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Inhibitory Effect of Hydrogen Peroxide (H_2O_2) and Ionic Silver (Sanosil-25®) on Growth of a Pathogenic Bacterium (*Vibrio harveyi*) Isolated From Shrimp (*Litopenaeus vannamei*)

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Abstract

Vibrio harveyi is a bacteria. It is a pathogen of shrimp which can cause vibriosis. It cause serious disease in the shrimp post larvae and defect in post larvae production. The aim of this study was to determine the concentration of Sanosil-25® which able to inhibit the bacterium growth. Therefore, Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of sanosil-25 against *Vibrio harveyi* was determined by tube dilution method which recommended by National Committee for Clinical Laboratory Standards (NCCLS) in the laboratory condition. The results showed that MIC was 15 Part Per Million (ppm) and MBC was 20ppm during 48 hours. It showed that Sanosil-25 is an active antimicrobial agent against *vibrio harveyi* and can eradicate the bacteria from the water at concentration as well as 20ppm. Therefore, Sanosil-25 may be a useful chemical and for hygienic procedures of water against the bacterium. It didn't determined side effects of 20ppm of Sanosil-25 in shrimp.

Keywords: *Vibrio harveyi*; Sanosil-25®; Minimal inhibitory concentration (MIC); Minimal bactericidal concentration (MBC); Shrimp

Introduction

The commercial cultivation of shrimp (prawn) has become a major economic activity throughout the world as well as in Busherh province of Islamic Republic of Iran (I.R.I). The shrimp is widely commercially cultured in 5 provinces of Iran. In 2012, production of shrimp (cultured) was about 10000 metric-tons in Iran and it was about 6700 tons for Bushehr province alone. The commodity commands a high value because of its demand in both local and foreign markets. Shrimp farming in the world has been a major success in the 1980s until it's declined in the mid-1990s. The growth of the shrimp aquaculture industry increased the disease outbreaks. Thus, it is necessary to intensify farming practices to maximize profits. Problems of diseases often accompanied this intensification as environmental conditions deteriorated and brought the decline of the industry [1]. So, it is necessary to prevent disease by sanitary techniques and hygienic protocols.

Because of rapid development, coupled with mismanagement and the absence of intensive hygienic protocols, the global shrimp farming industry has been severely affected by various diseases resulting in huge economic losses. In Asia and South America, one of bacterial species, Vibrio harveyi, a Gram-negative bacteria and the causative agent of luminous *vibriosis,* has alone caused significant economic losses to the penaeid shrimp industry [2,3].

Vibrio harveyi is a species in the bacterial family: *Vibrionaceae. V. harveyi* are rod-shaped, motile (via polar flagella), facultatively anaerobic, halophilic, and competent for both fermentative and respiratory metabolism. It does not grow lower than 4°C or higher than 35°C.

Over the past decade, strains of this species have been reported to be significant pathogenic agents and one cause of the high rates of shrimp mortality in the shrimp culture industry worldwide. Mortalities of shrimps larvae associated with luminescence have been observed in hatcheries in Indonesia, Philippines and Taiwan. In Thailand, *V. harveyi* has been reported to cause 70-100% of deaths in shrimp larvae at the nauplii, mysis and postlarva stage with nauplii larvae being the

most sensitive. In southern parts of Thailand, *V. harveyi* is the most important pathogen of the shrimp, *Penaeus monodon*, in shrimp farms also found that when Vibrio and luminous bacteria exceeded 104 cells/ml in overcrowded cultured shrimp ponds, this caused serious health problems to the shrimp. Vibriosis in cultured shrimps causes severe economic losses in shrimp production [4].

Susceptibility testing is indicated for any organism that contributes to an infectious process. We can estimate MIC and MBC by it. Susceptibility tests are most often use when antimicrobial activity of an agent is unknown. A variety of laboratory methods can be used to measure the in vitro susceptibility of bacteria to antimicrobial agents. NCCLS M7-A3 document described methods (macrodilution and microdilution) and agar dilution techniques, and it includes a series of procedures to standardize the way the tests are performed [5,6].

Broth dilution susceptibility testing has become more frequently used with the popularization of efficient microdilution methods [7,8]. There are a few published data on the minimal inhibitory concentrations of antimicrobial agents for *vibrio harveyi* specially Sanosil-25. Sanosil-25 is a universally applicable disinfectant compound that is highly effective against pathogenic bacteria, fungi, algae, viruses and amoebae. The compound contains 48% hydrogen peroxide (H_2O_2) and 0.05% silver ion (Ag+) as a stabilizing agent [9]. The purpose of this study was to evaluate MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) values obtained with sanosil-25 against an isolate of *vibrio harveyi* from infected shrimp which cultured in pond.

Materials and Methods

Test organism

An isolated microorganism (*vibrio harveyi*) from an infected shrimp (*Litopenaeus vannamei*) was evaluated. The organism was isolated by cultivation of shrimp haemolymph on TCBS medium then the organism identified by microbiological diagnostic tests. *Vibrio harveyi* ferment glucose, cellobiose, threhalose but can't ferment *meso*-Inositol. The bacteria reduce nitrate to nitrite and was oxidase positive [10].

Vibrio harveyi have been isolated from sea water and infected shrimp which cultured in ponds of Bushehr province in Iran. So, there is need to an antimicrobial agent to inhibit growth of the bacteria in the water and kill it. Therefore, we evaluated susceptibility of *Vibrio harveyi* at the different concentration of Sanosil-25 as well as a disinfectant agent for water sanitary.

Antimicrobial agent

Sanosil-25 was obtained from the manufactured (SANOSIL®, ltd) as standard reference liquid, then diluted with distilled water for the experiments and added to liquid media. It claimed that Sanosil Super-25 is highly effective, universally applicable disinfectants by the factory. Sanosil® disinfectants eliminate all pathogenic bacteria, biofilms, fungi, mould, virus, amoeba, etc. without side effects. The two main components, hydrogen peroxide (H_2O_2) as the oxidizing agent and silver (Ag+) with its powerful oligodynamic and catalytic effect, are combined with stabilizers to form a complex solution. Due to the patented manufacturing process of the two basic elements, an excellent enhancement of the disinfecting effect is obtained.

Susceptibility testing

Antimicrobial susceptibility testing standards exist in NCCLS documents. NCCLS publishes guidelines for susceptibility testing that define the organisms, conditions, methods, and antimicrobial agents that have been validated as accurate, reproducible, clinically relevant, and predictive of clinical efficacy based on reliable pharmacokinetic and outcome data. Antimicrobial susceptibility testing has become highly standardized in the efforts of the NCCLS subcommittee on Antimicrobial Susceptibility Testing [11].

Tube dilution method testing was performed according to NCCLS M7-A3 guideline and procedures which introduced in the Diagnostic microbiology [5,6]. After preliminary test, some stock solutions concentrations were adjusted. The organism was grown in trypticase soy broth medium (E.Merck) and tested in salinity-adjusted muller-hinton broth with NaCl (3%). The tube dilution method achieved by preparing bacterium *vibrio harveyi* solution at a concentration of 5×10^5 Cell/milliliter. Glass tubes were used and MIC test was incubated at 30 °C for 24-48 hours in air (no added CO_2) incubator [5,12]. Tubes show no visible growth identified and they sub-cultured on trypticase soy agar (TS-Agar) which prepared with sea water and incubated overnight for 48 hours at 30°C then MBC determined.

Results and Discussion

Table 1: The effect of Sanosil-25 to inhibition growth of the bacterium (MIC), in the test tube cotaining broth medium.

Species of Bacterium	Time	Concentrations of Sanosil-25ppm					
		Control (0ppm)	5	10	15	20	25
Growth of *vibrio harveyi*	24 Hours	+	+	+	-	-	-
	48 Hours	+	+	+	-	-	-

(+): Growth, (-): No Growth.

At the primary stage, susceptibility of the bacterium (*Vibrio harveyi*) evaluated in the presence of five concentrations of Sanosil-25 as well as 5, 10, 15, 20 and 25ppm. Each concentration consists of three repeated test sample. However, there were three test tubes as well as control, they have not any Sanosil-25 and its concentration was 0 in each of them. Bacterial growth assessed by turbidity which happened due to growth of bacterium in each test tubes after inoculation and incubation after 24 and 48 hours.

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The results show that the bacterium can tolerate 10ppm of the Sanosil-25 and growth at the same concentration (Table 1). The test tubes which have more than 10ppm of the Sanosil-25 were be clear and show that the bacterium couldn't growth. Thus, the bacterium couldn't tolerate 15ppm of the Sanosil-25 at least and its growth inhibited.

After primary stage, Secondary stage was started. It started after 48 hours from the beginning of the primary stage. In the second stage, the bacteria transferred from test tubes to solid media (TS-Agar) which have not any Sanosil-25. It found that bacteria which have growth on the TS-Agar media were be related with tubes which its sanosil-25 concentration was less than 20ppm. Thus, It was determined that 15ppm of the Sanosil-25 can inhibit growth of the *Vibrio harveyi* but can't kill it. The trials show that Sanosil-25 no only inhibited the bacterial growth but also kill it at a concentration as equal as 20ppm and more (Tabel 2).

Table 2: The bactericidal effect of Sanosil-25 on the bacterium (MBC), growth of the bacterium after cultivation on the solid medium.

SPECIES OF BACTERIUM	Time	Concentrations of Sanosil-25ppm					
		Control (0ppm)	5	10	15	20	25
Growth of vibrio harveyi	24 Hours	+	+	+	+	-	-
	48 Hours	+	+	+	+	-	-

(+): Growth, (-): No Growth.

Results of the in-vitro susceptibility test are shown in the Table 1 & 2. The Sanosil-25 was active against *vibrio harveyi*; its MIC value was 0.015%. MBC value was higher than MIC value, it was 0.02%. It was important that compared two concentration 15 and 20ppm. The bacteria have not growth in the presence of the 15ppm Sanosil-25 while the bacteria is alive whenever it transferred to fresh media which have not any Sanosil-25 the bacteria getting to growth and its colony appears.

The results of the present study is consistent with the notion that sanosil-25 can kill *Vibrio harveyi* at 0.02% (20ppm) concentration. The bacteria have not growth on fresh media after the transferring it from test tube which had been contained 20ppm Sanosil-25. So, the bacteria had killed at the test tube and did not be able to growth after transferring to afresh media without Sanosil-25. In summary, Sanosil-25 is active in-vitro against *V. harveyi* and may be a potential disinfectant against it at a concentration as equal as 0.02%.

It is very important that mentioned all test tubes and media contained 2% NaCl because the bacteria need to it for growth. Fortunately, antibacterial activity of the Sanosil-25 evaluated at the same concentration of NaCl. However, we can conclude that 2% NaCl have not neutralized Sanosil-25. It is a good property for Sanosil-25 and makes it suitable for sea water sanitary.

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