ORIGINAL PAPER

EFFECTS OF A NOVEL CHitosan-LOADED Bacteriophage ANTIBACTERIAL GEL AGAINST FUSOBACTERIUM ULCERANS FOR TROPICAL SKIN ULCER TREATMENT

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Abstract. This study aims to evaluate the chitosan-loaded bacteriophage gel (CLBG) for the treatment of tropical skin ulcer (TSU) against Fusobacterium ulcerans. The sewage was collected, isolated and suspended to obtain the phage. The plaque formed from phage propagation was then collected and recorded. The chitosan gel was prepared and mixed with activated bacteriophage lysate and tested using the Minimum Inhibitory Concentration (MIC) and disc diffusion tests. Physico-chemical evaluation of the loaded gel was observed including viscosity, texture, TEM and pH. The plaque-forming units (PFU) of the bacteriophage were 6.8x10^4 PFU/mL, 2.3x10^4 PFU/mL, 1.1x10^4 PFU/mL and 4.0x10^4 PFU/mL. Physical evaluation revealed a milky yellowish formation of a gel texture with a pH of 4.63. Microscopic evaluation showed the morphology of chitosan and live tailless bacteriophage. The MIC values of CLBG against the selected pathogens were 1.0 x 10^-5 mL/mL and 1.0 x 10^-6 mL/mL, respectively. The zone of inhibition (ZI) for the CLBG was greater (>48mm) compared to gentamicin (positive control >25mm). Statistical analysis using One-Way Anova showed the highly significant value of this study with p<0.001. The CLBG demonstrated a greater synergism effect against the selected pathogen. Hence, the CLBG has a great potential to be a novel drug delivery for the treatment of tropical skin ulcer.

Keywords: Bacteriophage, chitosan gel, tropical skin ulcer, Fusobacterium ulcerans.

1. INTRODUCTION

Skin disease is one of the most common illnesses among human which is presented in up to 15% of all patients in health clinics. A study in 2010 by Global Burden of Diseases reported that skin conditions were one of the main culprits, ranging from second to 11th rankings, for living impairments and were in the third rank in terms of non-fatal disease burden [1].

Tropical skin ulcer (TSU), also known as Tropical Phagedenic Ulcer, is endemic to village communities throughout the tropics and in some subtropical regions. It is mostly common in wet tropics and its incidence rose sharply during the wet season. Most cases were secondary to insect bites or scratches and usually occur on the legs [2]. World Health Organization in 2010 reported that TSU occurred mostly on children and teenagers in some

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tropical zones, although it presumably endemic in numerous damp tropical zones [3]. High incidence was reported in South America, West Indies, Africa, India, Vietnam, Philippines, Malaysia, Indonesia, Melanesia, and the West Pacific. In 2013, the disease was diagnosed in up to 100,000 cases in Thailand [4]. Majority of the cases (96%) occur on the lower leg or foot and more prevalent in males [5].

Tropical skin ulcers were caused by different types of bacteria including *Fusobacterium ulcerans* in patients with a poor immune system, compromised mucosal integrity or co-infection with other microbes, including virus [5]. Furthermore, the disease can lead to chronic phase through significant tissue interruptions such as sclerosis and osteitis that have a higher risk for removal of whole or some parts of an appendage by a surgical evacuation [6].

In most rural areas in tropical countries, western medicine is not always readily available. If clinics are present, the staff is often very busy and may not be cognizant of the diagnosis or treatment of skin conditions. The appropriate medication was often not available or of poor quality, and all too often, prohibitively expensive [2]. Availability of suitable natural alternative that are effective for tropical skin ulcer is still needed.

Bacteriophages, also referred to as “phage”, are natural bacterial viruses abundant in all environmental locations and can be found in soil, sediments, water, as well as in living or dead plants/creatures. The evaluated worldwide phage populace size is exceptionally high [7]. The ability of phage in not just to target and obliterate a particular bacterium, but to duplicate exponentially which underscores their potential part in treating infectious disease [8].

A phage is commonly made up of a capsid head and a tail [9]. It shows its antibacterial activity in their ability to increase in numbers in the presence of bacterial targets. In lytic infections, developed phages are discharged from the infected bacteria by means of debasement of the bacterial cell envelope. In the course of this lysis, the tainted bacterium is physiologically and to a large extent, also structurally destroyed. Of comparative significance, phages just minimally affect non-target bacteria or body tissues [10]. Phage therapy tremendously proven to have positive effects to patients without any harmful reaction observed during clinical trials which exhibit potential treatment for serious diseases including multi-drug resistant bacteria. Topical applications do not show any adverse effects or any immunological inconveniences [10-13]. Suitable natural biopolymer should be utilized for the use of skin recovery to enhance drug infiltration and penetrability [2, 14] especially in phage delivery.

Chitosan, a versatile hydrophilic polysaccharide derived from chitin via deacetylation, had a broad antimicrobial spectrum to which gram-negative, gram-positive bacteria and fungi are highly susceptible [15]. Chitin is the second most essential common polymers on the planet. It is a normally copious mucopolysaccharide and a fundamental part of the exoskeleton of shellfish, such as shrimps and crabs, as well as insects [16]. The structure of chitosan (deacetylated chitin) is fundamentally the same as that of cellulose. Chitosan is an immediate polysaccharide made of arbitrarily circulated N-acetyl-D-glucosamine, which is an acetylated unit and β-(1-4)-connected D-glucosamine, a deacetylated unit. It is produced by treating shrimp and shells of other shellfish with an antacid substance, similar to sodium hydroxide [14]. However, less consideration has been paid to chitin than cellulose, largely due to its inactivity. Therefore, for the most part, it remains an unutilized asset [17].

Recently, reviews from Radulescu et al. (2020) and David et al. (2019) had shown that the combination of active ingredients with biopolymer will improve drug delivery [19-20]. Furthermore, consolidating chitosan with antibacterial agents in the dermal application will enhance drug delivery [2]. Over the years, reviews found that chitosan plays an important role as a vehicle in delivering antibacterial medication, either topically or orally. The
accessibility of chitosan in a variety of structures and its remarkable biochemical qualities make it an extremely appealing biomaterial for a broad range of uses in different fields [18-20].

In the quest of searching for new alternatives to treat tropical bacterial infections which occur on the surface of the skin, the researcher aims to evaluate the compatibility, effectiveness as well as the physicochemical characteristics of chitosan-loaded bacteriophage gel against *Fusobacterium ulcerans*.

## 2. MATERIALS AND METHODS

### 2.1. MATERIALS

Deacetylated chitosan powder of A.R Grade was obtained from Chemical Solution Sdn Bhd. Acetic acid glacial; Ammonia solution and Folic acid were obtained from Bendosen Laboratory Chemicals. Methanol A.R (99.98%) grade was acquired from PC Laboratory Chemical and the *F. ulcerans* was provided by the Microbiology Department, Management and Science University. Mueller Hinton agar powder, nutrient broth agar, Trypticase Soy (TS) Agar and TS Broth Agar were purchased from Becton, Dickinson and Company.

### 2.2. METHODS

#### 2.2.1. Preparation of viral suspension

5-litre containers of marsh water were obtained from within the Shah Alam area and a viral suspension was prepared from the marsh water sludge. The sludge (10mL) was then transferred to a sterile 25mL tube and centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant was aseptically transferred to a sterile 15mL tube without disturbing the pellet. A viral suspension was prepared by aseptically filtering the supernatant through a 0.8 mm pore-sized cellulose filter to remove particulates, followed by filtration through a 0.45 mm pore-sized filter to remove bacterial cells and cellular debris [21].

#### 2.2.2. Viral isolation

Viral isolation was done by placing the sewage water mixed with soft agar and on the hard agar. The plates were subsequently incubated at 37°C for 24 hours. The plates were then checked for any plaque formation. Clear zones indicated the presence of a phage. The phage assay was repeated twice using all the bacterial strains isolated from brine water and marsh water sludge. When plaques were identified, a pure suspension was prepared by carefully removing a portion of the plaque using a sterile pipette tip and transferring the plaque to 10 mL sterile TS broth. The broth was vortex to free viral particles from the agar and residual cells removed by aseptically transferring the broth to a sterile 25 mL tube and centrifuging at 5000 rpm for 5 min. The supernatant was aseptically transfer to a sterile 15 mL tube and
stored at 5°C. The phage assay was repeated using isolated bacterial strains and stored viral suspensions to ensure the presence of phage. After which, determination of phage numbers and assess host specificity. A serial dilution (100-10-9) of viral filtrate was prepared. As previously described, 1.0 mL of viral dilutions and 3 drops of a 24 hours’ bacterial broth culture was added to soft agar (TS) and pour on top of the hard agar. Plates were incubated for 24 hours and were examined for plaques [21]

2.2.3. Preparation of chitosan gel

The chitosan gel without bacteriophage was prepared by dissolving 1 g of chitosan in 20 mL of 10% acetic acid and then diluted with 80 mL of methanol, acetylated with acetic anhydride, precipitated, rinsed with 300 mL of methanol and dried. The 10% acetic acid to methanol ratio was fixed at 20:80 to combine the efficiency of reaction with a low risk of irreversible gelation [22]. The solution was stirred at room temperature for 1 hour to allow complete dissolution of chitosan. At the same time, precautions were taken to avoid evaporation of methanol by covering it with aluminium foil.

2.2.4. Preparation of chitosan loaded with bacteriophage gel

Chitosan-loaded bacteriophage was prepared by solubilising the chitosan (4% w/v) in a dilute acetic acid solution as the aqueous phase first to complete the dissolution at 4°C. The prepared chitosan-dilute acetic acid solution (aqueous phase) was then mixed with liquid paraffin (oil phase) at a volume ratio of 1:2 and activated bacteriophage lysate (3.6 x 10^3 PFU per mL) at a volume ratio of 1:8 with chitosan solution. Then, the mixture was magnetically stirred at a rate of 800 rpm at 40°C for 15 min [23].

The gel was set up by dispersing 1% carbopol in 25mL of hydrogel for 24h and neutralizing it with sufficient amount of triethanolamine. The solution was then stirred properly with a glass rod and kept for 15 minutes. The chitosan-loaded bacteriophage solution was added to the neutralized carbopol while magnetically stirring it for about one hour, continuously [24].

2.2.5. Physico-chemical evaluation

The chitosan loaded bacteriophage solution was undergoing several centrifugation processes. At first, 1.0 mL of chitosan loaded bacteriophage solution was put in a centrifuge tube and mixed with 0.5 mL of sterilized phosphate buffer solution. The solution was centrifuged (Hermle Z206, Compact,USA) at 6000 rpm for 10 minutes. After the centrifugation, the supernatant was taken and put to a tube and was centrifuged at 16,000 rpm for 7 minutes and the steps were repeated for 6 times. Then, the supernatant was taken and mixed with distilled water. The solution was centrifuged again. The supernatant was taken and filtered using 0.45 mm millipore syringe filter. The sample was observed under TEM equipment (H-9500, Hitachi, Japan) at 80 kV, and phages were examined at 20,000-50,000 times magnification [25].
Viscosity of chitosan loaded bacteriophage gel was resolved utilizing Brookfield R/S-CC Plus rheometer (Ametek Brookfield, Middleboro, Massachusetts, USA) with shaft # C 50-1 having a speed of 20, 40, 60, 80 and 100 rpm. All estimations were done at room temperature [26].

2.2.6. Minimum inhibitory concentration (MIC)

The MIC of the chitosan-loaded bacteriophage gel was tested by a 10-fold serial dilution using 96-well plates with Brain Heart Infusion broth. 150 µL of chitosan and 30 µL of bacteriophage were subsequently diluted by transferring half of the solution from the first well up to the 9th well. An equal amount of bacteria was added to each well accordingly. The mixtures were allowed to incubate overnight, and the turbidity was observed comparing with McFarland standard [27]. The presence of turbidity indicates the presence of bacteria.

2.2.7. Kirby-Bauer’s disc assay

The antibacterial activity of the chitosan-loaded bacteriophage gel was identified using the Kirby-Bauer’s disc diffusion method [28]. The selected bacteria were tested against the formulated chitosan-loaded bacteriophage gel, positive control (Gentamicin), and negative controls (chitosan gel without bacteriophage and bacteriophage without chitosan gel). The agar plates were incubated for 24 hours, and the zone of inhibition was measured.

2.2.8. Statistical Analysis

The results obtained were subjected to statistical analysis by utilizing the SPSS One-Way ANOVA test using post hoc Tukey tests. Results were expressed as mean ± standard deviation and p-value. Statistically, significant difference was denoted by p<0.05 and if p<0.001 indicating highly significant results.

3. RESULTS AND DISCUSSION

3.1. RESULTS

The results revealed that chitosan loaded bacteriophage gel (CLBG) was clear yellow, opaque, and presented a dense outer layer. The texture of CLBG was jelly and in semi-fluid form. This was due to the fact that chitosan has dissolved in the acetic acid and then mixed with triethanolamine and carbopol which was alkaline in characteristic, thus, it became a semi-fluid in texture. Upon the mixing the chitosan loaded bacteriophage solution with triethanolamine-carbopol solution, the colour of the formulated gel changed from milky yellowish to clear yellow which is presented in Table 1.
Table 1. Physical characteristics of formulated chitosan loaded bacteriophage gel.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Results</th>
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<tbody>
<tr>
<td>Weight (g)</td>
<td>46.04</td>
</tr>
<tr>
<td>Colour</td>
<td>Clear yellow</td>
</tr>
<tr>
<td>Texture</td>
<td>Jelly and semi-fluid</td>
</tr>
<tr>
<td>pH</td>
<td>4.63</td>
</tr>
<tr>
<td>Odour</td>
<td>Slightly pungent smell</td>
</tr>
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The viscosities shown are the steady shear viscosities calculated from the torque measured after 5 min of continuous shear. All materials exhibited shear thinning behaviour over the range of shear rates studied. The relatively constant slope of these curves indicate that, under these flow conditions, all product mixtures can be approximated by the power law equation for the viscosity (cP) as a function of shear rate (rpm). Fig. 1 below shows the viscosity of the formulated gel according to the speed of the rotation per minute. The graph below shows the graph of speed vs viscosity of the CLBG.

![Graph of Speed vs Viscosity](image)

**Figure 1.** The viscosity (cP) of the gel in the increasing rate of speed (rpm).

The morphologies and compatibility of chitosan and bacteriophage were investigated using Transmission Electron Microscope (TEM). Fig. 2(a, c, d) show the morphology of chitosan. It can be observed that the diameter of chitosan was about 200 nm. Fig. 2b shows the morphology of bacteriophage.
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Figure 2. TEM morphology of chitosan loaded bacteriophage gel: a) the morphology of chitosan under 200 nm TEM observation; b) the morphology of tailless bacteriophage under 2000 nm TEM observation; c) the tailless bacteriophage that surrounded by chitosan under 500 nm TEM observation; d) two tailless bacteriophage stick together with chitosan under 500 nm TEM observation.

Minimum inhibitory concentration (MIC) is assumed as ‘gold standard’ in order to determine the lowest concentration of the gel formulation that could inhibit the growth of the selected bacteria. MIC of chitosan loaded bacteriophage against the selected bacteria was determined prior conducting antimicrobial study.

Figure 3. The graph of the mean zone of inhibition against *F. ulcerans*.

The formulation was subjected to serial dilution during the procedure. A single 96-well microdilution plate was used in this study. The wells were prepared in the order of decreasing concentration of formulation of chitosan loaded bacteriophage solution. 100 µL of chitosan was consequently transferred and diluted into the rest of wells in which contained 30 µL of bacteriophage and 15 µL of the selected bacteria in each of the wells. The MIC was identified on the absence and presence of turbidity in the wells.
The MIC results obtained from this study were similar for each row of the wells. In the wells number 1-6, clear yellowish liquid was found. Though there were presence of the clear yellowish liquid, the solution in the wells were not turbid indicating the absence of bacteria. The formulated gel has an MIC of $0.5 \times 10^{-6} \text{ mL/} \mu \text{L}$ against the *F. ulcerans*. The antibacterial study was carried out by performing Kirby-Bauer’s disc diffusion method against the *F. ulcerans* using the formulated CBLG, positive control (gentamicin), chitosan gel without bacteriophage and bacteriophage alone as a control. The zone of inhibition of discs for each formulation was recorded by measuring the inhibited area using a ruler.

Based on the graph, the formulated CBLG has higher antibacterial activity compared to the positive control (gentamicin) against *F. ulcerans*. From the results mentioned above, it can be deduced that the formulated chitosan loaded bacteriophage gel has more efficacy than the compared antibiotic discs, which was gentamycin. This statement can be further strengthened by statistical analysis conducted using one-way ANOVA in SPSS [29-33]. Based on one-way ANOVA using data of ZI, it can be proven that the formulated chitosan loaded bacteriophage gel has higher efficacy as compared with antibiotic disc. The formulated chitosan loaded bacteriophage gel shows a significantly difference (*p<0.001*) as compared to the positive and negative controls.

3.2. DISCUSSION

The chitosan-loaded bacteriophage gel was observed using TEM to determine the compatibility between the chitosan and the bacteriophage. The chitosan-loaded bacteriophage observed under the TEM was clearly visible. The results showed that the chitosan diameter (0.5 µm) was much smaller and spherical in shape. This was because when observed under the TEM, the image showed a very small object or entity similar to a virus. Based on the results, the tailless viruses appeared to be surrounded and covered with chitosan. The image was much greater than when it was observed under the Scanning Electron Microscope (SEM). The previous study showed that the bacteriophage microspheres were spherical and had a smooth surface texture, with diameters mostly measuring at 25 µm [34].

Moreover, chitosan gel was heterogeneous, with well-interconnected pores. According to the study conducted by New *et al.* (2010), elongated pores were observed perpendicularly in chitosan derived from shrimp shells. This might be contributed by the growth of highly parallel ice crystals in between the layers created by the hydrogen bonds between the long chains of polymers during lyophilisation [35]. When the chitosan powder was dissolved in acetic acid, the alignment and arrangement of the chitosan molecules fell apart, collapsed, and disappeared. This was subsequently followed by a freezing process at 40°C which led to the reorganization of polymer chains in the course of the construction of intersecting network pores as well as scaffold layers by means of hydrogen bonds present in the polymer chains and initial crystalline formation.

Fig. 2 illustrated in the result showed that the virus is tailless, with a polygon-shaped head. The first evidence which indicated that the T-even head is a bipyramidal hexagonal prism observed in a hexagonal profile, where the head appeared to be nearly parallel to its long axis [36]. Based on the results, the viruses mostly showed a diameter of 500 nm, without a tail and with an empty head.
According to Chai et al. (2016), among all the viruses collected, electron microscopy revealed that the φHN161 virus particle was tailless with a circular capsid; the isometric capsid had a diameter of 40 nm. The genome formed a firmly pressed loop in some virus particles, while virus φHN161 in different phases showed an empty centre [37].

The chitosan-loaded bacteriophage gel had a pH of 4.63, which is slightly acidic in terms of its properties since the chitosan was initially dissolved in acetic acid. Based on previous research, the pH of the chitosan arrangements caused a significant decrease on a large portion of the experimented surrogates at 37°C for 3 hrs showing a 0.70-0.69 log PFU/mL and 0.46-0.47 log PFU/mL reduction at pH 4.5 and 5.6, respectively. The results showed a 0.89-1.02 PFU/mL and 0.58-0.64 log PFU/mL reduction at pH 4.5 and 5.6, respectively for the bacteriophage [38].

The effect of chitosan gel against *F. ulcerans* that was shown in the results of the disc diffusion confirmed the antimicrobial properties of chitosan, but the result showed no zone of inhibition between chitosan gel and bacteriophage alone. As demonstrated by previous studies, restraint zones do not show when the agar dissemination technique is utilized for the assurance of chitosan film movement [39].

However, there is an adequate proof that chitosan films do exert antimicrobial effect but not on *F. ulcerans*. Previous studies observed a reduction in bacterial growth when chitosan was incorporated into the agar medium or when the agar medium was under chitosan coating and film. Furthermore, the researchers found that the level of restraint of bacterial growth relied on the deacetylation degree (DD) estimation of chitosan [39].

The significant antibacterial activity of CLBG was comparable to the broad-spectrum gentamicin. This method institutes the specific concentration that is effective to act against the selected pathogen in order to prevent further spreading and eventual infection due to the bacteria. The lower MIC value is a significant indication of the great potential and effectiveness of the formulation. The Kirby-Bauer method was used to conduct the disc diffusion procedure. This approach is commonly and widely accepted as a relatively simple methodology while at the same time accurate and reproducible. The mechanism of the disc diffusion can be explained by the formulation or diffusion of the antibiotic in the agar medium. The existence of growth inhibition zones proves the effectiveness of the chitosan-loaded bacteriophage gel as well as the antibiotic. The size of inhibition zone is influenced by a few factors [40], such as the density and/or viscosity of the agar medium, concentration of chitosan in the formulation, rate of diffusion from the gel and disc, as well as the sensitivity of the selected bacteria towards the chitosan-loaded bacteriophage gel and antibiotic.

A larger ZI indicates higher antibacterial activity and sensitivity of the bacterial strain against chitosan-loaded bacteriophage gel. Based on the results obtained, the negative controls, which were chitosan gel without bacteriophage and bacteriophage without chitosan gel, exhibited the smallest ZI on the tested bacteria. The results showed that the antibacterial activity was much lower in comparison to the chitosan-loaded bacteriophage gel. The chitosan itself does in fact possess antibacterial activity [41], but our results showed that it does not work efficiently alone against *F. ulcerans*.

When chitosan powder is dissolved in acetic acid, the acylation process occurs. Chitosan contains a numbers of amino molecules [14], which create easier acylation process than that in chitin, and thus the presence of a catalyst is not necessary. The reaction medium can be either methanol or ethanol. In this case, the medium used was methanol.
Generally, acylation reactions are frequently carried out in mediums such as aqueous acetic acid/methanol. When comparing acylation with N-alkylation, the former is reported to be more adaptable since it permits the addition of hydrophobic moieties of alcohol, amino or both residues [42-46].

The pungent odour mentioned above was due to the involvement of acetic acid in the formulation of chitosan gel-loaded bacteriophage. Acetic acid, also known as ethanoic acid, is widely recognized for its sour taste as well as its pungent smell, besides its identifiable presence in vinegar. Chitosan is reported to dissolve in an acidic medium as opposed to a basic or neutral medium. This is due to the existence of amino groups in the chain structure of chitosan, which enables it to dissolve in aqueous acid solution such as acetic acid [47].

The protonation of chitosan changes it into a polyelectrolyte in acidic solutions. The positive charge in chitosan is attained after the amino groups undergo protonation in the form of NH$_3^+$

The presence of acetic acid in the gel and methanol in chitosan-loaded bacteriophage gel makes it highly possible for them to react with each other, forming the ester methyl acetate, even though it is present in small quantities. The esterification linkage also contributed to the structural organization and arrangement of polymer chains in the chitosan-loaded bacteriophage gel. Esters generally have lower pH compared to carboxylic acids. This also explains the pH value of the chitosan-loaded bacteriophage gel [50].

According to the statistical analysis conducted using the One-Way ANOVA test with Tukey HSD, the antibiotic disc, chitosan gel without bacteriophage, bacteriophage without chitosan gel and chitosan-loaded bacteriophage gel were divided into four subsets. The chitosan-loaded bacteriophage gel and antibiotic disc fell under a separate subset, indicating that the antibacterial activity is much more significant in comparison with the chitosan gel without bacteriophage and bacteriophage alone.

4. CONCLUSIONS

In conclusion, chitosan-loaded bacteriophage gel plays an excellent role as an antimicrobial agent against the selected bacteria and has been proven to have a broad-spectrum antibacterial effect. This preliminary study further enhances our understanding regarding the compatibility between chitosan and bacteriophage and their potential to exert antimicrobial effect in order to act against tropical skin ulcer. However, extensive preclinical
studies which includes toxicological, pharmacological and stability testing in the development of novel drug delivery system in the future.

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