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PREVALENCE, ANTIBIOTIC-RESISTANCE AND GROWTH PROFILE OF VIBRIO SPP., ISOLATED FROM IMPORTED FISH IN THE LOCAL MARKETS

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Food Science

Under the Supervision of Dr. Mutamed Ayyash

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Declaration of Original Work

I, Tarafa Ali Mohamed Abdalla, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Prevalence, Antibiotic-resistance and Growth Profile Of Vibrio spp., Isolated From Imported Fish In The Local Markets*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Mutamed Ayyash, in the College of Food and Agriculture at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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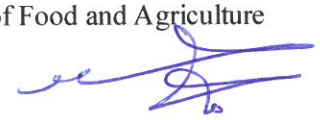
   Signature  Date 24/11/2019

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   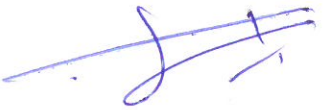

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Abstract

Seafood and fish are important food components for a large section of the world population. Seafood are prone to bacterial contamination, many are pathogenic to human and marine animals, and three species, *Vibrio mimicus*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, are responsible for most cases of seafood related human illness caused by *Vibrio* species. The study on prevalence of these microorganisms in seafood of United Arab Emirates is vital due to the cultural background of the Emiratis as a coastal heritage. A study was conducted to assess the prevalence of *Vibrio spp.* in imported fishes from local markets, identify the *Vibrio spp.*, examine the antimicrobial resistance and profile growth conditions of the isolated *Vibrio*. In the present study, 200 fish samples were collected from four different main markets at four cities (Al-Ain, Dubai, Fujairah and Abu Dhabi) in United Arab Emirates. *Vibrio spp*. were isolated from the collected fish samples and identified by the standard culture method. DNA was extracted from all the isolates and used for molecular characterization by Polymerase Chain Reaction (PCR). The antibiotic study was also performed to find out the resistance and sensitivity of the *Vibrio* species. The factors affecting growth rate and survival of the isolated *Vibrio spp.* was studied by analyzing the effect of different parameters such as temperature, pH and salinity. Results showed that *V.paraheamolyticus* was predominant in the isolates. The presence of *Vibrio spp.* was confirmed in 129 (64.5%) of the 200 isolates collected from different cities. The isolates from Al-Ain showed an occurance of 1 (2%) for *Vibrio mimicus* and were 3 (6%) for each of *V.vulnificus* and *V.paraheamolyticus.* An occurrence of 5 (10%) for *V.paraheamolyticus, V. mimicus* and *V.vulnificus* was not detected in isolates from Dubai. *Vibrio* isolates from Fujairah showed an occurrence of 4 (8%) for *V. vulnificus* and *V.paraheamolyticus*, 2 (4%) for *V.mimicus*. The prevalence of *Vibrio* in isolates from Abu Dhabi was 3% for *V.vulnificus* and *V.paraheamolyticus* and 0% for *V.mimicus.* Antibiotic sensitivity of the isolates were evaluated by measuring the zone of inhibition against 6 common antimicrobial agents. *Vibrio parahemolyticus, Vibrio vulnificus* and *Vibrio mimicus* isolates were resistant to penicillin G, daptomycin, vancomycin, ampicillin and erythromycin while all the three *Vibrio spp.* were susceptible to sulfamethoxazole-trimethoprim. The effect of various parameters such as temperature, pH and salinity

on growth and survival of *Vibrio* isolates showed *Vibrio parahemolyticus, Vibrio vulnificus* and *Vibrio mimicus* isolates exhibited maximum growth rate at 37°C, while increasing the temperature to 47°C the growth percentage was decreased. The three *Vibrio spp.* were grown significantly at alkaline pH (pH 5 and 7). Increasing the concentration of NaCl from 0.5% to 2%, the growth rate of *Vibrio* isolates were increased and optimum growth rate was showed in 1% NaCl. From the results, we can conclude that the *Vibrio* isolates from different cities of UAE showed antibiotic resistance and it is a threat to public health as the antibiotic resistant determinacies transferred to other bacteria of the clinical significance.

Keywords: *Vibro spp.*, Fish, Antibiotic-resistance, Growth profile, Survival.

Title and Abstract (in Arabic)

انتشار ومقاومة المضادات الحيوية وملامح النمو لـ *VIBRIO SPP.* ، المعزولة من الأسماك المستوردة في الأسواق المحلية

الملخص

تم العثور على المأكولات البحرية والأسماك لتكون عنصرا غذائيا هاما لقطاع كبير من سكان العالم. الأطعمة البحرية عرضة للتلوث الجرثومي، والعديد منها مُمْرِض للبشر و المخلوقات البحرية، و تم العثور على ثلاثة أنواع هي *Vibrio mimicus* و *Vibrio parahaemolyticus* و *Vibrio vulnificus*، هي المسؤولة عن معظم حالات الأمراض البشرية المرتبطة بالمأكولات البحرية التي تسببها أنواع بكتيريا الڤيبريو.

أهمية دراسة آثار هذه الكائنات الحية الدقيقة على البشر في دولة الإمارات العربية المتحدة أمر حيوي بسبب الخلفية الثقافية للإماراتيين كتراث ساحلي. تم إجراء دراسة لتقييم مدى انتشار بكتيريا الڤيبريو في الأسماك والمنتجات السمكية التي تباع في دولة الإمارات العربية المتحدة، وتحديد محيط البكتيريا، ودراسة مدى حساسية مضادات الميكروبات وظروف نمو الڤيبريو المعزولة. تم جمع 200 عينة سمكية من 4 أسواق رئيسية مختلفة في مختلف الإمارات (العين ودبي والفجيرة وأبو ظبي) في الإمارات العربية المتحدة. بكتيريا الڤيبري تم عزلهم من عينات الأسماك التي تم جمعها والتي تم تحديدها بواسطة طريقة الاستزراع القياسية. تم استخراج الحمض النووي من جميع العزلات واستخدم للتوصيف الجزيئي بواسطة تفاعل البلمرة المتسلسل (PCR) كما تم إجراء دراسة المضادات الحيوية لمعرفة مقاومة وحساسية أنواع البكتيريا .

تمت دراسة العوامل التي تؤثر على معدل النمو والبقاء على بكتيريا الڤيبريوالمعزولة من خلال تحليل تأثير المعلمات المختلفة مثل درجة الحرارة، ودرجة الحموضة والملوحة. أظهرت النتائج أن *paraheamolyticus* كان سائدا في العزلات. أظهرت العزلات من العين حدوث 1 (2%) للـ *Vibrio mimicus* وكانت 3 (6%) لكل من *V.vulnificus* و .*V.paraheamolyticus*. كما لوحظ حدوث 5 (10%) لـ *V.paraheamolyticus* و لـ *V.mimicus* و *V. vulnificus* في عزلات من دبي.

أظهرت العزلات المعزولة من الفجيرة حدوث 4 (8%) بالنسبة لـ *V.vulnificus* و *V.paraheamolyticus*، 2 (4%) بالنسبة لـ*V.mimicus*. كان معدل انتشار *Vibrio* في عزلات من أبو ظبي 3% لل *V.vulnificus* و *V.paraheamolyticus* و 0% لل *V.mimicus*.

تم تقييم حساسية المضادات الحيوية من العزلات عن طريق قياس منطقة تثبيت ضد 6 عوامل مضادة للميكروبات المشتركة. كانت عزلات *Vibrio parahemolyticus* و *Vibrio vulnificus* و *Vibrio mimicus* مقاومة للبنسلين G و daptomycin و vancomycin و ampicillin و erythromycin بينما كانت جميع *Vibrio spp.* الثلاثة عرضة للسلفاميثوكسازول-تريميثوبريم. وأظهر تأثير مختلف العوامل مثل درجة الحرارة، ودرجة الحموضة والملوحة على نمو وبقاء العزلات *Vibrio parahemolyticus*، *Vibrio vulnificus* و *Vibrio mimicus* المعزولة أظهرت أقصى معدل نمو عند 37 درجة مئوية ، بينما زادت درجة الحرارة إلى 47 درجة مئوية وكانت نسبة النمو انخفض. نمت الـ *Vibrio spp.* الثلاثة بشكل كبير عند درجة الحموضة القلوية pH 5 و 7 .بالاضافة الى زيادة تركيز كلوريد الصوديوم من 0.5% إلى 2%، وزاد معدل نمو العزلات *Vibrio* وأظهر معدل النمو الأمثل في 1% كلوريد الصوديوم. من النتائج، يمكننا أن نستنتج أن عزلات *Vibrio* من مدن مختلفة في الإمارات العربية المتحدة أظهرت مقاومة للمضادات الحيوية وأنها تشكل تهديدًا للصحة العامة حيث إن محددات مقاومة المضادات الحيوية تنتقل إلى البكتيريا الأخرى ذات الأهمية السريرية.

مفاهيم البحث الرئيسية: بكتيريا الڤيبريو، الأسماك، الإمارات العربية المتحدة، مقاومة مضادات الميكروبات، معدل النمو، نجاة.

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Dedication

To my beloved parents and family

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Chapter 1: Introduction

Pathogenic non-cholera *Vibrio* species, especially *Vibrio parahaemolyticus,* represent an emerging cause of several diseases due to consumption of contaminated seafoods. It can cause mild to moderate gastrointestinal infections, which are usually self-limiting and critical. The pathogenicity factors of *V. parahaemolyticus* are known to be caused by the presence of thermostable direct haemolysin (*tdh*) and thermostable direct haemolysin-related haemolysin (*trh*) genes (Raghunath *et al*., 2008).

Tan *et al*. (2017) reported the density of *V. parahaemolyticus* strains ranging from 3.6 to >105 MPN/g and microbial loads of *V. parahaemolyticus* strains positive ranging from 300 to 740 MPN/g in short mackerels (*Rastrelliger brachysoma*) from different retail markets in Malaysia. Kang *et al*. (2017) studied the changes in the environmental parameters and occurrence of *V. parahaemolyticus* in oyster aquaculture sites and found that 75.0% of the 44 isolates exhibited resistance to vancomycin. Yang *et al*. (2017) reported that the prevalence of *V. parahaemolyticus* was more common in summer than winter among the 98 strains identified in sea food from South China with 8.16 and 12.24% of prevalence to tdh and trh genes and 79.59% of isolates were resistant to ampicillin. Yaashikaa *et al*. (2016) isolated and identified *Vibrio cholerae* and *Vibrio parahaemolyticus* from prawn (*Penaeus monodon*) seafood using different enrichment and selective plating methods. Alaboudi *et al*. (2016) examined the prevalence of pathogenic strains of *V. parahaemolyticus* in marketed fish and water and sediment samples from the Gulf of Aqaba and results showed that both 16S rRNA had same sensitivity and tested isolates had high nucleotide similarity irrespective of their sources. Xie *et al*. (2016) studied the features of *V. parahaemolyticus* in ready-to-eat (RTE) foods in China and found 39 strains of *V.*

parahaemolyticus with 33.3% isolates of serotype O_2 having negative results for tdh and trh which are resistant to streptomycin (89.7%), cefazolin (51.3%), and ampicillin (51.3%). Kang *et al*. (2016) found that *Vibrio parahaemolyticus* isolated from oysters in Korea exhibited resistance to cephalothin (52%), rifampin (50.7%), streptomycin (50.7%) and (53.5%) of the total 71 isolated strains showed the presence of tox gene confirmed by PCR analysis. Xie *et al*. (2015) investigated the prevalence of *Vibrio parahaemolyticus* in aquatic products of South China and found that among the 224 samples analysed, 150 isolates were negative for tdh, 61 strains were trh positive and 88.67% isolates were resistant to streptomycin. Letchumanan *et al*. (2015) investigated the antimicrobial resistance of *V. parahaemolyticus* strains in shrimps from wet markets and supermarkets in Malaysia in which 57.8% isolates were positive for *V. parahaemolyticus*. Lopatek, Wieczorek and Osek (2015) evaluated the occurrence of *V. parahaemolyticus* in live bivalve molluscs in Polish market and *V. parahaemolyticus* was identified in 70 (17.5%) of the 400 samples, and the *toxR* gene was confirmed in 64 (91.4%) of these isolates. Yu *et al*. (2015) investigated the prevalence and drug resistance of *V. parahaemolyticus* isolated from retail shellfish in Shanghai and results showed that tdh gene was positive in two isolates and the trh gene was not detected in all isolates, 33 out of 96 isolates were resistant to cephazolin (31.3%). Oramadike and Ogunbanwo (2015) investigated prevalence of *Vibrio parahaemolyticus* in food samples prepared using croaker fish, shrimps, blue crab collected from landing sites along the Lagos Lagoon in Nigeria.

Xu *et al*. (2014) reported 37.7% of *V. parahaemolyticus* with bacterial densities less than 100 MPN/g in studied shrimp samples from Chinese retail markets. Five trh-positive isolates were identified from 247 isolates, and none of the isolates were tdh-positive. Yano *et al*. (2014) investigated the prevalence and antimicrobial resistance

of pathogenic *Vibrio cholera* (62-252,000 MPN/g) and *Vibrio parahaemolyticus* (370-6,300,000 MPN/g) which are resistant to ampicillin and oxytetracycline and *Vibrio vulnificus* (16-1300 MPN/g) resistant to 20% nalidixic acid in shrimps cultured at inland ponds with low salinity in Thailand. Al-Othrubi *et al*. (2014) studied the antibiotic profile of *V. parahaemolyticus* gastroenteritis associated with the consumption of contaminated shrimp and cockles marketed in Selangor Malaysia and found that eight isolates were positive for tdh virulence gene whereas twenty six isolates were positive for trh virulence gene. Jones *et al*. (2012) investigated biochemical profiles, serotype, and the presence of potential virulence factors (tdh, trh, and type III secretion system [T3SS] genes) in *Vibrio parahaemolyticus* isolates from oyster and established that all isolates were positive for oxidase, indole, and glucose fermentation with 27% were negative for tdh and trh, while 45% contained both genes. Koralage *et al*. (2012) investigated the prevalence and molecular characteristics of *Vibrio spp.* in 170 farmed shrimp (*Penaeus monodon*) samples in Sri Lanka and found that 98.1% of the farms and 95.1% of the ponds were positive for *Vibrio spp*. The *Vibrio parahaemolyticus* isolates were not positive for the virulence-associated tdh and trh genes. Rodriguez-Castro *et al*. (2010) reported that *Vibrio parahaemolyticus* was present in 35.3% and 535 strains were isolated in a study conducted in coastal waters of Galicia, Spain. Yang *et al*. (2008) identified 8 isolates of *V. parahaemolyticus* positive in seafood samples from fishing farm, retail markets, restaurants and cooking rooms of hotels in Jiangsu province and Shanghai city of China. Jun *et al*. (2012) investigated the incidence, risk assessment, antibiotic resistance, and genotyping of *Vibrio parahaemolyticus* in Korean seafood. Adebayo-Tayo *et al*. (2011) studied the occurrence of pathogenic *Vibrio* species in sea foods and water samples obtained from Oron creek and the results showed *Vibrio* was

recovered from 44.2% of samples, with 90.0% of fish, and in water *Vibrio cholerae* was the most predominant *spp*. Raghunath *et al*. (2008) studied levels of total and tdh+ *Vibrio parahaemolyticus* were estimated in 83 seafood samples from southwest coast of India by colony hybridization.

This study aims to determine the prevalence of *Vibrio* species isolated from imported fish in local markets of UAE, identify the *Vibrio spp*., and examine the antimicrobial resistance and growth profile of the isolated *Vibrio.*

Chapter 2: Literature Review

2.1 Background about *Vibrio spp.*

Based on the classification rules, *Vibrio* is the genus name for a gram-negative bacteria that belongs to the family *Vibrionaceae*. The genus *Vibrio* comprises facultative and fermentative bacilli with a single polar flagellum (Farmer & Hickman-Brenner, 2006). One of the main features of this group of bacteria is that they are halophilic, meaning that they require salt for survival. Research has shown that the members of this genus inhabit marine coastal waters (Farmer & Hickman-Brenner, 2006). There are also instances of them being found in the inland streams and lakes that are brackish in nature. The concentration of the various species of *Vibrio* generally depends on the environmental factors such as salinity and the temperature of the water (Oliver *et al*., 2013). While defining this genus, it is important to note that various species belonging to it are aerobic and gram-negative; furthermore, they are chemo-organotrophic. They have the ability to grow in the absence of air.

2.1.1 Prevalence of *Vibrio* in GCC and MENA Countries

M Kurdi Al-Dulaimi *et al*. (2019) studied the Multiple Antibiotic Resistance (MAR), plasmid profiles, and DNA Polymorphisms among *Vibrio vulnificus* Isolates from from clams (*Mercenaria mercenaria*) in Qatar and results demonstrated the high MAR index and genomic heterogeneity of *V. vulnificus* are of great concern to the human health. A study conducted by (Ghenem & Elhadi, 2017) confirmed the presence of *V. parahaemolyticus* in the Eastern coast of Saudi Arabia. Alsalem *et al*. (2018) isolated 17.95% *Vibrio vulnificus* isolates in sea water collected from the Coastal areas of Eastern province of Saudi Arabia and antibiotic susceptibility test indicated high

resistance to ampicillin (96%), cephalothin (73%), rifampicin (63%), and amoxicillin-clavulanic acid (56%) by the isolates. Elhadi (2018) studied the clonal relationship among the *Vibrio parahaemolyticus* isolates from coastal water in Saudi Arabia and the genetic fingerprints patterns comprised by ERIC-PCR evidenced the strong genetic relationships of isolated *V. parahaemolyticus*. Ibrahim *et al*. (2016) identified *Vibrionaceae* (58.4%), followed by *Aeromonadaceae* (10.4%), *Shewanellaceae* (3.57%), *Pasteurellaceae* (2.9%), *Caulobacteriaceae* (2.0%), *Pseudomonadaceae* (1.56%), *Enterobacteriaceae* (1.56%) and *Burkholderiaceae* (1.33%) in seafoods obtained from the Eastern Province of Saudi Arabia. The prevalence of *Vibrio* in market seafood samples of Kuwait by using biochemical (API 20E) strips and 16s rDNA-based molecular methods and found that *Vibrio* occurrence in the seafood samples was 77.99%. Elhadi (2018) has found that pathogenic *Vibrio* are present in coastal waters of the Eastern province of Saudi Arabia with 38% of *V.alginolyticus,* 13.3% of *V.parahemolyticus*, 7.6% of *V.vulnificus*, 5.6% of *V. cholerae* non-O/non-O139 and 0.33% for *V. mimicus*. Al-Sunaiher *et al*. (2010) identified the presence of *Grimontia (=Vibrio) hollisae* (54.5%), *Vibrio. fluvialis* (20.5%), *Photobacterium (=Vibrio) damselae* (12.6%), *V. alginolyticus* (6.8%) *and V. vulnificus* (4.5%) in some cultured fishes in the Kingdom of Saudi Arabia. Kelly (1982) investigated the effect of temperature and salinity on *Vibrio* (Beneckea) *vulnificus* occurrence in a Gulf Coast environment and found that *V. vulnificus* is commonly found in Gulf Coast environments and that the occurrence of the organism is favored by warm temperatures and relatively low salinity.

Fattel *et al*. (2019) studied the prevalence of *Vibrios* in the isolates recovered from stool specimens of gastroenteritis infected patients in Lebanon, characterized the

spp. using whole-genome sequencing and found that the isolates were O3:K6 serotype which exhibited identical resistance, virulence, and phylogenetic patterns. Youssef *et al*. (2018) conducted a study for the molecular characterization of *Vibrio parahaemolyticus* isolated from shellfish harvested from Suez Canal area, Egypt and revealed that overall prevalence of *V. parahaemolyticus* in shellfish was 9.27%. Al-Taee *et al*. (2017) investigated the occurrence of potentially pathogenic species of *Vibrio* in seven types of fish sampled from fish farms located in different districts in Basra governorate, Iraq and found that *V. alginolyticus* was the predominant species, followed by *V. cholerae, V. furnisii, V. diazotrophicus , V. gazogenes and V. costicola* The prevalence of *Vibrio* species was 37.1% in fish species; 47.1% in *Mulloidichthys vanicolensis* , 34.3% in *Lethrinus lentjan* and 30.6% in *Siganus rivulatus* collected from Red Sea in Egypt (Abdel-Azeem *et al*., 2016). Abd-Elghany and Sallam (2013) investigated the occurrence and molecular identification of *Vibrio parahaemolyticus* in retail shellfish in Mansoura, Egypt and found that 16.7% of shellfish samples were positive for *V. parahaemolyticus*. Alaboudi *et al*. (2016) reported the prevalence rates of pathogenic *V. parahaemolyticus* were 4%, 8%, and 12% in sediment, water, and fish samples collected from Gulf of Aqaba in Jordan.

Some members of this genus are saprophytes while others possess a parasitic mode of nutrition (Faulkner *et al*., 2003). There are close to 100 species of this genus. The available research indicates that it is difficult to determine the exact number of the known species since the list is continually updated. These updates result from the continued discovery of the new species of the genus. Different species of the genus *Vibrio* have a negative impact on humans but they serve the purpose of ensuring that the aquatic milieu is maintained (Oliver *et al*., 2013). The variability of the aquatic environment tends to determine the fitness of each species of the *Vibrio*. There is a

difference between the species found in freshwater bodies and those found in saline conditions. Most importantly, the species that inhabit freshwater environments have low sodium ions as a requirement for their growth and starvation survival.

2.2 History of *Vibrio spp.*

Research has shown that *Vibrio* species were the first kinds of bacteria to be identified and isolated from the environment (Farmer & Hickman-Brenner, 2006), The *Vibrio* species called *Vibrio parahaemolyticus* were isolated for the first time in the 1950s by a group of Japanese medical researchers (Letchumanan, Yin, Lee, & Chan, 2015). The subsequent research showed that these species have an annual cycle of abundance in estuaries and near the shore marine. *Vibrio vulnificus* is the third type of species belonging to this genus. It was first identified as a disease agent in 1979, after the first registered disease instance (Ceccarelli & Colwell, 2014). During that time, the causes associated with the infection by the bacterium were wound infections and a syndrome called primary septicemia.

2.3 *Vibrio* Species

Vibrio cholera is the most commonly known one in the world among the vibrio *spp*. These species are gram-negative, oxidase-positive, and in the shape of a bean rod (Drasar & Forrest, 1996). The freshly isolated kinds of species are prototrophic (Albert, 1994). When in a suitable medium, they exhibit a faster breeding rate and a possible maximum growth rate of 30 minutes. Such growth is best achieved in an aerobic environment even though they are facultative in nature (Abd *et al*., 2007). This strain also survives better in alkaline conditions however, it can be destroyed when the pH of the environment falls below 6 (Drasar & Forrest, 1996). These species of *Vibrio*

can be found in the intestines, stool of humans, and in an aquatic environment. *Vibrio cholerae* species are subdivided into *Vibrio cholerae* O1 and *Vibrio* cholerae O139 (DiSalvo, Blecka & Zebal, 1978). There are cholera toxin producing groups in both O1 and O139 strains, which causes cholera itself. There are also the non-toxigenic groups of O1 and O139 (Faruque *et al*., 2003). These groups cause wound infections, non-epidemic diarrhea, gastroenteritis, skin infections, and septicemia (Table 2.1).

According to Faruque *et al*. (2003), the non-toxigenic strains in this environment are mostly found within the exoskeleton of the zooplankton and phytoplankton. This is a mode of their adaptation to the aquatic environment. Most of the structures in the cholerae species, such as their pili which are strong, and gives them the ability to colonize the surface (Drasar & Forrest, 1996). The preserved and mutable genetic factors are the key concerns in this area of study. *Vibrio* cholerae have a fundamental habitual routine, which is an add-on to the chitin outer walls (Pruzzo *et al*., 2008). These species need the formation of biofilm as it is critical to their ecological existence (Pruzzo *et al*., 2008).

Table 2.1: *Vibrio species* associated with human diseases

Species	Source of Infection	Clinical Disease
V. alginolyticus	Seawater	Wound infection, external otitis
V. cholerae	Water, food	Gastroenteritis
V. cincinnatiensis	Unknown	Bacteremia, meningitis
V. fluvialis	Seafood	Gastroenteritis, wound infection, bacteremia
V. furnissii	Seawater	Gastroenteritis
V. harveyi	Seawater	Wound infection (shark bite)
V. etschnikovii	Unknown	Bacteremia
V. mimicus	Fresh water	Gastroenteritis, wound infection, bacteremia
V. parahaemolyticus	Shellfish, seawater	Gastroenteritis, wound infection, bacteremia
V. vulnificus	Shellfish, seawater	Bacteremia, wound infection, cellulitis

2.4 Emergence of *Vibrio cholerae* O139

For years, the Indian subcontinent suffered deaths as a result of severe dehydration without knowledge of the possible cause of the primary illness. Only in 1849, an English scientist John Snow proved that the *Vibrio* cholerae strain could be naturally transmitted through water (Faruque *et al*., 2003). The disease became common and started spreading across the world from 1817. The categorization of the CT-producing strain into classical and El Tor variant was done in 1992. The first discovery of the classical strain happened in 1883 while the El Tor strain was first isolated in the early 1900s, from one of the Mecca-bound pilgrims in the Sinai Peninsula (Oladokun & Okoh, 2016). The two strains are similar in almost every aspect except for the fact that El Tor strain causes blood haemolysis according to a Greig test.

The El Tor carrier did not show any symptoms even after it had been discovered, a factor that led to it being disregarded. However, in the 1930s, a similar species was isolated due to a diarrhea disease outbreak in Celebes (Finkelstein, 1996; Olsvik *et al*., 1993). This strain was called Para-Cholera. It was followed by an outbreak of the strain in 1991 in Hong Kong. During that time, the El Tor was declared pandemic. The classical strain, on the other hand, was associated with the pandemic that occurred between the years 1899 and 1923 (Faruque *et al*., 2003).

The research has further shown that the El Tor strain is more pandemic and dangerous as compared to the classical strain (Faruque *et al*., 2003). This conclusion was derived based on certain comparative characteristics. Firstly, it was determined that the El Tor strain can live for a longer period in the host after infection than the

classical strain (Faruque *et al*., 2003). At the same time, the El Tor strain is more asymptomatic as compared to the classical strain. These properties allow the El Tor strain to spread within the unaware host. The carriers of the strain are extremely contagious and they have the ability to infect everything that they contact. The El Tor strain also tends to endure harsh environmental conditions for longer periods as compared to the classical strain once they have been released into the environment from the intestines.

The classical strain reappeared in 1982 in Bangladesh (Blackwell & Oliver, 2008). The severity of the strain was so intense that it overshadowed that of the El Tor. The strain, however, was restricted to the regions where it had reappeared and thus the world did not experience a severe outbreak. Peru was another country to experience an outbreak of El Tor biotype after spending over 100 years free from a cholera epidemic (Pruzzo *et al*., 2008).

2.5 The Taxonomy and Classification of *Vibrio*

2.5.1 The Genomic Taxonomy

Taxonomic relationship can be explored based on various tools. These tools include Multilocus Sequence Analysis (MLSA), Average Amino Acid Identity (AAI), genomic signatures, and Genome BLAST atlases.

2.5.1.1 Average Amino Acid Identity (*AAI)*

This is one of the most important genomic features used when trying to determine the taxonomy of *Vibrio* species. This technique measures the relatedness of genetic materials between a pair of genomes (Thompson *et al*., 2009). The AAA method is applied, especially when trying to identify the relationship that exists

between the shared gene content and the genetic relatedness between the pair under comparison. AAI allows the evaluation of the robustness of the alternative genetic makers in a given species. The results are normally calculated based on the genes conserved between each pair of genomes. The Blast algorithm is applied to the whole genome in a pairwise sequence.

2.5.1.2 Genome Signature Dissimilarity

The genetic signature for the *Vibrio* species has been determined to be more similar between the closely related species as compared to the distantly related species. The method assumes that there is a possibility that the species belonging to different genera might have similar signatures (Thompson *et al*., 2009). The relative dinucleotide abundance is an aspect that is evident in the genomic signatures. Despite the diversity that might exist between the *Vibrio* species, the variation is small and in most cases, it lies between 50-kilo bases on a given genome (Thompson *et al*., 2009). The cause and the functional significance of the variation are illuminated by determining the scale of the level of persistence. Genomes can be identified through their signatures (Thompson *et al*., 2009). The dissimilarities between the signatures are the features used in estimating the evolutionary relationship between the species. Large deviation on the signature scale is a likely indication of a horizontal transfer of a segment from another species (Thompson *et al*., 2009). This technique helps highlight the closeness between the species of the *Vibrio* genus.

2.5.1.3 Genome Blast

This method is applied in depicting the compositional difference between the genomes of different *Vibrio* species. During the process of analysis, the differences

are observed in terms of the gene content and features of the DNA in each species. This technique is applied as a measure to validate the outcomes of the techniques used in the identification and classification of the members of the *Vibrio* species during the scientific research (Thompson *et al*., 2009).

2.6 *Vibrio parahaemolyticus*

This species of the genus *Vibrio* was discovered by Fujino Tsunesaburo in 1950 as a major causative agent of foodborne diseases, after a large outbreak in Japan (Letchumanan *et al*., 2015). In rare cases, these species have been known for causing wound infections, septicemia, and ear infections. Since the discovery of the species, the research has attributed it to 20% –30% of cases of food poisoning (WHO, 2019). Similar to the *Vibrio* cholerae, this type of species is found in the aquatic environment. The species can cause gastroenteritis as a result of consumption of raw or even partially cooked food (Su & Liu, 2007). The onset period for this species is between 4 and 48 hours. The disease is mostly mild, accompanied by symptoms such as vomiting, nausea, abdominal pain, and diarrhea (Di Pinto *et al*., 2008).

Seafood-associated diarrhea is mainly caused by the pathogenic *Vibrio parahaemolyticus* (Whitaker *et al*., 2010). The emergence of the O3:K6 of *V.haemolyticus* strain was originally witnessed in the Southeast regions of Asia (Indonesia, Philipines, East Malaysia), resulting in an increased number of cases of seafood-associated diarrhea across the world (Whitaker *et al*., 2010). In 1995, a strain of the species emerged worldwide, causing the first known pandemic of this species. Originally, it comprised clonal autochthonous bacteria that dwelled in the ocean, and its evolution was realized to have occurred in the ocean environment (Whitaker *et al*., 2010). There was a low sequence diversity in its population, thus enabling the

discovery of the information concerning its origin and the evolution hidden in those clones that had been experiencing evolution for a long time. According to Whitaker *et al*. (2010), the founder clone for this species of *Vibrio* was the O3: K6 nonpathogenic strain. It shows that most of the genetic changes in the species occurred through gene conversion and horizontal transmission of the DNA. When the core genomes from the founder strain are compared, it becomes clear that only several hundred single nucleotide variations exist between the isolated types (Letchumanan *et al*., 2015). However, when applying a method of comparison to the entire genome, it appears that the number of DNA with the clonal frame reaches up to 4.2% (Letchumanan *et al*., 2015). The number of variations in the single nucleotides can be hundreds of thousands. The differences in clonal genealogy and the diversification of the genome have been a key contributor to *Vibrio* parahaemolyticus evolution. According to Letchumanan *et al*. (2015), the emergence of new pathogens of *Vibrio parahaemolyticus* species is a result of the horizontal transfer of genes. The extent of the horizontal gene transfer appears to have depended upon the vicissitude of the bacterium life.

2.6.1 Ecological Condition

According to Blackwell and Oliver (2008), the water temperature of the aquatic environment is a major predictor that has both a negative and a positive correlation with salinity if measurements are taken across a variable range (Blackwell & Oliver, 2008). The salinity of approximately 10%–23% has been determined as optimal since the abundance of the species tends to decrease as the water becomes too saline or too fresh (Blackwell & Oliver, 2008).

The optimal temperature for the growth of *Vibrio parahaemolyticus* is between 35 and 37°C. The lowest temperature that has ever been reported for the growth of this type of species is between 3 and 130°C (Whitaker *et al*., 2010). pH level also tends to affect the species' survival at the lower temperature limits. According to the research conducted by Thomson and Thacker, the multiplication rates for the species could be dangerous when the being they are attached to, such as an oyster, is stored at a temperature above 80°C (Thompson *et al*., 2009).

2.6.2 Epidemiology of *V.parahaemolyticus*

According to Chowdhury *et al*. (2004), the epidemiology of the species has undergone drastic changes in February 1996. They were indicated by an increase in atypical infections by *Vibrio parahaemolyticus spp*. The infections were witnessed in Kolkata city of India and the infection was linked to strains belonging to the O3:K6 serotype. Furthermore, the clone rapidly spread throughout the northeastern parts of Asia within a year. After several years, the strains similar to those from Kolkata were reported within the Gulf coasts and Atlantic regions. Europe, Africa, as well as North and Central America also reported similar strains during diarrhea outbreaks in the subsequent years. In the previous years, there were no widespread reports of the species, meaning that a big number of cases during that period was the clear evidence of a pandemic emergence (Chowdhury *et al*., 2004). Since then, several serotypes of the species have been discovered and isolated. They include the O1:K25, O1:K56, and O3:K75, among others that have been marked as the predominant groups causing outbreaks in different parts of the world since 1996 (Chowdhury *et al*., 2004).

2.7 *Vibrio vulnificus*

This type of species forms a part of the natural flora found in the marine coastal environments across the world. The isolation of the bacterium has been done based on the sediments, water, and seafood, including fish, oysters, and shrimp. Infection by the bacteria can cause a severe type of a fulminant systemic infection (Wellard-Cole *et al*., 2019). The disease caused by the infection through this bacterium is characterized by such symptoms as hypertensive septic shock, chills, fever, and nausea (Strom & Paranjpye, 2000). There can also be the formation of lesions in the patient's extremities. The most lethal infection resulting from *Vibrio vulnificus* is called septicemia. On average, the mortality rate of the illness is 50%. Furthermore, this species is prone to causing wound infections. They can progress into ecchymosis, cellulitis, and even bullae, and these infections, in turn, can progress into necrotizing fasciitis on the infection site (Strom & Paranjpye, 2000).

There are two biotypes of the *Vibrio* species, a classification that is mainly based on the biochemical characteristics of the species. Most human infections are associated with biotype 1 (Strom & Paranjpye, 2000). The strains belonging to biotype 2, on the other hand, are associated with eel pathogens. A third strain that has been discovered is deemed to have the biochemical characteristics of both biotype 1 and biotype 2 (Strom & Paranjpye, 2000). There are numerous genes located within the genomic island that are considered to be involved in the species pathogenesis (Strom & Paranjpye, 2000).

2.7.1 Ecological Requirements

The temperature of the waters is supposed to exceed 18°C, with a level of salinity being 15–25 parts per one thousand (Blackwel & Oliver, 2008). For this reason, Blackwel and Oliver attribute most incidents of infections caused by this bacterium to tropical climates. It has been determined that in order for the species to cause an infection in the human body, it must first survive within the inhospitable conditions provided by the human body. Secondly, the species must be able to overcome the human immune system for the disease to emerge (Blackwel & Oliver, 2008). It is explained by the fact that the species' innate virulence factors that tend to enhance its pathogenicity provide it with the ability to survive in the human body long enough to cause infection and disease.

2.7.2 The Occurrence of *Vibrio vulnificus*

The countries where the existence of the species have been reported include Denmark, Sweden, Germany, Turkey, Spain, and Belgium, as well as the United States of America. This species has been determined to be the leading cause of seafood fatalities in the United States. Based on the dietary differences there is a geographical variation is the primary source of the infection. In South Korea, between 2001 and 2010, a total of 588 cases of this strain were reported. The fatality rate was relatively high, with 285 of the 588 patients reported to have died as a result of the infection. The implication is that the occurrence of this species of the *Vibrio* has been witnessed in almost all parts of the world.

2.8 *Vibrio* Diseases

Vibrio parahaemolyticus infection is one of the diseases associated with *Vibrio* species. It commonly results in the appearance of gastroenteritis, with the symptoms including diarrhea that is at times accompanied by blood, nausea, fever, vomiting, headache, and abdominal cramps. *Vibrio parahaemolyticus* might occasionally cause wound infections. *Vibrio cholera*, in turn, causes a disease termed cholera (Drasar & Forrest, 1996). It is associated with symptoms such as diarrhea and dehydration. In severe cases, this disease causes death; other symptoms include the loss of skin elasticity, muscle cramps, and low blood pressure. Furthermore, *Vibrio* infections are the result of consumption of contaminated food; they are called food-borne diseases and have a high yearly prevalence.

2.8.1 Gastrointestinal Illness

This disease is caused by infection from *Vibrio parahaemolyticus*. It is mostly self-limited and lasts for approximately three days. The symptoms of the infection include diarrhea, abdominal pain, vomiting, nausea, fever, chills, and abdominal cramping. Severe conditions of the disease can only be found in an individual with a weak immune system. The main method of preventing this infection is by cooking food properly. Raw seafood should be kept separate from all other products. The exposure of open wounds to warm seawater should be steadily avoided (WHO, 2019).

2.8.2 *Vibrio vulnificus* Infection

Vibrio vulnificus species can cause a range of symptoms. In particular, vomiting, diarrhea, and abdominal pain are associated with the infection (CDC, 2018). When an open wound is exposed to warm seawater, skin infections might occur. For

people with chronic liver diseases and those with weak immune system, this infection can reach a severe degree. It can also cause bloodstream infection if it invades the human bloodstream. In such case, severe symptoms such as decreased blood pressure, fever, and skin lesions might ensue. The CDC provides relevant pieces of advice for preventing this infection. They include properly cooking seafood, keeping other food substances separately from raw seafood, and avoiding eating raw seafood (CDC, 2018).

2.8.2.1 Cholera

This infection is known to cause severe diarrhea that might lead to dehydration and possible death. The infection is mainly caused by eating food or drinking water contaminated by *Vibrio* c*holerae* species. The signs and symptoms associated with the infection include the increased heart rate, low blood pressure, loss of skin elasticity, thirst, muscle cramps, and severe diarrhea (CDC, 2018). Cholera is treated by three main methods, the first one being rehydration therapy. This process involves mechanisms aimed at restoring the lost fluids and salts. Oral rehydration with low-osmolarity is used for malnourished patients (CDC, 2018). The second method is antibiotic treatment, which aims to reduce the requirement for fluids in the body and the duration of the illness. Zinc treatment is the third method and it is mainly used for treating the illness' symptoms in children (CDC, 2018).

2.8.2.2 Known *Vibrio* Outbreaks

One of the well-documented *Vibrio* outbreaks is the cholera outbreak on the African continent between 1991 and 1996. The number of cases during this period ranged between 70000 and 160000 as per the World Health Organization records (WHO, 2019). The 1991 cholera outbreak in Latin America has also been severe.

During the outbreak that lasted for two years, 750000 cases were reported, out of which 65000 deaths occurred (WHO, 2019). Finally, there was an outbreak of *Vibrio* infection between April 2018 and July 2018. According to the U.S. Food and Drug Administration, this outbreak was linked to people eating fresh crabs imported from Venezuela (U.S. Food & Drug Administration (FDA, 2019). The *Vibrio* species identified as responsible was *Vibrio parahaemolyticus*. During the research conducted by the CDC, 24 people were interviewed and 22 of them indicated that they had consumed crabs either at their homes or in a restaurant. Another most recent outbreak happened in 2013: it was associated with the consumption of shellfish (CDC, 2018). It affected thirteen states in the United States. There were104 cases with six people being hospitalized, with no deaths reported. It is notable that cholera outbreaks have been experienced on the African continent since 1971 (CDC, 2018). Yemen also still reports the incidences of cholera. It means that the outbreak of *Vibrio* infections remains a threat to the world public health.

2.9 *Vibrio spp.* in the Seafood

Seafood has been considered a major constituent of a healthy diet. However, one major health risk associated with it is caused by the consumption of raw or undercooked seafood (Froelich & Noble, 2016). This could result in infections caused by the *Vibrio* species since the aquatic environment is their natural ecological niche. It implies that they form a part of the human pathogens present in the marine environment. *Vibrio* species have the ability to remain attached to the surface of the organisms in the marine environment. At times, they can be found as free swimmers (Blackwell & Oliver, 2008). However, there are higher chances that the *Vibrio* species can be found attached to the surface of the seafood products.

There is a possibility that the species can increase exponentially in case they are mishandled during the processing of seafood (Froelich & Noble, 2016). The fact that *Vibrio* species have the aquatic environment as their natural ecological niche means that they are most commonly associated with seafood diseases. *Vibrio parahaemolyticus* and *Vibrio cholerae* are the most common types of such species (Oliver *et al*., 2013; Su & Liu, 2007). Despite the fact that a lot of research has been conducted on *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are currently the leading cause of seafood poisoning in the world. Approximately 76.9% of bacteria-associated food poisoning cases have been linked to the *Vibrio parahaemolyticus* species between 1996 and 2002 in Taiwan (Su & Liu, 2007; Oliver *et al*., 2013).

Oyster is a type of seafood that can easily lead to the infection caused by the *Vibrio* species (Froelich & Noble, 2016). This species of marine creatures feed by constantly consuming the objects in the water along with the water itself. When the oyster is feeding, both bacteria and viruses are attracted. During summer periods when the waters are warm, *Vibrio* species tend to increase in population since this temperature is favorable for their growth and survival (Froelich & Noble, 2016). Thus, the concentration of *Vibrio* bacteria and other forms of bacteria and viruses also increases (Froelich & Noble, 2016).

Consuming raw or undercooked oyster creates the risk of ingesting the *Vibrio* species, which leads to an attack on the body's immune system. Once the bacteria overpower the human's immune system, an infection occurs (Oliver *et al*., 2013). The United States food-borne diseases statistics indicate that over 80000 people are infected by *Vibrio* species and 100 of them die on a yearly basis (CDC, 2018). This

issue evidently requires attention since there is no palpable difference between harmful and non-harmful oysters: they smell, taste, and even look the same (Froelich & Noble, 2016). This case presents an example of one of the ways through which *Vibrio* species can migrate from the water bodies into the human body.

2.10 *Vibrio spp.* and Food System

2.10.1 Water-borne Diseases

For a long time, freshwater bodies have been the main source of water for communities living in the rural areas of most of the developing and underdeveloped countries (Osunla & Okoh, 2017). The main uses of water in such areas included drinking, agricultural irrigation, and cooking. In such areas, the water sources are subjected to a higher rate of pollution as a result of their fast-growing population. According to Osunla and Okoh (2017), the continuous pollution of these water bodies has resulted in water and food-borne epidemics in both the developed and undeveloped countries across the world. The contamination of the freshwater bodies leads to the contamination of the drinking water, which can be linked to insufficient hygiene practices in various communities (Osunla & Okoh, 2017). Approximately 80% of wastewaters across the world is channeled back into the ecosystem without being treated or even reused. This results in a situation where 1.8 billion people have to use the contaminated water for their domestic purposes (Osunla & Okoh, 2017).

2.10.2 *Vibrio* Prevalence in Food

Seafood acts as a transmission vehicle for *Vibrio* infection. Food pathogens such as *Vibrio* species have been determined to be a major cause of most of the food-borne outbreaks across the world (WHO, 2019). *Vibrio parahaemolyticus* was first

recognized in the Asian region in 1951(Letchumanan *et al*., 2015). Since that time, this species has been isolated from foods such as shrimp and oysters in the southeastern markets of Asia (Su & Liu, 2007). *Vibrio parahaemolyticus* strain has also been isolated in cockles and shrimps in Thailand and Malaysia, and the same species have been identified as the main cause of food-borne infections in China (Letchumanan *et al*., 2015). In addition, between 2001 and 2012, 13607 cases of diarrhea associated with *Vibrio parahaemolyticus* were reported in the slum areas of Kolkata India (Letchumanan *et al*., 2015).

In Europe, this strain has been isolated from seafood brought from the Baltic Sea, Black Sea, and the Mediterranean Sea. According to Letchumanan *et al*. (2015), a research conducted along the coastal waters of Guadeloupe showed a significant presence of *Vibrio parahaemolyticus* in the foods collected from the sea. In 1997, in France, there was a severe outbreak of this species that affected 44 people (Letchumanan *et al*., 2015). Other European countries where the cases of food poisoning caused by this strain were witnessed include Denmark, Turkey, Greece, Britain, Scandinavia, Yugoslavia, and Spain (Qadri *et al*., 2005). The same cases have also been reported in the United States, with the first one happening in 1971 in Maryland. Since then, there have been intermittent outbreaks within the American coastal regions associated with the consumption of uncooked seafood (WHO, 2019).

Various countries have reported the cases of cholera associated with food poisoning in the recent past. In 2016 alone, 132121 cases of *Vibrio cholera* infection were reported. The analysis of the reports reveals that 17 of the cases were from Africa, 4 from Europe, 12 from Asia, 4 from the United States, and one from Oceania (WHO, 2019). 80% of these cases were drawn from DRC Congo, Yemen, Haiti, and

Republic of Tanzania. However, the research suggests that the true number of cholera cases associated with food contamination is much higher than the presented figures claim (WHO, 2019). The prevalence of *Vibrio vulnificus* has been determined to be higher in oysters than in any other types of seafood across the world (Blackwell & Oliver, 2008). In terms of the level of food prevalence, the research has shown that *Vibrio parahaemolyticus* is dominant among the three species, with *Vibrio vulnificus* being the second and *Vibrio cholerae* being the third. There is not much research conducted yet on the prevalence of *Vibrio harveyi* in food worldwide.

2.11 *Vibrio spp.* in fish

The existence of *Vibrio* species in various fish species poses a considerable health risk and is thus becoming a problem to the fish consumers and the global fish economy at large (Ping-Chung *et al*., 1996). Furthermore, the contamination of the fish and fish products by the *Vibrio* species results in an increased burden on the global healthcare system due to the possible disease outbreaks. Despite the fact that fish is considered a part of a healthy diet, it is responsible for a greater percentage of food-borne diseases across the world. There are two main species of shellfish that contain *Vibrio vulnificus* during the warm seasons, which increases the chances of its consumers being infected by this strain. Apart from the existence of the vulnificus species in the shellfish, *Vibrio parahaemolyticus* species has been linked to the majority of the seafood-borne diseases in China and Malaysia (Malcolm *et al*., 2015). It implies that there are bigger chances of most of the fish products from China being contaminated with various strains of *Vibrio* species. The economists view China as a major producer of fish and fish products in the world, with the increasing incidences of fish poisoning related to *Vibrio* species across the world. According to Halpern and

Izhaki (2017), there is the possibility that fish could be a reservoir for *Vibrio* species, especially *Vibrio cholerae*.

According to Sharma *et al*. (2017), *cholera* strain has in some cases been isolated from the diseased fish, which is an indication that the fish have the potential to harbor *Vibrio* species. In fact, the *cholera* occurrences in India are an example of cholera outbreak being associated with hilsa fish (Sharma *et al*., 2017). Shellfish and shrimp, among others kinds of fish, have a high prevalence of the *Vibrio* species, including the rare species such as *Vibrio harveyi* and the least documented species known as *Vibrio carchariae.* According to WHO (2019), shellfish was the transmission vessel for *cholera* that travelled from Latin America to the United States in the 1960s. It means that such species of fish can cause food-borne diseases and disease outbreaks.

Tetrodotoxin, a harmful toxin produced by the *Vibrio* species, has been isolated from some species of fish, such as starfish and puffer fish. On a theoretical ground, *Vibrio* species and fish share the same ecological niche. Apart from the *Vibrio* species being free swimmers, they can attach themselves to other organisms in the water and move with them (Di Pinto *et al*., 2008). The fish are not the exception here, meaning that in a contaminated water environment, it is likely that any species of the fish drawn from the water will be *Vibrio*-contaminated, capable of spreading the infections if eaten raw or undercooked. Malcolm *et al*. (2015), therefore, recommend an implementation of the routine screening of the fish and fish products to help reduce the risk of *Vibrio* infections.

2.12 Fish Production in the World

In 2016, the global production of fish attained an all-time high of approximately 171 million tons (Odeyemi, 2016). 88% of the total production was directly consumed by humans (FAO, 2018). With this record, a per capita consumption of 20.3 kg was reached in 2016. Recently, the aquaculture sector has experienced a considerable economic growth mainly based on the contribution of Africa and Asia. The value of the global fish export thus increased to USD 152 billion in 2017 (FAO, 2018).

2.12.1 Challenges in Fisheries Sector

According to FAO (2018), the fisheries sector is facing challenges in ensuring that the percentage of the fish stocked regardless of the biological sustainability is reduced. Secondly, addressing the animal disease and biosecurity issues remains a problem. The third challenge involves maintaining accurate and complete statistics that support the development of appropriate policies and their implementation.

2.12.2 Major Fish *spp.* and Statistics

In the production of stocked fish existing within the range of biologically sustainable levels, the United States has increased its production from the 53% in 2005 to 74% in 2016 (FAO, 2018). Australia, on the other hand, has upped its production from 27% in 2004 to 69% for the year 2015. The North East Atlantic has experienced an increase from 34% in 2003 to 60% in 2015 (FAO, 2018). The graph below shows the fish species that have increased the contamination by *Vibrio* throughout the years.

2.13 Fish and Fish Products in the UAE

The UAE waters have been assessed as holding approximately 280 species of fish. Approximately 20 species are used for commercial purposes. In this country, fishing is artisanal in nature and most of the fish caught are sold to the local markets and nearby processing plants. The largest fishing industry is located in the oil-rich emirates of Abu Dhabi. The Dubai Sharjah and Fujairah possess the second largest industry in the country. There has been a general decline in the fish caught there associated with the reduced amounts of fish caught in Abu Dhabi. Various species of fish are sheltered in the gulf waters, including such kinds as kingfish, cobia, queenfish, barracuda, and trevally.

The UAE occupies the second position in terms of per capita fish consumption in the world's ranking. The country experienced an increase in population by 125%, a factor that has led to an increase in fish consumption among the young protein-demanding population (Environmental Agency - Abu Dhabi, 2017). The UAE is one of the countries that have managed to establish food security within the area of fish production (Figures 2.1 & 2.2).

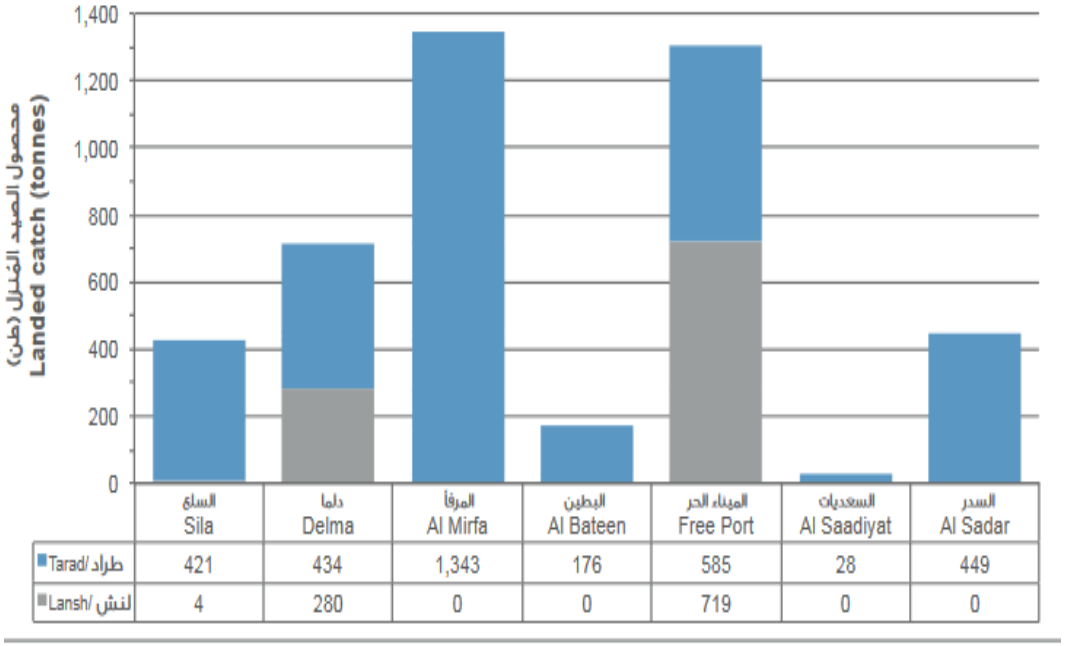


Figure 2.1: Total catch by landing site and boat type (EnvAgency - Abu Dhabi, 2017)

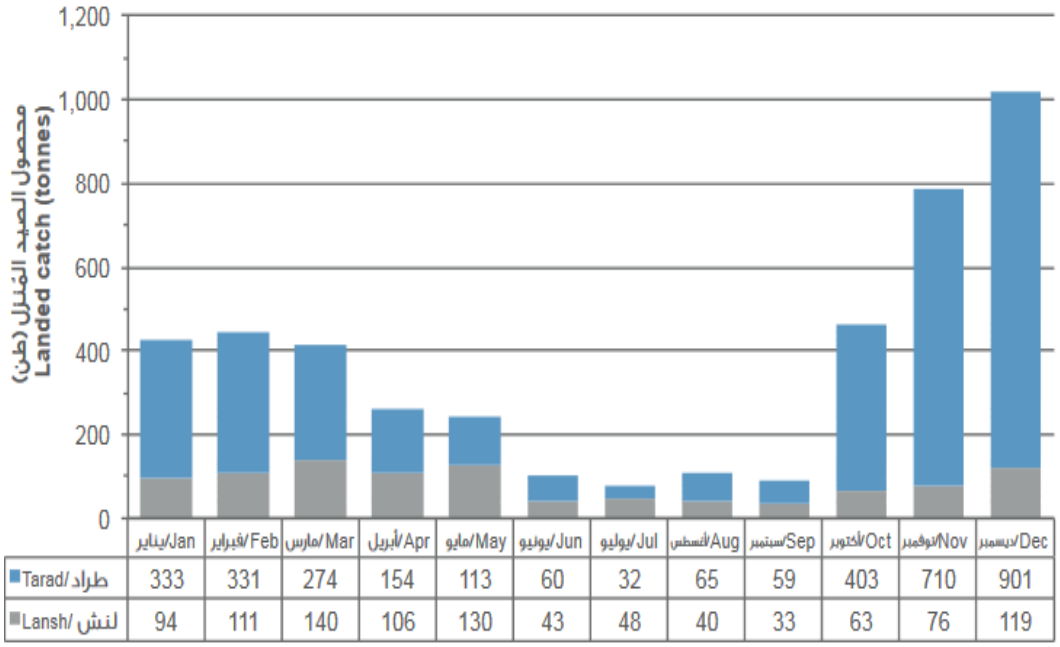


Figure 2.2: Total catch by month per boat (Env Agency - Abu Dhabi, 2017)

2.13.1 Lack of Research on *Vibrio*

The incidences of *Vibrio* infection outbreaks such as cholera and wound infections have been reported in most continents around the world (Oladokun & Okoh,

2016; Oliver *et al*., 2013; Osunla & Okoh, 2017). There are countries from different regions with cases and incidences of *Vibrio* infection. However, no study has been conducted in the UAE. There have been outbreaks in some countries in the Middle East, as indicated in the letter addressed to the UAE Ministry of Health. According to the letter, the country was concerned about the *cholera* cases in DRC Congo, Iraq, and Tanzania. The government, however, indicated that at that time, there were no outbreaks reported in the UAE and that the chances of an outbreak were extremely low. Nevertheless, the contamination in Tanzania could easily spread through the ocean to the UAE. It could also happen through migration into the country, which poses a public health risk as people might not be aware of some of the infections that could arise from eating raw or undercooked fish products. The UAE waters could be subjected to pollution like any other water body across the world. With the growing demand for fish and fish products, the consumption practices will change over time. This requires conducting the studies on fish and fish products to determine the safety level of the UAE fish products as far as *Vibrio* species are concerned.

Chapter 3: Materials and Methods

3.1. Study Area and Sample Collection

A total of 200 fresh local fish samples were imported from four different main markets at different cities (Al-Ain, Dubai, Fujairah and Abu Dhabi) in United Arab Emirates. Samples were collected throughout an 9-month period during summer extended from June to September, 2017 at early morning. The samples were placed in individually labeled and sealed in plastic bags and transported in sealed containers with dry ice to UAEU laboratory for microbial analysis. Fish samples in Figure 3.1. A layout of experiments conducted in the study is shown in Figure 3.2.

Figure 3.1: Fish for isolation of *Vibrio*

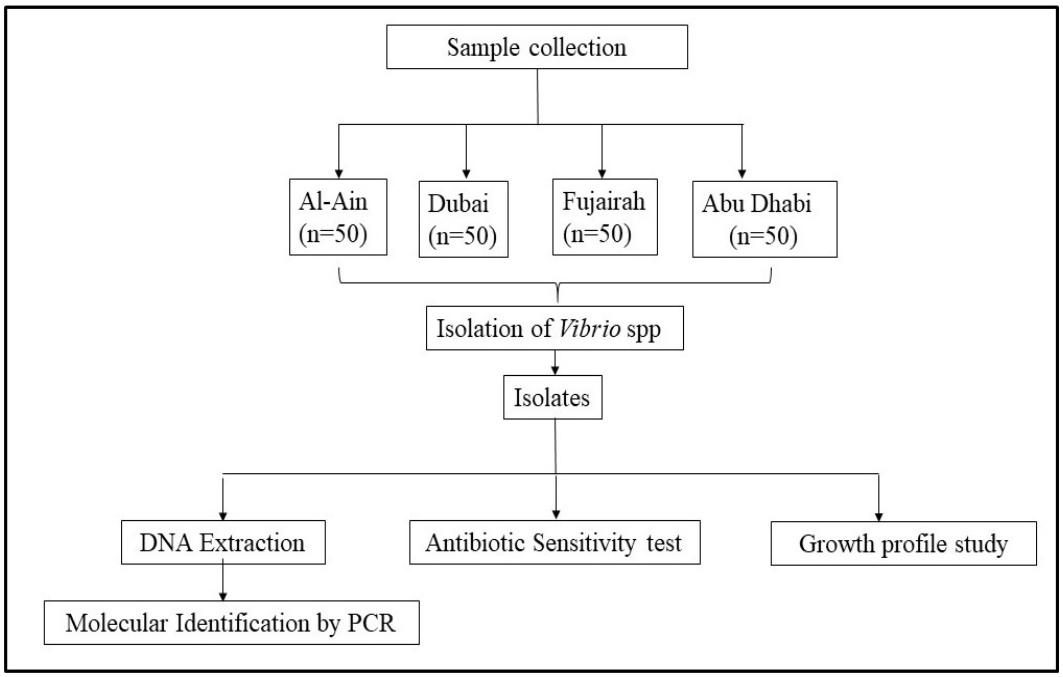


Figure 3.2: Flow of the experiments

3.2 Isolation of *Vibrio*

Vibrio spp. was isolated and identified by the standard culture method according to Sujeewa, Norrakiah and Laina (2009).

Twenty-five gram of imported fish flesh samples were homogenized in 225 mL alkaline peptone saline water (APSW, Hi Media, Bombay, India). The homogenate was mixed thoroughly for 1 min at 260 rpm using Stomacher Circular Unit 400 (Seward Ltd., London, UK), and incubated at 42°C for 8 h. Then 10 ml of the incubated homogenate was streaked in duplicate on thiosulfate citrate bile salts sucrose agar (TCBS, Hi Media) and mPCP (modified cellobiose-polymyxin B-colistin) agar. The inoculated plates were incubated at 37°C for 18 to 24 h (Figure 3.3).

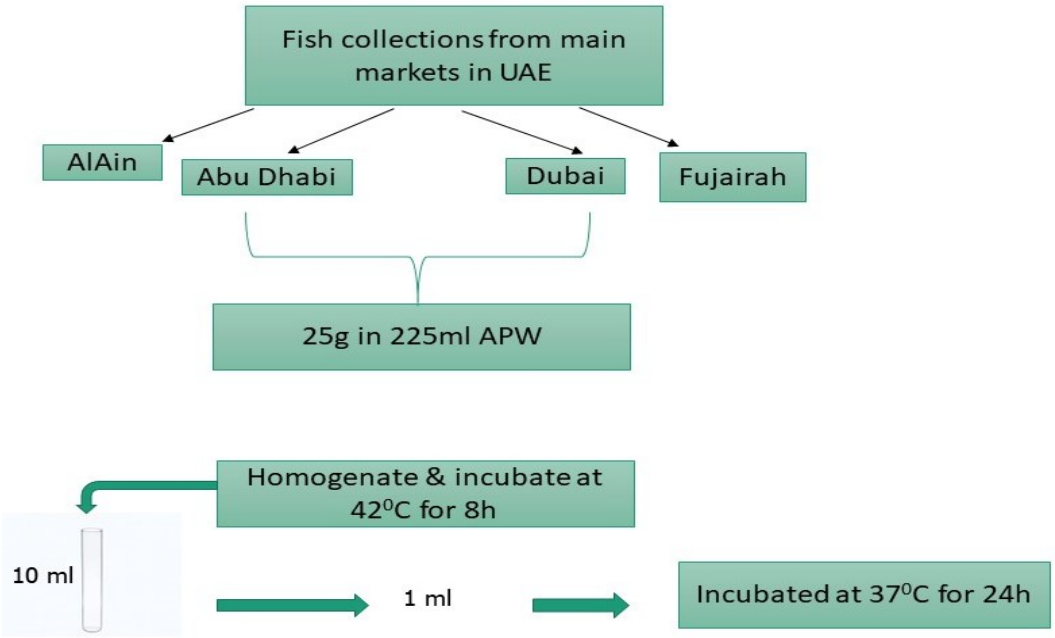


Figure 3.3: Schematic representation of isolation of *Vibrio*

3.3 Molecular Identification of *Vibrio*

3.3.1 DNA Extraction

Tissue homogenate (10 ml) incubated at 37°C was streaked in duplicate on thiosulfate citrate bile salts sucrose agar (TCBS, Hi Media) and tryptone soy agar (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 3% NaCl (TSA + 3% NaCl). The inoculated plates were incubated at 37°C for 18 to 24 h. Suspected colonies were streaked again on TSA + 3% NaCl to obtain a pure isolate.

3.3.1.1 Reagents

Solution CB1 - an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residues of salt, and other contaminants while allowing the DNA to stay bound to the silica membrane

Solution IRS - IRS solution contains a reagent to precipitate non-DNA organic and inorganic material including cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

Solution SB- Solution SB is a highly concentrated salt solution. It sets up the high salt condition necessary to bind DNA to the Spin Filter membrane

3.3.1.2. Procedure

DNA was extracted by QIAGEN DNA extraction kit. Briefly, 1.8 ml of bacteria culture was added to a 2 ml collection tube and centrifuge at 10,000 x *g* for 30 s at room temperature. Decant the supernatant and spin the tubes again at 10,000 x *g* for 30 s at room temperature. Supernatant was removed, the cell pellet was resuspended in 300 µl of Power Bead Solution and vortexed gently. Resuspended cells were then transferred to Power Bead Tube and 50 µl of CB1 solution was added and vortexed for 10 min. The tubes were centrifuged at a maximum of 10,000 x *g* for 30 s at room temperature and the supernatant was transferred to 2 ml collection tube. 100 µl of IRS Solution was added to the supernatant, vortexed for 5s and incubated at 4°C for 5 min. The tubes were then centrifuged at 10,000 x *g* for 1 min at room temperature. 900 µl of SB solution was added to the supernatant and vortexed for 5s. In the next step, 700 µl of supernatant with SB solution was loaded into a MB Spin Column and centrifuge at 10,000 x *g* for 30 s at room temperature. Then, 300 µl of CB solution was added and centrifuged at 10,000 x *g* for 30 s at room temperature. The MB Spin Column was placed in a new 2 ml collection tube, 50 µl of elution buffer was added in the center of white membrane. Centrifuged at 10,000 x *g* for 30 s at room temperature. The MB Spin Column was discarded and DNA was collected.

3.3.2. Polymerase Chain Reaction (PCR)

PCR assay was performed separately for general (*Vibrio spp*.) and specific (16 S rRNA) genes (Table 3.1) of the suspected *Vibrio* isolates. The amplification conditions were 35 cycles of amplification, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The reaction mixtures were resolved by electrophoresis in 2% agarose gel and visualized under UV light. The presence of the gel bands compared with the DNA molecular weight standard (100 bp marker) was recorded.

Table 3.1: Primers used for PCR analysis

Primer code	**Sequences (5' to 3')**
Vibrio spp. F	CGGTGAAATGCGTAGAGAT
Vibrio spp. R	TTACATGCGATTCCGAGTTC
V.16S-700F	CGG TGA AAT GCG TAG AGA T
V.16S-1325R	TTA CTA GCG ATT CCG AGT TC

3.4. Factors Affecting Growth Rate of *Vibrio*

The effect of temperature, pH and salinity on the growth and survival rate of *Vibrio* isolates were studied by the method of Yaashikaa, Saravanan & Kumar (2016)

3.4.1. Effect of Temperature

The nutrient broth was taken in a boiling tube and sterilized. The organism was inoculated in the medium at different temperatures (0°C, 37°C, 50°C and 70°C) and incubates. Growth of organisms was observed at 620 nm at regular intervals of time.

3.4.2. Effect of pH

The nutrient broth was taken in a boiling tube and sterilized. The organism was inoculated at different pH (3, 5, and 7) and incubated at 37°C for 24 h. Growth of organisms was observed at 620 nm at regular intervals of time.

3.4.3. Effect of Salinity

The nutrient broth was taken in the boiling tube at different concentration of NaCl (0.5%, 1%, and 2%). The organism was inoculated in the medium and incubated. Growth of organisms was observed at 620 nm at regular intervals of time.

3.5. Antibiotic Sensitivity Test

Antibiotic sensitivity of *Vibrio* isolates were studied by the method of Yaashikaa, Saravanan & Kumar (2016). The test culture was transferred into a sterilized broth. The broth is then incubated at 35°C till it becomes slightly turbid. By using a sterile cotton swab the standardized bacterial test suspension was inoculated evenly on the entire surface of sterile Muller Hinton Agar plates. Antimicrobial susceptibility test discs (Oxoid, Thermofischer scientific) (Table 3.2) were placed on the surface of the medium and plates were incubated on 37°C for 24 h. The antimicrobial activity was interpreted from the diameter of zone of inhibition which was measured in millimeter.

Table 3.2: Antimicrobials for sensitivity study

Antibiotics	**Concentration/disc**	**MIC break point (mm)**		
		S	**I**	**R**
Penicillin G	10iu	10	11-19	20
Vancomycin	2 mcg	12	-	13
Daptomycin	30 mcg	14	20	15
Ampicillin	10 mcg	14		15
Erythromycin	15 mcg	13	18	16
Sulphamethoxazole/Trimethoprim	25 mcg	13	14-16	17

Breakpoints as recommended by the CLSI M45-A (2010). iu- international unit, mcg-micro gram. S, I and R stand for susceptible, intermediate and resistant, respectively**.**

3.6 Statistical Analysis

Growth profile data were subjected to the analysis of variance (ANOVA) using general linear model (GLM) and mean comparisons were performed using Duncan's multiple range test to compare significant differences between means for all analyses. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago).

Chapter 4: Results and Discussion

4.1. Isolation of *Vibrio spp*. in Fish Samples of UAE

A total of 200 fish samples were imported from 4 different main markets at different cities. Twenty-five gram of fish flesh were used for isolation of *Vibrio spp.* Results showed that a total of 129 (64.5%) isolates were *Vibrio* positive in which the number of *Vibrio* positive isolates in each city is in the order of Fujairah (47) > Al-Ain (46) > Dubai (21)> Abu Dhabi (15). The percentage occurrence of *Vibrio* in Fujairah was 94% while in Al-Ain, Dubai and Abu Dhabi the percentage was 92, 42 and 30% respectively (Table 4.1). The results showed that the prevalence of *Vibrio* was higher in flesh of fish. This prevalence is also higher than that found in *Vibrio* isolates from Saudi Arabia (14%) (Elhadi, 2018). According to epidemiologic reports, *Vibrio spp.* is a major cause of bacterial infections associated with the consumption of imported fish from local markets (Tan *et al*., 2017). Raissy *et al*. (2014) revealed that 29.3% of the examined fish samples were *Vibrio* positive. Letchumanan *et al*. (2015) found a high level ($\geq 10^4$ MPN/g) of *Vibrio* in fish samples purchased from wet markets compared to supermarkets. Among the *Vibrio spp*., isolated, *V. paraheamolyticus* was predominant. The prevalence of *Vibrio* isolates detected off the coastal water of the eastern province of Saudi Arabia was also reported to be less than that observed in The study (Elhadi, 2018). The conventional method by biochemical means indicated that 33.3% of samples were positive for *Vibrio* in retail shellfish in Mansoura, Egypt (Abd-Elghany & Sallam, 2013).

Table 4.1: Occurrence of *Vibrio spp*. in fish

Location	**Number of fish samples**	**Number of *Vibrio* positive samples**	**% of *Vibrio* positive samples**
Al-Ain	50	46	92
Dubai	50	21	94
Fujairah	50	47	42
Abu Dhabi	50	15	30

4.2 Molecular Identification of *Vibrio*

Results showed that prevalence of *Vibrio parahemolyticus* was higher when compared to *V.mimicus* and *V.vulnificus* (Table 4.2). An incidence of 11.62% for *V. paraheamolyticus* was observed in isolates from different cities while for *V.vulnificus* the prevalence was 7.75% and for *V.mimicus* the prevalence was only 2.32%. Ghenem and Elhadi (2018) reported that 90% of studied samples from coastal water in the Eastern Province of Saudi Arabia were positive for *Vibrio* among the identified species *V.parahaemolyticus* was predominant. This data is in agreement with the present study. Seawater samples collected from 17 different locations along the coastal areas of the Eastern Province of Saudi Arabia showed the presence of *V. vulnificus* (17.95%) (Alsalem *et al*., 2018). Youssef, Farag and Helal (2018) reported that overall prevalence of *V. parahaemolyticus* in shellfish was collected from Suez Canal area, Egypt was (9.27%), whereas in water was 12/48 (25%). Most studies demonstrated a predominance of *V. alginolyticus* in shrimp or seafood samples (Chitov *et al*., 2009), but Chen *et al*. (2011) found *V. parahaemolyticus* was the predominant *Vibrio spp*. which is similar to the data. Similar results were reported by Yucel and Balci (2010). *Vibrio cholerae* and *Vibrio parahaemolyticus* were present in the gills, skin, intestine of fish and overlying water (Amirmozafari *et al*., 2005). The study by Gopal *et al*. (2005) revealed the dominance of *V. alginolyticus*, followed by *V. parahaemolyticus*

in east and west coast of India samples. Some studies reported lower infection rates of *V. parahaemolyticus* in seafood. The percentage of *V. parahaemolyticus* in shrimps harvested from Dardanelles Market in Turkey was zero (Colakogu *et al*., 2006).

Table 4.2: Prevalence of *Vibrio spp*. in fish

Isolates	**Prevalence of *Vibrio* in different cities**				**% Prevalence**
	Al-Ain	Dubai	Fujairah	Abu Dhabi	
V.paraheamolyticus	3	5	4	3	11.62
V.vulnificus	3	0	4	3	7.75
V.mimicus	1	0	2	0	2.32
Others	39	16	37	9	78.29

The *Vibrio* positive isolates were used for the molecular identification by using PCR. Results showed that the presence of *Vibrio* was confirmed by using both general and *Vibrio* specific sequences. Recently, many *Vibrio* PCR assays have been reported for the identification of the major pathogenic *Vibrio* species (Izumiya *et al*., 2011). Ghenem and Elhadi, (2018) confirmed the presence of 6% of *Vibrio* isolated from coastal water in the Eastern Province of Saudi Arabia. Kim *et al*. (1999) characterized *V16.S* involved in regulation of gene expression in *Vibrio*. *V16.S* is present in all of the *Vibrio* isolates and could be used as marker genes for specific detection of this bacterium (Zhang & Orth, 2013). *V. paraheamolyticus* was present in mussels in Qatar as confirmed by the cluster D. 16S rDNA-based identification. With the use of a specific primer set for *V*.16S, target bands of 370 bp were obtained by PCR amplification and gel electrophoresis. The major target of this microorganism has been identified as a wide variety of aquatic animals, such as mollusc, crustaceans and fish. *Vibrio* also causes zoonoses by affecting humans (Baker-Austin, 2010). An increased occurrence of *Vibrio spp.* has been confirmed in other sea food samples including

cockles (50%) from Indonesia (Zulkifli *et al*., 2009), oysters (44%) from Alaska (Nordstrom *et al*., 2007), shellfish (85%) from Chile (Fuenzalida *et al*., 2007), natural oysters (51.5%) from the Gulf of Mexico, Alabama, USA (Ward & Bej, 2006) and oysters (83%) from the Gulf of Mexico, USA (Panicker *et al*., 2004). Another study determined the incidence of food borne pathogens in some European fish (France, Britain, Greece and Portugal) and reported the presence of *V. parahaemolyticus* in 35% of samples from Portugal and 14% from Greece but no *Vibrio spp.* in samples from Britain (Davies *et al*., 1993). Karunasagar *et al*. (1994; 1997) found that atypical strains of *Vibrio* could be recognized using 387-bp fragment of chromosomal region with PCR. Later studies showed that a correlation was established between the results of PCR with *V.16S* fragment suggesting that for molecular identification of microbial species genetic methods are widely used in research. In this study, presence of *Vibrio* in fish samples were atypical in different location. The result also support that the *V.16S*-based approach is a reasonable method to identify the presence of *Vibrio* cluster (Figure 4.1).

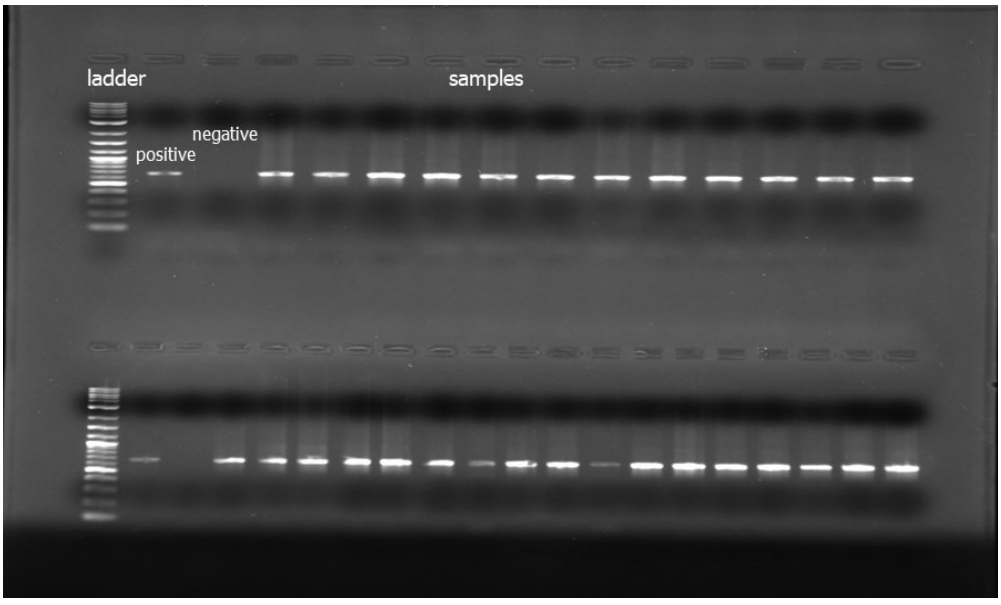


Figure 4.1: Gene amplification profile of *Vibrio* isolates

4.3. Antimicrobial Resistance

Antibiotic resistance study showed that *Vibrio parahemolyticus* isolates were resistant (100%) to penicillin G, daptomycin, vancomycin, ampicillin and erythromycin. Among the isolates 6 (40%) of *V. parahemolyticus* were resistant to Sulfamethoxazole-trimethoprim. *Vibrio vulnificus* isolates showed resistance (100%) to penicillin G, daptomycin, vancomycin, ampicillin and erythromycin while 4 (40%) of isolates were resistant to Sulfamethoxazole-trimethoprim. 1 (33%) of *Vibrio.mimicus* were resistant to Sulfamethoxazole-trimethoprim while the isolates were 100% resistant to penicillin G, daptomycin, vancomycin, ampicillin and erythromycin (Table 4.3). This finding is in agreement with the results reported by Letchumanan *et al*. (2015) where 92% of the *Vibrio* isolates from shrimp samples were resistant to penicillin, erythromycin, daptomycin and ampicillin. *V. vulnificus* isolates in mussels of Qatar showed resistance to antibiotics with the most common resistances were demonstrated towards penicillin (93%), ampicillin (70%), cephalothin (65%), clindamycin (66%), vancomycin (64%), and erythromycin (51%) (M Kurdi Al-Dulaimi *et al*., 2019). The antibiotic susceptibility test against *Vibrio vulnificus* isolated from the coastal areas in the eastern province of Saudi Arabia indicated high resistance to ampicillin (96%), cephalothin (73%), rifampicin (63%), and amoxicillin-clavulanic acid (56%) (Alsalem *et al*., 2018). Han *et al*. (2015) reported the susceptibility of *V. parahaemolyticus* and *V. vulnificus* isolates in oysters from the United States for ampicillin showed decreased exposure which was MIC50 ¼ 32 mg/ml. In cultured seafood products, the *V. parahaemolyticus* isolated were resistant to penicillin G, vancomycin and ampicillin (Elexson *et al*., 2014). In microbes mainly Gram-negative bacteria, due to the intricacy of their outer

membrane which inhibits the passage of antibiotic compounds through the outer membrane (Blair *et al*., 2014). Antimicrobials like penicillin G, vancomycin, erythromycin and ampicillin are ineffectual for the treatment of *Vibrio* infections.

Table 4.3: Antimicrobial resistance of *Vibrio* isolates

Antimicrobial Agents	**Disc Conc.**	**MIC break point (mm)**			**Number of isolates resistant to antibiotics (%)**		
		S	**I**	**R**	***V.parahemolyticus***	***V.vulnificus***	***V.mimicus***
Penicillin G	1 iu	10	11-19	20	15 (100)	10(100)	3(100)
Daptomycin	2 mcg	12	-	13	15 (100)	10 (100)	3 (100)
Vancomycin	30 mcg	14	20	15	15 (100)	10 (100)	3 (100)
Ampicillin	10 mcg	14		15	15 (100)	10 (100)	3 (100)
Erythromycin	15 mcg	13	18	16	15 (100)	10 (100)	3 (100)
Sulfa methoxazole-trimethoprim	25 mcg	13	14-16	17	6 (40)	4 (40)	1 (33.3)

Results expressed as the number of positive sample; the numbers in bracket indicate the percentage. Disc conc: - Disc concentration, iu-international units, mcg-microgram. Breakpoints as recommended by the CLSI M45-A (2010). S, I and R stand for susceptible, intermediate and resistant.

Han *et al*. (2015) found that *V. vulnificus* isolates were susceptible to all antimicrobial agents, including penicillin G. Susceptibility tests show that isolates *V. parahaemolyticus* in South China appear to a high level of resistance to penicillin G. This result is similar to a report by Letchumanan *et al*. (2015) in which 82% of the isolates from shrimp samples were also resistant to penicillin. As there is an increase in the number of resistance genes and the spread of antimicrobial-resistant *V. parahaemolyticus* isolates worldwide, the misuse and overuse of antibiotics are considered the most important factors (Tan *et al*., 2017).

Three isolates in Al-Ain (FA15, FA 21 and FA 34) were susceptible to daptomycin and the zone of inhibition ranges from 10 to 18 mm. Results showed that *Vibrio* isolates in fish samples from Dubai were not susceptible to daptomycin. In Fujairah, 11 *Vibrio* isolates were susceptible to daptomycin and the zone of inhibition was 7.5 to 16.5 mm. 10 mm was the zone of inhibition for the one daptomycin susceptible *Vibrio* isolate from Abu Dhabi. Susceptibility profiles to antibiotics such as cefotaxime, imipenem and daptomycin were studied and found some isolates were sensitive to these antimicrobials, which are first-line drugs used in clinical treatment (Akins & Rybak 2000).

Ampicillin was the only tested antimicrobial in the Gulf Coast study to which a large percentage of *V. parahaemolyticus* isolates demonstrated intermediate resistance to resistance. This trend was seen in a study conducted by Joseph *et al*. (1978) where the resistance of *V. parahaemolyticus* to ampicillin and b-lactamase inhibitors was 90%. In contrast to the present study, Han *et al*. (2015) found no resistance in either *Vibrio* species to ampicillin, chloramphenicol, cefotaxime, and vancomycin, while we observed intermediate resistance against ampicillin by the *Vibrio* positive isolates from different locations in UAE.

Alam *et al*. (2015) reported that 17 isolates of *V. cholerae* O1 from aquatic environments were susceptible to doxycycline, erythromycin, and ampicillin. In another collection of 1029 *V. cholerae* O1 strains collected from 18 towns in Haiti, the 115 *V. cholerae* tested by CDC Atlanta showed 100% susceptibility to erythromycin (Steenland *et al*., 2013). Baker-Austin *et al*. (2009) reported higher percent intermediate susceptibility among *V. vulnificus* against sulfamethoxazole-trimethoprim compared to that of the isolates reported in this study.

Susceptibility results to antibiotics such as daptomycin, vancomycin and sulfamethoxazole-trimethoprim were similar with many other studies reported in different seafood sources in several countries (Ottaviani *et al*., 2013). *V. vulnificus* from the Coasts of Tripoli, Libya were sensitive to doxycyclin, polymyxin, and oxytetracyclin, while resistant to florfenicol, sulfamethoxzole-trimethoprim and ampicillin (Eissa *et al*., 2017). Motaweq (2017) reported that *Vibrio* isolated from Najaf Province of Iraq showed susceptibility to ampicillin (100%), nalidixic acid (89%) and ciftazidime (85%) while lower resistance toward azithromycin (37%), erythromycin (33%), ceftriaxone (33%), chloromphenicol (22%), tetracycline (11%) and ciprofloxacin (7.5%). Similar antimicrobial resistance profiles were also reported in studies using large numbers of *V. parahaemolyticus* isolates from coastal environments of Korea (Baker-Austin *et al*., 2009), and from farmed fish in Korea (Oh *et al*., 2011). The results of the present study were supporting previous studies in seafood sample isolates, except for the incident of resistance to vancomycin. A prior study by Chanratchakool *et al*. (1995) on diseased black-tiger shrimps cultured in Thailand established that the rate of resistance to oxytetracycline by the *Vibrio* isolates was >70% among *V. parahaemolyticus* isolates with the zone of inhibition ranging from 22.5 to 38.6 mm.

4.4 Factors Affecting Growth Rate of *Vibrio*

The more antibiotic resistant *Vibrio* isolates from different locations of UAE were used to study the effect of different factors such as temperature, salinity and pH on survival and growth rate of the bacterium.

4.4.1 Effect of Temperature on Growth Rate

4.4.1.1. Growth Rate of *Vibrio* Isolates at 25°C

The growth and survival of antibiotic resistant *Vibrio* isolates from different locations of UAE at different temperature were studied. *Vibrio* isolates were incubated at different temperature (25- 45°C) and the growth rate was determined.

A gradual increase in growth rate was observed in isolates during the incubation period (0 to 6 h). Among the isolates, *V. parahemolyticus* and *V. Vulnificus* showed maximum growth rate at 25°C. A growth rate of 82% was observed in *V. parahaemolyticus* F36 and F46 (Figure 4.2a & Figure 4.2c) and *V. vulnificus* AD11 (Figure 4.2d) attained a growth rate of 82.8% at 25°C. When compared to *Vibrio parahemolyticus* and *V. Vulnificus, V. mimicus* exhibited lowest growth rate at 25°C which was 70% by *V.mimicus* F4 (Figure 4.2b).

a

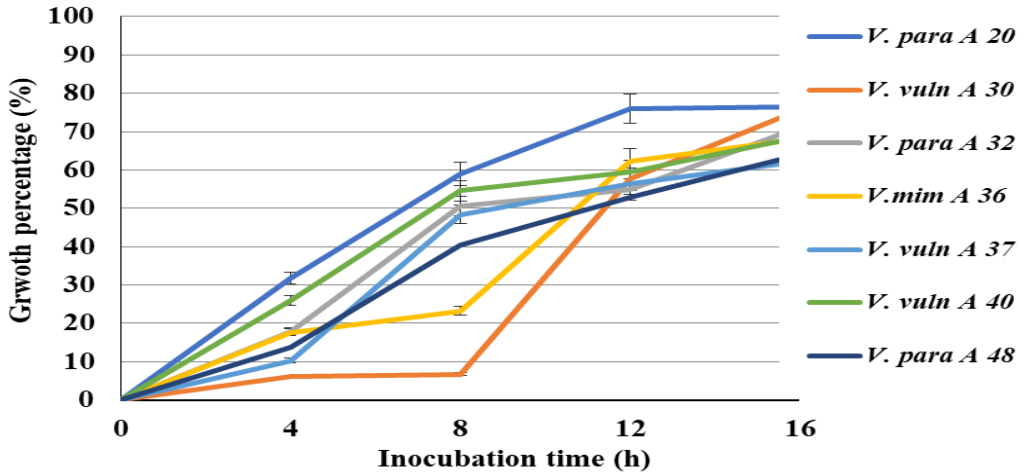
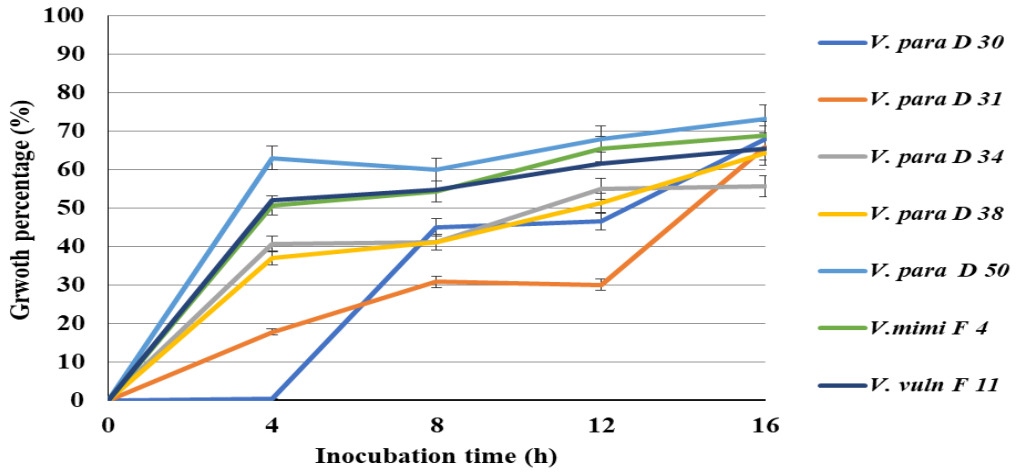


Figure 4.2: Growth rate of *Vibrio* at 25°C

Values are expressed as average of 3 samples ± SE

b



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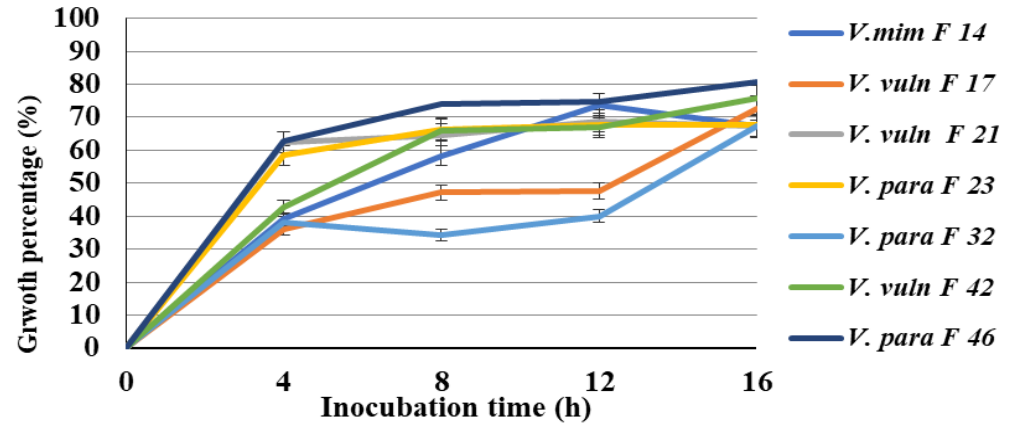

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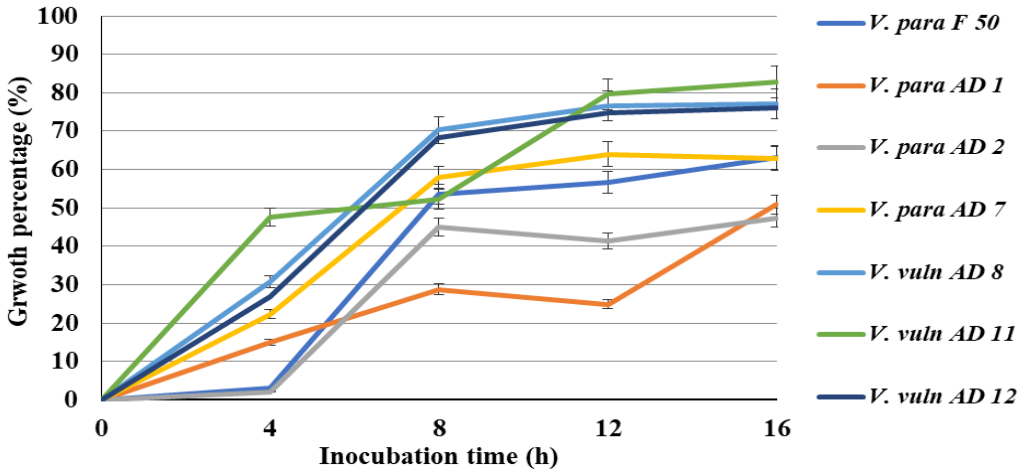


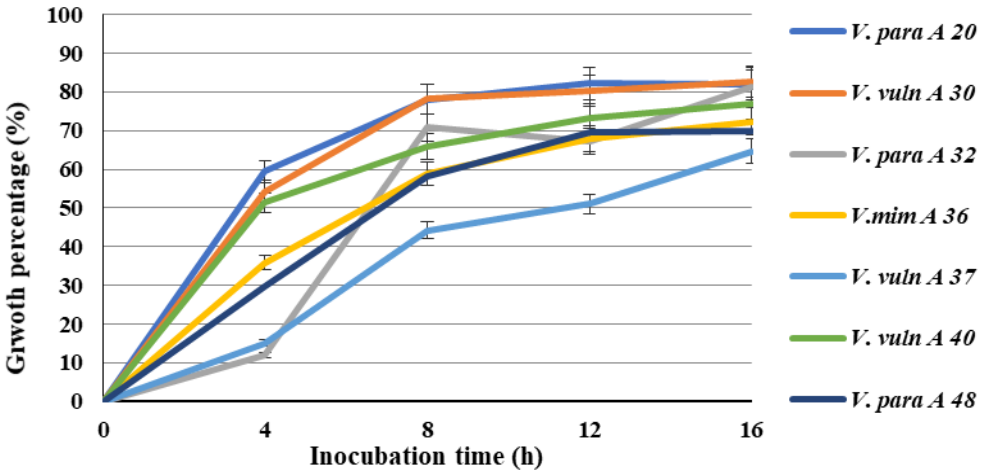
Figure 4.2: Growth rate of *Vibrio* at 25°C (Continued)

Values are expressed as average of 3 samples ± SE

4.4.1.2. Growth Rate of *Vibrio* Isolates at 37°C

In the present study, results showed that all the three types of *Vibrio spp.* isolated maximum growth rate at 37°C. Among the isolates, *V.parahemolyticus* A20 (Figure 4.3a) and *V.parahemolyticus* AD1 (Figure 4.3d) attained a growth rate of 82.6% and 81.4% at 37°C on 16 h of incubation. *V.vulnificus* A30 (Figure 4.3a) and *V.vulnificus* AD 8 (Figure 4.3) attained a growth rate of 80% at 37°C. The *V.mimicus* F4 (Figure 4.3b) and *V.mimicus* F 14 (Figure 4.3c) attained 83% growth rate at 37°C.

a



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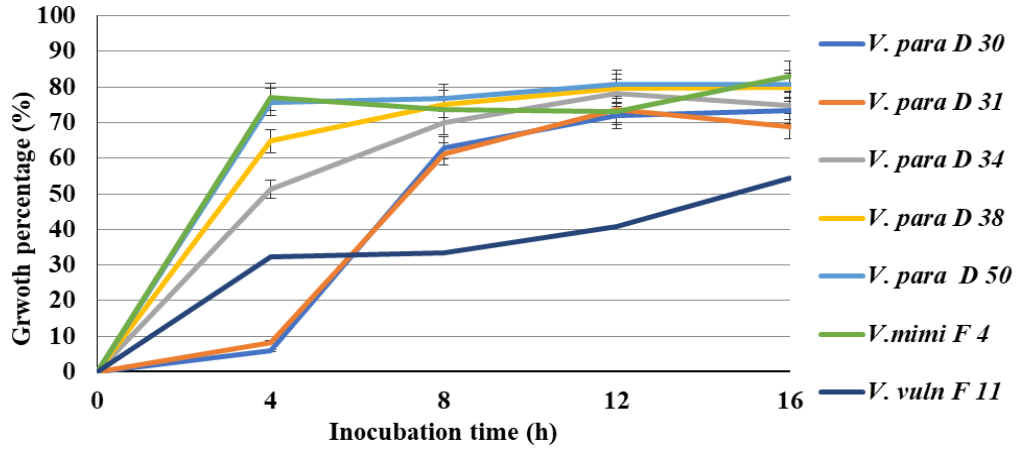
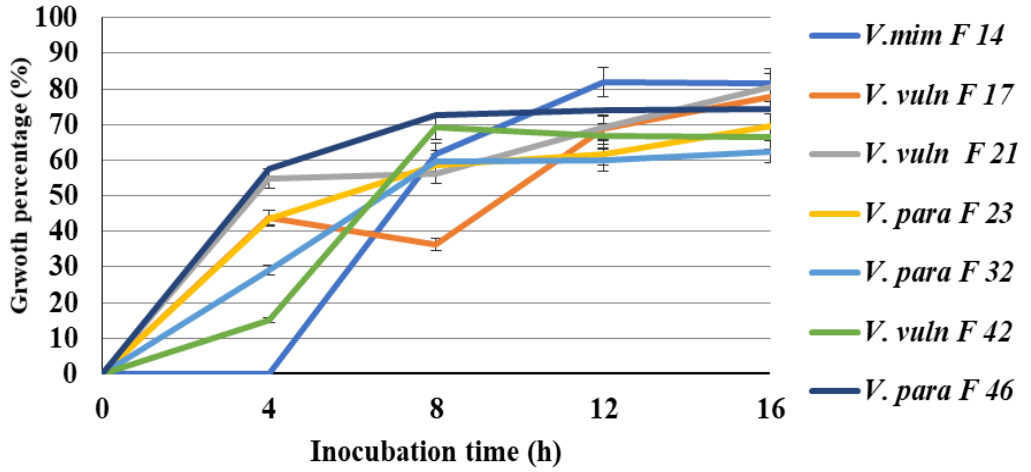


Figure 4.3: Growth rate of *Vibrio* isolates at 37°C

Values are expressed as average of 3 samples ± SE

c



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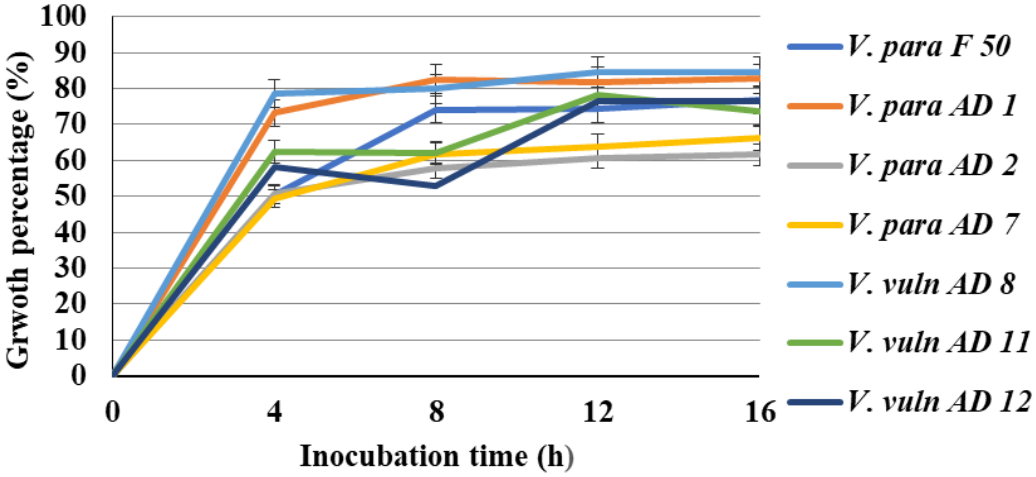


Figure 4.3: Growth rate of *Vibrio* isolates at 37°C (Continued)

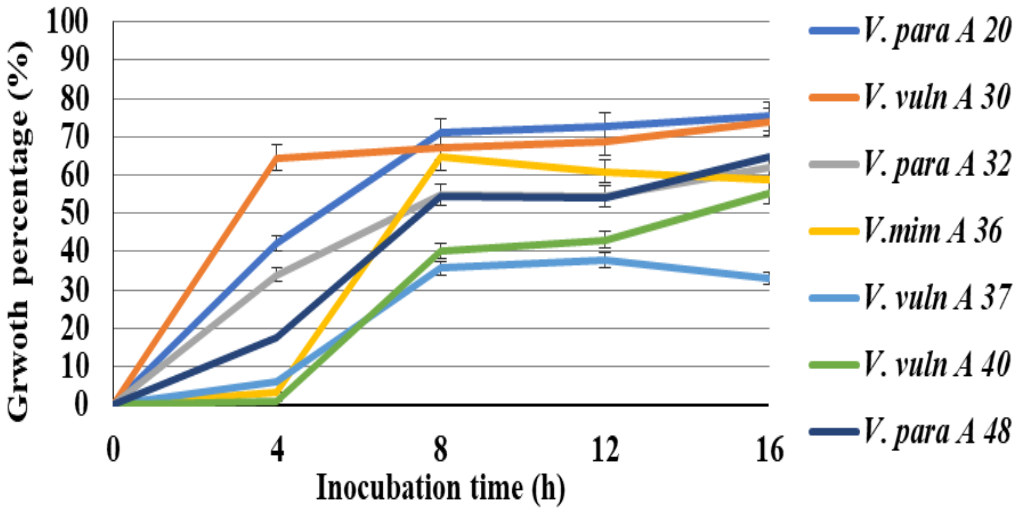
Values are expressed as average of 3 samples ± SE

4.4.1.3. Growth Rate of *Vibrio* Isolates at 45°C

At 45°C, *Vibrio* isolates attained decreased growth rate when compared to the growth rates at 25°C and 37°C. Among the isolates, a growth rate of 75.3% at 16 h was observed in *V. parahaemolyticus* A20 (Figure 4.4a) and *V. parahaemolyticus* AD7 (Figure 4.4d). The maximum growth rate attained by *V.vulnificus* at 45°C was 78% by

V.vuln F11 (Figure 4.4b) and 76% by *V.vuln* F17 (Figure 4.4c). When compared to growth rate of *V.parahemolyticus* and *V.vulnificus* at 45°C, *V.mimicus attained* a decreased growth rate. *V.mimi* F4 (Figure 4.4b) attained a growth rate of 68% while the growth rate of other *V.mimicus* isolates were less than 60%.

a



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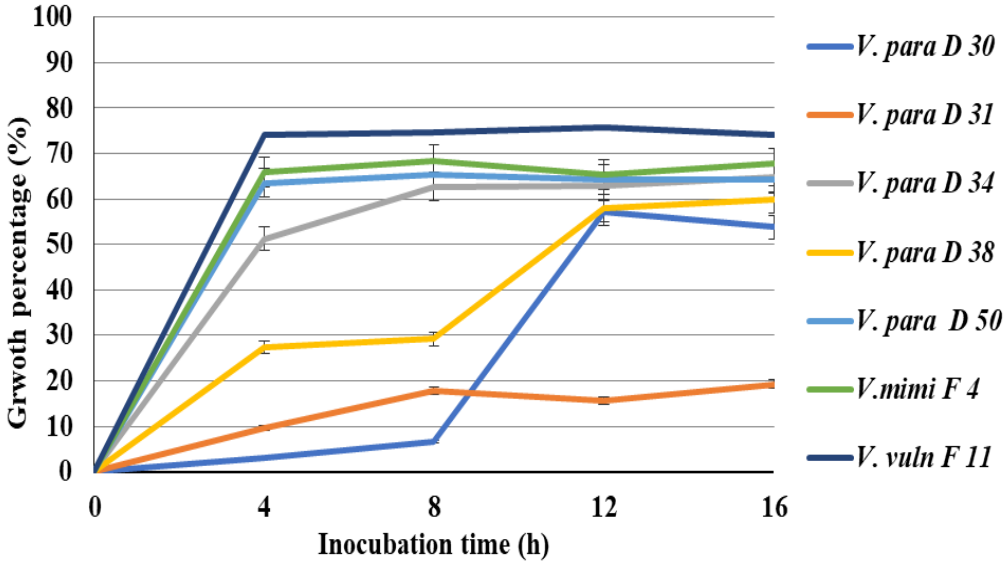
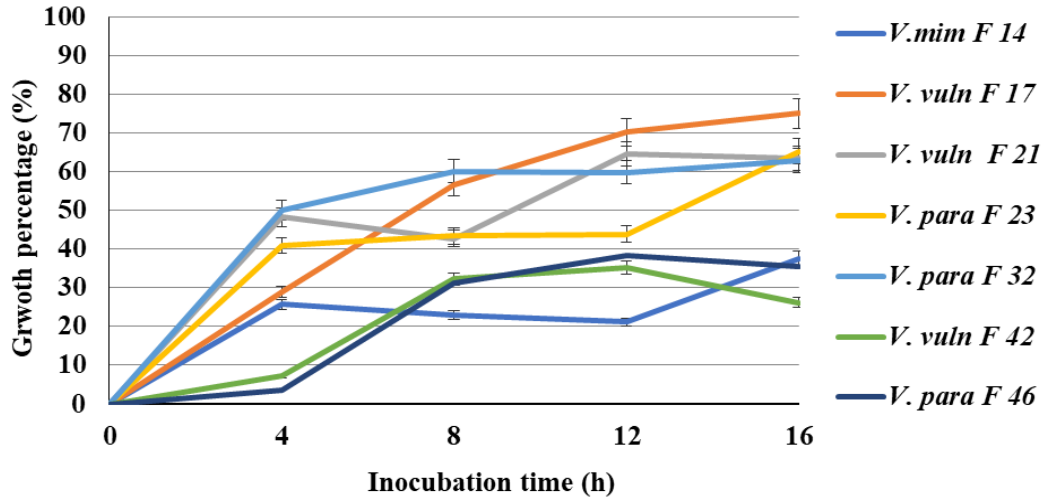


Figure 4.4: Growth rate of *Vibrio* isolates at 45°C

Values are expressed as average of 3 samples ± SE

c



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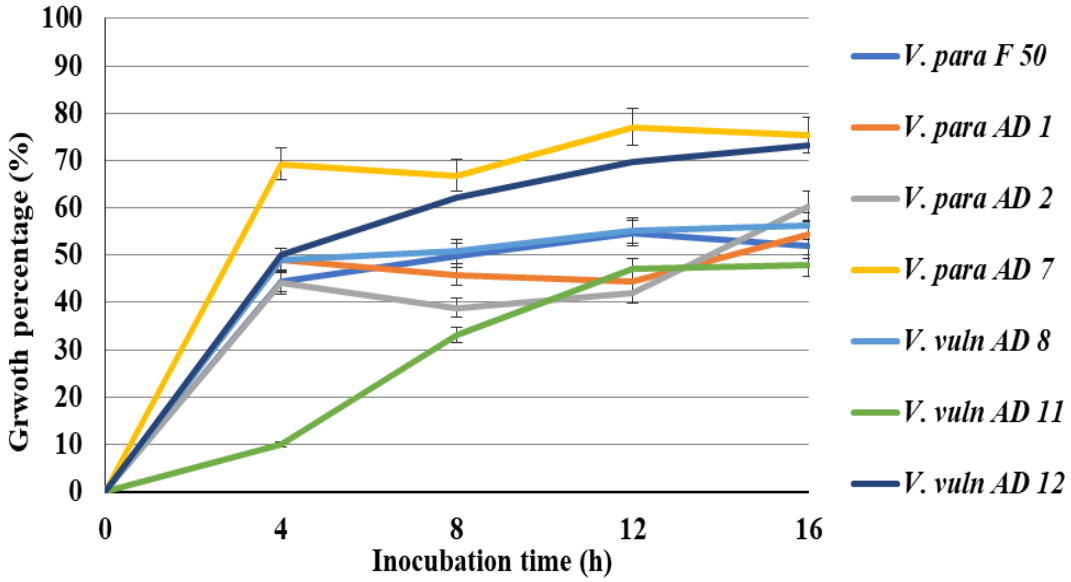


Figure 4.4: Growth rate of *Vibrio* isolates at 45°C (Continued)

Values are expressed as average of 3 samples ± SE

Research regarding the effect of temperature on the growth of *Vibrio*. *spp.* were also reported by Miles *et al*. (1997); Yoon *et al*. (2008) and Fernandez-Piquer *et al*. (2011). Yang *et al*. (2009) inoculated *V. parahaemolyticus* on salmon meat over a temperature range from 0°C to 35°C for studying the growth and survival curves of the *Vibrio spp*. Kim *et al*. (2012) evaluated the growth and survival of *Vibrio* samples

in ready-to-eat seafood such as sashimi and raw oyster meat and found that specific growth rate (SGR) values between flounder and salmon sashimi were at temperatures ranging from 13°C to 30°C. The pathogenic *V. parahaemolyticus* showed continuous growth under 15, 25, and 35°C, while a decline in growth was found under 5°C (Wang *et al*., 2018).

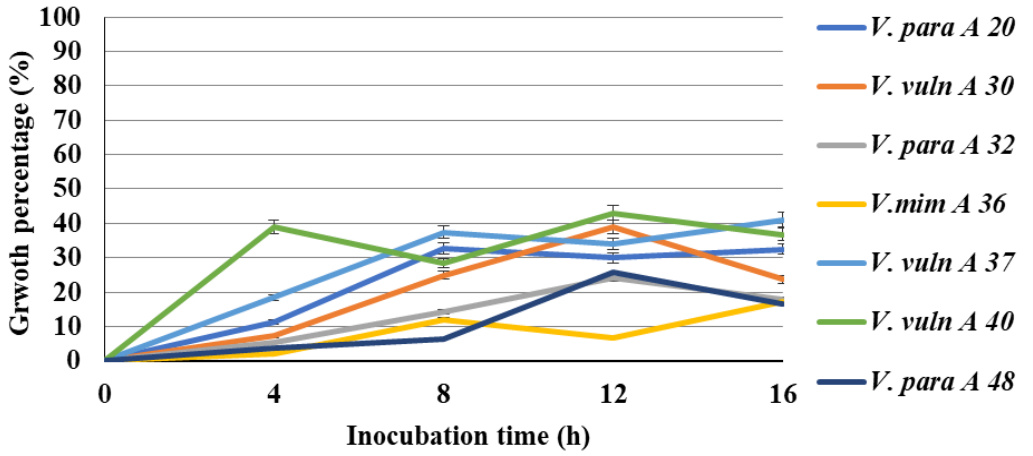
4.4.2 Effect of pH on Growth Rate of *Vibrio* Isolates

The effect of different pH level on growth rate and survival of *Vibrio spp.* was studied. *Vibrio* isolates were incubated at different pH (3- 5) at different time period (0 to 16 h).

4.4.2.1. Growth Rate of *Vibrio* Isolates at pH 3.0

At pH 3.0 most of the isolates showed decreased growth rate in which *Vibrio parahemolyticus* D31 attained a highest growth rate of 42% (Figure 4.5b). The growth rate of other *V. parahemolyticus* isolates are less than 40% at pH 3.0. *V. vulnificus* AD12 (Figure 4.5d) attained highest growth rate of 60% which was the maximum growth rate of *V.vulnificus* isolates at pH 3.0. *V.mimicus* also attained decreased growth rate at pH 3.0 which was 41% by *V.mimi* F4 (Figure 4.5b).

a



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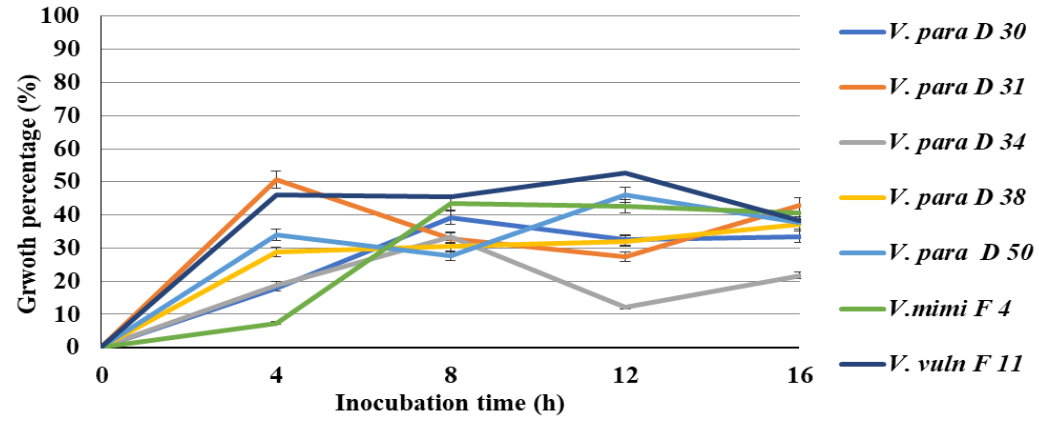

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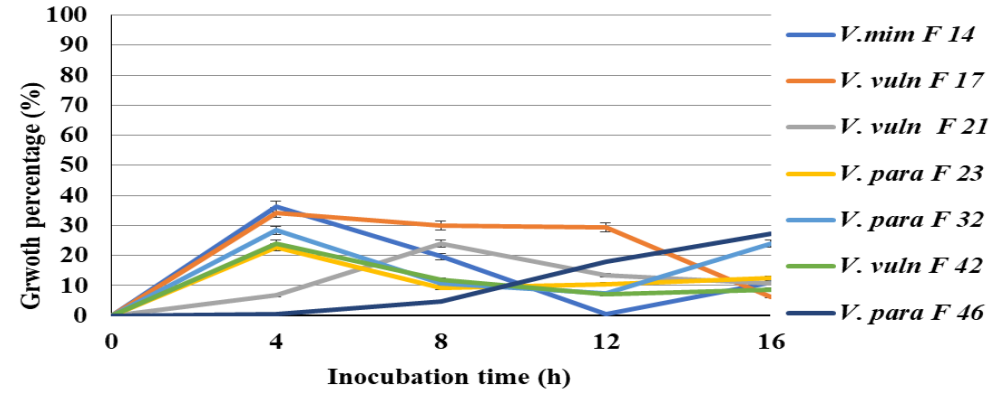


Figure 4.5: Growth rate of *Vibrio* isolates at pH 3.0

Values are expressed as average of 3 samples ± SE

d

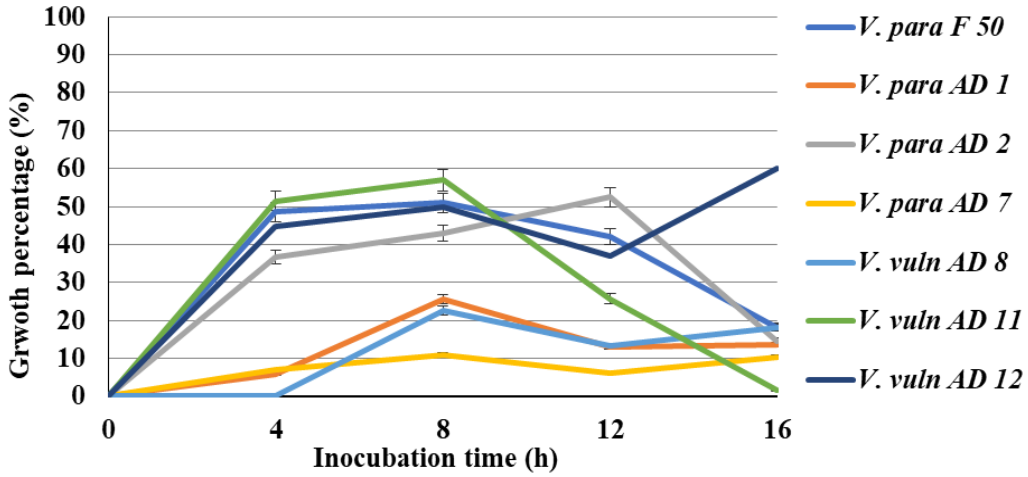


Figure 4.5: Growth rate of *Vibrio* isolates at pH 3.0 (Continued)

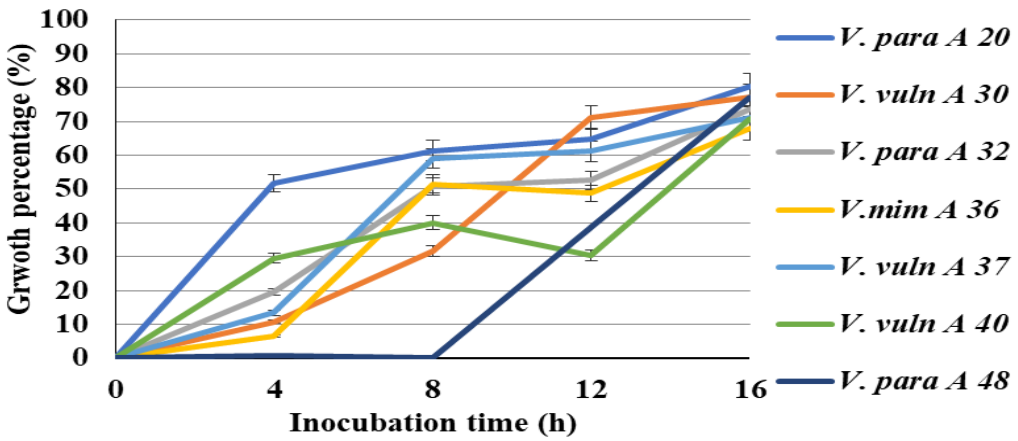
Values are expressed as average of 3 samples ± SE

4.4.2.2. Growth rate of *Vibrio* Isolates at pH 5.0

In the present study, results showed that when the pH of the system was increased from pH 3.0 to pH 5.0, the growth percentage was also increased. Most of the *Vibrio* samples attained a maximum growth rate of 80% at pH 5.0 on 16 h of incubation.

The growth rate of *Vibrio parahemolyticus* isolates was in the range of 60% to 80%. Among them isolate *V. para* A20 (Figure 4.6a) and *V.para* F32 (Figure 4.6c) showed maximum growth rate of 80%. *Vibrio vuln* A30 (Figure 4.6a), *Vibrio vuln* AD 11 and AD12 (Figure 4.6d) attained 80% growth rate at pH 5.0. Among the *V.mimicus* isolates only *V.mim* F14 (Figure 4.6c) showed 80% growth at pH 5.0.

a



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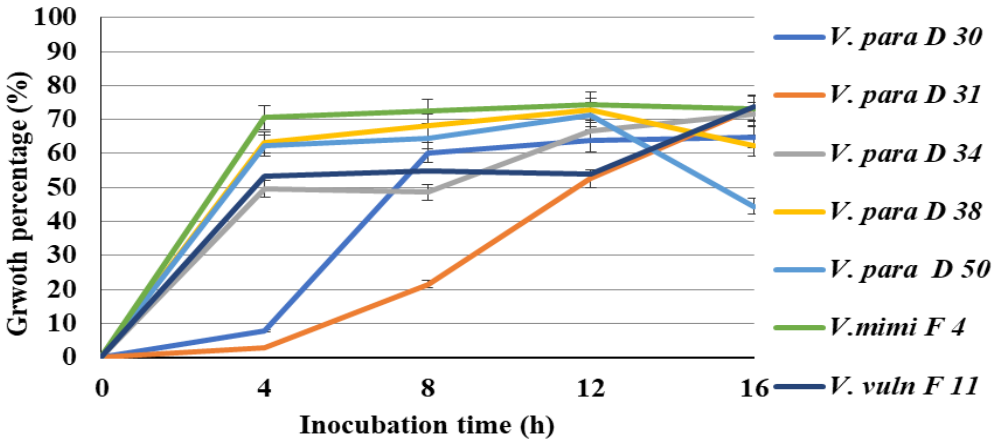

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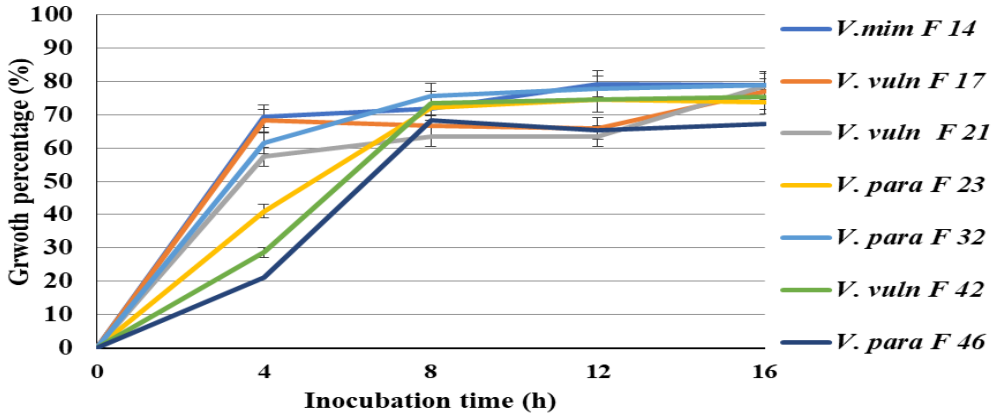


Figure 4.6: Growth rate of *Vibrio* isolates at pH 5.0

Values are expressed as average of 3 samples ± SE

d

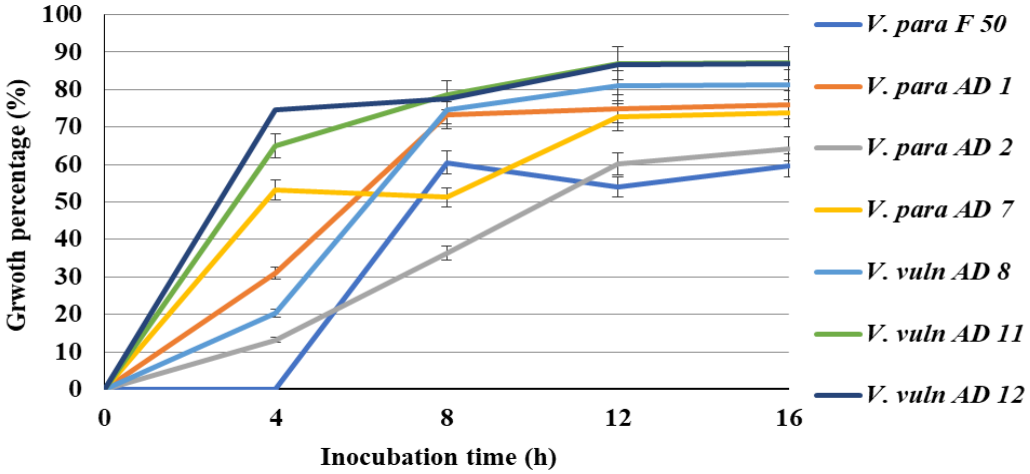


Figure 4.6: Growth rate of *Vibrio* isolates at pH 5.0 (Continued)

Values are expressed as average of 3 samples ± SE

4.4.2.3 Growth Rate of *Vibrio* Isolates at pH 7.0

At pH 7.0, *Vibrio parahemolyticus* attained growth rate from 72 to 81%. Among the isolates, *V. parahaemolyticus* A 20 showed growth rate of 81.6% on 16 h of incubation (Figure 4.7a). *V. vulnificus* F11, F42 and AD8 showed growth rate of 80% at pH 7.0 (Figure 4.7b, 4.7c & 4.7d). At pH 7.0, *V.mim* A36 showed a growth rate of 70% which was the highest growth rate of *V.mimicus* isolates (Figure 4.7a).

a

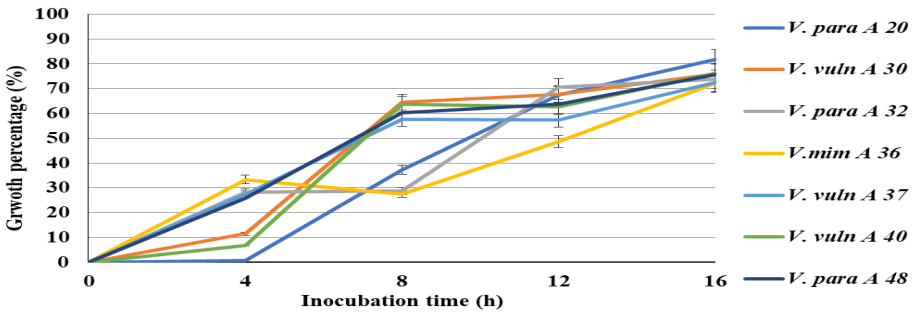
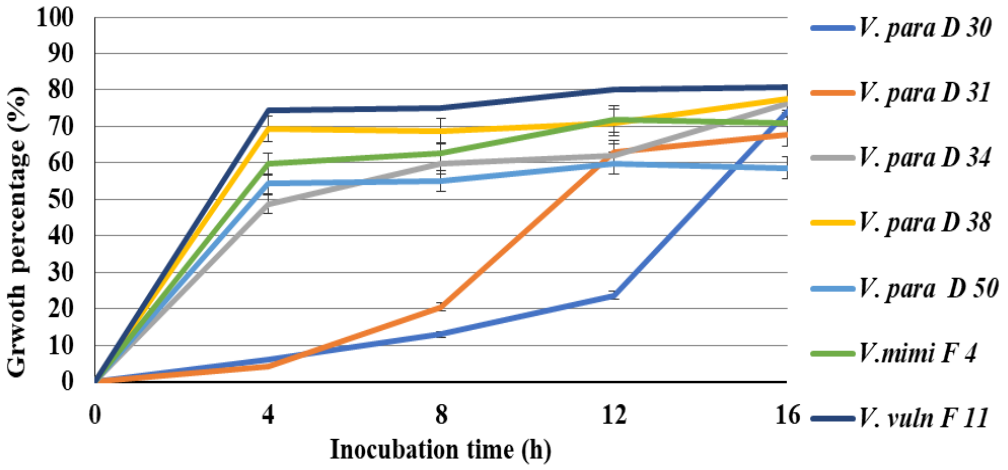


Figure 4.7: Growth rate of *Vibrio* isolates from at pH 7.0

Values are expressed as average of 3 samples ± SE

b



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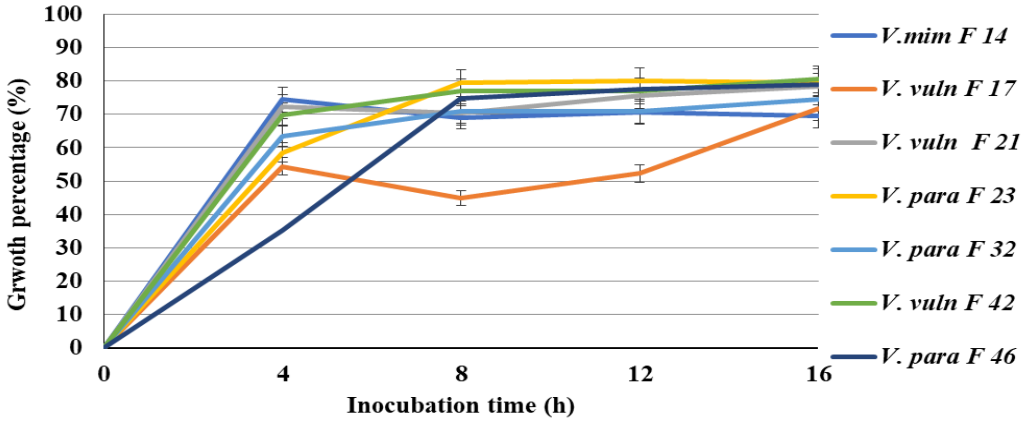


d

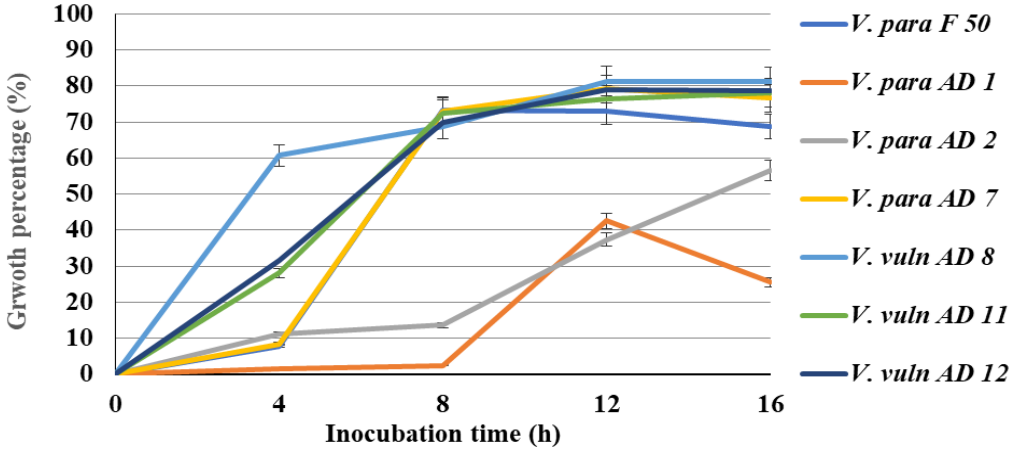


Figure 4.7: Growth rate of *Vibrio* isolates from at pH 7.0 (Continued)

Values are expressed as average of 3 samples ± SE

Beuchat (1973) studied the influence of growth conditions on survival rate of six strains of *Vibrios* and found that the lowest pH permitting growth was pH 7.3. The *Vibrio spp.* isolated from prawn (*Penaeus monodon*) seafood grows best at alkaline pH and the maximum growth rate was observed at pH 9 (Yaashikaa *et al*., 2016). *Vibrio parahaemolyticus* was able to grow at pH 5.0 to pH11, and at NaCl concentrations of 1 to 7% (Twedt, 1969).

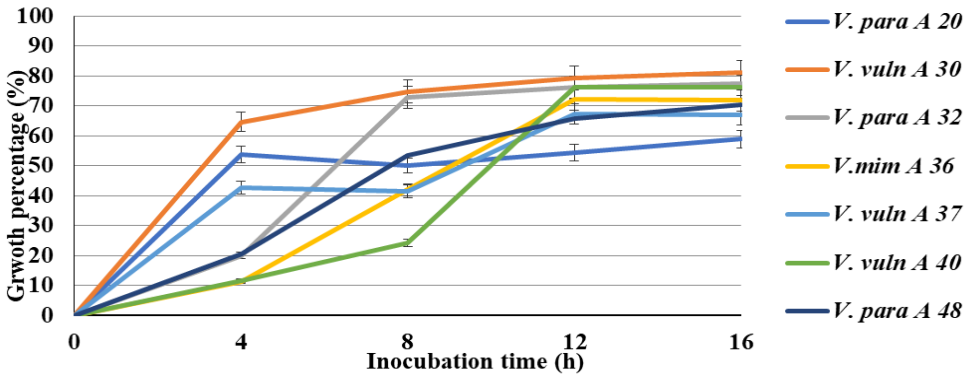
4.4.3 Effect of NaCl on Growth Rate of *Vibrio* Isolates

At different salinity level, the maximum growth rate and survival of *Vibrio spp.* was studied. *Vibrio* isolates were incubated with different concentration of NaCl (0.5% - 2%) at different time period (0 to 16 h).

4.4.3.1 Growth Rate of *Vibrio* Isolates at 0.5% NaCl

At 0.5% NaCl concentration, *V.parahemolyticus, V.Vulnificus* and *V.mimicus* isolates showed highest growth rate from 4 h of incubation and the rate reaches to a maximum growth rate at 16 h. *V. parahaemolyticus* isolates *V para* F23 (Figure 4.8c) showed growth rate of 92% while *V. para* D38 (Figure 4.8d) and *V.para* AD7 (Figure 4.8d) showed 82% growth rate. Among *V.vilnificus* isolates, *V.vul* F21 (Figure 4.8c) showed highest growth rate (83.1%) at 0.5 NaCl. The growth rate of *V.mimicus* was less when compared to *V.parahemolyticus*. *V.mimi* F4 showed highest growth rate of 82% at 0.5% NaCl concentration (Figure 4.8b).

a



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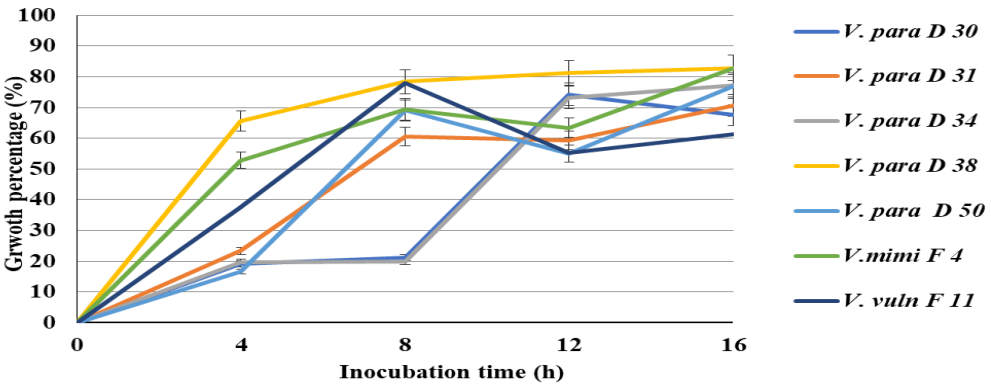

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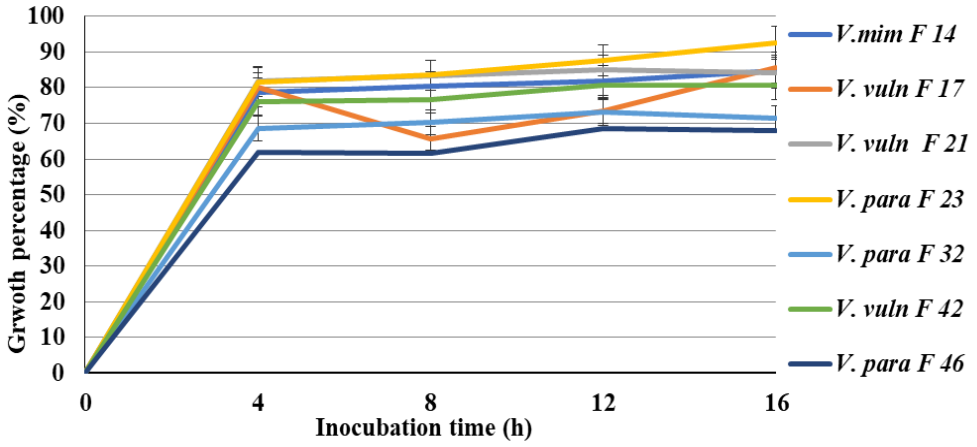


Figure 4.8: Growth rate of *Vibrio* isolates at 0.5% NaCl

Values are expressed as average of 3 samples ± SE

d

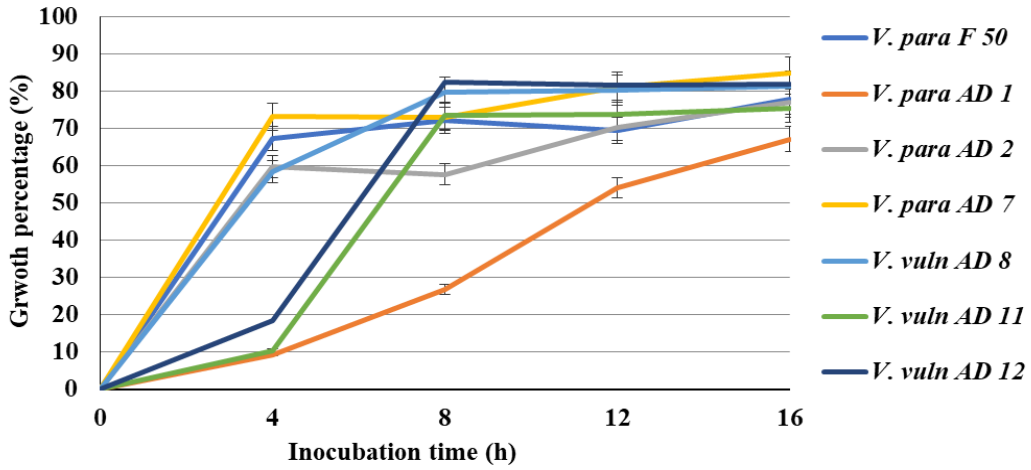


Figure 4.8: Growth rate of *Vibrio* isolates at 0.5% NaCl (Continued)

Values are expressed as average of 3 samples ± SE

4.4.3.2 Growth Rate of *Vibrio* Isolates at 1.0% NaCl

At 1% NaCl, *Vibrio* isolate *V. para* F23 (Figure 4.9c) showed highest growth rate of 83%. Among the *Vibrio vulnificus* isolates, *V.vul* AD8 (Figure 4.9d), showed 85% growth rate which was high growth rate when compared to *V.parahemolyticus* and *V.mimicus*. *V.mimi* F4 (Figure 4.9b) showed a growth rate of 78% when compared to other *V.mimicus* isolates.

a

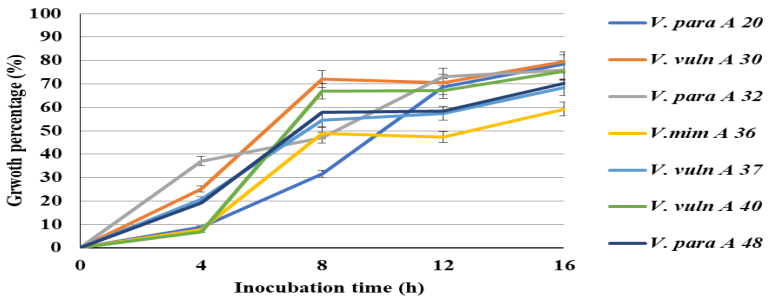
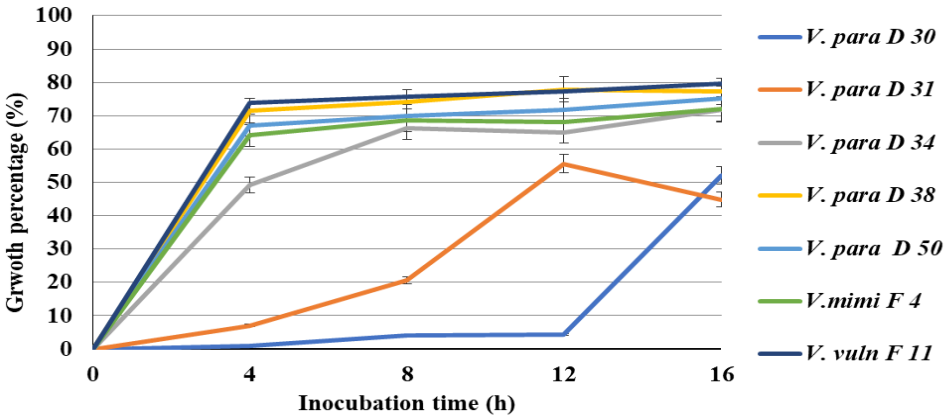


Figure 4.9: Growth rate of *Vibrio* isolates at 1.0% NaCl

Values are expressed as average of 3 samples ± SE

b



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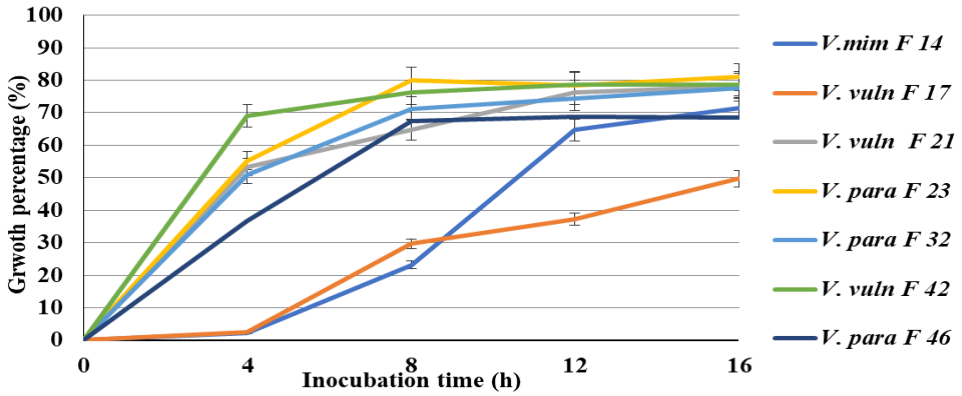

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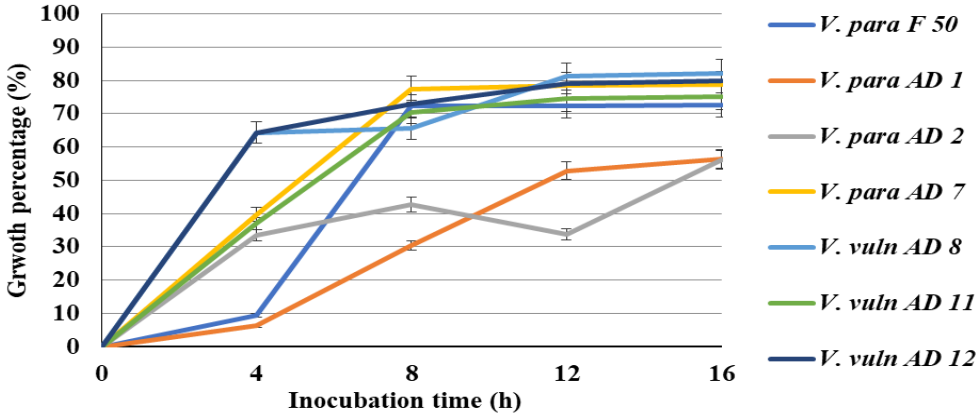


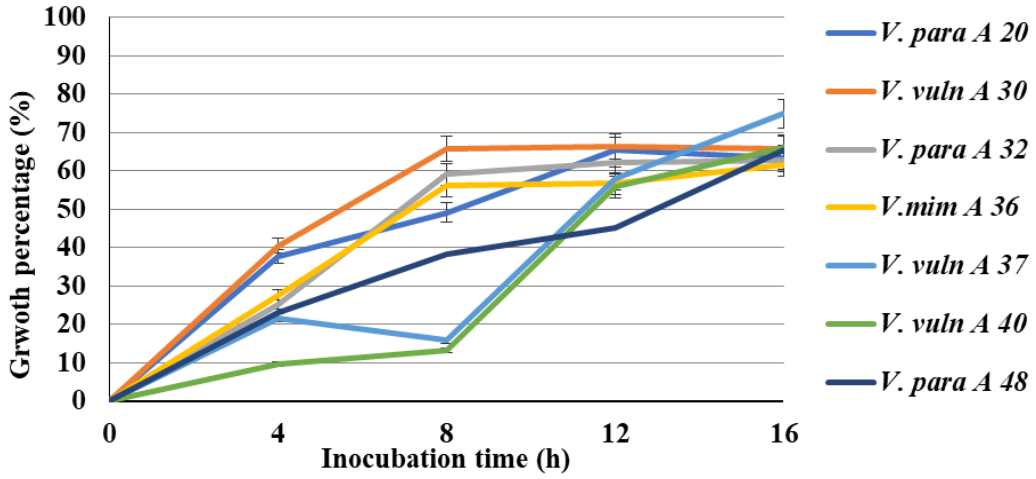
Figure 4.9: Growth rate of *Vibrio* isolates at 1.0% NaCl (Continued)

Values are expressed as average of 3 samples ± SE

4.4.3.3. Growth Rate of *Vibrio* Isolates at 2.0% NaCl

At 2.0% NaCl, *V. para* AD7 (Figure 36) attained growth rate of 87%, while *V.vulni* F21 (Figure 4.10c) showed 85% of growth rate on 16 h of incubation. The growth rate of *V.mimicus* was less at 2.0% NaCl ranging between 60 and 75%. *V.mimi* F4 (Figure 4.10b) showed a growth rate of 74%.

a



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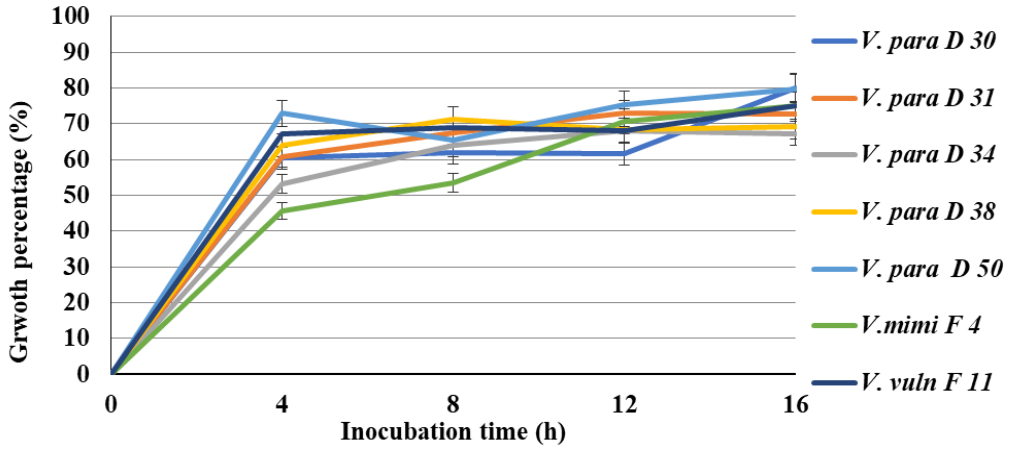
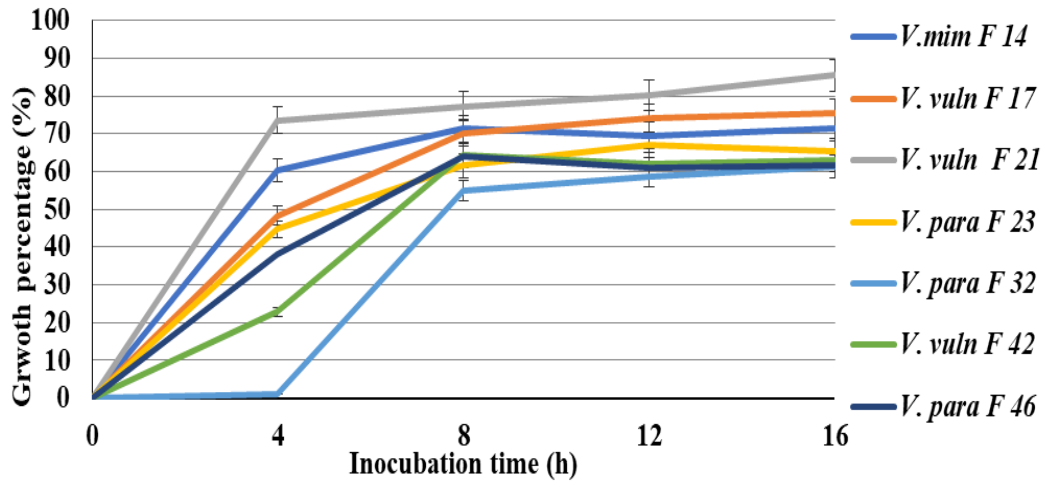


Figure 4.10: Growth rate of *Vibrio* isolates at 2.0% NaCl:

Values are expressed as average of 3 samples ± SE

c



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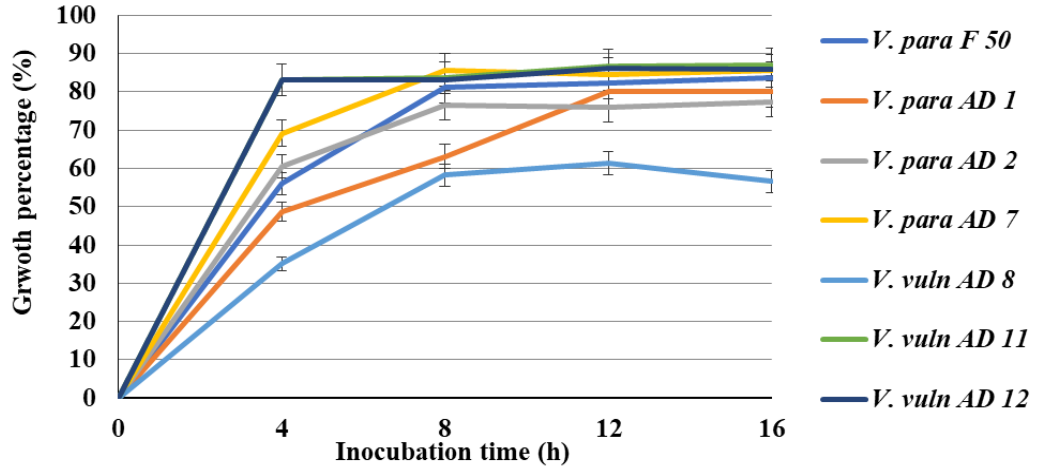

Figure 4.10: Growth rate of *Vibrio* isolates at 2.0% NaCl (Continued)

Values are expressed as average of 3 samples ± SE

Vibrio parahaemolyticus is moderately halophilic in nature and requires a minimum of 0.086 M (0.5%) NaCl for growth (Palasuntheram, 1981). Whitaker *et al*. (2010) reported that growth of *V. parahaemolyticus* in 1% NaCl was significantly less when compared to growth in 3% NaCl. The present study confirmed that increasing the concentration of NaCl results in an increase in the growth rate of *Vibrio spp.* which

was in agreement with the results of study conducted by Yoon *et al*. (2017) who established that *V. parahaemolyticus* and *V. vulnificus* were rapidly reached the viable-but-nonculturable (VBNC) state with increasing levels (≤30%) of NaCl. *V. alginolyticus* strains showed most favorable growth rate in a 3% NaCl solution, while the growth in a 6% solution was lower and the lowest growth was found in the 0.5% solution. (Farid and Larsen, 1981). *V. cholerae* and *V. Pelagius* were able to grow with cell densities ranging from 80%–100% of the maximum at intermediate concentrations (100–400 mM) of NaCl.

Chapter 5: Summary

In UAE, fish harvesting is a prevalent practice since the Emirates is connected to the sea. Trading of fish both locally and internationally has also increased due to new and modern fishing methods Thus, the *Vibrio* pathogen has a high likelihood of existing in the fish and its products, and this has caused a great concern internationally due to the export. Therefore, it is imperative to assess vibrio risk in the fish products in UAE.

The present study assessed the prevalence of *Vibrio spp.* in fish and fish products sold in UAE. Among 50 samples from different cities, the samples showed presence of *Vibrio*. The prevalence of *Vibrio* isolate in fish samples collected from Al-Ain showed that the incidence of 1 for *Vibrio mimicus* and 3 for each of *V. vulnificus* and *V.paraheamolyticus.* Results showed that in *Vibrio* isolates from Dubai, the prevalence for *V. paraheamolyticus* was 5 and 0 for *V. mimicus* and *V. vulnificus*. *Vibrio* isolates from Fujairah showed an incidence of 4 for *V. vulnificus* and 2 for *V. V.paraheamolyticus and V.mimicus*. The prevalence of *Vibrio* isolates in Abu Dhabi was 3 for *V. vulnificus* and *V.paraheamolyticus* and for *V. mimicus*.

The *Vibrio* isolates *V.parahemolyticus*, *V.vulnificus* and *V.mimicus* were resistant to penicillin G, daptomycin, vancomycin, ampicillin and erythromycin as evidenced by the results. 40% of *V.parahemolyticus* and *V.vulnificus* were resistant to sulfamethoxazole-trimethoprim while only 1% of *V.mimicus* were sulfamethoxazole-trimethoprim resistant.

The effect of temperature on survival and growth rate of the *Vibrio* isolates showed that a gradual increase in growth rate was observed in *V.parahemolyticus*,

V.vulnificus and *V.mimicus* during the incubation period at different temperature and growth rate attained maximum at 37°C. In the present study, results showed that when the pH of the system was increased from pH 3.0 to pH 5.0, the growth percentage was also increased. Most of the *V.parahemolyticus* and *V.vulnificus* attained a maximum growth rate of 80% at pH 5.0 on 16 h of incubation. At different salinity level, the growth rate and survival of *Vibrio spp.* was studied and results showed that the growth rate of *V.parahemolyticus* and *V.vulnificus* isolates were increased while increasing NaCl concentration from 0.5% to 2.0%.

Chapter 6: Conclusion and Recommendations

6.1. Conclusion

Fishes are a candidate vehicle for transfer of *Vibrio spp.* also these bacteria can survive in the gastrointestinal tracts of both human and animals. Rapid development of antibiotic resistance in bacteria and emergence of drug resistant microbial disease possess serious problems in environmental, economic and management and in addition create human health hazards.

The present study found that among the 129 *Vibrio* positive isolate in fish flesh imported from different markets, 15 isolates showed the presence of *Vibrio paraheamolyticus. Vibrio vulnificus* was present in ten isolates while three isolates showed the presence of *Vibrio mimicus*. The identified *Vibrio* isolates were more resistant to pencillin G, daptomycin, vancomycin, ampicillin and erythromycin. The *Vibrio* isolates were susceptible to sulfamethoxazole-trimethoprim. At 37°C, all the identified *Vibrio spp.* attained 80% growth rate. Incubation temperature of above 37°C is recommended. At higher temperature, the survival rate of *Vibrio spp.* will be reduced. Alkaline pH (pH 5 to pH 7.0) promotes the growth of *Vibrio* isolates. So acidic pH is suggested by this study, at acidic pH the survival rate of *Vibrio* will be less. The effect of different salt concentration on growth and survival of *Vibrio* confirmed that higher salt content increased the survival rate as evidenced by the study. NaCl concentration of less than 0.5% is recommended.

6.2 Recommendations

To the best of our knowledge, the study findings represents the first comprehensive report about the prevalence, antibiotic resistance profile, and antibiotic susceptibility of *Vibrio* isolates from fish samples in United Arab Emirates. The fish samples from different cities of UAE are contaminated with *V. parahaemolyticus*, *V.vulni*ficus and *V. mimicus spp*. All *Vibrio* isolates are highly pathogenic showing multiple drug resistance and are being potential to cause food borne illness thus posing risk to human consumers. The occurrence of pathogenic *Vibrio* isolates in fish samples requires extended surveillance across the UAE. Hence, continuous monitoring of *Vibrio* strains in food samples and their antibiotic susceptibility by food control authorities in UAE is necessary to ensure the best treatment for consumers to avoid diseases like gastroenteritis and thereby ensuring seafood safety. Also attention should be paid to farmers' markets and local fish markets. The simple and effective control of the pathogen by using effective antimicrobials is recommended as a better choice for avoiding *Vibrio* contamination in future risk assessment.

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Appendices

Appendix 1

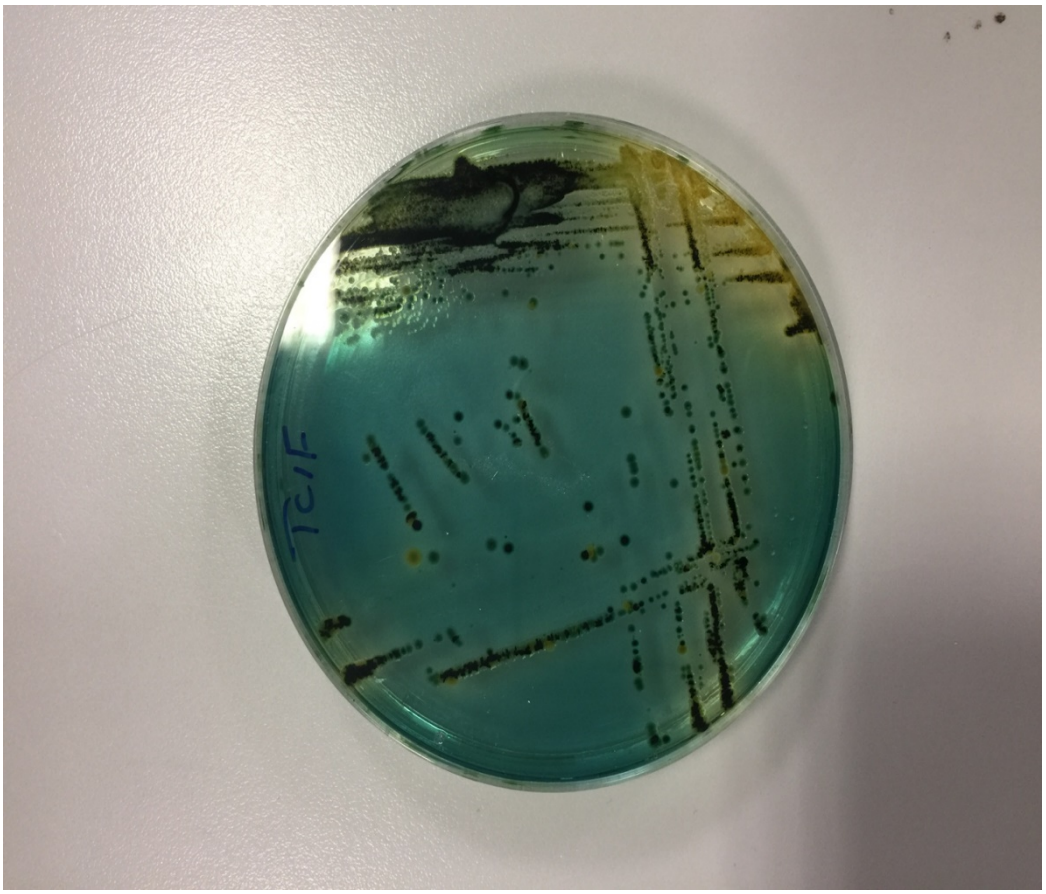

Figure A1.1: Culture plate of isolated *Vibrio spp.* in TCBS Agar

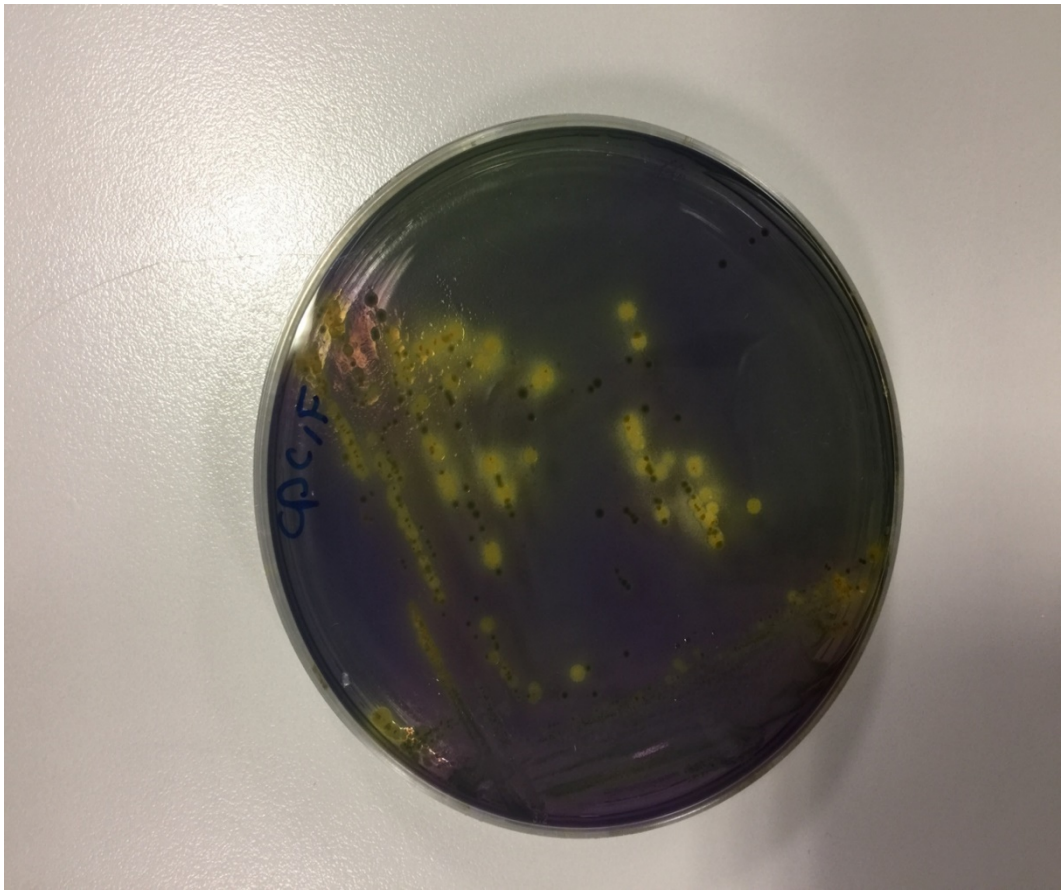

Figure A1.2: Culture plate of isolated *Vibrio spp.* in CPC Agar

Appendix 2

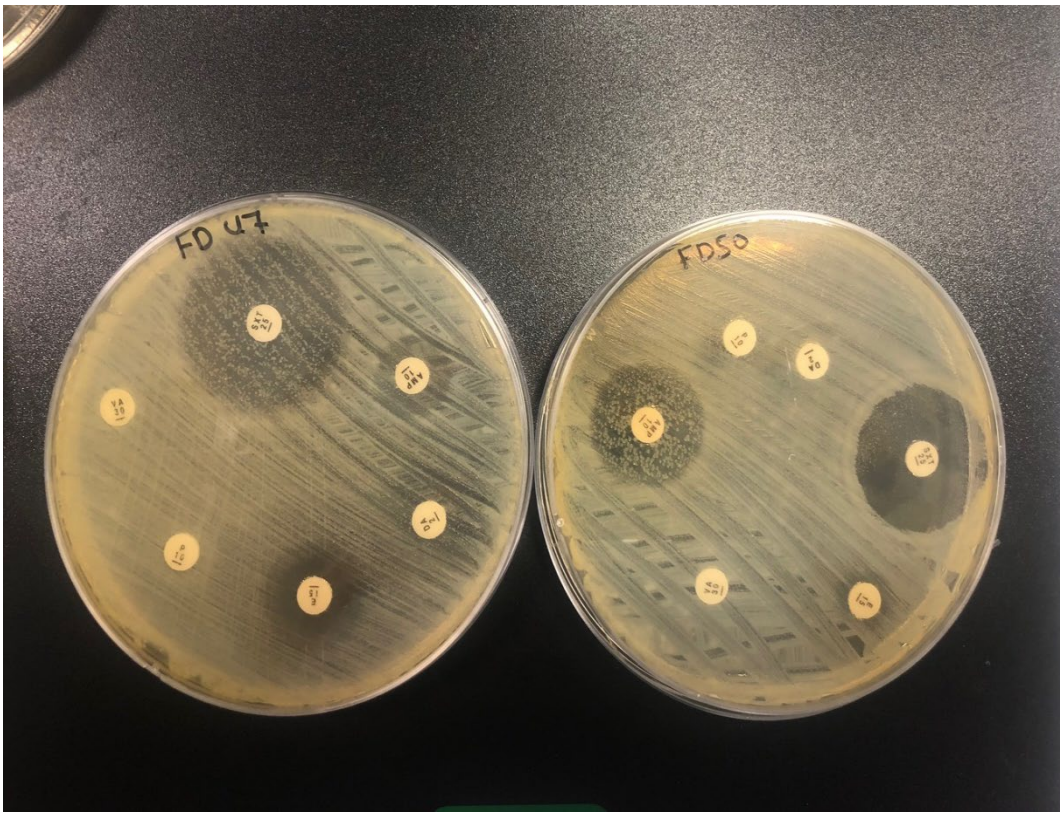

Figure A2.1: Antibiotic sensitivity of *Vibrio* for six different antibiotics

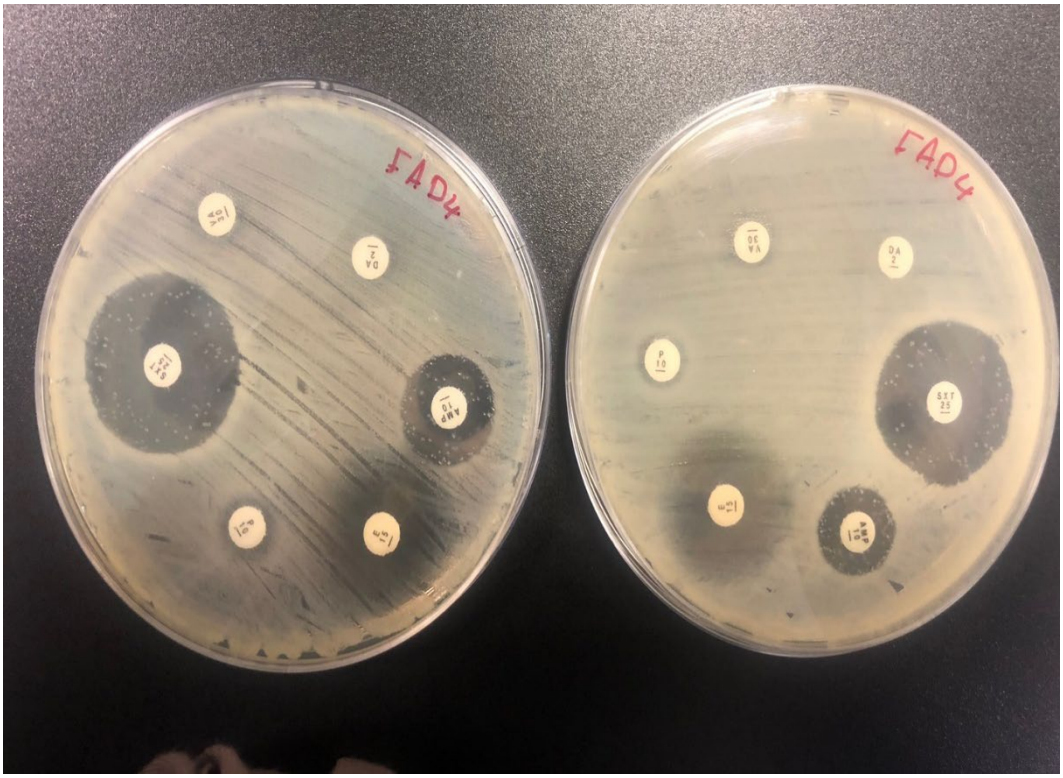

Figure A2.2: Antibiotic sensitivity of *Vibrio* for six different antibiotics

Appendix 3

Experienced User Protocol for DNA Isolation Kit Sample; DNeasy UltraClean Microbial Kit (Qiagen, Carlsbad, CA, USA)

1. Add 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x g for 30 seconds at room temperature. Decant the supernatant and spin the tubes at 10,000 x g for 30 seconds at room temperature and completely remove the media supernatant with a pipette tip. Note: Based on the type of microbial culture, it may be necessary to centrifuge longer than 30 seconds.

2. Resuspend the cell pellet in 300µl of MicroBead Solution and gently vortex to mix. Transfer resuspended cells to MicroBead Tube.

3. Check Solution MD1. If Solution MD1 is precipitated, heat the solution at 60°C until the precipitate has dissolved. Add 50µl of Solution MD1 to the Glass Micro Bead Tube.

4. Secure Micro Bead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

5. Make sure the 2 ml Micro Bead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at 10,000 x g for 30 seconds at room temperature.

6. Transfer the supernatant to a clean 2ml Collection Tube (provided).

7. Note: Expect 300 to 350µl of supernatant.

8. Add 100µl of Solution MD2, to the supernatant. Vortex for 5s. Then incubate at 4°C for 5 minutes.

9. Centrifuge the Tubes at room temperature for 1 minute at 10,000 x g.

10. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2ml collection tube (provided).

11. Shake to mix Solution MD3 before use. Add 900µl of Solution MD3 to the supernatant and vortex for 5 s.

12. Load about 700µl into the Spin Filter and centrifuge at 10,000 x g for 30 s at room temperature. Discard the flow through, add the remaining supernatant to the Spin Filter, and centrifuge at 10,000 x g for 30 s at room temperature.

13. Add 300µl of Solution MD4 and centrifuge at room temperature for 30 s at 10,000 x g.

14. Discard the flow through and centrifuge at room temperature for 1 minute at 10,000 x g.

15. Add 50µl of Solution MD5 to the center of the white filter membrane.

16. Centrifuge at room temperature for 30 s at 10,000 x g.

17. Discard Spin Filter column. The DNA in the tube is now ready for any downstream application. No further steps are required.

18. Storing DNA frozen (-20°C). Solution MD5 contains no EDTA.

Appendix 4

Gel electrophoresis images for virulence genes detection

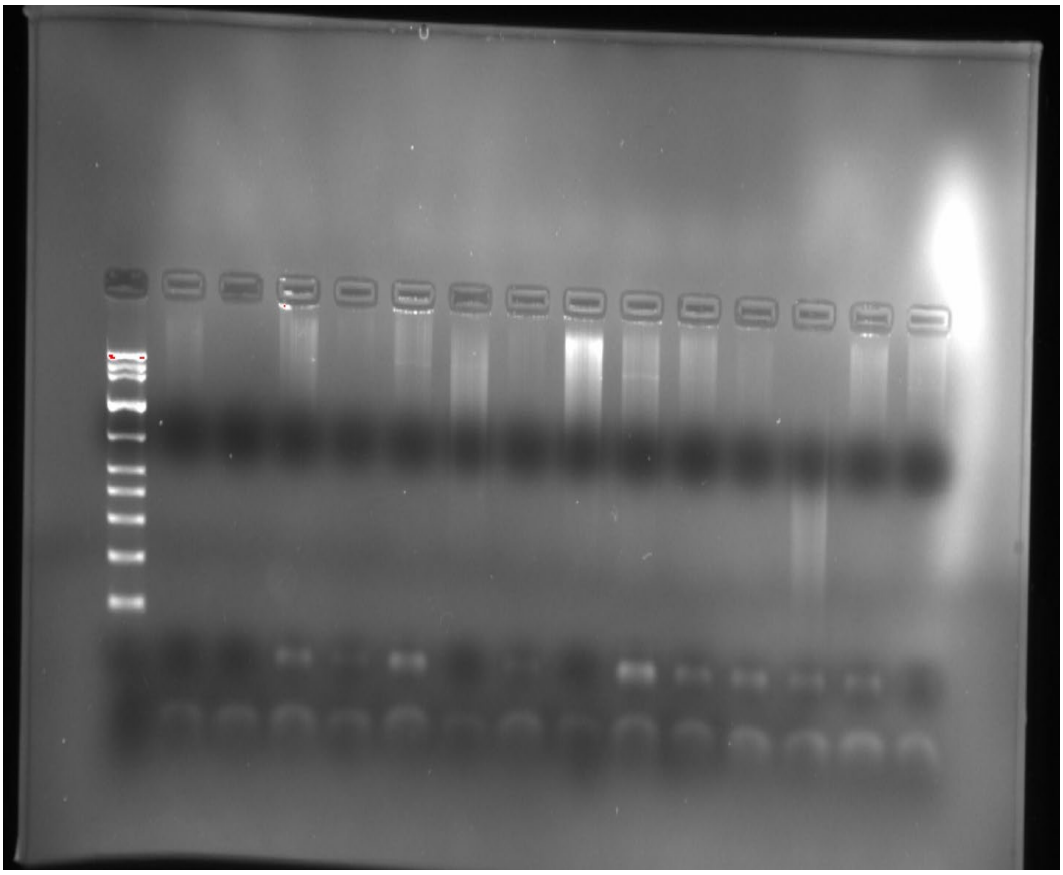

Figure A4.1: PCR product of amplified toxR gene of *V. parahaemolyticus*

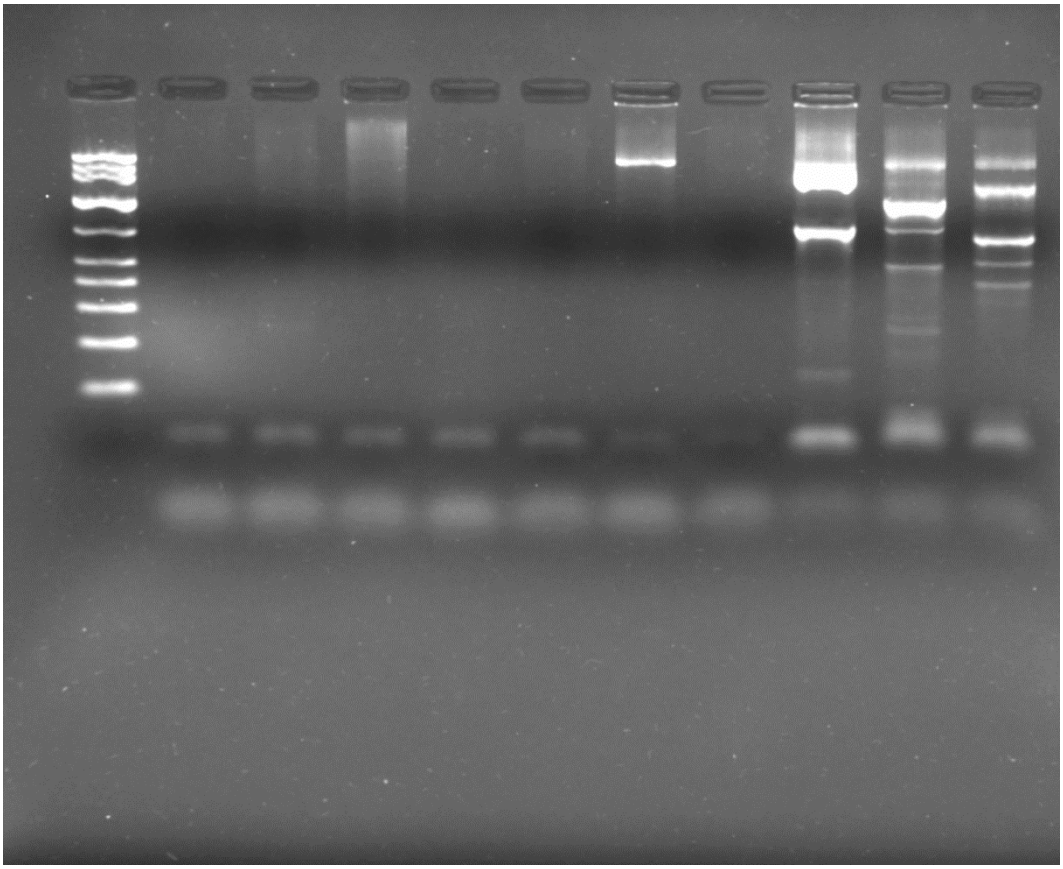

Figure A4.2: PCR product of amplified toxR gene of *V. Vulnificus*

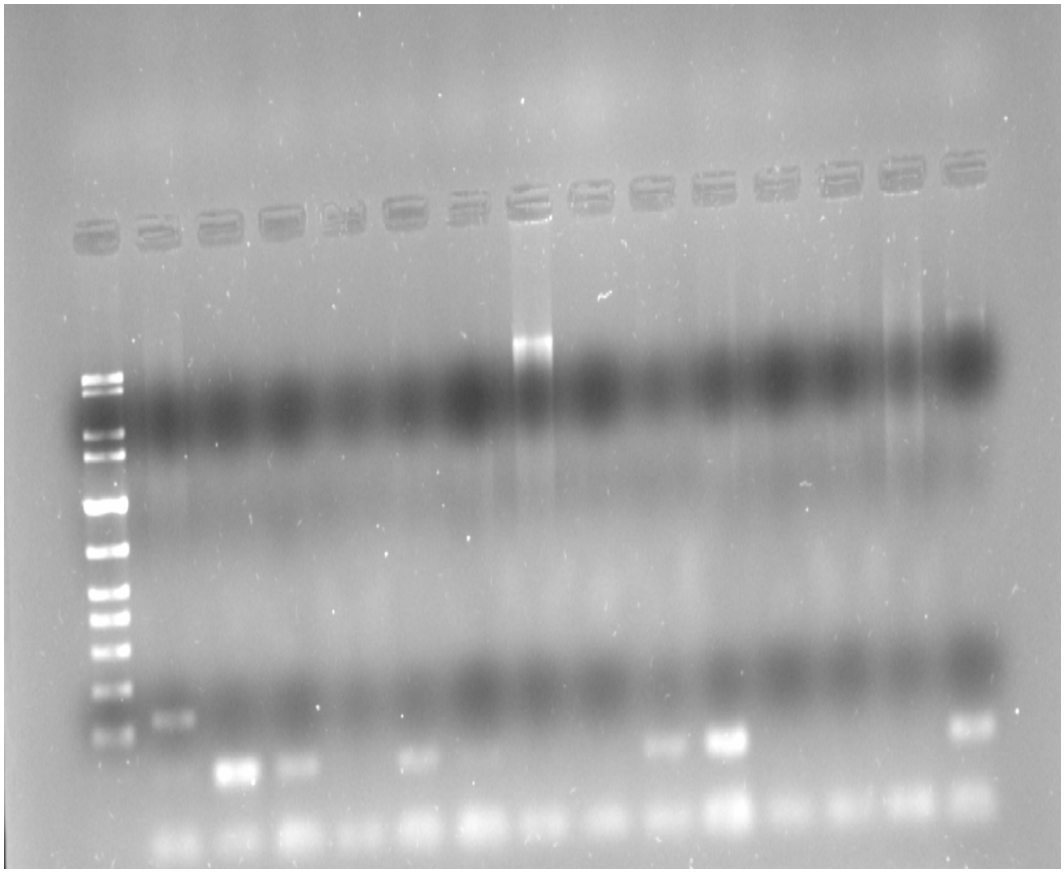

Figure A4.3: PCR product of amplified toxR gene of *V. Mimicus*