Environmental Research 111 (2011) 21–24

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Antimicrobial resistance profile of *Vibrio* species isolated from marine shrimp farming environments (*Litopenaeus vannamei*) at Ceará, Brazil

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ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form
24 July 2010
Accepted 27 September 2010

Keywords:
Vibrio sp.
Multiresistance
Antibiotics
Plasmids
Aquaculture

ABSTRACT

Brazilian shrimp culture industry has a great economic importance mainly to the northeast region. However, the accelerated development of this activity has resulted in the emergency of outbreaks of diseases from farming shrimp, and as a consequence the use of antimicrobial drugs to minimize the potential adverse effect under the shrimp production. The inappropriate use of antibiotics in aquaculture is one of the causes for the high incidence of antimicrobial resistant bacteria isolated from aquatic environments that represent a danger for aquatic organisms and human health. There is little information available on the level of antimicrobial resistance in pathogenic bacteria from shrimp farming environment. Therefore, this study aimed to evaluate the phenotypic resistance profile among *Vibrio* isolates from hatcheries water samples and from cultivated marine shrimp hepatopancreas (*L. vannamei*). Antimicrobial susceptibility testing was carried out by a standard disc diffusion method and the minimum inhibitory concentration (MIC) of oxytetracycline (OTC) for resistant *Vibrio* isolates was determinate by broth dilution method. The results showed a high incidence of resistance to ampicillin (45.2%) and to the tetracycline class (38.7%). Florfenicol and nitrofurantoin were 100% effective against *Vibrio* isolates. In this study, the OTC-resistant *Vibrio* spp. showed MIC values of more than 400 mg/L and the presence of seawater did not influence the oxytetracycline bioactivity. The occurrence of antimicrobial multiresistance patterns was observed in 29% of *Vibrio* isolates. Fifty-five percent of multiresistant isolates of *Vibrio* lost one or more antibiotic resistance phenotype after procedure to curing of resistance plasmids. The oxytetracycline resistance was the phenotype most often lost among plasmid-cured isolates.

1. Introduction

Large-scale marine aquaculture has been associated with environmental issues worldwide as a consequence of accelerated development and high stocking density. In marine shrimp farming this is particularly evident in the disquieting increase in disease incidence as shrimp farmers around the world are coping with extremely damaging virus epidemics and vibriosis (Chiu et al., 2007).

Vibrios are natural inhabitants of aquatic environments with high salinity and temperatures ranging from 10 to 30 °C (Manjusha et al., 2005; Strohl et al., 2004; Murray et al., 2004). Several species, including *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum* and *V. splendidus*, are known to induce severe infections in aquaculture livestock, especially shrimp (Jayasree et al., 2006).

Chemicals and antibiotics are widely used to prevent or treat such infections. However, according to Nogueira-Lima et al. (2006), evaluating the risks associated with the use of chemicals in aquaculture is difficult due to the lack of quantitative data from most countries involved in this activity. Most available information on metabolization efficiency, tissue withdrawal time and environmental impact has been collected in temperate climate zones and may not apply to tropical environments and species.

Over time vibrios exposed to antibiotics inside or outside the shrimp farming environment can acquire antimicrobial resistance transferable by mobile genetic elements and horizontal gene transfer (Serrano, 2005). Thus, due to the presence of R-factors in the population, resistance developed through gene regulation of plasmids and chromosomes may be transferred vertically (by heredity) or horizontally (Madigan et al., 2003).

The transfer of multiple resistances by plasmids is a major concern in aquatic bacterial chemotherapy. To face the challenge,

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doi:10.1016/j.envres.2010.09.012

much more research is needed regarding the incidence of multiresistant isolates and the use and effect of antibiotics in shrimp and humans (Manjusha et al., 2005).

The present study evaluated the response of *Vibrio* isolates from shrimp farm water and livestock to a range of antibiotics, some of which are regularly used in humans. Research on antimicrobial resistance in vibrios should be encouraged. Some species of the genus *Vibrio* are opportunistic pathogens. When infecting marine livestock they strongly impact productivity and pose a potential health risk to human consumers.

2. Material and methods

2.1. Bacterial isolates

Isolates investigated are currently maintained within the bacterial collection of the Laboratório de Microbiologia Ambiental e do Pescado, Instituto de Ciências do Mar(LABOMAR/UFC). Thirty-one *Vibrio* isolates were previously isolated from hatcheries water samples ($n=13$) and shrimp hepatopancreas ($n=18$) from three (3) farms at Ceará State, NE Brazil. Samples were collected from August 2005 to October 2006, from shrimp farms located at three (3) important sites of marine shrimp farming along the Ceará costline, in Acaraú, Coreaú and Jaguaribe rivers estuary. Isolates were isolated from representative colonies grown on selective medium Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS Agar). Pure cultures were identified by biochemical tests following protocol described by Alsina and Blanch (1994).

2.2. Disc diffusion susceptibility test

Commercially available antibiotic disks were used to test the susceptibility patterns. Antimicrobial classes used in panel screens included: AMINOGLYCOSIDE: gentamicin 10 μg (GEN); CHLORAMPHENICOL: florfenicol 25 μg (FLF); TETRACYCLINES: tetracycline 30 μg (TET) and oxytetracycline 30 μg (OTC); AMINOPENICILLINS: ampicillin 10 μg (AMP); CEPHALOSPORIN: cefoxitin 30 μg (FOX); QUINOLONE: nalidixic acid 30 μg (NAL); MONOBACTAM: aztreonam 30 μg (ATM); CARBAPENEMS: imipenem 10 μg (IMP); SULFONAMIDE: trimethoprim-sulfamethoxazole 25 μg (SXT); NITROFURAN: nitrofurantoin 300 μg (NIT). All antimicrobial disks were supplied by Laborclin®.

This assay was carried out according to the CLSI (2009b) guidelines. *Escherichia coli* ATCC 25922 was used as control.

2.3. Determination of minimum inhibition concentration (MIC) of oxytetracycline

The minimum inhibitory concentration (MIC) of oxytetracycline was estimated by broth dilution technique (Macrodilution) (CLSI, 2009a) for multiresistant *Vibrio* sp. Bacterial suspensions corresponding in turbidity to McFarland 0.5 were diluted 1:10 ratio to reach a density of 10^7 CFU/mL and then were used as inocula. A 50 μL aliquot was inoculated into tubes containing Mueller-Hinton broth (1% NaCl) supplemented with different OTC concentrations. Tubes containing Mueller-Hinton broth (1% NaCl) without antibiotics were used as control group.

To verify the influence of seawater ions on antibiotic bioactivity, another batch of tests was carried out using Mueller-Hinton broth diluted in seawater adjusted to reach at 10 ppm salinity. Tubes were inoculated and then incubated at 30 °C for 16–20 h. After incubation period, bacterial growth was visually detected by turbidity (CLSI, 2009a). Oxytetracycline concentrations ranging from 25 to 2000 μg/mL were evaluated.

2.4. Plasmid curing

Multiple resistant isolates were submitted to a curing treatment with 200 μg/mL of Acridine Orange (AO). Isolates were grown on Triptone Soy Broth (TSB) supplemented with 1% NaCl at 30 °C for 18–24 h. After incubation time, 200 μL aliquots were added to tubes containing Luria Bertani broth (LB) 1% NaCl (control) and to tubes containing Luria Bertani (LB) 1% NaCl+AO. Tubes were incubated in water bath under agitation at 30 °C for 18–24 h. After treatment with curing agent, to verify changes in resistance profiles, antibiograms were again performed for the antibiotics to which multiresistant isolates were originally resistant (Molina-Aja et al., 2002).

3. Results

A total of thirty-one *Vibrio* isolates were tested for their susceptibility to antimicrobial agents. Among these isolates, thirteen *Vibrio* were isolated from hatcheries water from shrimp farms: *V. mimicus* (8), *V. hispanicus* (2), *V. marinus* (1), *V. tubiashii* (1) and *V. mediterranei* (1); and eighteen isolated from shrimp hepatopancreas (*L. vannamei): V. mimicus* (5), *V. metschnikovii* (3), *V. fluvialis* (2), *V. hispanicus* (1), *V. mediterranei* (1), *V. harveyi* (1), *V. vulnificus* (1), *V. alginolyticus* (1) and *Víbrio* sp. (3).

In Table 1 are presented the antibiotic profiles of *Vibrio* isolates. The bacteria were tested for susceptibility to 11 antimicrobials representing 10 antimicrobial drug classes. Among the *Vibrio* isolates, 61.3% (19/31) showed resistance to at least one of the 11 tested antibiotics. When data were analyzed taking into account the source from which samples were obtained, 53.8% of the isolates from water and 66.6% of the isolates from hepatopancreas were found to be resistant to at least one antimicrobial agent. The *Vibrio* isolates from both shrimp hepatopancreas and hatchery water were resistant to AMP, FOX, NAL, OTC and TET. Resistance to NAL was detected only in two isolates from hepatopancreas. Most of the isolates were resistant to AMP (14) and OTC (8). Sixteen isolates exhibited intermediate resistance to AMP, ATM, FOX, GEN, IPM and OTC. Most frequent intermediate resistance phenotype was to AMP and ATM. Antimicrobial agents FLF and NIT were 100% effective against *Vibrio* isolates and AMP was the drug less effective.

Bacterial isolates from Coreaú river showed the highest frequency of resistance (10/12) followed by Acaraú (5/9) and Jaguaribe (4/10) rivers. It was possible to verify that the antimicrobial resistance patterns of the *Vibrio* were not related to specific species. In general, evident differences in antimicrobial resistance patterns were observed among isolates from the same estuary.

The occurrence of simultaneous resistance to multiple antimicrobial drugs was observed in 29% (9/31) of *Vibrio* isolates. Eight different profiles were identified among multiresistant isolates: AMP+FOX; AMP+OTC; FOX+NAL; AMP+OTC+TET; AMP+FOX+TET; AMP+OTC+SXT; AMP+FOX+OTC+TET and FOX+NAL+OTC+TET.

The MIC values for all resistant *Vibrio* isolates were up to 400 mg/L of oxytetracycline in both broths tested (without and with seawater). The presence of seawater did not influence, in this case, the oxytetracycline bioactivity.

Changes in antibiotic resistance patterns among multiresistant bacterial isolates after plasmid curing are shown in Table 2. Of the total multiresistant *Vibrio* isolates submitted to plasmid curing, five (55.5%) lost 1 or more resistance profile while four isolates (44.5%) did not lose their resistance. Resistance to OTC was the most frequently lost phenotype after plasmid curing. Two bacterial isolates, *V. marinus* (HW57) and *V. mimicus* (HW133), from Coreaú estuary lost all resistance phenotypes indicating that these profiles were encoded by plasmidal genes.

4. Discussion

Continuous and improper use of antibiotics in aquaculture favors the selection of resistant isolates and the dissemination of resistance genes within bacterial populations in the environment, reflecting the pattern of drug use (Tendencia and Peña, 2002).

In this study, *Vibrio* isolates from shrimp pond water and hemolymph displayed resistance to six antibiotics belonging to five different classes. Most isolates (64.52%) presented an intermediate resistance profile and resistance to ampicillin, while 45.16% were resistant to antibiotics of the tetracycline group.

Akinbowale et al. (2006) investigated the occurrence of resistance to antibiotics among the bacteria isolated from the aquaculture environments and different types of livestock. Their antibiogram included the antibiotics most commonly used in aquaculture settings in the US, Denmark, Norway, the UK, Canada

Table 1
Resistance profile of *Vibrio* spp. isolated from hatcheries water and from cultivated marine shrimps.

Estuary	Isolates	Identification	Susceptible	Intermediate	Resistant
Coreaú River	HW57	*V. marinus*	SXT; IPM; NAL; FLF; GEN; ATM; NIT	–	AMP; FOX; OTC; TET
	HW59	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; TET	–	AMP; OTC
	HW60	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; FOX; NIT; TET	ATM; OTC	AMP
	HW61	*V. tubiashii*	SXT; IPM; NAL; FLF; GEN; FOX; NIT; TET	ATM; OTC	AMP
	HP19	*Vibrio* sp.	SXT; IPM; AMP; FLF; GEN; ATM; NIT; OTC; TET	IPM	FOX
	HP20	*Vibrio* sp.	SXT; IPM; AMP; FLF; ATM; NIT	GEN	FOX; NAL; OTC; TET
	HP21	*Vibrio* sp.	SXT; IPM; AMP; FLF; GEN; NIT; OTC; TET	ATM	FOX; NAL
	HP51	*V. fluvialis*	SXT; IPM; NAL; AMP; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	–
	HP52	*V. fluvialis*	SXT; IPM; NAL; AMP; FLF; GEN; FOX; ATM; NIT; TET	–	OTC
	HP54	*V. hispanicus*	SXT; IPM; NAL; AMP; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	–
	HP101	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT	–	AMP; OTC; TET
	HP110	*V. vulnificus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	AMP
Acaraú River	HW65	*V. hispanicus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; TET	AMP;OTC	–
	HW66	*V. hispanicus*	SXT; IPM; NAL; FLF; GEN; ATM; NIT; OTC; TET	AMP;FOX	–
	HW68	*V. mediterranei*	SXT; IPM; NAL; AMP; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	–
	HW133	*V. mimicus*	TET; IPM; NAL; FLF; GEN; ATM; NIT;	FOX	AMP; OTC; SXT
	HP70	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	AMP
	HP71	*V. metschnikovii*	SXT; IPM; NAL; FLF; GEN; NIT; OTC	ATM	AMP; FOX; TET
	HP77	*V. metschnikovii*	SXT; IPM; NAL; FLF; GEN; ATM; NIT; OTC; TET	–	AMP; FOX
	HP120	*V. metschnikovii*	SXT; IPM; NAL; AMP; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	–
	HP123	*V. alginolyticus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	AMP
Jaguaribe River	HW86	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	AMP
	HW87	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; ATM; NIT; TET	AMP; FOX	OTC
	HW89	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; TET	AMP; OTC	–
	HW91	*V. mimicus*	SXT; IPM; NAL; AMP; FLF; GEN; FOX; ATM; NIT; TET	OTC	–
	HW134	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; OTC; TET	AMP	–
	HP78	*V. mediterranei*	SXT; IPM; NAL; FLF; GEN; FOX; NIT	ATM; TET	AMP; OTC
	HP82	*V. mimicus*	SXT; IPM; NAL; FLF; GEN; FOX; ATM; NIT; OTC; TET	AMP	–
	HP83	*V. harveyi*	SXT;IPM;NAL;FLF;GEN;FOX;NIT;OTC;TET	ATM	AMP
	HP84	*V. mimicus*	SXT; IPM; NAL; AMP; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	–
	HP85	*V. mimicus*	SXT; IPM; NAL; AMP; FLF; GEN; FOX; ATM; NIT; OTC; TET	–	–1

AMP=ampicillin 10 μg, ATM=aztreonam 30 μg, FOX=cefoxitin 30 μg, FLF=florfenicol 25 μg, GEN=gentamicin 10 μg, IPM=imipenem 10 μg, NAL=nalidixic acid 30 μg, NIT=nitrofurantoin 300 μg, OTC=oxytetracycline 30 μg, SXT=sulfamethoxazole−trimethoprim 25 μg, TET=tetracycline 30 μg, HW=hatcherie water sample, HP=hepatopancreas sample.

Table 2
Resistance profiles of *Vibrio* spp. isolates prior and after plasmid curing.

Estuary	Strain	Identification	Resistance profile	
			Before curing	After curing
Coreaú River	HW57	*V. marinus*	AMP; FOX; OTC; TET	–
	HW59	*V. mimicus*	AMP; OTC	AMP
	HP20	*Vibrio* sp.	FOX; NAL; OTC; TET	NAL
	HP21	*Vibrio* sp.	FOX; NAL	FOX; NAL
	HP101	*V. mimicus*	AMP; OTC; TET	–
Acaraú River	HW133	*V. mimicus*	AMP; OTC; SXT	AMP; OTC; SXT
	HP71	*V. metschnikovii*	AMP; FOX; TET	AMP; FOX; TET
	HP77	*V. metschnikovii*	AMP; FOX	AMP
Jaguaribe River	HP78	*V. mediterranei*	AMP; OTC	AMP; OTC

AMP=ampicillin 10 μg, FOX=cefoxitin 30 μg, NAL=nalidixic acid 30 μg, OTC=oxytetracycline 30 μg, SXT=sulfamethoxazole−trimethoprim 25 μg, TET=tetracycline 30 μg, HW=hatcherie water sample, HP=hepatopancreas sample.

and other countries. Some of the isolates were resistant to ampicillin, tetracycline and oxytetracycline and susceptible to florfenicol and sulfametoxazol-trimetoprim. According to Wang et al. (2008), antibiotics like oxytetracycline, tetracycline and chloramphenicol are commonly added to fodder to stimulate growth, due to their low cost and ample spectrum of action against bacteria.

In our study, the most efficient antibiotics against *Vibrio* isolates were FLF and SUT-TM (100% susceptibility), matching results published by Roque et al. (2001) for 144 *Vibro* isolates from shrimp farms (59.03% and 92.36%, respectively).

Many cases of multiple antimicrobial resistance have been reported from shrimp farms in countries where the activity is well developed, such as China (Dang et al. (2006)), Korea (Kang et al., 2005) and Chile (Miranda and Rojas, 2007). In our region most cases of multiple antimicrobial resistances among *Vibrio* spp. come from the estuary of the Coreaú river (60%), with resistance to AMP and OTC as the most frequent. Thus, in (2008) Costa et al. reported multiple antimicrobial resistances in 15.4% of their *vibrio* isolates from pond water and shrimp farmed in this estuary.

Five of our isolates were resistant to two antibiotics (SUT+CRO and SUT+AMP) while one isolate was resistant to three antibiotics

(SUT+AMP+CRO). In a study by Sarter et al. (2007) involving sutchi catfish from three farms in Vietnam, some of the isolated *Vibrio* isolates presented multiple resistance to AMP+OTC+SXT+NAL and AMP+NIT+OTC.

Resistance to OTC was observed in 25.8% of our isolates, compared to 54.3% in a study by Kim et al. (2003) surveying the diversity of *Vibrio* species in seawater and soil from aquaculture areas in Japan.

Although OTC is not approved for use in shrimp culture in Brazil, the drug is widely used in the treatment of bacterial infections in aquatic livestock. A United Nations publication on the use of antibiotics in aquaculture highlights the risk of using OTC in marine environments. The molecules in OTC bind to Ca^{++} and Mg^{++} ions in the seawater thereby significantly reducing the drug's biological activity (FAO/OIE/WHO, 2006).

The minimum inhibition concentration (MIC) of OTC-resistant isolates was lower when the medium was diluted in seawater than when it was merely enriched with NaCl. Contrasting with other studies (Choo, 1994; Lunestad and Goksayr, 1990; Lunestad and Samuelsen, 2001), seawater at the concentration used in our study did not affect the bioactivity of OTC. MIC values for all the tested isolates were above 400 μg/mL. Roque et al. (2001) reported an average MIC of 304 μg/mL for OTC-resistant *Vibrio* isolates from shrimp farmed in Mexico.

When submitted to plasmid curing, multiresistant isolates lost their resistance to some antibiotics, the most frequent of which was OTC. Two isolates (AV57 and HP101) became susceptible to all the antibiotics they had been resistant to, indicating resistance was related to plasmids.

Molina-Aja et al. (2002) reported that *Vibrio* isolates lose resistance to ampicillin and cephalotin, but not to oxytetracycline, if submitted to plasmid curing. Kim et al. (2004) found genes of resistance to drugs of the tetracycline group to be ubiquitous in aquatic organisms and seawater, suggesting marine aquaculture environments may serve as a reservoir for such genes.

Due to the occurrence of diseases on local shrimp farms and the disquieting increase in antimicrobial resistance, the use of fodder with added antibiotics requires extensive investigation. The establishment of appropriate therapeutic doses of antibiotics can help minimize potential impacts on the environment and on human health (Nogueira-Lima et al., 2006).

5. Conclusions

Most of the *Vibrio* isolates from the shrimp farm environment were resistant to at least one of the tested antibiotics, and a significant percentage exhibited simultaneous resistance to multiple antibiotics, indicating a serious risk to public and animal health. R-plasmid-mediated resistance was also observed. The widespread resistance of *Vibrio* isolates to antibiotics such as oxytetracycline and ampicillin is mostly the result of careless use of drugs on shrimp farms. Further research will clarify how the presence of microorganisms carrying drug resistance genes affects the incidence of infection in aquatic livestock and how it impacts human health and antimicrobial therapy.

Surveillance of antimicrobial resistance and monitoring of drug use in aquaculture should be encouraged in order to improve management of antibiotics to the benefit of public health and food safety associated with the activity.

Acknowledgment

The authors are thankful to Fundação Cearense de Apoio à Pesquisa – FUNCAP, for the financial support to this research.

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