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MULTIRESISTANCE IN VIBRIOS ISOLATED FROM TISSUES AND HEMOLYNPH OF *Litopenaeus vannamei* FARMED IN NORTHEASTERN BRAZIL

Multiresistência em vibrios isolados de tecidos e hemolinfa de *Litopenaeus vannamei* cultivado em fazendas do Nordeste do Brasil

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ABSTRACT

The objective of this study was to establish the antibacterial resistance profile of vibrio strains isolated from the hemolynph, hepatopancreas and muscle of Pacific white shrimp, Litopenaeus vannamei, *collected from farms in Northeastern Brazil. Nine (43%) out of 21 strains presented a multiple antibiotic resistance (MAR) index of ≥0.2, indicating an overall pattern of multiresistance. MAR values were higher for strains from hemolynph (0.19), followed by strains from hepatopancreas (0.143) and muscle (0.095). The highest MAR values observed (0.62) came from two strains (one hemolynph, one hepatopancreas) resistant to six classes of antibiotics. Two strains isolated from muscle were resistant to five classes of antibiotics. In conclusion, the emergence of multiresistant strains in the microbiota of shrimp reared on farms in Northeastern Brazil may be the added effect of extensive use of antibiotics by aquafarmers and contaminated discharge from neighboring hospitals, sewers, crops and livestock.*

***Keywords*:** Litopenaeus vannamei, *vibrio, hemolynph, hepatopancreas, muscle, resistant bacteria.*

RESUMO

O objetivo dessa pesquisa foi traçar o perfil de multirresistência a antimicrobianos, de 21 cepas de vibrio pertencentes a diferentes espécies, isoladas de hemolinfa, hepatopâncreas e músculo do camarão Litopenaeus vannamei *cultivado em fazendas dos estados do Ceará, Piauí e Rio Grande do Norte. Entre as estirpes bacterianas testadas, 42,8% apresentaram o índice MAR igual ou maior que 0,2 caracterizando multirresistência. Os isolados da hemolinfa apresentaram os maiores percentuais de MAR (19,0%), seguidos dos isolados do hepatopâncreas (14,3%) e os do músculo (9,5%). Os maiores índices de MAR (0.62) foram detectados em duas amostras (da hemolinfa e do hepatopâncreas) com resistência a seis diferentes classes de antimicrobianos. Duas estirpes (V18 e V21) isoladas do músculo apresentaram resistência a cinco classes de antimicrobianos. Conclui-se que o aparecimento de cepas MDR na microbiota de animais aquáticos reflete a somatória do uso de drogas que pode ser tanto originária do ambiente das fazendas, como das circunvizinhanças na forma de detrito hospitalars, esgoto doméstico e resíduos de atividades agropecuárias.*

***Palavras-chaves**:* Litopenaeus vannamei, *vibrio, hemolinfa, hepatopâncreas, músculo, bactérias resistentes.*

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INTRODUCTION

Epizootics involving infectious agents have caused considerable losses to shrimp farming worldwide. Viroses may not be as common as bacterial diseases, but they are responsible for greater economic losses (Gesteira, 2006). However, bacteria, especially vibrios, can seriously threaten systems of shrimp larviculture and grow-out. Vibrios can induce a range of diseases and have in some cases led to 100% mortality in shrimp ponds (Sung et al., 2001). In addition, due to their chitinolytic action, *Pseudomonas* spp. and *Aeromonas* spp. can cause damage to shrimp cuticula resulting in focal and multifocal necrosis (Guzmán & Valle, 2000).

Shrimp farming is further threatened by the presence of pathogens resistant to antibacterial drugs, in part as a result of improper use of antibiotics (Weston, 1996). In Brazil, although the use of antibiotics by shrimp farmers is extensive, it remains unregulated. As observed in other shrimp-producing countries, the careless use of antibiotics to control disease in cultured shrimp is a growing concern for public health authorities and regulatory agencies alike.

The objective of this study was to establish the antibacterial resistance profile of strains of different vibrio species isolated from the hemolymph, hepatopancreas and muscle of Pacific white shrimp, *Litopenaeus vannamei*, collected from farms in the Brazilian shrimp belt during an outbreak of infectious myonecrosis virus (IMNV).

MATERIAL AND METHODS

Isolation and identification of strains

During an outbreak of IMNV (October, 2003 to January, 2004), shrimp (*L. vannamei*) were collected from five farms located in the country's largest shrimp-producing region (Acaraú, Aracati and Camocim/Ceará, Parnaíba/Piauí and Mossoró-Pendências/Rio Grande do Norte, respectively) (Figure 1) and transported live to the laboratory. Following anesthesia by immersion in ice-cold water, hemolymph, hepatopancreas and muscle were sampled and inoculated in vibrio-selective agar (TCBS/Difco) (Kaysner & Depaola, 2010). After incubation at 30°C for 24 hours, colonies were selected for purification and identified following the procedures described by Noguerola & Blanch (2008).

Antibiograms

The antibiogram used commercially available disks (LABCLIN, Paraná, Brazil) representing the following classes of antibiotics:

The β-lactam family: cephalothin (CET; 30μg), cefotaxime (CTX; 30μg), ceftriaxone (CRO; 30μg) and ampicillin (AMP; 10μg)

The tetracycline family: tetracycline (TCY; 30μg)

The aminoglycoside family: gentamicin (GEN; 10μg) and amikacin (AMK; 30μg)

The chloramphenicol family: chloramphenicol (CHL; 30μg)

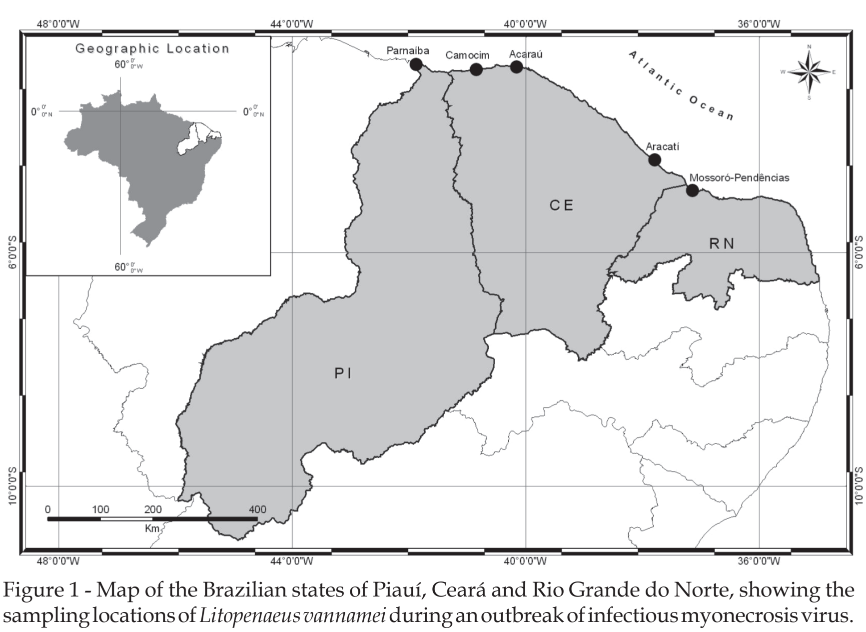


Figure 1 - Map of the Brazilian states of Piauí, Ceará and Rio Grande do Norte, showing the sampling locations of *Litopenaeus vannamei* during an outbreak of infectious myonecrosis virus.

The fluoroquinolone family: ciprofloxacin (CIP; 5µg)

The nitrofuran family: nitrofurantoin (NIT; 300µg)

The quinolone family: nalidixic acid (NAL; 30µg)

The sulfonamide family: sulfametoxazol-trimetoprim (SXT; 30µg)

The pefloxacin family: Pefloxacin (PEF; 30µg)

The authors followed the guidelines published by the Clinical and Laboratory Standards Institute (CLSI, 2010).

Antibiotic resistance index

Based on the observed resistance patterns, the antibiotic resistance index (ARI) (Jones *et al*., 1986) and the multiple antibiotic resistance (MAR) index (Krumperman, 1983) were calculated.

RESULTS AND DISCUSSION

Twenty-one vibrio strains were identified in the study (Table I). Although vibrios are opportunistic pathogens manifesting mainly as secondary infection in debilitated animals, some strains are virulent enough to cause primary infection (De La Peña *et al*., 1993) and induce considerable losses in shrimp culture (Vieira *et al*., 2000). Abraham *et al*. (2004) and Karunasagar *et al*. (2001) reported *V. harveyi* and *V. campbelli* to have a preference for shrimp larvae while infection with *V. penaeicida* and *V. parahaemolyticus* is more commonly observed in juveniles and adults (Ishimaru *et al*., 1995; Roque *et al*., 1998).

The genus *Vibrio* includes both pathogens capable of infecting humans and aquatic animals and species involved in the biogeochemical cycle. Some vibrios can break down chitin or even degrade extremely toxic polycyclic aromatic hydrocarbons (Thompson *et al*., 2004). *V. harveyi*, an important pathogen in shrimp culture, has been responsible for great losses on shrimp farms in the Philippines and other countries in Southeast Asia (Gräslund and Bengtsson, 2001). In a larval bioassay, while some *V. harveyi* strains failed to kill *Penaeus monodon* larvae at densities above 10^8 cells/mL (1,000-10,000 times above normal levels in larviculture ponds), other strains induced mortality under ideal conditions at

Table I - Multiresistant vibrio strains isolated from samples of hemolymph, hepatopancreas and muscle of Pacific White shrimp reared on farms of the states of Ceará (CE), Rio Grande do Norte (RN) and Piauí (PI), Brazil, during an outbreak of infectious myonecrosis virus.

Strain	Species	Sample type	Origin	Resistance profile	MAR	ARI
V1	*Vibrio alginolyticus*		Parnaíba (PI)	AMP, CET, CRO, SXT	0.31	
V2	*V. fisheri*		Parnaíba (PI)	AMP, CET , CLO, NAL, NIT, SXT, TET	0.54	
V3	*V. gigantis*		Natal (RN)	CET, TET	0.15	
V4	*V. gigantis*		Natal (RN)	CET, TET	0.15	
V5	*V. coralliilyticus*	Hemolymph	Parnaíba (PI)	CET	0.07	
V6	*V. proteolyticus*		Parnaíba (PI)	NAL	0.07	
V7	*V. proteolyticus*		Parnaíba (PI)	AMP, CET, CLO, NAL, NIT, PEF, SXT, TET	0.62	
V8	*V. proteolyticus*		Parnaíba (PI)	*	#	
V9	*V. mimicus*		Camocim (CE)	AMP, CET, CLO, NAL, NIT, PEF, SXT, TET	0.62	0.12
V10	*V. calviensis.*		Natal (RN)	AMP, CET, CLO, NAL, NIT, PEF, SXT, TET	0.62	
V11	*V. mediterranei*		Natal (RN)	NIT	0.07	
V12	*V. mimicus*	Hepatopancreas	Parnaíba (PI)	AMP, CET	0.15	
V13	*V. alginolyticus*		Aracati (CE)	CET	0.07	
V14	*V. logei*		Acaraú (CE)	AMP, CET, NAL, NIT, SXT	0.38	
V15	*V. logei*		Acaraú (CE)	AMP, NAL, NIT, PEF	0.31	
V16	*V. superstes*		Parnaíba (PI)	NAL	0.07	0.08
V17	*V. diazotrophicus*		Natal (RN)	TET	0.07	
V18	*V. cincinnatiensis*		Aracati (CE)	AMP, CET, CLO, NAL, NIT, SXT	0.46	
V19	*V. mimicus*	Muscle	Camocim (CE)	NIT	0.07	
V20	*V. mimicus*		Camocim (CE)	NIT	0.07	
V21	*V. mimicus*		Aracati (CE)	AMP, CET, CLO, NAL, NIT, SXT	0.46	0.04

* Resistance not detected; # number not calculated. SXT=sulfametoxazol-trimetoprim; AMK=amikacin; CET =cephalothin; PEF=pefloxacin; GEN=gentamicin; NIT=nitrofurantoin; CTX=cefotaxime; CIP=ciprofloxacin; CHL=chloramphenicol; CRO=ceftriaxone; AMP=ampicillin; NAL=nalidixic acid; TCY=tetracycline

densities below 10^2 cells/mL (10-100 times below normal levels). Thus, virulence in marine vibrios appears to be lineage-specific rather than species-specific (Harris *et al*., 2000).

Hao *et al*. (1997) isolated *V. parahaemolyticus, V. vulnificus, Aeromonas* spp.*, V. alginolyticus, Proteus* spp. and *Pseudomonas* spp. from samples of diseased tiger prawn (*P. monodon*) collected in central and southeastern Vietnam. Likewise, *V. proteolyticus*, a known marine shrimp pathogen, has been isolated from the hemolymph of shrimp reared on a farm in Parnaíba (PI) (Table I) and, in a recent study, the same species was detected in samples from three shrimp farms in Pernambuco with a relative abundance surpassed only by *V. anguillarum, V. fluvialis, V. parahaemolyticus, V. damsela, V. vulnificus* and *V. carchariae* (Mendes *et al*., 2009)

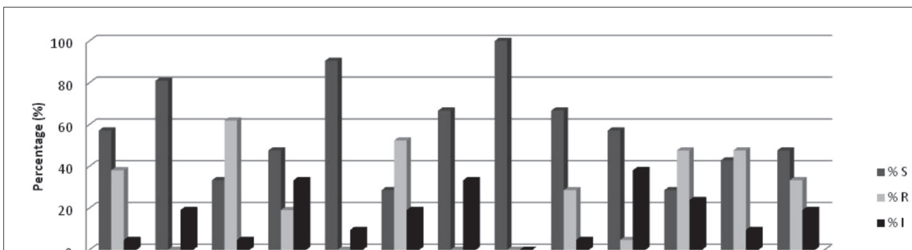
More than half (12 out of 21) the isolated vibrio strains were resistant to at least two classes of antibiotics. Three strains were multiresistant to six classes: V7 (*V. proteolyticus)*, V9 (*V. mimicus*) and V10 (*V. calviensis*) (Table I). According to Noga *et al*. (1996), Johnson (1976), Lee and Pfeifer (1975) and Bang (1970), crustacean hemolymph is normally sterile, except as a result of stress or injury. Lightner (1977) isolated bacteria from asymptomatic cultured shrimp and concluded that, without causing disease, a small number of bacteria may enter the hemolymph through wounds in the cuticula. However, the shrimp in our sample were infected with IMNV and were scarcely alive when they arrived at the laboratory. Whether the vibrios isolated from hemolymph in this study were the main cause of death or not, they appear to have made the animals more vulnerable to IMNV. The presence of vibrios in crustacean hemolymph is evidence of systemic infection (Mendes *et al.,* 2005).

Figure 2 shows the antibacterial resistance profile of the 21 strains isolated from samples of muscle, hemolymph and hepatopancreas. Twelve strains (57%) were resistant to at least two classes of antibiotics (Rabatsky-Her *et al*., 2004; Gibbs *et al*., 2006). Thirteen (62%) were resistant to CET and 10 (48%) were resistant to AMP (both from the β-lactam family). According to Alterthum (2004), bacteria usually become resistant to this class of antibiotics by producing β-lactamases. Over 30 types of β-lactamases encoded and transferred by plasmids have been identified in Gram-negative bacteria isolated from shrimp samples. TEM-1, the most well-known of them, is encoded by plasmids and transposons.

Seven (33%) multiresistant strains were resistant to TCY (Table I), a widely used drug in shrimp farming. Tetracyclines are broad-spectrum antibiotics effective against Gram-positive and negative bacteria, mycoplasmas, mycobacteria and protozoan parasites (Roberts, 1996). Based on a study of *V. harveyi*, Teo *et al.* (2002) described two genes of tetracycline resistance and their cloning process. One was identical to the *tet*A gene present in transposon Tn*10* of *Shigella flexneri*.

Eight strains (38%) were resistant to drugs belonging to the quinolone, sulfonamide and nitrofuran families. Resistance to quinolones is due to mutations affecting RNA polymerase and gyrase, against which rifampicins and quinolones are ineffective, respectively. Some mutations can even change bacterial permeability to quinolones. Bacterial resistance to sulfonamides may be due to plasmid acquisition or mutation.

In Brazil, drugs of the nitrofuran and chloramphenicol families are no longer allowed for veterinary use (Directive #9 of the Ministry of



	SXT	AMK	CET	PEF	GEN	NIT	CTX	CIP	CHL	CRO	AMP	NAL	TCY
% S	57	81	33	48	90	29	67	100	67	57	29	43	48
% R	38	0	62	19	0	52	0	0	29	5	48	48	33
% I	5	19	5	33	10	19	33	0	5	38	24	10	19

Antimicrobials

Figure 2 - Antibacterial susceptibility and resistance of 21 vibrio strains isolated from samples of hemolymph, hepatopancreas and muscle of Pacific White shrimp reared on farms in Ceará (CE), Rio Grande do Norte (RN) and Piauí (PI), Brazil, during an outbreak of infectious myonecrosis virus.

Agriculture, Livestock and Food Supply, 2003). Surprisingly, several years after the ban the microbiota of cultured shrimp is still resistant to these drugs. The reason for this is not clear but, according to Nygaard *et al*. (1992), some antibiotic residues remain stable in marine sediments for at least one year. In a study establishing the minimum inhibitory concentration of antibiotics (oxytetracycline, oxolinic acid, norfloxacin, chloramphenicol and kanamycin) commonly used by Thai shrimp farmers to treat vibriosis, Ruangpan *et al*. (1997) found strains resistant to very high concentrations, some of which with genes encoded by R plasmids.

Table I also shows ARI and MAR values for the 21 strains tested. The overall level of resistance differed between the three sampling locations.

In general, strains isolated from hemolymph presented higher average ARI values (0.12) than strains from hepatopancreas (0.08) or muscle (0.04). In comparison, Vieira *et al*. (2010) reported ARI values in the range 0.068–0.077 for strains isolated from shrimp of the Acaraú and Jaguaribe rivers farming system, according to whom such levels indicate a dissemination risk of resistance genes.

The MAR index was ≥0.2 in 9/21 strains (43%), indicating a pattern of multiresistance. Again, strains isolated from hemolymph presented higher average MAR values (0.19) than strains from hepatopancreas (0.143) or muscle (0.095), while its highest value (0.62) was observed for two samples (one hemolymph and one hepatopancreas) resistant to six classes of antibiotics. Two strains (V18 and V21) isolated from muscle samples presented multiresistance to 5 classes of antibiotics. This is an expected finding in environments exposed to frequent administration of antimicrobials (Lima *et al*., 2006).

In conclusion, the emergence of multiresistant strains in the microbiota of shrimp reared on farms in Northeastern Brazil may be the added effect of extensive use of antibiotics by aquafarmers and contaminated discharge from neighboring hospitals, sewers, crops and/or livestock.

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