


RESEARCH ARTICLE

ARAŞTIRMA MAKALESİ

## Pathogenicity and antibiotic susceptibility of two bacterial pathogens associated with the clam *Tapes decussatus* in some Egyptian fisheries

Bahaa Abdella<sup>1,2</sup> • Eman El-Wazzan<sup>2</sup> • Nermeen A. El-Sersy<sup>3</sup> • Soraya A. Sabry<sup>4</sup> • Ehab R. El-Helow<sup>4\*</sup> 

<sup>1</sup>Aquatic Microbiology Department, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafr El-Shiekh, Egypt

<sup>2</sup>Aquaculture Division, National Institute of Oceanography and Fisheries, Alexandria, Egypt

<sup>3</sup>Marine Environmental Division, National Institute of Oceanography and Fisheries, Alexandria, Egypt

<sup>4</sup>Department of Botany and Microbiology, Faculty of Science, Alexandria University, Alexandria, Egypt

\* Corresponding author: [elhelow@fulbrightmail.org](mailto:elhelow@fulbrightmail.org)

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**Abstract:** Bivalve aquaculture is generally influenced by bacterial pathogens that cause high mortality-related losses in hatcheries. Six bacterial phenotypes had been previously isolated as the most predominant microorganisms in carpet shell clam (*Tapes decussates*) samples collected from natural beds in representative Egyptian clam fisheries. The main aims of this study were to investigate the nature of the pathogenic strains that significantly affect clam survival and test their susceptibility to commercially available antibiotics. Based on their 16S rRNA sequences and some biochemical features, two potent clam pathogens were emerged; one of which is gram-positive and the other is gram-negative. The former isolate was identified as *Micrococcus luteus* and the latter as *Vibrio alginolyticus*. Experimental challenges with the two bacterial pathogens introduced at different initial cell concentrations ( $2.5 \times 10^4$  -  $7.5 \times 10^4$  cfu ml<sup>-1</sup>) showed markedly diverse clam mortality results. However, the pathogenic interaction of *M. luteus* with clam survival was generally higher than that of *V. alginolyticus*. When introduced at a relatively low initial cell density, the infectious records of *V. alginolyticus* were significantly increased suggesting that the expression of its key virulence factors is mainly triggered as a response to host contact. Antibiotic susceptibility tests suggested chloramphenicol and tetracycline as markedly effective agents that can be used to control the spread of these two bacterial pathogens in aquaculture applications.

**Keywords:** *Micrococcus luteus*, *Vibrio alginolyticus*, *Tapes decussates*, pathogenicity, fisheries and Aquaculture

## INTRODUCTION

Bivalves are traditionally used in most of the coastal regions as food. They are rich in proteins with essential amino acids and polyunsaturated fats with significant amounts of omega-3 fatty acids required for the growth and development of the human body (livestrong.com, 1969; Dong, 2010). However, due to feeding by filtering large volumes of water, bivalves can concentrate different types of pollutants including trace metals and microorganisms and exert health risks to consumers (Collin et al., 2008; Ramos, et al., 2012; Tubiash, 1975).

Microbial diseases affecting marine cultured bivalves have been previously revised and analyzed taxonomically and phylogenetically (Paillard et al., 2004). These included *Pecten* larvae-*Vibrio pectinica*, brown ring disease, juvenile oyster disease, Pacific oyster nocardiosis and summer mortalities of oysters.

Disease-associated mass mortalities of bivalves were responsible for declining bivalve populations in commercial clam beds and fisheries in many areas such as the Yellow Sea of Korea, China as well as in Europe (Paillard, 2004; Park, et al., 2006). Aquaculture of molluscs is greatly influenced

worldwide by bacterial pathogens that cause high mortalities and losses in hatcheries as well as in natural beds. *Vibrio* species are considered the main bacterial pathogens being responsible for the mortality outbreaks in aquaculture over the past few years (Romalde and Barja, 2010, Chatterjee and Haldar, 2012). As well, a new bacterial pathogen identified as a *Vibrio* spp has been recently isolated from bivalves (Dubert et al., 2016).

Therefore, the control of bacterial diseases in aquaculture is still an active research field. It involves investigating alternative treatment and disease prevention methods such as the use of disinfectants, antibiotics and vaccines (Romero, et al., 2012). However, governmental controlling actions are required for responsible use of antimicrobial agents in commercial scales. Based on preliminary research results, it has been also suggested that probiotics enhance the immune response of fish and confer tolerance against different stressors (Dawood and Koshio, 2016).

Investigations regarding the occurrence of pathogens in bivalves and their possible effects on bivalve survival and

production in some countries and water areas are still needed. The present work was mainly conducted to uncover some necessary microbial information related to marine bivalves collected from Egyptian commercial fisheries using the carpet shell clam, *Tapes decussatus* as a model.

The main aims of the present study were to identify major clam-associated bacterial pathogens and assess their potentials to cause clam mortalities in addition to their sensitivity to some commercially available antibiotics.

## MATERIALS AND METHODS

### Test animal

Healthy clams of *Tapes decussatus* were collected from natural fishery of Ismailia coast with the aid of divers. In each experiment, about two hundred clams were used. The shell lengths of clams ranged between 19.2-38.5 (28.3±6.1) mm, whereas the whole weight range was between 1.28 - 10.33 grams.

### Bacterial growth medium

Bacterial isolates were kept in refrigerator on nutrient agar medium. Nutrient broth was used for cultivation of bacterial strains. Both medium was prepared by seawater instead of distilled water.

### Bacterial isolates and mortality assay

Six distinct bacterial phenotypes have been previously isolated from Egyptian clam fisheries on TCBS agar plates (Abdella, et al., 2015). Bacterial isolates were purified and kept at -80 in cryo-tube. The assessment of the bacterial potential to infect clam was conducted using a modified method after Gómez-León et al., (2008). Three replicates of 4 L containers were used for each of the six bacterial isolates in addition to three containers as control without bacteria. Containers were filled with two liters of seawater sterilized by chlorination followed by de-chlorination (Kemp, 2006). Thirty randomly chosen clams were added to each container at room temperature. Bacterial cells were introduced with a final concentration of approximately  $5 \times 10^4$  cfu ml<sup>-1</sup>. Clam survival was checked twice in a day and gaped or dead clams were removed and counted. The water was replaced in 50% weekly.

### Statistics

All statistical analyses were conducted using Microsoft Excel 2013. The differences in survival between clam samples exposed to different concentrations of experimental pathogenic bacteria were analyzed by the one-way ANOVA test to evaluate their global significance. This was followed by LSD post-hoc test to determine the specific pairs of means that differ significantly at  $\alpha = 0.05$ .

### Molecular characterization of bacterial isolates

DNA was obtained by harvesting up to  $2 \times 10^9$  bacterial cells in a 1.5 ml micro-centrifuge tube by centrifugation for 10 min at 5000 x g. The pellet was re-suspended in 180  $\mu$ l of lysis

Solution (Sigma). Proteinase K (20  $\mu$ l) was added and mixed. The mixture was incubated at 56°C with shaking till complete lysis (about 30 min). RNase A (20  $\mu$ l) was added, mixed and incubated for 10 min at room temperature. After centrifugation (10 min, 5000 x g), clear filtrate was transferred to a new microfuge tube for ethanol precipitation. DNA was then purified using genomic DNA purification columns (GeneJET™). PCR reaction was performed with Maxima® Hot Start Master Mix. The PCR components were mixed with F27 and R1492 16S rDNA universal primers and DNA template in nuclease free water (Lane, 1991). PCR was then performed using the thermal cycling of 94, 94, 50, 72, 72°C for 10 min, 20 s, 1 min, 1.5 min, 10 min respectively. PCR product was loaded on 1% agarose gel against 1 Kb ladder for analysis, purified using GeneJET™ purification column and sequenced by an ABI 3730xl DNA sequence analyzer. Homology search for the obtained sequences was performed using BLAST through NCBI. A maximum likelihood phylogenetic tree was constructed using the software Molecular Evolutionary Genetics Analysis version 7.0 (Kumar et al., 2016)

### Phenotypic characterization

In addition to microscopic examination of cell morphology and gram staining, potent clam pathogens were subjected to biochemical characterization using API 20E for Gram-negative and the API STAPH for Gram-positive isolates following the manufacturing instructions (biomerieux).

### Antibiotic susceptibility test

Antibiotic susceptibility test was carried out using disc diffusion method (Kayser et al., 2005; Jorgensen and Ferraro, 2009) on Muller-Hinton Agar. Sodium chloride was added to the medium (1%) to give optimal growth for *Vibrio* spp. (Ottaviana et al., 2001). The antibiotics tested included Rifampicin (RD 5 $\mu$ g), Chloramphenicol (C 30 $\mu$ g), Gentamicin (CN 10 $\mu$ g), Amoxicillin (AML 10 $\mu$ g), Metronidazole (MTZ 5 $\mu$ g), Piperacillin (PRL 100 $\mu$ g), Fusidic acid (FD 10 $\mu$ g), Streptomycin (S 10 $\mu$ g), Cephalexin (CL 40 $\mu$ g) and Tetracycline (TE 30 $\mu$ g)] (CLSI, 2007; Jorgensen and Ferraro, 2009; Kayser et al., 2005). Plates incubated at 37°C for 24 h and the inhibition zone diameter was measured and recorded to the nearest millimeter. Observed diameters were translated into susceptible, intermediate and resistant categories according to the inhibition zone diameter standard table of Clinical and Laboratory Standards Institute (CLSI, 2007; Jorgensen and Ferraro, 2009).

## RESULTS

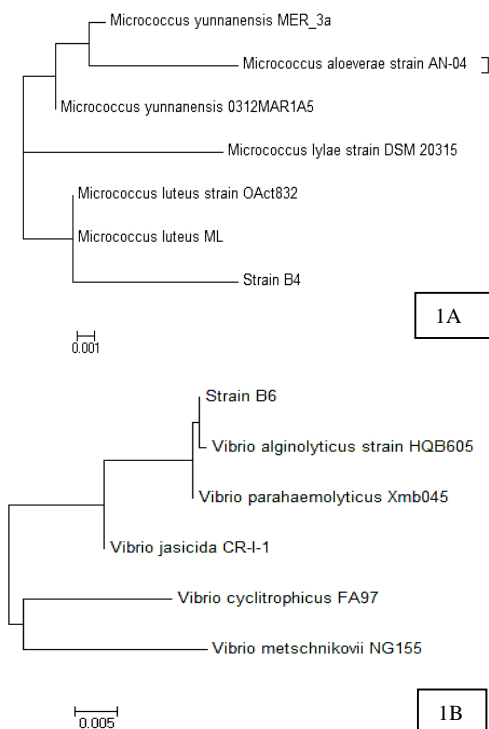
### Experimental bacterial challenges

We evaluated the relative survival of *Tapes decussatus* under experimental challenges using six phenotypically distinct bacterial isolates (B1-6) that had been previously isolated as the most predominant microorganisms in some Egyptian carpet shell clam samples. After 15 days of bath exposure, considerable differences in susceptibility to bacterial challenges were observed. However, among the six examined bacterial isolates, only B4 and B6 showed significant clam

mortality records that exceeded 50% (data not shown). Until the end of the incubation period, no mortality was detected in the control cultures.

### Characterization of the potent clam pathogens B4 and B6

After Gram staining, microscopic examination demonstrated that B4 cells are Gram-positive cocci and that B6 cells are Gram-negative and rod-shaped. PCR amplification of the DNA extracted from each of the two pathogenic isolates resulted in approximately a 1400 base pair fragment of the 16S rDNA region. Based on nucleotide homology analysis of the obtained sequences, the isolates B4 and B6 have been identified as members of the species *Micrococcus luteus* and *Vibrio alginolyticus* which are well known causative agents of severe diseases such as meningitis (Altuntas et al., 2004) and vibriosis (Laganà et al., 2011), respectively. The two sequences were submitted to DNA Data Bank of Japan (DDBJ) with the accession numbers AB859252 and AB859251 for isolate B4 and B6 respectively. The phylogenetic relationships between each of the two isolates and some closely related genotypes showed in Fig 1.



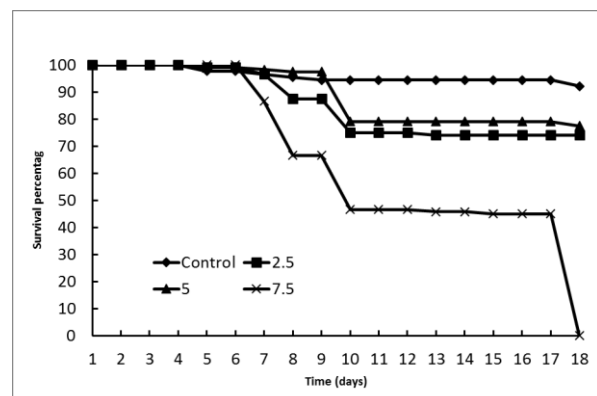
**Figure 1.** A maximum likelihood phylogenetic tree based on 16S rDNA genes of the isolates B4 (A) and B6 (B) and closely related sequences. The branch lengths indicate the number of substitutions per site

The two pathogenic isolates were further subjected to biochemical tests which confirmed the molecular characterization results of B4 as a *M. luteus* strain and B6 as a *V. alginolyticus* strain (data not shown).

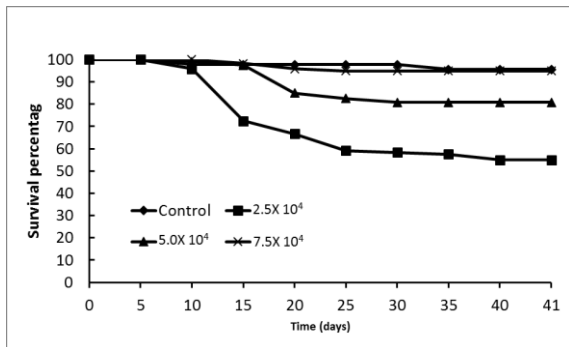
### Effect of *M. luteus* and *V. alginolyticus* dosages on survival of *Tapes decussatus*

Carpet shell clam cultures were separately exposed to three different concentrations of the experimental pathogenic strains of *M. luteus* and *V. alginolyticus*. As shown in Fig 2 and 3, mortalities were absent or very low under unchallenged control conditions. However, all bath exposure challenges showed that *M. luteus* and *V. alginolyticus* induced considerable mortalities. The highest examined *M. luteus* cell concentration ( $7.5 \times 10^4$ ) induced *T. decussatus* mortalities up to more than 50% death after 17 days of exposure and 100% in the 18<sup>th</sup> day (Fig 2). Lower mortality records were observed with the lower concentrations ( $2.5 \times 10^4$  and  $5.0 \times 10^4$ ). On the other hand, the lowest examined level of *V. alginolyticus* cells ( $2.5 \times 10^4$ ) appeared to be the most effective dose as it induced the highest *T. decussatus* mortality records (more than 30%) within 17 days of exposure (Fig 3). The number of dead clams was then continued to increase to reach 45% within 39 days. On the other hand, lower mortality records were observed in the presence of higher bacterial cell concentrations.

The one-way ANOVA test revealed that there are significant variations among the observed mortality records (Table 1) with  $P=0.00818$  for *M. luteus* and  $P=0.000283$  for *V. alginolyticus*. Performing post-hoc tests and plotting the means of number of dead clams against concentration of bacteria (Fig 4) confirmed that the challenging effects caused by the highest examined *M. luteus* dose and the lowest introduced *V. alginolyticus* level on clams are significant.



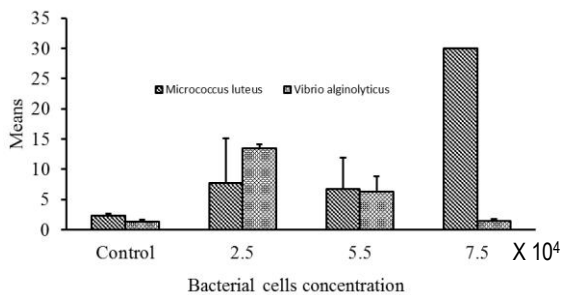
**Figure 2.** Survival percentage of *T. decussatus* challenged with three different concentrations of *M. luteus* (conc. 1=  $2.5 \times 10^4$ , conc. 2=  $5 \times 10^4$  and conc. 3= $7.5 \times 10^4$  cfu ml<sup>-1</sup>)



**Figure 3.** Survival percentage of *T. decussatus* challenged with three different concentrations of *V. alginolyticus* ( $2.5 \times 10^4$ ,  $5 \times 10^4$  and  $7.5 \times 10^4$  cfu ml<sup>-1</sup>)

**Table 1.** ANOVA test for the effect of different bacterial cell concentrations of *M. luteus* and *V. alginolyticus* on *T. decussatus* mortalities

Organism	SS	df	MS	F	P-value
<i>M. luteus</i>	1757.167	3	585.7222	6.60025	0.00818
<i>V. alginolyticus</i>	371.5167	3	123.8389	15.58316	0.000283

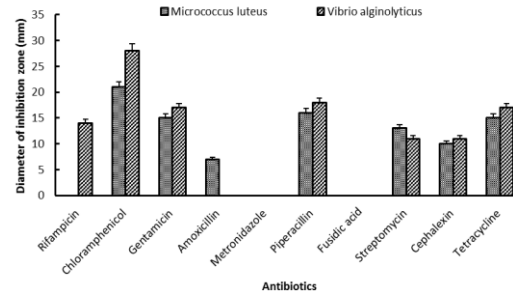


**Figure 4.** Means of dead *T. decussatus* as a response to exposure to different bacterial concentrations

### Screening for antibacterial susceptibility

Intensive aquaculture applications lead to the spread of several bacterial pathogens, which in turn necessitate the use of antimicrobials. In the present work, the experimental *M. luteus* and *V. alginolyticus* isolates were assayed for their susceptibility to ten different antibiotics currently used in aquaculture practices. Based on the inhibition zone diameters shown in Fig 5, the phenotypes of B4 and B6 can be described as sensitive, intermediate or resistant to an antibiotic. The results indicated that *M. luteus* and *V. alginolyticus* isolates respond similarly with most antibiotics; for instance, both are resistant to metronidazole and fusidic acid and highly sensitive to chloramphenicol and tetracycline. However, the effects of gentamicin, piperacillin, streptomycin and cephalixin on the two pathogens are relatively intermediate. On the other hand, the two isolates responded oppositely to rifampicin and

amoxicillin where the former was not effective against *M. luteus* and the later was resisted by *V. alginolyticus*.



**Figure 5.** Inhibition zone diameters expressed in mm corresponding to ten antibiotics against *M. luteus* and *V. Alginolyticus*

### DISCUSSION

In the present study, two microbial pathogenic strains that significantly affect clam survival have been isolated and identified as members of the gram-positive species *M. luteus* (B4) and the gram-negative species *V. alginolyticus* (B6). These results support the findings reported by other workers who suggested the ability of these two-bacterial species to cause disease and induce mortality in bivalves (Wu et al., 2013). They examined the ability of *M. luteus* and *V. anguillarum* to induce an immunological response in the mussel *Mytilus galloprovincialis* such as osmotic stress, disturbance in energy metabolism and alteration of metabolic biomarkers. They found that both of these two bacteria are able to induce osmotic regulation disturbances, oxidative stress, cellular injury but with different levels of severity in mussels.

Many *Micrococcus* spp. and closely related genera are generally harmless saprophytes. However, *M. luteus* can infect different hosts including humans, mammals, and some marine animals such as fish, sharks, crustacean shellfish, shrimps, and prawns (PHAC, 2011). *M. luteus* has been reported as a causative agent in cases of meningitis, intracranial abscesses, pneumonia and septic arthritis in immune-suppressed or immune-competent persons (Altuntas et al., 2004; PHAC, 2011). The study conducted by Aydın et al. (2005) also confirmed that *M. luteus* must be considered as a potential bacterial pathogen for fish.

The cell wall teichuronic acid (TUA) of *M. luteus* is an acidic long-chain polysaccharide polymer and has some similarity with that of the so-called capsular polysaccharides which are usually considered to be essential and virulent factors for pathogenic bacteria, such as *Streptococca* and *Staphylococca* (Deng et al., 2010). Therefore, the more the doze of *M. luteus* cells faced by the host, the more their infectious severity.

The second isolated pathogenic bacterium, *V. alginolyticus* has been reported as a causative agent of vibriosis which is the most commonly encountered disease associated with intensive bivalve cultures in hatcheries and nurseries (Miossec et al., 2009). In the South China Sea, *V. alginolyticus* was reported to be the dominant causative agent of high mortality vibriosis in the large yellow croaker, sea bream, grouper, kuruma prawn and shellfish species (Liu et al., 2004). *V. alginolyticus* reported to be common in adult bivalve over the bivalve in the hatcheries (Wang et al., 2016). Besides, it was reported that other vibrios such as *Vibrio europaeus* cause mortality in larvae of other kinds of clams (Dubert et al. 2017).

In contrast to the case of *M. luteus*, our results demonstrated that the clam mortality caused by the *V. alginolyticus* isolate is inversely proportional to initial challenge concentrations of the cells. A similar observation has been previously reported with respect to susceptibility of cod yolk-sac larvae to different concentrations of *Vibrio* spp (Sandlund et al., 2010). They found that the larvae challenged with relatively low doses ( $10^4$  cfu ml<sup>-1</sup>) suffered mortality significantly more than those infected by higher bacterial cell densities. This unpredicted mortality impact of the relatively low bacterial doses observed in the present work could be explained as a consequence of host and/or pathogen behavior and interaction. With respect to the host, it has been reported that bivalves have the ability to regulate ingestion of particles present at high concentrations by rejecting the excess as pseudofaeces (Arapov et al., 2010). Concerning the bacterial pathogen, it can be speculated that the community of *Vibrio* cells developed on the host as a growth environment is more physiologically adapted as pathogens when compared to seed culture cells grown on the experimental growth medium.

Moreover, the metabolic and behavioral activities of a bacterial community is affected by quorum sensing; a cell-to-cell bacterial communication which involves intercellular signaling mechanisms in response to cell density (Sifri, 2008). It is also known that most bacterial cells have a remarkable ability to adapt to enormously diverse environmental conditions. Pathogenic bacteria, for example, have to cope with adverse conditions, such as the host environment and host defense reactions. This normally takes place by triggering alternative metabolic and energy generation pathways in bacterial cells (Kidd, 2011).

Another considerable issue is that pathogens have to induce complex regulatory networks that ensure adequate expression of their virulence genes (Duprey et al., 2014). This is mainly because many virulence factors are costly metabolic products and their production is under strict regulatory control mechanisms including quorum sensing (Ruwandeeepika et al., 2012). For instance, the alkaline serine protease was shown to be a virulence factor of *V. alginolyticus* and its expression takes place at late growth stages to reduce the possibility of effective host defenses (Rui et al., 2009). As well, the development of capsule is involved in attachment of the vibrios to the host cells (Hsieh et al., 2003) and plays an important role in immune

avoidance (Chen et al., 2010). Therefore, it can be concluded that expression of key virulence factors in *V. alginolyticus* is not constitutive but rather depends on regulatory mechanisms controlled by bacterial cell-density as well as the nature of the host as a growth environment. Thereby, infection of the clam body with few numbers of *V. alginolyticus* cells may allow a chance for a lag adaptation phase followed by generating a huge number of bacterial cells that have evolved with specialized metabolic regulations customized for interaction with the host environment. On the contrary, invasion of the clam with a large number of *Vibrio* cells that are not pre-adapted to operate with the host body as a growth environment would not be as much effective.

From genomic point of view, a study conducted by Castillo et al. (2015) who worked on two draft genomes of *V. alginolyticus* has identified different virulence factors that could be related to pathogenicity. They have succeeded by using computational approach in the detection of the genes that encodes virulence factors such as collagenases, proteases, and hemolysin, and toxins (RTX, YafQ).

Intensive aquaculture applications have promoted the growth of several bacterial diseases and consequently increased the need for appropriate microbial control methods (Carvalho et al., 2012; Romero et al., 2012). Vaccination was suggested as an ideal method for preventing infectious diseases in the aquaculture field (Deep et al., 2011). However, many aquaculture vaccines are predicted to prompt the evolution of more virulent strains because of being developed for preventing disease symptoms and mortality rather than avoiding infection and transmission (Kennedy et al., 2016). Therefore, it is still necessary to consider chemotherapeutic agents as effective tools against bacterial infections in aquaculture practices.

The results of antibiotic sensitivity test carried out in this work showed that the two experimental *M. luteus* and *V. alginolyticus* isolates are highly sensitive to chloramphenicol, and tetracycline which are protein synthesis inhibitors. In addition, the *V. alginolyticus* isolate was also sensitive to gentamicin which interferes with protein synthesis as well. Similar sensitivity results have been reported for *M. luteus* (Fazlani et al., 2011) and *V. alginolyticus* isolates (Mustapha et al., 2013). However, the use of such chemotherapeutics in aquacultures must be controlled by government agencies, particularly with respect to dose rates and limitations.

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