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**Lactobacillus acidophilus and Bifidobacteria spp having antibacterial and antiviral effects on chronic HCV infection**

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Hepatitis C virus is a serious hepatic disease that could be developed into hepatocellular carcinoma. Previously, some probiotic strains showed a natural therapeutic activity against the fatty liver disorder. Therefore, it could make sense to evaluate the antiviral and antibacterial responses to probiotics as *Lactobacillus acidophilus* and *Bifidobacteria* spp. in patients with chronic hepatitis C virus. Twenty (20) patients with chronic hepatitis C (both gender in age 47± 5 years) were treated with capsule that contains probiotics (*L. acidophilus* and *Bifidobacterium* spp.). They administered one capsule per day for a month before HCV treatment; blood and urine samples were collected before and after the given treatment and they were processed for a quantitative estimation of HCV by PCR, identification of bacteria by VITEK2 system and 16S r RNA gene sequencing assay. Moreover, the estimation of antibacterial activity of probiotics by antibiotic sensitivity test, counts of leukocytes, CD3+ T cells and CD56+ natural killer cells by flowcytometry, DGAT1 by ELISA. Administration of probiotics enhanced the treatment response rate to HCV treatments pegylated IFN-α and ribavirin by 25% and showed an antibacterial activity against five species of the most common infectious bacteria in chronic HCV patients which were identified by this study. CD3+ cells counts and CD56+ natural killer cells were increased. *L. acidophilus* and *Bifidobacterium* spp. can act as a supportive supplement with antiviral and antibacterial activities; we recommend Probiotics side by side with HCV treatments.

**Key words:** Hepatitis C virus, *Lactobacillus acidophilus* and *Bifidobacteria* spp., CD3+, CD56+, pegylated IFN-α and ribavirin.

INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of pathogenesis of liver disease including chronic hepatitis, fibrosis, cirrhosis, hepatocellular carcinoma, liver failure and death. Currently, no effective vaccine is available for

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HCV infection (Dore et al., 2014). The most recent estimates of disease burden show about 185 million infections worldwide (Hanafiah et al., 2013).

One of the most common HCV drug therapies was pegylated interferon (IFN) and ribavirin combination therapy, but it was limited to about 55% of patients with a lower efficacy and associated with severe side effects (Lam et al., 2014). Recently, Sovaldi®, alone or in combination with IFN and ribavirin, has also been introduced as a new treatment modality with promising antiviral effects (Heathcote and Main, 2005; Gane et al., 2013).

However, exploring new therapeutic protocol, especially those that can improve the existing ones and lower their toxicity can have significant impact on the anti-viral responses. One of the promising modalities is natural products as well as probiotics that have potential anti-toxic effects on liver functions (Gratz et al., 2010). Probiotics are defined as live microorganisms which are consumed as food or therapeutic supplements with beneficial effects on the health (Bermudez-Britet et al., 2012; Kechagia et al., 2013; Martin et al., 2013). For instance, Lactobacilli and bifidobacteria are beneficial in the treatment of intestinal micro-flora disturbance by increasing gut permeability and re-establishment of the microbial equilibrium (Deye et al., 2016; Bischoff et al., 2014; Goldenberg et al., 2013). With this regard, it was found that Lactobacilli and bifidobacteria provide antibacterial activity against common pathogenic bacteria such as Escherichia coli, Staphylococcus aureus and Salmonella typhimurium ((Bali et al., 2011; Butel et al., 2014), Enterobacteriaceae species, Klebsiella and E. coli. Indeed, these gram negative bacteria as well as gram-positive bacteria including S. aureus are the most common pathogenic bacteria in patients with chronic HCV, which is considered as secondary bacterial infections (Carrión et al., 2009; Jalan et al., 2014).

Triacylglycerol synthesis is catalyzed by acyl-CoA: diacylglycerol acyltransferase (DGAT) enzymes, DGAT1 and DGAT2 (Harris et al., 2011). DGAT1, an enzyme involved in triglyceride synthesis and luminal LD maturation, targets core to lipid droplets LDs and bounded with NS5B in active HCV replication (Camus et al., 2013). Its absence or inhibition leads to the inhibition of viral assembly and production (Herker et al., 2010).

Besides their anti-bacterial activities, recent studies showed that both Lactobacilli and bifidobacteria have different immunomodulatory effects, including increasing phagocytosis NK cell activity (Yuan and Walker, 2004; Candore et al., 2008; Rask et al., 2013), IgG and IgA synthesis (Stagg et al., 2004; Tsai et al., 2012), production of cytokines (Th1 or Th2) in both in vivo and in vitro systems, the development and maturation of mucosal and systemic NKT cells (Villamil et al., 2002; Pagnini et al., 2010). The cytotoxic effects of natural killer (NK; CD3 CD56dim) cells against virally infected cells caused inhibition of HCV replication as well as their cytoxicity against hepatocellular carcinoma (HCC) (Doskali et al., 2011). The effects of probiotics on these cells are paramount significance; especially these NK cells can activate multiple elements of janus kinase/signal transducer and transcription (JAK/STAT) pathway, resulting in an induction of endogenous IFN-alpha/beta expression in hepatocytes and enhancement of anti-HCV cell-mediated immunity (Ye et al., 2009). Additionally, probiotic bacteria differentially activated dendretic cells (DCs) in vitro and induced CD4+ T cells (Hart et al., 2003). With these beneficial effects, probiotics have been recommended for use as biological safe product drug system by FDA (Degnan, 2008; Snydman, 2008).

This study aimed to enhance utilize L. acidophilus and Bifidobacteria spp. as a supportive supplement to the treatment strategy based on Pegylated IFN-α and ribavirin which provides antibacterial, antiviral effect, reduce DGAT1 and improving the immune response rate. Subsequently, increase the treatment response rate to HCV treatment.

MATERIALS AND METHODS

Patients

This study was adopted on 40 selective patients (males and females) with chronic HCV who were diagnosed by a specialist physician that selected them according to a mean age of 47± 5 years and their positive results of HCV antibody antigen test and polymerase chain reaction (PCR) estimation which showed high viral load. The exclusion criteria included liver cirrhoses, HCC, hepatitis B virus (HBV), diabetes and/or renal impairment. Patients were recruited from the Virology Unit, El-Obour Hospital, Kafr El-sheikh, Egypt.

Ethical approval

This study had an approval letter from the ethical committee, Faculty of Medicine, Tanta University, Egypt.

Treatment strategies involved in the study

L. acidophilus and Bifidobacteria spp combination (1.5 billion cells) were used in this study in the form of a capsule (Phillips® Colon Health, Bayer Health Care, USA). The ingredients included potato starch, gelatin, and silicon dioxide. The probiotics with long term benefit takes longer within the host body to colonize in the gut, colon or the small intestine. The process may take days to months with the least reported being 2-3 weeks as mentioned by Swanson (2013). Three groups (A, B and C) were designed in this study. Group A included healthy volunteers as controls who took the capsule once a day for one month. Group B included chronic hepatitis C patients who took the capsule once a day for one month before treatment with pegylated IFN-α and ribavirin (single interferon injection weekly for 12 weeks). Group C of chronic hepatitis C patients was treated with only pegylated IFN-α and ribavirin (12 weeks). Patients were followed up clinically during the time course of the experiment.

Samples collection and duration of study

Blood and urine samples were collected from the patient groups in
the time range from October 2013 to April 2014. Ten samples were collected before and after capsule supplementation on (day 1, and day 30) from healthy volunteers as controls (group A). Twenty samples were collected before and after the given treatment strategies on day 1, 30 then day 120 in the groups B and C.

Reagents and materials

The following media were used. 1) Nutrient agar (Thomas et al., 1977) pH was adjusted to 5.0; 2) Mannitol salt agar (Koch, 1942) (Final pH: 7.4 ± 0.2 at 25°C); 3) MacConkey agar (pH 7.4 ± 0.2 at 25°C). Gram stain (crystal violet, gram’s iodine solution, acetone/ethanol (50:50 v: v), 0.1% basic fuchsin solution). VITEK 2 system (Funke, 1998), (bioMérieux Inc., Hazelwood, MO) VITEK 2 Cassette Loaded with Cards and Suspension Tubes being loaded into the Automatic Transport System.

Antibiotics used for antibacterial sensitivity test (Barry, 1979) included the following: nitrofurantoin (F), doxycyclin (DA), OFloxicin (OFX), Nalidixic acid (NA), Vancomycin (VA), Tetracycline (TE), Erythromycin (E), Amplicilin sulbactam (SAM), Imipenim (IPM), Amikacin (AK), chlorphenicol (C), Ciprofloxacin (CIP), Levofloxacin (LEV), Piperacillin-tazobactam (TPZ), sulfamethoxazole (SXT), Cefuroxime (CXM), Cefataxime (CTX), Amoxicilin calvulinic acid (AMC).

The QIAGEN gel extraction kit for 16S rRNA gene sequencing (Stackebrandt, 1992) was purchased from Sigma (Sigma Scientific Services Co., Cairo, Egypt).

Antibodies used were Anti-human CD3 and CD56 monoclonal antibodies (BD Biosciences, SanJose, CA, USA) were used for immunophenotype analysis.

BD FACS was used for lysing solution for RBCs lysis and PBS buffer solution for washing and suspension. EDTA tubes were used as anticoagulant tubes for blood collection. Sterilizing Petri dishes and swabs were used.

Quantitative estimation of HCV RNA

Blood samples were used for RNA extraction. 20 μL of the extracted RNA was added to 30 μL of Master Mix in each 0.1 mL microtube and test was performed in automated instrument (HVD Auto Q server) (Bieche, 1998). A standard curve was automatically drawn with software using five quantification standard concentrations of HCV-RNA to analyze the viral RNA load.

Isolation of bacteria from patients with chronic HCV and antibacterial responses to Lactobacillus acidophilus and Bifidobacteria spp.

Isolation of bacteria from urine samples was performed on nutrient agar inoculated with 100 μL of each urine sample and incubated at 37°C for 18-24 h. Investigation of the isolated bacteria by Gram reaction was performed, eventually pure subculture of gram positive and gram negative were cultured in 20 mL of sterilized Macconky and Mannitol media, respectively, at 37°C for 18-24 h. The load of pathogenic bacteria was observed by detection of CFU/mL based on this equation CFU/mL = (no. of colonies x dilution factor) / volume of culture plate. Moreover, the numbers of the infected patients were recorded before and after the capsule administration in the three groups.

Identification of isolated bacteria

**VITEK 2 system**

This system provided 64 identification tests processed for identification of isolated bacterial strains while, BioMerieux VITEK® 2 system version: 06.01 was used as lactase, alkalization, growth under inhibition conditions like oxidase, enzyme hydrolysis acidification test. Sufficient numbers of pure colonies were transferred into 3.0 mL of sterile saline, a special rack (cassette) of the suspended microorganism; the identification cards were placed in slots that moved to the optical system where readings were observed each 15 min. Then the data were recorded.

**Antibacterial sensitivity test (AST) (Kirby Bauer technique)**

Sterile Petri plates with 20 mL of sterilized MacConkey and Mannitol agar were inoculated with 100 μL of each isolated bacteria; the antibiotics discs were placed on plates and incubated for 18-24 h at 37°C. Inhibition zones diameters were recorded in mm according to the Clinical and Laboratory Standards Institute (CLSI, 2013).

**16S r RNA gene sequences identification**

PCR was performed in a thermal cycler (Bio-Rad MJ Research, Hercules, USA). The 50 μL reaction mixture consisted of, 5 μL of 10 x Taq buffer (100 mM Tris- HCl, 500 mM KCl pH 8.3), 2.5 U of Taq DNA polymerase, 20 ng of genomic DNA, 200 μM dNTP, 10 p moles each universal primers, Forward primer CAAACAGATTAGACCCCTG, reverse primer CGGGAAGACCTTACC and 2.0 mM MgCl2. Amplification started with initial denaturation for 5 min at 94°C, followed by 25 cycles of denaturation for 30 s at 94°C, annealing temperature of primers for 30 s at 50°C, and extension for 1 min at 72°C. The last extension for 15 min at 72°C was used. Submarine agarose gel electrophoresis in 1.2% agarose gel pre-staining with ethidium bromide at 8 V/cm was used for analyzing 5 μL of the amplified product, gel doc UV transilluminator was used for visualizing the PCR product (Imran and Abd-Al-Kareem, 2016). The QIAGEN gel extraction kit serves as a gel purified for the amplified PCR product. GATC Company using ABI 3730xl DNA sequence used forward and reverse primers for the sequencing of a total of 100 ng/μL concentration of 16S rRNA amplified product (Sigma Scientific Services Co., Cairo, Egypt).

**Flow cytometric analysis**

Fresh venous blood samples were collected on EDTA tubes and100 μL was stained by human mAbs in staining tubes using the recommended concentrations by the manufactures. The stained samples were incubated at 4°C in the dark for 20 min, and then RBCs were lysed by adding BD FACS or ACK lysing solution (1x) for 15 min then centrifugation at 1250 rpm for 5 min. Then, the supernatant containing lysed RBCs was discarded and the cells were washed using PBS buffer solution. The cells were acquired on BD FACSCalibur or FACS Canto II (BD Biosciences, San Jose, CA, USA) and analyzed using FACSDiva, Cell Quest (BD Biosciences) and Flow jo software (Givan et al., 2011).

**White blood cells**

A total number of leukocytes, differential lymphocytes, monocytes and neutrophils were identified by using fully automated instrument (CBC Swelab Alpha three part differential).

**Determination of DGAT1 enzyme level**

Enzyme-linked Immunosorbent Assay Kit for Diacylglycerol-O-
Acyltransferase Homolog 1 (DGAT1). Organism Species: Homo sapiens (Human). This assay has high sensitivity and excellent specificity for detection of DGAT1.

Statistical analysis

The clinical data were recorded for the study and analyzed for each patient. The statistical presentation analysis of the present study was conducted using the mean, standard deviation, Chi-square test by software SPSS V.20. Standard student 't test' was used to test significance of the differences between means *P≤ 0.05, **P≤ 0.01.

RESULTS

The antiviral effect of probiotics by HCV PCR assays result

The antiviral effect of L. acidophilus and Bifidobacteria spp. capsule was determined by HCV PCR assay for three groups (Group A, B and C). The outcome data illustrated that L. acidophilus and Bifidobacteria spp increased the number of responded patients to the IFN-α and ribavirin treatment about 95 % in group B compared to their number in group C about 70 % which was treated with only IFN-α and ribavirin treatment compared to the control group A which was negative (Figure 1; Table 1A,B).

Antibacterial activity of treatment with Lactobacillus acidophilus and Bifidobacteria spp.

The secondary infectious bacteria in patients with chronic HCV (groups B and C) were compared to that in the group A of healthy people which revealed no bacterial growth. It was found that, the number of patients and the load of isolated bacteria before oral administration of the probiotic capsules were higher than after administration. The loads of Gram positive and negative bacteria were reduced to 43.2 and 76%, respectively in urine samples as a response to probiotic administration in group B; the number of infected patients was reduced by 60% after probiotic supplementation. On the other hand, group C did not show any enhancement as it is nearly the same percentage of both the number of patients and the bacterial load after as well as before any treatments.

Identification of isolated bacteria using VITEK 2 system

The microscopic investigation of Gram reaction revealed presence of G+ve isolates in cocci form especially Staphylococci form while G-ve isolates were bacilli. They were cultured on selective media for further biochemical identification by VITEK 2 system. Five main bacterial
Table 1A. Quantitative estimation of HCV PCR before and after the treatment strategy with only IFN-α and ribavirin.

<table>
<thead>
<tr>
<th>Patient (GpC)</th>
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<th>Sex</th>
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<th>PCR IU/ML (After)</th>
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<td>F</td>
<td>428.847</td>
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<td>M</td>
<td>997.631</td>
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<td>M</td>
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<td>5</td>
<td>48</td>
<td>M</td>
<td>500.000</td>
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<td>6</td>
<td>50</td>
<td>M</td>
<td>250.593</td>
<td>Nil</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>F</td>
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<td>53</td>
<td>F</td>
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</tr>
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<td>M</td>
<td>629.462</td>
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</tr>
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<td>54</td>
<td>M</td>
<td>292.170</td>
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<td>13</td>
<td>55</td>
<td>M</td>
<td>70.244</td>
<td>Not responded</td>
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<td>53</td>
<td>F</td>
<td>428.897</td>
<td>Not responded</td>
</tr>
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<td>Not responded</td>
</tr>
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<td>20</td>
<td>40</td>
<td>M</td>
<td>233.98</td>
<td>Nil</td>
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</table>

M=Male, F=Female, Nil=too high response rate to revealed no HCV RNA observed (negative result), non-responded = high viral load ≥ the initial load. It's shown that the response rate to IFN-α and ribavirin treatment about 70%.

Table 1B. Quantitative estimation of HCV PCR before and after the treatment strategy including probiotic administration before IFN-α and ribavirin.

<table>
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<tr>
<th>Patient's name (GpB)</th>
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<th>HCV PCR IU/ML (After)</th>
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<td>45</td>
<td>1.016.250</td>
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</tr>
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<td>4</td>
<td>M</td>
<td>44</td>
<td>1.581.138</td>
<td>Nil</td>
</tr>
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<td>M</td>
<td>56</td>
<td>1.787.737</td>
<td>Nil</td>
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<td>50</td>
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<tr>
<td>10</td>
<td>M</td>
<td>45</td>
<td>138.772</td>
<td>Nil</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>50</td>
<td>193.035</td>
<td>Nil</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>48</td>
<td>7.684</td>
<td>Nil</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>45</td>
<td>13.561.362</td>
<td>Nil</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>55</td>
<td>23.207</td>
<td>Not responded</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>45</td>
<td>1.190.245</td>
<td>Nil</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>43</td>
<td>170.727</td>
<td>Nil</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>40</td>
<td>711.710</td>
<td>Nil</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>53</td>
<td>1.356.13</td>
<td>Nil</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>52</td>
<td>1.250.23</td>
<td>Nil</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>40</td>
<td>234.95</td>
<td>Nil</td>
</tr>
</tbody>
</table>

M=Male, F=Female, Nil=too high response rate to revealed no HCV RNA observed (negative result), non-responded = high viral load ≥ the initial load.
Figure 2. Inhibition zones diameters of different antibiotics that were used against *Escherichia coli* and *Pseudomonas aeruginosa* and showed their resistance.

Figure 3. Inhibition zones diameters of different antibiotics against *Staphylococcus aureus* strains.

**Antibacterial sensitivity of the isolated bacteria**

It was found that the investigated antibiotics against isolated bacterial species revealed different inhibition zones with variable degrees of resistance. *P. aeruginosa* was resistant against the following antibiotics: Ofloxacin (OFX), nalidixic acid (NA), chloramphenicol (C), levofloxacin (LEV), norfloxacin (NOR), furantoin (F), and ampicillin+sulbactam (SAM). Moreover, among 5 isolated *S. aureus*, isolate number 2 was the most resistant against doxycyclin (DA), Nalidexic acid (NA), erythromycin (E), chloramphenicol (C), amikacin (AK), piperacillin-tazobactam (TPZ), amoxicilin calvulinic acid (AMC) (Figures 2 and 3).

**16S rRNA bacterial identification**

The 16S rRNA gene sequence proved that *S. aureus* number 2 (highly antibiotic resistant) is subsp. strain N315 16S ribosomal RNA with identity about 89%, and the phylogenetic tree was constructed as illustrated in Figure 4.
Figure 4. Phylogenetic tree of *staphylococcus aureus* by gene bank access and the highest identity was *staphylococcus aureus* N315 strain N315 16Sr ribosomal RNA.

**Analysis of CD3\(^+\) and CD56\(^+\) cells counts**

Treatment with *L. acidophilus* and *Bifidobacteria* spp. resulted in significant enhancement in the numbers of CD56\(^+\) NK cells (P values = 0.001) and CD3\(^+\) T cells (Figures 5 and 6; Table 2).

It was found that the counts of CD3\(^+\) and CD56\(^+\) cells in healthy individuals group A were more than group B before oral administration of probiotics. CD3\(^+\) and CD56\(^+\)cells counts increased after probiotic capsule administration in both groups A and B. In group B, their production slightly increased than group A after probiotic administration, but level of CD3\(^+\) cells in Group A was more than Group B, group C did not undergo this test as they did not take probiotics (Figure 7; Tables 3, 4).

**White blood cells**

The differential count of Leucocytes (WBCs), including monocytes, lymphocytes and granulocytes, increased after oral administration of the probiotics capsules in group B by 8.7%, while total WBCs count before probiotics capsule administration was 6.33% compared to group A (healthy individuals) controls as they did not show observed change after probiotics administration. This parameter was not measured in group C as it did not take probiotics (Figure 8).

Subsequently, probiotics enhanced the immune responses by increasing the immune cells that produce T lymphocytes (CD3\(^+\)), natural killer cells (CD56\(^+\)), monocytes, Basophiles and eosinophiles, thus strengthening immune defenses against both bacterial and viral infections.

**Inhibition of DGAT1 reduces HCV viral production**

The outcome data illustrated that the concentration of DGAT1 enzyme was reduced as a result of probiotics capsule administration inside the same group B (chronic HCV patients) from 528 up to 373 ng/ mml (Table 5). More evidence was observed after three months of the treatment protocols in group (B) DGAT1 concentration was about 373 ng/mml lesser compared to group (C).
who treated only with pegylated IFN and it was 1038 ng/mml higher with a significance P. value = 0.05*.

On the other hand, the presented study revealed the correlation between the level of DGAT1 and HCV infection which was determined in group (C) of patients treated with only pegylated IFN-α and ribavirin without

Figure 6. A, B. Analysis of CD3+ (FL4) cells count and CD56+ (FL1) cells count and total populations before and after administration of probiotics capsule by using FLOWJOW software.
Table 2A, B, C. The immune response in patients with chronic hepatitis c virus before and after oral administration of probiotics capsule, group (B).

<table>
<thead>
<tr>
<th>(A):- CD3⁺ cells populations</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 3⁺ cells % Gp B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>12 – 25</td>
<td>11 – 24</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>18.53 ± 4.26</td>
<td>15.43 ± 3.58</td>
</tr>
<tr>
<td>t. test</td>
<td></td>
<td>4.659</td>
</tr>
<tr>
<td>P. value</td>
<td></td>
<td>0.040*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B):- CD56⁺ cells populations</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 56⁺ cells % Gp B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>22 – 38</td>
<td>27 – 39</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>29.2 ± 4.06</td>
<td>33.2 ± 3.84</td>
</tr>
<tr>
<td>t. test</td>
<td></td>
<td>7.692</td>
</tr>
<tr>
<td>P. value</td>
<td></td>
<td>0.010*</td>
</tr>
</tbody>
</table>

| (C):- both CD3⁺ CD56⁺ cells populations before and after probiotic capsule | Before | After |
| Both CD3⁺ and CD56⁺ cells % Gp B |        |       |
| Range                        | 10 – 29| 16 – 38|
| Mean ± SD                    | 18.76 ± 5.43 | 29.87 ± 5.77 |
| t. test                      |        | 29.470 |
| P. value                     |        | 0.001* |

P. value = 0.01-0.05 showed significant result, gpB = chronic HCV patients. As its shown probiotics increased the populations of CD3⁺ (A)=0.04* and CD56⁺(B)=0.01* and both (C) with high significant value = 0.001*.

Figure 7. CD56⁺ cells (1), CD3⁺ CD56⁺ cells (2) and CD3⁺ cells (3) in group B compared to control group A oral administration or probiotics capsule.
Table 3. Populations of CD3⁺ and CD56⁺ cells in both healthy individuals (group A) and patients with chronic HCV (group B) before any treatments.

<table>
<thead>
<tr>
<th>CD 3⁺ cells %</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>18 – 27</td>
<td>12 – 25</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>23.0 ± 4.58</td>
<td>18.53 ± 4.26</td>
</tr>
<tr>
<td>t. test</td>
<td>2.699</td>
<td></td>
</tr>
<tr>
<td>P. value</td>
<td>0.120</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CD 56⁺ cells %</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>24 – 36</td>
<td>22 – 38</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>30 ± 6.11</td>
<td>29.0 ± 4.06</td>
</tr>
<tr>
<td>t. test</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>P. value</td>
<td>0.962</td>
<td></td>
</tr>
</tbody>
</table>

A. Mean ± SD showed slightly decreasing of CD 3⁺ cells % in group B (chronic HCV patients) than group A (healthy individuals) before any treatment.

Table 4A, B, C. The immune response in healthy people (group A) compared with its response in patients with chronic hepatitis c virus (group B) after oral administration of probiotics capsule.

A):- CD3⁺ cells populations after probiotics capsule administration for a month

<table>
<thead>
<tr>
<th>CD 3⁺ cells % After</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>21 – 28</td>
<td>11 – 24</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>24.67 ± 3.51</td>
<td>15.43 ± 3.58</td>
</tr>
<tr>
<td>t. test</td>
<td>16.708</td>
<td></td>
</tr>
<tr>
<td>P. value</td>
<td>0.001*</td>
<td></td>
</tr>
</tbody>
</table>

(B):- CD56⁺ cells populations after probiotics capsule administration for a month

<table>
<thead>
<tr>
<th>CD56⁺ cells After</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>26 – 35</td>
<td>27 – 39</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>31.33 ± 4.73</td>
<td>32.2 ± 3.84</td>
</tr>
<tr>
<td>t. test</td>
<td>0.555</td>
<td></td>
</tr>
<tr>
<td>P. value</td>
<td>0.467</td>
<td></td>
</tr>
</tbody>
</table>

(C):- CD3⁺ CD56⁺ cells populations after probiotic capsule administration for a month

<table>
<thead>
<tr>
<th>Both CD3⁺ CD56⁺ cells % After</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>19 – 22</td>
<td>16 – 38</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>20.67 ± 1.53</td>
<td>29.87 ± 5.77</td>
</tr>
<tr>
<td>t. test</td>
<td>7.197</td>
<td></td>
</tr>
<tr>
<td>P. value</td>
<td>0.016</td>
<td></td>
</tr>
</tbody>
</table>

A: P.value= 0.01-0.05 showed significant result, gpA = healthy individuals, gpB = chronic HCV patients. As its shown probiotics increased the populations of CD3⁺ with high significant value = 0.001*. B: P. value= 0.01-0.05 showed significant result, gpA = healthy individuals, gpB = chronic HCV patients. As shown, probiotics increased the populations of CD56⁺ cells with significant p. value 0.46. C: P. value= 0.01-0.05 showed significant result, gpA = healthy individuals, gpB = chronic HCV patients. As shown, probiotics increased the populations of CD3⁺ and CD56⁺ cells with 0.016 significant values.

probiotics capsule, while the concentration of DGAT1 enzyme increased from 624 up to 1038 ng/mml. DGAT1 concentration in group (A) was 302 ng/mml less than its concentration in group (B) which was 528 ng/mml (Table
Table 5. Concentration of DGAT1 enzyme in healthy individuals (gpA) compared to its concentration in patients with chronic hepatitis C (gp B) before any treatments.

<table>
<thead>
<tr>
<th>DGAT1 (Before any treatments)</th>
<th>gp A</th>
<th>gp B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>68.9 – 1011</td>
<td>42.3 – 1819</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>302.1 ± 264.2</td>
<td>528.4 ± 438.7</td>
</tr>
<tr>
<td>t. test</td>
<td>2.214</td>
<td></td>
</tr>
<tr>
<td>P. value</td>
<td>0.148</td>
<td></td>
</tr>
</tbody>
</table>

P. value = 0.01-0.05 showed significant result, Mean ± SD showed the mean and standard error, gpA = healthy individuals, gpB = chronic HCV patients, according to the mean data, it increased in gp B.

Table 6. Concentration of DGAT1 enzyme after probiotics administration and IFN-α injection treatment protocol in group (B) compared to its concentration in group (C) after IFN-α injection only.

<table>
<thead>
<tr>
<th>DGAT1 After</th>
<th>gp B</th>
<th>gp C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>126.2 – 1027.0</td>
<td>172.7 – 2162</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>373.63 ± 262.99</td>
<td>1038.1 ± 806.03</td>
</tr>
<tr>
<td>t. test</td>
<td>9.471</td>
<td></td>
</tr>
<tr>
<td>P. value</td>
<td>0.005*</td>
<td></td>
</tr>
</tbody>
</table>

P. value = 0.01-0.05 showed significant result, Mean ± SD showed the mean and standard error, gpc = chronic HCV patients (only IFN), gpB = chronic HCV patients (probiotics plus IFN), as shown it increased with significance P. value = 0.05*.

DISCUSSION

In this work, we performed a study providing a principle and clinical guidance that aimed to reduce HCV severity in a representative group of Egyptian patients with chronic HCV infection (where HCV genotype 4 was dominant). The patients were treated with *L. acidophilus* and *Bifidobacteria* spp. a month before starting the conventional therapy with IFN-α and ribavirin which was the available therapy at the time of study. However the
benefits of probiotics were the main objective of this study and developing new treatments which are various latterly (Servin, 2004). It was assessed the probiotic treatments on three main parameters, including the viral titer, the secondary bacterial infections, as well as the count of immune cells and their differential numbers and phenotypes. It was found that treatment with L. acidophilus and Bifidobacteria spp. induced antiviral and antibacterial activities and increased the immune cell numbers. These pilot studies indicate that probiotics can be used as an adjuvant system during conventional HCV therapy.

The outcome data in vivo demonstrated that the most common secondary bacterial infections associated with chronic HCV patients were S. lentus, S. aureus, K. pneumonia, P. aeruginosa, and E. coli from the investigated HCV patients in the current study showed resistance against common used antibiotics especially P. aeruginosa and strain No 2 S. aureus. The bacterial load and the numbers of the infected patients with bacteria remarkably reduced in response to oral administration probiotic capsules. This is in line with prior studies which detected antibacterial activity of Lactobacilli and Bifidobacteria in the laboratory against common disease pathogens such as S. typhimurium, E. coli and S. aureus that proved what Hor and Liong (2014) and Hütt (2006) noted. The identification with VITEK 2 system as a precise automated technique was used to identify the isolated pathogens into P. aeruginosa, E. coli, S. lentus, S. aureus, K. pneumonia. This is consistent with prior studies (Garcia-Valdecasas et al., 2009; Kawano et al., 2015) which showed that E. coli, Klebsiella, and Enterobacteriaceae species were the most common pathogenic bacteria in patients with chronic HCV infection. As expected, the group of healthy people A showed no bacterial growth on both of media. In the same connection, Jacobs et al. (2009); (Sikorska and Smoragiewicz, 2013) illustrated and explained the mechanism of the antibacterial activity of Lactobacillus strains. The activity was correlated to production of metabolites such as lactic and acetic acid and that reduced the pH. In the same context, Reis et al. (2012), Ibrahim et al. (2010) explained the activity of Probiotic cell free supernatant by presence of acetic acid, lactic acid, organic acids, hydrogen peroxide, diacetyl, phenols and bacteriocins using GC-Mass.

It has been established that HCV patients are immunocompromised since viral infection causes several symptoms by time related to severity of the infection and progression of the disease. Subsequently, the immunocompromised patients develop fibroses followed by cirrhosis and end up by HCC. Throughout all these stages several biochemical changes have been noticed including decreasing of albumin, increasing of liver enzymes, liver dysfunction, immune dysfunction, diarrhea, respiration and digestion difficulties (Ibrahim et al., 2010). Due to these dysfunction several other side effects emerge which are related to secondary bacterial infection.

Another objective of this study was to investigate whether treating with probiotics can also enhance the immune responses through increasing the counts of immune cells CD3⁺ and CD56⁺. Also it was to investigate the antibacterial effects of probiotics on the load of pathogenic bacteria that included G +ve or G-ve bacteria which was an evidence. Moreover, treatment with probiotics capsule (one per day for a month before IFN-α) resulted in improving the treatment response rate of chronic HCV patients that reflected by PCR assay result. These data suggest that probiotic can be used as a supportive therapy to the treatment of HCV infection whether it based on IFN-α treatment only or with Sovaldi and IFN-α protocol. With this regard, it has been found in preclinical and clinical studies that administration of probiotics enhanced the innate and adaptive immune defenses (Ye et al., 2009), including phagocytosis, NK cell activity, IgG, IgA (Chen and Morgan, 2006) as well as induction of Th1 and Th2 both in vivo and in vitro systems, while maintaining the balance between the strength of the Th2-like response, and Th1-like response. In line with these studies on the effect of exogenous probiotics, endogenous probiotics (gut microbiota) have been reported to control systemic NKT cells (Tsai et al., 2012). Other studies showed that probiotics, including, Lactobacillus and Bifidobacterium, increased expression level of the activation molecule CD69 on CD4⁺ (Th) and CD3⁺CD56⁺ (NKT cells) (Takeda et al., 2013; Kim et al., 2008).

Additionally, probiotics are considered by FDA as biological products which are safe (Doskali et al., 2011; Snydman, 2008) with protecting and supporting the liver and the immune function (Gratz et al., 2010). As a result of this clinical study, the level of DGAT1 after probiotic capsule and treatment by IFN-α injection group (B) was less than its level after treatment by IFN-α injection only group(c) with P value = 0.005* significance. DGAT1 enzyme level catalyzes the fat droplet formation, where probiotics decrease the fat droplet formation that in agree with (Hung et al., 2017) and DGAT1 enzyme which involved in lipoprotein coat structure of HCV particle (Blaising and Pécheur, 2013) and as a result the virus could not replicate or escape from the immunity defense.

**Conclusion**

In conclusion, oral administration of L. acidophilus and Bifidobacteria spp. before treatment with IFN-α and ribavirin associated with significant antiviral activity, and antibacterial effects result in enhancement of the immune status and antibacterial metabolites.

**Recommendation and Future work**

This study recommends prescription of probiotic side by
side with HCV treatment due to the antiviral and antibacterial effects of probiotics. Moreover it enhanced the immune response against the viral and bacterial infection. It is recommended to work on other probiotic species and study its role in the treatment of HCV infection.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

REFERENCES


Full Length Research Paper

Molecular characterization of silver resistant *E. coli* strains isolated from patients suffering from diarrhea

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Silver nanoparticles (AgNPs) are considered a good alternative for antibiotics due to emerging Multidrug Resistance (MDR) crisis. Resistance to AgNPs is approximately limited among Gram-positive and Gram-negative pathogens. Low toxicity to human cells permits its safe use as a new antimicrobial with broad spectrum. Bacterial cells are used as a factory for AgNPs synthesis supplying a powerful antimicrobial with eco-friendly way. In this study, MDR *Escherichia coli* strains were recovered from patients attending Minia University hospital. Biogenic synthesis of AgNPs was performed using *E. coli* cells. Transmission Electron Microscopy (TEM) was used to characterize AgNPs size and shape. Antibacterial activity of AgNPs was tested against the MDR *E. coli* isolates. Screening for Sil and Omp genes was done using polymerase chain reaction (PCR). A total of 13 MDR *E. coli* bacterial culture supernatant isolates were recovered from patients under study. Biosynthesis of AgNPs was observed after addition of supernatant to AgNO₃ by color change from yellow to brown. TEM characterization indicated the presence of silver nanoparticles with 15-75 nm particle size range. Eleven of MDR *E. coli* isolates were sensitive to biogenic AgNP under study. *SilB* and *SilE* genes were encoded by the two AgNPs-resistant *E. coli* isolates which were negative for *OmpF* and *OmpC* genes, respectively demonstrating the role of Sil efflux pump genes and porin deficiency in AgNPs resistance. As indicated, the emergence of silver resistance due to the wide spread of biocides including silver has become a great challenge for the treatment of different infections.

Key words: MDR *Escherichia coli*, *SilB*, *SilE*, silver resistance, *OmpF*, *OmpC*.

INTRODUCTION

Bacterial infections caused by *Escherichia coli* are the most common between hospitalized patients including septicemia, urinary tract infections, enteritis and neonatal meningitis (Allocati et al., 2013). Prevalence of antimicrobial resistance among clinical bacterial isolates is growing everyday representing a great medical challenge for microbiologists and health care professionals all over the world (Prestinaci et al., 2015). Trials for finding effective alternatives for routinely used antibiotics are being made to overcome antimicrobial resistance crisis. AgNPs are promising alternatives which possess great antibacterial and antiviral activity. Antibacterial activity of AgNPs was greater against Gram-negative bacteria than Gram-positive ones, due to
attraction between negative charges on Gram-negative lipopolysaccharide and weak positive charge on AgNPs (Franci et al., 2015). Green synthesis using bacterial cells is considered a friendly approach for creating AgNPs in a more economic and safe way (Ghorbani, 2013). Bacterial cell enzymes such as nitrate reductase reduce Ag\(^+\) ions to AgNPs, which are indicated by colorimetric reaction (Rajesh et al., 2014). Antibacterial activity of biosynthesized AgNPs has been screened against different Gram-negative isolates including \textit{E. coli} indicating observable inhibitory activity (Abu-Zaied, 2016). Resistance to AgNPs can be correlated to decrease permeability either due to porin deficiency in Gram-negative cell wall or due to presence of \textit{Sil} genes which encodes efflux pumps (Gupta et al., 1999). Porins (outer membrane proteins) are encoded by \textit{Omp} genes. \textit{OmpF} and \textit{OmpC} are two major proteins of \textit{E. coli} outer membrane, these proteins have a role in small hydrophilic molecules passive diffusion through bacterial membrane (Matsuyama et al., 1986). \textit{Sil} efflux genes consist of \textit{SilRS} (transcriptional regulatory system which consists of two components), this system controls \textit{SilE} (protein binds to periplasm). Another components are two efflux pumps \textit{SilP} (a P-type ATPase) and \textit{SilCBA} (three protein chemiosmotic RND Ag (I)/H* exchange system) (Silver, 2003). In the present study, AgNPs were biosynthesized using \textit{E. coli} cells, antibacterial activity of AgNPs against clinical MDR \textit{E. coli} isolates, recovered from patients suffering from gastroenteritis, was studied using agar well diffusion method and the presence of \textit{OmpC}, \textit{OmpF}, \textit{SilB} and \textit{SilE}, \textit{SilP}, \textit{SilS} genes were screened among recovered isolates.

MATERIALS AND METHODS

Collection of samples

A total of 30 stool samples were collected from patients suffering from bacterial gastroenteritis attending department of accident and emergency in Minia University hospital (Minia, Egypt), samples were collected from March 2018 to April 2018.

Isolation of \textit{E. coli} strains

Fecal samples were processed as follow: 3 g of fecal samples were mixed with normal saline, and centrifuged. The supernatants were discarded and the deposit was suspended in peptone water. Aliquots were used for the inoculation of MacConkey agar (Oxoid, UK), followed by an overnight incubation at 37°C. \textit{E. coli} positive cultures were confirmed using biochemical tests (Himedia, India) (catalase positive, methyl red positive, indole positive, nitrate reduction positive and citrate negative) and the formation of metallic sheen on Eosin methylene blue agar.

Antimicrobial susceptibility testing

Amoxycillin/clavulanic acid, norfloxacin, azithromycin, cefoperazone levofloxacin, ceftriaxone, sulfamethoxazole/trimethoprim, cefuroxime, imipenem, tetracycline, cefipime, amikacin were used for testing antimicrobial activity against \textit{E. coli} isolates. Disk method using Muller-Hinton agar plates was applied for antimicrobial susceptibility testing. By inoculating nutrient agar plates with suspension of inoculum and streaking of bacterial suspension using cotton swab. Antibiotic discs (Oxoid, UK) were applied after drying of inoculated nutrient agar plates. Diameters of inhibition zones were measured indicating sensitive or resistant isolates according to CLSI standards (CLSI, 2008).

Silver nanoparticles biosynthesis

\textit{E. coli} culture was obtained from Microbiology and Immunology Department, Faculty of Pharmacy, Minia University. Nutrient broth was used to inoculate \textit{E. coli} isolate. Supernatant was obtained by centrifugation of \textit{E. coli} culture for 15 min at 5000 rpm and added to the flask of sterile aqueous AgNO\(_3\) (1 mM). The reaction was performed for 10 min in bright conditions (Ghorbani, 2013).

Silver nanoparticle isolation and purification

By centrifugation of mixture of supernatant and AgNO\(_3\) for 20 min at 10000 rpm, AgNPs were isolated. The AgNPs pellet was washed by centrifugation with sterile distilled water twice for 20 min at 1000 rpm, so the culture filtrate and excess silver ions were removed. Freeze drying was used to obtain AgNPs pellet as powder for further characterization and applications (Rajesh et al., 2014).

AgNPs characterization

Transmission electron microscope (TEM) (JEM1010, JEOL, Tokyo, Japan) was used to characterize shape and size of the isolated AgNPs. A nanoparticle solution drop was placed over carbon-coated copper grids and left for water evaporation. Then, size and shapes of AgNPs were examined (Rajesh et al., 2014).

AgNPs antibacterial activity

Antibacterial screening was performed using disc diffusion method by preparing nutrient agar and nutrient broth culture of tested isolates. Both nutrient agar and broth cultures were mixed together. The mixture was poured onto sterile petridishes. AgNPs discs were prepared by sterilizing filter paper discs in autoclave, dipping into AgNO\(_3\) solution (10 \(\mu\)g/ml) and drying in air in sterile area. AgNPs discs were placed onto the nutrient agar after seeding with broth culture. After incubation for 24 h at 37°C, inhibitory activity of AgNO\(_3\) discs was indicated by presence of inhibition zones around the discs (Rosanoainvo and Ratsimamamanga-Urvery, 1993; Malabadi et al., 2005). Data entry and analysis were all done using software called Statistical Package for Social Science (SPSS) (IBM, U.S.A).

DNA extraction

The DNA template was prepared by boiling suspension of bacterial pellet for 10 min and directly used in the PCR assay. Genomic DNA was extracted from \textit{E. coli} overnight culture by method described by Wilson (1987).

PCR primers and condition

PCR reaction was performed in a total of 50 µl reaction as follows: DNA extract (5 µl), each primer (50 pmole), Go Taq Green Master
Table 1. Primers in the study with corresponding amplification products.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
<th>Amplification product (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmpF</td>
<td>Forward: ACCTGGCAGCGAATACG</td>
<td>191</td>
<td>Vinson et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Reverse: AACATCACCAGATACCTCTACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OmpC</td>
<td>Forward: CAGGATGTTGGTTCTTTTG</td>
<td>162</td>
<td>Vinson et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAAGTCAGTGTTACGGTACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SilB</td>
<td>Forward: CAAAGAACAGCGGTGATTA</td>
<td>233</td>
<td>Woods et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTCAGACATTGCTGGCATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SilE</td>
<td>Forward: GTACTCCCGCGACATGCTTAATT</td>
<td>400</td>
<td>Percival et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGCCAGACTGACCGTTATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SilS</td>
<td>Forward: GGAGATCGCGGATGCAATGAA</td>
<td>1500</td>
<td>Percival et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTTTGCTGACAGCGCTGAAGCATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SilP</td>
<td>Forward: CATGACATATCGGAGACACAGAATAAG</td>
<td>2500</td>
<td>Percival et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGGGCAGACCCAGCATAAACAGATA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mix (Promega) (25 μl). Conditions for OmpC and OmpF genes were: initial denaturation step at 94°C (3 min), 35 cycles of denaturation at 94°C (45 s), annealing of primers at 53°C for OmpC / 54°C for OmpF (45 s) and extension step at 72°C (1 min) then final extension at 72°C (5 min) (Vinson et al., 2010). Conditions for SilB, SilS and SilP genes were 95°C (5 min) and 30 cycles at 95°C (30 s), at 57°C (30 s) and 72°C (30 s) then final elongation at 72°C (5 min) (Losasso et al., 2014). Conditions for SilE gene were 94°C (5 min), 30 cycles at 94°C (30 s), 55°C (30 s), 72°C (30 s) then final elongation at 72°C (12 min) (Shutterlin et al., 2014). Agarose gel (2%) with ethidium bromide (Sisco Research Laboratories Pvt, Ltd., India) staining was used to analyze PCR products; gel document system was used to visualize DNA bands (Gel Doc 2000; Bio-Rad, USA)(Table 1).

RESULTS

Prevalence of isolates

In the present study, out of thirty stool samples, 13 (43.3%) E. coli isolates were recovered.

Antimicrobial susceptibility testing

Table 2 indicates that all E. coli isolates are resistant to most tested antimicrobial agents such as amoxycillin/ clavulanic acid, norfloxacin, azithromycin, cefoperazone, levofloxacin, ceftriaxone, sulfamethoxazole/ trimethoprim, cefuroxime and imipenem.

Silver nanoparticles synthesis

Silver nanoparticles formation were detected by the change of color of AgNO₃ solution after addition of bacterial supernatant to yellow, orange then brown (Figure 1).

Silver nanoparticle characterization

AgNPs characters were detected by TEM, the TEM micrographs of biogenic AgNPs showing that particles are spherical with size ranges from (15-75) nm (Figure 2).

Antibacterial activity of AgNPs against MDR E. coli isolates

Our study shows that out of 13 E. coli isolates tested, 11 (84.6%) were sensitive to AgNPs which indicated by presence of inhibition zone with a diameter ≥ 1.6 cm around prepared AgNPs discs (Table 3).

Screening of Omp and Sil genes

Figures 3a and b show that among 13 E. coli isolates tested, one isolate was positive for SilB gene and another was positive for SilE gene but No strain was positive for SilP or SilS genes. Figure 4 shows that 11 E. coli isolates tested were positive for OmpC and OmpF genes and only two isolates were negative for OmpF (SilB positive) and OmpC (SilE positive). All isolates were negative for SilS and SilP genes.

DISCUSSION

Silver was known for longtime by its antibacterial and antifungal activity. By increasing the incidence of antimicrobial resistance between clinical pathogens, silver use as AgNPs has found a great application in healthcare facilities such as wound dressings and antibacterial agents (Shutterlin et al., 2012). Bacterial cells are found to supply a good factory for AgNPs synthesis (Ghorbani, 2013). In the present study, AgNPs...
Table 2. Antimicrobial susceptibility of the tested *E. coli* isolates.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Resistance pattern</th>
<th>Sensitivity pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM</td>
<td>TE, FEP, AK</td>
</tr>
<tr>
<td>2</td>
<td>AMC, NOR, AZM, CFP, LEV, CRO, SXT</td>
<td>TE, FEP, AK, CXM, IPM</td>
</tr>
<tr>
<td>3</td>
<td>AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM, TE</td>
<td>FEP, AK</td>
</tr>
<tr>
<td>4</td>
<td>AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM</td>
<td>TE, FEP, AK</td>
</tr>
<tr>
<td>5</td>
<td>AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM, IPM, AK</td>
<td>TE, FEP</td>
</tr>
<tr>
<td>6</td>
<td>AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM</td>
<td>IPM, TE, FEP, AK</td>
</tr>
<tr>
<td>7</td>
<td>AMC, NOR, AZM, CFP, LEV, SXT, CXM, IPM</td>
<td>AK, TE, FEP</td>
</tr>
<tr>
<td>8</td>
<td>AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM</td>
<td>IPM, TE, FEP, AK</td>
</tr>
<tr>
<td>9</td>
<td>AMC, NOR, AZM, CFP, LEV, CRO, SXT, CXM</td>
<td>IPM, TE, FEP, AK</td>
</tr>
<tr>
<td>10</td>
<td>AMC, NOR, AZM, CFP, LEV, SXT, CXM, IPM</td>
<td>TE, FEP</td>
</tr>
<tr>
<td>11</td>
<td>AMC, NOR, AZM, CFP, LEV, SXT, CXM, IPM, AK</td>
<td>FEP, AK</td>
</tr>
<tr>
<td>12</td>
<td>AMC, NOR, AZM, CFP, LEV, SXT, CXM, IPM, AK</td>
<td>TE, FEP</td>
</tr>
<tr>
<td>13</td>
<td>AMC, NOR, AZM, CFP, LEV, SXT, CXM, IPM</td>
<td>AK, TE, FEP</td>
</tr>
</tbody>
</table>

AMC, Amoxycillin/clavulanic acid; NOR, Norfloxacin; AZM, Azithromycin; CFP, Cefoperazone; LEV, Levofloxacin; CRO, Ceftriaxone; SXT, Sulfamethoxazole/trimethoprim; CXM, Cefuroxime; IPM, Imipenem; TE, Tetracycline; FEP, Cefipime; AK, Amikacin.

Figure 1. Colorimetric reaction due to formation of AgNPs.

were synthesized biogenically from *E. coli* strain by addition of bacterial supernatant to AgNO₃ solution and the reaction color change to brown within 10 min in the light suggesting that nitrate reductase enzyme of *E. coli* reduced Ag⁺ ions to AgNPs. Formation of brown color was suggested to be due to surface plasmon vibration excitation in AgNPs. Extracellular reduction by *E. coli* was applied for AgNPs synthesis (Ghorbani, 2013). *Lactobacillus acidophilus* culture was filtered and used for AgNPs synthesis by Ag⁺ reduction at room temperature within one day time (Rajesh et al., 2014). Also, Nitrate reductase of *B. licheniformis* has a role in AgNPs synthesis (Kalimuthu et al., 2008). AgNPs isolated in this study and characterized by TEM micrographs showed spherical shape with size ranges from 15-75 nm. Smaller size AgNPs shows antimicrobial activity greater than larger ones (Rai et al., 2012). Also, shape of AgNPs is of great influence as rod-shaped and spherical AgNPs are less effective as biocidals against *E. coli* than triangular ones (Pal et al., 2007; Sharma et al., 2009). Particle size (4-50 nm) with spherical shape also indicated for AgNPs synthesized from *Lactobacillus acidophilus* (Rajesh et al., 2014). In another study, it was reported that AgNPs size ranges from 10-100 nm (Ghorbani, 2013).

The previous findings are in agreement with the results obtained in the present study as AgNPs were biosynthesized extracellularly with particle size ranges from 10-80 nm. As illustrated in our study, AgNPs discs showed inhibitory activity against 11 (84.6%) MDR *E. coli* clinical isolates. Gram-negative bacteria such as *Klebsiella pneumoniae*, *E. coli* and Gram-positive such as *Staphylococcus aureus* and *Bacillus subtilis* are affected by antibacterial activity of AgNPs (Malabadi et al., 2012). Confirmation with high antimicrobial activity of biogenic AgNPs against MDR *E. coli* was reported (Abu-Zaid, 2016). It was indicated that *K. pneumoniae* was affected...
Figure 2. TEM micrographs of biogenic AgNPs.

Table 3. Antimicrobial activity of AgNPs against tested *E. coli* isolates.

<table>
<thead>
<tr>
<th><em>E. coli</em> isolated in the study</th>
<th>Zone of inhibition (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate no. 1</td>
<td>1.6</td>
</tr>
<tr>
<td>Isolate no. 2</td>
<td>0</td>
</tr>
<tr>
<td>Isolate no. 3</td>
<td>1.6</td>
</tr>
<tr>
<td>Isolate no. 4</td>
<td>1.7</td>
</tr>
<tr>
<td>Isolate no. 5</td>
<td>1.8</td>
</tr>
<tr>
<td>Isolate no. 6</td>
<td>1.6</td>
</tr>
<tr>
<td>Isolate no. 7</td>
<td>1.7</td>
</tr>
<tr>
<td>Isolate no. 8</td>
<td>0</td>
</tr>
<tr>
<td>Isolate no. 9</td>
<td>1.7</td>
</tr>
<tr>
<td>Isolate no. 10</td>
<td>1.6</td>
</tr>
<tr>
<td>Isolate no. 11</td>
<td>1.6</td>
</tr>
<tr>
<td>Isolate no. 12</td>
<td>1.6</td>
</tr>
<tr>
<td>Isolate no. 13</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Mean = 1.39, Standard deviation = 0.59.
Figure 3. PCR products of SilB gene (A), all lanes are negative for SilB gene except lane 7 showed band of 233 bp and SilE gene (B), all lanes are negative for SilE gene except lane 2 showed a band of 400 bp.

Figure 4. PCR products of OmpF (lanes 2-6 from left to right) and OmpC (lanes 7-11 from left to right), Lane 6: OmpF negative and Lane 11: OmpC negative.

by *Lactobacillus acidophilus*-synthesized AgNPs antibacterial activity (Rajesh et al., 2014). AgNPs antibacterial activity against *E. coli* was reported (Cunha et al., 2016; Li et al., 2010). Mode of action of AgNPs as antimicrobial may be attributed to free radicals generation, also after penetration of AgNPs into the cells, oxidative stress was reported (Hussain et al., 2006). Particle size of AgNPs is influential in its antibacterial
action as *E. coli* was killed by low concentration of 16 nm-
size AgNPs, which was found inside bacteria and it is cell wall adherent (Raffin et al., 2008). Also, *E. coli* membrane disruption by 20 nm-size AgNPs was illustrated after few minutes’ exposure indicating effect of large surface area of AgNPs on its antimicrobial efficiency (Raffin et al., 2008). Yeast, *Staphylococcus aureus* and *E. coli* were inhibited and killed by AgNO₃ and Ag⁺ ions (Kim et al., 2007). Heat shock protein expression of *E. coli* was altered by short AgNPs exposure (Lok et al., 2006). Due to safe AgNPs use against human cells and effective low concentration, AgNPs are more applicable as antimicrobial agent (Abo-Neima and El-Khaly, 2016). In addition to antibacterial activity, also cells infected with HIV were killed due to AgNPs antiviral activity (Sun et al., 2005). It was found that combination of AgNPs with antibiotic increase its antibacterial activity (Ingle et al., 2009, Rathod and Ranganath, 2011). MDR *E. coli* and *Streptococcus* species showed great response to AgNPs antimicrobial action (Lara et al., 2010). All previous reports agree with our results as AgNPs showed good antibacterial activity against MDR isolates. In another finding, *E. coli* isolates showed no response to AgNPs indicated by absence of inhibition zone which is in a disagreement with results obtained in our study (Inbaneson et al., 2011). Our study shows that 11 isolates were positive for OmpC and OmpF genes as it confirms the presence of porin channels among 11 AgNPs-sensitive tested *E. coli* isolates. Also, absence of OmpC and OmpF genes is observed among AgNPs-resistant *E. coli* isolates. Ag⁺ ions enter the bacterial cell thorough porins and in case of porin protein mutation, *E. coli* becomes resistant to silver antibacterial activity (Li et al., 1997). When OmpF or OmpC porins are absent due to mutation, *E. coli* becomes 4-8 times less susceptible to the antibacterial activity of AgNPs (Radzig et al., 2013). The previous reports agree with our findings with respect to the role of Omp genes in *E. coli* response to antibacterial activity of AgNPs. The present study illustrated the absence of SilB and SilE genes among 11 tested *E. coli* isolates and only two isolates were positive for SilB and SilE genes for each. These two isolates were resistant to AgNPs antibacterial activity while other 11 isolates were AgNPs sensitive as confirmed by the absence of Sil genes. Bacteria resist silver by uptake decrease, cell membrane alteration and efflux pump that cause silver to be expelled out of cell (Silver and Phung, 2005). SilB is a fusion protein in periplasmic space which links SilA (pump protein present in inner membrane) to SilC (porin channel). SilB gene is an indicator for resistance of Gram-negative to silver (Silver et al., 2006). Whether in presence or absence of nanosilver, SilB gene is constitutively expressed among *Salmonella senftenberg* (Losasso et al., 2014). SilE gene was common among *E. coli* isolates producing CTX-M after exposure to silver (Shutterlin et al., 2014). The previous findings are in agreement with our results as Sil operon plays a role in AgNPs resistance. As illustrated in our study, one *E. coli* isolate was found negative for OmpF and positive for SilB and another *E. coli* isolate was found negative for OmpC and positive for SilE, these two isolates were AgNPs resistant confirming the role of efflux pump encoded by Sil gene and the effect of porin absence in *E. coli* resistance to AgNPs. In another report, Sil genes were not correlated to resistance of *E. coli* to silver (Shutterlin et al., 2012) which contradicts with the findings of the present study.

**Conclusion**

Although silver resistance was not frequent among bacterial isolates for long time, the presence of Sil genes and absence of Omp genes have a great influence in *E. coli* response to AgNPs antibacterial activity indicating that silver resistance among MDR isolates is a possible behavior which threatens the power of silver as a strong old biocidal.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

**ACKNOWLEDGMENT**

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**REFERENCES**


Related Journals:

2. Journal of Microbiology and Antimicrobials
3. African Journal of Microbiology Research
4. International Journal of Biotechnology and Molecular Biology Research
5. Journal of Bioinformatics and Sequence Analysis
6. Journal of Biophysics and Structural Biology
8. Journal of Microbiology and Antimicrobials

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