



ISSN: 2319-9490

## RESEARCH ARTICLE

# IDENTIFICATION OF PHAGE THERAPY CANDIDATES AGAINST *STAPHYLOCOCCUS AUREUS* ISOLATED FROM COMMONLY USED VEGETABLE GARNISHES

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Received 21<sup>st</sup> March, 2018; Accepted 13<sup>th</sup> April, 2018; Published 18<sup>th</sup> May, 2018

### ABSTRACT

Vegetable food garnishes are used for the decoration purposes and that too, most often, in an uncooked form. This increases the chances of contamination of the food items that they are added to. This research work was performed to isolate *Staphylococcus aureus*, which is known to widely cause food borne infection and food spoilage, from six different vegetable garnishes. Of the six vegetable food garnishes analysed, four were found to regularly harbour *S. aureus* as a contaminant. *S. aureus* has been known to cause food poisoning as well as food spoilage and in case of the former, the course of treatment would involve being prescribed an antibiotic. However, these organisms have been found to be developing antibiotic resistance due to non-adherence to the prescribed time course of antibiotics and rampant use of antibiotics. This has led to wide-spread development of antibiotic resistance in recent times. To check if the isolated *S. aureus* is resistant to any of the antibiotics, antibiotic sensitivity test was performed. It was found that the tested *S. aureus* isolates were resistant to at least one of the tested antibiotics. As the organism was found to have been developing antibiotic resistance, an alternative wherein the chances of developing antibiotic resistance is the least, is the use of bacteriophages to combat organisms like *S. aureus* specifically, without any harm to human beings. This therapy is referred to as phage therapy. To isolate phage therapy candidates, we have isolated bacteriophages from sewage samples against the *S. aureus* isolates obtained in this study. The cultivated and purified phage therapy candidates were found to be effective against not only their parent *S. aureus* isolate but also against non-parent *S. aureus* isolate, thus, proving to have better range and effectiveness as phage therapy candidates. Thus, our studies have shown a novel use of bacteriophages against pathogenic and/or food spoilage causing *S. aureus* present in food garnishes as a method of phage therapy.

**Key words:** *Staphylococcus aureus*, phage therapy, antibiotic resistance, vegetable garnishes.

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**Citation:** Payel Sarkar, Sudeepthi, V., Sugata Bhowmick and Swathi B., 2018. "Identification of phage therapy candidates against staphylococcus aureus isolated from commonly used vegetable garnishes" *International Journal of Current Research in Life Sciences*, 7, (05), 1989-1993.

### INTRODUCTION

Garnish is a substance which is added to a food to enhance its texture, appearance, color or taste. Food garnishing can be as simple as a single fruit slice or as complicated as sculpture carved from vegetables and fruits. Fresh fruits, herbs, vegetables and edible flowers add color to food and tempt the appetite. Food garnishes can also be non-edible. They are items which do not play any major role with the rest of the ingredients. The non-edible garnishes are usually used only for the purpose of decorating food and to increase the visual impact and presentation. Our focus of study would be the edible vegetable garnishes from a microbiological point of view. Usually food garnishes are added onto the food without cooking thus they can carry numerous microorganisms and have the potential to cause food spoilage and/ or food poisoning.

Staphylococcal food-borne disease, caused by *Staphylococcus aureus*, is one of the most common food-borne diseases worldwide. However widespread overuse of antibiotic has provoked an extraordinary increase of multi antibiotic resistant *S. aureus* strains. Few options to treat these multidrug-resistant bacteria are: 1. Decontamination by using natural products such as vinegar, salt, organic acids etc. (Sarkar et al., 2017), or 2. Phage therapy by using bacteriophages and their product as bioagent and used for bacterial infection treatment. (Matsuzaki Shigenobu et al., 2005). Phage therapy is the natural use of lytic phage for the therapeutic use of pathogenic bacteria. Bacteriophages can lyse only specific range of host or bacteria and they have less harm to the normal human body flora whereas commonly used antibiotic destroy normal gastrointestinal flora as well. (Havelaarand Hogeboom,1984; Mahadevan et al. 2009). The objective of this study was to isolate *Staphylococcus aureus* from commonly used vegetable garnishes and to identify phage therapy candidates against those *S. aureus* isolates.

Following isolation and characterization of *S. aureus* from the vegetable garnishes analyzed in this study, antibiotic

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sensitivity of the isolated *S. aureus* was also determined. As an alternative to antibiotic therapy against these potentially pathogenic and/or food spoilage causing isolates, we attempted to isolate and identify bacteriophages as phage therapy candidates. For this, we isolated bacteriophages against the isolated *S. aureus* from sewage samples. These bacteriophages were purified, cultivated and re-purified, and then analyzed for their effectiveness against both parent *S. aureus* isolates and non-parent *S. aureus* isolates. Thus, our studies have shown a novel use of bacteriophages against *S. aureus* present in food garnishes, with the potential for causing food poisoning and/or food spoilage.

## MATERIALS AND METHODS

**Sample collection:** A total of 18 samples of 6 different kinds of vegetable garnishes (chilli, coriander, curry leaves, mint, onion, spring onion) were purchased from different places in and around Bangalore, India. Of these 3 samples for each kind of garnish, one sample was obtained from Hebbal market, another sample was from backyard of house garden in Srirampuram and the third sample was obtained from street vendor at Rajajinagar, all different localities in Bangalore. Approximately 50-100g of unpacked garnish samples were collected in sterile aluminium foil pouches. Samples were taken to the laboratory and analysed as soon as possible.

**Isolation:** The isolation procedure was carried out in laminar air flow (LAF) chamber. The required glasswares and media were autoclaved. BPA – Baird Parker Agar (HiMedia – M043) was used for the isolation of *Staphylococcus aureus* from the garnishes. The media was poured on to the sterile petriplates and allowed to solidify. The petriplates were labelled and each plate was made into 6 divisions. On each petriplate 6 different samples of the selected food garnishes were plated. The samples were plated onto the plates containing the media by using sterile forceps. The plates were incubated at 37°C for 24 hours and the plates were observed for colonies. The colony morphology of each plate was noted down and the results were tabulated.

**Subculturing and Characterization:** The colonies were selected and picked up from BPA media and streaked onto LB – Luria Bertani (HiMedia – M1151) agar slants under aseptic conditions for subculturing. Characterization was done using Gram staining, catalase test and mannitol salt test following standard protocol.

**Antibiotic Sensitivity Test:** Antibiotic sensitivity test was performed for the isolated *Staphylococcus aureus*. The commercially available antibiotic discs that were used were: Amoxycylav - HiMedia – SD063 – 30mcg/disc, Amphotericin - HiMedia – SD111 – 100 units/disc, Ampicillin – HiMedia – SD002 – 10mcg/disc, Bacitracin – HiMedia- SD003 – 10mcg/disc, Chloramphenicol - HiMedia – SD006 - 30mcg/disc, Erythromycin – HiMedia –SD013 - 10mcg/disc, Kanamycin – HiMedia – SD223 – 5mcg/disc, Nystatin – HiMedia- SD271 -50mcg/disc, Penicillin-G HiMedia - SD028- 10units/disc, Streptomycin – HiMedia – SD031 – 1VL – 10mcg/disc, Tetracycline – HiMedia – D037 – 30mcg/disc. LB agar medium was prepared and autoclaved, poured onto sterile petriplates and allowed to solidify. The test organism was picked and swabbed onto the media. Antibiotic discs were placed onto the media under aseptic conditions and incubated for 24 hours at 37°C and the plates were observed for zone of

inhibition. The zone of inhibition was measured and the results were noted down and tabulated.

**Bacteriophage Isolation and Visualization:** For the isolation of the phage, the confirmed organism (*S. aureus*) from 2 different samples, coriander and curry leaves, were taken and the sewage sample was collected from a nearby source. Double agar layer technique was performed for both the samples for each of the confirmed *S. aureus* isolates. 2 ml of sewage sample was incubated with 20 ml of LB broth at 37°C for 2 hours. 1 ml of the sewage- LB broth incubated sample was mixed with 10 ml of soft agar (HiMedia; 50% LB broth mixed with 50% LB agar), mixed and poured over a layer of hard agar (HiMedia LB agar). The plates were incubated at 37°C for 24 hours. Plaques were observed as zones of clearing by holding against light. For better visualization, the plates were stained with crystal violet (Spectrum reagents & chemicals Pvt Ltd). 5 ml crystal violet was poured onto each plate and incubated for 30 seconds, poured off and washed with distilled water. The plaques were visualized as clear zones after drying.

**Bacteriophage Purification:** For purification, unstained isolated plaques were picked up under aseptic conditions by using sterile microtips into 1.5 ml Eppendorf tubes containing 1 ml LB broth (HiMedia). The tubes were vigorously shaken and the contents were filtered through 0.22 µm filter papers (Merck: Durapore PVDF GVWP04700).

**Bacteriophage Cultivation:** 10 ml LB broth was prepared and inoculated with the confirmed organism (*S. aureus*) isolated from coriander and curry leaf samples and incubated at 37°C for 24 hours. For cultivation of the purified plaques, the purified filtrate from the purification step was inoculated into the overnight grown 10 ml *S. aureus* culture. This was incubated at 37°C until the culture gradually decreases in the turbidity due to the lysis of the bacteria by the phage. These isolates were purified by centrifugation of the broth culture at 10,000rpm for 10 mins and filtered using 0.22 µm filter paper (Merck: Durapore PVDF GVWP04700) under aseptic conditions. This filtrate was cultured by double agar layer technique and checked for plaques as mentioned above.

**Cross-parent Analysis of Phage Therapy Candidates:** For the cross-parent analysis of the phage therapy candidates, cultivated and purified phages were used to infect both parent *S. aureus* isolate as well as non-parent *S. aureus* isolate using the double agar layer technique as described above. The plaques were visualized by crystal violet staining as mentioned above.

## RESULTS AND DISCUSSION

**Several of the vegetable food garnishes screened were found to contain *Staphylococcus aureus***

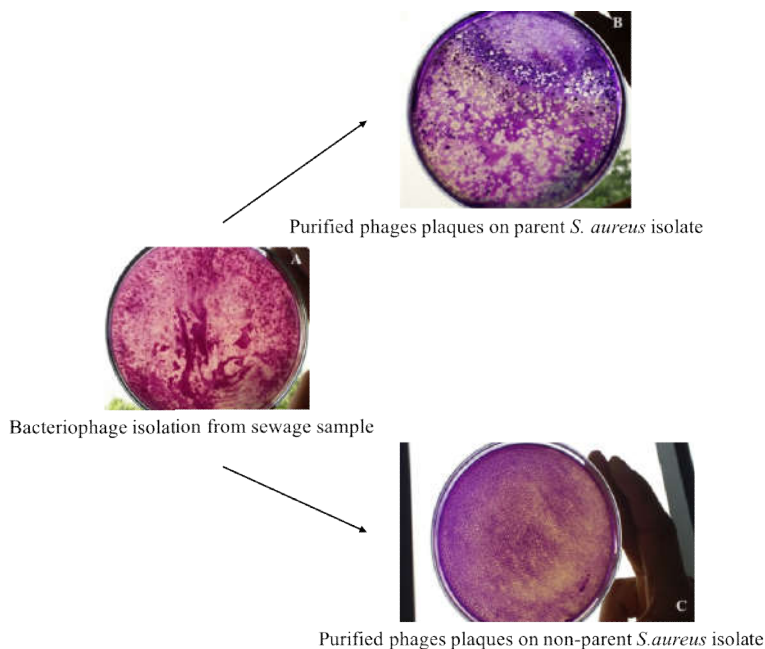
Food garnish studies done by far have shown that many food garnishes harbor one or more potentially pathogenic and spoilage causing organisms (Oluwafemi F, *et.al* 2013., Elviss, *et.al* 2009., Sarkar *et.al.*2017). Six vegetable food garnishes were screened for the bacterium, *S. aureus*, which has the potential to cause food spoilage and food-borne infections. Three samples of each of the six vegetable food garnishes (Table 1) were analyzed for the presence of *S. aureus* on Baird Parker medium. Post incubation, four of the food garnishes used in this study showed colonies on the BPA medium. Of the six garnishes analyzed, all samples of chilli and onion showed

**Table 1. Vegetable garnishes used for isolation of *S. aureus*. The scientific names, sources of the samples and positive results identified as black colonies on Baird Parker Agar (BPA) plates have been shown**

Sl. No.	Food Garnish	Scientific Name	Positive on BPA medium		
			Sample 1 (Street side vender)	Sample 2 (Market)	Sample 3 (Home garden)
1	Chilli pepper	<i>Capsicum Annuum</i>	(No growth)	- (No growth)	- (No growth)
2	Coriander	<i>Coriandrum Sativum</i>	+ Black, Shiny, Irregular, Opaque)	+ (Black, Round, Shiny, Opaque) (Black, Irregular, Shiny, Opaque)	+ (Black, Round, Irregular, Opaque)
3	Curry leaves	<i>Murraya Koenigii</i>	+ (Black, Shiny, Round, Opaque)	- (No growth)	+ (Black, Shiny, Round, Opaque)
4	Mint	<i>Mentha</i>	+ (Black, Round, Opaque) (Black, Irregular, Opaque) (Green, Round, Shiny, Opaque)	+ (Black, Shiny, Round, Opaque) (Black, Shiny, Irregular, Opaque)	+ (Black, Irregular, Opaque)
5	Onion	<i>Allium cepa</i>	- (No growth)	- (No growth)	- (No growth)
6	Spring onion	<i>Allium fistulosum</i>	+ (Black, Round, Opaque)	- No growth	+ (Black, Shiny, Round, Opaque)

**Table 2. The inhibition zone size (diameter in mm) interpretation was based on HiMedia instruction sheet (the following values are upper and lower cut-off lines for R and S, respectively): 19 and 20 Amoxyclav; 35 and 37 (for *S. aureus*) for Ampicillin; 8 and 13 for Bacitracin; 12 and 18 for Chloramphenicol; 13 and 23 for Erythromycin; 13 and 18 for Kanamycin; 19 and 28 for Penicillin - G; 14 and 21 for Streptomycin; 14 and 19 for Tetracycline (Banerjee, 2004)**

Isolated Bacterium: <i>Staphylococcus aureus</i>										
	Antibiotic Disc	Amoxyclav	Ampicillin	Bacitracin	Chloramphenicol	Erythromycin	Kanamycin	Penicillin - G	Streptomycin	Tetracycline
Source	<i>S. aureus</i> isolated from Coriander	S (30)	R (26.3)	S (16.8)	S (26)	I (17.8)	R (11.5)	I (23.8)	S (21)	S (24.3)
	<i>S. aureus</i> isolated from Curry leaves	S (37.1)	R (34.8)	S (19.3)	S (31.1)	S (29.3)	I (14)	S (37)	I (18.1)	S (28.3)



no growth on BPA medium and thus were not considered for further analysis. The observed result in relation to these two food garnishes could be owing to their anti-microbial property which has been previously documented (Eltaweel, 2013). Food garnishes that showed growth on BPA were observed to have characteristic BPA *S.aureus* specific colonies (Table 1). Colonies from all the four different garnishes, coriander, curry leaves, mint and spring onion, were subjected to further characterization.

**Colonies from four different vegetable food garnishes were isolated and characterized to confirm for *S. aureus***

Four food garnishes, coriander, curry leaves; mint and spring onion, were used for the characterization of the bacteria on BPA. All BPA plates showed characteristic grey-black shiny colonies which were subjected to gram staining, catalase test and mannitol fermentation test for the identification of *S. aureus* as per the identification scheme based on the Bergey's

Manual of Determinative Bacteriology (Gaithersburg, Md. 1998). Colonies isolated from the four different vegetable food garnishes (Coriander, Curry leaves, Mint, Spring Onion) were observed to be Gram positive cocci in bunches and showed positive results for catalase test and mannitol fermentation test, i.e. effervescence and change in colour from red to yellow, respectively. Based on these results, all colonies isolated on BPA medium in this study were tentatively identified as *S. aureus*. We have assigned the tentative designation because in our study, *S. aureus* was characterized only based on phenotypic analysis (staining and biochemical tests) and not by genotypic analysis (16S rRNA studies). (Clarridge, 2004)

### *S. aureus* isolated from selected vegetable food garnishes were found to be resistant to antibiotics







To check for antibiotic resistance of *S. aureus*, antibiotic sensitivity tests were performed using the disc diffusion method against nine different antibiotics, (Amoxycylav (Amoxycillin/ Clavulanic acid), Ampicillin, Bacitracin, Chloramphenicol, Erythromycin, Kanamycin, Penicillin – G, Streptomycin and Tetracycline). Two *S. aureus* isolates (those from coriander and curry leaves) were selected for further analysis. The zones of inhibition were measured and the results were interpreted (Table 2) as R (resistant to the particular antibiotic), S (sensitive to the particular antibiotic) or I (intermediate sensitivity to the particular antibiotic). It was found that the *S. aureus* analyzed were resistant to and showed intermediate sensitivity to a few antibiotics. *S. aureus* isolated from coriander was resistant to ampicillin and kanamycin and showed intermediate sensitivity to erythromycin and penicillin-G. *S. aureus* isolated from curry leaves was resistant to ampicillin and exhibited intermediated sensitivity to kanamycin and streptomycin.

Thus, the isolated bacteria from vegetable garnishes were found to be resistant to one or more antibiotics. Even though the *S. aureus* isolated in this study were sensitive to few antibiotics, the problem of antibiotic resistance has been on the rise at an alarming rate due to the rampant use of antibiotics (Alanis, 2005). Therefore, the need of the hour is to find alternate remedies. The inhibition zone size (diameter in mm) interpretation was based on HiMedia instruction sheet (the following values are upper and lower cut-off lines for R and S, respectively): 19 and 20 Amoxycylav; 35 and 37 (for *S. aureus*) for Ampicillin; 8 and 13 for Bacitracin; 12 and 18 for Chloramphenicol; 13 and 23 for Erythromycin; 13 and 18 for Kanamycin; 19 and 28 for Penicillin - G; 14 and 21 for Streptomycin; 14 and 19 for Tetracycline (Banerjee, 2004).

### Bacteriophages were isolated and purified from sewage sample against the isolated *S. aureus*

Antibiotic resistance by pathogenic bacteria is posing to be a threat to the entire human and animal community. Hence, there is a growing need to find an alternative method for the use of antibiotics used to treat or get rid the pathogen and not develop any resistance as well. As an alternative method phage therapy can be used. To identify candidates for phage therapy against the isolated *S. aureus*, double agar layer technique was used to isolate bacteriophages from the sewage sample. Both coriander and curry leaves isolated *S. aureus* were plated with the sewage sample in the soft agar layer and incubated. After incubation, plaques were observed on both the *S. aureus* plates. This indicated that the bacteriophages from the sewage sample had lysed the *S. aureus* from their respective plates, which lead to the plaque formation (Figure 1). In order to use the isolated phages as phage therapy candidates against isolated *S. aureus*, these phages need to be purified.

**Table 3. Plaque assay using purified bacteriophages (phage therapy candidates have been indicated by plaque isolate numbers) isolated from either coriander and curry leaves against their respective parent *S. aureus* isolates. The CIPIN2 phage therapy candidate was tested against both parent (*S. aureus* isolated from curry leaves) and non-parent (*S. aureus* isolated from coriander) *S. aureus* isolates. - denotes that the indicated plaque assay has not been tested in this study. [CoPIN1: coriander plaque isolate number 1; CoPIN2: coriander plaque isolate number 2; CIPIN1: curry leaves plaque isolate number 1; CIPIN2: curry leaves plaque isolate number 2; CIPIN3: curry leaves plaque isolate number 3]**

<i>S. aureus</i> isolation source	Purified Phage Therapy Candidates	Plaque Assay with Purified Phages	
		<i>S. aureus</i> isolated from Coriander	<i>S. aureus</i> isolated from Curry leaves
Coriander	CoPIN1		-
	CoPIN2		-
Curry leaves	CIPIN1	-	
	CIPIN2		
	CIPIN3		

For purification, two candidate plaques from coriander *S. aureus* isolate plate (CoPIN1 and CoPIN2) and three candidate plaques from curry leaves *S. aureus* isolate plate (CIPIN1, CIPIN2 and CIPIN3) were picked using sterile microtips (Table 3). The plaque-agar plugs were purified by passing through 0.22 µm filter paper.

#### **Cultivated and purified bacteriophages were found to be effective against their respective parent *S. aureus* isolates**

To cultivate the purified bacteriophages, the purified phages were grown by infection of their respective *S. aureus* host cultures, i.e. CoPIN1 and CoPIN2 phage candidates were grown by infection of *S. aureus* isolated from coriander and CIPIN1, CIPIN2 and CIPIN3 phage candidates were grown by infection of *S. aureus* isolated from curry leaves. The phage-host culture lysate was again passed through 0.22 µm filter paper for purification. To check for purity of phages after cultivation and purification, the purified phage therapy candidate isolates were plated with their respective parent *S. aureus* strains using double agar layer technique (Figure 1). After incubation, plaques of uniform sizes were observed on all the five plates (Table 3). These results indicate that the purified phage therapy candidate bacteriophages have the capability to infect and lyse their respective host *S. aureus* stains. Thus, these candidates can be safely considered as candidates for phage therapy. Further studies would be required to optimize their effectiveness and utility against their host bacterium.

#### **The phage therapy candidates were also found to be effective against non-parent *S. aureus* isolates**

We have demonstrated that our phage therapy candidate bacteriophages were effective in infection and lysis of their parent *S. aureus* isolate host strain. These can, therefore, be taken up for further studies to optimize them for use against food garnish-borne pathogenic or food-spoilage causing bacteria. However, every time a new phage would be required to be isolated for use against different strains of the same bacterium. For example, in this case, bacteriophage isolated against *S. aureus* from coriander may or may not be effective against the *S. aureus* isolated from curry leaves. To test this, the bacteriophage isolated against *S. aureus* from curry leaves was used to infect *S. aureus* isolated from coriander using double-agar layer technique. After incubation, it was observed that both plates contained plaques (Figure 1, Table 3). This indicated that the bacteriophage that acts on *S. aureus* isolated from curry leaves can also act on *S. aureus* isolated from coriander. We have tested the cross-effectiveness of one isolate. Further studies with different crosses would corroborate this point. Thus, these phage therapy candidates would have a wider application than just being effective against their parent bacterium strains. For characterization and optimization of these candidate phages, sequencing of the phages, identification of storage buffer and conditions of storage, and application methodologies would need to be worked out.

Another interesting aspect would be to identify their effectiveness against other species of *Staphylococcus*, besides being effective against different *S. aureus* strains. Thus, the present study along with further characterization and optimization studies would help in designing strategies for effective phage therapy against pathogenic and food-spoilage causing bacteria that could get transmitted from contaminated food garnishes.

#### **Acknowledgement**

This work was supported by the Gokula Education Foundation and Department of Microbiology, Ramaiah College of Arts, Science and Commerce. We thank the department and the college for their support.

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