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Isolation and identification of phage vB_SaP-AZ2 against multi drug resistant staphylococci of animal origin

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Abstract
Bacteriophages are considered as alternative approach to combat with multi drug resistant bacterial infections. Phage vB_SaP-AZ2 is anti-staphylococcal bacteriophage belonging to podoviridae, which possesses specific and potent bactericidal activity against multi drug-resistant *Staphylococcus aureus.* The study was carried out with an aim of isolation and identification of a lytic bacteriophage against multi drug resistant staphylococci from animal origin. The lytic bacteriophage was isolated from sewage source, enriched with specific MDR Staphylococci, concentrated and purified using PEG precipitation techniques. The phage morphology, structure and stability were determined using electron microscopy (SEM & TEM) and other physicochemical methods. The phage (vB_SaP-AZ2) belonged to family *Podoviridae* with icosahedron head with diameter 86 nm and a non-contractile tail measuring about 36 nm in length. The thermo-stability of phage was observed to be up to 45 °C, higher temperatures resulted in progressive inactivation. The phage was stable in pH range of 6-8. UV exposure for 1 hr and chloroform treatment for 24 hrs did not affect the phage stability. The host range of phage was determined by plaque assay. The phage had lytic activity against 52.5% of clinical isolates of *Staphylococcus* spp. whereas it was effective against 73.33% of MRS isolates *in-vitro*.

Keywords: Phage, staphylococci, bacteriophage, vB_SaP-AZ2

Introduction
Staphylococcus aureus is a pathogen of pyogenic inflammatory diseases, food poisoning, and toxic-shock syndrome; it is also a major causative agent for opportunistic and/or nosocomial infections, often with a high mortality rate (Noble, 1998) [1]. Clinical isolates of *S. aureus* have evolved to become resistant to many commonly used antibiotics including methicllin (MRSA). Moreover, certain MRSA strains also have already acquired resistance to vancomycin [vancomycin-resistant *S. aureus* (VRSA)], a unique antibiotic previously considered to be effective against MRSA (Yayan *et al*., 2015) [2].
Use of antibiotics in treatment and control of bacterial infections is widely adopted strategy since the discovery of antibiotics in late 1920s. Indiscriminate antibiotic usage including overuse, abuse or misuse (misdiagnosis), substandard antimicrobials/counterfeit drugs and the adaptation evolution governed by natural selection has lead to development of antimicrobial resistance in bacteria. So, there is an urgent need to develop new therapies against this important pathogen. Phage therapy may be an alternative to antibiotics, because it has proved to be medically superior to antibiotic therapy in many ways (Kazmierczak *et al.,* 2014) [3].
The use of phage therapy with host specific bacteriophages is cost effective and acceptable approach to control multidrug resistant bacteria (Sulakvelidze and Barrow, 2005) [4]. Bacteriophages are natural predators of bacteria and are ubiquitous in nature. The advantages of host specific bacteriophages over chemotherapy are that; they are self limiting and replicating only on the specific bacteria without affecting harmless flora in body. Considering these advantages host specific bacteriophages were isolated against MDR staphylococci. This study was also intended to investigate sewage water sources for the isolation and characterization of indigenous bacteriophages to assess their lytic activity against MDR staphylococci.

Material and Methods
Isolation and identification of *Staphylococcus aureus*
Samples from TVCC (Teaching Veterinary Clinical Complex, Junagadh) clinical cases like

milk, pus, skin lesion wash etc. were cultured initially in Brain Heart Infusion (BHI) agar and incubated at 37 °C for 18-24 hr for primary isolation. Samples were also inoculated on the Mannitol Salt Agar petri plates and were incubated at 37 °C for 48 hours. Biochemical confirmation of bacteria was carried out using Gram's stain, catalase test, oxidase test and carbohydrate utilization test. Antibiotic sensitivity test and mPCR with gene specific primers of (16S *rRNA*, *Nuc, Coa* & *Mec A* gene) carried out to confirm MDR *Staphylococcus aureus.*

Isolation of bacteriophage

Drainage water from various TVCC departments (Medicine, Gynaecology & Surgery) and CBF (Cattle Breeding Farm) were collected for bacteriophage isolation. Samples were centrifuged at 12000 rpm for 15 minutes and then supernatant collected & filtered through 0.22 µm pore size polycarbonate membranes. The filtrates have been added to a fresh known MDR Staphylococci lawn to screen lytic phages via plaque assays using the soft agar overlay technique.

Phage enrichment

A 20 µl of filtrate containing phage was mixed with the 200 ml of host bacteria (in log phage) and allowed for incubation overnight at 37 °C. The homogenate was centrifuged at 2000 rpm (Remi motor Ltd.) and filtered using sterile syringe filters with pore size of 0.22 µm to make them bacteria-free. After filtration 100µl of the filtered sample was serially diluted in salt of Magnesium (SM) buffer, by 10 fold dilution. This filtrate was screened for the presence of phage and was stored at 4 °C for the further use (Texas A & M University, 2011) [5].

Phage purification

The corresponding Staphylococcal host strain was grown to $0.1OD_{600}$ at 37 °C. Cells were infected with phage at a multiplicity of infection (MOI) of 0.5 and incubated at 37 °C until the culture was clear. The lysate was cleared via centrifugation at 10,000 g for 10 min and 10% w/v of polyethylene glycol 8000 (PEG) was added to the supernatant and precipitated overnight at 4 °C. The solution was centrifuged at 5,000 g for 1 h, the supernatant decanted, and the pellet re-suspended in 5 ml of SM buffer. Then suspension was dialyzed using a 12 000-14 000 Daltons MW dialysis membrane-70 in 4 L of dialysis buffer (100 mM NaCl, 8 mM MgSO4, 50 mM Tris-HCl). After 24 h the dialysis buffer was exchanged and dialyzed for 4 h. Phage was collected from the dialysis membrane and titred.

Double agar overlay method

The titre of lysate was then assayed according to the double agar overlay method of Adams (1959) [6]. The 100 µl of the host bacterial strains in TSB were mixed with 10 µl of the serially diluted lysate and were incubated at 37 °C for 10 min. After incubation, 10 ml of sterile soft agar was added to this, mixed well and was immediately overlaid on MH plates. Phage free cultures (containing only bacterial host) and host-free cultures (containing only phage) were used as controls. Then the Petri plates were kept in laminar air flow till solidification, and incubated at 37 °C for overnight to obtain clear zone of plaques.

Genetic characterization of phage

For the identification of nucleic acid type, purified phage genomic DNA was treated with *DNAse* I, *RNAse* A and Mung bean nuclease at 37 °C for 1 h.

Electron microscopy

Scanning and Transmission electron microscopy of concentrated bacteriophage was carried out using standard protocols to determine morphological features. For SEM Phage fixed with glutaraldehyde and dehydrated with acetone, then observed with ZEISS Evo-18 Scanning Electron Microscope. For TEM Phage spotted on carbon-coated copper grid and observed with Tecnai-20, Philips (Holland) Transmission Electron Microscope (TEM). It was stained by 1.6% uranyl acetate.

Study the effect of phages on different clinical isolates

Effect of bacteriophage on clinical isolates including MDR was studied on suitable solid media for appearance of definitive plaques (spot assay).

Results

Isolation and identification of *Staphylococcus* spp. strains from clinical samples

A total of 30 Staphylococci have been identified and characterized on the basis of phenotypical properties like, colony morphology, staining characteristics, growth pattern, production of enzyme like catalase, oxidase and fermentation of various carbohydrates.

The biochemical profiles of all the bacterial isolates were examined as per (Collee *et al*. 2008) [7]. Biochemical characteristics are described in Table 1 in which, out of 30 isolates 18 have fermented mannitol and produced yellow colonies. Other 12 isolates were non mannitol fermenters. All isolates were catalase positive, and oxidase negative. A total of 18 isolates were identified as *Staphylococcus aureus* according to their carbohydrate utilization patterns shown in Table 2.

Antibiotic susceptibility profile of *Staphylococcus* spp.

All 30 isolates showed resistance towards Penicillin-G (100%), Methicillin (80%), Amoxicillin-sulbactam (60%), Ceftriaxone (100%), Cefoperazone (80%), Gentamicin (100%), Enrofloxacin (100%), Levofloxacin (80%), Oxytetracycline (100%) and Chloramphenicol (70%).

Molecular confirmation of staphylococcal isolates by mPCR.

For molecular confirmation of *Staphylococcus* spp. and MRSA identification, DNA of isolates extracted with *Proteinase* K-SDS method and column based method. Multiplex PCR with 4 gene specific primers (*16S rRNA, Nuc, Coa & mecA* gene) revealed that all 4 products were amplified by 12 isolates. The amplicon size of *16S rRNA, mecA, Coa* and *Nuc* genes are 370 bp, 454 bp, 117 bp and 279 bp respectively. (Plate 1)

Isolation of phage

Drainage water samples were centrifuged at 12,000 rpm for 15 minutes and supernatants were collected & filtered through 0.45-µm pore size polycarbonate membranes. Filtrate was added on lawn of known MDR staphylococcal isolates and incubated. After incubation three types of plaques formed by bacteriophages according to size: Small (1-2 mm), Medium (3-5 mm) and Large (5-7 mm). (Plate 2)

According to Bacterial and Archeal viruses Subcommittee (BAVS) of the International Committee on Taxonomy of

Viruses (ICTV) naming guidelines, name was given to above phages as vB_SaP-AZ1, vB_SaP-AZ2 and vB_SaP-AZ3 respectively. Phage vB_SaP-AZ2 was then used for further characterization for convenience.

Phage titration

Isolated phage was enriched and purified. Purified phage stock was used further for titration to determine concentration in pfu/ml using double agar overlay method with host bacterial strain. In SM buffer $10^{-1}$to 10^{-3} dilutions of phage were made for titration and the optimum dilution factors for ideal counting (20-200 plaques) were optimised. Serially diluted phage had shown 260, 30 and 3 plaques at 10^{-6}, $10^{-7}$and 10^{-8} dilutions respectively. The suitable dilutions were used for further study. (Plate 3)

Genetic characterization of phage

Phage nucleic acid was treated with *DNase*-I, *RNase*-A and Mung bean nuclease in different tubes with appropriate buffers. Gel electrophoresis after treatment revealed that nucleic acid material of phage was completely cleaved by *DNase*-I and remained intact in *RNAse*-A treatment indicated the phage had DNA as its genetic material. Treatment with Mung bean nuclease which cleaves ssDNA showed no effect with intact band of original size on gel electrophoresis, which confirmed further dsDNA as the genetic material of isolated phage. (Plate 4)

Electron microscopy of phage

Electron microscopy of phage was carried out to determine morphology and for phage classification. The purified & concentrated phage preparations with required processing were use in both scanning & transmission electron microscopy.

In Scanning electron microscopy host bacteria were infected by phage, then phage morphology was visualized using ZEISS Evo-18 scanning electron microscope. The samples of different hours of post infection showed relative number of phage adsorbed to the surface of staphylococcal cells and the dimensions of adsorbed phage were determined. Infecting phages were observed on staphylococci bacteria and having size of 132.3 nm at 87.08 K magnification. (Plate 5)

Transmission electron microscopy was carried out to study detailed structure of phage. Phage structures corresponding to head diameter of 86 nm, tail length of 36 nm were observed. The tail was observed to be non-contractile. These observations suggested isometric symmetry of phage. The finer details and dimensions revealed that the phage was morphologically similar to phages of family *Podoviridae* according to ICTV. (Plate 6)

Host range determination of phages

Bacteriolytic activity of phage was tested on heterologous (*non-Staphylococcus*) Genera. The phage failed to infect gram negative bacteria and gram positive bacteria other than staphylococci**.** MTCC strains of *Staphylococcus* spp. were tested for activity of phage. No any strain other than *S. aureus* found susceptible to phage. Different phages may have different specificity to different host bacteria strains as well (Garcia *et al*. 2009) [8]. (Table - 3)

Susceptibility of clinical staphylococcal isolates to phage

A total of 120 clinical *Staphylococcus* spp. isolates were tested for phage susceptibility using spot lysis test. Out of 120 isolates, 63(52.5%) were found to be susceptible to phage and produced clear plaque on bacterial lawn.

Susceptibility of clinical MRS isolates to phage

A total of 45 clinical MRS isolates were identified among 120 clinical staphylococcal isolates and were tested to study susceptibility to phage. Out of 45 isolates, 33 were found to be susceptible to phage on plaque assay. Percentage of susceptibility calculated was 73.33%. The isolated phage was found to be specific for MRS only (Table - 4)

Table 1: Biochemical characterization of each *Staphylococcus spp*. isolates.

Total 30 isolates	Biochemical tests		
	Mannitol Fermentation	Catalase Test	Oxidase test
Positive	18	30	0
Negative	12	0	30

Table 2: Carbohydrates utilized by *Staphylococcus* spp. isolated from clinical samples.

Sr. No.	Carbohydrate discs	Carbohydrate fermentation (%) (n=30)
1	Arabinose	0%
2	Dextrose	100%
3	Dulcitol	0%
4	Fructose	100%
5	Galactose	100%
6	Inositol	0%
7	Lactose	100%
8	Maltose	100%
9	Mannitol	60%
10	Melibiose	0%
11	Raffinose	0%
12	Rhamnose	0%
13	Salicin	0%
14	Sucrose	100%
15	Trehalose	80%
16	Xylose	0%

Table 3: Susceptibility of non-*Staphylococcus* spp. and MTCC stains of *Staphylococcus* spp. to phage.

Bacterial species tested	Activity of phages
Micrococcus spp.	0
Corynebacterium spp.	0
Bacillus spp.	0
Pseudomonas spp.	0
Serratia spp.	0
Proteus spp.	0
Pasteurella spp.	0
S. epidermidis (MTCC-3382)	0
S. haemolyticus (MTCC-3383)	0
S. simulans (MTCC-3610)	0
S. xylosus (MTCC-6149)	0
S. sciuri (MTCC-6154)	0
S. saprophyticus (MTCC-6155)	0
S. chromogens (MTCC-3545)	0

++, Full activity of phage on plaque assay
+, Partial activity of phage on plaque assay
0, No activity of phage on plaque assay

Table 4: Susceptibility of clinical *Staphylococcus spp*. and MRSA isolates to phage.

Tested isolates	No. of isolates tested	No. of isolates susceptible to phages on plaque assay	Percentage of susceptibility
Staphylococcus spp.	120	63	52.5%
MRSA	45	33	73.33%

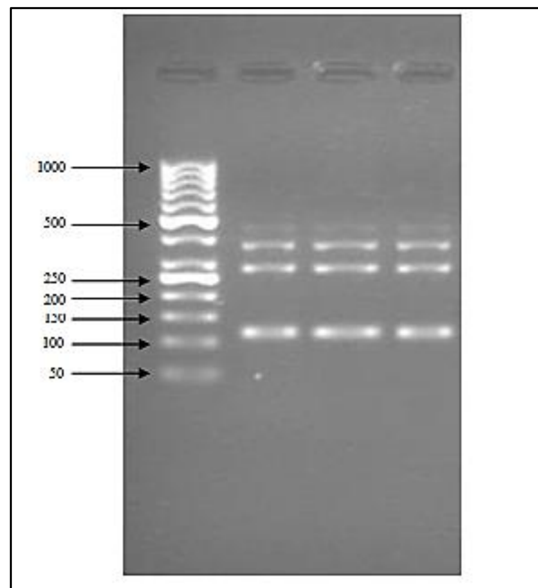

Plate 1: Detection of Methicillin resistant *Staphylococcus aureus* by m-PCR (Lane 1= 50bp DNA marker, Lane 2= 966, Lane 3= 967, Lane 4= 1503) (16s rRNA gene=370bp, Mec-A=454bp, Coa=117bp and Nuc= 279bp)

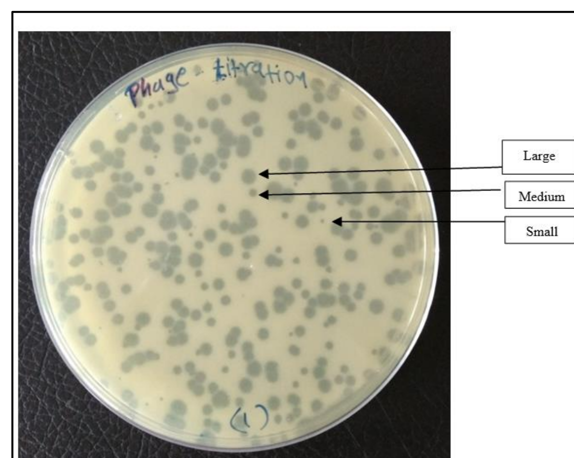

Plate 2: Diverse plaque morphology in double agar overlay: According to size three types of plaques formed by bacteriophages - Small, Medium and Large.

Plate 3: Plaque counts in serial 10 fold dilutions of bacteriophage stock on double agar overlay. Serially diluted phage (Dilution 1, 2 & 3) showing 260, 30 and 3 plaques respectively. Titre of phage shown below:
Dilution-1: 2.6×10^7 pfu/ml
Dilution-1: 3.0×10^6 pfu/ml
Dilution-1: 3.0×10^5 pfu/ml

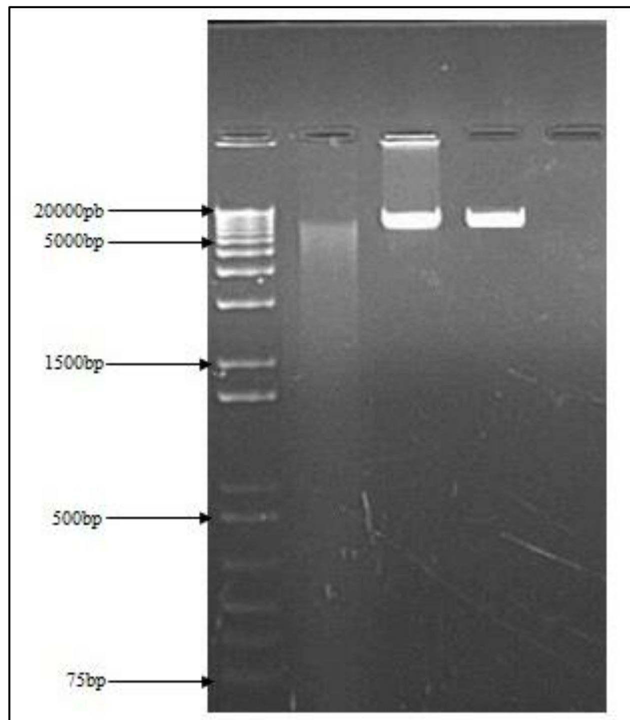

Plate 4: Gel electrophoresis image of bacteriophage genome with various enzyme treatments
Lane-1: 1kb plus DNA ladder
Lane-2: *DNase* I treated DNA
Lane-3: *RNase A* treated DNA
Lane-4: Mung bean nuclease treated DNA

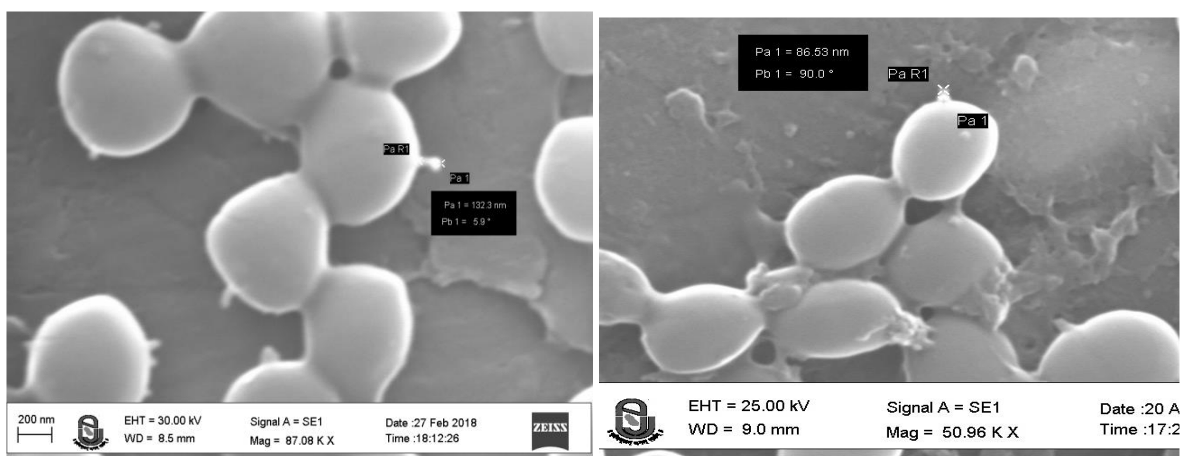

Plate 5: Scanning electron microscopy of phage vB_SaP-AZ2 with measurements

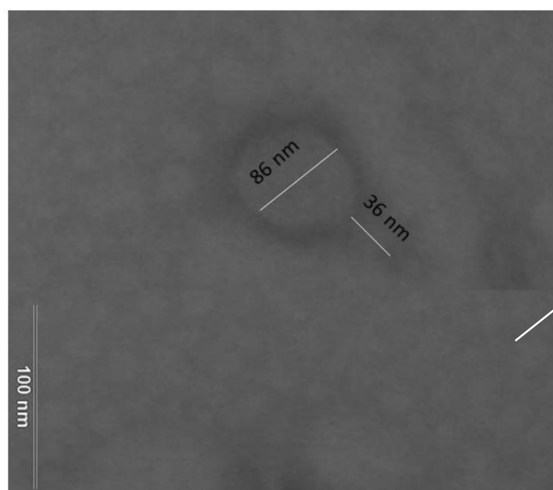

Plate 6: Transmission electron micrograph of phage vB_SaP-AZ2

Discussion

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major pathogen responsible for pyogenic inflammatory diseases, viz. mastitis, food poisoning and toxic-shock syndrome. Mastitis is the single most important factor contributing to the economic losses to the dairy industry, resulting in reduction in milk yield and quality of the milk. Mastitis is one of the most common diseases of high yielding dairy animals and having great economic importance to the dairy industry. The staphylococcus prevalence in this region in bovine mastitis is reported to be 62% encompassing clinical and sub-clinical form (Nimavat, 2015) [9]. The average cost of a clinical mastitis case is US $ 179 per lactating season (Bar *et al.,* 2008) [10]. In India, 50% of milking animals are reported to be affected with clinical or sub-clinical mastitis (Dua, 2001) [11]. Bacteria evolved to acquire resistance to several antimicrobial agents. Diseases caused by these drug resistant microbes are very difficult to treat. In these aspects *Staphylococcus* spp. is leading the list in acquiring the antimicrobial resistance.

The application of lytic bacteriophages for bio-sanitation and therapeutic aspects is one of the novel alternatives for antibiotic usage, as fast emergence of antibiotic resistance in the pathogenic organisms is there. Successful phage therapy was reported in human bacterial infections worldwide, but very few reports are available for animal pathogens. In this perspective the focus of our work was to isolate and characterize the lytic bacteriophages against MDR staphylococci of animal origin. MDR staphylococci were isolated from the samples of clinical cases presented in TVCC, Junagadh. Bacteriophage isolation was carried out from drainage water of TVCC, various clinical departments and Cattle breeding farm (CBF), JAU, Junagadh. We have isolated 3 types of phage from sewage water against MDR *Staphylococcus aureus*. In several previous reports of isolation and characterization of phages, the plaque morphology and size were used as important criteria for identification. Han *et al.* (2013) [12] reported plaques of two types, clear and turbid. A similar morphology and plaques sizes were also been reported in the same study. The turbid plaques were of medium size, while the clear plaques were pinheads, medium sized and, in some cases, large. The properties like morphology, genetic nature, and efficacy against the clinical isolates was determined during the present study. In other ways this research work also generated the important information that provides broad picture of multi-drug resistant staphylococci in this region including pathogens of animals, possible diversity of staphylococci specific phage populations, its morphological & physico-chemical characteristics, host range, reproduction strategy, growth parameters, stability and efficiency against the staphylococci under laboratory conditions.

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