CHARACTERIZATION OF VIRULENT BACTERIOPHAGES FOR *STREPTOMYCES GRISEOFLAVUS* ISOLATED FROM SOI

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ABSTRACT

Streptomyces griseoflavus and two of its specific phages were isolated from free clay soil samples obtained from Al-Sharkia Governorate, Egypt. *Streptomyces* phages were detected after their enrichment by the spot test. The specific phages were isolated by the single plaque isolation technique; propagated by the liquid enrichment method and purified by the polyethylene glycol, dextran sulfate two phases system. The phages were signed as S_1 and S_2, referring to the genus *Streptomyces*. S_1 phage appeared with head (91 x 66 nm) and long contractile tail (158 x 66 nm) and S_2 phage had isometric head (112 nm) and a long non-contractile tail with length of about 200 nm. The phages formed plaques differing in their sizes 3.0 and 7.0 mm for S_1 and S_2 phages, respectively. UV-absorption properties of the phages were determined. Maximum absorption (A_{max}) of S_1 and S_2 phages was at 275 nm while their minimum absorption (A_{min}) was at 245 nm for S_1 phage and 250 nm for S_2 phage. The ratio between $A_{max/min}$ was 1.407 for S_1 phage and 1.553 for S_2 phage and the ratio between $A_{280/260}$ was 1.186 and 1.371 for S_1 and S_2 phages, respectively. The host ranges were investigated with 5 species of *Streptomyces.* Results showed that S_1 phage, differ from S_2 phage as it did not infect *S. noboritonesis* and *S. lincolnesis* in contrast with S_1 phage, which infect all the tested *Streptomyces* species. The antisera specific for S_1 and S_2 phages were prepared and serological relations were studied. Serological data revealed that, the two phages were serologically related to each other

INTRODUCTION

The actinomycetes comprise an order of bacteria, which exhibit wide physiological and morphological diversity. The majority of species are aerobic, saprophytic, mesophilic forms whose natural habitat in soil (Lechecalier and Pine, 1977). It was reported that actinomycetes and in particular strains of *Streptomyces* were the source of useful and consequently profitable antibiotics and antiviral directed unprecedented attention to this genus (Mansour *et al*., 1988). *Streptomyces* are also known as producers of many secondary metabolites, such as antibiotics and other bioactive compounds (Williams *et al*., 1989)

Phages attack actinomycets commonly known as actinophages, since they were first isolated; many such phages have been reported, particularly for members of the genus *Streptomyces* (Matthews, 1982). Actinophages have been isolated and investigated for reasons, including the problems cause in the fermentation industry and as a taxonomic tool for typing of streptomycetes (Wendisch and Schneider, 1992). Many *Streptomyces* phages have been isolated from soil (Onanong *et al*., 2004) while Actinophages and phages were obtained by enrichment procedure. The phages were plaque purified and morphologically

examined under a transmission electron microscope. All actinophages possess tails and contain double-strand DNA. They comprise six morphological types (Ackermann *et al*., 2000) and belong to three families, *Mycoviridae, Siphoviridae* and *Podoviridae* (Matthews, 1982).

Bacteriophages consist of nucleic acid packaged in a protective shell composed of proteins. Therefore, antibodies can be raised against this antigenic material for producing antibodies, concentrated purified phage particles were injected into experimental animals (rabbits). Richard *et al*., (1969) showed that phage specific antisera obtained to be used in different immunological technique as described by Anna *et al*., (1990). Pringsulaka *et al*., (2004) prepared antiphage sera specific for actinophages to study their relatedness or diagnosis. Onanong *et al*., (2004) prepared antisera against 21 *Streptomyces* phages and investigated the serological relevance between selected phages and antiserum. It was found that some phages showed closely related group patterns and some showed moderate serological relevance to antiserum while others were slightly related to each other.

Based on the little knowledge about the actinophages in Egypt, the present study aimed to detect the presence of phages specific for actinomycetes (actinophages) isolated from an Egyptian soil followed by identification of such actinophage(s).

MATERIALS AND METHODS

Collection of soil samples: Soil samples were collected from different regions of Hehia, Minia El-Kamh, Kafr Saker and Abo Kabir, El-Sharkia Governorate, Egypt. Soil samples were directly taken from rhizosphere zones around roots of cultivated plants into clean plastic bags. The collected soil samples were sieved to remove various contaminant materials, then part was kept in plastic bags for isolation of phages and other was used for isolation of actinomycetes.

Isolation of actinomycetes: Actinomycetes were isolated and counted using plate count technique (Clark, 1965). The total viable counts of different soil microflora were determined. Soil samples were air dried and mixed with calcium carbonate ($CaCO_3$) before plating. A suspension of the soil sample was prepared by shaking 10 grams of the soil in 90 ml sterile distilled water and then stirred for 15 minutes until a soil suspension was obtained. Different serial dilutions from each soil sample were prepared. One ml aliquot of each dilution was transferred aseptically to a Petri dish containing 15 ml of starch nitrate agar medium (Mariana and Denitsa, 2003). The dishes were rotated by hand to ensure homogenous distribution of soil suspension with the agar medium. The plates were then incubated for 7 days at 28°C. After incubation, the plates were counted to determine colony-forming unit per gram soil (cfu/g). Actinomycete isolates were picked and purified by repeat streaking and maintaining on starch casein agar medium (Mariana and Denitsa, 2003).

Identification of *Streptomyces* isolates:

Spore chain morphology: The direct microscopic method of agar cultures described in the International *Streptomyces* Project (I.S.P) (Shirling and Gottlieb, 1966) was used to identify the isolated *Streptomyces*.

Spore surface: The spore-print technique described by Tresner *et al*., (1961) was applied on the prepared materials for electron microscopy to observe the mature sporephores and spore chains.

Cultural properties: The cultural properties expressed as color of sporulated aerial mycelium, color of reverse side of colony and production of diffusible pigments were

determined for 14 days-old cultures maintained on four media as described by Shirling and Gottlieb (1966).

Physiological properties: Producing of melanoid pigments, gelatin liquefaction, hydrogen sulphide production, nitrate-reduction and detection of amylase were carried out as reported by Gottieb (1974) and Mariana and Denitsa (2002) for determination of the physiological properties of the selected *Streptomyces* isolate. In addition, the abilities to utilize different carbon sources such as filter-sterilized D-Glucose, D-Fructose, D-galactose, Sucrose, D-xylose, L-Arabinose, L-rhamnose, Raffinose, D-Manitole and I-Inositol at concentrations of 1% were also tested.

Preparation of indicator strain for virus assaying: *Streptomyces* sp. cultures used for the background growth were grown in 250 ml Erlenmeyer flasks and incubated under shaking conditions at 28-30°C for 72-96 h. *Streptomyces* was prepared at density of 10^8 cell/ml of liquid medium before using.

Isolation of *Streptomyces* phages: Isolation of phages was carried out according to Dowding (1973) with some modifications as following: Spores (10^7cfu/ml) of streptomycete hosts and twenty-five grams of soil sample were inoculated into 250ml Erlenmeyer flasks containing 50ml of nutrient broth and incubated at 200rpm on a rotary shaker for 72h at 30°C. The suspension was clarified by centrifugation for 30min at 3000rpm. Chloroform was added to the supernatant with a rate of 1/10 (v:v) with vigorously shaking and phages were obtained from the upper layer. The presence of phages was determined by the spot test. Quantitative assaying of the phages was carried out as described by the soft-agar overlay (double layer) method as reported by Onanong *et al*., (2004). A single plaque was picked up using a sterile needle and immersed into 2 ml of liquid broth. Subsequently, 10 ml of the original host spore suspensions were added and incubated for 72 h. Plaque formation was assayed and the purification step was repeated 3 times until a high degree of plaque purity was achieved.

Preparation of high titer phage lysate: High titer phage stock of the actinophage lysate was obtained using the culture method as following: Erlenmeyer flasks (250 ml) containing 100 ml of liquid culture medium were prepared and each flak was inoculated with a loop full of appropriate *Streptomyces* sp. After 72-96 h incubation at 28-30°C under shaking, phage particles (10^9pfu/ml) were added in a ratio of (1:10, v/v) to each flask. Flasks were then incubated at 28-30°C, after incubation, cultures were centrifuged at 6000 rpm for 15 minutes and then chloroform was added to the supernatant (1:10, v/v). The mixture was vigorously shaked for 3-5 minutes and left to clarify for 30 minutes. The suspension containing phage was then transferred into sterile flasks and stored at 4°C with traces of chloroform.

Purification and concentration of the isolated actinophages: Dextran sulfate-polyethylene glycol two phase liquid system was used (Othman, 1997), 222.3, 0.48, 15.8 and 4.2 grams of phage lysate, dextran sulfate 500, polyethylene glycol (PEG 6000) and NaCl, respectively, were mixed in a separating funnel to give a mixture containing ratio 6.5%, 0.2% and 1.7% (w:w) respectively. After mixing, the funnel was allowed to stand at 4°C overnight. A heavily turbid bottom layer was slowly collected into a clean tube and centrifuged at 2000 rpm for 10 minutes. The clear top and bottom phases were removed by pipette and the remaining interface “cake” was suspended in 2.5 ml of a 1% (w:w) dextran sulfate solution. Then 0.15 ml of a 3 M KCl was added to each ml of suspension, the mixture was allowed to stand for 2 h, at 4°C and centrifuged at 2000 rpm for 10 minutes. After centrifugation, the supernatant containing phages was obtained and dialyzed against saline solution (0.85%

NaCl) at 4°C for 48 h. The phage suspensions were then centrifuged at 15000 rpm for 2 h at 4°C, and the supernatants were discarded and the pellets were re-suspended in a saline solution and then assayed.

Ultraviolet extinction spectra of purified phages: Purified phage preparations were diluted and measured in the range of 230 to 300 nm (Unico-U.V. 2100 spectrophotometer) in order to determine the optical properties, evaluate purity and yield of the purified phages.

Electron microscopy: According to El-Tarabily *et al*. (1995), phage particles were examined. A drop of each phage suspension (10^7 pfu/ml) was placed on 200-mesh copper grids with carbon-coat formvar films and the excess was drawn off with filter paper. A saturated solution of uranyl acetate was then placed on the grids and the excess drawn off as before. Specimens were observed with a Jeol-1010 Transmission Electron Microscope.

Host range: In this experiment, a number of five *Streptomyces* sp. obtained from Botany and Microbiology Dept., Faculty of Science, Al-Azhar University, Cairo, Egypt were used. The host range of the phages was studied by spotting 0.2 ml of phage suspensions containing 10^8 pfu/ml on to agar plates each previously seeded with suspension (10^6 cfu/ml) of one of the type strains. The plates were then incubated for 48 h at 28°C and examined for lysis (El-Tarabily *et al*., 1995).

Preparation of the antisera specific to actinophages: Two rabbits were used for each phage isolate to prepare the phage specific antisera prior to rabbit immunization. Normal serum was taken as a control (normal serum) before immunization. The purified phage (10^{12} pfu/ml) and freunds adjuvant were mixed to make emulsion. The rabbits were immunized by injection subcutaneously two times a week for 3 consecutive weeks followed by injection in the leg muscle one week later. Sera have collected by bleeding the rabbits one-week after the last injection (Onanong *et al*., 2004). Ouchterlony double diffusion test was used as described by Watter (1976) to investigate the specification between phages as antigens and their resulted antisera.

RESULTS AND DISCUSSION

Occurrence of phage specific for *Streptomyces* in clay soil samples: As shown in Table –1, phage specific for *Streptomyces* presented in some free soils of Al-Sharkia Governorate (Hehia, Menia El-Kamh and Abo Kabier) when the phages were assayed qualitatively by the spot test. Total number of actinomycetes in the tested soils ranged from 3.9×10^3 to 7×10^3cfu/g, total number of bacteria ranged from 2.5×10^2 to 8.0×10^4cfu/g and total number of fungi ranged from 2.5×10^2 to 8.0×10^2cfu/g. Obtained data indicated that no relation observed between the number of the main host of phage or the microorganisms related to them and the presence of phages. Data also indicated that no relation observed between the presence of *Streptomyces* phage and pH of the soil. The predominant *Actinomyces* genus in soil, are readily detected and it appears that streptomycete phages are widespread in the soil environment

Table-1: Occurrence of phage specific for *Streptomyces* in some clay soil samples obtained from different regions of Al-Sharkia Governorate, Egypt.

Soil samples	Locations	pH	Total count of bacteria* ($x10^4$)	Total count of fungi ($x10^2$)	Total count of action-mycetes ($x10^3$)	Presence of phages
1	Hehia	8.0	5.5	8.0	7.0	+
2	Menia El-Kamh	8.5	2.5	5.0	4.5	+
3	Awlad Sakr	8.6	8.0	2.5	6.4	-
4	Kafr Sakr	7.8	2.9	8.0	5.0	-
5	San El-Hagar	7.0	2.5	4.0	3.0	-
6	Abo Kabier	7.7	6.5	7.0	4.3	+
7	Abo Kabier	8.4	4.5	6.0	3.9	+
8	Abo Kabier	7.9	8.0	8.0	4.4	+
9	Al-Zakazik	7.9	2.5	5.0	4.5	-

*** cfu/ml. +: positive. -: Negative.**

Isolation and identification of *Streptomyces:* Four isolates of actinomycetes were isolated from the soil and signed A, B, C and D. The four actinomycetes were tested for their ability to phage infection by using them individually as an indicator to detect the actinophages in the crude phage suspensions prepared directly from soil samples. Actinomycete A was the most sensitive one to actinophages and its lysis occurred by crude actinophages prepared from five out of nine soil samples. The most sensitive isolated actinomycete A was completely identified as a main host to actinophages.

According to the color of sporulated aerial mycelium the isolate follows gray series. Results in Figure -1 indicated that the mature sporophores and spore chains are retinoculiaperti; where it consists of short spirals and often form loop and the spore surface was spiny. The isolate was characterized by gray aerial mycelium formed on the tested media. Reverse side of colony colored by green to yellow on oat meal agar, grayish on starch-nitrate agar and yellow on Czapek's solution.

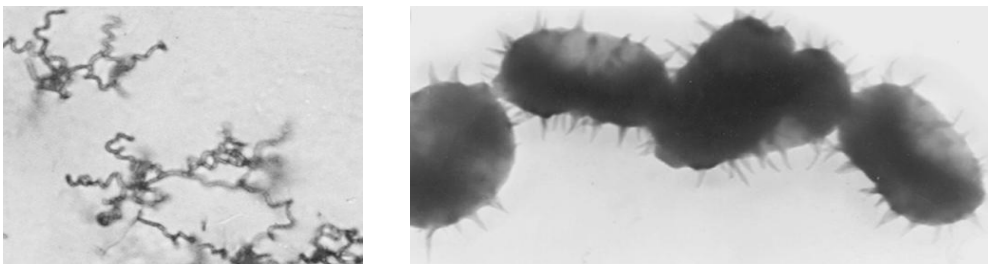

Figure-1: Mature sporophores and type of *Streptomyces* spore chains (left) and *Streptomyces* spore with spiny surface (right).

The isolate was able to utilize D-glucose, D-Fructose, D-Galactose, D-Xylose, L-Rhamnose, D-Manitole and I-Inositol if compared with medium free from carbon source. No production of melanoid pigment was detected on any of the standard media used. The isolate showed liquefication of gelatine, hydrolysis of starch, coagulation of milk but was unable to produce H_2S and reduction of nitrate. The optimum growth temperature of isolate was 30°C. The *Streptomyces* can tolerate NaCl reaching to about 7%, the isolate showed antimicrobial potentialities against *Bacillus subtilis, Staphylococcus aureus* and not *Aspergillus niger*. Following the diagnostic key of Bergey's Manual (1989). It has been found that the isolate under study (indicator) gave homogenous to the isolate *Streptomyces griseoflavus*, so it can be classified as *S. griseoflavus* Sha I (where Sha I is abbreviation to El-Sharkia Governorate the source of soil of the isolate).

Isolation of some phages specific for *Streptomyces:* Isolation of phages was carried out by some authors (Diaz *et al*., 1989, Godarny *et al*., 1996, Pringsulaka *et al*., 2004) from most soils of pH higher than 5.0 either directly or after a short period of enrichment by incubation with freshly germinated *Streptomyces* spores in a conventional soft-agar overlay containing spores of an indicator strain and plaques were usually seen after overnight incubation at 28-30°C. Balan and Padilla (1973) also isolated *Streptomyces* phages by the specific enrichment liquid method when 2 g of soil sample was enriched by incubating with 50 ml (10^7cfu/ml) of *Streptomyces* sp. for 60 h at 30°C. Dowding (1973) also isolated group of 28 phages acting on *S. coelicolor* by the specific enrichment technique.

In this study, crude phage suspension prepared from the free soil from Hehia region was assayed qualitatively by the over-layer agar technique. Single plaques from Petri dishes (Fig. -2) resulting from the high dilutions of phages (10^{-6}) were selected and picked up based on their morphology (size and shape). Three plaques with diameters of 3.0 and 7.0 were chosen and signed as S_1, and S_2, respectively. Each one was added to 3 ml of liquid culture of *Streptomyces* (10^8cfu/ml) and incubated at 30°C for 96 h, then, phage lysates were prepared and assayed quantitatively.

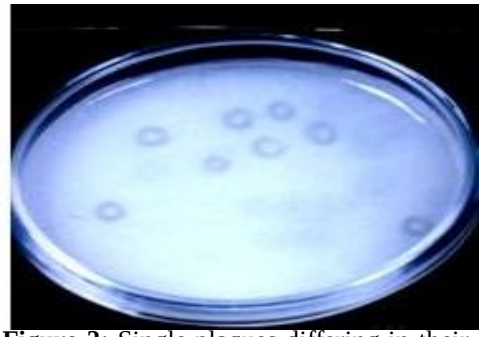

Figure-2: Single plaques differing in their sizes resulting from 10^{-6} dilution by overlayer agar technique.

Propagation,purification and concentration of *Streptomyces* phage: The two isolated *Streptomyces* phages (Figure-3) were propagated by the liquid culture method. Phages were added to the main sensitive host (*S. griseoflavus* Sha I) in 1000 ml Erlenmeyer flasks with ratio of 1:10 (v/v) and incubated at 30°C for 7 days. The propagated phages (1200 ml of each) were purified by dextran sulfate-polyethylene glycol two phases system. As shown in Figure-4, turbid layers were precipitated in the separating funnel with 90 and 80ml in case of S_1 and S_2 phages. The cake layer containing the phage (Figure -4B) was 29 and 26 ml in case of S_1 and S_2, respectively. Phages in the cake layers were separated and concentrated by high-speed centrifugation

(15000rpm for 2h). Phages were assayed quantitatively after concentration, and their titers were about 10^{12} and 10^{11}pfu/ml for S_1 and S_2, respectively.

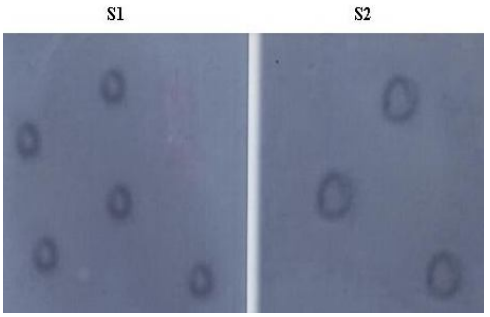

Figure- 3: Plaques formed by *S. griseoflavus* S_1 and S_2 phages.

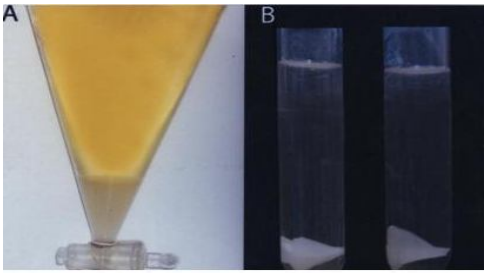

Figure-4: Purification of *S. griseoflavus* phage with dextran sulfate-polyethylene glycol two phases system. A) Turbid precipitate in separating funnel containing the phage particle, Intermediate phase (cake) containing the phage particles.

Brownell and Adams (1967) found that young cultures gave the best yields of phage and the maximum yield of phage was dependent upon the absolute number of host organisms, the growth stage of the host and ratio of phage to host. Anné *et al*. (1984) prepared high titer phage suspension by the specific liquid enrichment method.

UV-absorption properties of *Streptomyces* phages: Purified preparations of S_1 and S_2 phages were scanned in unico-UV, 2100 spectrophotometer in-between 230 to 300 nm to determine the UV-absorption properties of *Streptomyces* phages and also to evaluate the purification process of phages. Data in Table -2 showed that A_{max} of S_1 phage was at 275 nm, A_{min} was at 245 nm, $A_{280//260}$ was 1.186, $A_{260/280}$ was 0.842 and $A_{max/min}$ ratio was 1.407. For S_2 phage, A_{max} was at 275 nm, A_{min} was at 250 nm, $A_{280/260}$ was 1.371, $A_{260/280}$ was 0.729 and $A_{max/min}$ was 1.553. Comparing with ratio of $A_{260/280}$ and ratio of $A_{max/min}$ for S_1 and S_2 phages, it was noted that S_1 phage ratio of $A_{260/280}$ was higher than S_2 phage. It was also noted that the ratio of $A_{max/min}$ of S_2 phage was higher than S_1 phage indicating that it contained higher nucleic acid.

Table-2: Extinction spectra of S_1 and S_2 actinophages.

Purified phages isolates	UV ratios				
	Maximum (nm)	Minimum (nm)	$A_{max/nim}$	$A_{260/280}$	$A_{280/260}$
S_1	275	245	1.407	0.842	1.186
S_2	275	250	1.553	0.729	1.371

Morphology of *Streptomyces* phages: Some actinophages have a long, flexible, non-contractile tail. The phage was found to have a hexagonal head of length 55 nm and a tail 220 nm in length (Dowding, 1973). Anné, *et al*., (1984) stated that all the phage heads were of an icosahedral form observed by electron micrographs, but head size and length of the tail varied. Balan and Padilla (1973) reported that electron microscopy of purified *S.* virions revealed that phages have icosahedral heads of 60-70nm in diameter, and tails of 290-300nm in length.

In this study, purified, concentrated phages specific for *Streptomyces* (S_1 and S_2) were negatively stained with uranayl acetate and examined by transmission electron microscope to determine the

morphotype of phages. As shown in Figure -5, the particles of S_1 phage appeared with head and long contractile tail. Diameter of the head is about 91 x 66 nm and the tail has length of about 158.33nm and its width of about 1.66 nm. Particles of *Streptomyces* S_2 phage appeared as well as S_1 phage like tadpole shape, but having isometric head with diameter of about 112.5 nm and tail with length of about 200nm and width of about 2.5nm.

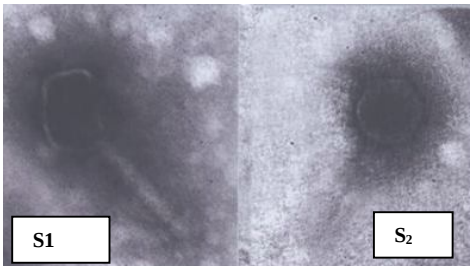

Figure -5: Electron micrographs show the morphology of S_1 and S_2 phages.

Host range of *Streptomyces* phages: Host range specificity of streptomycete phages infected only *Streptomyces* sp. could be applied to be as a tool for identification of streptomycete strains. Some phages had a very broad host range and the other displayed a narrow host range (Onanong *et al*., 2004 and Anné *et al*. (1984).

In this investigation, host range of S_1 and S_2 phages specific for *S. griseoflavus* was determined against five species of *Streptomyces* (Table -3). Results showed that both S_1 and S_2 phages has large host range on the level of the species which they reacted positively resulting lysis with *S. antibiotics, S. meiolani, S. gresiofulum and S. minutiscleroticus*. S_1 phage reacted with *S. lincolneisis* and *S. noboritonesis* whereas S_2 phage failed to react positively with them. That means that the two phages differ in their lytic patterns.

Table-3: Lytic patterns of the isolated phages specific for *S. griseoflavus*.

Host range	Spot test results*	
	S_1 phage	S_2 phage
S. antibiotics	+	+
S. mediolani	+	+
S. lincolnesis	+	-
S.minutiscleroticus	+	+
S. noboritonesis	+	-
S. griseoflavus	+	+

+: positive -: Negative * Results of three replicates for each treatment**.**

Serological properties of *S. griseoflavus* phages: Ouchterlony double diffusion test is one of the most widely used technique for phage assay, detection and diagnosis, it has the advantages of simplicity, low amount of antigen used provides information about homogeneity and purity of the viruses. As shown in Figure -6, the immunodiffusion test revealed that the antiserum prepared to S_1 phage reacted with the two antigens: S_1 and S_2 while the antiserum prepared to S_2 phage reacted with the two antigens. It means that the two phages are serologically related to each other.

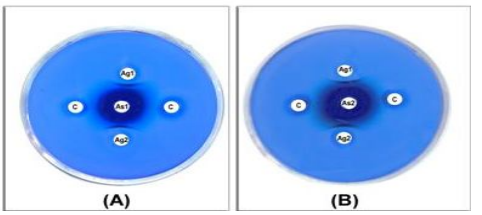

Figure-6: Serological detection and relation of *S. griseoflavus* viruses by immunodiffusion tests. Central wells contain antisera to S_1 phage (A) and S_2 phage (B). Peripheral wells contain antigens (S_1 and S_2 phages).

Mathews (1992) reported that, the advantages of using serological techniques, when antisera can be stored and tests made over periods of year in different laboratories.

The specificity of the reaction allows detection of antigen even in the presence of the host materials. Results can be obtained with in short time comparing with infectivity assay, which requires few days. Small volumes of antiserum and phage suspension are sufficient.

The concentration of S_1 and S_2 phages specific for *S. griseoflavus* was determined serologically by the immunodiffusion test as showed in Figure -7. Serial two fold dilutions of purified virus suspension was prepared and placed in the wells, 0.9 cm distance from the central well containing the specific antiserum, the antigens S_1 and S_2 diffuse from the peripheral wells towards the antisera of S_1 and S_2 phages, respectively. The precipitation bands were observed at the position where the lines meet after 24 h, the highest dilution at which precipitation occurred was 1/128 and 1/16 for S_1 phage and S_2 phage respectively.

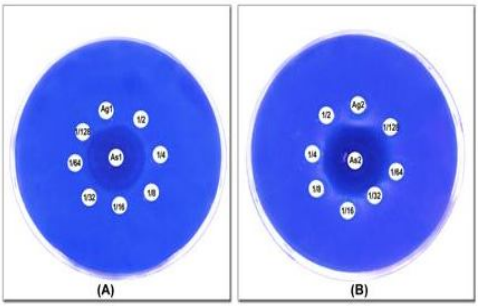

Figure-7: Serological determination of the phage concentration by immuno-diffusion test. Central wells contain antisera of S_1 phage (A) and S_2 phage (B). Peripheral wells contain concentrated phage 1/2, 1/4, 1/8, 1/16,1/32,1/128 dilutions (S_1 and S_2 phages).

Kurp and Heinzen (1978) prepared antiserum for *Thermoactinomyces* and *Micropolyspora* phages by immunizing the rabbits subcutaneously weekly for 6 weeks at the presence of the incomplete freund's adjuvant. It was mentioned that the cross neutralization tests showed complete non-relatedness between *T. candidus* phage φ11SA and *M. faeni* phage φ-ISOA. Those antigens (phages) were useful in detecting antibodies in patients' sera and hence are important in the diagnosis of the disease. In the present work phages were related to each other.

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