6%) was found to be associated with cholelithiasis (75.8%), followed by hepatitis B (13.6%). Auto-immune disease, diabetes mellitus, contra-ceptive pills and obesity are considered risk factors for cholelithiasis.

Conclusion: Cholelithiasis tends to occur more frequently in patients with decompensated CLD. The higher incidence of cholelithiasis in CLD appears to be associated with HCV infection. This is an important parameter to be considered in a country with high prevalence of HCV as Egypt.

INTRODUCTION

Gallstones (GS) are a major cause of morbidity and mortality throughout the world [1]. Gallstone disease (GSD) is responsible for about 10,000 deaths per year in the United States. About 7000 deaths are attributed to acute GS complications, such as acute pancreatitis. About 2000-3000 deaths are caused by gallbladder cancers (80% of which occur in the setting of gallstone disease with chronic cholecystitis) [2]. The prevalence of GS in patients with chronic liver disease (CLD) is 20-40%, while it is 10-15% among the general population [3]. Moreover, the incidence of gallstones increased significantly with the progression of liver disease [4]. In Eygpt, it was found that the prevalence of GSD in patients with CLD was 21.8% [5]. GSD is a multifactorial disease based on a complexinteraction of environmental and genetic factors. Gallstones are principally formed due to abnormal bileconstituents (eg, cholesterol, phospholipids and bilesalts) [6]. When bile is concentrated in the gallbladder, it can become supersaturated with such substances, which then precipitate as microscopic

crystals. The crystals are trapped in gallbladder mucus, producing gallbladder sludge. Over time, the crystals grow, aggregate, and fuse to form macroscopic stones [7]. Moreover, the increase in gall bladder wall thickness by hyperemia, edema, decreased contractility or impaired gallbladder emptying contributes to gallstone formation [8]. The most accurate and non-invasivemethod of predicting gallstone disease was achieved with the advent of the ultrasound, which has a sensitivity and a specificity of greater than 95%. However, the true prevalence of the disease remains hard toderive as the majority of patients remain asymptomatic.

According to the NIH guidelines, removal of thegallbladder is the treatment of choice for symptomatic GSD [9]. However, less focus has been directed on patient selection and typical or common symptom characteristics of this disease.

The primary aim of this study is to determine the frequency of cholelithiasis in CLD patients admitted to Zagazig university hospitals. The secondary aim is to determine the risk factors and their association with the underlying etiology and severity of liver disease.

PATIENTS AND METHODS

The study included 131 patients with CLD who were selected from Zagazig University hospitals from May to December, 2013. The study was approved by the local institutional review board. Informed consent was provided by all participants.

For each patient; demographic, clinical and etiological data were recorded by using a pre-coded questionnaire. A number of laboratory tests as fasting plasma glucose, total cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phos-phatase (ALP), hepatitis B surface antigen (HBsAg), and antibody to hepatitis C virus (HCV-Ab) were analyzed. All patients underwent ultrasound abdominal scanning.Ultrasonographic findings suggesting cirrhosis include; hypertrophy of the caudate lobe with concomitant atrophy of the posterior segments of the right lobe, coarse and heterogeneous echo texture, portal vein diameter> 13 mm, splenomegaly, and ascites [10]. Gallstones appear as echogenic foci in the gallbladder. They move freely with positional changes and cast an acoustic shadow [11,12]. Cirrhosis of the liver was diagnosed based on typical clinical features and sonographic findings according to the following criteria; (a)surface

nodularity of the liver (b) coarsening and nodularity of the liver parenchyma with ascites (c)evidance of splenomegaly, and (d) evident collateral circulation shown in US. The severity of cirrhosis was categorized according to the Child– Pugh classification.

Patients were subsequently classified into compensated group (N=22 patients) and decompensated group (N= 109 patients). Decompensation means cirrhosis complicated by one or more of the following features; jaundice, ascites, hepatic encephalopathy, bleeding varices, hepatorenal syndrome, hyponatremia and spontaneous bacterial peritonitis.

Statistical analysis:

The Sample size was calculated using Epi info version 6.04. According to the statistical data, the average total number of registered patients was 1500 patient per year, and the prevalence of cholelithiasis was 40 %in CLD patients in the study of Acalovschi et al.[8] at confidence interval 95%, and the power was 80%. Our study included 131patients. We used SPSS (Statistical package for social science) version 21 to obtain descriptive statistics that were calculated in the form of: A) Mean ± Standard deviation (SD) for quantitative parametric data, B) Median and range for quantitative non-parametric data, C) Frequency (Number and percent) for qualitative data. Significance level for all statistical tests has a threshold of significance is fixed at 5% level (p-value).

RESULTS

This study included 131 patients with CLD; Their mean age was 52.9, SD (±11.7) years. There were 55 (42%) males and 76 (58%) females. Chronic HCV infection was found in 101 (77%) patients and 21 (16%) had chronic HBV infection. Table (1) summarizes the demographic and clinical characteristics of patients included in the study. The clinical presentation of the patients included in the study was summarized in Table (2).

The prevalence of cholelithiasis in the examined patients was 50.4% (66 of 131 patients). Our study showed that Cholelithiasis is more associatied with decompensated than compensated liver diseases (54.1% Vs 30.4%; p value <0.05).

In compensated patients with cholelithiasis; the mean serum Direct bilirubin level was significantly lower than in patients without cholelithiasis

(3.5mg/dL vs 0.5mg/dL, p value = 0.031).Other factors including AST, ALT, GGT, cholesterol and TG were not significantly associated with cholelithiasis.

In decompensated patients with cholelithiasis; the mean serum cholesterol and TG level was significantly lower than in patients without cholelithiasis (179mg/dL vs 155mg/dL, p value <0.001) and (109mg/dL vs 91mg/dL, p value = 0.017) retrospectivly. There was no statistically significant difference between ultrasonographic finding of cholelithiasis in compensated and decompensated patients in term of CBD, number and size of gallbladder stones (p value > 0.05) (Table 3).

There was no statistically significant difference between patients with or without cholelithiasis in term of spontaneous bacterial peritonitis, hepatic encephalopathy, hematemesis and melena (p value > 0.005) (Table 4).

The etiology of liver disease did not differ significantly between theose patient with or without cholelithiasis. Common causes included HCV infection, HBV infection and autoimmune disease (p value > 0.05) (Table 5).

The prevalence of cholelithiasis increased with the severity of the disease according to Child-Pough classification as the fellowing; In Child-Pugh A (10.6%), Child-Pugh B (21.2%), and in Child-Pugh C (68.2%). For all child-pugh grades, the difference between patients with or without cholelithiasis was statistically significant (p value = 0.004) as shown in Table (6).

The risk of cholelithiasis increased in patients with liver disease who were smokers, diabetic or have a history of contraceptive pills intake (OR= 0.86, 4.71 and 18.69 retrospectively. Diabetis mellitus and history of contraceptive pills intake have a significant association with cholelithiasis (p value > 0.001) (Table 7).

In the compensated group; we found three patients with cholecystitis. Two of them developed obstructive jaundice and one patient had Cholangitis. While, in decompensated group; we found seven patients with cholecystitis. Two of them developed obstructive jaundiceand onepatient had Cholangitis. Obstructive jaundice and cholecystitis showed a statistically significant increase in the compensated group (p value >0.008).

Table (1): Baseline characteristics of enrolled patients

Variables	Compensated group		Decompensated group	
	Patients with cholelithiasis N=7	Patients without Cholelithiasis N=15	Patients with Cholelithiasis N=59	Patients without Cholelithiasis N=50
Age (Mean±SD)	48.1±12.9	51.6±7.3	55.9±7.6	53.3±8.9
Sex Female (%)	6(85.7)	4(25.0)	36 (61)	30(60)
DM No.(%)	5(28.6)	3(18.8)	35(59.3)	13(26.0)
Smoking No. (%)	1(14.3)	9(56.3)	25(42.4)	19(38.0)
RBS (Mean ± SD)	203.3±79.3	136.5±34.6	178.6±11	157.5±10
Cholesterol(mg/dl)	186.2±44.4	138.3±43.7	179.1±41.2	155±23.1
TG (mg/dl)	114.8±52.8	62±39.3	109±42.6	91.3±20.3
WBC (cells/L)	5.5±4.1	5.6±1.4	7.6±4.7	7±5.1
HB(g/dl)	11.7±1.8	10.3±3.9	11.9±12.4	10.1±2
Platelet (cells/L)	80.5±56.5	117±112.7	86.9±49.1	107.9±83
T. bilirubin (mg/dl)	7.5±3	0.8±0.1	5.7±6.9	5.5±7.1
D.bilirubin (mg/dl)	3.5±1	0.5±0.1	3.3±5.2	4.2±5.3
T. protein (gm/L)	7.7±1.1	7.6±0.6	6.3±1.5	6.4±0.8
Albumin (gm/L)	3.3±0.5	3.2±0.5	2.4±1.2	2.3±0.9
Creatinine (mg/L)	0.9±0.3	0.6±0.2	1.1±0.7	1.7±3.1
INR	1.2±0.2	1.3±0.1	1.7±0.5	1.8±0.5
PT(seconds)	53.7±26.5	44.4±15.2	46.6±17.7	41.3±19.3
AST(IU/L)	57±15	49.4±14.8	66.8±62.4	74.4±90.4
ALT(IU/L)	55.5±17.8	47.2±14	80.2±119.1	64±42.8

Table (2): Clinical presentation of patients with chronic liver disease

Variables	Decompensated group			Compensated group		
	Patients with cholelithiasis N=59	Patients without cholelithiasis N=50	P value	Patients with cholelithiasis N=7	Patients without cholelithiasis N=15	P value
Abdominal pain	42(71.2)	31(62.0)	0.309	7(100.0)	4(25.0)	<0.001**
Nausea	30(50.8)	27(54.0)	0.743	3(42.9)	9(56.3)	0.554
Vomiting	18(30.5)	20(40.0)	0.3	1(14.3)	6(37.5)	0.265
Anorexia	54(91.5)	38(76.0)	0.026*	7(100.0)	11(68.8)	0.094
Heart burn	46(78.0)	36(72.0)	0.472	3(42.9)	9(56.3)	0.554
Dyspepsia	58(98.3)	49(98.0)	0.906	5(71.4)	12(75.0)	0.857
Rt. Hypo-chondrium pain	29(49.2)	9(18.0)	<0.001**	7(100.0)	12(75.0)	0.146
Fever	39(66.1)	24(48.0)	0.06	3(42.9)	0(0.0)	0.004*
LL edema	40(67.8)	25(50.0)	0.06	6(85.7)	5(31.3)	0.016*
Bleeding tendency	30(50.8)	22(44.0)	0.475	5(71.4)	0(0.0)	<0.001**
Jaundice	33(55.9)	33(66.0)	0.284	7(100.0)	4(25.0)	<0.001**
HE	45(76.3)	32(64.0)	0.161	3(42.9)	9(56.3)	0.554

Table (3a): Ultrasonographic characteristic of cholelithiasis in compensated versus decompensated group

Variables	Compensated group N=7	Decompensated group N=59	P value
Number of GS			
single	4(57.1)	25(42.4)	
multiple	2(28.6)	11(18.6)	0.735
mud	1(14.3)	14(23.7)	
Size of GS			
Small	3(42.9)	25(42.4)	
Moderate	2(28.6)	5(8.5)	0.229
Large	2(28.6)	29(49.1)	
CBD			
Normal	5(71.4)	57(96.6)	0.191
Dilated	2(28.6)	2(3.4)	

Table (3b): Ultrasonographic presentation of enrolled patients

Variables	Decompensated group			Compensated group		
U/S Presentation	Patients with cholelithiasis N=59	Patients without Cholelithiasis N=50	P value	Patients with cholelithiasis N=7	Patients without Cholelithiasis N=15	P value
Spleen						
Removed	3(5.1)	1(2.0)		3(42.9)	1(6.3)	
Average	0(0.0)	0(0.0)	0.393	0(0.0)	6(43.7)	0. 32
Enlarged	56(94.9)	49(98.0)		4(57.1)	8(50.0)	
Liver (cirrhotic)						
Shrunken	43(72.9)	36(72.0)		3(42.9)	4(25.0)	
Average	12(20.3)	10(20.0)	0.97	4(57.1)	7(50.0)	0.315
Enlarged	4(6.8)	4(8.0)		0(0.0)	4(25.0)	
Gall bladder						
Shape						
Pear	57(96.6)	48(96.0)		6(85.7)	15(100.0)	
Distended	2(4.0)	0(0.0)	0.132	1(14.3)	0(0.0)	0.122
Contracted	0(0.0)	2(3.4)		0(0.0)	0(0.0)	
Wall						
Normal	0(0.0)	2(4.0)	0.121	1(14.3)	3(18.8)	
Thick wall	59(100.0)	48(96.0)		6(85.7)	12(81.2)	0.795
Ascitis						
Mild	2(3.4)	13(26.0)				
Moderate	40(67.8)	27(54.0)	0.003			
Marked	17(28.8)	10(20.0)				
Focal lesion	6(10.2)	15(30.0)	0.009			
IHBRRID	1(1.7)	0(0.0)	0.355			
PV	Mean±SD	Mean±SD	P value	Mean±SD	Mean±SD	P value
	1.8±0.2	1.4±0.3	<0.001**	1.3±0.2	1.4±0.3	0.605

Table (4): Complications in decompensated group

Variables	Patients with cholelithiasis N=59	Patients without cholelithiasis N=50	P value
Spontaneous bacterial peritonitis	32(54.2)	26(52.0)	0.816
Hepatic encephalopathy	45(76.3)	32(64.0)	0.161
Hematemesis and melena	25(42.4)	15(30.0)	0.182

Table (5): The etiology of liver disease

Etiology of liver disease	Patients with cholelithiasis N= 66(%)	Patients without cholelithiasis N=65(%)	P value
HCV	50(75.8)	51(78.5)	**0.713**
HBV	9(13.6)	12(18.5)	0.451
HCV+HBV	6(9.1)	2(3.0)	2(3.0)
Autoimmune	1(1.5)	0(0.0)	0.319

Table (6): Child Pugh classification of cirrhosis in patients with and without cholelithiasis

Variables	Patients with cholelithiasis N=66(%)	Patients without cholelithiasis N=65	Odds ratio (95%CI)	P Value
Child A	7(10.6)	15(23.1)	0.4(0.13-1.14)	0.06
Child B	14(21.2)	22(33.8)	0.53(0.22-1.23)	0.105
Child C	45(68.2)	28(43.1)	2.83(1.31-6.17)	0.004

Table (7): Frequencies of risk factors for patients with chronic liver disease

Variables	Patients with cholelithiasis N=66	Patients without cholelithiasis N=65	Odds ratio (95%CI)	P Value
Smoking	26(39.4)	28 (43.1)	0.86(0.4-1.83)	0.669
DM	40(60.6)	16(24.6)	4.71(2.09-10.74)	<0.001**
history of CCP intake	38(57.6)	4(6.2)	18.69(5.67-68.21)	<0.001**

DISCUSSION

Gallstone disease is a multifactorial disease based on a complexinteraction of environmental and genetic factors. The incidence rate of gallstones in the general populationwas found to be 0.60% per year. The current systematic review of Shabanzadeh et al. additionally identified some dietary factors,comorbidities, and parity to be positively associated and consumptionof coffee, fish, and whole meal bread to haveinverse associations to incident gallstones [13]. Impaired gallbladder contractility as in cirrhosis was found to be in direct relation with the severity of liver disease.

The current study revealed 66 patients with cholelithiasis out of 131 patients with chronic liver disease with a proportion 50.4%. This prevalence is higher than the previously reported in an Egyptian study by Eljaky et al. (21.8 %) [5]. Also, many studies confirmed the relation between liver cirrhosis and GSD with varying percentage (23-40%) [4,8,14]. This variability in the frequency may be explained by the different sample size, patients' characters and the stage of liver disease.

Our study revealed 22 compensated patients and 109 decompensated patients. Of them, 30.4% and 54% had cholelithiasis respectively. This result is consistent with the study of Naheed et al. which reported a higher incidence of cholelithiasis with cirrhosis [15]. Furthermore, Acalovschi et al., reported that the incidence of gallstones is five times higher in decompensated patients [8].

In this study, female predominance among patients with cholelithiasis was statistically significant in the compensated group (p=0.006). The high frequency of cholelithiasis in compensated females may be due to childbearing age of these females which explain excessive secretion of cholesterol into bile under the influence of estrogen. Therefore, the incidence of gallstones in women is significantly reduced after menopause due to decline in estrogen levels [16].

Our result revealed that 28.6% compensated patients with cholelithiasis had diabetes mellitus versus 18.8% without cholelithiasis. Also, 59.3% of decompensated patients with cholelithiasis had diabetes mellitus versus 26% without cholelithiasis. This results is consistent with the study of Shizuka et al., which proved a higher incidence of GSD in diabetics and explained that by the disturbed lipid profile and diminished gall bladder motility in diabetic patients [17]. Moreover, advanced liver disease may add to the disturbed lipid profile and also associated with disturbed glucose homeostasis [18].

The body mass index of compensated patients with cholelithiasis showed a statistically significance increase in comparison with patients without cholelithiasis, suggesting that obesity is a risk factor for cholelithiasis. This result is consistent with the study of Sahi et al. which reported that obese subjects (BMI >30 kg/m2) are at twice the risk of gallbladder disease than those with a normal BMI [19].

The history of contraceptive pills usage was frequently reported among female patients with cholelithiasis in decompensated group with a statistically significant pattern when compared to females with no history of contraceptive pills usage (p<0.001). This result was consistent with the study of Cirillo et al. which proved that women on long term oral contraceptives have a two folds increased incidence of cholelithiasis over controls and postmenopausal women taking estrogen-containing drugs have a significant increase frequency (around 1.8 times) of cholelithiasis [20].

It was reported that gallstones are twice as common in CLD with portal hypertension due to prolonged congestion and increase in venous hydrostatic pressure, which results in edema of the gallbladder and reduce gallbladder contractility [21].

As regard complication of cholelithiasis among our compensated and decompensated patients. Obstructive jaundice and cholecystitis showed statistically significant increase in compensated group. This finding is consistent with Acalovschi et al. suggesting that hypo-contractility could promote gallstone formation in advanced stage of liver disease.

Regarding to the etiology of CLD in patients with and without cholelithiasis, out result showed that hepatitis C in the most common cause for cholelithiasis (75.8%) followed by hepatitis B (13.6%) and autoimmune disease(1.5%).This result is consistent with the study of Eljaky et al., which proved that the prevalence of GSD in patients with chronic HCV infection was 24.7% versus 10.4% in patients with chronic hepatitis B infection [5]. The high prevalence of gall stones in patients with chronic HCV infection may be attributed to HCV which was detected in the biliary epithelium and it may potentially impair gall bladder function and contribute to gall stone formation [22].

CONCLUSION

The higher incidence of cholelithiasis in CLD appears to be associated with HCV infection, portal hypertension, gallbladder stasis, and obesity. The risk is increased with the severity of CLD. This is an important parameter to be considered in a country with high prevalence of HCV as Egypt.

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A molecular Approach for Evaluation of Experimental Trials of Anti Schistosomal Vaccination in Murine Models

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FCA, modified Kato
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PCR and egg DNA*

Background and study aim: Current schistosomiasis control strategies are mainly based on chemotherapy, but many researchers believed that the best long term strategy to control schistosomiasis is through immunization with anti-schistosomiasis vaccines. This study aims at assessment of the efficacy of different potential anti-schistosomal vaccines (as crude soluble egg antigens (SEA), soluble worm antigen preparation (SWAP) and combined SEA & SWAP) by parasitological and molecular studies in experimental murine models.

Materials and Methods: Sixty male laboratory bred Swiss Albino mice were used and divided into six groups; control normal (G1), control infected by ± 80 cercariae by S.C. route (G2), Freund's adjuvant (adj.) received then infected (G3), SEA+adj. received then infected (G4), SWAP+ adj. received then infected (G5) and combined (SEA+SWAP) + adj. received then infected (G6). A schedule of sensitization, immunization and schistosomiasis challenge were followed and performed on different mice groups. Mice were euthanized 10 weeks post-infection. Potential vaccine efficacy was investigated by parasitological and molecular studies including egg count/gram stool using modified Kato thick smear, liver egg load, oogram pattern in the liver and stool PCR to detect *S. mansoni* egg DNA in stools of studied mice.

Results: The combined (SEA+SWAP) vaccine caused the highest significant reduction in the fecal egg count followed by SWAP then SEA antigens. On the other hand, the highest percentage reduction in eggs/gram liver tissue was attributed to the combined (SEA+SWAP) followed by SEA then SWAP antigens. Regarding oogram results, the combined (SEA+SWAP) antigens were more efficient in increasing the number of dead ova with highly significant reduction in the number of mature & immature ova, followed by SEA then SWAP antigens. The lowest percentage of *S. mansoni* egg DNA detected by PCR in stool samples was encountered with the combined (SEA+SWAP), followed by SEA then SWAP antigens.

Conclusion: The parasitological and PCR-based assessment studies denoted that the combined (SEA+SWAP) vaccine candidate was the most effective in protection against schistosomiasis challenge. The results of parasitological and molecular studies were nearly similar but the molecular study was more sensitive, definite and accurate.

INTRODUCTION

Schistosomiasis is a parasitic disease caused by blood flukes of the genus *Schistosoma.* After malaria and intestinal helminthiasis, schistosomiasis is the third most destructive tropical disease in the world, being a major source of morbidity and mortality for developing countries in Africa, South America, the Caribbean, the Middle East, and Asia **[1]**. Mature patent schistosome infections are associated with chronic local inflammatory response to schistosome eggs trapped in host tissues **[2].** In the context of schistosomiasis, an effective anti-schistosome vaccine would contribute greatly to a decrease in morbidity

associated with schistosomiasis via protective immune responses leading to reduced worm burdens and decreased egg production [3]. *Schistosoma* parasites secrete & excrete a number of different antigens into circulation of the host; these antigens are classified according to the stage of development of the parasite into cercarial; adult worm and egg antigens [4]. In order to develop an accurate immunization procedure, many different antigens have been prepared and tested as adult worm and egg antigens [5] and irradiated cercarial antigen [6]. The combination of two vaccines provides augmentation of the protective immunity and reduction of hepatic immunopathology [7].

Microscopic examination of stools remains the gold standard test for diagnosis of *schistosoma mansoni* infection, either by using a direct fecal smear or after the application of a concentration technique. *Schistosoma mansoni* eggs are easy to be detected and identified on microscopy owing to their characteristic size and shape with a lateral spine [8].

Kato technique is widely used in field studies and recommended by WHO for diagnosis of intestinal schistosomiasis when the intensity of infection is high, as it could detect the egg load as low as 40 eggs per gram stool [9]. The Kato-Katz technique was found to be more sensitive than the formalin ether concentration technique and the wet smear preparation [10].

Polymerase chain reaction (PCR) is a relatively simple technique that can detect a nucleic acid fragment and amplify its sequence, with high sensitivity and specificity [11]. Pontes et al. reported the first use of PCR for the diagnosis of *S. mansoni* DNA in fecal samples [12].

The current study aims at assessment of the efficacy of different potential antischistosomal vaccines as crude SEA, SWAP and combined SEA & SWAP by parasitological and molecular (PCR) studies experimentally, in murine models.

MATERIALS AND METHODS

This experimental study was conducted during the period between February 2013 and May 2014 at the Departments of Medical Parasitology and Biochemistry, Faculty of Medicine, Zagazig University, and Theodor Bilharz Research Institute, Imbaba, Giza, Egypt.

Infective cercariae: According to Liang et al. [13], *S. mansoni* cercariae (Egyptian strain) were obtained from infected laboratory bred *B. alexandrina* snails which were purchased from Schistosome Biological Supply Center at Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. After exposure to light for at least 4 hours, *S. mansoni* cercariae shed from the snails were used to infect the studied experimental animals (± 80 cercariae/mouse) by subcutaneous injection.

Animals: The current work was carried out on male laboratory-bred, parasite-free Swiss albino mice, about eight weeks's old age and 18-20 grams in weight for each mouse at the beginning of the experiment. Mice were obtained from Schistosome Biological Supply Center at Theodor Bilharz Research Institute (TBRI). The mice were maintained on a standard commercial pelleted diet with free accessible water and in an air-conditioned animal house at 20-22°C all over the time of study.

Materials:
- Schistosomal antigen preparations: Schistosomal crude antigens (SWAP and SEA) were obtained from Schistosome Biological Supply Center at Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt.
- Adjuvants: Freund's complete adjuvant (FCA) was obtained from Sigma Chemical Co., St Louis, Mo, USA and emulsified in phosphate-buffered saline (PBS) at a ratio of 2:1.

Experimental design:
Sample size:
Sixty albino mice. Mice were divided into six groups (of ten mice each).

Preparation of Antigens:
SWAP was prepared according to the method of Salih et al. [14], and SEA was prepared according to the method of Boros and Warren [15], the protein content was estimated using Bio-Rad kit (Bio-Rad Laboratories, Hercules, California, USA) [16] and the final concentration was adjusted with PBS to a concentration 50 μg / ml and stored at -70°C until use.

Antigen administration regimens:
The immunization schedule was performed according to Nabih and Soliman [17]. Each mouse was sensitized with an initial S.C. injection of 200 μl of the extracted antigen with a total antigen concentration of 30 μg protein. After two weeks, a second S.C. injection of 200

μl of the same antigen was taken and diluted to contain 20 μg protein. The antigen was combined with complete Freund's adjuvant at a 1:1 ratio and injected S.C. **[18].**

Infection of mice:

Infection of mice was done by subcutaneous injection with about ±80 *S. mansoni* cercariae/ mouse, suspended in 0.2 ml solution (cercariae in distelled water) 3 weeks after the initial S.C. antigen injection. The suspension was injected into the loose skin of the back of the mouse using an insulin syringe (1 cm length) **[19].**

Animal groups:

Group 1: control non infected group.

Group 2: control infected group (infected by ± 80 *S. mansoni* cercariae/ mouse by subcutaneous injection).

Group 3: mice were subcutaneously injected with complete Freund's adjuvant (CFA) and then infected by ±80 *S. mansoni* cercariae (adj. + infected group).

Group 4: mice were subcutaneously injected with soluble egg antigen (SEA) + complete Freund's adjuvant, and then infected (SEA+ adj. + infected group).

Group 5: mice were subcutaneously injected with soluble worm antigen (SWAP) + complete Freund's adjuvant, and then infected (SWAP+ adj. + infected group).

Group 6: mice were subcutaneously injected with SWAP and SEA +complete Freund's adjuvant, and then infected (SEA+ SWAP + adj. + infected group).

Vaccination efficacy assessment:

Animals were sacrificed by cervical dislocation 10 weeks post infection. Efficacy of the vaccination was assessed by parasitological and molecular studies.

1- Parasitological studies:

a) Egg count/gram stools using the modified Kato thick smear **[20]** daily starting from the 7th week post-infection, the average egg count for each mouse and group was calculated and tabulated.

b) Tissue (liver) egg load **[21]**:

c) Oogram pattern in the liver **[22]:** for each liver specimen, 100 eggs were microscopically counted & classified into viable (either immature or mature) and dead.

2- Molecular studies:

Detection of *S. mansoni* egg DNA in stool samples of studied mice by Polymerase chain reaction "PCR" **[12]**:

(A) DNA extraction :

In this study, Extraction of DNA from stool samples was done using **QIAamp® DNA stool mini Kit .Cat. No. 51504 (QIAGEN, GmbH, Hilden, Germany)**.

(B) DNA amplification and PCR reaction:

The PCR was done using a forward primer (5′-GAT CTG AAT CCG ACC AAC CG-3′) and reverse primer (5′-ATA TTA ACG CCC ACG CTC TC-3′) that were designed to amplify the 121-bp tandem repeat DNA sequence of *S. mansoni*. Briefly, for a 25 μL final volume of PCR mixture, 5 μL of DNA extract was used as template, 12.5 μL 2X QiagenTag PCR Master Mix (0.05 u/μL Taq DNA Polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTPs), 1.5 μL of each primer and finally 4.5 μL of molecular biology grade water. The amplification reaction was carried out for 35 cycles, with each cycle consisting of a denaturation step at 95 °C for 40 s, an annealing step at 60 °C for 30 s and an extension step at 72 °C for 1 min. The first cycle had an extended denaturation step for 5 min and the reaction was ended with an extension step at 72 °C for 5 min. Amplified PCR products were then analyzed by electrophoresis in 2.5% agarose gels and detected by UV trans-illumination after staining with ethidium bromide. Stool samples positive for *S. mansoni egg* DNA showed bands at the level of 121bp in the DNA ladder, while Stool samples negative for *S. mansoni egg* DNA showed <u>no</u> bands at the level of 121bp in the DNA ladder **[12].**

Statistical analysis:

Data were entered, checked and analyzed using statistical computer program Statistical package for Social Sciences (SPSS version 16 windows). Data were expressed as the mean ± standard deviation (SD). Comparison between the mean values of different parameters in the studied groups was performed using one way analysis of variance (ANOVA) test, with paired (t) test for comparison between means of two groups. Chi square test was used for comparing between the qualitative data.

RESULTS

I- Results of the parasitological studies:

Table (1): The results of Kato technique, Liver egg load and Liver oogram in the different groups

Group	No. of eggs/g stool Mean ± SD	Liver egg load Mean ± SD	Liver oogram		
			Immature Mean ± SD	Mature Mean ± SD	Dead Mean ± SD
G2	361.83±37.66	1036.17 ± 72.43	25.3 ± 3.2	70.5 ± 1.6	4.2 ± 0.7
G3	234.83±27.25	1010.83 ± 21.25	24.6 ± 5.6	69.1 ± 2.9	6.3 ± 1.6
G4	64±7.4***	356.83 ±36.18***	18.1±2.1*	35.5± 1.8**	46.4 ± 2.3***
G5	56.33± 8.62***	462.83 ±41.22***	19.6±1.3*	44.1 ±2.4**	36.3 ±1.8**
G6	35.17 ± 8.2***	108.8 ± 34.13***	6.3±1.7***	21.3± 2.8***	72.4± 3.1***

*Significant difference from infected control at p<0.05.
**High significant difference from infected control at p<0.01.
***Very high significant difference from infected control at p<0.001.

II- Results of the molecular (PCR) study:
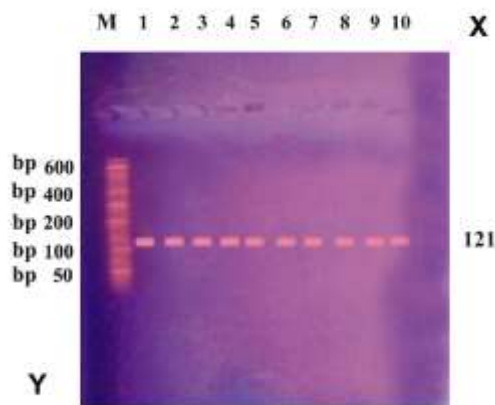
Figure (1): PCR results of (G2) showing that all fecal samples were positive for *S. mansoni* egg DNA (10 bands at 121 bp).
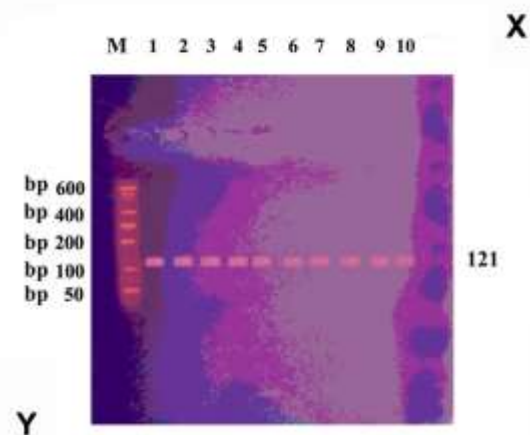
Figure (2): PCR results of (G3) showing that all fecal samples were positive for *S. mansoni* egg DNA (10 bands at 121 bp).
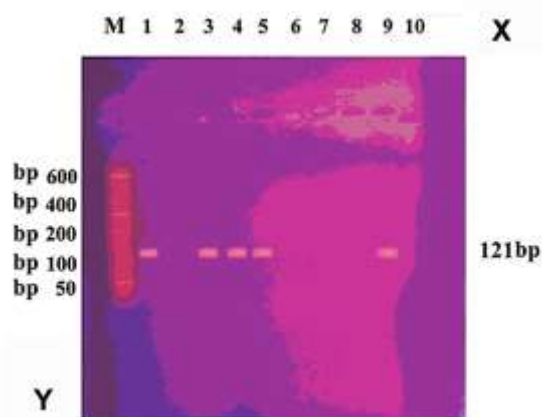
Figure (3): PCR results (G4) showing that **five** out of ten fecal samples were positive for *S. mansoni* egg DNA (at 121 bp).
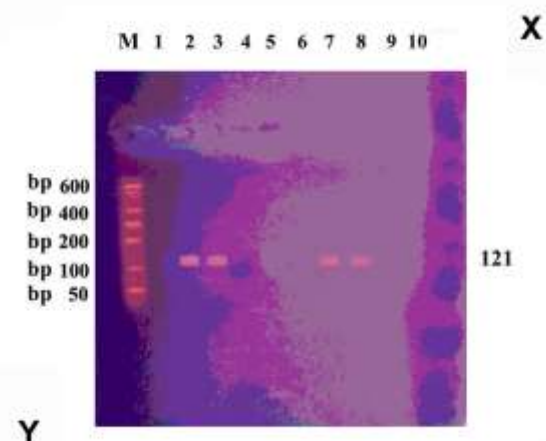
Figure (4): PCR results of (G5) showing that **four** out of ten fecal samples were positive for *S. mansoni* egg DNA (at 121 bp).
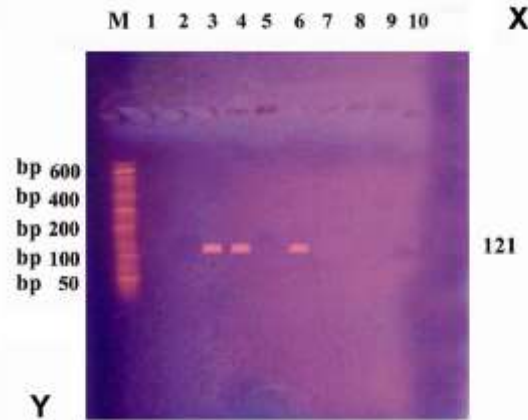
Figure (5): PCR results of (G6) showing that **three** out of ten fecal samples were positive for *S. mansoni* egg DNA (at 121 bp).

- **Axis (Y)** represented the number of DNA base pairs in the used DNA marker (ladder).
- **Axis (X)** represented the stool samples of each group.
- **M:** molecular weight marker.
- **Lanes 1 to 10:** represented the PCR results of fecal samples of each group.

Table (2): PCR results of studied control and vaccinated groups

Group	Number of stools samples	+ve stools samples by PCR	-ve stools samples by PCR	% of +ve samples	Chi sequare χ^2	P-value
G1	10	0	10	0 %	G2VsG4=6.7	0.01**
G2	10	10	0	100 %	G2VsG5=8.5	0.003**
G3	10	10	0	100%	G2VsG6=10.8	0.001**
G4	10	5	5	50%	G3VsG4=6.7	0.01**
G5	10	4	6	40%	G3VsG5=8.5	0.003**
G6	10	3	7	30%	G3VsG6=10.8	0.001**
					G4VsG5=0.2	0.6
					G4VsG6=0.8	0.4
					G5VsG6=0.2	0.6

**High significant difference between the groups at P-value <0.01

G2VsG4	**G2VsG5**	**G2VsG6**
G3VsG6	**G3VsG4**	**G3VsG5**

Insignificant difference between the groups at P-value > 0.05

G4VsG5	**G4VsG6**	**G5VsG6**

Table (3): Comparison between PCR and Kato technique results for evaluation of the efficacy of the different potential antischistosomal vaccines regarding the percentage of positive samples

	No. of samples	No. of +ve samples	No. of –ve samples	% of +ve samples
Kato technique	60	26	34	43 %
PCR	60	32	28	53 %

Table (4): The diagnostic value of PCR technique using (Sensitivity %, Specificity %, PPV %, NPV % and Accuracy %) as compared to Kato technique

PCR				
Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
100 %	82 %	81.25%	100%	90%

Table (5): Parasitological and molecular results in studied groups

Group \ Item	G2	G3	G4	G5	G6
No. of eggs/g stool Mean±SD	361.83±37.66	234.84±27.25	64±7.4***	56.33±8.62 ***	35.17±8.2***
Liver egg load Mean±SD	1036.17±72.43	1010.83±21.25	356.83±36.18***	462.83±41.22***	108.8±34.13 ***
Immature Mean±SD	25.3±3.2	24.6±5.6	18.1±2.1*	19.6±1.3*	6.3±1.7***
Mature Mean±SD	70.5±1.6	69.1±2.9	35.5±1.8**	44.1±2.4**	21.3±2.8***
Dead Mean±SD	4.2±0.7	6.3±1.6	46.4±2.3***	36.3±1.8**	72.4±3.1***
% of +ve stool samples by PCR	100 %	100 %	50 %	40 %	30 %

*Significant difference from infected control at p<0.05.

**High significant difference from infected control at p<0.01.

***Very high significant difference from infected control at p<0.001.

DISCUSSION

Schistosomiasis is a parasitic disease caused by the digenetic trematodes of the genus *Schistosoma* members which are commonly known as blood flukes [23]. It is one of the most prevalent parasitic diseases in the world, second to malaria, it is estimated that schistosomiasis causes about 200,000 deaths per year [24].

In this work, vaccination of mice with different schistosomal antigens (SEA, SWAP and combined SEA & SWAP) showed very high significant results which were supported by previous studies that demonstrated the ability of antigens obtained from different life stages of the parasite to induce protective immune response in mice [25]. On using the crude vaccines, we are putting all the parasite stage components in one preparation because that parasite stage is parasitizing man as one unit by its whole components. So, the current study aimed to evaluate the efficacy of the potential antischistosomal vaccines using crude preparation of (SEA, SWAP and combined SEA+SWAP) by parasitological and molecular methods.

A significant reduction in the number of *S. mansoni eggs* in stool samples was detected 10 weeks post infection in comparison to the control infected group. The highest percentage reduction "90.3 %" was encountered with the combined SEA & SWAP-vaccinated group. On the other hand, there was insignificant reduction in ova count in (Freund's adjuvant + infected) group when compared to the control infected group.

These results were in agreement with those reported by Gundersen et al. [26] who found a positive correlation between the number of eggs per gram stools (using Kato technique) and the schistosomal antigen level.

The present results were in agreement with those of Ismail [27] who used also crude antigens and reported very high significant reduction in fecal egg count in mice vaccinated by adjuvant and combined schistosomal antigens (CAP + SWAP +SEA). The current results were in accordance with Etewa et al. [28], who found that a significant reduction in the mean egg counts/g stools was recorded by Kato technique in combined (CAP+SEA+SWAP) - vaccinated group, as compared to control infected group. On the other hand, a statistically insignificant reduction in the mean egg counts/g stools was recorded in the adjuvant- received group as compared to control infected group (P>0.05).

The present work cleared that the most effective antigen with very high significant reduction in liver egg load (the mean egg count/gram liver) was the combined vaccine + FCA, followed by SEA + FCA then SWAP + FCA. On the other hand, insignificant reduction was detected in the FCA- received group as compared to control infected group. These results nearly coincided with those of Etewa et al. [29] who detected that

the most effective antigen with significant reduction in tissue (liver) egg load was the combined (CAP, SWAP & SEA) + Freund's adjuvant with 93.67% percentage reduction as compared to control infected group in spite of using an extra antigen (CAP). On the other hand, the current results were not near to those of Romeih et al. [30] who noted that there was a significant reduction (41.53%) in the mean number of eggs/g liver of the multivalent DNA-vaccinated mice group, as compared to the control group. This may be attributed to the difference in antigen preparation. They also added that a significant reduction (29.90%) in the fecundity of female worms was observed after vaccination, denoting that the multivalent DNA vaccine permitted a better growth of mice and reduced the worm burden, egg number and worm fecundity.

In agreement, Fallon and Dunne [31] & Ismail [27] reported that administration of individual or combined schistosomal antigens led to significant reduction in worm burden with subsequent decrease in the number of deposited eggs. Besides, Teixeira de Melo et al. [25] explained that immunization of mice with adult worm tegument (Smteg) together with Freund's adjuvant induced a Th1 type of immune response associated with a significant reduction in eggs trapped in the liver by 65%.

On the other hand, results of the present study were higher than that of El-Ahwany et al. [32] who used SEA without Freund's adj. and Rezende et al. [33] in SWAP vaccinated group; they reported a percentage reduction in the liver tissue egg load of 42.8% and 8.4%, respectively.

This contrary may be explained by the higher protective effect of the combined (cocktail) antigens together with the augmenting role of Freund's adjuvant in nonspecific induction of the immune system.

In this work, the most effective antigen resulting in remarkable oogram changes with highly significant reduction in immature & mature ova and highly significant increase in dead ova was the combined vaccine + Freund's, followed by SEA + Freund's then SWAP + Freund's. On the other hand, insignificant oogram changes were detected in the adjuvant + infected group, as compared to control infected group. The present findings were partially agreed with those of Ismail [27] and Etewa et al. [29] who reported marked oogram changes in the vaccinated groups with highly significant reduction in immature and mature ova together with a highly significant increase in dead ova in combined vaccine (CAP + SEA + SWAP) + Freund's, followed by SEA + Freund's, then SWAP + Freund's.

In the present study, it was noticed that the decrease in *Schistosoma mansoni* egg count in stools of vaccinated groups (G4, G5 and G6) was directly proportional to the decrease in liver egg load, mature and immature egg numbers, while it was inversely proportional to the number of dead eggs in the oogram pattern.

These results were similar to that of Ismail [27] and Etewa et al. [29] who found marked reduction in fecal egg count, liver egg load, immature and mature eggs number in oogram and marked increase in dead ova in the vaccinated groups which demonstrated positive efficacy of the tested antigens.

Techniques for identifying circulating S. mansoni DNA have been widely used and such methods have demonstrated that it is possible to detect infections as early as one day after parasite exposure [34].

PCR-based diagnosis has been shown to be highly sensitive and specific and should therefore be considered as alternative methods for the diagnosis of *S. mansoni* infections [35].

The present study revealed that the combined (SWAP + SEA) potential vaccine was the most protective regarding detection of *S. mansoni* egg DNA in stools of *S. mansoni* - challenged mice with 70% success, followed by SWAP then SEA antigens with 50% and 40% success, respectively, as compared to control infected group. On the other hand, the adjuvant alone (G3) exhibited no protection and all fecal samples were positive for egg DNA by PCR.

Considering that the Kato technique is the gold standard test, we found that the PCR sensitivity was 100 %, specificity was 82 %, PPV was 81.25% and NPV was 100%. So, PCR was more accurate in evaluating the tested antigens as potential vaccines.

The obtained results are nearly in agreement with Pontes et al. [36] who compared between Kato technique and PCR for diagnosis of *S. mansoni* infection and evaluation of the efficacy of Praziquantel in treatment of infection. They found that the PCR technique showed a sensitivity of 96.7%, a specificity of 88%, PPV

was 78.4% and NPV was 98.3%, using the parasitological examination as the reference test.

Moreover, our results were near to those of Gomes et al., who compared between the Kato technique and PCR for diagnosis of *S. mansoni* in stool samples. They studied 67 cases and found that Kato technique detected infection in 42% of samples while, PCR detected infection in 61% of samples. However, they reported that PCR sensitivity was 92.9% and PCR specificity was 61.5% **[37]**.

The current results were also in agreement with those of Oliveira et al., who studied fecal samples using 2 PCR assays utilizing distinct primer pairs. One of the primer pairs was targeted to a highly repeated 121-base pair sequence of *S. mansoni*, and the other was targeted to *Schistosoma* 28S rDNA. The results obtained with stool samples from individuals with schistosomiasis showed a high sensitivity for PCR as *S. mansoni* DNA was detected in 91% of the samples analyzed **[38]**.

The obtained results are also supported by de Carvalho et al., who reported that the assessment of a single faecal sample by PCR detected more cases of infection than the analysis of one sample with two slides using the Kato-Katz technique, suggesting that PCR can be a useful diagnostic tool, particularly in areas with low endemicity. They found that the PCR sensitivity was 93.8%, specificity was 70%, PPV was 100% and NPV was 99.3 % **[39]**.

The present findings partially differ from those of Carneiro et al., who analysed and compared the results from a conventional PCR-based method with results from serological (ELISA) and parasitological tests. *S. mansoni* was detected by the KK technique in 33.9% of cases. In contrast, the PCR-based method was able to detect *S. mansoni* in 68.4% of cases i.e. more than two-fold increase in sensitivity. However, PCR failed to detect DNA in six samples that were confirmed to be positive by parasitological examination and ELISA **[35]**. Pontes et al. **[36]** reported that missed cases after PCR were certainly misdiagnosed by the DNA amplification assay due to many factors such as: inhibition of the amplification reaction by fecal compounds and/or DNA degradation during transportation from the field, variation in egg output and uneven distribution in feces.

CONCLUSION

On using combined antigens as a potential vaccine, more stimulation of the immune system against different antigenic components will occur, thus increasing the vaccine efficacy. This suggests that development of a multivalent (cocktail of antigens) vaccine may be the way forward, while the molecular method (stool PCR) for vaccine evaluation was more sensitive, accurate and definite.

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Hydatidosis in Morocco: Review of literature and epidemiology

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Hydatidosis or hydatid cyst is a prevalent zoonosis all over the world due to larval forms of the tapeworm of the genus Echinococcus. Echinococcus granulosus is the most frequent form of echinococcal infection in humans. The disease generally results from an oral ingestion of the parasite ova eliminated with feces of dogs leading to the emergence of hydatid cysts.

Hydatidosis is a health problem that may remain asymptomatic for several years. This disease is endemic in Morocco and is recorded to be a serious problem that requires great caution.

INTRODUCTION

Hydatidosis or hydatid cyst is a widespread zoonosis throughout the world due to larval forms of the tapeworm of the genus Echinococcus present in the small intestines of carnivores **[1]**. Infection with *Echinococcus granulosus* is the most frequent form of echinococcal infection in humans **[2]**. It is actually endemic in numerous areas of the world, like nations near to the Mediterranean Sea, in addition to northern and eastern Africa, China, South America, central as well as western Asia, and Australia **[3]**. The disease commonly results from an oral ingestion of the ova parasite eliminated with feces in dogs and transmitted via close contact with infected animals. Moreover, the consumption of contaminated food, which is under scrutiny, is a potential means of infection, especially in developing countries **[4]**. The disease is endemic in Morocco and presents a serious problem that increasingly draws attention.

Echinococcus granulosus is, known as a tapeworm, belongs to the Taeniidae family .Its adult resides in the small intestine of carnivorous definitive hosts, including dogs, coyotes, or wolves. It is a minute worm of 2 to 9 mm in length. It contains three or four segments and seems to have a globular scolex of 0.3 mm in diameter possessing a rostellum and four cup-like oval suckers. This rostellum structure is armed with a double crown of large and small hooklets. The amount of hooklets can range between 25 and 40 **[5]**. The scolex is succeeded by a short neck and generally only one or two immature segments; after that there is the mature segment which is the broadest and the longest. The uterus includes up to 500 eggs which are released into the feces from the ruptured segment.

Echinococcal cyst stages take place in herbivorous intermediate hosts, like camels, sheep and cattle. echinococcal cyst is a fluid-filled, rounded, unilocular cyst which contains an inner germinal layer of cells reinforced by a characteristic acellular, laminated membrane of variable thickness. Every cyst is enclosed by a host-produced layer of granulomatous adventitial reaction. Small vesicles named brood capsules bud internally from the germinal layer and generate multiple protoscolices via asexual division **[6]**. Humans get infected by eating tapeworm eggs transferred from an infected carnivore.

CLINICAL FEATURE

Accidental human infestation by larval form leads to the emergence of hydatid cysts. Generally, primary infections in humans are represented by a single cyst; nevertheless, 20-40% of persons have multiple cysts or multiple organ involvement [7]. The liver and lungs are the most commonly affected organs [8]. Less frequently involved organs are the spleen, the kidney, the heart, the bone, central nervous system, and other organs [9]. Hydatidosis is a health problem that may remain asymptomatic for several years, with symptoms generally appearing only when the cysts increase in size [10].

DIAGNOSIS

The ultimate diagnosis for mosthuman forms of hydatid disease is through physical imaging techniques, like ultrasonography, radiology, computed tomography and also magnetic resonance imaging [11]. Antibody assays are helpful to check presumptive radiologic diagnoses; however, few affected individuals with cystic echinococcosis do not reveal a detectable immune response [12]. In current times, significant efforts are already made to improve most of these techniques employing new recombinant proteins, synthetic peptides, and combinations of identified antigens. Nonetheless, regardless of the fact that immunoglobulin levels are undetectable, solely 60%-80% of affected people are seropositive [13]. The indirect hemagglutination test is sensitive but is currently been changed by the enzyme immunoassay (ELISA) for preliminary checking of sera. Confirmation of reactivity can be acquired by illustration of specific echinococcal antigens by immunoblot assays. Eosinophilia is found in <25% of infected people. In seronegative persons, a presumptive diagnosis could be proved by showing protoscolices or hydatid membranes in the liquid extracted by percutaneous aspiration of the cyst [14].

EPIDEMIOLOGY

Around the world, the important endemic regions are localized in the Mediterranean peripheries: North Africa, Middle East, Greece, Turkey, Cyprus, and southern Italy and

Spain (The human infestation incidence is about 10 cases per 100,000 habitants), as well as East Africa, especially Kenya, where the incidence is the highest in the world with 220 cases per 100,000 inhabitants [15].

Current reports reveal the endemic occurrence of *E. granulosus* in dogs as well as in livestock and of human cases of CE in almost all North African Arabic countries which include Algeria, Libya, Egypt, and Tunisia [16]. In Algeria,annual incidence according to hospital cases has been recorded 3.6–4.6/100,000 populations [17]. The incidence of surgically proved cystic echinococcosis in eastern Libya was expected to be less than 4.2 cases per 100,000 inhabitants. [17].

This incidence is less than Tunisia which is estimated of15 cases per 100,000 inhabitants [18]. Christine M. Budke et al reported in systematic reviews an incidence rate of 2.3,3.1, 3.35, and 18.0, 32.0/100,000 persons-year in Jordan, Palestine, Bulgaria, Kyrgyzstan and Peru respectively [19].

EPIDEMIOLOGY ON MOROCCO

The exact data on the prevalence of this disease is unknown. It is certainly underestimated due to the existence of asymptomatic forms and the low level of medicalization of the population. Thus, studies on the prevalence of hydatid disease in humans are rare and are mostly devoted to rural and operated cases [20].

The distribution of operated hydatid cyst cases in different regions of Morocco shows that the highest incidence is found in two regions: Rabat-Salé-Zemmours-Zaers and Meknès-Tafilalet fig1 [21].

Fig (1): distribution of operated hydatid cyst cases between 1980 and 1992 in public hospitals

Another study was carried out by Derfoufi et al to evaluate the epidemiological profile of hydatidosis in Morocco between 1980 and 2008declared an increased number of annual cases estimated to 5,2 per 100 000 habitants **[22]**.

In Morocco, the transmission of the parasite through a synanthropic cycle including dogs and livestock (sheep, cattle, camels, and Equidae). As outlined by Gemmell et al. **[23]**, 8,470 eggs are eliminated by infected dogs daily. Therefore, there is an increased pollution of the environment with eggs. A large number of dogs (estimated of around 2 millions) and which keep the livestock in the farms live in close contact with the rural population.

The number of stray dogs in the villages and on the outskirts of cities is also a major factor in the transmission; the high percentage of the population who hardly have any knowledge about the disease and the factors of its transmission is another cause of the disease spread **[24]**.

Sheep and cattle are the major intermediate hosts in Morocco, but sheep are considered the main source of infection to dogs and subsequently to human **[25]**.

There are a lot of celebrations or occasions, like weddings and Aid El Kebir (the great feast) in the Muslim countries, in which the ritual home-slaughter of millions of sheep takes place (5 million sheep and goats in Morocco). This habit leads to multiplying the parasitein owned and farm dogs, which helps to preserve high infection rates in these dogs, in ruminants and in humans **[26]**.

CONCLUSION

The risk of hydatid infection is high in rural regions where the close contact between dogs and humans is frequent. To control the disease, the measures should focus on dogs as the main factor of transmission. So it is necessary to get rid of stray dogs, deworm the suspected infected dogs as pets at home, andincinerate infected organs from dead intermediate animal hosts in addition to individual hygiene based on washing hands after gardening or touching dogs.

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