Isolation and Characterization of Bacteriophage Against Methicillin Resistant Staphylococcus aureus

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ABSTRACT- Methicillin resistant Staphylococcus aureus (MRSA) is a major human pathogen responsible for several life threatening conditions. MRSA has the ability to acquire resistance to several antimicrobial agents and phage therapy is one potential option to treat this pathogen. The aim of the study was to isolate and characterize bacteriophages effective against a wide range of methicillin-resistant Staphylococcus aureus (MRSA). A mixture of ten MRSA isolates was used for the isolation of phage from wastewater treatment plants. Three phages were selected for further characterization. All three phages belong to the Siphoviridae family and have long non-contractile flexible tails. The three phages showed a wide host range against S. aureus. Phages $\Phi$SA1 and $\Phi$SA2 were resistant to a pH range from 4-10 while $\Phi$SA3 has a pH range from 3-11. DNA from all three phages were resistant to digestion by endonuclease enzymes such as EcoRI and AccI. There was a high degree of mosaicism among the three virulent phages and with their ancestor phages of Siphoviridae due to their non-uniform access to the common genetic pool by horizontal gene transfer and recombination. Since some of the staphylococcal toxins are phage encoded, the presence of genes for such toxins was tested by performing polymerase chain reaction and all three phages lacked genes for any of the staphylococcal toxins, including staphylococcal enterotoxins (sea, seb, sec and see), exfoliating toxins (eta and etb) and the toxic shock syndrome toxin (tst), therefore these bacteriophage are suitable candidates for future use in phage therapy against MRSA.

KEY WORDS- Staphylococcus aureus, phage therapy, MRSA phage, Siphoviridae, Anti-restriction mechanism, Mosaicism

1. INTRODUCTION

Methicillin-resistant Staphylococcus aureus (MRSA) is a worldwide pathogen that is responsible for a variety of diseases ranging from soft tissue and skin infections to life threatening conditions such as pneumonia, bacteremia and sepsis [1]. MRSA is one of the major human pathogens that may cause community and hospital acquired infections [2]. These organisms are frequently resistant to most of the commonly used antimicrobial agents, including β-lactam antibiotics [3]. The emergence and spread of strains resistant to oxacillin, methicillin and even vancomycin has made therapy of these multi drug resistant bacteria a global challenge [4]. MRSA has a wide variety of virulence factors that include structural and secreted factors [5]. These factors include superantigens, cytolytic toxins, exoenzymes and miscellaneous proteins [6]. Superantigens are a group of powerful immuno-stimulatory proteins implicated in a variety of human diseases including gastroenteritis and toxic shock syndrome [7].

One possible approach to treatment of methicillin resistant S. aureus is phage therapy, defined as the application of phage to selectively reduce or eliminate susceptible pathogens from specific environments [8]. Phage therapy may be a suitable alternative to antibiotic treatment due to the high specificity of and effectiveness against multi drug resistant bacteria [9, 10]. The use of bacteriophages in clinical medicine was first introduced by Félix d’Herelle [11]. Many therapeutic phage have been isolated against MRSA, most of which belong to the Myoviridae family such as the well-known phage K and MR-10 [12, 13]. Since all tailed phages are believed to share common ancestors, a high frequency of chimeric and mosaic structures can be observed among different tailed phage families due to their access to a common genetic pool [14, 15]. Such mosaicism has resulted in a high degree of similarity among phages in both nucleic acids and proteins [16]. The main aims of this study were to isolate a set of bacteriophages effective against a wide range of MRSA isolates and characterize these phages according to their morphological features, host range, endonuclease enzyme digestion pattern, molecular identification, the presence of undesirable toxin encoding genes.

2. MATERIALS AND METHODS

CULTURE MEDIA AND CHEMICALS

The following media and chemicals were used for the study: brain heart infusion agar (Salucea, Netherlands), brain heart infusion broth (HIMEDIA, India), blood agar (HIMEDIA, India), mannitol salt agar (OXOID, England), Mueller Hinton agar (HIMEDIA, India), Agar agar (HIMEDIA, India), agarose (Bio Basic, Canada), peptone (DIFCO, USA), beef extract (OXOID, England), yeast extract (Sigma-Aldrich, Switzerland), sodium chloride (Sigma-Aldrich, Switzerland), sodium hydroxide (Sigma-Aldrich, Switzerland), potassium dihydrogen phosphate (Sigma-Aldrich, Switzerland), potassium dihydrogen phosphate (Sigma-Aldrich, Switzerland), Gelatin (BDH, England), barium chloride (Hopkins & Williams Limited, England), Gram stain (HIMEDIA, India), catalase test reagent (HIMEDIA, India), coagulase plasma (HIMEDIA, India), API Staph (bioMérieux, France), EcoRI (promega, USA), AccI (BioLabs, New England), sulfuric acid (BDH, England), hydrochloric acid (BDH, England), tris-(hydroxymethyl)-aminoetane (pH 7.5) (Riedel-deHaën, Germany), Chloroform (Sigma-Aldrich, Switzerland), glycerol (Chem-supply, Australia), absolute...
ethanol (BDH, England), nuclease free water (Promega, USA), ethidium bromide (Fisher, USA), bromophenol blue (Fisher, USA), TBE buffer (Promega, USA), TE buffer (Promega, USA), (Bioanalyse, USA), cefoxitin disc (30 µg) (Bioanalyse, USA), DNA ladder marker (100-1000 bp) (Promega, USA) and DNA ladder marker (100-10000 bp) (KAPA, USA).

**SAMPLE COLLECTION**

A total of 100 isolates of staphylococci were obtained from various clinical sources such as skin, anterior nares, ear wound, sputum, blood and urine all according to standard methods of sample collection [17]. The isolates were randomly collected from healthcare workers, hospital patients and patient’s escorts at Al-Sadar hospital, Al-Basra General Hospital, Ibn Ghazwan Hospital and day care centers in Basra.

**MRSA IDENTIFICATION**

Staphylococcus aureus isolates were identified by standard methods described [17]. Staphylococcus aureus isolates were characterized as methicillin resistant (MRSA) using the cefoxitin disc (30 µg) diffusion method [18]. The diameter of the zone of inhibition was measured and the results were interpreted according to CLSI criteria [19].

**ISOLATION OF BACTERIOPHAGE**

Bacteriophages were isolated from raw sewage samples obtained from Al-Sadar hospital raw sewage in Basra according to previously described methods [20, 21]. A 200 ml volume of fresh sewage was mixed with 20 ml of bacteriophage broth (peptone (100 g/L), beef extract (30 g/L), yeast extract (50 g/L), sodium chloride (25 g/L), potassium dihydrogen phosphate (80 g/L)), 20 ml of a mixture of 2 broth culture containing MRSA in a test tube and 100 µl of the selected host bacterium for 20 min at 37 ºC, then 3 ml of top agarose was added, mixed and poured onto a BHI agarose plate which was incubated for 6-8 h at 37 ºC. A 2 ml volume of SM buffer was then added to each plate in order to harvest the phages and then was transferred into tubes containing 0.2 ml of chloroform. The mixture was kept at 4 ºC for 2-3 h and gently vortexed and centrifuged at 4500 x g for 15 min. The supernatant was recovered and a drop of chloroform was added. The supernatant was filtered through a sterile 0.45 µm membrane filter (chm, Spain) and the filtrate was stored at 4 ºC and the titres of the phage stocks were determined by plaquing 10-fold dilutions using the soft agarose overlay method. The phage stock was purified for further studies by layering 4 ml of 5% glycerol solution in SM buffer over 40% glycerol and adding the sample on the top, followed by ultracentrifugation at 25000 x g, for 2 h at 4 ºC. The phage-containing pellet was resuspended in 1 ml SM buffer.

**ELECTRON MICROSCOPY**

Phage particles were negatively stained with 2% uranyl acetate (Sigma-Aldrich, Switzerland) on carbon-coated copper grid using standard procedures. TEM images were captured in Zeiss EM10C (Zeiss, Germany) at Khajeh Nasir Toosi University of Technology (IRAN). Three phages that were isolated were classified according to the International Committee on Taxonomy of Viruses ICTV [22].

**HOST RANGE**

The host range of each of the isolated bacteriophages was determined against a number of MRSA isolates as described by [20]. A lawn of a single MRSA isolate was inoculated on a BHI agar plate and the plate was divided into four squares by marking the surface of the plate. The plate was left to dry for a few minutes and then 10 µl of each phage suspension (109 pfu/ml) was dropped in the center of each square except for one square which was left as control. Following incubation at 37 ºC for 24 h, these plates were examined for lysis. A clear zone in the bacterial lawn was recorded as complete lysis.

**RESISTANCE OF PHAGES TO ACIDITY AND ALKALINITY**

Resistance of phages to acidity and alkalinity was done according to [20]. A 100 µl volume of phage suspension (109 pfu/ml) was added to 900 µl of saline adjusted to a specific pH. The mixture was incubated at 37 ºC for 16 h. A control sample (phage suspension and normal saline, pH 7.2) was also incubated at 37 ºC for 16 h. The titre of the surviving phages was determined by plaquing 10-fold dilutions by the soft
agarose overlay method.

**EXTRACTION OF BACTERIOPHAGE DNA**

The bacteriophage DNA was extracted using the QIAprep Spin M13 kit (QIAGEN, Germany) and according to the manufacturer’s instruction. DNA was detected with the Nano drop (Optizen, Korea) and was visualized by agarose gel electrophoresis [21].

**RESTRICTION ENDONUCLEASE ENZYME DIGESTION PATTERNS**

A (5-10) µl volume of DNA was digested with EcoRI and Accl enzymes according to the manufacturer’s instructions.

**MOLECULAR IDENTIFICATION OF BACTERIOPHAGE**

In an attempt for molecular identification of the three phages, conserved genomic sequences of Staphylococcus aureus phage type 3A, 11, 77, 187 and Twest like phages representing serogroups A, B, F, L and D were used. Each conserved sequence encodes a certain protein such as head protein, major capsid protein, packaging protein, tail protein or tail fiber protein [23]. Taq PCR master mix used and was supplied by Bioneer (Korea); the primers were designed by Macrogen (Korea). The primer sequences and their lengths are shown in Table 1.

Table 1 Staphylococcal phage type, sero-group, primer sequence, PCR product length (bp) and type of protein.

| Phage type | Sero-group | Primer sequence | PCR product length (bp) | Sequence coding for |
|------------|------------|-----------------|-------------------------|---------------------|
| 34-like phage | A | SG04 1 | TATCGGAGCAGGAAATTCAGG | 744 | Tail fibers |
| | A | SG05 1 | CATGGAGGATTTCAGCAACAGA | 405 | Hypothetical tail protein |
| 11-like phage | B | SG06 1 | GTAGTGGCGCGATCTGACCA | 555 | Hypothetical tail protein |
| | C | SG07 1 | TACGTGCAAGGAATCCCTTCACC | 584 | Packaging protein |
| 77-like phage | F | SG08 1 | GAGACATTTATGAGGACGATA | 1477 | Packaging protein |
| | F | Sub-group 1 | GAGACATAATATGAGGACGATA | 648 | Hypothetical capsid protein |
| | F | Sub-group 2 | GAGACATAATATGAGGACGATA | 331 | Major capsid protein |
| 187-like phage | L | SG09 1 | GCAATGCAAGGAAATTCAGG | 648 | Hypothetical capsid protein |
| TMV-like phage | D | SG10 1 | TGGCCAATTATGAGGACGATA | 331 | Major capsid protein |

**DETECTION OF POSSIBLE TOXIN GENES BY POLYMERASE CHAIN REACTION (PCR)**

Undesirable genes including staphylococcal enterotoxins and exfoliating toxins as well as toxic shock syndrome toxin of the isolated phages were searched by using QIAGEN multiplex PCR kit (QIAGEN, Germany) using a thermo cycler from Eppendorf (mastercycler, personal 5332, Germany) and according to the manufacturer’s instructions. The primers were designed for this study by Eurogentec (Belgium). A 100 µM (100 pmol/µl) stock of each primer was prepared according to the technical data sheet for each primer and kept in TE buffer at -70 °C. Staphylococcus aureus specific genes, primers and their exact sequence as well as the size of the amplified product (bp) are listed in Table 2 [24].

3. RESULTS

**BACTERIOPHAGE ISOLATION**

The isolation of bacteriophage from sewage was successful on the first attempt in October 2014 (Figure 1). Initially 20 phages were isolated and of these about 8 phages were tested for their host range. Out of 8 phages only three bacteriophages (ϕSA1, ϕSA2, and ϕSA3) were selected for further characterization for showing a strong lytic activity against all MRSA isolates. The titre of the three bacteriophages (ϕSA1, ϕSA2, and ϕSA3) was (5x1012, 2x1011 and 4x1013) respectively. A stock of each of the three bacteriophage sample was prepared and purified. All three phages produced clear large to medium sized plaques (4-6 mm in diameter) when propagated on MRSA isolate.

Figure 1. Plaques caused by bacteriophage ϕSA1.
MORPHOLOGY OF BACTERIOPHAGE

The appearance of the three phages by transmission electron microscopy is shown in Figure 2. All three phages have an icosahedral head and a long thin non-contractile flexible tail with tail fibers. Based on their morphology all three phages belong to the family Siphoviridae (order Caudovirales).

The dimensions of the three phages are shown in Table 3. Five images of each phage were measured and the mean values were recorded. All phages have long non-contractile flexible tail ranging from 159 nm to 167 nm in length.

**Table 3.** Estimated dimensions of phages ϕSA1, ϕSA2 and ϕSA3. Each value is the mean of five independent measurements.

| Phage | Head dimensions (nm) | Tail dimensions (nm) |
|-------|----------------------|----------------------|
|       | Length | Width | Length | Width | Length | Width |
| ϕSA1  | 39     | 37    | 167    | 9     |
| ϕSA2  | 42     | 39    | 160    | 10    |
| ϕSA3  | 50     | 52    | 159    | 16    |

**HOST RANGE**

The host range of bacteriophage samples was determined against 100 isolates of methicillin resistant Staphylococcus aureus (MRSA). All three phages showed lytic activity against all 100 MRSA isolates. The characteristic clear zone of inhibition is shown in Fig. 3.

**RESISTANCE OF BACTERIOPHAGE TO ACIDITY AND ALKALINITY**

Differences between the three phages in the pH range in which they were active were observed. All phages were resistant to pH 4 to pH 9. Phages ϕSA1 and ϕSA2 were also resistant to pH 10 while ϕSA3 was resistant to pH 3 (Table 4).

**Table 4.** Titre of phages (ϕSA1, ϕSA2 and ϕSA3) after exposure to a range of pH from 1 to 11

| pH range | Titre of phages that survived (pfu/ml) |
|----------|--------------------------------------|
| 1        | ND*                                 |
| 2        | ND                                  |
| 3        | 2 x 10^3                            |
| 4-9      | 10^3                                |
| 10       | 1 x 10^9                            |
| 11       | ND                                  |
| Control  | 10^9                                |

*ND= Not Done

**RESTRICTION ENDONUCLEASE ENZYME DIGESTION PATTERNS**

All three phages were highly refractory to restriction by EcoRI and AccI endonucleases enzymes. No migrating fragments could be detected (data not shown).

**MOLECULAR IDENTIFICATION OF BACTERIOPHAGE**

Based on the multiplex PCR results 4 genes out of 7 were detected. The PCR products were 155 bp, 405 bp, 548 bp and 744 bp (Fig. 4). These genes are common among staphylococcal phages of Siphoviridae family sero-groups A, B
and F which include mostly temperate phages infecting Staphylococcus aureus.

**MULTIPLEX PCR FOR DETECTION OF POSSIBLE STAPHYLOCOCCAL TOXINS**

The aim of the study was to isolate and characterize bacteriophages that have lytic activity against MRSA. Phages are known to be the most abundant viruses in the environment [25]. They are widely spread and can appear in many different environments including marine and soil environments [26, 27]. Waste-water treatment plant was considered as a potential source of phages in the current study. The isolation of Siphoviridae phages was successful from the first attempt with high phage titre ≥10¹⁰ since these phages are known for their ability to withstand adverse conditions due to their morphology [28, 29].

Based on the morphological features of the bacteriophages observed by electron microscopy all three phages belong to the Siphoviridae family with icosahedral head and long non-contractile flexible tail. The name Siphoviridae comes from the Greek word Siphon which means tube referring to the long tail [22]. This family includes eight genera that are characterized by having long (65-570 x 7-10 nm), non-contractile, thin tails which are often flexible and built of stacked disks of 6 subunits [22].

The newly isolated phages are virulent against a broad range of S. aureus isolates while most staphylococcal phages of this family are temperate phages detected as prophage or may have lytic activity due to mutation in the lysogeny functions [30, 31]. However there are several Siphoviral phages with lytic activity against a wide range of S. aureus such as YMC/09/04/R1988 MRSA BP [32]. Other virulent phages against MRSA have been isolated such as SEW, M1M4, CJ11, CJ12, CJ16, CJ17, CJ18 and CF6 [33].

The three phages were susceptible to low pH levels of 1 and 2 while their susceptibility to pH 3 varied. Phages are usually influenced by the acidity of the environment but some phages can withstand low pH environment (pH≤3.5) [34]. All three phages were stable within a wide pH range from 4-10 resembling other phages of Siphoviridae family such as the λ phage [35]. ϕSA3 was stable at a wider pH range (3-11) while ϕSA1 and ϕSA2 were stable at pH (4-10). Extremely high pH environments (pH≥12) were unfavourable for all three phages. The ability of these phages of Siphoviridae to survive such a wide range of pH make them suitable for oral administration as possible bacteriophage-based DNA vaccines as suggested by [35].

Interestingly, all three phages were refractory to digestion by endonuclease enzymes such as EcoRI and Accl. This phenomenon is referred to as an anti-restriction mechanism which is common among Siphoviridae phages [36, 37]. There are several anti-restriction strategies by which phages can evade destruction by their bacterial host restriction systems [38]. These anti-restriction strategies include alteration of phage DNA sequence such as the loss of recognition sites in the phage genome for a particular endonuclease enzyme and the incorporation of unusual bases in the phage DNA making it insensitive to digestion by endonuclease enzymes, transient occlusion or blocking of restriction sites by phage encoded proteins, subversion of restriction-modification activities and direct inhibition of restriction enzymes by phage encoded anti-restriction proteins [38-41].

The molecular identification of the three phages showed remarkable results. A high degree of homology between the newly isolated virulent phages and their temperate ancestors was observed in which four genes all of temperate phage origin were detected. Therefore our data suggest that the genomes of these virulent phages have undergone recombination or horizontal gene transfer from a common genetic pool.

Such mosaicism has been reported several times among members of all three tailed phage families. Due to such mosaicism some lytic phages can be derived from temperate phages by rearrangement and deletion of lysogeny modules [42]. A virulent mycobacteriophage D29 appeared to be
derived from the temperate phage L5 due to deletion resulting in 80% homology of their nucleotide sequences [43].

Certain staphylococcal phages encode virulence factors such as staphylococcal toxin which include staphylokinase ( sak), enterotoxin A (sea), enterotoxin E (see), enterotoxin P (sep), exfoliative toxin A (eta), Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin (tst), the innate immune modulators SCIN and CHIPS [44-46]. These toxins are responsible for a wide range of life threatening illnesses such as scalded-skin syndrome, food poisoning and toxic shock syndrome [44]. Since these toxins are harmful to mammalian cells it is important to ensure that they are not encoded by therapeutic phages. Based on the PCR results, all three phages were unable to encode for enterotoxins (sea, seb, sec and see), exfoliating toxins (eta and etb) and the toxic shock syndrome toxin (tst). The absence of these genes from the phages needs to be ensured by further studies including complete genome sequencing and clinical trials to ensure their safety as an alternative therapeutic agent against MRSA.

In conclusion, the three isolated phages are highly effective against a wide range of methicillin resistant S. aureus isolates and have the ability to survive a pH range from 4-10. Moreover, these phages lacked genes for the staphylococcal enterotoxins although they show some similarity with their ancestors of the Siphoviridae family. The strong lytic activity of these phages against MRSA, the ability to withstand a wide pH range, and the absence of toxin encoding genes all indicate that these Siphoviridae phages maybe a possible alternative for antibiotic therapy but further studies are required to assess their activity in clinical trials.

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