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Research Article

In Vitro Activity of Bacteriophage and Chitosan Against Sea Water Isolated Bacteria

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Abstract

The primary cause of morbidity and mortality is the soft tissue infections after bacterial infection with saltwater exposure. Bacteriophages were being considered to be bacteria that absorb viruses, so active bacteriophages can be applied theoretically to prevent bacterial infections. Chitosan, a partly deacidified natural polymer derived from a crustacean shell or fungus, which has antimicrobial potential. The present study aims to investigate bacterial and chitosan antibacterial activity against isolates of marine water. Five lytic phages in the allocated area of Shah Alam, Malaysia were isolated from marsh water and tested against five different seawater-isolated bacterial strains. Phage and disk diffusion experiments are used to assess the sensitivity profiles of the five isolated strains of marine water. The five isolates of bacteria, including small plaques and chitosan, were found to be susceptible to isolated phage. In particular, *Klebsiella terigeni* was > 22mm ZI, with *streptococcus spp* achieving > 18mm ZI, chitosan showed susceptibility

to maritime water isolates. A bacteriophage and chitosan combination may potentially be more effective in coping with seawater infections than by individual therapy alone, which is why future studies may also suggest.

Introduction

Seawater harbours a great diversity of living organisms, going from tiny microscopic bacteria, archaea, fungi and protista to naturally visible shellfish and fishes, and every assuming a noteworthy part in the biogeochemical cycle. As indicated by a few studies, the assorted microbial qualities increment as one gets closer to the coastal area [1]. Water-borne pathogen contamination in water resources and related diseases are a significant water quality concern throughout the world. Increasing interest in controlling water-borne pathogens in water resources evidenced by a large number of recent publications attests to the need for studies that synthesize knowledge from multiple fields covering comparative aspects of pathogen contamination, and unify them in a single place in order to present and address the problem as a whole. Providing a broader perceptive of pathogen contamination in freshwater (rivers, lakes, reservoirs, groundwater) and saline water (estuaries and coastal waters) resources, in which attempts to develop the first comprehensive single source of existing information on pathogen contamination in multiple types of water resources. In addition, a comprehensive discussion describes the challenges associated with using indicator organisms. Potential impacts of water resources development on pathogen contamination as well as challenges that lie ahead for addressing pathogen contamination are also discussed [2,3].

Chitosan, an N-acetylated derivative of the polysaccharide chitin, is a biopolymer that originated from exoskeletons of arthropods and crustaceans like shrimp, crabs and lobster. Chitosan is a weak base and insoluble in water but soluble in dilute aqueous acidic solutions. It is commonly solubilized with acetic acid at the concentration of 0.1 M or 1%, which is dependent on the pK value of acid to achieve a degree of protonation [4]. These cause the chitosan polymer to swell due to electrostatic repulsion of the polymer's internal groups having the same charge that is then soluble. Chitosan has reported several pharmacological actions, including anti-inflammatory and anti-microbial properties. Since chitosan has natural antibacterial properties, it can also be used as bandages or in wound dressings [5]. Moreover, chitosan can be characterized as a bacteriostatic rather than bactericidal [6], even though several factors may contribute to the antibacterial action and also the precise mechanism is not fully understood [7]. Inversely, chitosan is the most sensitive with Gram-negative, which is the hydrophilicity is significantly higher than in Gram-positive bacteria. Previous studies have shown that molecular weight (MW) and degree of acetylation (DA) give effect to the biological activity of chitosan significantly. Furthermore, MW has a significant influence on antimicrobial activity than DA, which may affect the antimicrobial activity of chitosan. Besides that, chitosan has been studied on yeast and moulds associated with food and plant spoilage then it is very effective in preventing spore germination, germ tube elongation and radial growth [8]. It is also useful in Botrytis cinerea in inhibiting the growth of decay-causing fungi and induces defence response in the host [9].

Chitosan has a considerable object of interest due to its biocompatibility, biodegradability, and bioactivity, which is relevant biomaterials for drug delivery system. It is mucoadhesive and releases of active ingredients in a period. It can be formulated in the hydrogel systems that can be designed to deliver drugs locally to the

locally to the stomach or the upper part of GIT to improve bioavailability [10]. Incorporating antibiotics in chitosan could also provide alternative methods of treating musculoskeletal infections [11]. Chitosan, a non-toxic polymer is an excellent vehicle of the active molecule in the prevention and treatment of periodontal diseases [12].

Bacteriophage, also called phage, is a virus which is known to infect only the bacteria and causes it to lyse [13]. Bacteriophages are the most charitable entities on earth. These bacterial viruses are encapsulated by protein coats which have genetic material in the form of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The polyhedral capsid has appeared in all phage except filamentous phages. When phages infect bacteria, it is proliferating in two possible ways which are lytic and lysogenic life cycle. Furthermore, some phages are known as temperate phages which can develop vegetatively and also can integrate their genome into host chromosome duplicate with the host for many geneses. Bacteriophages are getting a tremendous amount of attention due to their potential to be used as antibacterial alternatives, phage display systems and vehicles for vaccines distribution [14]. Consequently, this research is carrying out to investigate the antibacterial activity of bacteriophage and chitosan against seawater isolates.

Materials and Methods

Isolation of Bacteria from Seawater

5 litres of seawater was collected from Pantai Remis, Jeram, Selangor, Malaysia. A combination of 60% seawater prepared brine water, and 40% distilled water, and it was stored at room temperature for the duration of the research. Bacterial strains were isolated from the brine water by streaking brine water samples onto Tryptic Soy agar (TS) by using a sterile cotton-tipped swab. Inoculate agar plates were incubated for 24 h at room temperature [14]. To ensure the purity of the culture, after incubation, an individual and distinct colony were identified and inoculated onto TS agar plates using the streak plate technique. After 24h incubation at room temperature, a well-isolated colony was transferred to TS agar slants. Before phage assays and identification tests, 18-24 h broth cultures were prepared by aseptically transferring a loopful of bacteria from the agar slant to TS broth. An inoculated broth tube was incubated at room temperature while shaking at approximately 150 rpm [15,14].

Identification of Strains

Colonies obtained from the seawater were identified according to the standard microbiological techniques [16].

Preparation of Bacteriophage from Marsh Water

Preparation of the Viral Suspension

Phages were isolated from marsh water sludge obtained in Shah Alam area, Selangor state. Sludge (10mL) was transferred to a sterile 25mL centrifuge tube and was centrifuged at 2000 rpm for 5min. After centrifugation, the supernatant was aseptically transferred to a sterile 15mL tube without disturbing the pellet. A viral suspension was prepared by aseptic filtration of supernatant through a 0.8mm pore sized

cellulose filter to remove particulates, followed by filtration through a 0.45mm pore sized filter to remove bacterial cells and cellular debris.

Viral Isolation

The Phage Assay was used to determine if phage were present in the marsh water sludge suspension. Underlay "hard" TS and TSBW agar were prepared as previously described [17]. Overlay "soft" TS and TSBW agar were prepared by adding half of the amount of agar used for "hard" underlay agar. Sterile underlay agar was poured into sterile Petri dishes and left to harden. After the underlying agar was solidified, 1.0mL of marsh water sludge suspension and 3 drops of a 24 h broth culture was added to 3.0mL soft agar, vortex then pours on top of the underlying agar. The soft overlay was allowed hardening before incubation of plates at room temperature. After 24 h incubation, the plates were checked for plaques (clearing zones) within the bacterial lawn, indicating the presence of 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 10mL sterile TS broth. The broth was vortex to free viral particles from the agar, and residual cells were removed by aseptically transfer the broth to a sterile 25ml centrifuge tube and centrifuging at 5000 rpm for 5 min. The supernatant was aseptically transferred to a sterile 15mL tube and stored at 5°C. The Phage Assay was repeated using isolated bacterial strains and the stored viral suspensions to ensure the presence of phage, determination of phage numbers, and assess host specificity. A serial dilution (100-10-9) of the viral filtrate was prepared. As previously described, 1.0mL of viral dilutions and 3 drops of a 24 h bacterial broth culture was added to soft agar (TS) and poured on top of the hard agar. Plates were incubated for 24 h and were examined for plaques.

Preparation of Chitosan

Chitosan powder was purchased from blablabla chitosan powder.

Preparation of Chitosan Stock Solution

Chitosan stock solution was prepared by adding 2.5g of powder chitosan to 50mL of acetic acid and left overnight. After chitosan powder was dissolved, 200mL of methanol was added to dilute the dissolve chitosan. Further, the solution was stirred and filtered through a 0.22μm millipore syringe filter to remove any impurity before it is used [18,19].

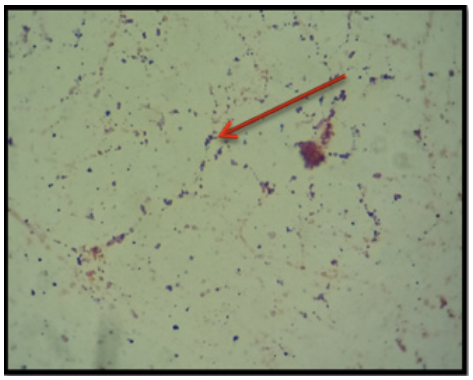
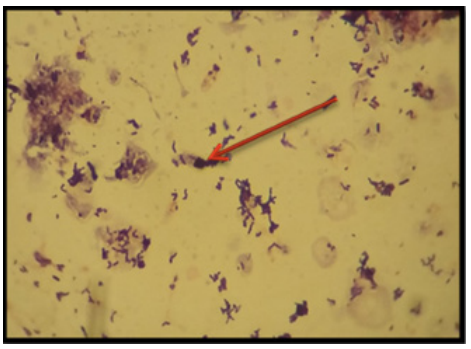
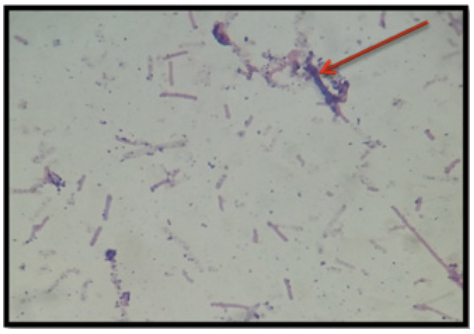
Disc Diffusion Preparation

The antibacterial activity of the chitosan was evaluated against isolated bacteria from seawater by the agar diffusion method with Muller Hilton agar (MH) as the medium. An isolated bacterium from seawater was cultured on MH agar. Place the Gentamicin antibiotic on MH agar as a control group. Place the plain disc that contains chitosan solution on MH agar and then was incubated for 24 h at 37°C. Antibacterial activity was measured in triplicate, as the diameter of the inhibitory zones in the plates, the mean of three measurements was taken then proceeded with data analysis which is by using Microsoft SPSS means that were expressed as mean ± standard deviation (SD) [18].

Results and Discussion

Isolated Seawater Bacteria Identification

Five bacterial strains were isolated from seawater. Three out of five isolates were determined to be Gram-negative and two Gram-positive. Both cocci and bacilli were represented (Figure 1). The bacteria strains were identified by using Gram staining to differentiate the shape of the bacteria and also to stain the colony. Table 1 and 2 showed that the biochemical tests were done to know the species of isolated seawater bacteria.

SEAWATER SAMPLE	GRAM STAINING RESULT
(a) 	Plate 1 • Shape: Coccus • Colour: Purple • Gram: Positive
(b) 	Plate 2 • Shape: Coccus (Short Chain) • Colour: Purple • Gram: Positive
(c) 	Plate 3 • Shape: Coccus • Colour: Purple • Gram: Positive

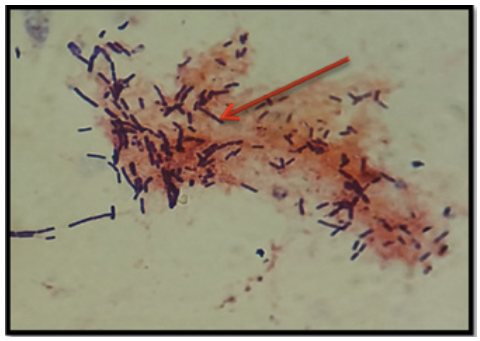
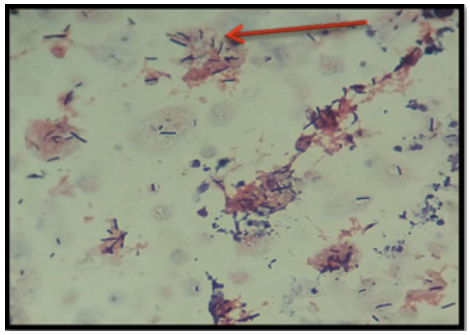
(d) 	Plate 4 • Shape: Rod / Bacillus • Colour: Pink to Red • Gram: Negative
(e) 	Plate 5 • Shape: Rod / Bacillus • Colour: Pink to Red • Gram: Negative

Figure 1: *Gram staining for isolated seawater bacteria: (a) Staphylococcusspp. and (b), Enterococcus spp. (c) Streptococcus spp. (d) Esherichiahermannii and (e) Klebsiellaterrigena.*

Table 1: *Biochemical test and name of species for Gram-positive bacteria*

NO.	TEST	NAME OF BACTERIA		
		Staphylococcus spp.	***Enterococcus spp.***	***Streptococcus spp.***
	CATALASE	+	+	-
	COAGULASE	-	-	-
	OXIDASE	-	-	-
	PENICILLIN ANTIBIOTIC TESTING	6mm (resistance)	6mm (resistance)	6mm (resistance)
	MANNITOL SALT AGAR (MSA)	+	+	-
	BLOOD AGAR	Gamma (ɣ) haemolysis	Alpha (α) haemolysis	Beta (β) haemolysis

Table 2: *Biochemical test and name of species for Gram-negative bacteria*

NO.	TEST	NAME OF BACTERIA	
		Escherichia hermannii	***Klebsiella terrigena***
	Indole	+	-
	Methyl red (MR)	+	-
	Voges – Proskauer (VP)	-	+
	Citrate	-	+
	Motility	+	-
	Urease	-	-
	Triple sugar iron (TSI)	A/A, Bubbles, No H_2S	A/A, Bubbles, No H_2S
	Oxidase	-	-
	GNA wells		
	• Lysine	+	+
	• Ornithine	-	+
	• Glucose	+	-
	• Mannitol	-	+
	• Xylose	-	-
	• ONPG	+	+
	• TDA	+	-

Phage Titer

Figure 2 showed that there was a presence of phage where are small clear spots that called plaques which infect and kill the seawater isolated bacteria in the plate. The plaques form because the phages have busted open the bacteria at that site on the plate. Determination of phage titer also showed in Table 3 that the number of the plaque-forming unit from phage isolates were found to be as high as 10^5 to 10^9pfu/ml.

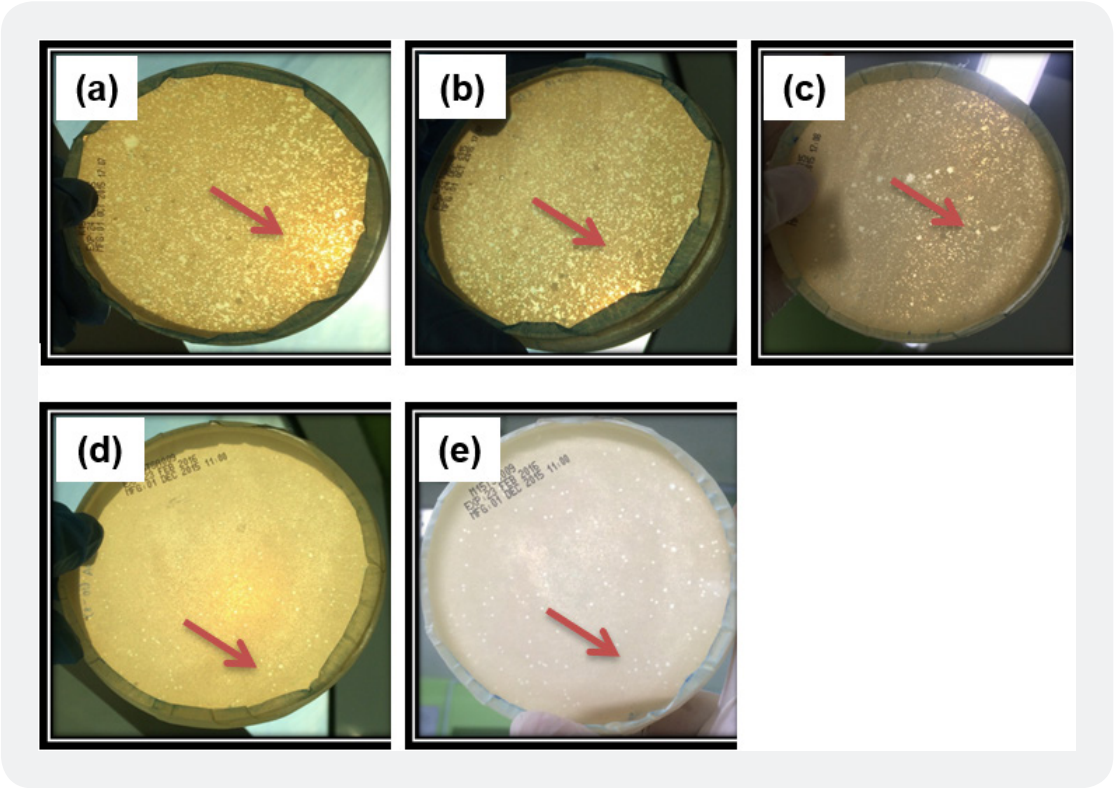


Figure 2: *Phage plaque plates: (a), Staphylococcus spp. (10-9): (b), Enterococcusspp. (10-6): (c), Streptococcus spp. (10-8): (d), Escherichia hermannii (10-5) and (e), Klebsiellaterrigena (10-8).*

Table 3: *Phage titre determinations: the viral titre is a quantitative measurement of the biological activity of a virus and is expressed as plaque-forming units (PFU) per ml*

Seawater Isolated Bacteria	Plaque per Plate	Dilution Factor (DF)	Volume of Phage Plates (ml)	Titre: Plaque - Forming Unit (PFU) per ml
Staphylococcus spp.	240	10^{-9}	0.1	2.4×10^{12}
Enterococcus spp.	210	10^{-6}	0.1	2.1×10^{9}
Streptococcus spp.	200	10^{-8}	0.1	200×10^{9}
Escherichia hermannii	270	10^{-5}	0.1	270×10^{6}
Klebsiella terrigena	140	10^{-8}	0.1	1.4×10^{11}

Disc Diffusion Test for Chitosan

Figure 3 shows results for disc diffusion test. Gentamicin antibiotic was used as a control group. All of the isolated seawater bacteria showed that indicates the sensitive result, which is the zone in inhibition more than 12mm of diameter. The diffusion test by Kirby - Bauer method shows that the chitosan solutions possess an effective and sensitive antimicrobial activity.

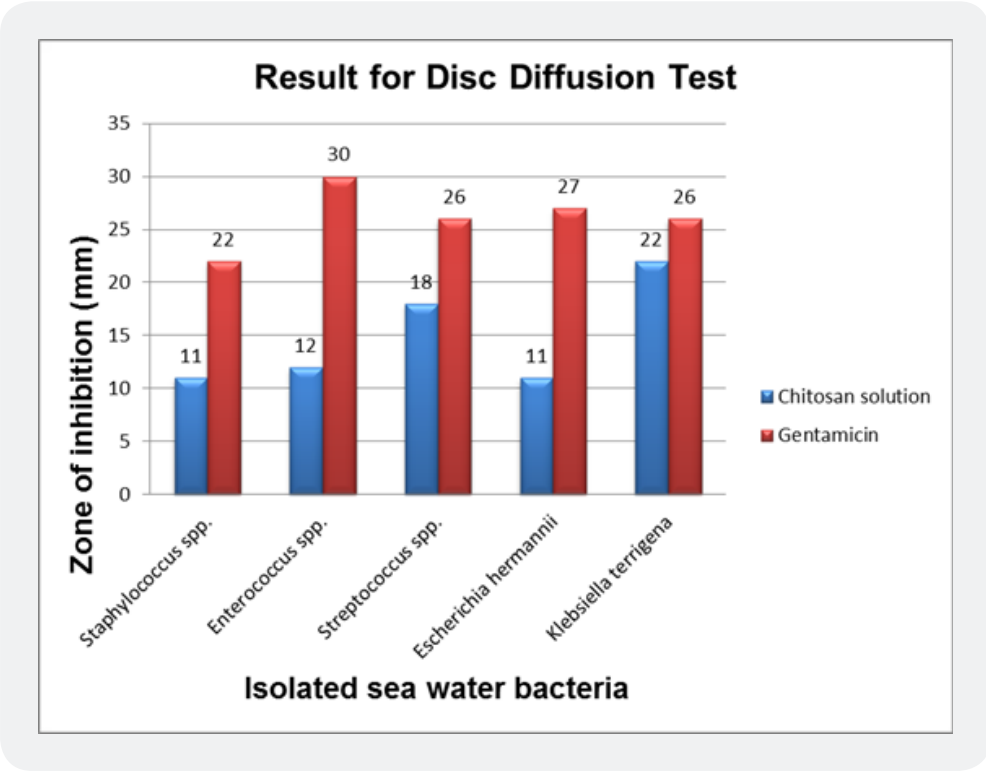


Figure 3: *Disc diffusion results of chitosan solution in the isolated seawater bacteria*

Discussion

Bacterial richness is large higher in the water column than in the sediment. It is most elevated in the O2-least zone of the water column and most minimal in the subseafloor sediment. The brine-seawater interface represents a unique microbial ecosystem mainly determined by a steep salt gradient (up to 26% NaCl) within only a few meters. It is, in addition, a significant cause of potentially life-threatening wound infections. Infections following ingestion of raw or undercooked seafood, commonly raw oysters, can lead to primary septicaemia with a fatality rate of 50-60%. An unusual symptom, occurring in 69% of 274 cases reviewed by [20], is the development of secondary lesions, typically on the extremities, which are generally severe (often necrotizing fasciitis) and require tissue debridement or amputation [20].

Based on the phage titer record in Table 3, the plaque created by the phages varies in size and is characterized by the round zone (Figure 2). The number of the plaque-forming unit from phage isolates were found to be as high as 10^5 to 10^9pfu/ml (Table 3). The high PFU observed in this study could be provided fresh growing cells resulting in enhanced production of progenies by the phages. For a substantial phage check, the quantity of plaques per plate should not exceed 300 nor be less than 30 [21]. Plates showing more than 300 PFUs are too numerous to count (TNTC) while plates demonstrating less than 30 PFUs are too few to count (TFTC). In plaque arrangement, it was observed the absence of bacteria against a background of dense bacterial growth, each zone of destruction being a phage plaque (hole) in the lawn of bacteria. Each hole represents to one phage in the example connected to the plate.

The disc diffusion results of chitosan solution showed that have possessed an effective and sensitive antimicrobial activity. The 50mg/ml concentrations of the chitosan solution used showed significant inhibition zone of 11mm to 22mm (see in Figure 2). Results obtained showed that the chitosan solutions were sensitive towards all seawater bacteria isolated.

Chitosan has several mechanisms involved in the process of the antimicrobial and antifungal property. The first mechanism involves the positively charge chitosan interacting with the negatively charged surface of the cell that significantly alters its permeability [22]. Ionic surface interaction results in wall cell leakage that eventually leads to cell death. The second mechanism is penetrating the nucleus of the bacteria and inhibiting RNA and protein synthesis by binding of the chitosan with the cell DNA via protonated amino groups, which leads to disruption of cell survival. Lastly, chitosan also exerts its antibacterial activity by acting as a chelating agent. It removes metals, trace elements or essential nutrients from bacteria causing distortion in cell growth and eventually death [7]. The molecular weight (MW) and the degree of acetylation (DA) are also important factors in determining such action [8].

Conclusion

As a conclusion, this research has the effectiveness of antibacterial activity of bacteriophage and chitosan against and also can be used to treat infectious diseases caused by both Gram-positive as well as Gram-negative seawater bacteria. Although further investigation is needed, the results might shed light on the application of bacteriophage and chitosan to control seawater-related bacterial infections. A combination of bacteriophage and chitosan could potentially be more efficient in the treatment of seawater infections than using individual therapy alone and therefore, can be suggested for future studies.

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