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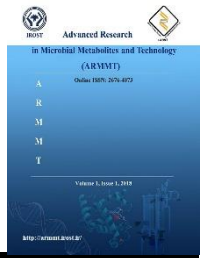

Potential application of two tectivirus and cystovirus isolated from the Caspian Sea for bio-control of dental plaque using in vitro assay

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Abstract

The aggregation of oral Streptococci results in the beginning of enamel calcium degradation of the teeth and subsequent emergence of dental caries. Bacteriophages have a considerable effect in controlling the populations of various bacteria. The isolation and characterization of specific lytic bacteriophages against oral streptococci were the main goals of this study. Brain Heart Infusion Broth (BHI) culture medium was used for oral streptococci enrichment from dental plaque. The Mitis Salivarius Agar (MSA) culture medium was applied to oral streptococci isolated from dental plaque samples. A 0.45 µm membrane filtrate of Caspian Sea water was added to the streptococcal isolate suspension and cultured using the overlay method. The isolated bacterium on MSA, according to macroscopic, microscopic and biochemical examinations, was identified as *Streptococcus salivarius*. The BLASTN of the *gtf*K gene after DNA sequencing reconfirmed that the isolate was *S. salivarius*. The strain was named *S. salivarius* KBM-ISF-2 and its *gtf*K gene deposited at the GenBank, National Center for Biotechnology Information, NCBI, under the accession number KJ634208.1. Transmission electron microscopy revealed two specific lytic bacteriophages that attacked *S. salivarius* KBM-ISF-2 isolated from dental plaques. The first lytic phage was identified as a Tectivirus with icosahedral symmetry measuring 46.67 nm in diameter. The second phage was identified as a Cystovirus with icosahedral symmetry and a diameter of 85.56 nm. This is the first report of isolation and characterization of a Tectivirus from the Caspian Sea and its specific inhibiting effects on *S. salivarius* KBM-ISF-2. Also, a Cystovirus against *S. salivarius* KBM-ISF-2 was isolated and identified.

1. Introduction

There are more than 750 types of bacteria living on the surfaces, fissures and cracks of teeth in a human oral cavity (Vesna, 2018; Schaechter, 2004). Although most of them have symbiosis in their environment, a few species are thought to be responsible for dental caries and subsequently gingivitis and periodontal diseases (Vensa, 2018; Bachrach et al., 2003; Loesche, 1986).

It is believed that oral Streptococci play a significant role in initiation and formation of dental plaque as starter species (Tanzer et al., 2001). The oral streptococci are comprised from twelve spp., *S. anginosus*, *S. constellatus*, *S. cristatus*, *S. gordonii*, *S. mitis*, *S. mutans*, *S.*

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oralis, S. parasanguis, S. pneumonia, S. salivarius, S. sanguis and *S. sobrinus* (Beheshti Maal et al., 2010; Holt et al., 1994).

S. salivarius as well as mutans and sanguinis streptococci reside on tooth and mucosal surfaces of the oral cavity in high concentrations (Loesche, 1986; Nyvad and Kilian, 1990). Among oral Streptococci, *S. salivarius* accompanied by *S. gordonii, S. oralis* and *S. sanguis* are the primary spp. that colonize teeth surfaces via their specific capsular polymers, fructan and glucan, following consumption of sucrose containing food stuffs (Van der Ploeg, 2008; Jacques, 1998; Milnes et al., 1993).

Biotechnological applications of bacteriophages, viruses that attack their bacterial hosts specifically, for treatment of bacterial infections that have been previously reported (Amiri Fahliyani et al., 2018; Campbell, 2003; Chanishvili et al., 2001) include: phage therapy for gastrointestinal infection *Escherichia coli* (Drozdova et al., 1998; Marks and Sharp, 2000; Smith et al., 1987); coliform removal in wastewater treatment (Beheshti Maal et al., 2014; Beheshti Maal et al., 2015b); curing of *Acinetobacter baumanii* and *Pseudomonas aeroginosa* in patients with grafts and skin burns (Soothill, 1994, 1992); treatment of bovine mastitis (Amiri Fahliyani et al., 2018); and phage therapy for corrosion-producing bacteria (Pedramfar et al., 2017).

The aims of this study were the isolation and molecular identification of oral streptococci as the main bacterial starters of dental plaque. The isolation and characterization of specific lytic bacteriophages against isolated oral streptococci will be discussed in this study.

2.Materials and methods

2.2.Sampling, enrichment and screening of oral Streptococci

Patients with mild gingivitis and periodontitis were selected for sample collection. The sampling was fulfilled under the permission of the Ethical Committee, Islamic Azad University, Isfahan, Iran. The Brain Heart Infusion Broth (BHI) and Mitis–Salivarius Agar (MSA) (proteose peptone, 10 g/l; enzymatic digest of protein, 10 g/l; sucrose, 50 g/l; dextrose, 1 g/l; K_2HPO_4, 4 g/l; Na_2TeO_3 solution, 1 ml; crystal violet, 0.8 g/l; trypan blue, 0.08 g/l; agar, 15 g/l; distilled water, 1000 ml) were used for enrichment and screening purposes, respectively, as previously described in Beheshti-Maal et al. (2012).

2.3. Identification of oral streptococci

The purified colonies in the MSA culture medium were characterized against macroscopic, microscopic and biochemical examinations as previously described in Beheshti-Maal et al. (2010) and Beheshti-Maal et al. (2015a).

2.4. Molecular identification of isolated oral *Streptococcus* sp. using amplification of the specific *gtf*K gene.

The identified *Streptococcus* sp. on MSA culture medium after bio-typing by biochemical examinations was used for DNA extraction. The DNA extraction followed using the method described by Hoshino et al. (2004). The specific primers of the glucosyltransferase gene (*gtf*K), a gene responsible for the synthesis of adhesive microbial capsules, were used for the molecular identification of *Streptococcus salivarius,* one of the main oral streptococci responsible for dental caries. These primers were 5’GTGTTGCCACATCTTCACTCGCTTCG G3’ (27 bp) as the forward primer and 5’CGTTGATGTGCTTGAAAGGGCACCAT T3’ (27 bp) as the reverse primer. The polymerase chain reaction of the *gtf*K gene was carried out by an Eppendorf Gradient Master Cycler (Germany) and under the specified condition as previously described in Beheshti-Maal et al. (2015a) and Hoshino et al. (2004).

2.5. Molecular analysis of the amplified *gtf*K gene in isolated oral *Streptococcus*

For confidence purposes in isolate identification, after PCR the amplified *gtf*K gene was sent to Taligene Pars Company, Isfahan, Iran for DNA sequencing. BLSTN software was first used to analyze the results of DNA sequencing and then again to examine its relationship to all genome sequences available in the GenBank. The phylogenetic tree of the *gtf*K gene of isolated oral *Streptococcus* was drown using the NCBI software of "BLAST pairwise alignments". Lastly, the *gtf*K sequenced gene of isolated oral *Streptococcus* was submitted to the GenBank, NCBI for further processing and deposition.

2.6. Sampling, enrichment and isolation of specific lytic phages against *S. salivarius*

A 500 ml sample of Caspian Sea water (pH, 8.1; salinity, 1.2%; Temp, 32°C, intermediate turbidity) from Nashta Rud (geographical coordinates: 36° 45' 15" North, 51° 0' 37" East), Tonekabon County, Mazandaran Province, Iran was gathered in a sterile container using the aseptic method and transferred to the Microbial Biotechnology Laboratory, Falavarjan Branch, Islamic Azad University, Isfahan, Iran in an aseptic manner. The purified *S. salivarius* individual colony from the MSA was cultured on BHI and activated overnight. The overlay method was applied to purify and titrate the enriched bacteriophages. The phage filtrate dilution of 10^{-1} to 10^{-12} using SM buffer were prepared and then 100 µl of each dilution and 100 µl of activated bacterial culture were mixed and added to 5 ml of 45°C molten MSA with 0.5% agar. After a brief vortex poured on MSA with 1.5% agar. The plates were incubated at 37°C for 24 h and the bacteriophage plaques on each plate were enumerated (Amiri Fahliyani et al., 2018; Pedramfar et al., 2017; Franco e Franco, 2007).

2.7. Preparation of transmission electron micrographs

A purified individual phage plaque zone on the S. salivarius MSA culture was selected and removed using the sterile method. The agar containing phages were transferred to a SM buffer [Tris.HCl (50 mM), NaCl (100 mM), $MgSO_4$ (8 mM), gelatin, 0.01% (w/v)] and kept at 4°C for 2h. After centrifugation at 8000 x g for 10 minutes, the supernatant was filtrated through a sterile 0.45 µm syringe filter. One milliliter of filtrate containing 1.5×10^9 pfu was applied for the preparation of TEM micrographs. Then 0.01 ml of filtrate was poured on a 300 mesh copper grid and stained with 2% (w/v) uranyl acetate. The dried grid was observed by a transmission electron microscope (Philips, CM10) (Amiri Fahliyani et al., 2018; Pedramfar et al., 2017).

3. Results and Discussion

3.1. The macroscopic, microscopic, and biochemical identifications of oral *Streptococci*

The colonies of oral streptococci were observed in the MSA culture media after 24h incubation. The macroscopic examinations of individual colonies on the MSA showed convex shiny blue intermediate colonies of 3.5 mm in diameter. The Gram staining revealed Gram positive streptococci with large and distinctive chains. The biochemical examinations results are shown in Table 1. The macroscopic, microscopic, and biochemical examinations clarified that the isolated sp. from the dental plaque sample was *Streptococcus salivarius*.

3.2. Molecular identification of isolated oral *Streptococcus* sp. using amplification of the specific *gtf*K gene

The electrophoresis of PCR products showed specific DNA bonds with the molecular size between 500 and 600 bp. With regard to the expected size of the *gtf*K gene, 544 bp, (Hoshino et al., 2004), the isolated oral *Streptococcus* from the dental plaque sample

was identified molecularly as *S. salivarius* (Figure 1).

Table 1. The biochemical examination results for isolated oral Streptococcus from dental plaque

Biochemical Examination	Results
Catalase	Negative
Arginine dehydrolase	Negative
Alpha-galactosidase	Negative
Beta-glucuronidase	Negative
Beta hemolysis	Positive
Alkaline phosphatase	Positive
Amino peptidase	Positive
Esculin hydrolysis	Positive
VP	Positive
Acid production from:	
Glycogen	Negative
Inulin	Negative
Sorbitol	Negative
Trehalose	Negative
Ribose	Negative
Lactose	Negative
Mannitol	Negative
Raffinose	Positive
Amidin	Positive

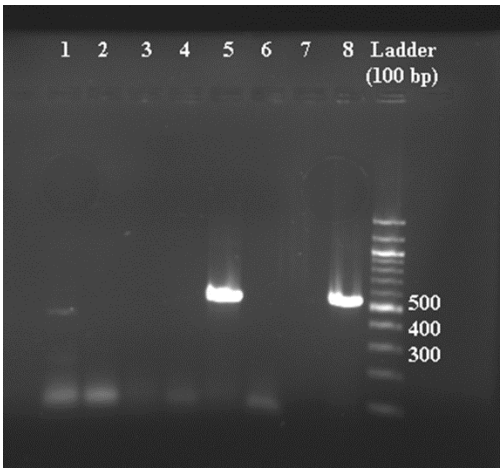

Figure 1. The electrophoresis of the PCR products of *S. salivarius* using the *gtf*K gene. The culumn 5 and 8 were DNA amplified by *S. salivarius* KBM-ISF-2 and *S. salivarius* PTCC 1448 as a positive control, respectively. The column 1, 2, 3, 4, 6 and 7 were DNA amplified by *S. mutan, S. oralis, S. sobrinus, S. sanguis, S. mitis* as a negative control (without the polymerase enzyme), respectively. The molecular size of the related bond was ~544 bp.

3.3. Molecular analysis of the amplified *gtf*K genome of isolated oral *Streptococcus*

The BLASTN of the *gtf*K gene after DNA sequencing reconfirmed that our isolate was *S. salivarius*. The *gtf*K gene of the isolated strain had 100% coverage and 95.92% query coverage with the *S. salivarius gtf*K gene of *S. salivarius* (GenBank accession No. Z11872). So the strain was named *S. salivarius* KBM-ISF-2 and its *gtf*K gene was deposited in the GenBank, NCBI under accession number KJ634208.1. Figure 2 shows the phylogenetic distance tree of *S. salivarius* KBM-ISF-2.

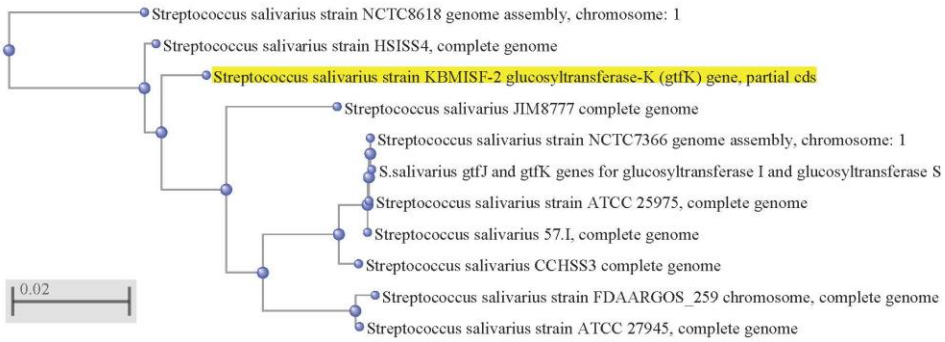

Figure 2. The phylogenetic distance tree of *S. salivarius* KBM-ISF-2. The tree was drown using the NCBI software of "BLAST pairwise alignments". The gtfK gene of *S. salivarius* KBM-ISF-2 had 100% coverage and 95.92% query coverage with the *S. salivarius gtf*K gene of *S. salivarius* (GenBank accession No. Z11872).

3.4. Isolation of specific lytic phages against *S. salivarius* KBM-ISF-2

The overnight incubated BHI containing the *S. salivarius* KBM-ISF-2 and purified specific lytic phages showed no growth of bacterial strain. The overlay method of phage filtrate on the MSA culture media containing *S. salivarius* KBM-ISF-2 showed clear plaques of specific lytic bacteriophages after 16h incubation at 37°C.

3.5. Identification of specific bacteriophages of *S. salivarius* KBM-ISF-2 Using TEM

The TEM micrographs of two purified phage plaques indicated two different lytic bacteriophages against *S. salivarius* KBM-ISF-2. The first lytic phage had icosahedral symmetry with a diameter of 47 nm. The morphological characteristic revealed that the first phage of *S. salivarius* KBM-ISF-2 isolated from the Caspian Sea sample was a member of the *Tectiviridae* family of viruses (Figure 3). The second lytic phage against *S. salivarius* KBM-ISF-2 had icosahedral symmetry with a diameter of 85.56 nm. The morphological characteristic revealed that the second phage of *S. salivarius* KBM-ISF-2 isolated from the Caspian Sea sample was a member of the *Cystoviridae* family of viruses (Figure 4).

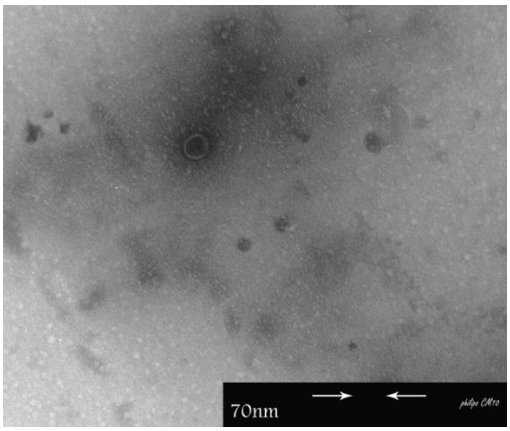

Figure 3. The TEM micrograph of the lytic Tectivirus with a 47 nm diameter isolated from the Caspian Sea sample specific for *S. salivarius* KBM-ISF-2 (Bar = 70 nm).

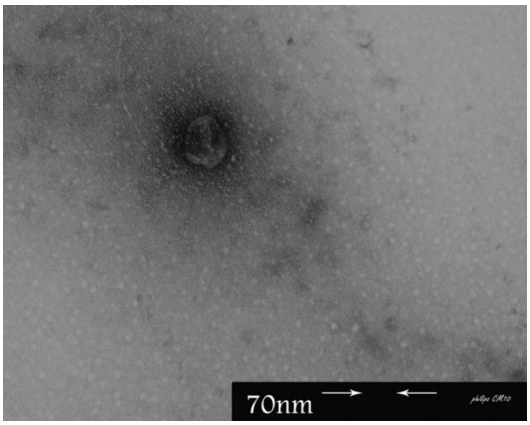

Figure 4. The TEM micrograph of the lytic Cystovirus with a 85.56 nm diameter isolated from the Caspian Sea sample specific for *S. salivarius* KBM-ISF-2 (Bar = 70 nm).

Delisle and Rostkowski (1993) isolated and identified three specific phages of f1, e10 and M102 from human saliva that had lytic effects on *S. mutans*. They reported that those phages were Siphoviruses. While Bacharach et al. (2003) tried to isolate the lytic phages against *S. salvarius, S. mutans* and *S. sobrinus* from human saliva, they were only able to isolated the lytic phages against *Enterococcus faecalis*. C1, as the first lytic phage of group C streptococci, was reported in a study and its genomic sequence was characterized, It was also shown to have a non-contractile short tail and recognized as a Podovirus (Nelson et al., 2003). Some reports described the isolation and identification of PK1, a lysogenic bacteriophage of *S. mutans* PK1, with 95 nm hexagonal heads and 150 nm tails (Higuchi et al., 1982). In this research, we isolated and identified two lytic bacteriophages against *S. salivarius*.

3.6. Phage therapy for oral streptococci

While there are several reports indicating oral streptococci and its impact on dentistry and dental disorders such as caries, gingivitis and periodontal diseases (Franco e Franco, 2007; Okada et al., 2002), only a few suggested potential roles for bacteriophages in the

microenvironment of the oral cavity and their probable functions for phage therapy of oral infections (Beheshti Maal et al., 2015a; Bachrach et al., 2003; Hitch et al., 2004). In this research the potential application of specific lytic bacteriophages against *S. salivarius* as one of the main bacterial spp. responsible for the origination of dental plaque (Van der Ploeg, 2008; Jacques, 1998; Milnes et al., 1993) was investigated.

3.7. The bacteriophages families against oral streptococci and their TEM characterization

There are few reports indicating the isolation of various families of bacteriophages that have lytic effects on oral streptococci spp related to Siphoviruses and Podoviruses (Delisle and Rostkowski, 1993; Nelson et al., 2003). The isolation and identification of a lytic phage related to Cystoviruses with lytic effects on *S. salivarius* from the Persian Gulf have been previously reported by Beheshti-Maal et al. (2010). Those bacteriophages had a hexagonal head and an average diameter of phage particle of ~ 83.33 nm, their morphological characterizations showed they were related to the *Cystoviridae* family of bacteriophages (Beheshti-Maal et al., 2010). In another study, the specific lytic bacteriophages related to Cystoviruses against *S. mutans* have been reported (Beheshti-Maal et al., 2015a). In this study, we isolated two specific lytic bacteriophages against *S. salivarius* KBM-ISF-2 from the Caspian Sea located in northern Iran. The first phage was hexagonal in shape and approximately 85.56 nm in diameter. According to approximate sizes as well as the morphological characterizations of bacteriophages clarified by Ackermann (2007, 2009), we suggested that the mentioned specific bacteriophage was most probably related to the family *Cystoviridae* of bacteriophages. The isolation of this phage from another its water source, the Caspian Sea, could be considered as a confirmation of the previous report. Also, we isolated another specific lytic bacteriophage for *S. salivarius* KBM-ISF-2 from the Caspian Sea whose capsid was hexagonal and approximately 47 nm in diameter. According to approximate sizes as well as the morphological characterizations of bacteriophages clarified by Ackermann (2007, 2009), we suggested that the mentioned specific bacteriophage was most probably related to the family *Tectiviridae* of bacteriophages. So far there is no report indicating that Tectiviruses, as linear dsDNA viruses with an inner lipid vesicle and size of 50-60 nm, could infect Streptococci (Ackermann, 2007, 2009). Beheshti-Maal et al. (2012) isolated and identified two lytic phages against *S. sobrinus*. They showed that those specific phages were related to the *Guttaviridae* and *Cystoviridae* families of bacteriophages.

3.8. The potential applications of phages in controlling infectious diseases

While identifying the role of bacteriophages as therapeutic agents in vivo is challenging, it has been indicated that bacteriophages are promising agents for controlling many infectious diseases, especially those emerged from multidrug resistant bacteria (Sabouri Ghannad and Mohammadi, 2012; Wittebole et al., 2014). Potential applications of phage therapy in bio-controlling of several infectious diseases has been reported in several studies such as gastrointestinal infection of *Escherichia coli* (Drozdova et al., 1998; Marks and Sharp, 2000; Smith et al., 1987), curing of *Acinetobacter baumanii* and *Pseudomonas aeroginosa* in patients with grafts and skin burns (Soothill, 1994, 1992), treatment of bovine mastitis (Amiri Fahliyani et al., 2018), and also in environmental issues such as the phage therapy of corrosion-producing bacteria (Pedramfar et al., 2017). The results of this research in isolating and evaluating the lytic effects of Tectivirus and Cystovirus against *S. salivarius* support our proposal that these lytic specific bacteriophages could be potentially

applied as a therapeutic measure to prevent infectious diseases in the human oral cavity.

4.Conclusion

This study reported the isolation and identification of two lytic bacteriophages from Caspian Sea water that had lytic effects against *S. salivarius* KBM-ISF-2. They were related to the families of *Cystoviridae* and *Tectiviridae,* respectively. Although there are few documents in the literature indicating the isolation of *S. mutans* lytic bacteriophages from different resources (Beheshti Maal et al., 2015a; Armau et al., 1988; Delisle and Rostkowski, 1993) the trials for isolation and purification of lytic bacteriophages against other oral streptococci have been unsuccessful (Bacharach et al., 2003). These bacteriophages could be practically applied as therapeutic agents in encapsulated liposome or might be added to drinking water (Colom et al., 2015). Therefore, it could also be suggested that the lytic bacteriophages of oral streptococci, such as *S. salivarius* KBM-ISF-2, could be applied for preventive measures of dental disorders using dentifrices, oral rinsing solutions, or as additive of drinking water.

Conflict of interest

The author declare that he has no conflict of interest.

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Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors. This article does/does not contain any studies with human participants or animals performed by any of the authors.

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