Research Article

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Isolation and Characterization of a Phage to Control Vancomycin Resistant *Enterococcus faecium*

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Abstract: *Enterococcus faecium*, is an important nosocomial pathogen with increased incidence of multidrug resistance (MDR) – specifically Vancomycin resistance. *E. faecium* constitutes the normal microbiota of the human intestine as well as exists in the hospitals and sewage, thus making the microorganism difficult to eliminate. Phage therapy has gained attention for controlling bacterial MDR infections and contaminations. We have successfully isolated from waste water and characterized a lytic bacteriophage STH1 capable of targeting Vancomycin resistant *Enterococcus faecium* (VREF) with high specificity. The phage was isolated from sewage water of a hospital at district Dera Ismail Khan, Pakistan. Initial characterization showed that magnesium and calcium ions significantly increased phage adsorption to the host. One step growth experiment showed a latent period of 18 min with burst size of 334 virions per cell. Optimal temperature and pH of the phage was 37°C and 7.0, respectively. Phage application to host strain grown in milk and water (treated and untreated) showed that the phage efficiently controlled bacterial growth. The study suggests that the phage STH1 can serve as potential control agent for *E. faecium* infections in medical facilities and in other environmental contaminations.

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1 Introduction

Enterococci, gram positive, known to be opportunistic pathogens are natural inhabitants of intestinal tract of animals and humans that may cause infective endocarditis, urinary tract infections (UTIs), bacteraemia and wound infections especially in immune compromised patients [1]. Over the time *E. faecium* has become an important pathogen due to the occurrence of high resistance to ampicillin and vancomycin, later being "drug of last resort" [2-3]. *Enterococcus* species are able to survive in harsh conditions and can be found in diverse environments including soil, sewage, hospitals, dairy products, and plants. These are considered as third most important cause of nosocomial infections [3]. Although *E. faecium* accounts for only 20% of the human infections caused by *Enterococcus* strains; it has emerged as a highly virulent strain associated with approximately 30% increased mortality rate [4-5]. *E. faecium* has acquired resistance to antibiotics either by intrinsic mechanisms or via acquired mechanisms primarily horizontal gene transfer [6]. Genes responsible for vancomycin resistance, vanA and vanB, are present on mobile DNA elements, considered responsible for dissemination of antibiotic resistance via horizontal gene transfer in *Enterococci* and other genera reported across the world [7-8]. Resistance genes can swap between *Enterococcus* strains originated from diverse niche for example hospital and community strains released into waster [9].

Although it is not considered safe, *Enterococcus* sp. Is frequently used in the food industry [10]. In dairy industry, milk gets contaminated by *Enterococci* either from animal feces directly or indirectly from contaminated water source, the bulk storage tank or milking equipment

[11]. Moreover, animal husbandries often cited to serve as a pool of resistant bacteria and antibiotic resistance genes [12-15]. Prevalence of VREF in contaminated food and water increases the risk of associated diseases in human population. Developing easy and cost-effective ways of ensuring food safety and control of VREF contamination in the environment is of prime importance.

Phage therapy has gained importance as bio-control strategy due to their high specificity, inertness and evolution with bacterial resistance development [16]. Biotechnological advances have expanded the use of phage therapy not only for targeted gene delivery and vaccine delivery but for bio-control of pests and food borne pathogens [17-18]. Biocontrol of bacteria with intact phages or purified lytic proteins has already been an intense area of research to target antibiotic resistant bacteria including VREF The bacteriophages for *Enterococcus* are isolated from sewage water and have narrow host range [19]. We intend to isolate and characterize the bacteriophage specifically targeting vancomycin resistant *E. faecium* and to observe its ability in controlling VREF growth in sewage water and milk.

2 Material and Methods

2.1 Bacterial Strain Identification

The clinical isolates were collected from the microbial culture facility of Railway General Hospital, Rawalpindi. The isolates were validated using established microbiological methods. Analytical profile index testing kit [API 20E; Biomérieux Direct] was used for standardized identification and Kirby Baeur method was employed for antibiotic susceptibility testing [20]. Vancomycin resistance was checked via polymerase chain reaction (PCR) analysis for vanA gene using vanA specific primers. For molecular identification 16S rRNA gene sequencing was performed for the clinical isolate. The 16S rRNA gene amplification was carried out by PCR using primers: RS-1 5′-AAACTC-AAATGAATTGACGG-3′, RS3 5′-ACGGGCGGTGTGTAC-3′ [21]. Amplified PCR product of approximately 0.5 kb was analyzed on 1% agarose gel and purified using gel extraction kit [Invitrogen Cat. K210012]. The sequenced product was aligned using NCBI-BLAST for identification of the sequence (www.ncbi.nlm.nih.gov/BLAST).

2.2 Bacteriophage Isolation and Enrichment

Clinical strain of VREF was selected as host for bacteriophage isolation. Bacteriophages specific to clinical strain VREF was enriched by previously described method with few modifications [21]. Sewage water sample of a hospital at district Dera Ismail Khan, Pakistan was centrifuged at 1,300 rpm for 10 min for removal of impurities and cell debris. The supernatant was filter-sterilized through 0.2 µm filter. About 5mL of sample was then added to 30 mL log phase (OD_{600} 0.4-0.6) *E. faecium* culture. The enriched culture was incubated at 37°C with shaking at 150 rpm. A drop of 1% chloroform was added to 1.5 mL of enriched culture for bacterial cell disruption and to release phages. Centrifugation at 14,000 rpm was then carried out for 15 min to settle down bacterial cell debris. Using 0.45 µm and 0.2 µm syringe filters sequentially, supernatant was filtered and transferred to a new Eppendorf tube.

Spot assay and plaque assay were used to isolate and detect phages on LB agar plates using soft agar. Overnight incubated bacterial culture and filtrate along with 0.7% soft agar were mixed and poured onto agar plates. Once solidified, plates were incubated overnight at 37°C to examine spots or plaques. For negative control only phage was added to soft agar. Amplification of phages was carried out by propagation of spots obtained after spot assay. Graph Pad Prism 5 was used to generate graphs of each test for phage characterization.

2.3 Host Range Determination

A wide range of clinical bacterial strains (Table 1) including *E. faecium*, *Acinetobacter, Citrobacter,* methicillin resistant *Staphylococcus aureus*, *E. coli*, *Pseudomonas* and *Enterococcus* were used to check the host specificity of bacteriophage STH1. To test the susceptibility of bacterial isolates, drop-on-lawn technique was used followed by plaque assay [22].

2.4 Morphology of the phage

The determination of phage morphology was done according to the previous reported methods [21]. A phage titer (≈10^{10} PFU/ml) was diluted 10 fold in 1× phosphate buffer saline in one liter distilled water having pH 7. On the surface of copper grids (formvar carbon), phage suspension was added. Uranyl acetate 2% was added for the negative staining of the samples. Through a filter paper the grids were instantly blotted and the grids were then air dried. The electron microscopy was done for the grid to analyze at 100 kV. For the classification of the phage STH1, morphological techniques were used according to the guidelines of international Committee on Taxonomy of Viruses.

Table 1. Host range determination of phage STH1 against different clinical bacterial species/isolates

S.No	Bacteria	Strain/ Isolate	Activity (+/-)
1	*E. faecium*	1969	+
2	*E. faecium*	U-523	_
3	*E. faecium*	U-781	_
4	*E. faecium*	U-790	_
5	*E. faecium*	FT-607	_
6	*E. faecium*	FT-549	Slight activity
7	*E.coli*	3086	_
8	*E.coli*	3183	_
9	*E.coli*	3056	_
10	*E.coli*	2997	_
11	*E. faecium*	U-520	Slight activity
12	*S.aureus*	2907	_
13	*S.aureus*	2941	_
14	*S.aureus*	2995	_
15	*S.aureus*	2962	_
16	*S.aureus*	2938	_
17	*P. aueroginosa*	3098	_
18	*P. aueroginosa*	2927	_
19	*P. aueroginosa*	2912	_

Table 1: + = Inhibition , _ = no Inhibition

2.5 Phage pH and Thermal Stability

Three consecutive experiments were conducted to determine appropriate phage pH stability using a range of values — 1, 3, 5, 7, 9, and 11 and thermal stability of phage STH1 according to the methods described previously [23-24]. Phage filtrate ($8.2x10^7$ PFU/mL) and LB media were mixed and incubated at 37°C for an hour (h). Phage viability was checked against the host for each treated sample by plaque assay. For thermal stability test, vials of phage filtrate were treated at a range of temperatures [37 (control), 45, 50, 55, 60, 65 and 70°C] for 1 h. Plaque assay was performed for each treated sample.

2.6 Effect of Calcium and Magnesium

E. faecium culture was equally poured into two sterile 50 mL tubes. Each of the two tubes were simultaneously inoculated with 0.25 mL (3.2×10^8 PFU) phages and $CaCl_2$ or $MgCl_2$ (250 µL and 10 mmol/L each). The samples from the culture were collected from each flask at regular interval of times (0, 10, 20, and 30 min) to calculate titers of free phages in untreated and Ca2+ or Mg2+ supplemented supernatant. The effect of Ca2+ or Mg2+ ions on adsorption potential was investigated according to previously described method [24].

2.7 One Step Growth Experiment

One step growth experiment was carried out as described previously [25]. *E. faecium* culture was grown to mid exponential phase and cells were harvested by centrifugation (10,000 rpm for 5 min). About 0.5 mL LB media was used to re-suspend the pellet obtained and mixed with 0.5 mL phage (8.2×10^7 PFU/mL). Phages were allowed to adsorb to bacteria for 2 min and free phages were later removed by centrifugation (13000 rpm for 30 s). About 100 mL fresh media was used to resuspend the pellet and the culture was incubated at 37°C. The incubated samples were taken at three minute interval and double layer agar assay was performed to determine the phage titer.

2.8 Bacterial Reduction Assay

Three consecutive bacterial reduction assays were conducted. Each experiment was performed by using *E. faecium* cultures in two flasks grown to mid exponential phase with absorbance (A_{600}) in the range of 0.4–0.6. One of the flasks had 1mL phage filtrate, while other flask was used as control without any phage. A_{600} of the samples were taken after every 2 h for 24 h using a spectrophotometer.

2.9 Effect of Bacteriophage on VREF Grown in Milk Samples

The experiment was performed for milk samples according to already described methods with few modifications [26]. Bacterial culture incubated overnight at 37°C in tryptic soy broth with CFU of $3.9x10^8$ was used. About 1.5 mL culture was added to sterile Eppendorf tube and centrifuged for 10 min at 13,000 rpm. After discarding supernatant, double distilled autoclaved water was used to wash the pellet twice. To dilute the culture ($2.0x10^5$ CFU), double distilled autoclaved water was used to dissolve the washed pellet. Diluted bacteria were added to 5 mL of each milk sample. The sample was transferred into two sterile flasks (sample and control) and incubated for 2 h. About of 500 µL phage filtrate ($8.2×10^7$ PFU) was added to sample flask and was vigorously shaken. Both sample and control flasks were incubated at 37°C and CFU was calculated after 24 and 48 h for comparison.

3 Results

3.1 Bacterial Strain Identification

Gram's staining showed that the bacterium *E. faecium* is a gram positive cocci. Biochemical tests indicated that it a catalase negative bacterium able grow at alkaline pH and temperature range of 10-50 °C under anaerobic conditions. Using PCR an amplicon of approximately 470 bp was obtained and sequenced. The sequence was submitted to NCBI and is available under accession number [GeneBank: KF386009]. BLAST analysis indicated 98% sequence homology to *E. faecium*. Kirby Bauer method showed *E. faecium* resistance to vancomycin and teicoplanin. PCR amplification of right sized *vanA* gene confirmed vancomycin resistance.

3.2 Bacteriophage Isolation

Lytic phage that was isolated against *E. faecium* gave a clear zone. Soft agar method reconfirm the phage potential. The appearance of clear plaque on bacterial lawn showed that this phage was lytic and had plaques within a range of 1.5 to 3.5 mm having well defined boundaries. This phage was named STH1 (Figure 1.A& B).

3.3 Host Range Determination

During host-range determination of STH1 phage, where non-host bacteria were also considered, nineteen pathogenic bacterial isolates were tested. Phage showed

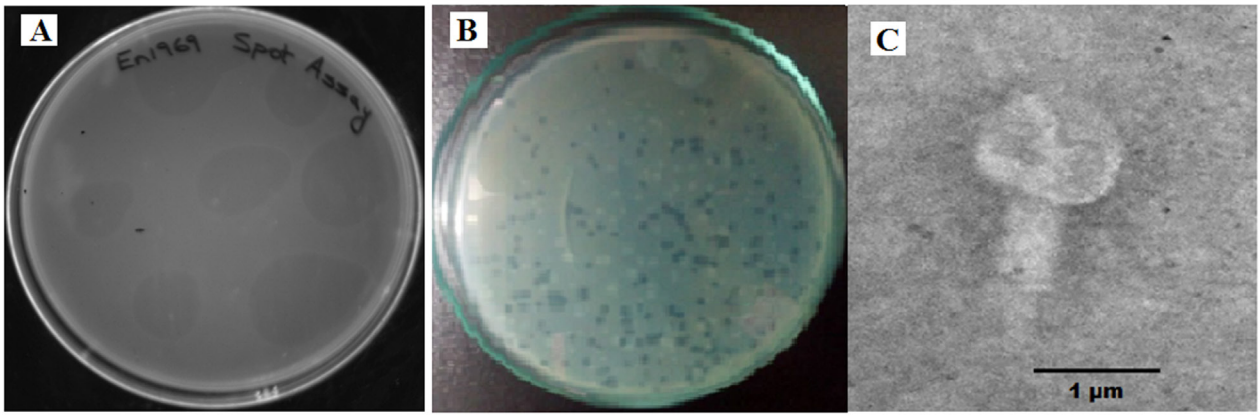

Figure 1. (A), Spot assay for detection of phage and (B), shows plaque assay for detection and confirmation of phage clearly showing plaques ranging in size from 1.5 to 3.5 mm, (C) Transmission electron micrographic (TEM) view of the purified STH1 phage particle (negatively stained preparations). Scale bars, 100 nm.

slightly lesser activity against two *E. faecium* strains U-520 and FT-549. However, no plaques were observed indicating narrow host range of the phage.

3.4 Morphology of Phage STH1

TEM analysis showed that phage head was isometric having contractile tail. The phage STH1 has a head of 132 nm with tail having a length of 120 nm and width of 20 nm. These morphological characteristics showed that phage STH1 belongs to family *Myoviridae* (Figure 1.C).

3.5 Characterization of Phage STH1

Reduced phage number was observed at extreme acidic conditions –pH 1, 3 and 5. Although with reduced growth, the phage exhibited plaques and that the phage was viable at the extreme pH. Optimum pH, however, was determined to be 7.0 with maximum number of plaques. The results suggest that extreme pH might be an obstacle to phage stability. Very few plaques were observed at pH 1. The number of plaques increased with increasing pH, reaching the highest number at pH 7.0. Gradual decrease in number of plaques was observed when pH elevated above 7.0 (Figure 2.A). To determine the heat resistant capability, phage thermal-stability test was performed at pH 7.0. The phage was incubated within a range of 35 to 70°C. Almost 100% infection activity was observed at 37°C. Although the phage was stable until 65°C, no plaque was observed at 70°C (Figure 2.B).

3.6 Effect of Calcium and Magnesium

Calcium chloride or Magnesium chloride (either with concentration of 10 mmol/L) was added to the mixture of STH1 and the *E. faecium* to determine their effects on STH1 adsorption. The number of free floating phageswas determined by plaque assay method at 0, 10, 20, and 30 min of the time intervals. A significant difference was observed in calcium or magnesium ion-treated phage STH1 groups. We observed a significant reduction in free phages in both calcium/magnesium chloride treated samples as compared with control by applying paired samples *t*-test, $p < 0.05$ for both Ca^{2+} and Mg^{2+} ions). Furthermore, calcium and magnesium ions were found to stabilize the phage adsorption process. The frequency of free phages declined when treated with calcium and magnesium ions as compared to the control group as shown in Figure 2.C.

3.7 Latent Time Period and Burst Size

By using single-step growth experiment, a triphasic curve was obtained that showed the latent phase, log phase, and stationary phase. Latent time period was found to be 18 min having burst size of 334 virions per cell (Figure 3.A).

3.8 Bacterial Reduction Assay

Phage infected culture of *E. faecium* was monitored for 24 h along with the control to observe bacterial lysis. The bacterial culture infected with phage showed drastic

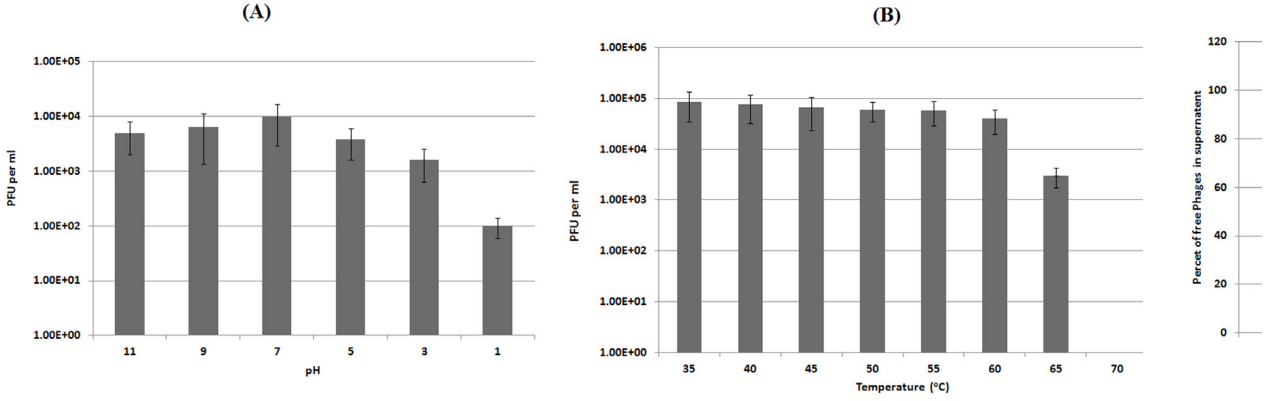

Figure 2. (A) pH stability test of phage at different pH values. At neutral pH maximum number of plaques were observed for STH1 (B) Thermal stability test of phage at different temperature. The optimal temperature for STH1 infection was found to be 37°C. (C)Test for adsorption rate of phage. The ability of divalent metal ions was observed by adding 10 mM $CaCl_2$ or $MgCl_2$ to the mixture of phage STH1 and host bacterium. A significant reduction in free phages in both Ca2+and Mg2+ ions treated samples was observed as compared with control (sample without Ca2+/Mg2+) by applying paired samples *t*-test ($p< 0.05$). All the values are mean with standard deviations.

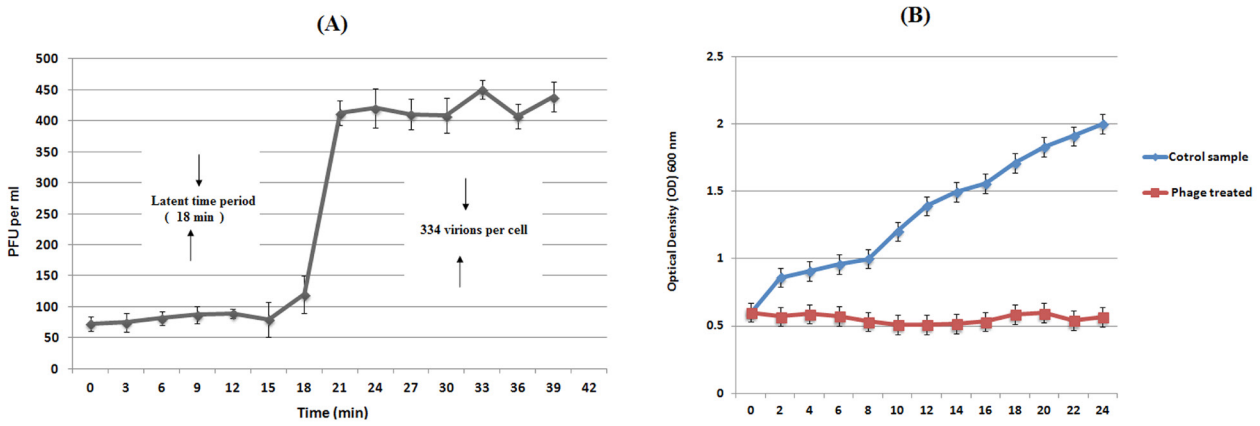

Figure 3. (A) One step growth experiment: Curve shows; latent period (18 min); log phase; stationary phase and burst size of 334 virions per cell. (B) Reduction in the growth of planktonic *E. faecium* by using phage STH1(8.0x10^{7}PFU/ml). Values are the means of 4 samples.

decrease in turbidity. However a gradual increase in turbidity was observed after 11 h which could be either due to bacterial cell debris or growth of phage resistant cells (Figure 3.B). On the basis of these results, we have reasons to believe that bacterial reduction strategy if practiced for therapeutic or environmental control of VREF, will yield promising results.

3.9 Applications of STH1 Phage

The inhibition of *E. faecium* growth was observed after 24 to 48 h in sterile milk samples. The sterile milk sample treated with phage showed 2.5 fold reductions after 24 hours incubation whereas the 48 hour incubated sample when treated with the same amount of phages showed 3.2 fold reductions (Figure 4). Reductions in both cases were found to be significant by applying paired samples t-test ($p < 0.05$) when compared with other control samples (sample without phages).

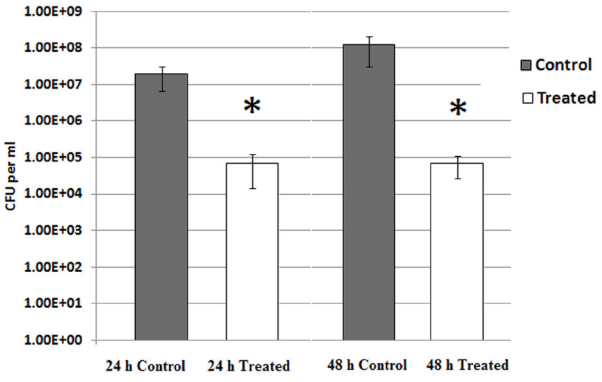

Figure 4. Effect of phage STH1 on reducing bacterial growth in milk samples when treated with 500 µL of phage filtrate (8.0x10^7 PFU/ml) post 2 hours of bacterial inoculation. About 2.5 and 3.2 fold reductions were observed in 24 and 48 incubated milk samples, respectively. Reductions in both cases were found significant by applying by t-test ($p<0.05$) when compared with other control (without phages) samples.

4 Discussions

Among all strains of *Enterococcus*, the most virulent strain with highest mortality rate known is *E. faecium* [4]. *E. faecium* is resistant to multiple antibiotics including aminoglycosides, beta-lactams, glycopeptides, vancomycin and teicoplanin [6]. With increasing prevalence of VREF as multidrug resistant (MDR) poses serious threats leading towards limiting and less viable treatment options [5]. MDR bacteria control through bacteriophages offers pathogen specific control with a potential to control human bacterial-infections. Bacteriophages have been used as a safe therapeutic option for the treatment of bacterial infections since long and is getting lime light in Western medicine [27]. Similarly, phages continue to be used in food industry [28, 29].

In the present study, VRE specific bacteriophage STH1isolated from sewage water suggests that phage exists in aquatic environment with its host. This strengthens the hypothesis that VREF are enriched in hospitals andare transferred to sewage water [30]. Phage produced clear zones or plaques within a range of 0.5 to 1 mm diameter as previously reported for phage vrep-5 isolated against VREF [26].

The phage STH1 was found to be highly lytic evident from its behavior thereby producing clear plaques (from 1.0 to 3.5 mm). However, the phage displayed a narrow host range by showing a slight activity against two clinical isolates *E. faecium* isolates U-520 and FT-549 when tested by spot assay. This slight activity of clearance against these two strains shown by spot assay maybe due to the presence of cytolysins in phage lysate. The cytolysins therefore may inhibit growth of *Enterococcal* species as described by Hickey et al. [31].

Temperature, pH and presence of divalent ions accelerate adsorption rate of phage to host [24, 32]. *Enterococcus* phages have been reported to be highly stable and can be maintained at 4°C for 2 years [33].In our study, the isolated phage STH1 showed optimum growth at 37°C suggesting its (*Enterococcus* sp.) optimal growth in its natural environment i.e. gastrointestinal tract and its microflora. Phages isolated in previous studies were reported to be stable ranging from pH 6 to 9 with few plaques at extreme conditions [34]. The phage STH1 has remarkable ability to withstand extreme pH condition. Large number of plaques were observed between pH 5 to 9 with maximum growth at pH 7. The survival of phage towards neutral to basic pH may be due to isolation from sewage water, known to be slightly basic.

Triphasic curve demonstrated stages of phage infection and multiplication. Previous studies for VREFs reported small sized burst ranging from 110-120 with latent period of 30 min [26]. However, in our study phage showed short latent period and pronounced burst size. These properties makes the phage suitable for phage therapy and other applications.

Besides other important parameters, calcium or magnesium ions also increased STH1 infectivity as compared to the control sample (without phage). Jamal et al. [21] have found a considerable increase in phage-absorption to host bacterium in the presence of 10 mM of $CaCl_2$ when compared with control (sample without cations). During the adsorption process, calcium/magnesium ions stabilize the weak virion interaction with the relevant bacterial host cell receptors. Thus in the presence of calcium ions an optimal phage infectivity can be obtained [35, 36].

Phages can be used for a wider range of applications, include biotechnological processes [33, 34]. *Enterococcus* sp. has been used in food industry for decades. However, it is not considered safe due to its opportunistic pathogenic nature and due to its ability to transfer resistant markers to susceptible strains [10]. *Enterococci* can also cause contamination in food-industry equipment difficult to sterilize. Also, VREFs are frequently isolated from sewage water known to cause serious environmental contamination all around the globe [31, 37]. In this study we applied phage STH1 to the milk samples having host bacteria to observe bacterial reduction. Bacteriophage based control of food spoilage and disinfection of equipment is relatively easy to handle and has high specificity against microbes [38].

In conclusion, waste water is a rich source for phage isolation against MDR bacteria. Isolation of lytic phage STH1 against VREF is an important approach for combating infections caused by MDR organisms. Phage characterization exhibits significant ability to withstand high thermal and pH conditions. The phage actively controlled the bacterial growth until 11 h. The phage has a narrow host range but "cocktail" of VREF specific phages might be a promising alternative therapeutic approach. Application of the phage to the bacteria grown in sewage water, and milk has promising results in controlling VREF in these samples. Our results, therefore, may open new horizons to combat MDR bacteria using host specific bacteriophages.

The study focuses on a lytic phage and its characterization. Future prospects include the use of cocktail as a biocontrol treatment and to study phage resistant cells, if any. Lastly, this study dealt with isolation and characterization of a bacteriophage (STH1) to control a bacteria (*Enterococcus faecium*) in a food (already spoiled milk) setting. Prevention of the bacteria, although is out of the scope of the present study, could be a better future direction.

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Conflict of interest: Authors state no conflict of interest

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