Genetic variability of NS5B region of Hepatitis C Virus patients in Egypt

M. A. Hegazy¹, M. A. Ahmed², M. M. Farag¹,*

¹Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt.
²Clinical Pathology Department, Military Medical Academy, Cairo, Egypt.

*Corresponding author E-mail: mohamed.farag@azhar.edu.eg (M. Farag)

ABSTRACT

Hepatitis virus C (HCV) is a serious public health issue worldwide. HCV is a cause of liver fibrosis, which may progress to cirrhosis or hepatocellular cancer. To assess the current study goal, we evaluated parameters including liver function, tumor marker, hematological, renal functions, lipid profile, and blood sugar levels in two groups (healthy controls and hepatitis C virus patients) to evaluate the current research’s aim. Based on the results of seven clinical tests, we observed insignificant changes in Cr, TSH, Alb, GCT, Alk.PH, Bill-total, Bill-direct, WBC, AFB, Cholesterol, and TG between the control and patient groups, there were significant differences for AST, ALT, Hb, platelets, INR, PT, PC percent, LDL, and FBS. The Hepatitis C virus profoundly affects blood parameters and body physiology in HCV patients. In the present study, 15 patients were sampled, and their HCV NS5B regions were directly sequenced and analyzed phylogenetically to learn more about HCV molecular epidemiology. According to the molecular epidemiology study, those who were treated with Sofosbuvir were found to have a high occurrence of certain genotypes.

Keywords: physiological; biochemical; hepatitis C virus; HCV; Genotyping; NS5B; Egypt

INTRODUCTION

HCV infection is a growing worldwide health concern. HCV is widespread in many countries and is a rising burden on communities and healthcare institutions. Cirrhosis and hepatocellular carcinoma (HCC) are becoming more prevalent long-term effects. (Wedemeyer et al., 2023)

The fast development of direct-acting antiviral (DAA) medicines for HCV infection has sparked significant excitement among HCV clinicians, who believe treatment approaches will soon be more effective, better tolerated, and last less time than present drugs. (Salama et al., 2022). The potential worldwide impact of therapeutic advances is today limited by a lack of effective treatments and pharmaceutical costs. Improved knowledge of the epidemiology and natural history of HCV infection is critical for guiding preventive and therapeutic public health efforts. (Mao et al., 2022)

There are seven distinct HCV genotypes, with nucleotide sequences that differ by 30% throughout the whole genome, and each genotype may be further classified into related subtypes (67 confirmed), with nucleotide sequence divergence ranging from 15% to 30%. (Smith et al., 2014 & Quer et al., 2015). Viral polymerases play an important role in the viral life cycle. They initiate processes required for the next stage of viral replication and appropriate transcription of the target DNA. (Sesmero and Thorpe 2015). Recent research has found direct-acting antivirals (DAAs) with a favorable safety profile and clinical effectiveness for treating chronic hepatitis C virus infection that targets particular HCV viral proteins. NS5B gene is in charge of viral RNA replication, and its catalytic region is substantially consistent among HCV genotypes, nucleotide inhibitors that target this protein are an intriguing therapy option (Sofia et al., 2010 & Stedman, 2014). The most accurate approach was thought to involve sequencing a genomic location that altered sufficiently to discriminate between types and subtypes (Neumann et al., 2022).

Consequently, the present research will compare the biochemical and physiological activities of the liver, kidney, lipid profile, blood sugar, and tumor marker in HCV patients receiving Sofosbuvir and a healthy control group. In addition, phylogenetic investigations of HCV strains derived from chronically infected individuals were used to develop a simple, accurate, and reliable genotyping map for HCV diagnosis.

PATIENT AND METHODOLOGIES

15 patients with a confirmed chronic hepatitis C virus diagnosis and a control group of 15 healthy people.

Liver function: -

AST: 

Kinetic method
Reagent 1 (AST substrate) 400 μL + Reagent 2 (Ast coenzyme) 100μL then (working reagent) incubate at 37 for 5 min. then add 50 μL serum samples and mix it well and read the absorbance at wave length 340 nm.

**Alt:**

Kinetic method

Reagent 1 (Alt substrate) 400 μL + Reagent 2 (Alt coenzyme) 100 μL

Then incubate the mix at 37 for 5 min, add 50 μL serum samples, mix it well, and then read absorbance at wave length 340 nm.

**Alb:**

Colorimetric method

Taking 2.5 ml albumin reagent + 10 μL serum samples, incubate the mix for 10 min at room temperature, then absorbance read at wave length 630 nm.

**Bilirubin total:**

Colorimetric method

Reagent 1 (sulphanilic acid) 100 μL + R2 (sodium nitrate) one drop + R3 (sodium benzoate) 500 μL + 100 μL serum samples then incubate the mixture at room temperature for 10 minutes then add Reagent 4 (titrate NaOH), then read absorbance against sample blank at wave length 578 nm.

(Sample blank)

Reagent 1 (sulphanilic acid) 100 μL + Reagent 3 (sodium benzoate) 500 μL + 100 μL serum samples, then carefully mix it for 10 minutes at room temperature then, add Reagent 4 (titrate NaOH), then read absorbance at wave length 578 nm.

**Bilirubin direct:**

Colorimetric method

Reagent 1 (sulphanilic acid) 100 μL + Reagent 2 (sodium nitrate) one drop + 1 ml saline + 100 μL serum samples, then incubate mix for 5 min at room temperature, then read absorbance against sample blank at wave length 546 nm.

(Sample blank)

Reagent 1 (sulphanilic acid) 100 μL + 1 ml saline + 100 μL serum sample. The absorbance is then measured after 5 minutes of incubation at room temperature at wave length 546 nm.

**GGT:**

Kinetic method

Reagent 1 (substrate) 400 μL + Reagent 2 100 μL. After 5 minutes at 37 °C, add 5 μL serum samples and read absorbance at wave length 405 nm.

**Prothrombin Time:**

By Semi-automated Coagulation analyzer

Take 200 μL Reagent and 100 μL plasma, incubate 15 minutes at 37 °C and add mix immediately, record clotting time

**Alk.ph:**

Kinetic method

Reagent 1 500 μL + Reagent 2 500 μL + 20 μL serum/plasma, incubate the mix for 1 min, then read at wave length 405 nm.

**Tumor marker:**

**AFP (alpha-fetoprotein):**

Using: ELISA for AFP, sample size: 25 μL, incubation time: 1.5 hours, and dynamic range: 5-200 ng/ml

**Hematology**

Take blood sample, then add it to (EDTA) anticoagulant and mix well, finally put it into a CBC device, and count hemoglobin, RBC, and WBC with differential and plt.

**Lipid profile**

**Cholesterol:**

Colorimetric method

1 ml cholesterol reagent + 10 μL serum samples, then incubate for 10 min at 37°C and read absorbance at wave length 546 nm.

**Triglycerides:**

Colorimetric method

1 ml triglycerides reagent + 10 μL serum sample, then incubate for 10 min at 37°C and read absorbance at wave length 546 nm at wave length 546 nm.

**High-Density Lipoprotein:**

R1 (diluted precipitant) 500 μL + 200 μL serum samples, mix, and let it sit at room temperature for ten minutes. Then spin for 10 minutes at 4,000 revolutions per minute. Finally, immediately remove the clear supernatant and test the cholesterol content using the CHOD-PAP technique.

**Blood sugar:**

Colorimetric method
Kidney marker:

Creatinine:

Jaffé colorimetric-kinetic

Reagent 2 (picric reagent) 500 μL + Reagent 3 (alkaline reagent)500 μL. micro, add 100 μL serum, shake well, and then read the result at wave length 505 nm.

HCV RNA Extraction and HCV-NS5B amplification.

HCV RNA extraction was carried out by using QIAmp reagent (QIAsymphony®SP/AS version 1), following the manufacturer’s instructions. The resultant HCV RNA (60 I) samples were stored at -70 0C until use. The presence of the HCV-NS5B gene was determined by nested PCR using the one step RT-PCR Master Mix Kit and the Hot start Taq plus PCR Master Mix Kit (QIAGEN) using the primers. The first round of RT-PCR amplification was performed according to the manufacturer’s instructions 1 HCV-RNA and 50 pmol each of primers using 10 NS5BOAS2. The cycling conditions for this were as follows: a reverse transcription step for 30 min at 50 0C, 15 min at 95 0C, followed by 35 cycles of denaturing for 1 min at 95 0C, annealing for 45 s at 59 0C, and an elongation step for 1 min at 72 0C, with a final extension period of 10 min at 720C. Nested PCR using products in the NS5BIS1 and NS5BIAS2 of the sample negative for primers was performed on 10 NS5BOAS2. The cycling conditions were as follows: a reverse transcription step for 30 min at 50 0C, 15 min at 95 0C, followed by 35 cycles of denaturing for 1 min at 95 0C, annealing for 45 s at 59 0C, and an elongation step for 1 min at 72 0C, with a final extension period of 10 min at 720C.

Chromas Pro 1.5 beta generated the final sequences (Technelysium Pty., Tewantin, QLD, Australia). Using the Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, the newly discovered HCV NS5BR gene sequences were compared with those in GenBank. MEGA 11.0 software was used to align the sequences using muscle alignment. Kimura’s two-parameter algorithm computes sequence divergences (K2P). N.J. trees use Kimura’s two-parameter approach to demonstrate patterns of species divergence (Kimura 1980). MEGA 11.0 was used for bootstrapping (Kumar et al., 2004)1000 times over. ITOL software improved visualization (Letunic and Bork, 2021). PopArt v.3.0 calculated the least spanning network for haplotype divergence.

Data analysis

The data were provided in tables as mean and standard deviation. The data were evaluated using an independent test. The data was coded and put into the SPSS V.22 statistical software. The mean and standard deviation were used for quantitative data, while the frequency was utilized for categorical data. P-value of less than 0.05 was statistically significant. R-studio V.4.1.3 now provides data visualization.

RESULT

Table (1) shows that the age difference between the control and patient groups was not statistically significant. There is no statistically significant difference (P> 0.05) in the female-to-male ratios of the control and patient group, according to the chi-square analysis (Table 2).

An HCV PCR test is performed on the patient group, with an average result of 1127685.4 1335832.51. (Table 2). Creatinine levels did not vary significantly in the control and group of patients (Table 2). TSH analysis revealed that the association between the control and patient groups was insignificant. (Table 2). The current investigation used seven clinical analyses to identify liver functions. There was an insignificant difference (P> 0.05) in ALB, GGT, ALK.PH, Bill. Total, and Bill. Dir between the control and patient groups. However, AST and ALT levels differed significantly (Table 2).

WBC showed an insignificant difference (P> 0.05). In contrast, when the control and patient group were compared, HB, platelets, INR, PT, and PC % showed a significant variation. (Table 2).

AFP levels didn't vary statistically between the patient and healthy groups (P> 0.05). (Table 2). There was no change in cholesterol, HDL, or TG levels; only a substantial difference in LDL (P 0.05) has been observed. (Table 2). FBS indicated a significant variation between the healthy and patient groups. Post. PBG shows no significant change compared to the control (Table 2 and Figure 9).

After 3 and 6 months of therapy, there were significant changes (P< 0.05) between negative and positive patients count. There was no
difference when comparing 3 months and 6 months of therapy. (Table 3).

The Neighbor-Joining approach was used to establish the evolutionary history. (Saitou and Nei 1987). The best tree is shown in (Figure 1). The proportion of duplicate branches with color-coded branches clustered together using the bootstrap test (1000 repetitions) (Felsenstein1985). The branch lengths are shown in the same units as the evolutionary distances used to estimate the tree. The 2-parameter Kimura approach was used to determine evolutionary distances. (Kimura 1980). This study uses 28 distinct nucleotide sequences (15 sequences from the present study and 13 donor sequences obtained from the NCBI database). The first, second, third, and noncoding codon positions have all been provided. The final dataset had 576 sites in total. MEGA11 was used for evolutionary investigations (Tamura et al., 2021).

Furthermore, the minimal haplotype spanning network mirrored the haplotype of the twenty-eight sequences studied in this study. The pi-value for nucleotide diversity was 1.9091; 199 segregating sites and 145 parsimony-informative sites exist. The D statistic for Tajima was computed to be 25.0206, with P(D ≥ 25.0206) = 0(Figure 2). Significant genotype incidence exists among those on Sofosbuvir.

DISCUSSION

HCV may cause acute or chronic hepatitis, with symptoms ranging from mild to severe, including cirrhosis and liver cancer. Hepatitis C is a virus transmitted by blood, and most infections are caused by exposure to unsafe injecting practices, unsafe medical treatment, and unscreened blood transfusions (Alter and Seeff, 2000). DAAAs successfully treat HCV infection in clinical practice and have resulted in a worldwide drop in its incidence, which is consistent with our research findings.

Assisting physicians in monitoring the course of liver disease requires simple, readily available approaches based on clinical/biochemical signs. In this view, albumin concentrations are characteristic of liver synthesis; our research shows that the ALT and AST scores are well-established indicators of liver function. Our data suggest that viral eradication impacts HCCV patients. In addition, the non-significance albumin levels, which could act as an indicator of disease improvement in cirrhotic patients, agree with El-Sherif et al. (2018), while our result was inconsistent with Maan et al. (2016). Hb, RBCs, and WBCs findings were inconsistent with Hussein et al. (2022). AFP levels in liver cirrhosis patients due to HCV infection are not increased compared to those with compensated cirrhosis (Daniela et al.,2020). HCV infection has been linked to host lipid dysregulation, with lower levels of LDL and TC when compared to healthy matched controls encounter to Corey et al. (2011). HCV infection was linked to an increased risk of developing CKD and renal progression in those with normal kidney function, highlighting the importance of viral diagnosis in preventing CKD and maintaining renal function (Park et al., 2018; Cheng et al., 2021).

HCV genotyping provides epidemiology and therapy monitoring guidance (El Hadad et al., 2017 & Cantaloupe et al., 2006). Studying viral variety improves our knowledge of viral diseases. HCV genetic variations are found all over the world. Present findings indicate that HCV patients have a heterogeneous genetic profile overall (Candotti et al., 2003). HCV genotype analysis within patients may be used to investigate the geographic distribution of HCV infection. HCV genotyping is especially relevant since it reveals strain differences and their potential association with disease severity (García-Montalvo and Galguera-Colorado 2008).

There is an actual genotype occurrence among Sofosbuvir patients in our research. Choosing the right genomic portion to be studied is critical to identify HCV genotypes and sub-genotypes. In phylogenetic analysis, the NS5B region of HCV gives the most information on genomic mutations and alterations (El Hadad et al., 2017). Using a phylogenetic tree and pairwise distance analysis of the NS5B nucleotide sequence, we matched 100 percent of amplified isolates to their respective HCV genotypes. This study’s HCV genotype isolates demonstrated nucleotide diversity with a pi-value of 1.9091, 199 segregating sites, and 145 parsimony-informative sites. Tajima’s D statistic was 25.0206, with P(D ≥ 25.0206) = 0, confirming our phylogenetic results. Numerous studies have found that the degree of accuracy of NS5B sequence variation correlates well with the HCV subtype definition, which is based on the highly informative nature of specific NS5B.

CONCLUSION

The current result demonstrates the Hepatitis C virus’s impact on HCV patients’ body physiology. Also, a substantial genotype
occurrence among Sofosbuvir patients has been proved in our research.

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Table 1: Average ± SD of participant age for control and patient group  

<table>
<thead>
<tr>
<th>Group</th>
<th>Age</th>
<th>Female</th>
<th>Male</th>
<th>X²</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.73 ± 9.42</td>
<td>7</td>
<td>8</td>
<td>0.07</td>
<td>0.796</td>
</tr>
<tr>
<td>Patient</td>
<td>40.4 ± 7.89</td>
<td>10</td>
<td>5</td>
<td>1.67</td>
<td>0.197</td>
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</table>

Table 2: Average ± SD of Biochemical markers for control and patient group  

<table>
<thead>
<tr>
<th>Biochemical</th>
<th>Group</th>
<th>Control</th>
<th>Patient</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quan-PCR</td>
<td>PCR-HCV</td>
<td></td>
<td></td>
<td>1127685.4 ± 1335832.51</td>
</tr>
<tr>
<td>Kidney function</td>
<td>Cr</td>
<td>0.92 ± 0.16</td>
<td>0.86 ± 0.18</td>
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<tr>
<td>Hormones</td>
<td>TSH</td>
<td>2.77 ± 0.98</td>
<td>2.38 ± 1.27</td>
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<tr>
<td>Liver function</td>
<td>ALB</td>
<td>3.99 ± 0.28</td>
<td>4.12 ± 0.43</td>
<td></td>
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<tr>
<td></td>
<td>GGT</td>
<td>27.73 ± 6.38</td>
<td>76.2 ± 121.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALK.PH</td>
<td>90.46 ± 28.06</td>
<td>98.06 ± 47.42</td>
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</tr>
<tr>
<td></td>
<td>AST</td>
<td>19.06 ± 2.83</td>
<td>49.73 ± 31.78</td>
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<tr>
<td></td>
<td>ALT</td>
<td>17.46 ± 3.77</td>
<td>56.4 ± 41.48</td>
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<tr>
<td></td>
<td>Bill. Total</td>
<td>0.66 ± 0.27</td>
<td>0.82 ± 0.65</td>
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<tr>
<td></td>
<td>Bill. Dir</td>
<td>0.18 ± 0.07</td>
<td>0.32 ± 0.38</td>
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<tr>
<td>Hematology</td>
<td>WBC</td>
<td>6.22 ± 1.19</td>
<td>5.26 ± 1.49</td>
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<tr>
<td></td>
<td>HB</td>
<td>20.6 ± 27.64</td>
<td>12.66 ± 1.25</td>
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<tr>
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<td>Platelet</td>
<td>323.6 ± 51.76</td>
<td>215 ± 66.24</td>
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<td>INR</td>
<td>1 ± 0.09</td>
<td>1.1 ± 0.1</td>
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<td></td>
<td>PT</td>
<td>12 ± 0.2</td>
<td>13.06 ± 1.1</td>
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<td></td>
<td>PC%</td>
<td>100 ± 6.64</td>
<td>91.98 ± 7.87</td>
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<td>Tumor marker</td>
<td>AFP</td>
<td>4.62 ± 2.14</td>
<td>8.29 ± 10.08</td>
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<td>Lipid profile</td>
<td>Cholesterol</td>
<td>139.6 ± 21.45</td>
<td>182.06 ± 69.38</td>
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<td>TG</td>
<td>109.13 ± 25.38</td>
<td>99.2 ± 42</td>
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<tr>
<td></td>
<td>HDL</td>
<td>47.06 ± 12.23</td>
<td>46.2 ± 16.14</td>
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<tr>
<td></td>
<td>LDL</td>
<td>77.86 ± 8.51</td>
<td>94.46 ± 23.36</td>
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<tr>
<td>Blood sugar</td>
<td>FBS</td>
<td>81.06 ± 6.35</td>
<td>101.53 ± 45.96</td>
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<tr>
<td></td>
<td>Post.PBG</td>
<td>103.66 ± 6.21</td>
<td>121.6 ± 52.43</td>
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Table 3: Average ± SD of PCR result after 3 and 6 months of treatment for a patient group

<table>
<thead>
<tr>
<th>Period</th>
<th>Positive</th>
<th>Negative</th>
<th>X²</th>
<th>P-Value</th>
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<tr>
<td>3 Months</td>
<td>0</td>
<td>15</td>
<td>15</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>6 Months</td>
<td>0</td>
<td>15</td>
<td>15</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 1: Neighbor-Joining phylogenetic evolutionary tree

Figure 2: Minimum haplotype spanning network
التنوع الجيني لميروس سي NS5B والعلامات البيوكيميائية لمرضى المصريين المصابة بفيروس سي محمد عادل جماعي 1، محمد عبد الرحمن أحمد 2، محمد منصور سعد فرج 1

قسم النيبات والكروميولوجي، كلية العلوم جامعة الأزهر، القاهرة، مصر.

قسم كيميائي بالكروميولوجيا الأكديمية الطبية العسكرية القاهرة مصر.

المؤلف الرئيسي: mohamed.farag@azhar.edu.eg

الملخص

تعتبر الإصابة بفيروس سي من أكبر المشاكل الصحية على مستوى العالم ومعلوم أن فيروس سي سبب شائع للإصابة بليف الكبد الذي قد يؤدي للإصابة بسرطان الكبد وتقلص الدائرة، فضلاً عن تلف الكبد، ولذلك من الأهمية المدنية تقييم هذه المشكلات. ففي البحث الحالي، قمنا بفحص و(Clone الكبد، ودلالات الأورام، وأعراض الدم، ووظائف الكبد، ووظائف العظام، وأعراض الدم، ووظائف الكبد، ووظائف العظام.

تم استخدام فحوصات سريرية Bill-direct و Bill-total و Alk.PH و GCT و Alb و TSH و Cr إلى استنتاج نتائجه. حيث يوجد فرق كبير بين مجموعتي الضايحة والمرضى من حيث Cr و TSH و Alb و GCT و Alk.PH و Bill-total و Bill-direct و WBC و AFB و Cholesterol و TG و PCR و PT و INR و Platelet و Hb و ALT و AST و FBS و LDH دلالة جوهرية بين المجموعتين. حيث يوجد فرق كبير بين مجموعتي الضايحة والمرضى من حيث Cr و TSH و Alb و GCT و Alk.PH و Bill-total و Bill-direct و WBC و AFB و Cholesterol و TG و PCR و PT و INR و Platelet و Hb و ALT و AST و FBS و LDH.

توضح هذه النتائج أن فيروس التهاب الكبد الوبائي سي له تأثير كبير على متابعة الدم ووظائف الكبد في الجسم. لانقاذ الامراضي المستمر، والتحلي بدراسة الجين فيفيروس التهاب الكبد الوبائي، حيث يتم استخدام فيروس التهاب الكبد الوبائي في الأبحاث القائمة على الخلايا البشرية.

الآفات الإسترشادية: فيروس سي، التنوع الجيني، الدلائل البيوكيميائية، الدلائل الفيسيولوجية، المصابين المصريين.