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PCR-based DNA Fingerprinting Analysis of Coliphages Isolated from Sewage Polluted Seawater in Alexandria

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Abstract: Three coliphage isolates were propagated and purified from sewage polluted water samples. Isolates were selected only on the basis of different plaque size and shape on *E. coli* host (ATCC 13706). Electron microscopy of purified isolates showed isolate CPS1 to belong to family Siphoviridae whereas isolates CPS2 and CPS3 both belong to family Myoviridae. DNA fingerprinting based on restriction endonucleases analysis showed the three isolates to have no *Eco*RI, *Pst* I or *Sac* I sites. The isolates have different patterns of digestion with *Hind* III and *Sal* I enzymes. RAPD-PCR using 10 different primers also indicated that the three isolates have different patterns and could belong to different species of both families.

Key words: Coliphages, Myoviridae, Siphoviridae, RAPD, DNA finger printing, *E. coli*

Introduction

Coliphages have been considered as indicators of the virological and hygienic water quality (Wentsel *et al.*, 1982; Settler, 1984; Jiang *et al.*, 2001). Because of phages' stability compared to their hosts, they appear to be better indicators of marine water quality than are fecal coliform bacteria as they are more persistent in the environment. They are considered as the best indicators of human enteric viruses in polluted water (Simkova and Cerrvenka, 1981). Coliphages were isolated at various beaches and are suggested to be used in the evaluation of fresh water and recreational water (Havelaar *et al.*, 1993; Richard *et al.*, 1994).

In Alexandria, sewage discharge into seawater in certain areas, requires continuous monitoring of pathogenic agents in such sites. Coliphages in sewage polluted waters were extensively studied and characterized (Paul *et al.*, 1997; Gantzer *et al.*, 1998; Stanek and Falkinham, 2001). Modern DNA-based techniques, including restriction fragment length polymorphism (RELP) demonstrated by hybridization with species-specific DNA probes (Fegan *et al.*, 1991; Richard *et al.*, 1994; Grundmann *et al.*, 1995), macro restriction analysis by pulsed field gel electrophoresis (PFGE) (Poh *et al.*, 1992; Struelens *et al.*, 1993; Kersulyte *et al.*, 1995) or field inversion gel electrophoresis (FIGE) (Grothuse *et al.*, 1988; Boukadida *et al.*, 1991) and restriction endonuclease analysis (REA) (Maher *et al.*, 1993) have been applied extensively in epidemiological studies of many diverse microorganisms.

However, these techniques are expensive, time consuming and some require specific gene probes. Among the PCR-based techniques, random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) is based on the amplification of DNA segments with a single primer of arbitrary nucleotide sequence and has been widely used for the epidemiological investigation and differentiation of many microorganisms (van Belkum, 1994). In this study, RAPD and restriction endonucleases based techniques were used to differentiate between three coliphages isolated from Kaiet Bay seashore in Alexandria near the site of sewage discharge. Such techniques were employed to reveal, to some extent, the genetic diversity among the isolates as an indication of the degree of relationship between them.

Materials and Methods

Isolation and purification of coliphage isolates from seawater sample: Coliphages were isolated from seawater samples collected from the seashore of Kaiet-Bay, Alexandria by the use of the double agar layer (DAL) technique (Rajala and Heinonen, 1994). TYG-agar was used as developing medium which contained: tryptone 10 g, yeast extract 5 g, glucose 2 g, NaCl 5 g and $MgSO_4.7H_2O$ 0.25 g supplemented with 1.2 or 0.65 % agar for hard and soft agar, respectively, in one liter of distilled water, pH was adjusted to 7.0 and the medium sterilized by autoclaving at 121 °C for 20 min. About 10 ml of the sample was centrifuged at 4000 rpm for 15 min. The pellet was discarded and the supernatant was further filtered through 0.45 µm pore size millipore filters. One ml of suitable dilution of the filtrate was added to 0.2 ml of exponentially growing host culture *Escherichia coli* (ATCC 13706) added to 2 ml of liquefied soft agar. The mixture was poured onto petri dishes containing TYG-hard agar, allowed to solidify and incubated at 37°C for 16 h.

Plaques of different size and morphology were selected for purification. Two ml overnight culture of the host bacterium was subcultured into 100 ml of the fresh liquid TYG medium and incubated with shaking at 37°C for about 1.5 h. Then a well-isolated plaque was picked with a sterile Pasteur pipette and put into the host culture which was further incubated for 4-5 h. Following incubation the bacterial cells were separated from the culture by centrifugation (4000 rpm, for 20 min), followed by membrane filtration with 0.45 µm pore size millipore filters. The titer and purity of the phage lysate was determined by the DAL-method and the purification procedure was repeated 2-3 times to obtain a pure one-phage lysate (Rajala and Heinonen, 1994).

Electron microscopy: The morphology of the 3 coliphage isolates CPS1; CPS2; and CPS3 was investigated with electron microscopy. Phages were negatively stained with 2 % sodium tungstate in bidistilled water, the pH was adjusted between 6.0-7.5. Five µl of the phage was dropped onto a carbon-coated grid. The excess liquid was removed with filter paper after 1 min. Five µl of dye was added and after 1 min the grid was then dried. The grids were examined and electron micrographs were taken with a T.E.M. (JEOL 100cx) operating at 80 kv.

Recovery of purified intact phage and phage DNA isolation and purification: To each lysate tube, RNAs A and DNAs I (Sigma Chemical Co.) were added each to a final concentration of 1 µg/ml and incubated at 37°C for 30 minutes. Polyethylene glycol (PEG, molecular biology grade, MW= 8,000) and sodium chloride were added to the tubes each at 9.3 and 5.8 g per 100 ml of lysate and the tubes were inverted several times to dissolve PEG and sodium chloride completely. The tubes were kept on ice for two hours to allow the precipitation of the bacteriophage. The precipitate was then recovered by centrifugation at 10,000 rpm for 20 minutes at 4°C. The supernatant was allowed to drain and the precipitate containing the purified intact coliphage isolates was resuspended by gentle vortexing in 2 ml of phage buffer (0.01 % gelatin, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$).

Phage DNA was isolated and purified as follows: each supernatant containing the purified coliphage was transferred to another tube and 100 µl of 10% SDS and 100 µl of 0.5 M EDTA, pH 8.0 per 100

ml of phage lysate were added. The tubes were incubated at 68°C for 20 min, after which phenol/chloroform extraction was carried out at 12000 rpm for 5 min at 4°C. The upper aqueous phase was then transferred to a clean tube and an equal volume of isopropanol was added and the tubes were kept at -20 °C for 1 h after which the DNA was collected by centrifugation at 12000 rpm for 20 min at 4 °C. The supernatants were drained carefully and pellets were washed with 1 ml of 70% ethanol, dried at room temperature for 15 min and finally resuspended in 200 µl of sterile distilled water (Sambrook *et al.*, 1989).

Restriction endonuclease analysis: Restriction digestion analysis for the purified DNA isolated from the phage isolates was carried out as follows: phage DNA 10 µg was digested with *Eco*RI, *Hin*dIII, *Pst*1, SalI and *Sac*I (promega, Southampton, UK). Fifty units of each restriction enzyme were used in the digestion reaction. The digestion reaction was carried out overnight at 37°C and the products of the reactions were analyzed by agarose gel (1%) electrophoresis (Sambrook *et al.*, 1989).

Analysis of phage DNA by RAPD-PCR: The purified phage DNA isolated from each phage isolate was analyzed using RAPD. Amplification reactions were performed in a total volume of 50 µl containing 100 µM each of dATP, dTTP, dCTP and dGTP, 0.2 µM of each RAPD primer indicated below, 25 ng of template DNA and 1.25 units of Taq polymerase in 1x PCR buffer containing 2.5 mM $MgCl_2$. The reaction mixtures were subjected to amplification as follows: 45 cycles of 1 min at 94°C, 1 min at 37°C and 1 min at 72°C. After the last cycle samples were maintained at 72 °C for 10 min. Amplification products were analyzed by agarose gel (1%) electrophoresis, stained with ethidium bromide and DNA profiles were documented and analyzed using Alpha Imager 1200 Tm (Williams *et al.*, 1990).
Faint (< 1% of total intensity) and inconsistent bands most likely the result of poor primer-template matching were excluded from the analysis. Amplification reactions were carried out using one of the following primers at a time:

OPA-03: 5` -AGTCAGCCAC-3` ;
OPA-16: 5` -AGCCAGCGAA-3` ;
OPB-14: 5` -TCCGCTCTGG-3` ;
OPB-16: 5` -TTTGCCCGGA-3` ;
OPB-09: 5` -TGGGGGACTC-3` ;
OPA-15: 5` -TTCCGAACCC-3` ;
OPA-19: 5` -CAAACGTCGG-3` ;
OPA-11: 5` -CAATCGCCGT-3` ;
OPB-05: 5` -TGCGCCCTTC-3` ;
OPB-13: 5` -TTCCCCCGCT-3` .

Results and Discussion

All of the three coliphage isolates were merely selected according to different shape and size of the plaques produced on their host bacterium *E. coli* (ATCC 13706). Isolates' phenotypes were examined by electron microscopy. Morphological criteria used for phage identification was as outlined by the International Committee for Taxonomy of Viruses (Murphy *et al.*, 1995). These isolates had icosahedral head and tail and thus belong to the order Caudovirales. Phage isolate CPS1 (Fig. 1A) belongs to family Siphoviridae, which contains phages that have icosahedral heads and flexuous tails. It belongs to morphotype 1 phage as it has no appendages. Both isolates CPS2 and CPS3 (Fig. 1B and 1C) belong to family Myoviridae that includes phages with icosahedral heads and long contractile tails. Both belong to morphotype 2 bearing appendages of which the collar is shown in the micrographs. None of the phage isolates belong to the third family of tailed phages known as Podoviridae with icorosahedral heads but short tails. Tailed coliphages usually predominate other morphotypes in sewage polluted samples (Wenstel *et al.*, 1982). Up to 1993, 96 % of the phages described in details had tails (Maniloff *et al.*, 1994). The virus species concept proposed by Murphy *et al.* (1995)

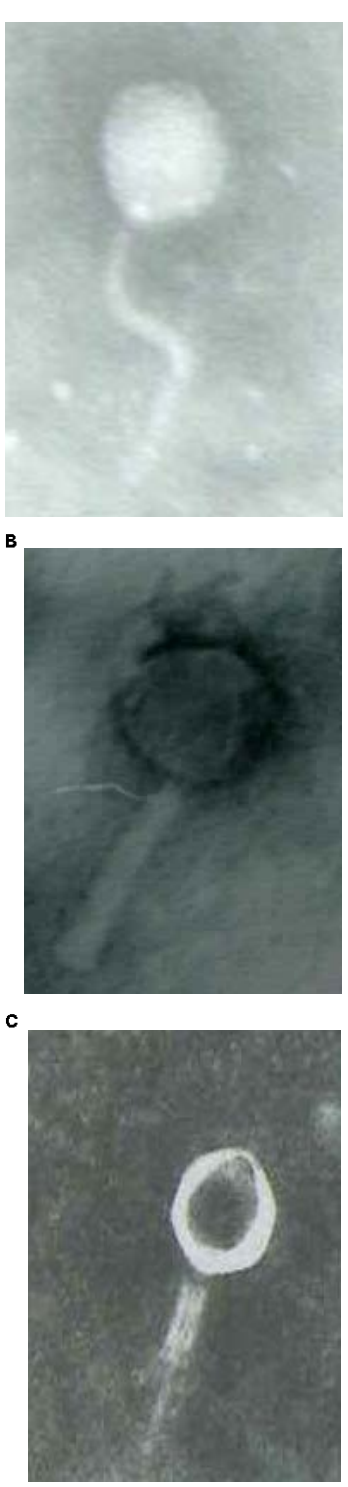

Fig. 1: Transmission electron micrographs of coliphages isolated from Kaiet Bay seashore.
(A) Phage CPS1 with icosahedral head a long flexuous tail. (B) Phage CPS2 with icosahedral head, collar and elongated tail. (C): Phage CPS3 with icosahedral head, collar, contracted tail and base plate.

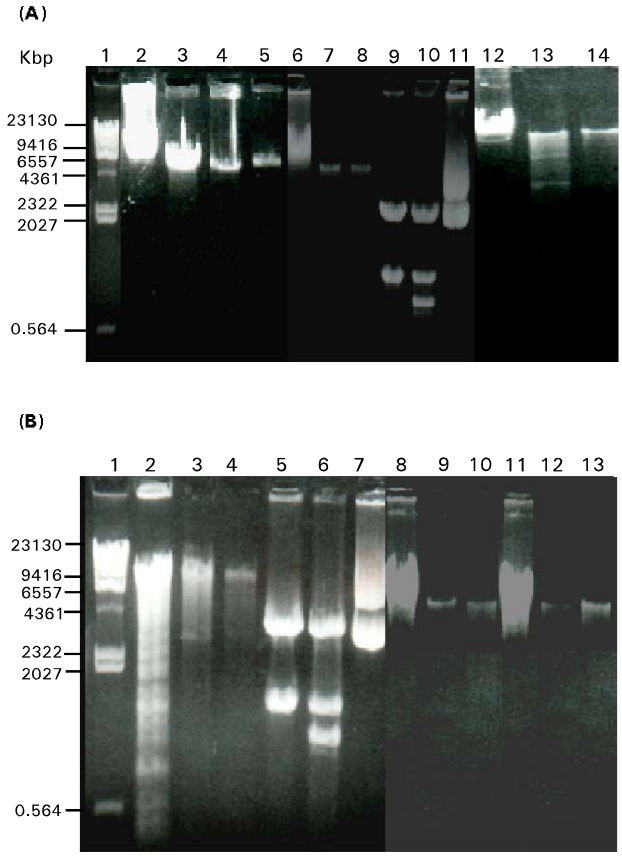

Fig. 2: Agarose gel (1 %) electrophoresis for DNA isolated and purified from coliphages CPS1; CPS2; and CPS3 lanes (2 – 4). Lane 5 represents DNA purified from phage λgt 11 as a control. Lanes (6-8) CPS1; CPS2; and CPS3 DNA cut with *Eco*RI and lanes (12-14) cut with *Sal*I. Lanes 9 and 10 pUC 8.0 plasmid carrying DNA insert cut with *Eco*RI under the same experimental conditions and lane 11 uncut pUC 8.0 plasmid. Lane 1 represents phage λ DNA *Hind*III cut molecular weight markers (A).
Agarose gel (1 %) electrophoresis for coliphages CPS1; CPS2; and CPS3 DNA cut with *Eco*RI and *Hind*III lanes (2 – 4), *Pst*I lanes (8-10) and *Sac*I lanes (11-13). Lanes 5 and 6 pUC 8.0 derivative plasmid cut with *Eco*RI and *Hind*III. Lane 7 represents control uncut pUC 8.0 plasmid. Lane 1 represents phage λ DNA *Hin*d III cut molecular weight markers (B).

delineates seven different families of bacteriophages based on morphological criteria and provides criteria for new phage species based on several traits such as DNA homologies, serological data protein profiles and host ranges. The restriction endonuclease digestion pattern with *Eco*RI for DNA isolated and purified from the three coliphage isolates are shown (Fig. 2A, lanes 6-8). The DNA isolated from these coliphages has no restriction site for *Eco*RI endonuclease, since it gave the same electrophoretic mobility pattern as compared with that of the undigested DNA (Fig. 2A, lanes 2-4). Lanes 9 and 10 in Fig. 2A show pUC 8.0 derivative plasmid carry different DNA insert cut with *Eco*RI under the same experimental conditions for the assay as a positive control, again lane 11 control undigested plasmid. Restriction endonuclease digestion pattern with *Hind* III and *Eco*RI (Fig. 2B, lanes 2-4), showed that the DNA purified from the three isolates gave different electrophoretic mobility pattern. Coliphage CPS1

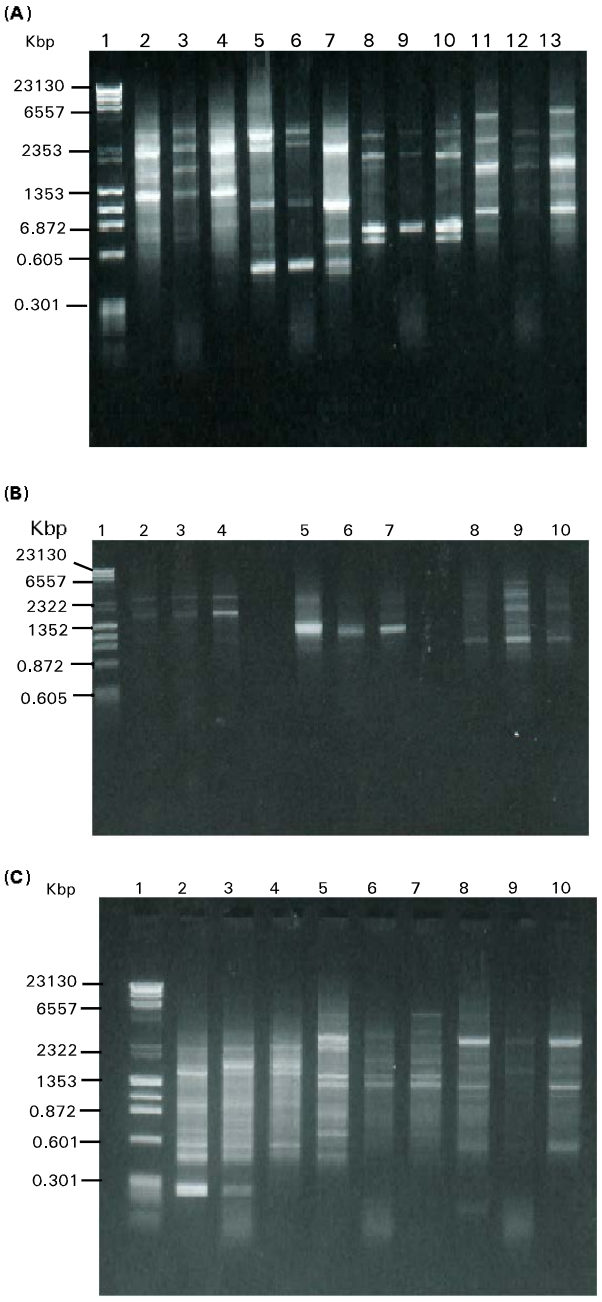

Fig. 3: Agarose gel (1 %) electrophoresis of RAPD products from CPS1; CPS2; and CPS3 coliphages template DNA with arbitrary primers; OPA-03 lanes (2-4); OPA-16 lanes (5-7); OPB-14 lanes (8-10) and OPB-16 lanes (11-13). Lanes 1 represents lambda/*Hind*III-ôX174/*Hae*III markers (A).
Agarose gel (1 %) electrophoresis of RAPD products from CPS1; CPS2; and CPS3 coliphages template DNA with arbitrary primers; OPB-09 lanes (2-4); OPA-15 lanes (5-7) and OPA-19 lanes (8-10). Lane 1 represent lambda/*Hind*III-ôX174/*Hae*III markers (B).
Agarose gel (1 %) electrophoresis of RAPD products from CPS1; CPS2; and CPS3 coliphages template DNA with arbitrary primers; OPA-11 lanes (2-4); OPB-05 lanes (5-7) and OPB-13 lanes (8-10). Lane 1 represent lambda/*Hind*III-ôX174/*Hae*III markers (C).

DNA is digested into several fragments with *Hin*d III, as *Eco*R1 was shown separately not to digest it. CPS2 DNA is also digested by *Hin*dIII giving only two digestion fragments. Coliphage CPS3 DNA, however, was not digested by both enzymes. The profile obtained with SalI endonuclease (Fig. 2A, lanes 12-14) shows that coliphage CPS2 has several *Sal*I restriction sites as compared with coliphages CPS2 and CPS3. All three isolates' DNA have no restriction sites for neither *Pst*I nor *Sac*I (Fig. 2B). Restriction mapping of small isometric-headed lactic streptococcal phages has shown possible evolutionary pathways (Jarvis and Meyer, 1986). In a homolous study between the DNAs of lytic and temperate phages however, only a low degree of relatedness was found, indicating that, in this case at least, lytic phages are not derived from temperate phages (Jarvis, 1984). Comparative phage genomics can retrace part of the evolutionary history of phage modules encoding phage-specific functions such as capsid build or establishment of the lytic or lysogenic state of the phage. Ackermann *et al.* (1983) differentiates phages into different species based on the occurrence of DNA homologies in phages belonging to the same family but not to different families. In this study, isolates CPS2 and CPS3 according to their morphology could belong to the same family Myoviridae, however, their restriction endonuclease pattern is not identical. This is an indication of difference in DNA homology among phages belonging to the same family. Wichels *et al.* (1988) reported that within the families studied not all the phages were genetically related. Some phages belonging to the same family based on other criteria such as morphology and size showed no or little DNA homology to phages belonging to the same family. The results obtained using either electron microscopic study or restriction endonuclease digestion analysis indicate that the three coliphage isolates were of different species.

To complement this study random amplification of polymorphic DNA (RAPD) technique was used to detect the polymorphic regions and the differences between these three isolates.

This PCR-based technique is used extensively for the epidemiological investigation and differentiation of many microorganisms (Jothikumar *et al.,* 1998, Schaper and Jofre, 2000; Dombek *et al.,* 2000; Walter *et al.,* 2000). Restriction enzyme analysis, PCR studies and partial sequencing of selected DNA regions in *E. coli* 0157:H7 phages has indicated frequent gene exchange between lambdoid phage genomes (Johansen *et al.,* 2001). Isolated and purified DNA from the three isolates were amplified using 10 different single primers of arbitrary nucleotide sequence. The results of the PCR amplification are shown in Fig. 3A, B and C. In RAPD with OPA-03, OPB-14, OPB-16, OPB-09, OPA-15, OPA-19, OPA-11 and OPA-13 primers showed identical mobility pattern for the three coliphage isolates and thus could not differentiate between them. RAPD with OPA-16 and OPB-05 primers could actually differentiate between these isolates. A specfic DNA band is shown at molecular size of 7,37 kbp for coliphage isolate CPS1 (Fig. 3A, lane 5). Coliphage isolate CPS3 with the same primer, OPA-16 (Fig. 3A, lane 7 showed two clearly distinguishable bands of molecular sizes 0.8 and 0.5 kbp which are completely absent in case of CPS1 and CPS2 isolates (Fig. 3A, lanes 5-6). The RAPD results with primers OPB-09, OPA-15, OPA-19 (Fig. 3B) and OPA-11, OPA-13 (Fig. 3C) were identical for the three-coliphage isolates but were not reproducible all the time. Moreover, RAPD with OPB-05 primer, showed different DNA profiles (Fig. 3C, lanes 5-7). Bands of molecular size 5.81 and 3.13 kbp were obtained with DNA isolated from coliphage CPS3 and bands of molecular sizes 2.55; 2.11; and 0.36 kbp were obtained with DNA isolated from coliphage CPS1 but not in case of coliphage CPS2 (Fig. 3C, lane 6).

Brussow and Desiere (2001), suggest that the diagnosis of relatedness is not exclusively on sequence similarity, but includes topological consideration of genome organization. Comparison of genomes of phages representative of families Siphoviridae, Podoviridae and *Myoviridae* revealed evidence of vertical and horizontal gene transfer among phage populations. However, the data obtained in this study have clearly demonstrated that coliphage isolate CPS1, CPS2 and CPS3 could belong to different species. The demonstration that it is possible to design and to use a set of random primers in RAPD-PCR is highly encouraging as this may provide another rapid, sensitive and specific tool for molecular differentiation between phages of different families or different species within the same family.

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