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# Studies on The Antibacterial Activity of Some Free and Entrapped Bacteriophages against Fish Pathogens

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THREE DIFFERENT bacteriophages namely VPS1, APS2 and APS3 were isolated from crab (*Callinectes sapidus*), clams (*Tapes decussatns*) and fish (*Tilapia* sp.) samples. They belong to families Siphoviridae, Myoviridae and Podoviridae, respectively. Statistical analysis based on analysis of phages DNA by using random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) revealed that the similarity levels were different among the three phages. Entrapment of VPS1 and APS2 was carried out into calcium alginate beads. The antibacterial activity of VPS1 and APS2 were tested before and after entrapment in Ca-alginate beads against some fish pathogenic bacteria such as *Vibrio anguillarum* and *Aeromonas hydrophila.* VPS1 seeded beads were superior (significant at P<0.05) from the free phages in reduction of the growth rate of *V. anguillarum* while, APS2 seeded beads exhibited lower efficiency in reduction of *A. hydrophila* growth rate. Antibacterial activity of entrapped VPS1 was studied during 7 successive cycles. VPS1 was successful (significant at P<0.05) in the reduction of *V. anguillarum* growth rate. A successful trial showed good applicability of entrapped VPS1 in reduction of 97% of the pathogenic *Vibrio* spp. in water sample from El-Mex fish farm after 3 h. Chemical characterization of plain and phage seeded beads was performed by using Fourier transform infrared spectroscopy (FTIR).

**Keywords:** Antibacterial, Bacteriophages, Ca-alginate beads. Entrapment.

Aquaculture often suffers from heavy financial losses (Subasinghe *et al*., 2001; Flegel, 2006 and Saksida *et al*., 2006) due to the development of infections caused by microbial pathogens, including multidrug resistant bacteria that are easily transmitted through water and therefore able to infect a great variety of fish species.

Different chemotherapeutic agents are used to control microbial diseases. The misuse of them lead to emergence of resistant bacteria in addition to presence of antibiotic residue in the aquatic environment which causing harmful effects on the existing microflora, therefore there is an urgent need to have alternative tools for pathogen control in aquaculture (Sihag & Sharma, 2012).

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Bacteriophages have long been recognized for their potential as biotherapeutic agents. The major advantages of phage therapy of bacterial disease are host specificity, self replication and environmental safety. The use of phages to control bacterial pathogen depending on the interaction between phages and their bacterial hosts in addition to phenotypic and genotypic diversity (Comeau *et al*., 2006 and Holmfeldt *et al*., 2007).

The use of bacteriophages to control bacterial pathogens has previously used with success to control bacterial pathogens in food industry (Greer, 2005; Bahador *et al*., 2007; Higgins *et al*., 2007 and Hussain *et al*., 2008). Phage therapy has been also used to control human and fish pathogens.

A number of phages have been isolated for the potential use in phage therapy in aquaculture (Kumar, 2002 and Nakai & Park, 2002) against *Aeromonas* spp. (Imbeault *et al*., 2006), *Vibrio* spp.( Vinod *et al*., 2006; Karunasagar *et al*., 2007; Shivu *et al*., 2007; Srinivasan *et al*., 2007 and Pasharawipas *et al.,* 2011) and *Pseudomonas* spp. (Park *et al*., 2000; Nakai & Park, 2002 and Park & Nakai, 2003).

The treatment of microbial diseases is still difficult and might involve environmental hazards. A possible method to confront this problem might be the oral administration of antimicrobial materials to the fish larvae through the food chain, using the bioencapsulation technique (Verpraet *et al*., 1992). It is a physico-mechanical process in which the active components are covered by a layer of another material such as alginate, carrageenan and agarose (Gluza & Kennedy, 2007).

Immobilized phage could be particularly useful to create antimicrobial surfaces (Cademartiri *et al*., 2010; Roiha *et al.,* 2010 and Tolab *et al.,* 2010). Immobilized phages were previously used in different applications (Fattouh *et al*., 2003; Gervais *et al.,* 2007 and Cademartiri *et al*., 2010).

El-Mex fish farm is a highly economic raring farm. Lately, El-Mex fish farm has been suffered from die-off of large amounts of fish. Different studies attributed this to the presence of high densities of fish pathogens especially *Vibrio* spp. which cause intestinal infections of fish (Abou-Elela *et al*., 2005).

The main objective of the present study is to study the antibacterial activity of the isolated phages before and after entrapment in Ca-alginate beads against A. *hydrophila* and *V. anguillarum* using the shake-flask method. The potential use of the entrapped VPS1 as bio-control agent of fish pathogenic bacteria in El-Mex fish farm will be investigated. The physico-chemical properties of the plain and encapsulated beads with the phages will be determined by FTIR analysis. RAPD-PCR will be also used for investigation and differentiation among the isolated phages. Cluster analysis also will be used to indicate the similarity levels among the isolated phages.

## Materials and Methods

*Sample collection and preparation*

Crab, clams and fish were purchased from the market. Kidneys of fish samples, crab and clams were held at 5 to 10°C during shipment for 24 to 30 hr prior to analysis. They were scrubbed, shucked, mixed with an equal (1:1) weight of Butterfield's phosphate-buffered saline and blended (APHA, 1970).

*Phage isolation*

Phages were isolated directly from supernatant of crab, clams and fish kidney homogenates as previously described (DePaola *et al.*, 1997). All media and diluents were prepared in seawater and diluted with deionized seawater. Casamino acids peptone marine (CPM) broth (5.0 g of Casamino Acids [Difco], 5.0 g of Bacto Peptone [Difco], and 1.0 liter of seawater, autoclaved for 15 min at 121°C) was used as a growth medium. Serial dilutions of each supernatant were prepared in sterile seawater. Aliquots (0.1 ml) of each dilution were adsorbed to 0.2 ml of log-phase host cultures for 15 min, and virulent phages were detected by using the soft-agar overlay technique (Adams, 1959). The plating medium and soft-agar overlay were prepared with CPM medium supplemented with 1.5 and 0.7% Bacto Agar (Difco), respectively. The plates were incubated at 30°C, and plaques were detected at 24-48 hr. The plaque types, different in shape and size were isolated, purified and propagated to represent different phage isolates. The titre of each isolate was determined using the double agar layer method.

*Electron microscopy examination of the phage isolates*

The morphology of the three phage isolates VPS1, APS2 and APS3 was investigated with electron microscopy. Phages were negatively stained with 2% sodium tungestate in bi-distilled water at pH 6-7.5. Five µl of each phage was dropped onto a carbon-coated grid. The excess liquid was removed with filter paper after 1 min. Five µl of dye solution was added and after 1 min, the grid was dried. The grids were then examined and electron micrographs taken with a transmission electron microscope (JEOL 100 CX) operating at 80 kv.

*Analysis of phage DNA by random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR)*

The purified phage DNA isolated from each phage isolate was analyzed using RAPD-PCR. Amplification reactions were performed in a total volume of 50 µl containing 100µM each of dATP, dTTP, dCTP, and dGTP, 0.2M of each RAPD primer, 25ng 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 Alfa Imager 1200 Tm. 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 done using one of the following primers 1, 6-d: 5`-(AAGAGCCCGT)-3`; 2, 6-d:5`(AACGCGCAAC)-3`; 3, 6-d: 5`(CCCGTCAGCA)-3`. The obtained results were further analyzed by Pearson Product (Autofit 4%) and clustering was also achieved.

*Entrapment in Ca-alginate beads*

Entrapment was done in 2% calcium alginate gel beads as described by Eikmeier & Rehm (1987). A 25 ml sodium alginate was prepared and then autoclaved at 121 °C for 10 min. 1 ml phage suspension was mixed with sodium alginate solution, then homogenized and dropped from a hydrodermic syringe to 100 ml of calcium chloride solution (2%) to obtain spherical beads of calcium alginate. The formed beads were maintained in the gelling bath to harden for 2 h., then, they were filtered through a Whatman No. 1 paper and washed with sterile distilled water. The resulted beads entrapping the phage were added to 50 ml CPM containing the exponentially growing bacterial cells. Flasks were incubated at 30$^{o}$C with shaking at 150 rpm.

*Beads characterization using Fourier transform infrared spectroscopy (FTIR spectra)*

A FTIR spectrophotometer (FTIR-8400S Shimadzu-Japan) was used. Samples were scanned from 500 to 4000 cm$^{-1}$.

*Antibacterial activity of free and entrapped phages*

The test microorganisms were grown at 30$^{o}$C for 24 hr on CPM medium. A cell suspension of each microorganism was used for the antibacterial test. The antibacterial activity was evaluated by using the shake flask method (Ye *et al*., 2005). In this test, 50 ml of each cell suspension and predetermined amounts of beads were placed in a sterilized flask and continuously shaken at 150 rpm on a rotary shaker. At prescribed time intervals, 1 ml of sample solution from bead/microbial suspension system was removed by pipetting and optical density (O.D) was measured at 550 nm. The optical density of free phage samples was also measured as mentioned above.

*Recycling of phage seeded beads*

Phage seeded beads were recycled for 7 times in 50 ml CPM containing the exponentially growing bacterial host. Flasks were incubated with shaking at 30oC. Optical density was measured at 550 nm as mentioned before.

*Effect of VPS1 seeded beads on the estimates of Vibrio spp. in water sample from El-Mex fish farm*

One ml of VPS1 seeded beads was added to 50 ml of water sample from El-Mex fish farm. The mixture was incubated at 30$^{o}$C. Estimates of *Vibrio* spp. was detected at different time intervals using thiosulphate citrate bile salt sucrose (TCBS) agar and was extended to 96 hr.

*Statistical analysis*

Data analysis was performed with the software package Microsoft Excel, Version 2003. Statistically significant difference was determined using paired Student's t-test and $P<0.05$ was used as a limit to indicate statistical significance.

## Results

Different phages namely VPS1, APS2 and APS3 were isolated from different sources including crab (*Callinectes sapidus*), clams (*Tapes decussatns*) and fish (*Tilapia* sp.) depending on the plaque morphology and size. VPS1 produced small circular clear plaques. APS2 plaques were circular with entire edges. APS3 produced large circular plaques with irregular edges.

The phages were identified by using morphological criteria. Morphological studies revealed that the two phages (VPS1 and APS2) have tails and thus belong to the order Caudovirales. As shown in Fig.1a, VPS1 has icosahedral head and long tail and thus belongs to family Siphoviridae morphotype 1 as it has collar like structure between the head and the tail with no additional appendages on its head or tail. APS2 (Fig. 1b) belongs to family Myoviridae morphotype 2 as it has icosahedral head and long tail with no special appendages. On the other hand, APS3 exhibited different morphology as it has no tail so, it belongs to family Podoviridae (Fig. 1c).

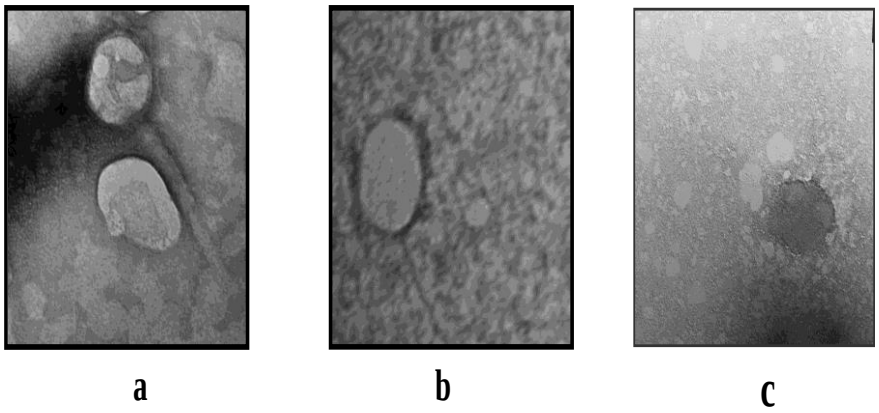


**Fig. 1. Electron micrographs showing a) VPS1 b)APS2 and c) APS3.**

*Cluster analysis based on molecular characterization using (RAPD- PCR)*

Analysis of phages DNA using Pearson Product (Autofit 4%) yielded the dendogram shown in Fig. 2. As observed, RAPD-PCR using primer 2 (Fig. 2B) showed that, APS2 was separated at 56.41% similarity level while VPS1 and APS3 were clustered in one group with 83.81% similarity. The similarity level increased between the two phages by using the primer 3 (Fig. 2C) to be 96.57% and APS2 was separated at 53.01% similarity level. On the other hand, RAPD-

PCR using primer 1 (Fig. 2A) showed that the similarity level between VPS1 and APS2 was 63.90% while APS3 was branched out at 52.08% similarity level .

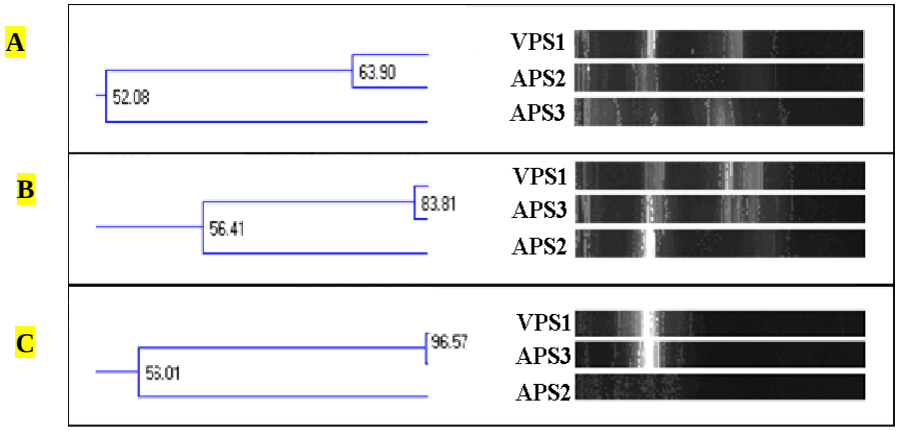


**Fig. 2. Similarity levels among VPS1, APS2 and APS3 based on molecular characterization using (RAPD -PCR).**

*FTIR spectrum of plain and phage seeded beads*

In this part of study, FTIR was used to reflect the molecular structure of plain and encapsulated calcium alginate beads. As shown in Fig. 3, Peaks of the plain calcium alginate beads appeared at 1614 $cm^{-1}$ and 1419 $cm^{-1}$ assigned to the asymmetric and symmetric carboxylate ($COO^-$) vibrations, respectively (Fig. 3a). The spectrum shows also some characteristic peaks at 804 $cm^{-1}$, 1276 $cm^{-1}$ (C-O), 2219 $cm^{-1}$, 2312 $cm^{-1}$ and 2925 $cm^{-1}$ ($CH_2$ stretching).

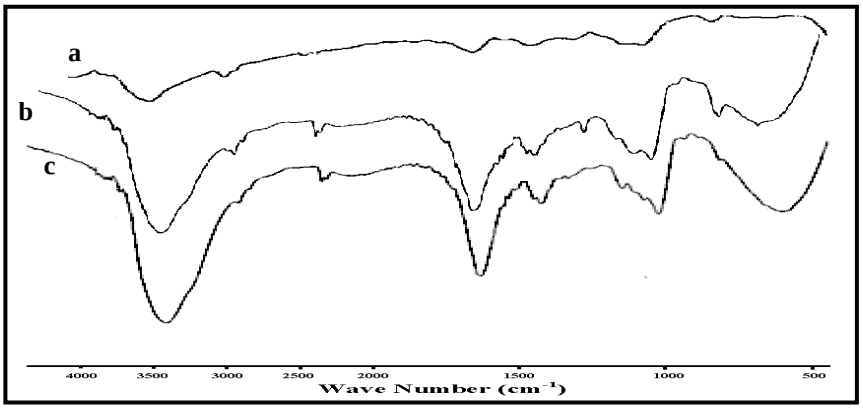


**Fig. 3. FTIR spectra of (a) plain Ca-alginate beads, (b) Ca-alginate beads encapsulated with APS2 and (c) Ca-alginate beads encapsulated with VPS1.**

Encapsulated beads with APS2 (Fig. 3b) showed new absorption peaks assigned to stretching vibration of OH and NH groups. New peaks were observed at 3834.1 $cm^{-1}$, 3798.3 $cm^{-1}$, 3733.9 $cm^{-1}$ and 3418.1 $cm^{-1}$. Also another new absorption peaks were appeared at 667 $cm^{-1}$, 1029.1 $cm^{-1}$, 1084 $cm^{-1}$, and 1867 $cm^{-1}$ (C-O bands). Another change was shifting in the position of the peaks such as (COO-) group which was shifted from 1419 $cm^{-1}$ to 1455.5 $cm^{-1}$. CH2 stretching groups (2312-2319 $cm^{-1}$) were also shifted

In case of encapsulated beads with VPS1 (Fig 3c), peaks at 2312 $cm^{-1}$ were extended to 2338.1 $cm^{-1}$ (CH2 stretching). Peaks representing (COO-) vibrations at 1419 $cm^{-1}$ was also shifted to 1431.3. There were appearance of new absorption beaks assigned to C-O bands at 559.9 $cm^{-1}$, 1028 $cm^{-1}$, 1078.5 $cm^{-1}$, 1152 $cm^{-1}$ and 1867.3 $cm^{-1}$ . The same for the groups at 3835.2 $cm^{-1}$, 3817 $cm^{-1}$, 3799.1 $cm^{-1}$ and 3742 $cm^{-1}$ which indicate the presence of stretching vibration of OH and NH groups.

*Antibacterial activity of free and entrapped phages*

The aim of this part is to study the effect of entrapment of phages on the interaction between them and their hosts. VPS1 and APS2 were chosen to complete the study based on that they are different and distinct from each other in the morphological and genotypic characteristics. Results showed that the entrapped VPS1 was superior (significant at $P<0.05$) in its effect on *V. anguillarum* than the free phage and caused complete elimination of *V. anguillarum* after 1 hr post addition of the beads as shown in Fig. 4. On the other hand entrapment of APS2 showed reverse effect and thus the free phage was better (significant at $P<0.05$) than the entrapped one in its reduction to the growth rate of *A. hydrophila* as shown in Fig. 5.

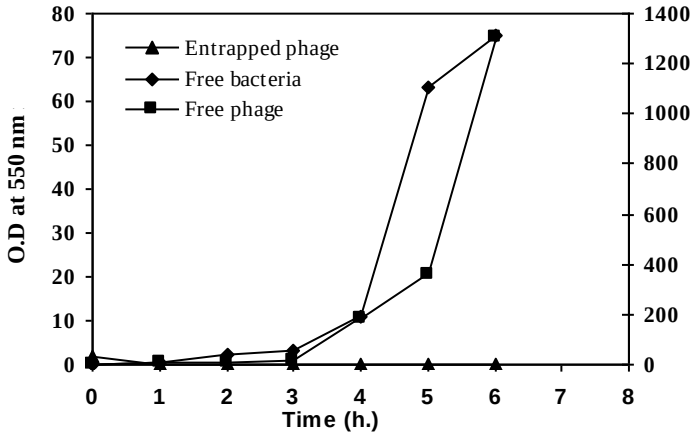


**Fig. 4. Effect of free and entrapped VPS1 on the growth rate of *V. anguillarum*.**

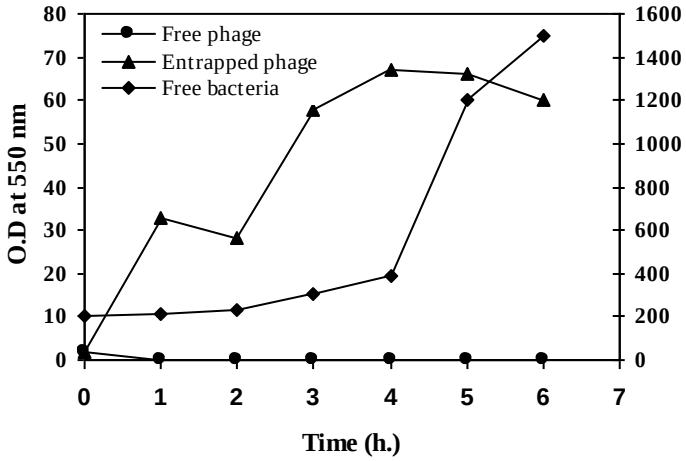


**Fig. 5. Effect of free and entrapped APS2 on the growth rate of *A. hydrophila*.**

VPS1 seeded alginate beads were tested for their efficiency in the reduction of the growth rate of *V. anguillarum* for 7 successive cycles. Results (Fig. 6) showed that, the entrapped VPS1 particles were active and caused complete elimination of the host growth rate until the end of the third cycle. They were also able to release and reduce the growth rate of the host bacterium during the rest of the cycles but with lower activity.

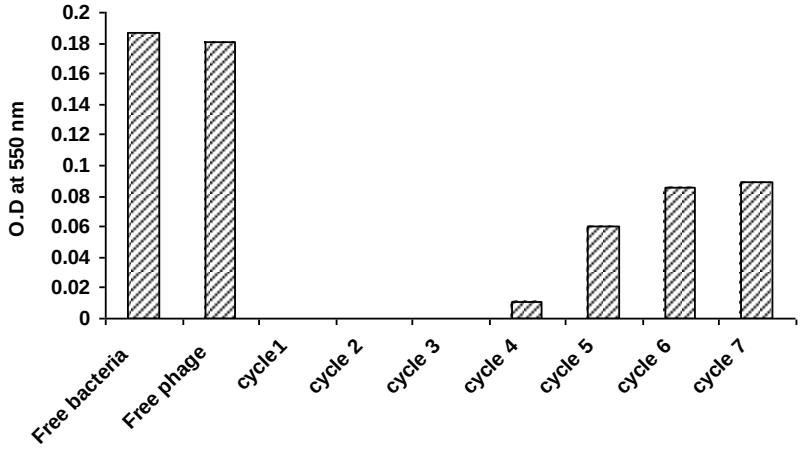


**Fig. 6. Recycling of VPS1 and its effect on the growth rate of *V. anguillarum* .**

The entrapped VPS1 was tested for their efficiency in biocontrol of *Vibrio* spp. invading the fish basins in El-Mex fish farm. Results in Fig. 7 indicated that VPS1 seeded beads were able to reduce the counts of *Vibrio* spp. 2 hr post

addition of the beads and reached its maximum reduction (97%) at 3hr compared to the control and this was confirmed with the statistical analysis using (t-test, $p < 0.05$). It was obvious that the effect of entrapped VPS1 extended until 96 hr but with lower efficiency.

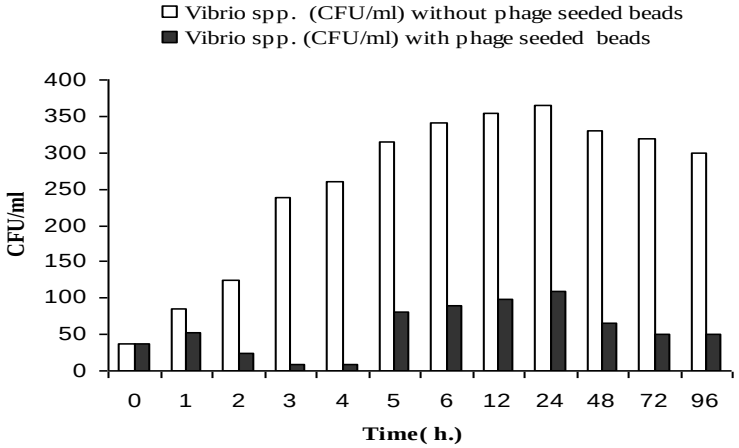


**Fig. 7. Effect of VPS1 seeded beads on the count of *Vibrio* spp.**

## Discussion

Bacteriophage therapy of infectious diseases in aquaculture has been suggested in different studies (Kumar, 2002; Nakai & Park, 2002 and Pereira *et al*., 2011).

Electron microscopy was used to classify the isolates into their appropriate taxonomic position (Prestel *et al*., 2012). VPS1 and APS2 assigned to order caudovirales as they have long tail and double stranded DNA. Our results are in accordance with that reported by Mitchell & Rouf (1983), Fattouh *et al.* (2002) and Pereira *et al.* (2011), where the tailed phages predominate other morphotypes. VPS1, APS2 and APS3 were found to belong to family Siphoviridae, Myoviridae and podoviridae, respectively as was proposed by Murphy *et al.* (1995). Frank & Moebus (1987) isolated a number of phages belonging to *Podoviridae* and also DePaola *et al.* (1998) reported the existence of phages belonging to *Podoviridae* in bivalves.

Random amplification of polymorphic DNA (RAPD) technique is used extensively for the epidemiological investigation and differentiation of many microorganisms (Jothikumar *et al*., 1998; Priscilla *et al*., 2000; Schaper & Jofre, 2000 and Walter *et al*., 2000). Presence of polymorphic DNA similarity among the three phages representing the families, Siphoviridae, Myoviridae and Podoviridae confirmed that they are not identical and do not seem to belong to the same species and also reveal evidence of vertical and horizontal gene transfer among populations. The same findings were reported by Fattouh *et al*. (2002).

FTIR spectra of plain and encapsulated Ca-alginate beads showed appearance and disappearance of some absorption peaks which indicated the modification in the molecular structure of phage seeded alginate beads compared to the plain beads which confirm the entrapment of the phages onto the beads as was reported by Shobier *et al.* (2010).

In the present work VPS1 seeded phage beads were efficient in causing complete growth elimination of *V. anguillarum* compared to effect of the free phage and thus representing promising agent for fish disease control in aquaculture. Conversely, other studies reported the better effect of free phage in its reduction to growth rate of its host (Fattouh *et al*., 2003 and Bielke *et al*., 2007). The efficiency of the entrapped phage may be attributed to the hydrophobicity of calcium alginate beads (Le-Tien *et al*., 2004), which retains the integrity of the beads and prevents their degradation over the course of experiment and thus allows more surface of contact between the phage and its host (Kim *et al*., 2007). In addition to the ability of the phages to release at once and causing infection to the host bacterium.

On the other hand, the entrapped APS2 showed lower efficiency in growth reduction which may be due slower release of phage particles from the beads or accumulation of phage particles in high densities without their immediate release into the surrounding medium as was reported by Fattouh *et al*. (2003).

Recycling of entrapped VPS1 during 7 successive cycles indicated sequential release of phage particles through the recycling period as was previously reported by Fattouh *et al*. (2003). The efficiency of the entrapped phage particles decreased upon reuse as was reported by Flood & Ashbolt (2000) and Fattouh *et al.* (2003).

The host-phage interaction is an important concept and was previously studied (Flood & Ashbolt, 2000). The present study showed extended efficiency of the phage in reducing the population of *Vibrio* spp. in the water sample of El-Mex fish farm up to 4 days compared to the water without inoculation of phage seeded beads which seems to be promising for biocontrol of fish diseases. Analogous results have been reported in some studies where virus sized fluorescent microspheres were dosed into wetland biofilms. Microspheres remained in the biofilm for a period of time of at least 7 months, while being rare in the water phase (Flood & Ashbolt, 2000). These results and the present findings could suggest that there is a potential for the release of the virus particles due to change in environmental factors or any disturbances in the surrounding medium that could result in active infection.

## Conclusion

The present study revealed that entrapment of VPS1 into calcium alginate beads proved an effective antibacterial activity against the tested fish pathogen

*V. anguillarum* and could be promising as antibacterial agent to control the growth of fish pathogenic bacteria whereas, the free APS2 showed superior effect against *A. hydrophila*. The use of bacteriophages as an alternative to synthetic drugs could be assayed for aquaculture and for biomedical purposes through further researches and investigations

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# دراسات على النشاط المضاد للبكتيريا لبعض اللاقمات البكتيرية الحرة و المقيدة ضد ممرضات الأسماك

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تم عزل ثلاث أنواع مختلفة من اللاقمات البكتيرية (VPS1, APS2, APS3) من الكابوريا (*Callinectes sapidus*)) و الجندوفلى (*Tapes decussatns*) والاسماك (*Tilapia* sp.) وقد وجدوا أنهم ينتموا للعائلات (Siphoviridae, Myoviridae , Podoviridae) على التوالي. اظهر التحليل الأحصائى وجود مستويات مختلفة من التشابه بين الثلاث لاقمات وذلك اعتمادا على تحليل الحمض النووي باستخدام random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR).

تم تقييد VPS1 و APS2 داخل كريات ألجينات الكالسيوم وقد تم التوصيف الكيميائي للكرات الحرة والمحملة بالفاجات عن طريق التحليل الطيفي باستخدام الأشعة تحت الحمراء (FTIR) وقد أظهرت وجود تغيرات مميزة. كذلك تم اختبار نشاط اللاقمات VPS1, APS2 ضد بعض البكتيريا الممرضة للاسماك مثل Aeromonas hydrophila و *Vibrio* anguillarum قبل وبعد التقييد داخل كريات ألجينات الكالسيوم. اتضح أن الكريات التى تحمل VPS1 فاقت ($P< 0.05$) اللاقمات الحرة فى النشاط ضد *V. anguillarum* بينما كان لكريات ألجينات الكالسيوم التى تحمل APS2 كفاءة اقل في تقليل معدل نمو *Aeromonas hydrophila* . تم دراسة نشاط VPS1 المقيد خلال سبع دورات متتابعة وقد لوحظ نجاحه في تقليل معدل نمو *Vibrio anguillarum* خلال هذه الدورات. اتضح من المحاولة نجاح VPS1 المقيد فى الحد من بكتيريا *Vibrio* الممرضة بمقدار ٩٧ ٪ في عينة ماء من مزرعة المكس بعد ٣ ساعات.