

Isolation and identification of bacterial contamination by phenotypic and genotypic methods in Artemia cysts and assessment of antibiotic susceptibility pattern of Vibrio species isolated in Artemia Research Center, Urmia

Yousefali Asadpour^{*1}, Ashkan Barzegar², Ehsan Soleimannezhadbari³ and
Amin Hashempour⁴

¹National Artemia Research Center, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Urmia, Iran

²Young Researcher and Elites club, Urmia Branch, Islamic Azad University, Urmia, Iran

³Young Researcher and Elites club, Urmia Branch, Islamic Azad University, Urmia, Iran

⁴Young Researcher and Elites club, Urmia Branch, Islamic Azad University, Urmia, Iran

Corresponding Email: asadnazlu@gmail.com

ABSTRACT

Shrimp farming is a sufficiently large and mature industry to have an effective range of antimicrobial agents for most bacterial diseases in shrimp culture. The objectives of this study were to investigate the occurrence of bacterial contamination by conventional and molecular techniques and assessment of antibacterial susceptibility of four antibiotic agents' ciprofloxacin, erythromycin, tetracycline and streptomycin by disc diffusion applied on them. Of 40 Brine shrimp (*Artemia urmiana*) samples analyzed 42.5% was recorded positive for vibrio Spp and 11%, 16.5%, 17%, 9%, 4% were positive for *Pseudomonas aeruginosa*, coccus spp, *E. coli*, *Bacillus cereus*, *Salmonella typhimurium* respectively. Of 17 (42.5%) vibrio spp 7 (41.17%), 4 (23.52%) and 6 (35.29%) identified as *V. parahaemolyticus*, *V. vulnificus* and *V. harveyi* respectively. Consequently of 17 isolates of vibrio spp 8 of them were resistant to all used antibiotic agents separately.

Keywords: Bacterial contamination, Vibrio species, Artemia cysts, Antibiotic susceptibility

INTRODUCTION

Propagation and breeding Artemia has a special and unique importance in developing and expanding of fishing industry especially in Propagation and breeding of shrimp, ornamental fishes, caviar fishes and all kinds of cold water and hot water fishes [1, 2]. Due to prohibition of harvesting Artemia cysts from Urmia Lake for provision of our country's extreme needs to this product, we are forced to seek another disposed areas and places to produce this product [1, 3]. Since the industry of propagation and breeding aquatic animals is largely use farmed Artemia cysts, due to lack of natural habitats and prohibition of harvesting them in nature, it is possible to transfer contaminations to aquatic beings that consume this farmed cysts and cause economic losses by hurting and killing them, determining contaminant bacteria in consumable Artemia cysts in aquaculture industry plays an important role. Because Artemia are used as an important and valuable food source in aquaculture and fishing industry and can transfer these contaminations through the aquatic animals [2]. Since the cysts nowadays are just bred in the pools and not harvested from nature they may have contaminations inside themselves and this contaminations can be transferred to the consumer aquatic animal and lead to their loss [3, 4].

Food security is a complex issue, where various factors pose a risk factor to fish food safety and they range from contamination from the environment where it is obtained to contamination by the consumer before eating [5]. Brine shrimp may contain many bacteria of public health importance like *Vibrio* species. Presence of such pathogenic bacteria in Brine shrimp beyond certain limits renders it unfit and dangerous for human consumption. Consumption of raw or undercooked seafood is the factor most commonly associated with infection [6]. Shrimps are one of the most important commodities of the global fishery trade [7]. As far as intensive shrimp culture goes, a large amount of shrimp food and antibiotics have been used to increase production and to protect shrimp from diseases [8]. One of the dangerous geniuses of pathogenic bacteria that cause disease and illness for shrimp is *Vibrio spp.* Vibriosis is a common disease of economically important marine invertebrates [9, 10]. The causative organisms are *Vibrio parahaemolyticus* in shrimp, oysters, mussels and other mollusks [11-13].

Infection is usually from exposure to seawater or consumption of raw or undercooked seafood [14, 15] Vibriosis is characterized by diarrhea, primary septicemia, wound infections, or other extra-intestinal infections [14-16]. Infection with pathogenic species of the family Vibrionaceae can cause two distinct categories of infection: cholera and vibriosis, both of which are nationally notifiable (site internet CDC). The development of antimicrobial resistance among pathogenic bacteria has emerged as a major public health concern, which has led to an intensification of discussion about the prudent use of antimicrobial agents, especially in veterinary medicine, nutrition and agriculture [17]. So the purpose of this investigation was to identify bacterial contamination by conventional and molecular methods in Brine shrimp cyst and also attempted to help the surveillance of the antimicrobial resistance by disc diffusion method in some of isolated bacteria.

MATERIALS AND METHODS

Sample collection and preparation

For this study, 40 *Artemia* cysts samples from brine shrimp farms of National *Artemia* Research Center timescales between May 1st and 30th of December 2015 were collected by using a 100 micron sterile mesh and were transported to the bacteriology laboratory of research center inside the containers.

Bacterial isolation

Cyst analyze

In this study samples of *Artemia* in their three stages (nauplius, larva and adult) were taken from *Artemia* husbandry pools of *Artemia* Research Center by using sterilized 100 micron laces and transferred into the central bacteriology laboratory inside sterilized sampling container. After transferring the samples into the laboratory, each sample was smashed separately inside the mortar and 1gr of the sample was put inside 9cc peptone water and the samples were placed inside incubator under 37°C degrees temperature for 24 hours. After completion of incubation, first of all, each of the samples were used in general culture of Mac Conkey agar. Then special culture of Muller Hinton Agar + 3% salt (NaCl) was used to study the growth of halophyte bacteria and principally *Vibrio*. After the completion of the preliminary stages, the sample was cultured and expanded on the media. After freezing and preparing the media in the plates, a certain number of the grown *Artemia* was separated and these samples were prepared and sieved using 150 micron screen. After separation, the same was smashed using the sterilized teance and an expansion of the smashed cysts on the special cultures were obtained, our expansion was conducted in the common method to prepare the standard linear culture and the samples were transferred into the incubator with 37°C temperature for growth and conducting further tests 6 [18]. In order to conduct the complementary tests for diagnosis purposes, the bacteria grown on the sample were Gram stained and other differential diagnostic tests were conducted as per the bacteriological standard methods, growth in special culture, hemolysis, gelatin and mobility, ureas, indole, catalase, MR, VP and bacterial shape [19].

For *vibrio* isolation and identification samples were smashed and added to alkaline peptone water (APW) containing 1 % NaCl (pH 8.6), and incubated at 37 °C for 6–24 h. Enrichment broth was streaked onto thiosulfate-citrate-bile salts-sucrose agar plates and incubated at 37 °C for 18–24 h. Colony morphology on TCBS agar was determined using API 20E (BioMérieux,-France) . The green or blue-green colonies were presumptively selected as *vibrio* colonies and transferred to trypticase soy agar plate containing %3 NaCl. After incubation at 37 °C for 24 h, the isolates were tested using conventional bacterial methods, including Gram's staining, culture sulfide indole motility and triple sugar iron tests media, cytochrome oxidase activity tests, lysine iron agar tests, urea tests, and tests for arabinose, lactose, mannitol, mannose, and sucrose fermentation [20].

DNA purification of specified *V. spp*

The exact identification of bacteria was done by polymerase chain reaction (PCR). Genomic DNA was prepared using a standard DNA extraction method and stored at -20°C. The purity of genomic DNA in each sample was evaluated by measuring optical densities at 260 and 280 nm wavelengths. The DNA concentration of each sample was adjusted to 50 ng μL^{-1} for PCR. Two sets of oligonucleotide primers were used for species-specific identification of *Vibrio* species. The PCR reaction was performed in a 50 μL reaction system consisting of 2 μL of purified genomic DNA (50 ng μL^{-1}), 5 μL of 10 \times PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 60 mM MgCl₂, 0.1% gelatin and 1% Triton X-100), 1 μL each of the primers (50 pmol μL^{-1}), 1 μL each of the 10 mM dNTPs, 0.2 μL units Taq DNA polymerase (5 units μL^{-1}) and 40 μL of sterile distilled water. The reactions were performed with a thermal cycler (Eppendorf, Germany) with the program described previously for the detection of *Vibrio* species [21-24]. [Table 1]

Table 1: The primer sequences, targeting genes and amplicon size are written in below in table

Sequence Target	Species (5'----- 3')	Amplicon size (bp)	Targeting gene	Reference
<i>V. parahaemolyticus</i>	GCAGCTGATCAAAACGTTGAGT ATTATCGATCGTGCCACTCAC	897 bp	flaE	[23]
<i>V. vulnificus</i>	GTCTTAAAGCGGTTGCTGC CGCTTCAAGTGTGGTAGAAG	410 bp	Hsp	[23]
<i>V. harveyi</i>	CTTCACGCTTGATGGCTACTG GTCACCCAATGCTACGACCT	235 bp	Vhh	[24]

All *vibrio spp* isolates were submitted to antibiotic susceptibility pattern tests by disk diffusion method [25, 26]. For the present study, antibiotics used in shrimp industry [27] and human clinical [28] were selected. The following antimicrobials (Luka) were tested: ciprofloxacin (CIP 5 μg), erythromycin (E 15 μg), tetracycline (TE 30 μg) and streptomycin (S 300 μg). For the antibiogram test, bacterial density was previously adjusted to a 10⁸ UFC mL⁻¹ concentration, by bacterial suspension in saline solution with 1% turbidity equivalent to the McFarland 0.5 Suspensions with standard densities were inoculated with swab in Petri dishes containing Mueller-Hinton Agar (Difco) medium with 1% NaCl, and then antibiotic disks (Luka) were applied. The plates were incubated at 35°C 24/h. The inhibition zone was measured (mm) and since there are no breakpoints defined for *Vibrio*, we use the zone diameter interpretive standards for Enterobacteriaceae cited by CLSI and company interpretation paper [11].

RESULTS**Table 2: Sample No and *Vibrio* species isolated from brine shrimps samples detected by PCR**

Vibrio species Sample No	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. harveyi</i>
2	+	-	+
3	-	+	-
5	-	-	-
10	+	-	+
14	-	-	+
23	+	+	+
24	-	-	-
25	+	-	-
29	+	-	-
31	-	-	-
35	-	+	-
40	+	-	-
43	-	-	+
47	-	-	-
49	-	+	+
50	+	-	-

In this study the investigation of *vibrio spp* and other bacteria conducted separately. First group of bacteria was investigated and isolated by conventional method and *vibrio spp* investigated by conventional methods and PCR. Although because of medical and pathogenic signification of *vibrio spp* antimicrobial susceptibility was just applied on them. By using of conventional and PCR method overall the average percentage and type of detected bacteria from general and specific mediums were 42.5% *Vibrio spp*, 11% *Pseudomonas aeruginosa*, 16.5% *coccus spp*, 17% *E. coli*, 9% *Bacillus cereus* and 4% *Salmonella typhimurium* (Fig 1).

A total number of 40 samples were studied and 17 samples (42.5%) contained one or two *Vibrio* species. In the present study, biochemical tests confirmed 17 green or blue-green colonies on TCBS agar as *Vibrio* positive samples. The molecular analysis carried out on the isolates gave positive results for all 17 strains using PCR assay. PCR products of 897, 410 and 235 bp were obtained for *V. parahaemolyticus*, *V. vulnificus* and *V. harveyi*, respectively. The specificity of the PCR products was confirmed by sequence analysis. According to the results, 7 samples contained *V. parahaemolyticus*, 4 contained *V. vulnificus* and 6 contained *V. harveyi*. [Table 2] and [Table 3].

Table 3: Susceptibility result of isolated vibrio specie against antimicrobial agents

V. spp Sample No	<i>V. parahaemolyticus</i>				<i>V. vulnificus</i>				<i>V. harveyi</i>			
	CIP	E	TE	S	CIP	E	TE	S	CIP	E	TE	S
2	-	-	-	-	-	-	-	-	S	I	S	R
3	-	-	-	-	S	I	S	S	-	-	-	-
5	S	S	I	S	-	-	-	-	-	-	-	-
10	S	S	S	S	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	S	S	S	R
23	S	S	S	I	-	-	-	-	-	-	-	-
24	-	-	-	-	S	I	S	I	-	-	-	-
25	S	S	S	S	-	-	-	-	-	-	-	-
29	S	S	S	S	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	I	S	R	R
35	-	-	-	-	S	S	S	S	-	-	-	-
40	R	I	R	S	-	-	-	-	-	-	-	-
43	-	-	-	-	-	-	-	-	I	I	S	S
47	S	S	S	S	-	-	-	-	-	-	-	-
49	-	-	-	-	S	R	R	I	S	S	S	S
50	S	S	S	S	-	-	-	-	-	-	-	-

I: Intermediate S: Sensitive R: Resistance

Of 17 isolates 14 (82.35%) were sensitive, 2 (11.76) were intermediate and 1 (5.88) was resistance ciprofloxacin, 11 (64.70%) were sensitive, 5 (29.41) were intermediate and 1(5.88) was resistance to erythromycin in addition 13 (76.47) were sensitive, 1 (5.88) was intermediate and 3 (17.64) were resistance to tetracycline although 11 (64.70%) were sensitive, 3 (17.64) were intermediate and 3 (17.64) were resistance to streptomycin. Furthermore major resistance appeared against streptomycin, tetracycline and minor appeared against ciprofloxacin and erythromycin.

DISUSSION

Shrimp is one of the most important fishery products of the coastal provinces at the Persian Gulf in Iran and other provinces those have potential of farming aquaculture. Whilst shrimp farming is a major economic characteristic of these provinces, a large part of the products are exported to other countries especially the European Union countries. Hygienic aspects of fishery industry have improved to the degree, which obtained HACCP certificate for their production. Nevertheless, seafood may be a vehicle for most of known bacterial pathogens [29].

Hameed and Balasubramanian also reported that 60% of the 336 isolates from *Artemia nauplii* were resistant to erythromycin, nitrofurazone and oxytetracycline. In the same study, the majority of isolates were resistant to several antibiotics used in human medicine and almost none to those employed in aquaculture [30].

In another study by Khamesipour et al aim was to detect the prevalence of *Vibrio* spp. in shrimp samples from farms in the southern provinces of Iran by polymerase chain reaction (PCR). A total number of 36 shrimp were caught from south coast of Iran and were studied to identify *Vibrio* spp. Three *Vibrio* species including *Vibrio parahemolyticus*, *V. alginolyticus* and *V. harveyi* were identified. A total number of 36 shrimps were studied and 7 samples (19.444%) contained one or two *Vibrio* species. In current study from 40 samples 17 samples contained one or two *Vibrio* species and it's done by biochemical and molecular methods [31].

The results of the study indicate that the tabulated bacteria are seen throughout the grown *Artemia* cyst. There is the possibility of infestation from consumable animal fertilizers and water and such infestation may be transferred into the consumer aquatic. Therefore, the most suitable solution is that the animal fertilizers used are exposed to direct sunlight for a long time and are mixed with 100Kg of lime powder per ton of animal fertilizer and is then used 32.

Considering the increasing consumption of Artemia and its importance in various aquatic larvae growing, in order to prevent the entrance of pathogenic agents into the larva growing systems in this manner certain scientific and healthy measures shall be taken, as the surface of the growing. May be one of the main factors in this regard, as various types of animal fertilizers such as animal and birds fertilizers are used in production of Artemia which is considered as one of the main sources of infestation. The results of this study indicate the existence of various types of infesting bacteria and are consistent with the results of previous research reported in aquatic husbandry industry [33, 34].

Acknowledgments

The authors would like to acknowledge the Dr. Ali Olfati (Chair man of this special issue, from University of Tabriz, A.olfati65@gmail.com; +98919 596 6273), thank you for keeping me on track, helping me to resolve issues and assisting me to get my dissertation completed.

REFERENCES

- [1] Agh, N., Noori, F. *Aqua Res.* **2011**.
- [2] Gunther, D.C., Catena, A. Belgium: Universa Press, **1980**.
- [3] Por, F.D. A classification of hypersaline waters, based on trophic criteria. *Mar Ecol*, **1980**.
- [4] Clinical Laboratory Standards Institute. Approved standard—9th Ed. CLSI document M2-A9. 26:1. Clinical Laboratory Standards Institute, Wayne, PA, **2006**.
- [5] A. Roque, A. Molina-Aja, C. Boln-Meja, and B. *International Journal of Antimicrobial Agents*, **2011**.
- [6] R. Helena Rebouças, O. Viana de Sousa, A. Sousa Lima, F. Roger Vasconcelos, P. B. de Carvalho, and R. H. S. *Environmental Research*, **2011**.
- [7] Van Stappen, G. Introduction, Biology and Ecology of Artemia. In: Manual on the production and use of live food for aquaculture. Lavens, P and Sorgeloos, P (Eds). FAO Fish tech paper, 1996.
- [8] Vieira, R. H. S. F. **1989**, 1: 1222-1272
- [9] Michel, C., Pelletier, C., Boussaha, M., Douet, D.G., Lautraite, A., Ailliez, P. *Appl. Environ. Microbiol.* **2007**.
- [10] Chan K.-Y., Woo M.L., Lam L.Y. & French G.L. (1989) *Vibrio parahaemolyticus* and other halophilic Vibrios associated with seafood in Hong Kong. *Jour Hosseini H., Cheraghali A. M., Yalfani R., Razavilar V., Food Control* **15**:187-190. *Journal of Applied Bacteriology*, **2004**, **66**, 57-64.
- [11] E.J.F. Risky Business: Vietnamese Shrimp Aquaculture Impacts and Improvements. London, UK7 Environmental Justice Foundation; **2003**, 44.
- [12] Maiti B., Shekar M., Khushiramani R., Karunasagar I., Karunasagar I., *J Genet*, **2009**, 88:273-279.
- [13] Butt, A.A. K.E. Aldridge and C. V. Sanders. *The Lancet Infect. Dis*, **2004**, 4: 201-212.
- [14] Bhaskar, N. T.M.R. Setty, G.V.S. Reddy, Y.B. Manoj, C.S. Anantha, B.S. Raghunath and M. A. Joseph. *Aquaculture*, **1995**, 138: 257-266.
- [15] Vanderzant, C. Nickelson, R. Parker, J.C. *Journal of Milk and Food Technology*, **1970**, 33, 161-162.
- [16] Egidius, E. A review. *Aquaculture*, **1987**, 67, 15-28.
- [17] Nolan, C.M. Ballard, J. Kaysner, C.A. Lilja, J.L. Williams, L.P. and Tenover, F.C. *Diagnostic Microbiology and Infectious Diseases*, **1984**, 2, 119-128.
- [18] Altekruse, S.F. Bishop, R.D. Baldy, L.M. et al. *Epidemiol Infect*, **2000**, 124:489-95.
- [19] Daniels, N.A. MacKinnon, L. Bishop, R. *J Infect Dis*, **2000**, 181:1661-6.
- [20] Nabi, A. Khalili, M.A. Halvaei, I. *Qom University of Medical Sciences Journal*, **2014**, 8(5): 54-67.
- [21] Léger, P. Bengtson, D. Simpson, K. et al. *Oceanography and Marine Biology*, **1986**, 24: 521-623.
- [22] Asadpour Y.A. *Iranian Fishery Sciences Research Center*, **2006**, 121.
- [23] Khamesipour, F. Khodadoustan Shahraki, A. Moumeni, M. Khadivi Boroujeni, R. Yadegari M. *Int J Biosci*, **2013**, 3(10):160-169
- [24] Hameed, A.S.S. Balasubramanian, G. *Aquaculture*, **2000**, 183:195–205.
- [25] Bauer, A.W. D.M. Perry, and W.M.M. Kirby. *A.M.A. Arch. Intern. Med*, **1959**, 104:208–21.
- [26] Hosseini, H. Cheraghali, A.M. Yalfani, R. Razavilar, V. *Food Control*, **2004**, 15:187-190.
- [27] Tarr, C.L. Patel, J.S. Pühr, N.D. Sowers, E.G. Bopp, C.A. Strockbine N. A. *J Clin Microbiol*, **2007**, 45:134-140.
- [28] Pinto, A. Ciccarese, G. Tantillo, G. Catalano, D. Forte, V.T. *J Food Prot* **2005**, 68:150-153.
- [29] Ausubel F.M. Brent R. Kingston R.E. Moore D.D. Sideman J. Smith J. Struhl K. Current protocols in molecular biology. Wiley, New York, **1987**.
- [30] Colakoglu FA, Sarmasik A, Koseoglu B. *Food Control* **17**:648–652.

- [31] Schilthuizen M. Springer Science and Business Media **2008**; 305.
- [32] Caprioli, A.L. Busani, J.L. Martel and R. Helmuth. *Int. J. Antimicrob. Agent*, **2000**, 14: 295-301.
- [33] Dechet, A.M. Yu, P.A. Koram, N. Painter, *J. Clin Infect Dis* **2008**, 46:970-6.
- [34] Abbaspour, M. Nazaridoust, A. *Int J Environ Stud*, **2007**.