Plant Archives Vol. 20, Supplement 2, 2020 pp. 450-464 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ISOLATION AND CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* AND *ENTEROCOCCUS FAECALIS* LYTIC BACTERIOPHAGES FROM WASTEWATER FOR CONTROLLING MULTIDRUG RESISTANT BACTERIAL STRAINS

Samir A. Mahgoub[1*], Marwa I. S. Muhammad[2], Sayed T. Abd-Elsalam[2], Maha M. Alkhazindar[2], and Hussein I. Abdel-Shafy[3]

[1]Microbiology Department, Faculty of Agriculture, Zagazig University, 44519, Zagazig, Egypt
[2]Botany and Microbiology Department, Faculty of Science, Cairo University, Cairo, Egypt
[3]Water Research and Polluted Control Department, National Research Centre, Dokki, Cairo, Egypt.
Corresponding author: mahgoubsamir@gmail.com&samahgoub@zu.edu.eg

Abstract

The aim of the current study was to isolate, identify and characterize lytic bacteriophages against multi-drug resistant strains of *Pseudomonas aeruginosa* and *Enterococcus faecalis* from wastewater. Plaque size, transmission electron microscopy (TEM), virulence factors, and *in vitro* lytic activity of bacteriophage isolates were investigated. The sewage water were collected from wastewater treatment plants (WTs) in El-Sharkia Governorate, Egypt. *E. faecalis* and *P. Aeruginosa* were the predominant pathogenic bacteria from wastewater. The highest percentage of multi-antibiotic resistance bacteria (MAR) in all sewage water samples were *P. aeruginosa* and *E. faecalis* (84%).The results showed that the presence of bacteriophages for *P. aeruginosa* strains named (PP1, PP2 and PP3) while phages infect *E. faecalis* strains named (EP1, EP2 and EP3). Isolated phages examined by TEM and their morphological shapes were detected. The bacteriophages had an isometric head and long-contractile tail and another phage with short tail with varying their dimensions from length, diameter of head and length, diameter of tail. They are belonging to *Myoviridae* and *Siphoviridae* families. TEM confirmed *P. aeruginosa* and *E. faecalis* bacteriophages belonging to the family *Myoviridae*. Among *P. aeruginosa* and *E. faecalis* bacteriophages with broad host range, 18 isolates (66.7%) did not harbor any *P. aeruginosa* and *E. faecalis* virulence factors. Among them, bacteriophage strains PP1, PP2, PP3, EP1, EP2 and EP3 effectively inhibited *P. aeruginosa* and *E. faecalis* in *vitro* within 1.0 h recorded 88٪, 91,7٪ and 92,3٪ and *E. faecalis* phages (EP1, EP2 and EP3) recorded 93,3،٪ 92,8٪ and 93,6٪ respectively. Therefore, they are considered potential candidates for controlling the contamination of *P. aeruginosa* and *E. faecalis* in water or other applications.

***Keywords*:** Bacteriophages, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, Isolation, Characterization, Attacking, Wastewater

Introduction

The use of phage therapy come from successful phage treatments to patients suffering from antibiotic resistant chronic infections by *Staphylococcus aureus* using phage cocktails (Barcelo *et al.,* 2017). Phage therapy has been proven to be more effective, in some cases than conventional antibiotic, especially regarding multidrug-resistant biofilm infections.

Water borne-pathogens, water scarcity and, in general, the ongoing environmental deterioration has pushed research into eco-friendly and cost efficient sanitation. With respect to growing urban, agricultural and industrial water demand, reuse of wastewater has an utmost priority to overcome water shortage issues. A renewed interest in phages therapy took shape predominantly due to the increase in multi-drug resistant pathogens (MDR) and the desire to find alternative treatment method to the use of chemical antibiotics in agriculture, veterinary science, industry and food safety (Sulakvelidze *et al.,* 2001). According to the national Nosocomial Infections surveillance system, *Pseudomonas aeruginosa* is the third most common pathogen associated with all hospital acquired infections that is associated with a high mortality rate (Moreau-Marquis *et al.,* 2008). The use of bacteriophages in containing biofilms comprises two strategies, one is to prevent biofilm formation, and the other is to disrupt existing biofilm (Azeredo and Sutherland, 2008).

Pseudomonas aeruginosa is one of the multidrug-resistant organisms most frequently isolated worldwide and because of a shortage of new antibiotics, bacteriophages are considered an alternative for its treatment. Previously, *P. aeruginosa* phages were isolated and used as the best candidate and chosen based on their ability to form clear plaques and their host range (Matsuzaki *et al.,* 2005 and Byrne *et al.,* 2015). *P. aeruginosa* isolates from sewage water were more susceptible to phage infection than isolates from clinical cases(Angela *et al.,* 2015; Alsaffar and Jarallah, 2017). *P. aeruginosa* phage AZ1 showed promising activity in the destruction of both planktonic cells and biofilm of *P. aeruginosa*-2995 (Jamal, *et al.,* 2017).

Enterococcus faecalis is a commensal Gram-positive facultative anaerobic bacterium inhabiting the gastrointestinal tract of humans and various animals, but is also found in environments like soil and water (Furtado *et al.,* 2014). *E. faecalis* is one of the most frequently isolated species from hospital-associated infections: it causes endocarditis, bacteremia, urinary tract infections, meningitis and other fatal forms of systemic and local infection in humans (Lee, 2013). *E. faecalis* phage (ECP3) belong to the family *Myoviridae* with contractile tail and lysed *E. faecalis* specifically (Kang *et al.,* 2015). *E. faecalis* phage φ 4D isolated from sewage capable of infecting a wide spectrum of enterococcal strains, including the vancomycin-resistant strain (Paisano *et al.,* 2004). *E. faecalis* phages isolated from environment named φ EF24C, has period and a larger burst size than ordinary tailed phages, indicating that φ EF24C has effective lytic activity against many *E. faecalis* strains (Uchiyama *et al.,* 2007). *E.faecalis* phage isolated from sewage water termed EFDG1 have high lytic activity that significantly reduced a 2-week-old *E. faecalis* biofilm, demonstrating the superiority of phage therapy over conventional antibiotic treatments in biofilms (De la fuente,

2013). *E. faecalis* phage isolated from sewage effluents, it belong to *Myoviridae* phages which includes promising candidates for therapy against Gram-positive pathogens (Khalifa *et al.,* 2015). It's have short latency period and fast release period indicate that phage EF- P29 possesses a highly efficient ability to infect *E. faecalis*. EF-P29 can lyse not only antibiotic-sensitive *E. faecalis* strains but also antibiotic-resistant *E. faecalis* strains EF-P29 for that use as a candidate therapeutic phage (Uchiyama *et al.,* 2008; Niu *et al.,* 2012 and Gu *et al.,* 2016). Five bacteriophages were isolated from concentrated waste water that belong to *Siphoviridae* family. They are specific to *E. faecalis* that they are lytic bacteriophages which candidate for therapy (Al-Zubidi, 2017), because bacteriophages show specificity for their bacterial hosts, there has been a growth in interest in using phages therapies to compact the rising incidence of multidrug-resistant bacterial infections using lytic phage (Cheng *et al.,* 2017; Erez *et al.,* 2017). This study intends to use bacteriophage therapy that are based on the use of specific phage against multidrug resistant bacterial strains of *Pseudomonas aeruginosa* and *Enterococcus faecalis*, to achieve complete disinfection. Therefore, the aim of this current study was to isolate and characterize of *Pseudomonas aeruginosa* and *Enterococcus faecalis* lytic bacteriophages (named as PP1, PP2 and PP3 & EP1, EP2 and EP3) from wastewater treatment plants and both phages have a high potential for phage application to control *P. aeruginosa* and *E. faecalis*, respectively.

Materials and Methods

Wastewater treatment sites and samples collection

The wastewater samples were collected from six municipal wastewater treatment effluents (MWTs) plants in EL-Sharkia Governorate, Egypt. On each sampling occasion, a one-litre volume of each sample was collected in pre-sterilised (autoclaved at 121ºC for 15 minutes) polyethylene bottles, stored in cooler boxes at approximately 4ºC and transported to the laboratory for further analysis within 4 hrs. At all sites, two different samples were collected on each occasion: (i) raw wastewater (RW) and (ii) final effluent (FE).

Enumeration of viable bacterial count

Water samples were shacked vigorously and serial dilutions were prepared in saline solution (8.0 g NaCl/l of distilled water). From three proper serial dilutions selected according to the nature of the sample, the standard plate count bacteria (SPC) was determined by pour plate method according to the method of APHA (2005). Plate count agar obtained in a dehydrated form (Difco, USA) was used for enumerating of SPC. Two sets of duplicate plates were used for each sample dilution. One set was incubated at 37°C for 2 days and the other set at 22°C for 5 days. Viable microorganisms developed in the form of colonies were calculated considering the dilution factor and the final estimate was taken as the average (cfu/ ml) of figures obtained from the countable plates.

Enumeration of indicator bacteria

Total coliform (TC) density was determined using membrane filter technique according to standard method No. 9222 B (APHA, 2005) on M-Endo Agar LES medium obtained from Difco, USA after incubation at 35°C for 24 hrs. Colonies showing pink to dark red color with metallic surface sheen were counted as total coliform bacteria. Results were recorded in cfu/100 ml using the following equation:

$$\text{Total coliform colonies/100ml} = \frac{\text{Coliform colonies counted} \times 100}{\text{ml sample filtered}}$$

Fecalcoliform (FC) density was determined using membrane filter technique according to standard method No. 9222 D (APHA, 2005) on M-FC agar medium obtained from Difco, USA after incubation at 44.5 ± 0.5°C for 24 hrs. colonies developing various shades of blue were counted as fecal coliform bacteria and recorded in cfu/100ml using the same equation in TC calculations.

Fecal streptococci (FS) density was determined using membrane filter technique according to standard method No. 9230 C (APHA, 2005) on M-Enterococcus agar medium obtained from BBL, USA after incubation at 35±0.5°C for 48hrs. colonies showing red to pink color were counted as fecal streptococci bacteria and recorded in cfu/100ml as mentioned previously. In this study, identification of bacterial isolates from collected sewage water samples were carried out according to Bergey's Manual of systematic Bacteriology.

Isolation of *Pseudomonas aeruginosa*

P. aeruginosa was isolated by membrane filter technique according to standard method No. 9213 E (APHA, 2005), Using M-PA-C agar medium obtained from BBL after incubation at 41.5±0.5°C for 72 hrs. colonies showing flat appearance with light outer rims and brownish to greenish black centers and 0.8 to 2.2mm in diameter were isolated as *P. aeruginosa*. These colonies were confirmed by streaking on cetrimide agar plates, a selective medium which inhibits other bacterial growth and enhances Fluorescein and pyocynin "Blue green" pigment production. Almost all microscopically examination and biochemical testing used for identification were carried out.

Isolation of *Enterococcus faecalis*

E. faecalis detection and isolation was done using M-Enterococcus agar medium. Colonies showing red to pink color were isolated as *E. faecalis*. Bacterial colonies developed from all previously mentioned media were chosen and picked up according to variation in culture characteristics and colony formation then purified by streak-plate method on Nutrient agar medium (Difco). Pure isolates were maintained on slants of the same medium at 4°C for subsequent identification. Almost all microscopically examination and biochemical testing used for identification were carried out according to (Collins and lyne, 2004 and cheesbrough, 2006).

Antibiotic sensitivity test for bacterial isolates

In this test, the standard Kirby-Bauer disk diffusion method (Bauer *et al.,* 1966) was performed, in which eight commercially prepared antibiotic discs (6 mm in diameter) belonging to eight different groups were chosen for their potency against bacteria isolated from water samples. These antibiotic classes are the most commonly used in human medicine, veterinary medicine, animal husbandry and in agriculture. The discs were obtained from Oxide, UK. Properties of the target antibiotics are listed in Table (1). Four to five similar colonies from overnight growth plate were transferred aseptically in sterile distilled water and vigorously agitated to give a turbidity that matches the 0.5 MacFerland standards (approximately 10^8cfu/ml). Within 15

minutes, sterile cotton swab dipped into the culture suspension was used for inoculating the surface of solidified Muller-Hinton agar plates (NCCLS/CLSI, 2007). Antibiotic discs were dispensed onto the inoculated plate surface and gently pressed down using a sterile. Forceps to insure complete contact with agar. Within 15 minutes of applying discs, the inverted plate were aerobically incubated at 37°C for 24hrs. The resulted diameters of inhibition zones around the antibiotic discs were measured to nearest whole mm and interpreted according to protocols standardized for the assay of antibiotic compounds as guided by NCCLS/CLSI(2007). The results were categorized as: R(resistant), I (intermediate sensitive), and S (sensitive).

Table 1 : Antibiotics used for sensitivity test.

No.	Group	Scientific name	Trade name	Symbol	Concentration (µg/disc)
1	Penicillins	Methicillin	Methicillin	MET	5
2	Cephalosporins	Cephalothin	Keflin	KF	30
3	Glycopeptides	Vancomycin	Vancocin	VA	30
4	Aminoglycosides	Tobramycin	Nebcin	TOB	10
5	Macrolides	Erythromycin	Erythromycin	E	10
6	Lincosamides	Clindamycin	Lincocin	DA	30
7	Sulfa drugs	Trimethoprim/ Sulfamethoxazole	Septrin	SXT	25
8	Chloramphenicol	Chloramphenicol	chloramphenicol	C	30

Pathogenicity test and virulence factors detected in the isolates

This test was carried out according to (Berkhoff and Vinal, 1986) using the Congo Red Agar (CRA) method. Single colonies from each isolates (*P. aeruginosa & E. faecalis*) of 24 hrs. the old pure bacterial cultures (*P. aeruginosa & E. faecalis*) were streaked on Congo Red Agar plates. After incubation at 35°C for 24hrs. the cultures were left at room temperature for 48hrs. to facilitate annotation of results. The colonies showing red color in appearance were recorded as positive result and consider as pathogen isolate. While, colonies remained white or grey were recorded as negative result and consider as non-pathogen isolates.

Blood haemolysis ability of the isolated bacterial colonies was tested using tryptic soy agar obtained from Difco, U.S.A., supplemented with 5% sterile human blood. Bacterial growth on blood agar medium showed the following features (A)- β-haemolysis, characterized by colorless zone of blood agar around the bacterial growth, (B)- α-haemolysis, characterized by production of grayish or green color around bacterial growth and (C)- No haemolysis on blood agar medium.

16S rDNA gene sequencing of *Pseudomonas aeruginosa*and *Enterococcus faecalis*

Bacterial genomic DNA for each bacteria were isolated using Applied BiosystemsPrepMan® Ultra sample preparation Reagent (PN 4322541) (QIAamp, Qiagen®, Hilden).The ideal colony size of each bacteria isolate is 2 to 3 mm. Each colony was re-suspended in 1ml deionized water. Five microliter of each suspension of bacteria isolates were added separately to 495µl of nuclease-free water (AM 9937) into a 1.5ml micro centrifuge tube (AM 12450) to get 1:100 dilutions. The tubes were vortexed to mix each solution and centrifuged for 10 minutes at 6000rpm. The supernatant for each bacteria was taken and stored at -20°C. At the end of the PrepMan® Ultra Protocol, a supernatant that contains bacterial genomic DNA was obtained.

PCR amplifies the first 500 base pairs of the 16S ribosomal RNA gene (16S rDNA) in the GeneAmp® PCR system 9700 as explained in the following procedure. Samples and controls in 0.2ml MicroAmp PCR tubes. Two primer sets PA-F (for forward) and PA-R (for reverse) were designed for 16S rDNA gene for each bacteria according to Tripathi *et al.* (2013) as follows:

PA-F: 5, GGGGGATCTTCGGACCTCA -3,

PA-R: 5, TCCTTAGAGTGCCCACCCG-3,

Primers are designated by the first three letters of the bacterial names followed by F and R for forward and reverse. The tubes were capped then placed in the thermal cycler (COT thermocycler II model 1105). In a 0.2ml micro centrifuge tube were added 7µl of the purified PCR product (~5ng) and 13µl of the forward and reverse sequencing mixture. The tubes were capped then placed in thermal cycler. The programme of the GeneAmp® PCR system 9700 using the following thermal-cycling conditions was applied.

After cycle sequencing excess dye Terminators and primers were removed from the cycle sequencing reactions using Dye Ex® 2.0 spin Kit (Oiagen PN 63204).The microcentrifuge tubes containing the purified extension products were centrifuged for 30-60 min. at 3000 rpm. The DNA pellet was re-suspended in 15µl of Hi-Di TM formamide (PN 4311320). The re-suspended DNA samples were loaded onto the sequencing platform. For maimed disrupts hydrogen bonds in dsDNA. This activity inhibits secondary structure and DNA conglomeration, resulting in cleaner and more consistent electrophoresis runs.

MicroSEQ® ID Analysis Software enables to analyze sequences obtained with any of the MicroSEQ® Microbial identification kits including the MicroSEQ® 500 16S rDNA Bacterial Identification Kits. The software assembles the 16S rDNA sequence for the unknown then compares the sequence with 16SrDNA sequence in the MicroSEQ® ID 16SrDNA 500 library (version 1.0 and 2.0). Based on the comparison, the software provides an ID for the unknown bacterial species.

Multiple alignments of sequences and nucleotide sequence statistics and variability were performed using DNAMAN software (Wisconsin, Madison, USA) and calustalow (Ver. 1.74) program (Thompson *et al.,* 1994). Secondary structure prediction, nucleotide diversity and melting temperature were performed using Molecular Evolutionary Genetics Analysis (MEGA) software (ver. 4.0)

(Tamura *et al.,* 2007). Phylogenetic relationships for two bacterial sequence variants were evaluated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) thought DNAMAN software and Neighbour joining (NJ) implemented thought MEGA 4.0 software.

Detection of bacteriophages

(i) Preparation of viruses lysate

The viruses specific for isolated and identified bacterial cultures including *P. aeruginosa* and *E. faecalis* from collected water samples were detected according to the method of Adams (1959). Erlenmeyer flasks (250ml) each containing 50ml nutrient broth were inoculated with 5ml of the collected water sample. Mixtures of each strain (1.0ml) were added and the flakes were incubated at the optimum temperature for 72hrs.Bacteriophages in these suspensions were assayed qualitatively using spot test and the date were recorded. Bacteriophages were qualitatively and quantitatively assayed by the spot test and the over layer agar techniques (plaque assay technique) according to the methods of Adams (1959).

(ii) Preparation of high titer phage stock

Single plaques of *P. aeruginosa* and *E. faecalis* phages resulting from the plaque assay technique were picked up using sterilized bacterial inoculation needle, then transferred to flasks containing 10 ml of liquid host culture (10^8cfu/ml), followed by incubation at 35°C for 48-72 hrs. with shaking at 150rpm using shaker incubator. After incubation, the liquid culture were centrifuged at6000rpm for 15min to remove the cell culture debris. Chloroform was added to the supernatant (1:10*v/v*), followed by a vigorous shaking for 3-5min, then the flasks were allowed to be settled for 30min to remove any contaminating bacteria and the clarified suspension was subjected to plaque assay technique. In the high dilutions, characteristics of the formed plaques were compared with the original plaques. The single plaque isolation was repeated three times to ensure the high purity of the single phage isolates.

(iii) Preparation of high titre phage lysates

High titre phage stocks of the phage lysates were obtained using the liquid culture method as follows: Erlenmeyer flasks (250 ml) each containing 100ml of liquid culture medium were prepared, each flask was inoculated with a Lopeful ($2x10^9$ cfu/ml) of appropriate *P. aeruginosa* and *E. faecalis*. After overnight (16-18 hrs.) incubation at 30-35°C with shaking, phages particles (10^9 pfu/flask) were added to each flask. The flasks were incubated at 30-35°C without shaking for 20 min to allow the phages to be adsorbed and then with shaking for 24-48 hrs. at the optimum temperature. After the incubation period, cultures were centrifuged at 6000rpm for 15min and then the chloroform was added to the supernatant (10:1 *v/v*). The suspension containing phages were then transferred into sterilized flasks and stored at 4°C with traces of chloroform.

Purification and concentration of *P. aeruginosa* and *E. faecalis* phages

Dextran sulfate-polyethylene glycol two phase liquid system was used (Watanabe *et al.,* 1970). The weight 222.3 ml, 0.44,14.45 and 3.78 grams of phage lysate, dextrane Sulfate 500, polyethylene glycol (PEG6000) and NaCl respectively were mixed in a separating funnel to give a mixture containing ratios 6.5% , 0.2% and 1.7%(*w/w*) of PEG 6000, dextran sulfate 500 and NaCl, respectively. After mixing the funnel was allowed to stand at 4°C overnight. A heavily turbid bottom layer was slowly collected into a clear tube and centrifuged at 2000rpm for 10min. The clear top and bottom phases were removed by pipette and the remaining interface "cake" was suspended in 2.5ml of a1% (*w/w*) dextran sulfate solution then 0.15ml of a 3m KCl solution was added to each milliliter of suspension. The mixture was allowed to stand for 24 hrs. at 4°C and centrifuged at 2000rpm for 10min. After centrifugation the supernatant containing phages was obtained and dialyzed against saline solution (0.85% NaCl) at 4°C for 72 hrs. after dialysis, the phage suspensions were centrifuged at 15000rpm for 2 hrs. at 4°C then the supernatants were discarded, then the pellets were re-suspended in 2-3ml of saline solution (0.85% NaCl) and then assayed.

Characterization of *P. aeruginosa* and *E. faecalis* phages

(i) Electron microscopy examination

One drop of isolated phages suspension (10^8pfu/ml) was placed on 200 mesh carbon coated cupper grid and allowed to absorb for approximately 20min. The excess liquid was removed with filter paper dick. The grids were negatively stained with 2% uranyl acetate (pH4.5) for 90 seconds and left for drying and then examined using a JOEL-JEM-1010 electron microscope operated at 80KV (Electron microscope unit, Regional Center for Mycology and Biotechnology, AL-Azhar Univ., Cairo) (Othman, 1997).

(ii) Bacteriophage host range

Agar double layer plates were used for host range assay three strains of *P. aeruginosa* named PS1, PS3 and PS4 and three strains of *E. faecalis* named EF1, F2 and F4 were used as different hosts in individual plates. Plates were prepared by pouring a base layer of nutrient agar medium with 1.5% agar in sterilized Petri dishes. The basal layer was allowed to solidify at room temperature. A mixture of 3.0 ml melted semi-solid agar and 1.0 ml of 3.01×10^7 CFU/ml of each host bacterial culture were poured into each plate. Once the top agar was solidified at room temperature, 10 to 20µl of a 10^7 PFU/ml of phage lysate suspension was spotted, incubated at 37°C overnight and examined for the presence of the clear zone of lysis.

(iii) Challenge test

To determine the multiplicity of infection (MOI) for wastewater phage treatment, challenge tests were performed with the following combinations: (i) Single phages and their respective hosts, and (ii) *Salmonella* strains mixture with phage cocktail. All trials were performed at 37°C maintaining a MOI of 10^1, $10^2$and 10^4pfu/cfu. The protocol recommended by O'Flynn, Ross, Fitzgeral, and Coffey (2004) was adapted. For the first trial, TSB (400 ml)was inoculated with a fresh overnight bacterial culture and incubated at 37°C with shaking. After 2 hrs. of incubation, the 400ml culture was split into four 100 ml volumes. Single phage was added to the culture maintaining a MOI value of 10^1, 10^2,and 10^4pfu/cfu.The efficiency of bacteriophages appear by counting bacterial colony before and after treatment *P. aeruginosa* and *E. faecalis* bacteria isolated and detected from collected sewage water samples using membrane filter technique according to Standard Methods (APHA, 2005). The identified phages placed in bottles

contained each sewage water samples separately (100ml/l), bottles incubated at 35°C for 48hrs. according to Adams (1959),after incubation period each bottle filtered, plates with specific media incubated with membrane for 48hrs. and then bacterial colony counted. The average results were recorded in (cfu/100ml).

Calculated parameters and data analysis

Multiple antibiotic resistance (MAR) index was suggested by Krumperman (1983) and Hinton *et al.* (1985) according to the following formula:

$$MAR=X/NY$$

X: number of resistance determined among population "Y".

N: number of tested antibiotics values of MAR higher than 0.25 pose a high risk source of contamination.

The correlation coefficient t matrix (r) between environmental factors and bacteriological parameters was carried out using the program Microsoft Excel (Ver., 2010).

Results and Discussion

Indicator bacteria in wastewater

Standard plate count (SPC) was used to indicate the total number of bacteria and the microbial status of water. Data showed obvious detectable differences in SPC levels among the six sites studied. SPC counts at different sites showed minimum and maximum values ($68x10^6$ and $180x10^6$ $cfu.ml^{-1}$) at 22°C and 37°C, respectively (data has not shown).These results indicate that all sites contain high numbers of bacteria and exceed the permissible limits. Total coliforms (TC) are commonly used indicators of sanitary quality of water, they belong to family *Enterobacteriaceae*. TC counts at different sewage sites showed minimum and maximum value ($72\ x10^5$ and $120x10^5$cfu $100ml^{-1}$), respectively (data has not shown). TC values in all sites were exceed the permissible limits. Fecal coliforms (FC) contain almost human enteric pathogen which considered greater risk to human health. In this study, all sewage sites taken were contaminated with highly undesirable levels of FC which exceed the permissible limits. FC counts at different sewage sites showed minimum and maximum value ($77x10^4$ and $56x10^5$cfu100 ml^{-1}) at 44.5°C, respectively (data has not shown). Fecal streptococci (FS) comprises bacterial group that are normally present in feces and gut of warm-blooded animals. The ratio FC/FS has been suggested as a method for tracing whether fecal pollution is from human or animal sources. A ratio greater than 4 was considered indicative of human fecal contamination, whereas a ratio of less than 0.7 was suggestive of contamination by non-human sources. However, some investigators have questioned the usefulness of this ratio since it is valid only for recent 24hrs. Fecal pollution and FS count shouldn't be less than 100 $cfu.100m^{-1}$ (Pourcher *et al.,* 1991; Bitton, 1994 and APHA, 2005). FS counts at different sewage sites showed minimum and maximum value ($128x10^4$ and $56x10^5$cfu$100ml^{-1}$) at 35°C, respectively. FS value in all sites were exceed the permissible limits (data has not shown).In this study the results indicated that values of standard plate count (SPC) determined at 22°C was higher than those determined at 37°C. The optimum temperature for the bacterial population which can grow at the human and animal bodies is 37°C while at 22°C most of air and soil bacteria can grow well. This explain why the numbers of bacteria counted at 22°C were much higher than those determined at 37°C. These results are in agreement with those reported by Sabae and Rabeh (2007), Ezzat (2008) and El-Bahnasawy (2013).Indicator organisms are used almost as a measure of sanitary quality of water, coliform group have been served as the traditional indicators of the presence or absence of enteric viruses in wastewater as reported by Duran *et al.* (2003). The main criterion for assessing the potential health risk of sewage water is the density of indicator bacteria. Although indicator bacteria do not cause illness, they are abundant in human waste where pathogenic organisms, TC, FC and FS most frequently used as indicators of fecal contamination. In this study total coliforms count was found to be higher than fecal coliforms and fecal streptococci in all collected sewage water samples and this result is similar with those obtained by El-Bahnasawy (2013), that total coliforms count from ($72x10^5$ to $120x10^5$cfu $100ml^{-1}$), fecal coliforms from ($24x10^5$ to $77x10^4$cfu $100ml^{-1}$) and fecal streptococci from ($14x10^5$ to $135x10^4$cfu $100ml^{-1}$). The fecal coliforms count showed strong relation to the total coliforms and increased with increasing the total coliforms and this due to fecal pollution from human sources in all collected sewage water samples. Among the three primary bacterial indicators total coliforms represented the highest values followed by fecal coliforms and fecal streptococci in all collected sewage water samples. The occurrence of fecal streptococci in water generally indicates fecal pollution source (Pinto *et al.,* 1999). This term refers to these *Streptococci* normally present in the faeces of human and animals. It includes *S. faecalis, S. faecaum, S. durans, S. bovis and S. avium*. These organisms rarely multiply in polluted water and they may be slightly more resistant to disinfection than coliform group. *P. aeruginosa* in this study present in range from ($11x10^2$ to $138x10^2$cfu $100ml^{-1}$) which is an opportunistic pathogens of humans and reported as a multi-drug resistant organism (Tsoraeva and Martinez, 2000). *S. aureus* present in range from ($6x10^4$ to $25x10^4$cfu $100ml^{-1}$) in all collected sewage water samples which is a matter of concern that cause a wide range of infections and Salmonella was obtained in five sites of sewage water samples ranged from (1 to 8 cfu $10ml^{-1}$) and this could be attributed to contamination of these samples by feces of infected humans or animals and especially from poultry farms (Geldreich, 1996).Results obtained from antibiotic sensitivity tests indicated that pollution levels indicated that physico-chemical and bacteriological analyses play an important role in the incidence of antibiotic resistant bacteria (ARB) and the dissemination of these bacteria depends on pollution extents as reported by Abo-state *et al.* (2012). Baquero *et al.* (2008) reported that in contaminated aquatic environments, resistant strains were transferred from human and animal sources to environment and these bacteria are able to spread their genes into native bacteria.

Susceptibility of bacterial isolates to different antibiotics

The resistant of the bacteria isolates to antibiotic appeared through calculated the total number and percentages of resistant bacteria isolates from sewage water samples from different sites as shown in Table (2). Resistance was (98.2%) for cephalothin, then followed by clindomycin (95.2%), methicillin (91.7%), erythromycin (79.4), tobramycin and chloraphenicol (66.2%), sulfa/trimethoprim (63.2%) and vancomycin (62.3%).The highest resistance of bacteria isolates recorded against cephalothin, clindomycin and methicillin and be over intermediate against other antibiotics.

Table 2 : Total number and percentages of resistant bacteria from sewage water samples isolated from different sites.

Antibiotic	Resistant isolates(Total 228)	
	No.	(%)
Methicillin (5µg)	209	91.7
Cephalothin (30 µg)	224	98.2
Tobramycin (10 µg)	151	66.2
Vancomycin (30µg)	142	62.3
Erythromycin (10µg)	181	79.4
Clindamycin (30µg)	217	95.2
Sulfa/Trimethoprim (25µg)	144	63.2
Chloramphenicol (30µg)	151	66.2

Bacterial isolates were classified as sensitive (S), intermediate(I) or resistance (R) to each antibiotic type according to NCCLS/CCLS (2007). The obtained results presented in Table (3). *P. aeruginosa* isolates were (100%) resistant to the used antibiotics methicillin and cephalothin and followed with vancomycin, clindomycin and sulfa/trimethoprim (98%), erythromycin (92%), chloramphenical (87%) and senisitive to tobramycin (2%). *P. aeruginosa* isolates was found to be resistant to 84% of the tested antibiotics. *E. faecalis* isolates from sewage samples from different sites were (100%) resistant to methicillin, followed with cephalothin and tobramycin (98%), sulfa/trimethoprim (96%), clindamycin and chloramphenicol (94%), erythromycin (92%) and sensitive to vancomycin. *E. faecalis* isolates was found to be resistant to 84% of the tested antibiotics (Table, 3). Multiple antibiotic resistance (MAR) from identified bacterial species isolated from sewage water samples from different sites presented in Table (4). Results of MAR from identified bacterial species showed that, the most pronounced MAR values were recorded by *Salmonella spp*. (0.95), then followed by *Staphylococcus aureus* (0.92), *Enterococcus faecalis and Pseudomonas aeruginosa* (0.84), *Proteus vulgaris* (0.76), *Shigella spp*. (0.68) and *E. coli* (0.44), respectively. According to Krumperman (1983) and Hinton *et al.* (1985), value of MAR higher than 0.25 pose a high risk source of contamination. Unfortunately, all the above calculated values of MAR index were obviously exceeding the high risk level (0.25) with different extents, demonstrating that our area of study is considered a high risk environment. Based on the calculation of MAR index in each sampling point, all sites taken under this study were facing high risk of contaminations with varying levels.

Table 3 : Resistance of *P. aeruginosa* and *E. faecalis* strains against individual antibiotics.

Antibiotic	*P. aeruginosa*			*E. faecalis*		
	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)
Methicillin (5µg)	0(0%)	0(0%)	45(100%)	0(0%)	0(0%)	48(100%)
Cephalothin (30µg)	0(0%)	0(0%)	45(100%)	1(2%)	0(0%)	47(98%)
Tobramycin (10µg)	44(98%)	0(0%)	1(2%)	1(2%)	0(0%)	47(98%)
Vancomycin (30µg)	1(2%)	0(0%)	44(98%)	48(100%)	0(0%)	0(0%)
Erythromycin (10µg)	2(4%)	2(2%)	41(92%)	2(4%)	2(4%)	44(92%)
Clindamycin (30µg)	0(0%)	1(1%)	44(98%)	3(6%)	0(0%)	45(94%)
Sulfa/Trimethoprim(25µg)	1(2%)	0(0%)	44(98%)	2(4%)	0(0%)	46(96%)
Chloramphenicol (30µg)	4(9%)	2(2%)	39(87%)	4(9%)	0(0%)	44(92%)

R, Resistant I, Intermediate S, Sensitive

Table 4 : Multiple antibiotic resistance (MAR) index for bacteria isolated from collected sewage water samples.

Bacterial Species	No. of Resistance	MAR %	MAR Index
E. coli	135	44	0.44
E. faecalis	322	84	0.84
S. aureus	221	92	0.92
Salmonella spp.	183	95	0.95
Shigella spp.	54	68	0.68
P. Vulgaris	201	76	0.76
P. aeruginosa	303	84	0.84

The most pathogenic bacteria are *Enterococcus faecalis* followed by *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus vulgaris*, *Staphylococcus aureus*, *Salmonella* spp. and *Shigella* spp. Results showed a presence of highly percent of multi-antibiotic resistance bacteria (MAR) in all sewage water samples collected from different sites, that *Salmonella* recorded high percent (95%) followed by *S. aureus* (92%), *P. aeruginosa* and *E. faecalis* (84%), *P. vulgaris* (76%), *Shigella* spp. (68%) and finally *E. coli* (44%), respectively Table(4). Results showed that high percent of antibiotic resistant bacteria, that recorded highly resistance percent for cephalothin (98.2%) followed by clindamycin (95.2%), methicillin (91.7%), Erythromycin (79.4%), tobramycin and chloroamphenicol (96.2%), sulfa/trimethoprim (63.2%) and finally vancomycin (62.3%), respectively. This study showed presence of Methicillin resistant *Staphylococcus aureus* (MRSA) and Vancomycin resistant *E.faecalis* (VRE), it's also showed no effect

appearing by different antibiotics to any bacterial species studied. Multi antibiotic resistant index (MAR) showed higher than permission value (0.25) on all used strains and this indicated that all sites of sewage-represent high pollution source Table (4). The pathogenicity test was used for monitoring virulence characteristics of wild isolated bacteria from aquatic environment. Table (5) showed the distinguish between pathogen and non-pathogen bacteria from the 228 total identified isolated bacteria from collected sewage samples. Results shown that positive Congo red (CR) was (100%) for *Shigella spp., Salmonella spp., Proteus vulgaris, Pseudomonas aeruginosa* and *Enterococcus faecalis*, then followed by *E. coli* (95%) and *Staphylococcus aureus* (90%). Results showed that the virulent of bacteria isolates detected by their ability to bind CR dye in CR media. CRD positive test appear as red culture. The characteristics of CR binding constitutes a moderately stable, reproducible and easily distinguishable phenotypic marker. The stability of the CR phenotype is greater in some isolates than in others. The loss of CR binding parallels the loss of virulence for isolates from water sources. Thus, results showed that each of *E. faecalis*, *P. aeruginosa*, *P. vulgaris*, *Salmonella* spp., *Shigella* spp. were pathogenic with percent (100%) then *E. coli* pathogenic with percent (95%) and finally *S. aureus* pathogenic with percent (90%) (Table, 5).In this study results showed high percent of antibiotic resistant bacteria and the resistance percent for each antibiotic arranged respectively as follows: Cephalothin (98.2%), clindamycin (95.2%), methicillin (91.7%), erythromycin (79.4%), tobramycin (63.2%) and finally vancomycin (62.3%). This emphasize the transfer of antibiotic resistant genes between different bacterial groups.

Multi-antibiotic resistance bacteria (MAR) detected for each bacteria isolates and their percent were for *Salmonella* (95%), *S. aureus* (92%), *P. aeruginosa and E. faecalis* (84%), *P. vulgaris* (76%). *Shigella* spp. (68%) and finally *E. coli* (44%) respectively. Also study recorded presence of methicillin resistant *Staphylococcus aureus* (MRSA) and Vancomycin resistant *Enterococcus faecalis* (VRE), its also showed no effect appearing by different antibiotics to any bacterial species studied. It is noticeable that the resistance was mostly directed generally to *B-lactam* antibiotics especially to ampicillin and methicillin. This phenomenon was obviously shown in *Enterobacteriaceae* and this could Gram-negative bacilli as reported by Sirot *et al.* (1986) and Samadpour *et al.* (2005). Lukasova and Sustackova (2003) categorized vancomycin-resistant enterococci as global threat to public health. It is obvious that, the presence of high number of antibiotic-resistant bacterial species in surface water may have ecological and public health implications which should not be ignored by decision makers. This amphasizes the need for further studies especially in relation to the genes encoding resistance in different bacterial species as well as the possibility of returning these genes to humans through water usage. Congo red binding phenotype (Crb+) used to differentiate between virulent and non-virulent bacterial isolates and commonly used as markers of hydrophobicity and linked directly to virulence and routinely used in vitro to assess the virulence of bacterial isolates (Swaminathan *et al.,* 2004). Virulent bacterial isolates of certain Gram-negative bacteria bind the dye Congo red from solid agar media, thus producing red colonies (Crb+), isolates which fail to bind the dye produce white colonies (Crb-) and are a virulent. Among organisms with virulence are associated with the ability to bind congo red is *E. coli, E. faecalis, P. aeruginosa, P. vulgaris, Salmonella spp., S. aureus* (Sharma *et al.,* 2006). and these result are in agreement with our results in this study, that percent of virulence of bacterial isolates (*E. coli, Shigella spp., Salmonella spp., Proteus vulgaris, P. aeruginosa, S. aureus* and *E. faecalis*) were (95%, 100%, 100%, 100%, 100%, 90% and 100%) respectively. These results indicated highly virulent for all tested bacteria isolated leading to great risk to human and environment. The molecular basis of Congo red binding with some bacterial isolates is unknown. But many authors observed that strong correlation exists between Congo red binding and hemin which serves as an iron source for the organisms and protoporphyrin which is the principal component of the cell surface protein. Because of their structural homology could be used interchangeably in solid media to distinguish virulent and non-virulent isolates (Ewers *et al.,* 2004). Sharda *et al.* (2010) used CR assay for in vitro pathogenicity testing of *E. coli* isolated from poultry and found that 95.38% and this result resemble with our result in this study.

Table 5 : Pathogenicity test for bacterial isolates from sewage water samples.

Bacterial strains	Pathogen		Non-pathogen	
	Total No.	%	Total No.	%
Escherichia coli	36	95	2	5
Shigella spp.	10	100	-	-
Salmonella spp.	24	100	-	-
Proteus vulgaris	33	100	-	-
Pseudomonas aeruginosa	45	100	-	-
Staphylococcus aureus	27	90	3	10
Enterococcus faecalis	48	100	-	-

16S RNA of *P. aeruginosa* and *E. faecalis* from sewage water

The counts of *P. aeruginosa* from six municipal wastewater treatment effluents (MWTs) Plants showed minimum and maximum value ($11x10^2$ and $138x10^2$cfu$100ml^{-1}$) at 41.5°C, respectively. *P. aeruginosa* in all sites were exceed the permissible limits.

Two *P. aeruginosa* and two *E. faecalis* isolates which isolated from sewage water samples from different sites, DNA extracted then study the nucleotide sequence for four strains using 16S rDNA gene depend on specific primer using PCR. Results of nucleotide sequence showed that *P. aeruginosa* PS3 (254bp), *P. aeruginosa*, PS4 (225bp), *E. faecalis* F2 (254bp) and *E. faecalis* F4 (248bp) Figure (1). The resulted sequences were compared with bacterial species

recorded on the Genbank using DNAMAN program and identified as *P. aeruginosa* PS3, PS4, *E. faecalis* F2 and F4, respectively as shown in Figure (2).The DNA sequences were subjected to several analysis using DNAMAN and MEGA.4 programmers (Wisconsin, Madison, USA). The sequences of each bacteria isolates were aligned for determining the sequence registered in GenBank as shown in Fig. (2) for two *P. aeruginosa* strains isolates and for two *E. faecalis* strains, Figure (3). Phylogenetic relationships between two strains of *P. aeruginosa* as shown in Figure (4 & 5) and for two *E. faecalis* strain as shown in Fig. (6 & 7). Multiple sequence alignment (MSA) was displayed in which the corresponding nucleotides occupy the same column when a sequence has no corresponding residue due to detection events, the position was displayed as "-" which is called a gap. Alignment of multiple genes shows the conserved sites and the percentage of conservation for each position. Aligned residues share evolutionary origin and sequence similarity to some extend. Based on MSA analysis, the phylogenetic tree was performed and shows four clusters in which the *P. aeruginosa* PS3, KX585259.1, KX778118.1 and KY355382.1 were found to be highly homologous with percentage (100%) while the *P. aeruginosa* PS3 showed distant homology (99%) with *P. aeruginosa* strain KY081667.1. Also percentage was (98%) between *P. aeruginosa* PS3 and KY238114.1 so it was represented as separate cluster. Two *P. aeruginosa* isolates were submitted to the DNA Data Bank of Japan (DDBJ), National Institute of Genetics, Shizuoka, Japan/European Molecular Biology Laboratory (EMBL), Heidelberg, Germany/GenBank, National Center for Biotechnology Information (NCBI), USA databases as follows: *P. aeruginosa* strain PS3 (Accession No. KY610281.1) and *P. aeruginosa* strain PS4 (Accession No. MG657025), respectively.

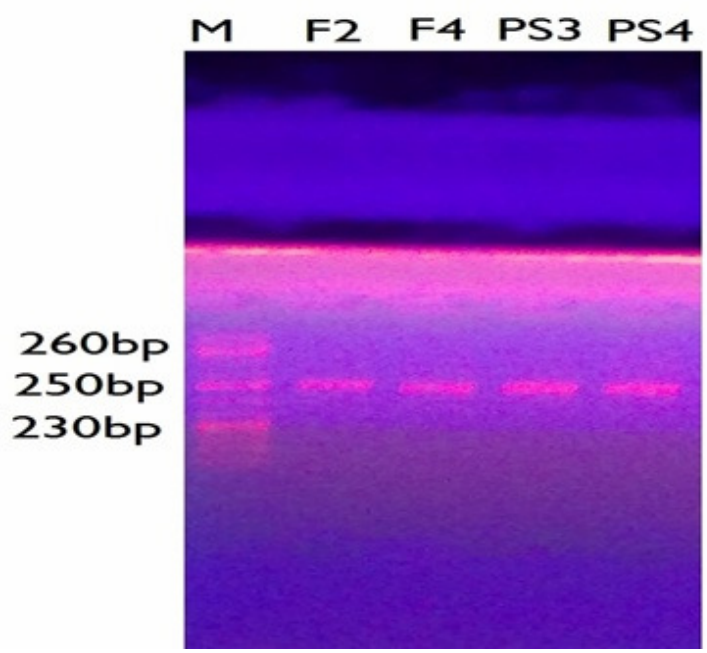

Fig. 1 : Agarose gel electrophoresis (1%) showing PCR products amplified fragment of 16S rDNA gene isolated from two *P. aeruginosa* isolates (PS3 and PS4) and two *E. facealis* isolates (F2 and F4) using specific sets.
Spot test assay (A) a higher dilution (10-6) of *P. aeruginosa* phage titer and (B) *E. faecalis* phage titer showing clear plaque of 0.3-1.0 mm in diameter.

Based on MSA analysis, the phylogenetic tree was performed and shows five clusters in which the *E. faecalis* strains registered in GenBank with accession No. KY711178.1, KY027194.1, KY786286.1, LC213017.1, KY858232.1, KY438200.1 and KY697092.1 were found to be highly homologous with percentage (100%) while the *E. faecalis* F2 and KY697092.1 showed distant homology (99%) with *E. faecalis* strain KY777745.1 on the other hand percentage was (88%) between *E. faecalis* F2 and other strains KY71117831, KY027194.1, KY786286.1, LC213017.1, KY858232.1 and KY438200.1 as well as percentage was (57%) between KY63067.1 and other strains so it was represented as a separate cluster. Two *E. faecalis* isolates were submitted to the DNA Data Bank of Japan (DDBJ), National Institute of Genetics, Shizuoka, Japan/European Molecular Biology Laboratory (EMBL), Heidlberg, Germany/GenBank, National Center for Biotechnology Information (NCBI), USA databases as follow: *E. faecalis* F2 (Accession No. KY711177.1) and *E. faecalis* F4 (Accession No. KY711178.1). Sequence of highly conserved gene region 16S rDNA data helps us for the prediction of correct taxonomy. The estimation of biodiversity was mediated at different levels correlated to environmental conditions and interpreted through the morphological, microbiological and molecular views. This study was carried out to sequence 16S rDNA based on PCR amplification for identification and genetic level conformation of *P. aeruginosa* and *E. faecalis* isolated from water (Kumaran *et al.,* 2010; Fontes *et al.,* 2011, De Abreu *et al.,* 2014 and Kumar *et al.,* 2015).

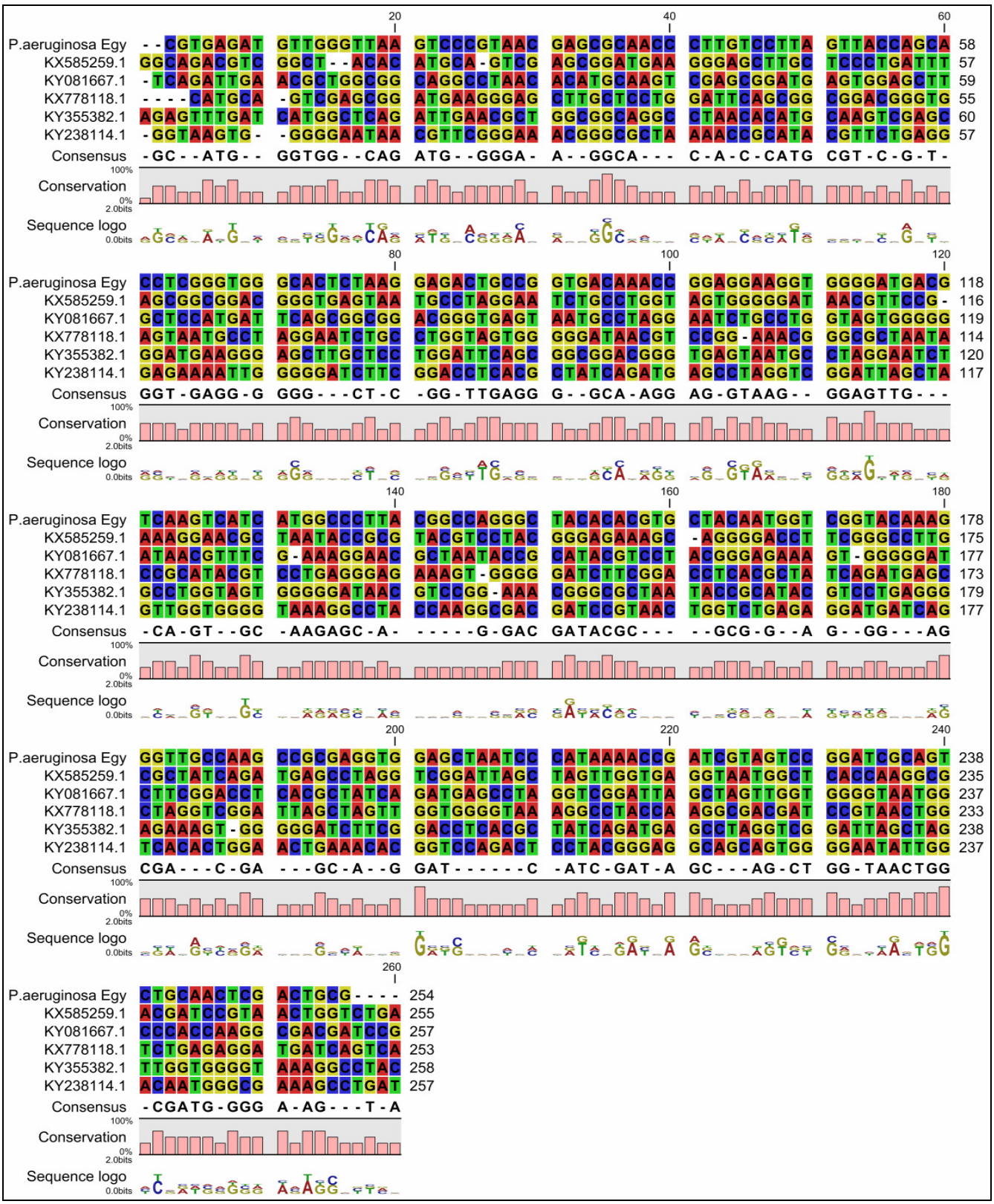

Fig. 2 : Multiple sequence alignment of *P. aeruginosa* partial nucleotide sequence of 16S rDNA gene.

In this study two strains of *P. aeruginosa* and two strains of *E. faecalis* isolated from sewage water samples from different sites. DNA of each bacteria isolate extracted and amplified by PCR conventional method using specific primers sequences for 16S rDNA gene for *P. aeruginosa* and *E. faecalis* different partial nucleotide sequence of 16S rDNA gene were identified as *P. aeruginosa* PS3, PS4 were (254 bp and 225 bp), respectively. *E. faecalis* identified as F2 and F4 with (254 bp and 248 bp), respectively. Based on multiple sequence analysis (MSA) between two *P. aeruginosa* strains and two *E. faecalis* strains and bacterial species recorded on the Genbank, 16S rDNA gene sequence variability was evaluated and sequence alignment showed homology bases were found that's attributed to base pair substitutions.

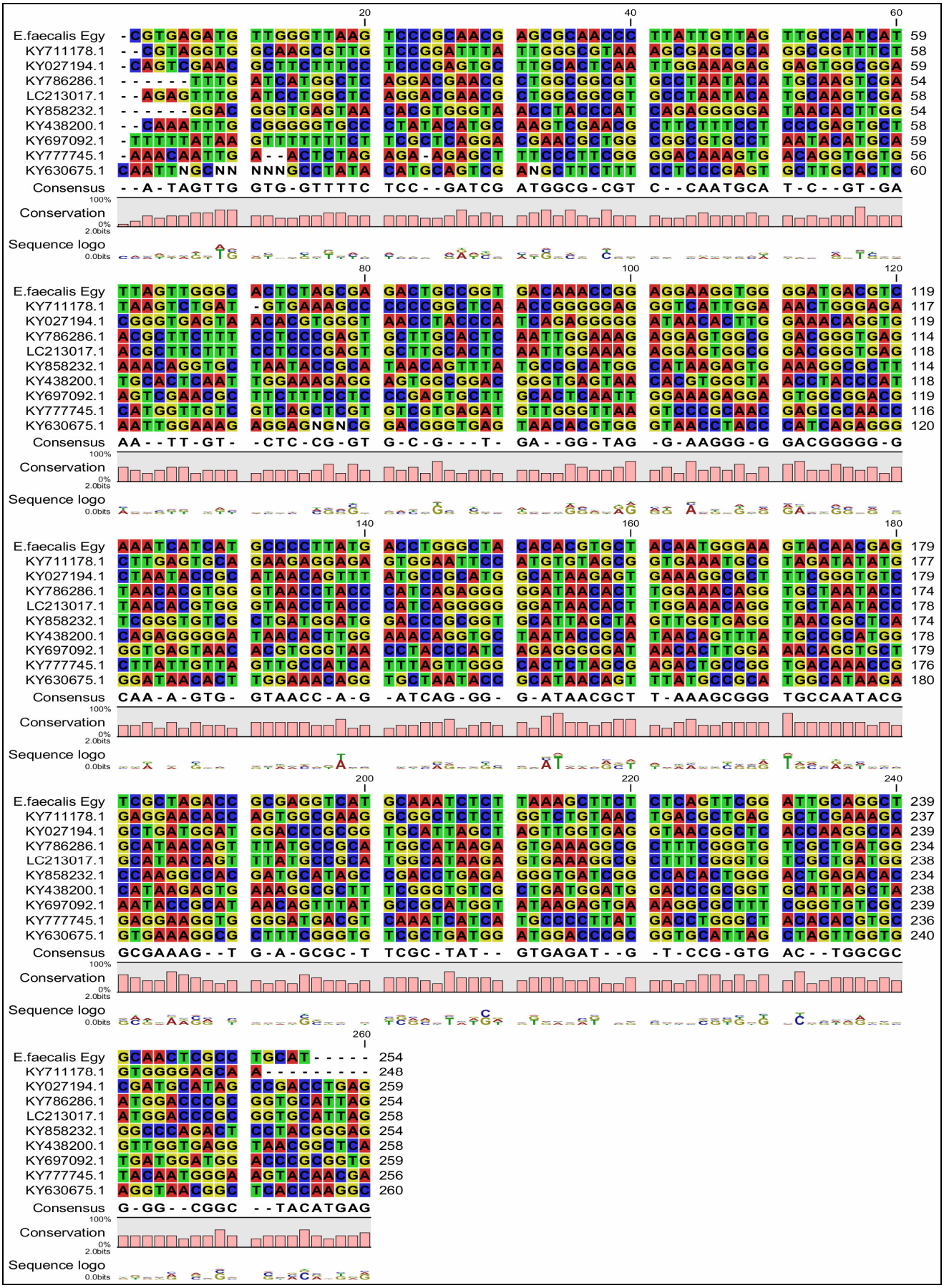

Fig. 3 : Multiple sequence alignment of *E. faecalis* partial nucleotide sequence of 16S rDNA gene.

In this study phylogenetic tree analysis for *P. aeruginosa* strains showed four clusters in which *P. aeruginosa* and KX585259.1, KX778118.1 and KY355382.1 were be highly homologous with percent (100%), (99%) for KY081667.1 and (98%) for KY238114.1 and phylogenetic tree analysis for *E. faecalis* showed seven clusters in which *E. faecalis* and KY711178.1, KY02719431, KY786286.1, LC213017.1, KY858232.1, KY438200.1 and KY697092) were be highly homologous with percent (100%), (99%) for KY697092.1 and (88%) for KY777745.1. The environmental factors appeared to exert the role of sequence variability to make such isolate more diverse, a matter that's found during phylogenetic analysis sequence homology of *P. aeruginosa* and *E. faecalis* strains were so far that such Egyptian isolates could be regarded as novel species minimal sequence homology must become 97% when comparing different strains within the same species as reported by many authors (Anzai *et al.,* 2000; Garau and Gomez, 2003 and Kulkarni and Kaliwal, 2015).

P. aeruginosa and *E. faecalis* phages isolation

A bacteriophage (*P. aeruginosa and E. faecalis* phages) was isolated from sewage water samples using spot test which showed clear plaques against their hosts and have negative effects on the wild strain which used as an indicator host, and using plaque assay test for detect their efficiency value. Morphology of six isolated phages plaques detected produce circular, clear, turbid, regular plaques. These plaques have just-defined frontiers generated by the potential lytic action of the phage and were ranging from 2.0 to 4.0 mm in diameter**.** The phage was designated as PP1, PP2 and PP3 (Figure 8: A, B &C) which lytic of *P. aeruginosa* strains while phages infect *E. faecalis* strains named EP1, EP2 and EP3 (Figure 8: D, E& F). The concentrations of phages were $50x10^6$, $33x10^6$ and $92x10^5$pfu.ml^{-1} for *P. aeruginosa* phages PP1, PP2 and PP3, respectively while the levels were $35x10^7$, $49x10^7$ and $28x10^6$ pfu.ml^{-1} for *E. faecalis* phages EP1, EP2 and EP3, respectively. Consequently, they can be a valuable additional tool to improve water resources management for minimizing health risks (Lin and Ganesh, 2013, Mookerjee *et al.,* 2014 and Yahya *et al.,* 2015). Phages in aquatic ecosystems have a high probability of encountering host bacteria due to continual movement in water (Kokjohn *et al.,* 1991). This means that the number and behaviour of phages in water environment is influenced by the densities of both host bacteria and physic-chemical properties such as temperature, pH, TDS, presence of organic matter, concentration and type of anions and cations and the metabolic activities of microorganisms (Goyal *et al.,* 1987). Bacteriophages stock lysate were prepared from these plaques by the single plaque isolation method. The propagated bacteriophages stock lysates were purified by polyethylene glycol sedimentation to obtain purified, concentrated bacteriophages stock lysates (Sundaram and Dhevagi, 2013). To obtain high-titer stock of phages isolated by using liquid culture propagation procedure which make the multiplicity of infection less than 0.1 failed, then phages purified by precipitation then resulting supernatant centrifuged to remove the pellet followed by filtration with Millipore Filter (Barcelo *et al.,* 2017).

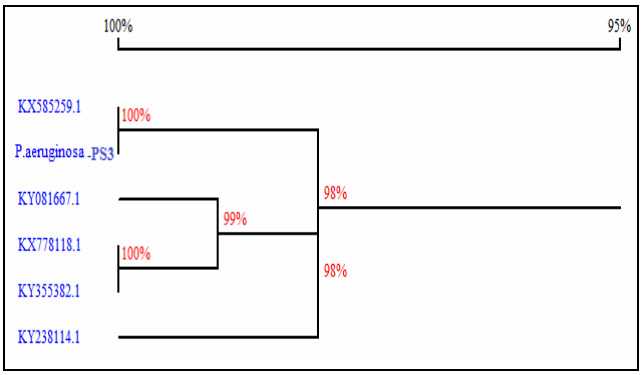

Fig. 4: Phylogenetic tree representing the relationship between the *P. aeruginosa* isolates and GenBank strains.

Distance matrix of 6 sequences

KX585259.1	0
KY081667.1	0.031 0
KX778118.1	0.032 0.014 0
KY355382.1	0.041 0.013 0.001 0
KY238114.1	0.043 0.017 0.008 0.023 0
P.aeruginosa Egy	0.000 0.000 0.000 0.000 0.000 0

Homology matrix of 6 sequences

KX585259.1	100%
KY081667.1	96.9% 100%
KX778118.1	96.8% 98.6% 100%
KY355382.1	95.9% 98.7% 99.9% 100%
KY238114.1	95.7% 98.3% 99.2% 97.7% 100%
P.aeruginosa Egy	100.0% 100.0% 100.0% 100.0% 100.0% 100%

Fig. 5 : Distance and homology matrix representing the relationship between two *P. aeruginosa* isolates and GenBank strains.

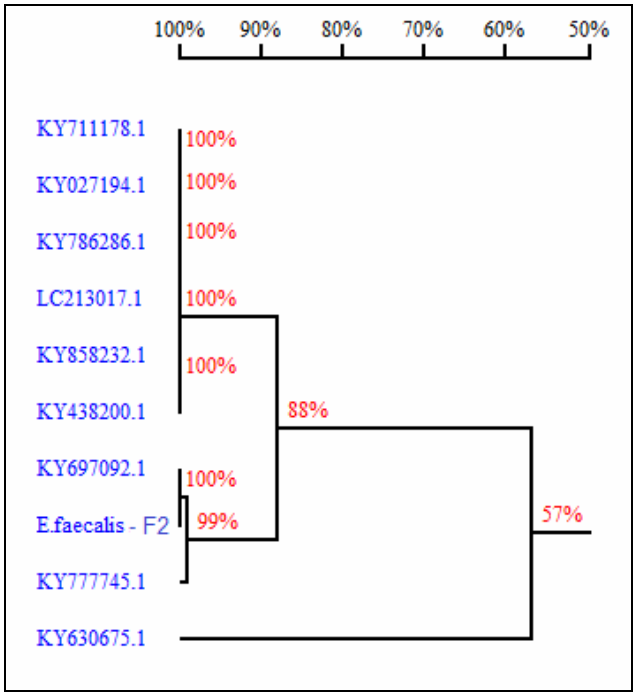

Fig. 6 : Phylogenetic tree representing the relationship between the *E. faecalis* isolates and GenBank strains.

Distance matrix of 10 sequences

KY711178.1	0
KY697092.1	0.004 0
KY027194.1	0.000 0.003 0
LC213017.1	0.000 0.010 0.002 0
KY786286.1	0.000 0.005 0.002 0.001 0
KY858232.1	0.000 0.002 0.002 0.002 0.001 0
KY438200.1	0.000 0.018 0.002 0.016 0.006 0.001 0
E.faecalis Egy	0.999 0.000 0.000 0.000 0.000 0.000 0.000 0
KY777745.1	0.999 0.012 0.011 0.015 0.015 0.013 0.015 0.000 0
KY630675.1	0.730 0.531 0.535 0.527 0.522 0.516 0.525 0.000 0.015 0

Homology matrix of 10 sequences

KY711178.1	100%
KY697092.1	99.6% 100%
KY027194.1	100.0% 99.7% 100%
LC213017.1	100.0% 99.0% 99.8% 100%
KY786286.1	100.0% 99.5% 99.8% 99.9% 100%
KY858232.1	100.0% 99.8% 99.8% 99.8% 99.9% 100%
KY438200.1	100.0% 98.2% 99.8% 98.4% 99.4% 99.9% 100%
E.faecalis Egy	0.1% 100.0% 100.0% 100.0% 100.0% 100.0% 100.0% 100%
KY777745.1	0.1% 98.8% 98.9% 98.5% 98.5% 98.7% 98.5% 100.0% 100%
KY630675.1	27.0% 46.9% 46.5% 47.3% 47.8% 48.4% 47.5% 100.0% 98.5% 100%

Fig. 7 : Distance and homology matrix representing the relationship between two *E. faecalis* isolates and GenBank strains.

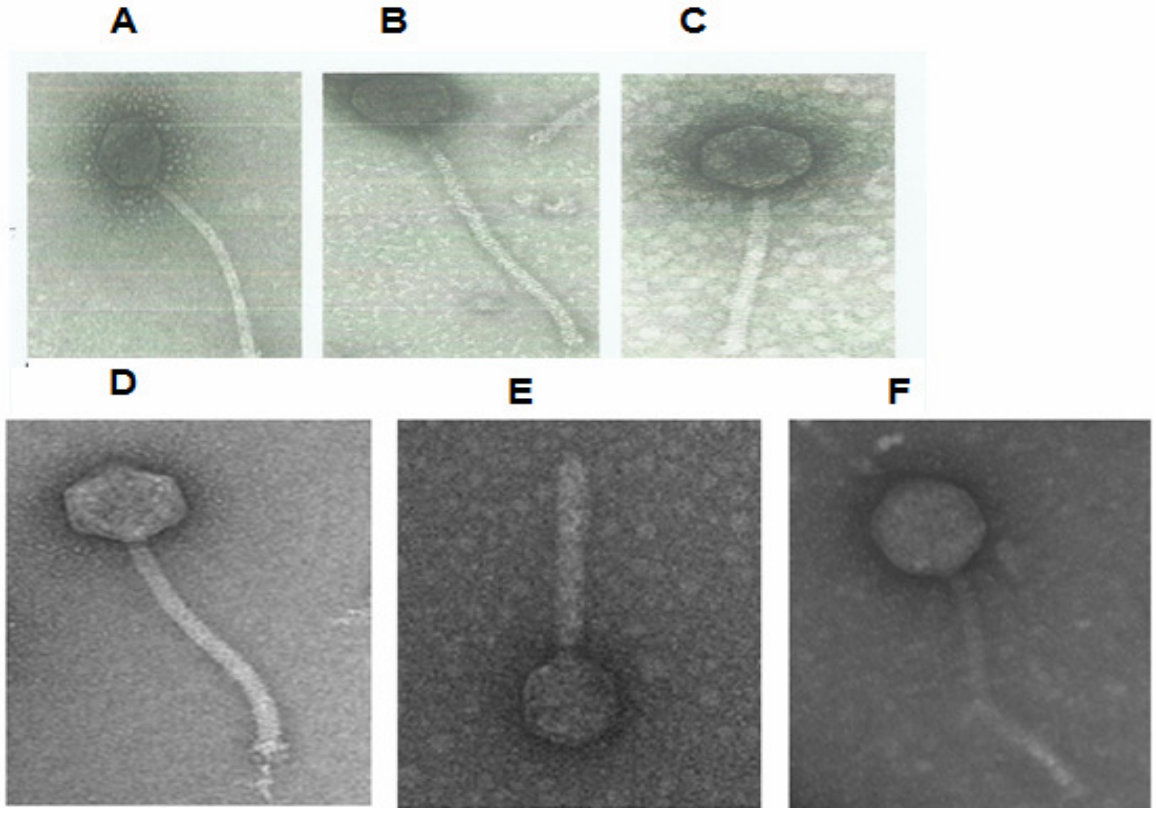

Fig. 8 : Transmission Electron microscopic (TEM) image of *Pseudomonas aeruginosa* phages (A, B and C) and *Enterococcus faecalis* phages (D, E and F) morphology. (A) Phage PP1, (B) Phage PP2 and (C) Phage PP3, (D) Phage EP1, (E) Phage EP2 and (F) Phage EP3.

Phage host range

The efficiency of phages appear by counting their specific bacteria before and after treatment with their phages. To determine the host range, the PP1, PP2 and PP3 as well as EP1, EP2 and EP3 phages were used to infect nine bacterial strains. Spot test method was used for initial selection of sensitive strains. The strains were further confirmed by plaque assay. Five different strains of *P. aeruginosa* were found susceptible to PP1, PP2 and PP3 phages Table (6). However, the *E. faecalis* strains were found resistant to these phages while these strains were susceptible to EP1, EP2 and EP3 Table(6). Our results indicated that all isolated phages often have wider host ranges (Jebra *et al.,* 2016). Niu*et al.* (2009) showed that many phages with a wide host range are able to lyse many strains and other pathogenic bacteria and generic strains.

Table 6 : Spot test of (PP1, PP2 and PP3 & EP1, EP2 and EP3) phages on different Strains collected Egyptian Microbial Culture Collection, Egypt and additionally two strains from.

S. No.	Bacteria strains	Activity (+/-)					
		φ PP1	φ PP2	φ PP3	φ EP1	φ EP2	φ EP3
1	*P.aeruginosa*PS3	+	+	+	-	-	-
2	*P.aeruginosa*PS4	+	+	+	-	-	-
3	*Pseudomonas aeruginosa*	+	+	+	-	-	-
4	*Pseudomonas aeruginosa*	+	+	+	-	-	-
5	*Pseudomonas aeruginosa*	+	+	+	-	-	-
6	*Enterococcus faecalis* F2	-	-	-	+	+	+
7	*Enterococcus faecalis* F4	-	-	-	+	+	+
8	*Enterococcus faecalis*	-	-	-	+	+	+
9	*Enterococcus faecalis*	-	-	-	+	+	+

+ = lysis , – = no lysis

Phage morphology

Images of TEM revealed that PP1, PP2 and PP3 & EP1, EP2 and EP3 phages have a different structural features and dimensions. The PP1, PP2 and PP3 phages have a isometric head with diameter (80±6, 120±10 and 80±13 nm), length (100±8, 120±13 and 100±11 nm) and tail diameter (40±6, 20±4 and 20±5 nm), length (240±23, 520±25 and 240± nm), respectively. The EP1, EP2 and EP3 phages have isometric head with diameter (120±12, 60±6 and 80±7nm), length (120±13, 80±9 and 80±8 nm) and tail diameter (20±3, 20±4 and 20±2nm), length (320±16, 160±11 and 240±14nm), respectively. On the basis of morphological features phages PP1, PP2 and PP3 most probably a member of *Myoviridae* family. The EP1, EP2 and EP3 phages based on morphological features are probably a member of *Siphoviridae and Myoviridae* families. All the values measured were means of ±SD from a triplicate experiments. According to International Committee on the Taxonomy of Viruses (2000), the *Myoviridae* and *Siphoviridae* families contain many phages which infect members of the *Enterobacteriaceae* and *Pseudomonadaceae* families, respectively. Morphological characteristics seen under an electron microscope are considered important in phage taxonomy and 96% of all phages investigated in the last 45 years have turned out to be members of the *Siphoviridae*, *Myoviridae* and *Podoviridae* families (Ackermann, 1996).

Killing rate after experimental challenge

The concentration of the purified identified phages used are given in Table (10). *P. aeruginosa* phages pp1, pp2 and pp3 used for treatment of *P. aeruginosa* strains collected from sewage water samples from different sites and its efficiency percent for lysis recorded (88%, 91.7% and 92.3%) respectively, as given in Table (7) *E. faecalis* phages Ep1, Ep2 and Ep3 used for the treatment of *E. faecalis* strains collected from sewage water samples from different sites and its efficiency percent for lysis recorded (93.3%, 92.8% and 93.6%) respectively, as shown in Table (8). In this study phages isolated showed high efficiency comparing with antibiotics in control of their specific bacterial host *P. aeruginosa* and *E. faecalis* strains collected from different sites of sewage water samples. The application test showed that the percent of lysis after treatment with *P. aeruginosa and E. faecalis* phages (PP_1, PP_2, PP_3, EP_1, EP_2 and EP_3) were (88%, 91.7%, 82.3%, 92.3%, 93.3%, 92.8%, 93.6%) respectively. Finally, many authors recommended that bacteriophages serves as reliable indicator of fecal contamination and should be considered as complementary to bacterial indicators. Nowaday, its must attention for the efficiency role in control and lysis of pathogenic bacteria which exceed the efficiency of antibiotic.

Table 7 : Concentration of purified phages (pfu ml^{-1}) isolated from collected sewage water samples.

Phages	*P. aeruginosa* (pfu ml^{-1})	*E. faecalis* (pfu ml^{-1})
PP1	$30x10^9$	ND
PP2	$49x10^9$	ND
PP3	$75x10^9$	ND
EP1	ND	$12x10^{10}$
EP2	ND	$80x10^9$
EP3	ND	$95x10^9$

ND, not detected

Table 8 : Concentration of bacterial species (cfu $100ml^{-1}$) in sewage water sample before and after treatment with identified phages.

Phages	Before treatment	After treatment	Percent of lysis (%)
PP1	750	90	88 %
PP2	480	40	91.7 %
PP3	130	10	92.3 %
EP1	4500	300	93.3%
EP2	2500	180	92.8 %
EP3	1100	70	93.6 %

References

Abo-State, M.A.; Mahdy, M.H.; Ezzat, S.M. and Abd El-Shakour, E.H. (2012). Antimicrobial resistance profile of *Enterobacteriaceae* isolated from Rosetta branch of River Nile, Egypt. World Appl. Sci. J., 19: 1234-1243.

Ackermann, H.W. (1996). Frequency of morphological phage descriptions in 1995. Arch.Virol., 141: 209-218.

Adams, M.H. (1959). Bacteriophages, Interscience Publishers: New York.

Alsaffar, M. and Jarallah, E.M. (2016). Isolation and characterization of lytic bacteriophages infecting *Pseudomonas aeruginosa* from sewage water. Int. J. Pharm.Tech. Res., 9(9): 220-230.

Alsaffar, M. and Jarallah, E.M. (2017). The molecular and biological study of the isolated bacteriophages infecting *Pseudomonas aeruginosa* from sewage water. J. Mesop. Environ., 64-82.

Al-Zubidi, M. (2017). Bacteriophages targeting *Enterococcus faecalis* strain a potential new root canal therapy. Journal of oral Microbiol., 9(1). Doi: 1325237.

Angela, V.H.; Guillermo, R.; Viviana, C.; Catalina, P.; Marcela, M.; Maria, C.G.; Elizabeth, K.; Corinda, T.; Peter, C.F.; Andres, F.G.B. and Martha, J.V. (2015). Phage-Pan70, a putative temprate phage, controls *Pseudomonas aeruginosa* in planktonic, Biofilm and Burn Mouse Model Assays. Viruses, 7: 4602-4623.

Anzai, Y.; Kim, H. and Park, J.; Wakabayashi, H. and Oyaizu, H. (2000). Phylogenetic affiliation of the *Pseudomonas* based on16S rRNA sequence. Int. J. Syst. Evol. Microbiol., 50:1563-1589.

APHA (American Public Health Association) (2005). Standard Methods For The Examination of Water and Wastewater (21st ed.), Washington, D.C., USA.

Azeredo, J. and Sutherland, I.W. (2008). The use of phages for the removal of infectious biofilms. Curr. Pharm. Biotechnol., 9: 261-266.

Bakaj, A.; Kulla, E.R. and Kalaja, J. (2017). Monitoring of microbiological parameters on the coast of Durres, Albania. Albanian J. Agric. Sci., 95-101.

Baquero, F.; Martinez, J. and Canton, R. (2008). Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol.,19: 260-265.

Barcelo, C.T.; Kaltz, I.; Froissart, R.; Gandon, S.; Ginet, N. and Ansaldi, M. (2017). French Phage Network-Second Meeting Report. J. MDPI., 9(4): 87.

Bauer, A.W.; Kirby, W.M.; Sherris, J.C. and Turk, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol., 45: 493-496.

Berkhoff, H.A. and Vinal, A.C. (1986). Congo red medium to distinguish between invasive and non-invasive *E. coli* pathogenic for poultry. Avian Diseases, 30: 117-121.

Bitton, G. (1994). Indicator microorganisms, In: Wastewater Microbiology. Wiley-Liss, Inc., U.S.A. pp. 101.

Bitton, G. (2005). Microbial indicators of fecal contamination: application to microbial source tracking. Report submitted to the Florida Storm water Assoc., 1-71.

Cheesbrough, M. (2006). District Laboratory Practice in Tropical Countries, 2nd ed., Cambridge University Press, New York.

Cheng, M.; Liang, J.; Zhang, Y.; Hu, L.; Gong, P.; Cai, R.; Zhang, L.; Zhang, H.; Ge, J.; Ji, Y.; Guo, Z.; Feng, X.; Sun, C.; Yang, Y.; Lei, L.; Han, W. and Gu, J. (2017). The bacteriophages Ef-P29 efficiency protects against lethal Vancomycin-resistant *Enterococcus faecalis* and alleviates gut microbiota imbalance in a murine bacteremia model. Front Microbiol., 8: 837.

Collins, C.H. and Lyne, P.M. (2004). Microbiological Methods. 8th ed. 450, Arnold, London.

De Abreu, P.M.; Farias, P.G.; Paiva, G.S.; Almeida, A.A. and Moaris, P.V. (2014). Persistence of microbial communities including *P. aeruginosa* in a hospital environment: a potential health hazard. BMC Microbiol., 14: 118-125.

Duran, A.E.; Muniesa, M.; Mace-Llivinal, L.; Campos, C.; Jofre, J. and Lucena, F. (2003). Usefulness of different groups of bacteriophages as model microorganisms for evaluating chlorination. J. Appl. Microbiol., 95: 29-37.

El-Bahnasawy, M.A. (2013). Assessment of gamma irridadiation on antibiotic resistant bacteria isolated from River Nile and drainage water in Egypt. M.Sc. Thesis. In Microbiol., Fac. of Sci., (Boys), Al-Azhar Univ., Cairo, Egypt.

Erez, Z.; Steinberger-Levy, I.; Shamir, M.; Doron, S.; Stokar-Avihail, A.; Peleg, Y.; Melamed, S.; Leavitt, A.; Savidar, A.; Albeck, S.; Amitai, G. and Sorek, R. (2017). Communication between viruses guides lysis-lysogeny decisions. Nature, 541: 488-493.

Ewers, C.; Janssen, T.; Kiessling, S.; Philipp, H.C. and Wieler, L.H. (2004). Molecular epidemiology of avian pathogenic *E. coli* (APEC) isolated from Coli septicemia in poultry. Vet. Microbiol., 104: 91-101.

Ezzat, S.M. (2008). Role of certain botanical extracts against bacteria isolated from Nile River and drainage water. Ph.D. Thesis In: Microbiology, Fac. of Sci., Ain Shams Univ., Cairo, Egypt.

Fontes, L.C.; Neves, P.R.; Oliveira, S.; Silva, K.C.; Hachich, K.C.; Sato, M. and Lincopan, M. (2011). Isolation of *P. aeruginosa* Co-producing Merallo-β-lactamase SPM-1 and 16S rDNA Methylase RmtD1 in an urban River. Antimicrobial Agents Chemotherapy, 55: 3063-3.

Furtado, I.; Xavier, P.C.N.; Tavares, L.V.M.; Alves, F.; Martins, S.F.; Martins, A. and Palhares, D.B. (2014). *Enterococcus faecium* and *Enterococcus faecalis* in blood of newborns with suspected nosocomial infection. Rev. Inst. Med. Trop. Sao Paulo, 56(1): 77-80.

Garau, J. and Gomez, L. (2003). *P. aeruginosa Pneumonia*. Curr. Opin. Infect. Dis., 16: 135-143.

Geldreich, E.E. (1996). Pathogenic agents in fresh water resources. Hydrol. Proc., 10: 315-333.

Goyal, S.M.; Gerba, C.P. and Bitton, G. (1987). Phage Ecology, 1st ed. New York: John Wiley and sons.

Gu, J.; Li, X.; Yang, M.; Du, C.; Cui, Z.; Gang, P.; Xia, F.; Song, J.; Zhang, L.; Li, J.; Yu, C.; Sun, C.; Feng, X.; Lei, L. and Han, W. (2016). Therapeutic effect of *Pseudomonas aeruginosa* phage YH30 on mink hemorrhagic Pneumonia. J. Vet. Microbiol., 190: 5-11.

Hinton, M.; Hedges, A.J. and Linton, A.H. (1985). The ecology of *E. coli* in market calves fed a milk substitute diet. J. Appl. Bateriol., 58: 27-35.

Jebra, S.; Hmaied, F.; Yahya, M.; Ben-Ammar, A. and Hamdi, M. (2016). Total coliphages removal by activated sludge process and their morphological diversity by teansmission electronic microscopy. J. Water Sci. and Technol., 76(2). Doi: 10.2166.

Kang, H-Y.; Kim, S. and Kim, J. (2015). Isolation and characterization of an *E. faecalis* bacteriophages. The Korean J. Microbiol., 51(3): 194-198.

Kokjohn, T.A.; Sayler, G.S. and Miller, R.V. (1991). Attachment and replication of *P. aeruginosa* bacteriophages under condition simulating aquatic environments. J. General Microbiol., 137: 661-666.

Krumperman, P.H. (1983). Multiple antibiotic resistance indexing of *E. coli* to identify high risk sources of fecal contamination of foods. Appl. Environ. Microbiol., 46: 165-170.

Kulkarni, A.G. and Kaliwal, B.B. (2015). Bioremediation of methomyl by soil isolate *P. aeruginosa*.J. Bioremed. Biodeg., 6: 281.

Kumar, A.; Asthana, M.; Gupta, P.; Yadav, S.; Sharma, D.; Singh, K. and Kumar, S. (2015). 16S rRNA sequencing of dye decolorizing bacteria isolated from water and soil. Bioinformatics, 11: 1-5.

Kumaran, S.; Deivasigamani, B.; Alagappan, K.M.; Sakthivel, M. and Guru Prasad, S. (2010). Isolation and characterization of *Pseudomonas sp.* KUMS3 from

Asian sea bass (Latescalcarifer) with fin rot, World J. Microbiol. Biotechnol., 26: 359-363.

Lee, G. (2013). Ciprofloxacin resistance in *Enterococcus faecalis* strains isolated from male patients with complicated urinary tract infection. Korean J. Urol., 54(6): 388-393.

Lin, J. and Ganesh, A. (2013). Water quality indicators: bacteria, coliphages, enteric viruses. Int. Environ. Health Res., 23: 484-506.

Lu, S.Y. (2010). High diversity of extended-spectrum lactamase-producing bacteria in an urban river sediment habitat. Appl. Environ. Microbiol.,76: 5972-5976.

Lukasova, J. and Sustackova, A. (2003). *Enterococci* and antibiotic resistance. Acta.Veter.Brno.,72: 315-323.

Matsuzaki, S.; Rashel, M.; Uchiyma, J.; Ujihara, T.; Kuroda, M.; Ikeuchi, M.; Fujieda, M.; Wakiguchi, J. and Imais (2005). Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. J. Infect. Chemother., 11: 211-219.

Mookerjee, S.; Batabyal, P.; Halder, M. and Palit, A. (2014). Specificity of Coliphages on evaluating marker efficacy: a new insight for water quality indicators. J. Virol. Methods, 208: 115-118.

Moreau-Marquis, S.; Stanton, B.B.; O'Toole, G.A. (2008). *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. Pulmon. Pharmacol. Therap., 21(4): 595-599.

National Committee for clinical laboratory standards/ Clinical and laboratory standards Institute (NCCLS/CLSI) (2007). Performance standards for antimicrobial susceptibility testing; Seventeenth informational supplement, M2-A9 and M7-A7. Wayne, P.A., U.S.A.

Niu, Y.D.; McAllister, T.A.; Xu, Y.; Johnson, R.P.; Stephens, T.P. and Stanford, K. (2009). Prevalence and impact of bacteriophages on the presence of *E. coli* 0157:H7 in feedlot cattle and their environment. Appl. Environ. Microbiol., 75: 1271-1278.

Niu, Y.D.; Stanford, K.; Kropinski, A.M.; Ackermann, H.W.; Johnson, R.P.; She, Y.M.; Ahmed, R.; Villega, A. and McAllister, T.A. (2012). Genomic, proteomic and physiological characterization of a T5- Like bacteriophages for control of Shiga toxin-producing *Escherchia coli* 0157:H7. J. Pone. Plos ONE, 7: 4.

Othman, B.A. (1997). Isolation of Lambdoid Bacteriophages B4 EC from sewage polluted drinking water. Proceedings of the 9th conference of Microbiology, Cairo, 25-27.

Paisano, A.F.; Spira, B.; Cai, S. and Bombana, A.C. (2004). *In Vitro* antimicrobial effect of bacteriophages on human dentin infected with *Enterococcus faecalis* ATCC 29212. Oral Microbiol. Immunol., 19(5): 327-330.

Pinto, B.; Pierotti, R.; Camale, G. and Reali, D. (1999). Characterization of fecal Streptococci as indicators of fecal pollution and distribution in the environment. Lett. in Appl. Microbiol., 29: 258-263.

Pourcher, A.; Devriese, L.A.; Hernandez, J.F. and Delattre, J.M. (1991). Enumeration by a miniaturized method of *E. coli, S. bovis* and *Enterococci* as indicator of the origin of fecal pollution of water. J. Appl. Bacteriol., 70: 525-530.

Sabae, S.Z. and Rabeh, S.A. (2007). Evaluation of the microbial quality of the River Nile waters at Damietta branch, *Egypt.* J. Aqua. Res., 33: 301-311.

Samadpour, M.; Roberts, M.C.; Kitts, C.; Mulugeta, W. and Alfi, D. (2005). The use of ribotyping and antibiotic resistance patterns for identification of host sources of *E. coli* strains. J. App. Microbiol., 40: 63-68.

Sharda, R.; Wilfred Ruban, S. and Thiyageeswaran, M. (2010). Isolation, characterization and antibiotic resistance pattern of *E. coli* isolated from poultry. Am. Eu. J. Sci. Res., 5: 18 - 22.

Sharma, K.; Soni, S. and Mcharchandani, S. (2006). Congo red dye agar test as an indicator test for detection of invasive bovine *E. coli.* Veter. Arhiv., 76: 363 - 366.

Sirot, D.; Sirot, J.; Saulnier, P.; Joly, B.; Chanal, M.; Cluzel, M. and Cluzel, R. (1986). Resistant to beta-lactams in *Enterobacteriaceae:* Distribution of phenotypes related to beta-lactamase production. J. Int. Med. Res., 14: 193-199.

Sulakvelidze, A.; Alavidze, Z. and Morris, J.G. (2001). Jr. Bacteriophage therapy. Antimicrob. Agents Chemother., 45: 649-659.

Sundaram, A. and Dhevagi, P. (2013). A novel approach for pathogen reduction in wastewater treatment. J. Environ. Health Sci. Eng., 11:2-9.

Swaminathan, T.R.; Chandran, N.D. and Dorairajan, N. (2004). Virulence attributes of *E. coli* associated with colisepticaemic Chickens. Ind. J. Anim. Sci., 74: 248-252.

Tsoraeva, A. and Martinez, C. (2000). Comparison of two culture media for selective isolation and membrane filter enumeration of *P. aeruginosa* in water. Rev. Lat. Am. Microbiol., 42: 149-154.

Uchiyama, J.; Rashel, M.; Maeda, Y.; Takemura, L.; Sugihara, S.; Akechik, M.A.; Wakiguchi, H. and Matsuzaki, S. (2007). Isolation and characterization of a novel *Enterococcus faecalis* bacteriophages φEF24C as a therapeutic candidate. FEMS MicrobiolLett., 278: 200-206.

Uchiyama, J.; Rashel, M.; Takemura, I.; Wakiguchi, H. and Matsuzaki, S. (2008). In Silico and in vivo evaluation of bacteriophages phiEF24C, a candidate for treatment of *Enterococcus faecalis* infections. Appl. Environ. Microbiol., 74: 4149-4163.

Watanabe, I.H.; Shimohashi, Y.K. and Mutai, M. (1981). Studies on Streptococci I. Distribution of fecal streptococci in man. Microbiol. Immunology, 25: 257-260.

Watanabe, K.; Takesue, S.; Jin-Nai, K. and Yoshikawa, T. (1970). Bacteriophage active against the lactic acid beverage. Producing bacterium *Lactobacillus casei*. Appl. Microbiol., 20: 409-415.

Yahya, M.; Hamaied, F.; Jebri, S.; Jofre, J. and Hamdi, M. (2015). Bacteriophages as indicators of human and animal fecal contamination in raw and treated wastewaters from Tunisia. J. Appli. Microbiol.,118: 1217-1225.