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ORIGINAL ARTICLE

Pathogenic and non pathogenic *Vibrio* species in aquaculture shrimp ponds

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ABSTRACT. Most shrimp farmers try to control *Vibrio* population in their ponds regardless of their pathogenicity to human or shrimp health. This study focuses on the biochemical diversity of *Vibrio* species found in a typical semi-intensive shrimp pond in the Mexican Northwest and it correlates environmental conditions to pathogenic *Vibrio* species detected in pond water. Bacterial diversity changed with time, depending on water temperature. Such changes could be associated to dissolved oxygen, temperature and the pond itself. Growth conditions for pathogenic highly salt-tolerant *Vibrio* species, are particularly favorable during the harvest period. Operational Taxonomic Units (OTU) associated to *Vibrio parahaemolyticus* or *V. harveyi* increased their population from 3 to 460 MPN ml -1at harvest time, while *V. cholerae* similar OTUs were only detected at initial stages of the production cycle at extremely low levels.

Key words: *Vibrio*, shrimp, aquaculture, water.

RESUMEN. La mayoría de las granjas camaroneras controlan la presencia de bacterias del género *Vibrio* en sus estanques, sin considerar su patogenicidad al camarón o al humano. El presente estudio se enfoca a determinar la diversidad bioquímica de especies del género *Vibrio*, encontradas en estanques de cultivo semi-intensivo en el Noroeste de México y a correlacionar la presencia de especies patógenas a las condiciones ambientales de los estanques. A lo largo del ciclo productivo se observaron cambios en la diversidad bacteriana de los estanques de camarón vinculados a la temperatura. Estos cambios fueron asociados tanto a la temperatura del agua en los estanques de camarón, como a los niveles de oxígeno disuelto y estanque analizado. En la etapa de cosecha, las condiciones en los estanques resultaron especialmente favorables para el crecimiento de especies patógenas del género *Vibrio* altamente tolerantes a la sal. Las unidades operacionales taxonómicas (OTU) aisladas de las muestras, asociadas a las cepas control de *Vibrio parahaemolyticus*/*V. harveyi* incrementaron su población de 3 a 460 MPN ml -1 al momento de la cosecha, mientras que aquellas similares a *V. cholerae* fueron detectadas a muy bajos niveles, únicamente en las etapas iniciales del ciclo productivo.

Palabras clave: *Vibrio*, camarón, acuacultura, agua.

INTRODUCTION

Vibrio spp occur naturally in aquatic environments and are one of the most commonly-occurring bacteria during shrimp farming (Vandenberghe, et al, 2003). The number of reported *Vibrio* species has increased rapidly in the last decade. Thompson, et al (2004) have reported 63 environmental species comprising the genus *Vibrio*. Ten of them are of human concern (Twedt, 1989) since they have been associated with skin infections and severe gastrointestinal disorders (Andrews, 2004, Pérez-Rosas and Hazen 1998, Venkateswaran et al 1998). Some of the pathogenic *Vibrio* species have also been reported as the causal agents of shrimp infections (Goarant et al, 1999). In contrast, other species such as *Vibrio alginolyticus* have been reported as probiotics for shrimp aquaculture (Vandenberghe, 2003, Direkbusaram, et al, 1998).

Shrimp ponds are stressful environments compared to estuaries or other enclosed water bodies (Direkbusararam, et al, 1998). This is mainly due to high organic matter and dissolved oxygen fluctuations which affect the composition of natural bacterial communities. Under normal conditions, temperature increments will also bring about a greater diversity of *Vibrio* species (Barbieri, et al, 1999, Pfeffer, et al, 2003). When high temperatures and high salinity conditions prevail, some species such as *Vibrio parahaemolyticus* will predominate (Williams and LaRock, 1985).

Nowadays, shrimp farming has become an important economic activity in many developing countries (FAO/

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NACA/UNEP/WB/WWF. 2006). Recently, an increasing number of shrimp farms have raised concern about water quality and shrimp health because of their economic impact on farm operations. Most farmers now recognize the relevance of *Vibrio* species in shrimp ponds, and safety levels for viable heterotrophic bacteria (VHB) and *Vibrio* species are commonly used as environmental health indicators. Routine counts of those bacterial groups in water and shrimp hepatopancreas and hemolymph are common (López-Torres, 2001). The routine determination of *Vibrio* species in farms is quite useful in terms of pond management decisions and in some cases the use of antibiotics is based on those results. On the other hand, preventive measures often include the use of disinfectant and antimicrobial solutions which alter the dynamics of bacterial communities and may induce antibiotic resistance (Verschuere, et al, 2000). This is a worldwide problem and has been documented in several Mexican shrimp farms (Thompson, et. al, 2004).

Numerous studies of seasonal variation of pathogenic *Vibrio* species in natural environments can be found in the scientific literature (Williams, and LaRock, 1985; Venkateswaran, et al, 1989; Barbieri, et al, 1999; Pfeffer, et al, 2003; Hosseini, et al, 2004). Other studies refer to isolated strains from marine aquaculture systems (Gomez-Gil, et al, 1998; Vandenberghe, et al, 2003; George, et al, 2005), but there is a notable lack of information regarding changes of *Vibrio* species in aquaculture systems along the growout period. This information is critical from a food safety as well as from an economic impact perspective. Therefore, the aim of this work was to study the variations of *Vibrio* species throughout the growout production cycle in shrimp pond water and soil and its association with pathogenic *Vibrio* species.

MATERIALS AND METHODS

Water and soil samples were collected during the growout period, from seeding to harvest, in a shrimp (*Litopenaeus vannamei*) farm located in the coastal area of Hermosillo, Sonora, Mexico. Pond selection was performed on the basis of pond size and age, seeding time, initial larvae density, water recharge, and history of antibiotic usage. A total of three ponds (A, B and C) were chosen for sampling. Five periodic sampling periods (S1 to S5) were considered, starting two days after seeding the first pond. Three samples were collected from each pond at different locations: a) incoming water, collected right after the filter in the intake water supply; b) a composite water sample prepared from a mixture of bottom and surface water collected in the middle of the pond and water collected before the pond discharge outlet; and c) a soil sample, collected in the wet portion of the pond wall. All samples were collected in sterile plastic containers and transported to the laboratory in insulated boxes to maintain low temperatures (below 5°C). Samples were analyzed the same day they were collected by traditional culture methods. The remaining water samples were frozen for further analysis. Shrimp farm management provided access to daily monitoring records of temperature and dissolved oxygen (two daily measures) and the historical feeding records to document antibiotic usage.

Samples were analyzed based on the Bacteriological Analytical Manual for the identification of *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and other *Vibrio* spp. (Elliot et al, 1992). Tenfold dilutions in alkaline peptone water (APW) were prepared in order to estimate the MPN of pathogenic *Vibrio* species. Partial (6-8 hr) and complete (18-24 hrs) incubation at 35-37°C was carried out before transferring to TCBS (thiosulfate-citrate-bile salts-sucrose, Difco) agar plates. All yellow, green, and blue green colonies were selected for biochemical characterization (0%, 3%, 6%, 8% and 10% NaCl growth, motility, indole production, lysine and ornithine decarboxilase, sucrose, glucose and fructose fermentation, arginine dihydrolase, oxidase and gelatinase). The following CAIM (Collection of Aquatic Important Microorganisms) strains were included in the analysis: CAIM320-V. *parahaemolyticus*; CAIM512-*V. harveyi*; CAIM516-*V. alginolyticus*; CAIM593-*V. fluvialis*; CAIM 602-*V. mimicus*; and CAIM610-V. *vulnificus*. Also a *V. cholerae* non O1 strain, provided by the Mexican Health Department was used.

Additionally, DNA extracted and purified from water samples was tested for *V. cholerae* and V. *parahaemolyticus* toxins gene using PCR. DNA extraction was conducted based in the Marmur Protocol (Johnson, 1991) and Barns et al (1994) with some modifications. Thawed samples were vigorously mixed to allow homogenization. Triplicates of 100 ml were centrifuged (15 min @ 3,000g), and the residue was washed with 10 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0), and centrifuged under the same conditions; re-suspended with 1 ml of buffer solution (500 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM sodium citrate) and the presence of lysozyme (15 mg, Sigma) and mutanolysin (4 U Invitrogen). The mixture was incubated for 18 - 24 hours at 37°C. Proteinase K (2 mg, Invitrogen) was added and incubated at 50°C for 30 min. One volume of lysis solution (200 mM Tris HCl pH 8.9, 100 mM NaCl SDS 8% and PVPP 1.5%) was added and mixed by inversion. The mixture was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol:vol), mixed by inversion and centrifuged at 10,000 g for 5 minutes. One volume of cold isopropyl alcohol was added,

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Table 1. Fluctuation of the three pond conditions during the experiment.

Pond	Sampling	Temp (°C)	DTF (C°)	DO (mg/l)	DDOF (mg/l)
A, B and C	S1-April	19.8–19.9	3.6–4.5	5.8–5.9	0.3-0.7
	S2-May	22.9–23.1	2.6-4.8	7.2–7.4	1.2-1.4
	S3-June	26.4-26.5	2.2-2.5	6.0–6.1	1.9 -2.0
	S4-July	29.2-29.3	1.9–2.0	3.9-4.0	1.9-2.1
	S5-Sept	31.0–31.1	1.9–2.0	3.0-3.3	1.5-2.0

DTF=Daily temperature fluctuation; DO=Dissolved oxygen; DDOF=Daily dissolved oxygen fluctuation

mixed and kept at -20°C overnight. Cold mixture was centrifuged at 13,000 rpm for 20 min and supernatant discharged, before 50 µl RNAse – TE (2 mg/ml) was added. Extraction was visually corroborated by gel electrophoresis in 1% agarose gel. PCR amplification was done in a thermal cycler (Perkin Elmer, DNA Thermal Cycler 480) under the following temperature profile: 96°C–6 min; 30 cycles: 94°C-1min, 50°C–1 min, 72°C-1 min; 72°C 5 min., using a 50 µl volume reaction (4 mM dNTPs, 50 pg primer, 2 mM $MgCl_2$, 5 µl buffer 10X, 2 U Taq polymerase (promega) and 5 µl template). Specific primers for tdh^+ *V. parahaemolyticus* (648R: 5´-GCT CTT AGC TGC GGC GGT GGT-3; 648F: 5´-CTG TCC CTT TTC CTG CCC CCG–3´) and ctx *V. cholerae* toxin (384R: 5´-gCA CCC CAA ATA gAA CTC gA-3; 384f: 5´-Cgg gCA gAT TCT AgA CCT TC-3´) were used.

Statistical analysis included principal component analysis (PCA) and cluster analysis. Pond conditions and biochemical responses were combined for the PCA analysis using the Unscrambler® v9.2 (1996-2005 CAMO PROCESS -DEMO). Dendrograms were obtained from the cluster analysis of biochemical responses using Bionumerics® - Version 3.0 (Applied Maths).

RESULTS

According to farm records, no antibiotic use was detected in any of the ponds under study, but lime (CaO) was used periodically (7 to 10 days, 50kg/ha) as a pond disinfectant. Variations in pond temperature and dissolved oxygen during the sampling period are summarized in Table 1. Those variables were monitored twice a day by the farm, and daily fluctuations were calculated. Average values were considered in the statistical analysis.

A total of 939 isolates from TCBS agar from 45 pond samples and control strains were biochemically characterized (data not shown). Loadings and scores from the PCA analysis considering pond conditions and biochemical responses are shown in Figure 1. Groups were obtained based on those variables with high correlation loading.

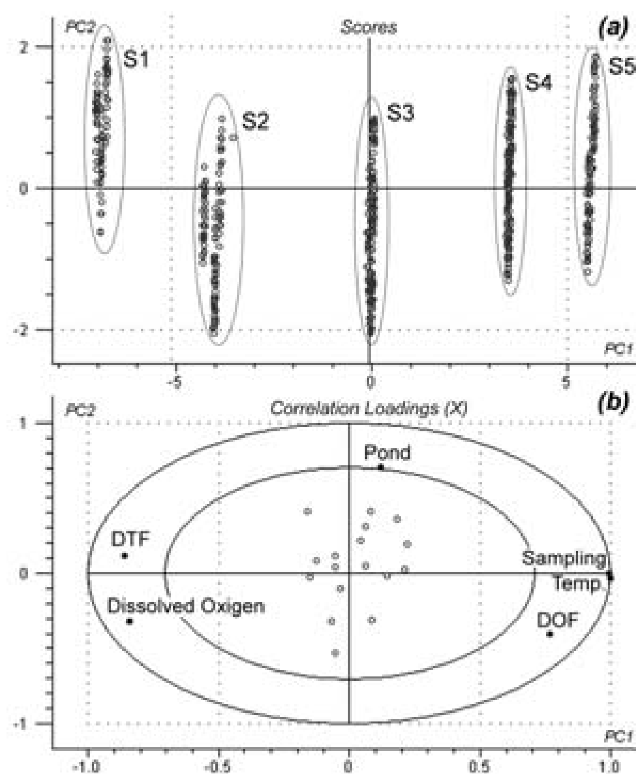

Figure 1. Principal component analysis (PCA) scores and loadings for PC1 and PC2: a) scores for samples grouped by average temperature and sampling time (S1 to S5), b) correlations loadings (DTF = Daily Temperature Fluctuation, DOF = Daily Dissolved Oxygen Fluctuation), shows OTUs variability highly influenced by temperature, dissolved oxygen and their fluctuations.

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Considering those results, cluster analysis was performed by pond and sampling time. Dendrograms obtained from cluster analysis of pond A at each sampling time (S-1 to S-5) are shown in Figure 2. Similar patterns were found for ponds B and C (not shown).

OTUs (Operational Taxonomic Units) which present 100% similarity with control strains in cluster analysis

were considered as presumptive pathogenic *Vibrio* species and MPN ml^{-1} calculated (Table 2).

A full cluster analysis including all 939 OTUs and control strains was obtained. Sixty-six subgroups of at least four OTUs with 100% similarity were identified. Those subgroups were associated at 60% similarity with the control strains and nine groups were observed (Figure 3). Major groups and subgroups present along the sampling periods are shown in Table 3.

PCR results did not detect *V. cholerae* (ctx) in any of the DNA extracts analyzed in water samples. *V. parahaemolyticus* (tdh+) was detected at pond C at S4 in composite water DNA extract (data not shown).

DISCUSSION

Water temperature increased gradually up to 30°C, while the daily average temperature fluctuations decrease resulting in a more stable temperature along the day (Table 1), which favored bacterial growth. Dissolved oxygen drastically dropped from S3 to the end of the sampling period, but the daily fluctuation increased favoring the development of facultative bacteria, capable of adjust to very low oxygen levels. Both conditions in conjunction with lime applications resulted in very stressful environment. Furthermore, the bacterial population dynamically adapted to the evolving conditions and their relative abundance changed over time giving rise to a new bacterial community profile (Figures 1a and 2).

Water temperature was negatively correlated with dissolved oxygen and average temperature daily fluctuation (Figure 1b). A positive correlation between *Vibrio* species and water temperature has been documented (Bariberi, et al, 1999; Pfeffer, et al, 2003); furthermore, temperature has shown a positive correlation with the presence of the cholera toxin gene (*ctx*) (Huq, et al, 2005). Pfeffer et al (2003)

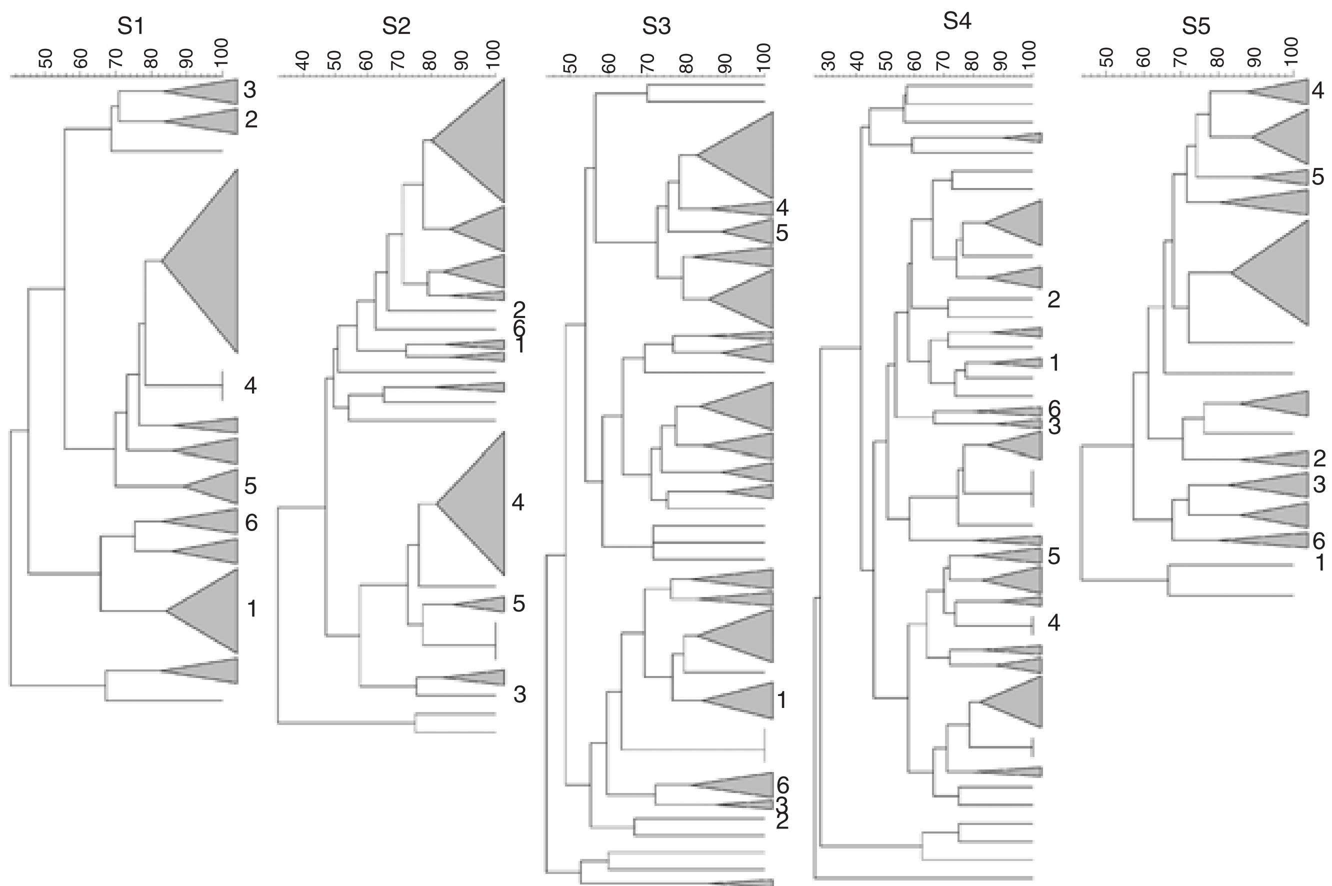

Figure 2. *Cluster analysis for water and soil OTUs found in pond A at different sampling times (S1-S5). OTUs are grouped based on 60% similarity, where 1:* V. fluvialis *group, 2:* V. vulnificus *group, 3:* V. mimicus *group, 4:* V. parahaemolyticus–V. harveyi *group, 5:* V. alginolyticus *group, 6:* V. cholerae *group.*

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found positive correlations for *Vibrio* spp. and water temperature and negative one for dissolved oxygen in estuarine waters, considering water temperature the most highly correlated variable to its abundance.

Results showed that temperature, sampling time and dissolved oxygen were the principal ambient conditions affecting OTUs distribution (Figure 1b). The combined effect of these variables can explain the bacterial population change throughout the growout period. The largest bacterial diversity for *Vibrio* species was found at the middle stage of the study. Once the new environmental conditions set in, diversity tends to decrease. Dendrogram comparison for all sampling periods (Figure 2), clearly show that at the earliest stages, suspected *Vibrio* populations were represented in a few large groups. By S3 the temperature rose about 6°C and population diversity increased substantially. From this point on, daily temperature fluctuations were the lowest and bacterial population stabilized. S4 results showed practically the same high bacterial diversity pattern than S3, which then diminished at the harvest period (S5), where less but more abundant groups were present. Seven of the ten reported *Vibrio* of concern to human health (Twedt, 1989), were used as control strains in this study. Five of them (*V. cholerae* non O1, *V. mimicus*, *V. alginolyticus* and *V. parahaemolyticus* - *V. harveyi*), were associated at 100% similarity with isolated OTUs (Table 2). The major factors that affect their distribution were temperature and salt tolerance. The most abundant group was that comprised by OTUs similar to *V. alginolyticus*, followed by those to *V. cholerae*, even though that PCR results were negative for *cholerae* toxin gen. Pérez-Casas and col. (2005) did not find the *V. cholerae*, toxigenic agent from *Vibrio* isolates in Mexican shrimp. Two of the control strains, *V. vulnificus* and *V. fluvialis*, were not similar to any OTU in this study, but *V. fluvialis* group was one of the major groups present at the initial stage (Figure 2). Low salinities and high temperatures have been associated to *V. vulnificus* in shrimp shell (Yano, et al, 2004).

Similar OTUs to *V. cholerae* and *V. mimicus* control strains, which are less salt tolerant (up to 3 and 6% respectively), were found only at S1, at the lowest recorded temperature (around 20°C). However, *V. mimicus* was identified in all periods using the Alsina and Blanch (1994) biochemical identification scheme. This difference could be explained by the biochemical variability in arginine dihydrolase and salt tolerance reported (Alsina and Blanch, 1994). Salinity and temperature have been reported as critical for *V. cholerae* in estuaries, with the best recovery rate between 16 and 24°C, and none at temperatures higher than 35°C (Williams and LaRock, 1985). On the other hand, *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus*, which are more salt tolerant could be found at the end of the sampling period (S4 and S5). No difference between

Table 2. Positive samples for pathogenic *Vibrio* species (100% similarity with control strain), sampling period where they were found, and estimated MPN ml^{-1}.

		V. cholerae	CAIM 602	CAIM 593	CAIM 516	CAIM 320 or 512	CAIM 610
Salt tolerance		3%	6%	6%	10%	8%	6%
Pond A	S1	1	1	—	—	—	—
	S2	—	—	—	—	1	—
Sub total		1	1	—	—	1	—
MPN ml^{-1}		43 – 3	3.6	—	—	43 – 3	—
Pond B	S1	1	1	—	—	—	—
	S2	—	—	—	—	2	—
	S4	—	—	—	1	—	—
	S5	—	—	—	—	1	—
Sub total		1	1	—	1	3	—
MPN ml^{-1}		43 – 3	3.6	—	43 – 3	9.2 – 3.6	—
Pond C	S1	—	—	—	1	—	—
	S2	—	—	—	—	2	
	S4	—	—	—	—	1	—
	S5	—	—	—	—	1	—
Sub total		—	—	—	1	4	-
MPN ml^{-1}		—	—	—	3.6	3 – 460	—
Total		2	2	—	2	8	—

— = Not similar OTU founded; CAIM320 = *V. parahaemolyticus*; CAIM512 = *V. harveyi*; CAIM516 = *V. alginolyticus*; CAIM593 = *V. fluvialis*; CAIM 602 =*V. mimicus*; CAIM610 = *V. vulnificus*; *V. cholerae* = *V. cholerae* no 01, reference strain from the Mexican Health Department

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V. *parahaemolyticus* and *V. harveyi*, could be detected with the biochemical tests performed in this study, as they were always grouped in the same cluster, nevertheless the presence of V. *parahaemolyticus* was confirmed by PCR at S4. Minimal water temperature for recovery of these bacteria has been reported at 27°C in estuaries (Williams and LaRock, 1985). A total of thirty-one OTUs were clustered with this pathogenic bacteria group and eight of them did not show differences in biochemical responses with the control strain used (100% similarity), but only three of them were found at S4 were PCR confirmed its presence and the temperatures were higher than 27°C. Even when the V *parahaemolyticus*-*V. harveyi* group was not as abundant as the *V. cholerar* or *V. alginolyticus* groups, this group encloses the major subgroup clustered at 100% similarity with control strains. Those suspected pathogenic *Vibrio* species were well distributed throughout the growout period and were found in water and soil samples. Most pathogenic *Vibrio* associated OTUs were present at low and stable concentration along the sampling period, except for *V. parahaemolyticus*-*V. harveyi* group, which increased drastically their estimated MPN ml^{-1} (from 3 to 460) at the harvest stage (S5). As indicated before, the presence of *V. parahaemolyticus* was confirmed by PCR only at S4. This species was not a major part of the total population, but it was the biggest pathogenic subgroup found. Its importance stems from its ability to affect human health since has been associated in up to 70% of the seafood gastroenteritis cases in Japan (Deepanjali et al, 2005).

Even when *V. alginolyticus* was found to be the major group (31 subgroups) (Figure 3) followed by those similar to *V. cholerae* (18 subgroups), only two fully similar OTUs were found. This could be explained by the high level of genetic variation reported among these bacteria in aquacultured shrimp (George et al, 2005). *V. alginolyticus* has also been reported as one of the main isolated *Vibrio* species in shrimp and shellfish (Hosseini et al, 2004; Parisi et al, 2004) at retail level in wild and aquacultured shrimp (Elhadi, et al, 2004). This human and shrimp pathogenic bacterium has been tested as a probiotic against other *Vibrio* species that can infect farmed shrimp (Sotomayor and Balcázar, 2003). Its variability and inhibitory effect against other *Vibrio* species could affect the high subgroup abundance, compared with all identified groups.

Seven subgroups were detected in all the sampling periods, all of which belong to those two more abundant groups (Table 3). Most of them, were highly salt tolerant. Subgroups that were detected at the beginning but disappeared in further samplings, also showed high salt tolerances except for those that were not detected or present in very small groups after S3. Also, those subgroups that were not present at initial stages but detected in later samplings, including harvest (S5), were highly salt-tolerant.

It becomes clear that pond conditions in shrimp farms may support the survival and growth of high salt-tolerant pathogenic *Vibrio* species, such as *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus* and potentially *V. cholerae*. Genotype studies have shown that *V. cholerae*, *V. vulnificus* and *V. alginolyticus*, do not always express the virulence factors in their natural environment. Baffone *et al* (2000) found *V. alginolyticus* as the most common patho-

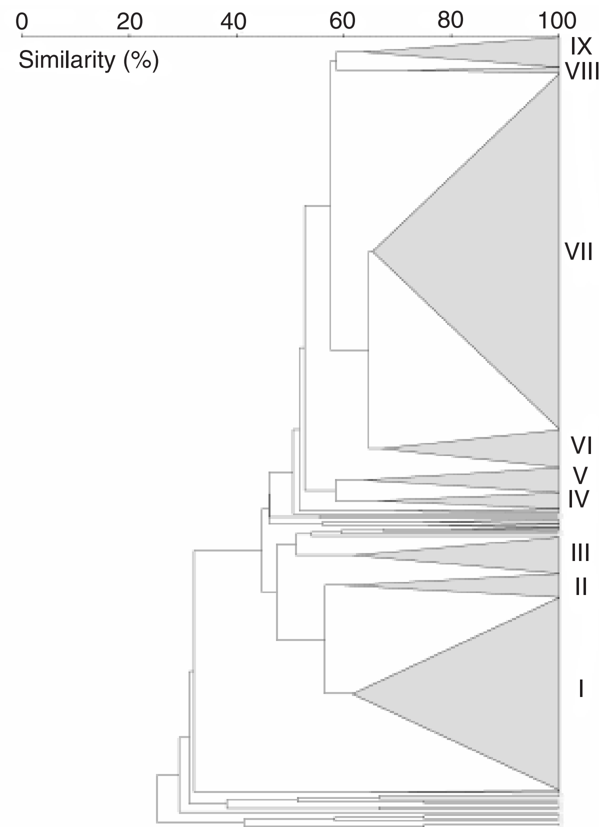

Figure 3. *Dendrogram for all OUT's found at different sampling time at three shrimp ponds. Groups were formed around control strains using at least a similarity value of 60%, where I =* V. cholerae *group, includes 18 subgroups (similarity 61.6%); II =* V. vulnificus *group, includes 3 subgroups (similarity 60.1%); III =* V. fluvialis *group, includes 3 subgroups (similarity 61.8%); IV = Unknown group, includes 2 subgroups (similarity 66.3%); V =* V. mimicus *group, includes 3 subgroups (similarity 63.7%); VI =* V. parahaemolyticus -V. harveyii *group, includes 5 subgroups (similarity 67.0%); VII =* V. alginolyticus *group, includes 30 subgroups (similarity 65.4%); VIII: Unknown group, includes no subgroups, only single OUT's (similarity 71.9%); IX = Unknown group, includes one subgroup (similarity 63.7%).*

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Table 3. Main subgroup variation whit yime, their group association and salt tolerance

Subgroup	No. OUT	Group	SALT	Sampling
Subgroups found throughout the sampling period				
B	71	*V. alginolyticus*	3 – 10	S-1 to 5
D	46	*V. alginolyticus*	3 – 10	S-1to 5
E	21	*V. alginolyticus*	0 – 10	S-1to 5
K	59	*V. cholerae*	3	S-1to 5
L	39	*V. cholerae*	3 – 6	S-1to 5
N	14	*V. cholerae*	3 – 6	S-1to 5
Subgroups that dissappear throughout the sampling period				
A	35	*V. alginolyticus*	0 – 10	S-1to 4
M	20	*V. cholerae*	0 - 3	S-1- 4
U	3	*V. cholerae*	0 - 3	S-1
44	5	*V. mimicus*	3 – 6	S-1 to 4
55	10	*V. cholerae*	0 – 3	S-1 to 3
W	4	Unknown	0 – 10	S-1 to 3
Subgroups that appear throughout the sampling period				
F	32	*V. alginolyticus*	3 – 10	S-2 to 5
G	29	*V. alginolyticus*	3 – 10	S-3 to 5
H	13	*V. parahaemolyticus*/*V. harveyi*	3 – 8	S-2, 4, 5
48	6	*V. fluvialis*	0, 3, 8	S-2 to 3
54	6	*V. cholerae*	3 – 6	S-2 to 4
61	12	*V. cholerae*	3 - 6	S-4 to 5

genic *Vibrio* in fresh seafood, but only 3 of 24 strains shown cytotoxic effects. Rivera et al (2001) isolated *V. cholerae* and concluded that in spite of the fact that those strains may not have their entire toxigenic factors; some environmental changes may trigger their virulent potential. If pathogenic *Vibrio* species are present in shrimp pond water, it is likely that they could also be present in the shrimp itself, with the implicit consumer health risk, particularly in regions where raw shrimp is consumed. They can also become a major vector for cross contamination when not properly handled.

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