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Full Length Research Paper

Identification of *Streptococcus salivarius* bacteriophage isolated from Persian Gulf as a potential agent for dental caries phage therapy

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The aim of this research was to detect oral *Streprtococci* bacteriophages from Persian Gulf. Dental plaque samples were collected using sterile explorer and cultured in brain heart infusion (BHI) Broth. The oral *Streptococci* were isolated in culture media. The Persian Gulf water sample was gathered using a sterile bottle from the depth of 50 cm under the inframarine surface at Boushehr Port, Boushehr state, Iran. The Persian Gulf water was centrifuged and its supernatant was filtered through a 0.45 micrometers membrane filter and with a sterile Millipore filtration system. The filtrates were added to activate oral *Streptococci* at their logarithmic phase and cultured in (BHI) Agar using overlay method. Bacteriophage plaque forming assay in (BHI) Agar and clearance of (BHI) Broth suggested the presence of specific bacteriophages in sample. Transmission electron microscopy revealed that the capsid of the isolated bacteriophage was hexagonal (diameter: ~ 83.33 nm) most probably related to *Cystoviridae* family. This is the first report of isolation and identification of oral *Streptococci* bacteriophages from Persian Gulf located in South of Iran. The applications of these lytic phages as a potential for phage therapy of dental plaque could be considered as the significance and impact of the present study.

Key words: Persian Gulf, *Streptococcus salivarius*, bacteriophages, phage therapy, dental plaque, pharmaceutical and medical biotechnology.

INTRODUCTION

The resident microorganisms of oral cavity especially those inhabit on tooth surfaces are responsible for dental plaque formation and conversion of dietary saccharides to organic acids. These acids decalcify the tooth enamel and lead to destruction of tooth hard tissue and consequently tooth decay (Loesch et al., 1986; Hitch et al., 2004; van der Ploeg, 2007). More than 500 species from 30 deferent genera reside in oral cavity (Schaechter et al., 2004). The most important species that play key roles in dental plaque formation are oral *Streptococci* (Tanzer et al., 2001). According to Bergey's mannual of systematic bacteriology, oral *Streptococci* are formed from 12 species including *Streptococcus salivarius, Streptococcus anginosus*, *Streptococcus constellatus*, *Streptococcus cristatus*, *Streptococcus gordonii*, *Streptococcus mitis* , *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus parasanguis*, *Streptococcus pneumonia*, *Streptococcus sanguis* and *Streptococcus sobrinus* (Holt et al., 1994; Schaechter et al., 2004). These species are the first bacteria that attach to salivary glycoproteins on tooth surfaces through their specific surface capsular polymers such as glucan and fructan (Freedman et al., 1974; Tanzer et al., 1974; Tanzer et al., 2001). *S. salivarius* as well as mutans *Streptococci* and nonmutans *Streptococci* or sanguinis *Streptococci* are present at high levels in tooth and mucosal surfaces some of which are highly acidogenic and few are acid tolerant (Nyvad et al., 1990; Tanzer et al., 2001). *S. salivarius* along with *S. sanguis*, *S. oralis* and *S. gordonii* are the first tooth colonizers however, *S. sobrinus* and *S. mutans* are more dealt with dental

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diseases (Milnes et al., 1993; Smith et al., 1993; Jacques et al., 1998; van der Ploeg, 2008). The bacteriophages, viruses that attack their specific bacterial hosts, have a great impact on controlling bacterial population throughout the world as well as micro environmental niches in human body (Marks et al., 2000; Chanishvili et al., 2001). In recent decades bacteriophages have been studied as biotechnological tools for treatment and eradication of bacterial pathogens such as *Escherichia coli* in gastrointestinal infections (Smith et al., 1987a; Smith et al., 1987b; Smith et al., 1982; Smith et al., 1983; Drozdova et al., 1998; Marks et al., 2000), *Pseudomonas aeroginosa* and *Acinetobacter baumanii* in skin burns and grafts (Soothill, 1992; Soothill, 1994).

Several aspects of oral Streptococci and their influences on dental disorders and dentistry have been studied (Jacques, 1998; Tanzer et al., 2001; Okada et al., 2002; Franco and Franco, 2007) but there are few reports indicating the role of bacteriophages in ecology of oral cavity as a micro environment or the attitude toward phages as strong biotechnological and natural therapeutic agents for phage therapy of oral *Streptococci* (Bachrach et al., 2003; Hitch et al., 2004). Some reports have indicated the isolation and identification of lytic bacteriophages of *S. mutans* from human saliva (Delisle and Rostcowski, 1993; Armau et al., 1998) and recently the complete genome sequence of one of them, *S. mutans* lytic baceriophage M102, has been revealed (van der Ploeg, 2007). The main goals of this research were isolation and identification of oral *Streptococci* from dental plaques of healthy individuals as well as patients with mild gingivitis, isolation and identification of their specific bacteriophages as potential agents for phage therapy of dental caries.

MATERIALS AND METHODS

Culture media and chemicals

The culture media used were Brain Heart Infusion (BHI) Broth, (BHI) Agar. Mitis - Salivarius Agar (sucrose, 50 g/l; agar, 15 g/l; enzymatic digest of protein, 10 g/l; proteose peptone, 10 g/l; K_2HPO_4, 4 g/l; dextrose, 1 g/l; trypan blue, 0.08 g/l; crystal violet, 0.8 g/l; Na_2TeO_3 solution, 1 ml; distilled water, 1000 ml) (Atlas, 2004) and Blood Agar Base medium, all from Himedia, India, NaCl, glycine, $CaCl_2$, anaerobic class A gas pack, all from Merck, 0.45 m membrane filter (Millipore, white gridded), api 20 Strep kit (bioMerieux, France) and H_2O_2 from Shimifan, Iran.

Dental plaque samples

The dental plaque samples were collected from healthy volunteers (10 students, aged 22 - 26, 8 females and 2 males at the Faculty of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran) and patients with mild gingivitis and periodontitis (aged 29 - 54, 6 females and 4 males). The dental plaque samples were obtained using sterile explorers from the upper right first molars in all individuals that had not applied antibacterial rinse and routine brushing 12 -14 h before sampling. The samples were taken in accordance with ethical guidelines and regulations prepared by Department of Operative Dentistry, Faculty of Dentistry, Isfahan University of Medical Sciences, which have been authorized by Iranian Ministry of Health and Medical Education.

Enrichment, isolation and primary identification of dental plaque *Stereptococci*

The dental plaque samples were cultured in BHI and then incubated at 37°C in 5% CO_2 for 24 h. After bacterial enrichment, the turbid broth media were cultured to Mitis – Salivarius Agar (MSA) using streak plate method and incubated at 37°C in 5% CO_2 for 48 h. The colonies were examined for catalase reaction using hydrogen peroxide.

Macroscopic, microscopic and biochemical characterizations

The individual colonies were examined for their macroscopic traits such as color, size, morphology, light reflection and hemolysis on 10% sheep blood agar. The microscopic morphology and arrangement of purified bacteria on selective media such as MSA were examined using gram staining method. For biotyping, the bioMerieux SA api 20 Strep kit, an identification system for Streptococcaceae was used. Before using api 20 Strep kit, a 48 h well isolated colony was picked from blood agar medium and suspended in 300 l sterile distilled water, homogenized well and then swabbed aseptically the entire surface of blood agar culture media. Next procedures were followed using api 20 Strep instruction.

Bacteriophage resource sampling

The water sample was collected using a sterile 1000 ml bottle from the depth of 50 cm inframarine surface of the Persian Gulf at shorelines of the Boushehr harbor, Boushehr State, South of Iran. The sampling was taken for 5 min and the cap of the bottle was fitted below the water surface. Then the Persian Gulf water sample was transferred to our laboratory at 4°C.

Preparation of bacteriophage samples and bacterial treatment

Fifty milliliters of well shaked Persian Gulf water pipetted to sterile falcons and centrifuged at 1000 g for 15 min. The supernatants were filtered through 0.45 m Millipore membrane filter using sterile Millipore filtration system and the filtrate was stored at 4°C. Then the identified bacterial isolate from dental plaque was cultured in 10 ml of BHI and incubated at 37°C for 24 h in order to be activated. After growth and obtaining appropriate turbidity, the bacterial inoculum was cultured to 250 ml Erlenmeyer flasks containing 100 ml BHI and incubated in a shaker incubator at 37°C and 120 rpm shaking speed for 16 -18 h. The shaking was then stopped at bacterial logarithmic phase and 10 ml of Persian Gulf filtrate were added to flasks aseptically. The shaking at 37°C was continued for another 45 - 60 min for attachment of probable bacteriophages to their specific bacterial hosts. Then 1 ml of BHI was added to 5 ml preheated 45°C BHA (0.7% agar), as top agar, vortexed and immediately over layed on BHA plates. The BHAs were incubated at 37°C for 24 - 48 h until lysis zones, bacteriophage plaques, were appeared.

Bacteriophage isolation and purification

After appearance of bacteriophage plaques, they were cut aseptically and washed with 1.25% glycine in sterile eppendorf tubes, vortexed well and the aforementioned procedures were repeated for 3 times. The overall BHI from the last trial after complete clearance, 24 h incubation at 37°C with 120 rpm shaking speed, were centrifuged at 1000 g for 15 min and supernatants were passed through 0.45 m Millipore membrane filter. Ten milliliters of the filtrates were used for further purification using a previously described method as follows: NaCl 1M was added to phage solution, vortexed for 2 min and was kept on ice for

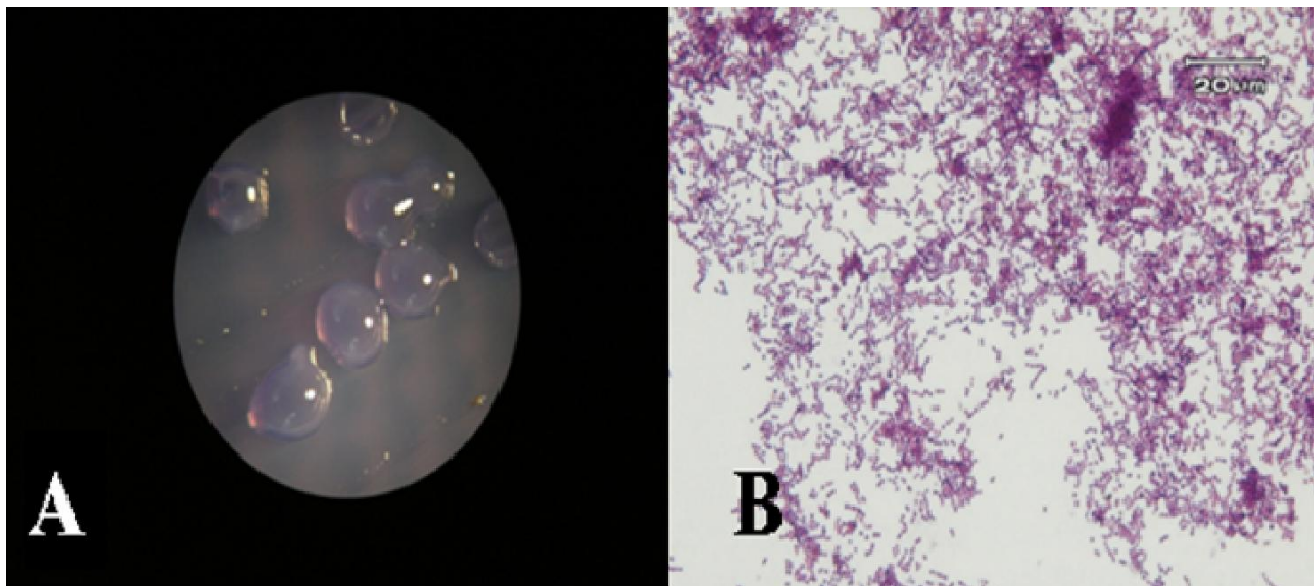

Figure 1. (A) Isolated oral *Streptococcus* from dental plaque in Mitis- Salivarius agar culture medium after 48 h incubation at 37°C and 5% CO_2. (B) The gram staining of *S. salivarius* on Mitis-Salivarius agar after 24 h at 37°C, isolated from dental plaque.

an hour. Then centrifuged at 11,000 g, 4°C for 10 min. The supernatant was transferred to a sterile tube and 10% (w/v) poly ethylene glycol 6000 was added to tube. The tube was kept on ice for another hour in order to phage precipitation. The suspension was centrifuged at 11,000 g, 4°C for 10 min. The supernatant was discarded and tube was placed in its reversal position for 5 min to be dried. Then SM buffer (NaCl, 5.8 g/l; MgSO4, 2 g/l; Tris 1 M, 50 ml; gelatin solution 2% (w/v), 5 ml; distilled water, 1000 ml) was added to pellet for soaking purposes and kept an hour in room temperature. The chloroform was added in equal volume of suspension, vortexed briefly for 30 s and centrifuged at 3,000 g, 4°C for 15 min. Organic phase discarded and aquatic phase, bacteriophage included, was kept at 4°C before TEM grid preparation (Sambrook et al., 1989).

Transmission electron microscopy

A drop of purified filtrated bacteriophage suspension transferred on a formvar coated grids (EM standard, 3.2 mm diameter). The additional suspension was removed by drying paper and grids were dried in front of light heat for 30 s. The grids were then negatively stained using 2% phosphotungstic acid (pH: 7.2 regulated with KCl, 0.5 molar) and dried following the same method and then observed through Transmission Electron Microscope (Philips, MC 10, Netherlands) at 78 K magnification.

RESULTS

The turbidity of BHI showed that the dental plaque bacteria were enriched after 48 h. The oral *Streptococci* were isolated on MSA at 37°C and 5% CO_2 after 48 h. The continual streak plate method on MSA at 37°C and 5% CO_2 confirmed the complete purification of isolates. The colonies were pale blue to bluish, smooth, mucoid with shiny reflection, convex and intermediate with 1 - 1.5 mm in diameter (Figure 1A). Microscopic observations showed large gram positive *Streptococci* and few diplococcic (Figure 1B). The negative catalase test indicated that the isolates were related to Streptococcacea family. The macroscopic and microscopic characterization of isolated oral *Streptococci* revealed that they were members of the genus *Streptococcus*.

The results of BioMerieux SA api 20 Strep kit after 4 and 24 h incubation periods at 37°C indicated two 7 – digit identification numbers, 5060045 and 5060445, respectively. Using the database (V7.0), analytical profile index and apiweb^{TM} identification software on the internet, it was confirmed that the isolated oral *Streptococcus* was *S. salivarius*. The main biochemical examinations that were used to identify the species of isolated oral *Streptococcus* as *S. salivarius* are shown in Table 1. This strain was isolated from patients with mild gingivitis. Using api 20 Strep kit, other Streptococci such as *Streptococcus ubris* and *Streptococcus thermophilus* were isolated from healthy volunteers (data not shown).

The addition of Persian Gulf filtrate to 16 - 18 h *S. salivarius* in BHI growth medium resulted in complete clearance of BHI after 12 h shaking incubation at 37°C. The cultivation of *S. salivarius*–Persian Gulf filtrate inoculums after 45 - 60 min shaking, on BHA plates showed the bacteriophage plaques after 24 h incubation at 37°C (Figure 2). The continuous contamination of new logarithmic growth of *S. salivarius* by bacteriophages obtained from individual plaques resulted in more purification of them. The results showed that these specific bacteriophages were lytic phages. Transmission Electron Microscopy of purified and concentrated bacteriohage suspension revealed that the capsid of the *S. salivarius* bacteriophage was hexagonal with

Table 1. The main biochemical characteristics of isolated *S. salivarius* from dental plaque on Mitis-Salivarius Agar by BioMerieux SA api 20 Strep kit after 4 and 24 h incubation periods at 37°C.

Biochemical tests	4 h	24 h
Voges Proskaur	+	+
Hipuric acid hydrolysis	-	-
Sculin hydrolysis	+	+
Pyrolidonyl arylamidase	-	-
- Galactosidase	-	-
– Glucuronidase	-	-
Alkaline phosphatase	+	+
Leucine amino peptidase	+	+
Arginine dihydrolase	-	-
Acidification of:		
D-Ribose	-	-
L-Arabinose	-	-
D-Mannitol	-	-
D-Sorbitol	-	-
D-Lactose	-	+
D-Trehalose	-	-
Inulin	-	-
D-Raffinose	+	+
Amidin	+	+
Glycogen	-	-
-Hemolysis	+	+
Catalase reaction	-	-

approximately 83.33 nm diameter (Figure 3). These results suggested that the specific bacteriophage of *S. salivarius* isolated from Persian Gulf is most probably related to family *Cystoviridae* of bacteriophages.

DISCUSSION

The bacteriophages specific for *Enterococcus faecalis* have been isolated from human saliva but the efforts to detect bacteriophages for gram positive oral pathogens such as *S. sobrinus*, *S. mutans* and *S. salivarius* from human saliva was not successful (Bachrach et al., 2003). Hitch et al. (2004) isolated bacteriophages from oral cavity. Although, their aim was isolation of lytic bacteriophages of oral pathogens from human saliva, dental plaque and mature biofilms originated from salivary bacteria, they obtained phages specific for non-oral bacteria such as *Proteus mirabilis* but did not find any phage specific for oral pathogenic bacteria. They suggested that the bacteriophages do not play a key role in regulating the nature of micro environmental ecology of oral cavity that was against previous hypothesis that clarified bacteriophages are vital in modifying the bacterial ecosystems in most of milieus (Campbell et al., 2003). Nelson et al. (2003) reported the genomic sequence of C1 as the first *Streptococcal* phage. They showed that C1, a lytic phage that contaminating group C S*treptococci*, was a member of *podoviridae* family of phages that have been recognized by short and noncontractile tails. Three lytic bacteriophages of *S. mutans* known as M102, e10 and f1 have been characterized (Delisle et al., 1993). They reported each phage had an icosahedral head with 67 - 68 nm diameters and a noncontractile flexible tail that are characteristics of *Siphoviruses*. The complete genome sequence of *S. mutans* bacteriophage M102 has been resolved and reported that the ORFs responsible for structural proteins in M102 and *S. thermophilus* bacteriophages show similarity (Van der Ploeg, 2007). The characterization of prophage PH15 of *Streptococcus gordonii*, another oral *Streptococcus*, has been reported and the complete genome sequence of this lysogenic phage has been analyzed (Van der Ploeg, 2008).

A lysogenic bacteriophage of *S. mutans* PK1 (mucoid strain) has been identified as bacteriophage PK1. It has been revealed that the most PK1 phage particles had 95 nm hexagonal heads and 150 nm tails (Higuchi et al., 1976; Higuchi et al., 1977; Higuchi et al., 1981). While there is no report for lytic bacteriophages of oral *Streptococci* except for *S. mutans*, we isolated a lytic bacteriophage from Persian Gulf located at the South of Iran that attacked specifically to *S. salivarius*, a member of dental-caries producing *Streptoccoci*. The TEM micrograph of isolated *S. salivarius* bacteriophage showed that it had hexagonal head. Regarding the average diameter of phage particle, ~ 83.33 nm, as well as morphological characterizations it could be most probably related to *Cystoviridae* family of bacteriophages. So far there is no report indicating that *Cystoviruses*, the enveloped dsRNA phages with the size of 85 nm, could attack the gram positive bacteria e.g. *Streptococci*.

The isolation of a specific bacteriophage for an oral *streptococcus, S. salivarius*, from Persian Gulf water with a high salinity level, 23 g/l salt, is another significance of this study. While the investigation of lytic effects of this phage on other oral *Streptococci* is challenging, it could be applied as a potential for phage therapy of dental caries and other dental and periodontal disorders. The *S. salivarius* was isolated from dental plaque of patients with mild gingivitis. Other *Streptococci* such as *S. ubris* and *S. thermophilus* were isolated from healthy volunteers, but we did not examine lytic effects of the isolated bacteriophages on them because they were not classified as oral *Streptococci*.

In conclusion this is the first time that a *Cystovirus* is reported for *S. salivarius* as a gram positive bacterium. Although, there are a few documents in literature indicating the isolation of *S. mutans* lytic bacteriophages from salivary samples (Armau et al., 1988; Delisle et al., 1993) there has not been any report so far showing the isolation and identification of lytic bacteriophages of other eleven oral *Streptococci* species. We suggested that the

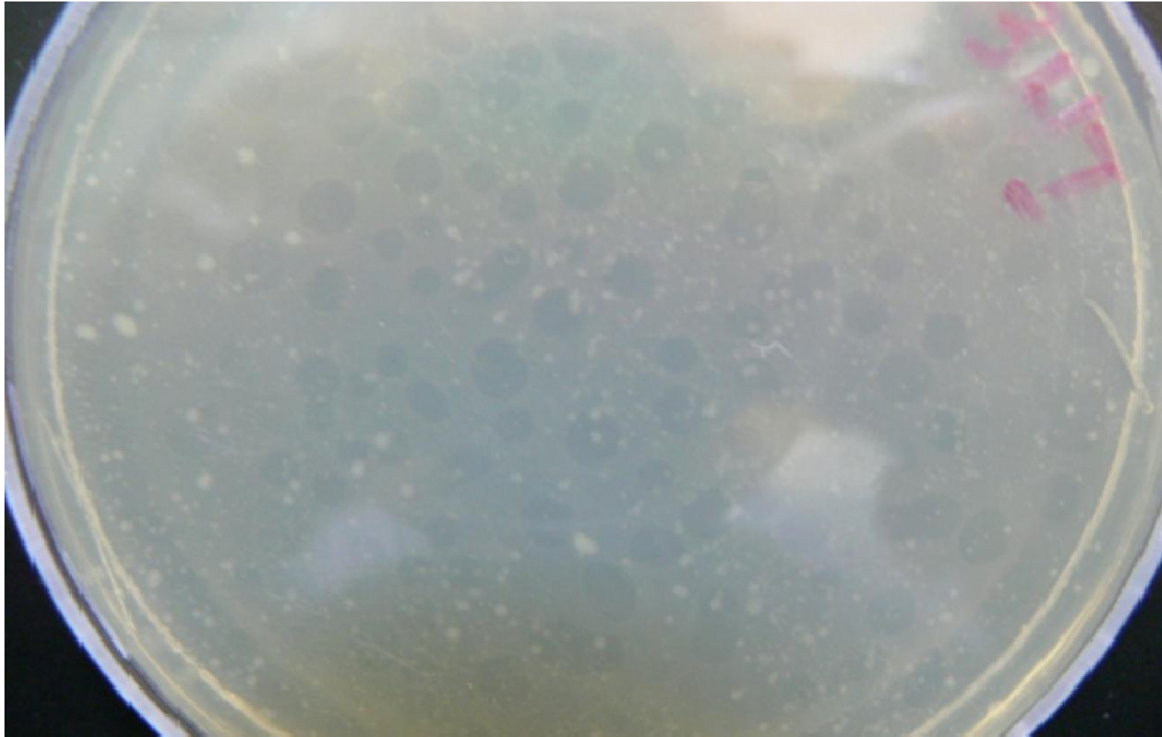

Figure 2. The plaques of *S. salivarius* bacteriophages isolated from Persian Gulf on Brain Heart Infusion Agar after 24 h incubation at 37°C.

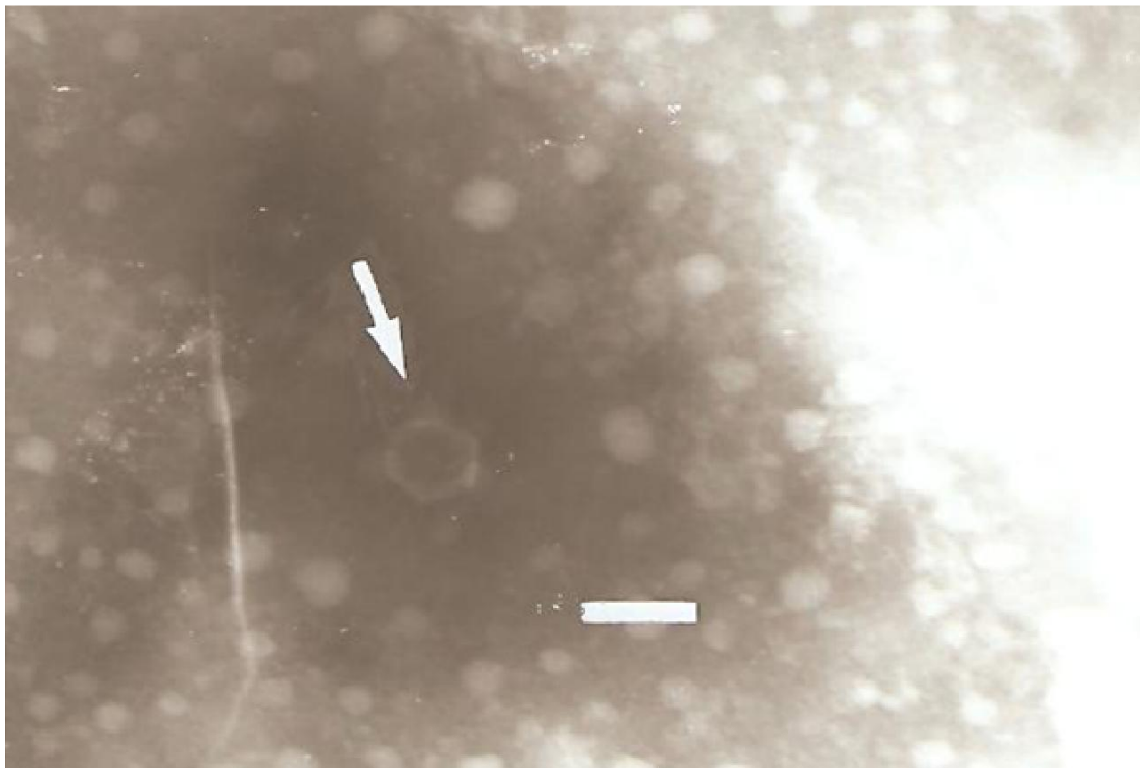

Figure 3. Transmission electron micrograph of the lytic hexagonal bacteriophage with approximately 83.33 nm diameter (arrow) isolated from Persian Gulf specific for *S. salivarius* isolated from dental plaque (Bar = 100 nm).

isolation and identification of new lytic baceriophages capable to eliminate oral *Streptococci*, starters of dental plaque formation, could be considered as a powerful approach for phage therapy of oral pathogenic bacteria in dentistry as well as modern medical and pharmaceutical biotechnology.

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