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Highly Sensitive Bacteriophage-Silver Nanoparticle Consortium for Enhanced Bacteriolysis of Multidrug Resistant *Salmonella enterica* subsp. *enterica* ser. *typhi*

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ABSTRACT

Increasing drug resistance among pathogenic bacteria is an emerging and potentially a serious threat to human beings. Despite various methodological limitations and regional prevalence in many studies, alarming resistance rates are noted in nearly all pathogens. High number of multiple resistance patterns towards the existing antibiotics is now more prevalent among Enterobacteriaceae, like *Escherichia coli* and *Salmonella typhimurium*. The increasing and unfettered use of antibiotics deteriorates the case further, and the development of alternative anti infection modalities has become one of the highest priorities of modern medicine and biotechnology. 'Bacteriophages' the natural viral predators, which target bacteria but leave mammalian and plant cells unscathed becomes the tool of interest in fighting against drug resistance. The present study suggests that a bacteriophage ϕS-41 belongs to myoviridae, found to have susceptibility against a multi-drug resistance strain of *Salmonella typhi*. A thorough screening of more than 60 samples, one phage titer ϕS-41 was found to have acute sensitivity and host specificity for *S. typhi*. An increased bacteriolysis observed when bacteria were challenged with this phage along with silver nanoparticles, thus it seems that nanoparticles were actually involved in the cell disintegration of the host and facilitate the phage infection that results into the increased bacteriolysis. Though, this finding has a significant improvement in curing MDR *S. typhi*, it is also very clear that the safe and controlled use of phage therapy will require detailed information on the properties and behavior of specific phage bacterium systems, both *in vitro* and especially *in vivo*.

Keywords: Bacteriophage, *S. typhi*, Silver nanoparticles, Drug resistance, Phage therapy, Nano-diagnostics

INTRODUCTION

Nano-biotechnology in majority of studies mostly focused on the bio-medical applications of nanoparticles, more precisely as anti-microbial either independently or in combination of antibiotics and clinical nano-diagnostics [1,2]. There are many studies that show the synergistic toxic effects between metal nanoparticles and the antibiotics when tested against infectious bacteria. Among most studied system is the *E. coli*, where there are a series of observations about the enhancement of the effect of some antibiotics like amoxicillin, penicillin, erythromycin, clindamycin and vancomycin was observed when tested against *E. coli* with silver nanoparticles [3,4]. Later on similar observations were also made for *S. aureus* [5]; this was significant because both of these organisms are frequently isolated with antibiotic resistance [6]. Thus, there is potential to breathe new life into failing anti-bacterial strategies through synergistic effects with nanoparticles.

However, these effects are not restricted only with the silver

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nanoparticles zinc oxide nanoparticles also increase the sensitivity for ciprofloxacin against *E. coli* in disc diffusion assays [5]. Initially the nanoparticle and the antibiotics were applied separately, but later on several reports come up with conclusion that a conjugated antibiotic with the nanoparticle surface had even greater activity compare to adding antibiotic alone. In this case, the observed synergistic effects may occur because of an enhanced reactivity of molecules presented at a nanoparticle surface. Nanoparticles are frequently observed in close association with *E. coli*, thus the efficacy of anti-bacterial conjugates may be increased where they are concentrated directly at the cell envelope [7-9]. But the increasing drug resistance among infectious bacteria put us on alert. We need some alternative approach to combat with the increasing incidences of drug resistance, and one such approach is the use of 'bacteriophage'. Bacteriophages are nanoparticles of diverse size, made of macromolecules of different charges and hydrophobicity, and since they are infective to their host we can harvest both of this virtue to cope with the drug resistance [10]. Bacteriophages, which are in equilibrium with bacterial world, are an important part of the micro-ecosystem. It is estimated that on earth there are approx. 1031 of such particles of a high genotypic diversity [11]. In such a large virus pool, one may expect that there are nanoparticles of broad application spectrum, and it's time now to think about this forgotten cure [12].

This study has been carried out to investigate cure for two strain of *Salmonella enterica* subsp. *enterica* ser. *typhimurium* (*S. typhi*, which is found to be resistance for most established antibiotics) using bacteriophage therapy in combination of silver nanoparticles as adjuvant. We perform an extensive survey for isolating and purifying bacteriophages against this strain from water sources of different habitats in and across the Varanasi district of North India, including river Ganga, from where the history of bacteriophage begins. The objectives of present work is: (i) isolation and purification of bacteriophages against *S. typhi* and their phage typing; (ii) assessing phages from divers sources for their potential bacteriolysis activity against the drug resistant *S. typhi*; (iii) evaluation of enhancement in bacteriolysis using phage in combination of silver nanoparticles; and (iv) evaluating synergistic toxic effect of phage-AgNPs combination against *S. typhi*.

MATERIALS AND METHODS

General materials

All reagents were purchased from Sigma unless otherwise stated, and used without further purification. Luria broth (LB), Muller-Hinton (MH) and standard microbiological agar were purchased from Oxoid. All solutions were prepared in ultrapure water (18 $m\Omega/cm^3$ at 25°C, MilliQ, Millipore).

Bacterial culture

The *S. typhi* strains (BHUST13 and BHUST29) used in this study was isolated from stool sample of a patient in OPD of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. We performed the molecular identification of this strain and deposit the sequences in the GenBank with accession no KJ740150 and KJ740151 (http://www.ncbi.nlm.nih.gov/genbank/). Bacterial culture (*S. typhi*) was carried out in Luria Broth (LB) or LB lacking sodium chloride (modified-LB) and/or Muller-Hinton (MH) Medium with 0.1% (w/v) glucose (LB was 10 gL^{-1} Tryptone, 5 gL^{-1} yeast extract, 10 gL^{-1} NaCl and pH 7.5). The solid medium, Muller-Hinton (MH) Medium contained 1.5% (w/v) standard microbiological agar. LB, MH agar were sterilized by autoclaving at 121°C for 15 min. All liquid cultures were incubated at 37°C, aerobically on a rotary shaker at 200 rpm. Optical density (OD) was preferentially used to monitor bacterial growth and replication unless in a medium containing nanoparticles, wherein optical density measurements were not possible due to the large scattering cross sections of the nanoparticles. The OD of bacterial cultures at 600 nm (OD600) was measured in 1cm optical path length cuvettes on a Hitachi dual-beam spectrophotometer (Hitachi, UV-2910) operated at a resolution of 1 nm. Alternatively, bacterial viable cell numbers were measured as Colony Forming Units (CFU). For this, a sample of culture was 10-fold serially diluted in ¼ strength Ringer's solution (7.2 mgL^{-1} NaCl, 170 mgL^{-1} $CaCl_2$ and 370 mgL^{-1} KCl in ultrapure water, pH 7.2) and a known volume of each dilution was spread onto MH Agar medium. Colonies were counted after an overnight incubation at 37°C.Sensitivity test for the *S. typhi* was also performed against 10 well established antibiotics and it is observed resistance against 6 out of 10.

Bacteriophage isolation

Bacteriophages were isolated from 60 water samples collected in Varanasi district of north India, from sewage, drainage and Ganga river water. Briefly, 5 ml of MH agar broth was inoculated with *S. typhi* and incubated at 37°C for overnight. A sewage water sample of 10 ml was used to inoculate with the 0.5 ml of *S. typhi* culture and incubate it for 24-48 h at 37°C. centrifuge the tube for 10 min at 2500 rpm and collect the supernatant. Filter the supernatant by using a 5 cc syringe filter attached with a 0.22 µm millipore filter. This 'enriched phage prep' can be stored for long time. Now fresh MH agar plates were prepared and seeded with a lawn of *S. typhi* using 100 ul of inoculum and a sterile spreader, added 10 ul of enriched phage prep and spread over the lawn and leave for overnight incubation at 37°C. The hundreds of plaques were observed which were further purified by adopting plaque streak protocol.

Nanoparticle synthesis and characterization

The silver nanoparticles were prepared by chemical reduction method according to the description of Lee and Meisel [13]. For TEM analysis, 2-4 µL drop of silver nanoparticle colloid was placed directly onto a 300 mesh carbon coated copper grid with a formvar/carbon support film and the solution was allowed to stand for 5 min excess solution was removed carefully and the grid was allowed to dry for an additional 5 min. The average size and size distributions of silver nanoparticles were determined by processing the TEM images with image processing software (Cyber View 4.7) on a Tecnai G-20 transmission electron microscope, a 200 kV TEM with a W-source and an ultra-high resolution pole piece with a point-point resolution of 1.9 A° (TEM, Tecnai [FEI]-12v.G-20). Surface topology was measured by atomic force microscopy (AFM) in the contact mode on a VEECO Digital Instruments multimode scanning probe microscope equipped with a Nanoscope IV controller. The characterization of silver nanoparticles was carried out by XRD (Cu-Kα radiation source) using a 12 kW rotoflux rotating Cu anode (Rigaku Tokyo, Japan) powder diffractometer (RINT 2000/PC series) operating in Bragg-Brentano geometry and fitted with a curved crystal graphite monochromator in the diffraction beam and a high temperature attachment.

Bacteria-nanoparticle-phage interactions

S. typhi were cultured in LB medium until mid-exponential phase (OD600=0.5) and then washed by sequential centrifugation (8000x g, 2 min) and suspended in PBS (8 gL^{-1} NaCl, 200 m gL^{-1} KCl, 1.44 gL^{-1} Na_2HPO_4, 240 mgL^{-1} KH_2PO_4, pH 7.0). Approximately 10^6 viable bacteria were added to 10 mL of the synthesized silver nanoparticles (5 mg mL^{-1}, aps 20 nm) in a colloidal suspension. For controls, bacteria were added to the supernatants after the nanoparticle colloids had been destabilized (by sonicating) and sedimented by ultracentrifugation (20,000x g, 30 min). To determine whether the nanoparticles were toxic to the bacteria, Colony Forming Units per mL (CFU mL^{-1}) were determined at several time points (10, 20, 40, 60 and 120 min) during the exposures.

TEM of bacterial-nanoparticle-phage interactions

The bacteria were exposed to the nanoparticles along with phages for 30 min. Then the cells were washed by sequential centrifugation (8000x g, 2 min) and suspension in phosphate buffered saline. Subsequently, the cells were fixed and stained by sequential suspension in 1% (v/v) glutaraldehyde, 1% (w/v) osmium tetroxide and 1% (v/v) uranyl acetate for 1 h each, washing 3 times between each step. Then the cells were dehydrated by suspending in ethanol solutions (30, 50, 75, 90 and 100%, 10 min in each) and propylene oxide (twice for 10 min). The bacteria were embedded in a low viscosity, medium grade epoxy resin (Taab, Agar Scientific), mixed (1:1) with propylene oxide for 24 h. Then the bacteria were transferred into pure resin for a further 24 h. Finally the samples were placed in gelatin capsules and cured at 60°C for 16 h in a dry heat oven. The specimens were viewed at between 50,000x magnification and 120,000x magnifications in a TEM, (Tecnai [FEI]-12v.G-20) and image capture was carried out using Digital Micrograph software.

RESULTS AND DISCUSSION

The present study deals with the bactericidal efficacy of bacteriophages in combination of silver nanoparticles. Salmonella has been known as most important causes of bacterial enteric illness in humans for many years [14]. The strain in question here, *S. typhi* was recovered from a patient that suffers with acute gastroenteritis and loosed sensitivity for antibiotic therapy. The stool samples were obtained and processed for the isolation and purification of the bacteria using standard protocol. The purified strain was then tested against 10 highly established antibiotics and we obtained that out of 10 only 4 antibiotics (Gentamicin, Amikacine, Chloremphenicol and Co-Trimoxazole) are having some sensitivity against *S. typhi* **(Table 1)**, while acquired resistance for rest of all. Use of excessive antibiotic therapy for infectious diseases are so common in these days, but the subsequent appearance and spread of antibiotic resistance have made many currently available antibiotics ineffective against them [15]. To cope with increasing number of drug resistant and multi drug resistance bacteria, apart from extensive studies of the molecular mechanisms of acquiring antibiotic resistance, one should must think about alternative ways to fight against multi drug resistant bacteria and one such approach might be a 'Phage therapy'. Bacteriophages are very unique in many respects that make them potentially attractive therapeutic agents, such as they are: (i) highly specific and very effective in lysing targeted pathogenic bacteria; (ii) safe, as underscored by their extensive clinical use in Eastern Europe and the Russia and the commercial sale of phages in the 1940s in the United States; and (iii) rapidly modifiable to combat the emergence of newly arising bacterial threats [16]. Thus we select this drug resistant strain of *S. typhi* phi to test this hypothesis again, we begin isolating bacteriophages for Salmonella from 60 different water samples sources in and around Varanasi region and we collected library of bacteriophages. Initially we perform the phage isolation through water samples collected from various locations in Varanasi district India representing a diverse set of habitats and the rationale behind this task is to get bacteriophages from deferent bacterial serotypes to have maximum effectiveness against the targeted bacteria. We seeded the bacterial suspension with water samples from different habitat after incubation we remove the bacteria expecting infection and releases of some bacteriophage in suspension. After ultracentrifugation we condense the spent and drop it on the bacteria seed plates and obtained thousands of plaques **(Figure 1a)**, then we re-suspended some of these plaques in normal saline and repeat the same

and we obtained plaques from pure bacteriophages **(Figure 1b)**. A heterogeneous mixture of plaques was evident, which were susceptible to lysis as shown in **Figure 1a**. This indicated the presence of different types of phages in the samples. Aside from differences in diameter, the clarity of the plaques also differed in some instances. Some plaques were clear zones while others were turbid or opaque plaques. The differences in lysis observed are probably due to the presence or absence of specific receptors on the bacterial cell wall to which the phage must bind. In addition, raw sewage sample contained different types of phages as indicated by the different plaque sizes. We use plaque streak protocol to purify the phages that have lysis activity against the *S. typhi* that is resistant against most of the antibiotics **(Table 1)**. After isolation and purification of susceptible phages against the strain, we performed extensive plaque assays with individual phages and then with some combination of phages (phage cocktail). In almost every case we find encouraging results, we observed acute and effective bacteriolysis with individual phages and phage cocktails. The plaques obtained are relatively bigger and clear in comparison to those antibiotics that have some sensitivity against bacteria **(Figure 1b and Table 1)**. The most effective phage that we obtained against *S. typhi* was ϕS-41, which represents a family of myoviridae phages. We noticed a long fully extended contractile tail and tail fiber attached with a hexagonal head **(Figure 2a)**, notice the contracted tail see the difference in the tail length that suggest a contraction with the tail plate **(Figures 2b and 2c)**. So, once we got a bacteriophage with acute bacteriolysis to *S. typhi*, against the strain in question, we start attempts to enhance the bacteriolysis by adding silver nanoparticles as adjuvant, since new aspects of bacteriophage application in treatment of bacterial infections are in under development, like recombinant phages causing bacterial cell death, but causing bacteriolysis [17]. There is also a new and most popular approach involving use of bacteriophages in antibiotic therapy. The advantage of such combined therapy is a less likely development of probability of occurrence of antibiotic-resistant bacterial strains [18,19]. So we decided to check efficacy of these bacteriophages in presence of silver nanoparticles (AgNPs). Why silver nanoparticle? Because it is well known for its antimicrobial properties [20]. Since the antibacterial activity of the AgNPs depends on many factors such as particle size, shape, concentration and its aggregation/dissolution in specific growth media [21-23], we decided to go for a complete characterization of the silver nanoparticles we synthesized. Recent studies showed that 10 mgL^{-1} AgNPs average particle size (aps=12 nm) reduced *Escherichia coli* growth by 70% [24] while AgNPs (aps=14 nm) caused 55% growth inhibition of *E. coli* at a much lower concentration (0.45 mgL^{-1}) [25]. **Figure 3** represents the characterization of silver nanoparticles. The TEM images suggests that most of the silver nanoparticles are spherical and hexagonal in their shape **(Figures 3a and 3b)** and their size distributed in between 20-100 nm range as analyzed by the zeta size analyzer. The three dimensional topographical view from atomic force microscopy also justifies the actual size measurement of the nanoparticles **(Figure 3d)**. The XRD crystallography confirms the face centered cubic (fcc) nature of silver nanoparticle, representing the faces for [111,200,220] correspond to fcc crystal structure **(Figure 3c)**. AgNPs of average particle size of 20 nm and spherical or hexagonal in shape was used for the rest of the study. We challenge the *S. typhi* with bacteriophage (ϕS-41) along with silver nanoparticles AgNPs. Our observation suggests that nanoparticles (AgNPs) facilitate the cell lysis by the bacteriophages **(Figure 4)**. The phages attack on bacteria that can be seen by the attached phage heads on the surface of bacterial cell **(Figure 4a)**, many phages can be seen on the surface of bacterial cell wall and hexagonal heads are clearly visible **(Figure 4b)**. Interesting observation was that most often the phages were found to attached with bacterial cell in the close vicinity of nanoparticles **(Figures 4c and 4d)**, this is clearly indicated by the increase bacteriolysis when compared with only bacteria and bacteriophage interactions **(Figure 1c)**. We observed many infection points where we obtained both nanoparticles and bacteriophages **(Figure 4d)**. One assumption is that first nanoparticle gets attached to the cell wall of bacteria and induces some deformity in cell membrane structure [26,27] that in turn facilitate phages to attack on them. Many metal oxide nanoparticles have similar actions to change bacterial cell properties. Many metal nanoparticles damage the bacterial cell wall and cause the increase of membrane permeability leading to accumulation of nanoparticles in the cell membrane and nanoparticle internalization [28]. Since nanoparticles are engaged in changing the cell membrane properties and in reducing cell activities, this may facilitate the phage ϕS-41 to infect the *S. typhi* host **(Figures 5b and 5c)**; however, this is still a premature speculation on the mechanistic aspect of this tripartite interaction in this particular case. Further resolution **(Figure 5)** of one 'infection point' actually gives us a bit more insight about this interaction leading to an increased bacteriolysis. A high resolution image of a single infection point revealed that phages were infecting to those points specifically were silver nanoparticles are localized and accumulated **(Figure 5a)** and this is more prevalence across the other infection sites as well **(Figures 5b and 5c)**. The phages are also known to disintegrate the cell wall of their host by using a low molecular weight protein or by releasing endogenous murein hydrolyses of the host cell [29,30], for this reason it seem that nanoparticles are actually facilitating the cell disintegration of the host followed by the phage infection that results into the increased bacteriolysis. The interactions of bacteria and viruses are complex and multifaceted, and adding metal nanoparticle makes it even more complex. So studying bacteria-phage-nanoparticle tripartite interaction is very complex in nature however, what we can note from this study for sure is that, (i) silver nanoparticles plays vital role in phage infection to the host

but yet we need to know more about the mechanism; (ii) the phage interact differently in presence of nanoparticles leading to increased cell lysis; (iii) and last but most important that we can engineered phages with nanoparticles as adjuvant for antibiotic therapy. Further we are in in vivo phase of our experiment and trying to fix the toxicity and other concern with both bacteriophage and nanoparticle **(Figure 6)** before using them as phage therapy against multidrug resistant *S. typhi*. The earliest *in vivo* studies suggested that the bacterial infection leads to severe infection in mice and cause heavy eye bleeding, this is probably because of the sudden release of endolysins due to the killing of bacteria by the phages or might be because of some uninfected bacteria. Our study clearly established that in the presence of AgNPs, exposure of phage (ϕS-41) to AgNPs prior to infection might facilitate virus uptake into the host cells, therefore causes faster and more intensive infection to *S. typhi* host. No doubt that we need extensive knowledge of specific receptors on integrated cell membrane of the host for recognition by bacteriophages to infect them precisely, but nevertheless we cannot ignore the importance of nanoparticles in destabilizing the cell membrane of host for successful infection by bacteriophage.

Table 1. The well-established antibiotics used for the treatment of Salmonellosis were tested against the isolated strain and most of them were find to have resistance.

Antibiotics	Name on Disc	Concentration (mcg/disc)	Efficacy (Zone of Inhibition in cm)
3-Ampicillin	AMP^{10}	10	R*
5-Amoxyclave	AMC^{30}	30	R
8-Cefuroxime	CXM^{30}	30	R
10-Ceftriaxone	CTR^{30}	30	R
12-Gentamicin	GEN^{10}	10	1.8
14-Amikacine	AK^{30}	30	1.6
18-Ceprofloxacin	CIP^{5}	5	R
19-Chloramphenicol	C^{30}	30	2.1
22-Co-Trimoxazole	COT^{25}	25	2.4
PTZ-Piperacillin/Tazobactum	$PIT^{100/10}$	100/10	R

* *R: Resistant*

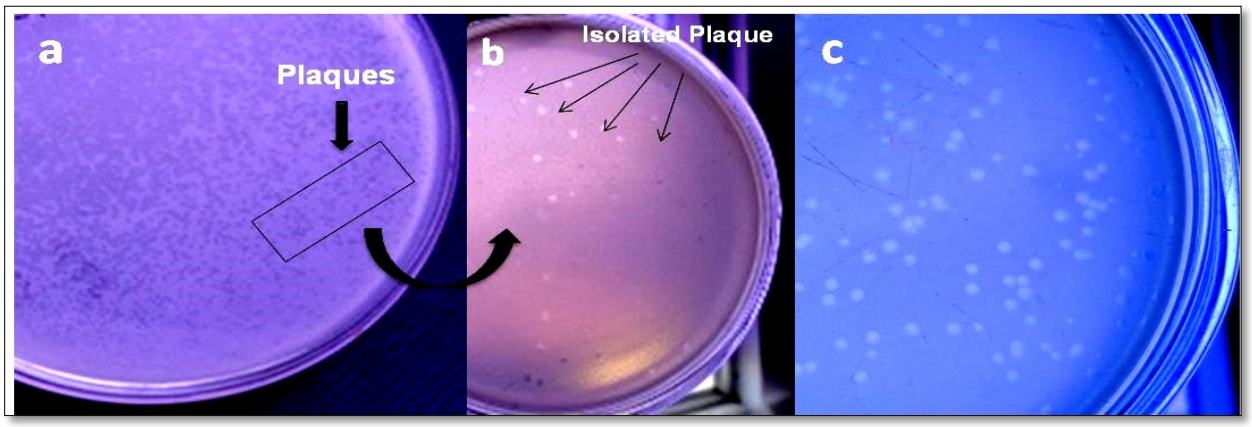

Figure 1. (a) Plaques obtained by concentrated impure 'enriched bacteriophage prep' suspension; **(b)** Pure bacteriophage plaque propagation isolated from single plaque; **(c)** The bacteriophage (ϕS-41) and Silver nanoparticle combination, notice the clear plaques due to bacteriolysis.

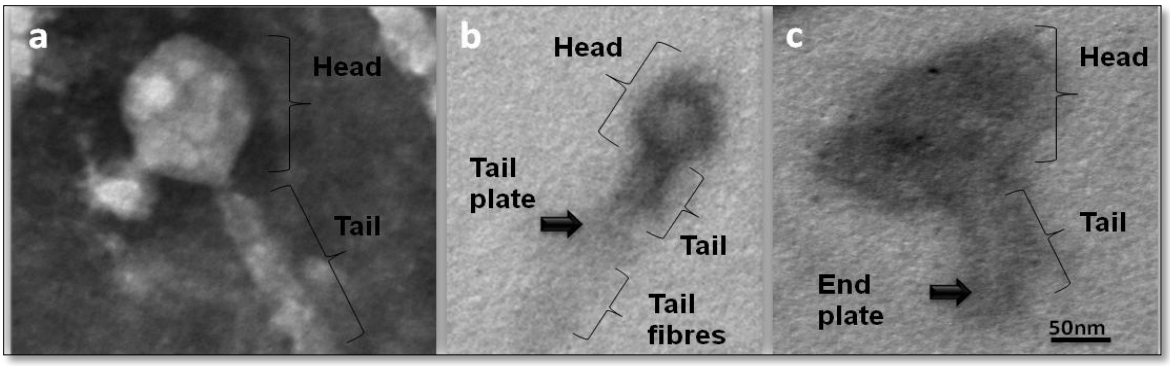

Figure 2. Bacteriophage (ϕS-41) characterization by TEM. The phages represent the myoviridae family because of their long tail and tail fiber. **(a)** The single bacteriophage represent the diagnostic morphological features of myoviridae family; **(b-c)** The clear head with a long tail and end plate. In some cases tail fibers can also be observed.

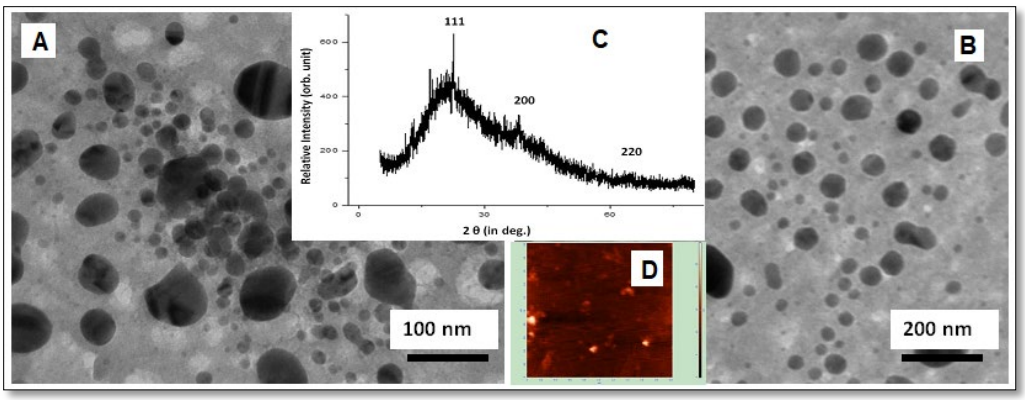

Figure 3. The silver nanoparticle characterization **(a-b)** the TEM images of silver nanoparticles mostly these are spherical and/or hexagonal **(c)** the corresponding AFM analysis shows the spherical nanoparticle with 30-120 nm size.

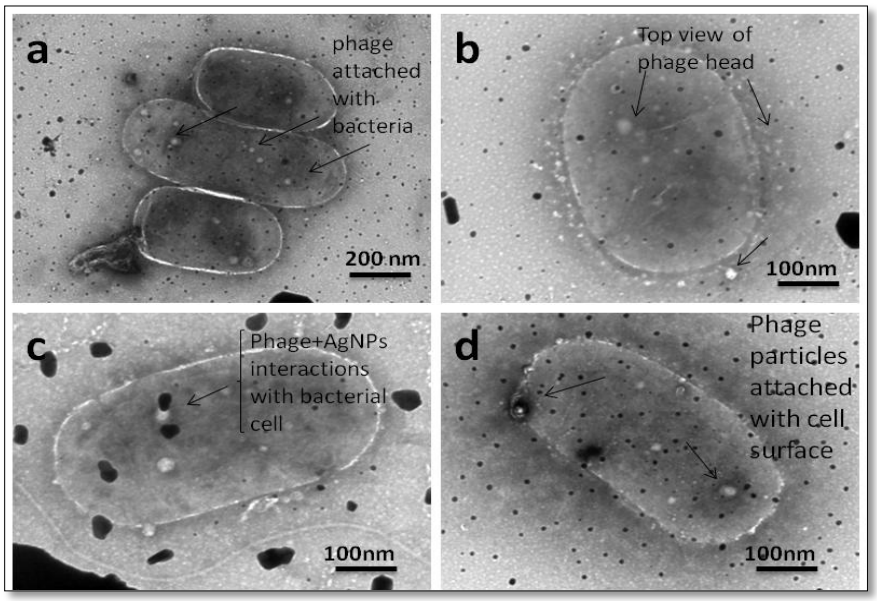

Figure 4. investigation of bacteria-nanoparticle-phage interactions: **(a)** The bacterial cells showing the surface attached phages, notice the top view of phage heads on the surface; **(b)** the single bacterial cell of *S. typhi* showing numerous phages on the surface; **(c)** the bacterial cell with phage and nanoparticles, notice the adherence of AgNPs in the vicinity of phage infection points; **(d)** the phage particles attached with bacterial cell along with nanoparticles facilitating the cell lysis.

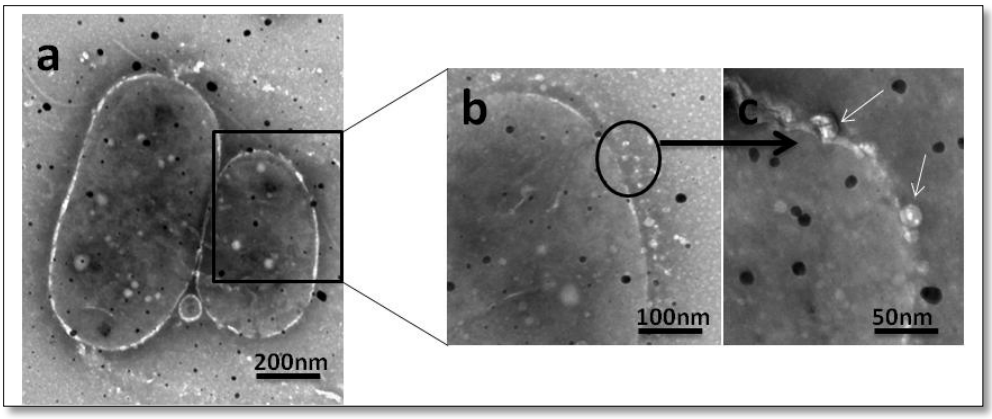

Figure 5. Transmission electron microscopy revealing the role of phage-nanoparticle combination in facilitating cell lysis, resolution of a single infection point. **(a)** The bacterial cells showing the adherence of phages and nanoparticles; **(b)** the high resolution image of cell surface shows the adherence of phages and localized nanoparticles surrounding the infection point; **(c)** the combination induced deformities of the bacterial cell wall, notice the phage and the nanoparticle both at the infection points.

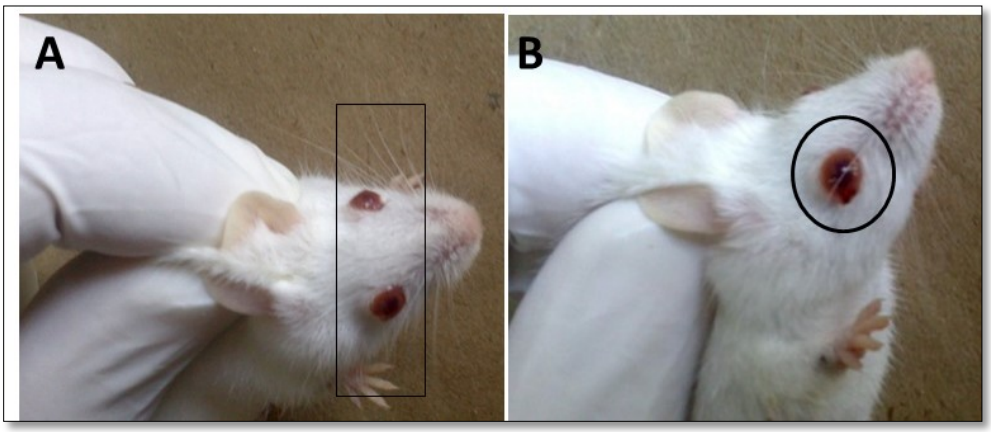

Figure 6. The *in vivo* toxicity assessment of *S. typhi* and phage ϕS-41 combination: (a) the bacterial infection at a concentration of 10^6 in combination of AgNPs leads to septicemic condition, witnessed eye bleeding; (b) However, bacteria alone is causing the more severe effects.

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CONFLICT OF INTEREST

Authors declare no competing financial interests. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter of materials discussed in this manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

AUTHOR'S CONTRIBUTIONS

AKV, GN and VCV hypothesized the experiment. VCV, MG, AKV, VK, SRP executed, collected and interpreted the data and wrote the manuscript. MY, performed S/TEM analysis, interpretation of the results and assisted in editing of manuscript. All authors have read and approved the final manuscript.

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