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Effect of Seawater on the Activity of Antibiotics Against *Vibrios* Isolated from the Hemolymph of Cultured Pacific White Shrimp

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Abstract. – The purpose of this study was to evaluate the effect of seawater (SW) on the activity of antibiotics belonging to 10 families (aminoglycosides, aminopenicillins, carbapenems, β-lactams, chloramphenicols, monobactams, nitrofurans, quinolones, sulfonamides, and tetracyclines) against *Vibrio* strains isolated from hemolymph of Pacific white shrimp, *Litopenaeus vannamei*, farmed in Northeastern Brazil and standard strain *Vibrio cholera* ATCC 19582. Susceptibility of the strains to antibiotics was determined by disk diffusion method and the minimum inhibitory concentration was determined by macrodilution method. The media Mueller–Hinton agar and broth used in the above methods were diluted in distilled water (control, 1% NaCl, pH 7.5) and SW (2.5% NaCl, pH 7.5). The antibiotics most affected by dilution in SW were tetracycline, penicillin, cephalothin, aztreonam, ampicillin, and imipenem, as indicated by a considerable increase in the number of strains classified as intermediate or resistance. Thus, in this study, the efficiency of these antibiotics on *Vibrio* strains was found to be reduced by contact with SW.

Antibiotics are extensively used in aquafarming. According to Hölmstrom et al. (2003), they are used in shrimp farming to prevent or treat disease although little has been published regarding patterns of use. The same authors interviewed 76 shrimp farmers along the coast of Thailand and found that 74% admitted to administering antibiotics. Gräslund and Bengtsson (2001) reported that an array of chemical and biological products are used by most shrimp farmers in Southeast Asia and pointed out the risk for human health represented by improper use of antibiotics.

The use of antibiotics for prophylaxis in aquaculture not only favors the selection of resistant bacteria in the pond environment, thereby changing the natural microbiota of pond water and sediments, but also increases the risk of transferring resistance genes to pathogens infecting humans and terrestrial animals (Cabello 2006). Likewise, Le and Munekage (2004) reported high levels of drug residues (sulfametoxazol, trimetoprim, norfloxacin, and oxolinic acid) in pond water and sediments from tiger prawn farms in Northern and Southern Vietnam due to indiscriminate use of antibiotics.

In Brazil, Costa et al. (2008) detected *Vibrio* strains resistant to ampicillin, sulfametoxazol–trimetoprim, and ceftriaxone in samples of pond water and cultured Pacific white shrimp, *Litopenaeus vannamei*, suggesting ponds and livestock might constitute a potential source of dissemination of resistant bacteria.

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In contrast, Alderman and Hastings (1998) believe that, although there is evidence that antibiotic resistance can be selected for in normal therapeutic use in aquaculture, the risks of transfer of such resistance to human consumers by any of the possible routes appear to be low.

Further studies investigating the possible effects of seawater (SW) on antibiotic activity and the interactions between drug and pond environment are required before recommendations can be made for responsible and sustainable use of antibiotics in shrimp farming. According to Lunestad and Goksoyr (1990), oxytetracycline (a drug commonly used in shrimp farming) is capable of forming complexes with divalent and trivalent cations. At a salinity of 35‰, SW contains 54 mM magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions capable of affecting the bioactivity of this drug in culture settings. The effect of other antimicrobial agents has previously been shown to be compromised by contact with SW (Pursell et al. 1995).

Certain components of SW have been shown to influence the susceptibility of bacteria to antibiotics in a number of ways. Changes in minimum inhibitory concentration (MIC) following the addition of SW may therefore be attributed to the influence of SW on both drug and bacterial physiology (Torkildsen et al. 2000).

Considering the relevance of *Vibrios* to aquaculture and the harm indiscriminate use of antibiotics can do to human health, this study was designed to (a) evaluate the susceptibility of *Vibrios* isolated from shrimp (*L. vannamei*) hemolymph to 14 commonly used antibiotics in growth media diluted with SW or distilled water (DW; control), (b) determine the nature of antibacterial resistance by way of plasmid curing, and (c) compare the MIC of tetracycline in growth media diluted with SW and DW.

Materials and Methods

Origin of Vibrio Strains

This study tested 25 *Vibrio* strains of the species *Vibrio navarrensis* ($n = 12$), *Vibrio brasiliensis* ($n = 6$), *Vibrio xuii* ($n = 4$), and *Vibrio coralliilyticus* ($n = 3$). As control, the standard strain *Vibrio cholerae* ATCC 19582 was used. Thus, 26 strains ($n = 26$) were analyzed. All the strains, except for the control, were isolated from healthy shrimp hemolymph (*L. vannamei*), and made available by the microbe bank of the Laboratory of Seafood and Environmental Microbiology (LABOMAR/UFC). All the selected strains presented the ability to express exoenzymes and hemolysin, noticeable potential virulence factors to shrimp: gelatinase, caseinase, phospholipase, lipase, and hemolysis of sheep erythrocytes (Liuxy et al. 1996; Rattanama et al. 2009).

Antibiogram

The susceptibility of the strains to antibiotics was determined with the disk diffusion method (Jorgensen et al. 2005) using Mueller–Hinton (MH) agar. The growth media were diluted with SW or DW, control, 1% NaCl, pH 7.5. The MH–SW preparation included the dilution of the medium in SW adjusted to 25 parts-per-thousand salinity (2.5%) and pH 7.5. The media was heated to dissolution and autoclaved at 121°C/15 min.

Each strain was challenged with 14 antibiotics belonging to 10 classes: aminoglycoside (gentamicin – Gen 10 μg and streptomycin – Str 10 μg), aminopenicillin (ampicillin – Amp 10 μg and penicillin G – Pen 10 U), carbapenem (imipenem – Ipm 10 μg), β-lactam (cephalothin – Cet 30 μg, ceftriaxone – Cro 30 μg, and ciprofloxacin – Cip 5 μg), chloramphenicol – Cho 30 μg, monobactam (aztreonam – Atm 30 μg), nitrofurantoin – Nit 300 μg, quinolone (nalidixic acid – Nal 30 μg), sulfonamide (sulfametoxazol–trimetoprim – Sut 25 μg), and tetracycline – Tcy 30 μg.

Following incubation, the inhibition halos were measured with a digital caliper (Digimess, São Paulo, Brazil) and each strain was classified as "susceptible," "intermediate," or "resistant," according to CLSI recommendations (2010).

Plasmid Curing with Acridine Orange

Resistant strains cultured in MH+DW were submitted to plasmid curing with acridine

TABLE 1. *Number of* Vibrio *strains (*n = *25) and* V. cholerae *ATCC 19582 (*n = *1) classified as "susceptible," "intermediate," and "resistant" to antibiotics when cultured in Mueller–Hinton (MH) agar diluted in distilled water (DW, control, 1% NaCl, pH 7.5) and seawater (SW, 2.5% NaCl, pH 7.5).*

	MH agar in DW			MH agar in SW		
Antibiotics	S	I	R	S	I	R
Tetracycline	26	0	0	10	11	5
Penicillin G	17	0	9	6	0	20
Cephalothin	20	3	3	15	1	10
Aztreonam	21	5	0	14	6	6
Ampicillin	26	0	0	25	0	1
Imipenem	26	0	0	25	0	1
Nalidixic acid	25	1	0	17	9	0
Streptomycin	26	0	0	23	3	0
Gentamicin	26	0	0	26	0	0
Chloramphenicol	26	0	0	26	0	0
Ceftriaxone	26	0	0	26	0	0
Ciprofloxacin	26	0	0	26	0	0
Sulfametoxazol–trimetoprim	26	0	0	26	0	0
Nitrofurantoin	26	0	0	26	0	0

I = intermediate; R = resistant; S = susceptible.

orange (SIGMA A-6014, St. Louis, MO, USA) (Molina-Aja et al. 2002). Resistance was classified as "chromosomal" when unaffected by plasmid curing, and as "plasmidial" when affected.

MIC

MIC levels were determined for the antibiotics most strongly affected by SW using the method of macrodilution (CLSI 2010) in both MH+SW and MH+DW. Each antibiotic was tested at concentrations of 2, 4, 8, 16, 32, 64, and 128 μg/mL.

Results

Table 1 shows the antibacterial susceptibility profile of *Vibrio* strains cultured in MH diluted with SW or DW. The antibiotics most affected by dilution in SW were Tcy, Pen, Cet, Atm, Amp, and Ipm, as indicated by a considerable increase in the number of strains classified as "intermediate" or "resistant." Thus, whereas all *Vibrio* strains ($n = 26$; 100%) were susceptible to Tcy in MH+DW, more than half were intermediate ($n = 11$; 42.3%) or resistant ($n = 5$; 19.2%) to Tcy when MH was diluted in SW.

Table 2 shows differences in the susceptibility of *Vibrio* strains cultured in MH+SW and MH+DW. The greatest difference between the two media was observed for strains of *V. navarrensis*, which were sensitive to all antibiotics in MH+DW, whereas one strain was resistant to Atm and two were resistant to Pen when cultured in MH+SW. Moreover, some strains displayed resistance to multiple drugs (PenAtm, PenIpm, PenAtmTcy, PenCetTcy, and PenAtmAmp). In MH+DW, the control strain *V. cholerae* showed resistance to Pen, whereas cross-resistance to PenCet was observed in MH+SW.

In this study, plasmid curing revealed resistance to be chromosomal (i.e., unaffected by plasmid curing) in the case of Pen and Cet.

MIC values for Tcy are presented in Table 3. Strains cultured in MH+DW yielded MIC values in the range 4–32 μg/mL. In comparison, 12 (48%) of the 25 strains cultured in MH+SW were inhibited at concentrations between 64 and >128 μg/mL.

Discussion

Discovered in the 1940s, the tetracyclines inhibit protein synthesis, preventing aminoacyl-RNAt from binding to the A site of the ribosome receptor (Chopra and Roberts 2001). Tetracyclines offer a broad spectrum of activity against Gram-positive and negative bacteria

TABLE 2. *Number of strains of* Vibrio xuii, Vibrio brasiliensis, Vibrio navarrensis, *and* Vibrio coralliilyticus *resistant to antibiotics when cultured in Mueller–Hinton (MH) agar diluted in distilled water (DW, control, 1% NaCl, pH 7.5) and seawater (SW, 2,5% NaCl, pH 7.5).*

Species	n	Resistance to MH agar diluted in DW
V. xuii	4	Pen ($n = 3$), PenCet ($n = 1$)
V. brasiliensis	3	Pen ($n = 1$), PenCet ($n = 2$)
V. coralliilyticus	1	Pen ($n = 1$)
Vibrio cholerae ATCC 19582	1	Pen ($n = 1$)
		MH agar diluted in SW
V. xuii	4	PenCet ($n = 3$), PenCflTcy ($n = 1$)
V. brasiliensis	4	Pen ($n = 1$), PenCet ($n = 3$)
V. coralliilyticus	2	PenAtm ($n = 1$), PenTcy ($n = 1$)
V. navarrensis	10	Atm ($n = 1$), Pen ($n = 2$), PenAtm ($n = 2$), PenIpm ($n = 1$), AtmTcyPen ($n = 1$), PenCflTcy ($n = 1$), PenAtmAmp ($n = 1$)
V. cholerae ATCC 19582	1	PenCet ($n = 1$)

Amp = ampicillin; Atm = aztreonam; Cet = cephalothin; Ipm = imipenem; n = number of resistance strains; Pen = penicillin G; Tcy = tetracycline.

and have been used extensively in human and veterinary medicine. The simplest form of tetracycline capable of antimicrobial activity, 6-deoxy-6-dimethyl-tetracycline, consists of a linear molecule fused with a tetracyclic structure (rings A–D) with a range of functional groups linked to the main molecule (Chopra and Roberts 2001).

The observed increase in the number of intermediate and resistant strains appears to be due to the greater availability of Ca^{2+} and Mg^{2+} in the admixed SW. According to Lambs et al. (1988), tetracyclines offer potential sites for binding to such cations. The electrical charge of the newly formed complex ($Tcy+Ca^{2+} + Mg^{2+}$) is different from that of Tcy prior to administration, preventing passive drug diffusion through cell membranes and compromising antibacterial activity. Alternatively, Chopra et al. (1992) suggest the efficiency of Tcy is influenced by binding to metal ions in the environment.

TABLE 3. *Minimum inhibitory concentration of tetracycline (Tcy) against* Vibrio *strains cultured in Mueller–Hinton (MH) agar diluted in distilled water (DW, control, 1% NaCl, pH 7.5) and seawater (SW, 2.5% NaCl, pH 7.5).*

	Species				
Tcy (μg/mL)	*Vibrio navarrensis* ($n = 12$)	*Vibrio brasiliensis* ($n = 6$)	*Vibrio xuii* ($n = 4$)	*Vibrio coralliilyticus* ($n = 3$)	*Vibrio cholerae* ATCC 19582
MH agar diluted in DW					
4	5	2	—	1	1
8	3	1	—	—	—
16	1	—	—	—	—
32	3	3	4	2	—
MH agar diluted in SW					
4	1	1	—	—	—
8	2	—	—	—	—
16	4	1	—	1	1
32	1	1	—	—	—
64	2	1	2	—	—
128	1	1	—	—	—
>128	1	1	2	2	—

n = number of strains.

A similar pattern was observed for other antibiotics in this study: when the medium was diluted in SW, the incidence of resistant strains increased from 34.6 ($n = 9$) to 76.9% ($n = 20$) for Pen, and from 11.5 ($n = 3$) to 38.5% ($n = 10$) for Cet. Resistance to Ipm, Atm, and Amp was observed only when strains were cultured in MH+SW (Table 1).

According to Smith (1998), the effect of SW on antibacterial activity may not be limited to inhibition induced by divalent cations: some drug components may interact with components in the growth medium affecting bacterial growth rates and compromising drug efficiency. During autoclaving of the medium, the interaction between phosphate (PO_4^{3-}) and magnesium (Mg^{2+}) can result in a variable degree of precipitation, probably of insoluble $Mg_3(PO_4)_2$. This precipitation and the increased availability of divalent cations influence the action of the antibiotic considerably.

In this study, differences regarding the susceptibility of *Vibrio* species cultured in MH+SW and MH+DW were observed (Table 2). Species-specific changes in susceptibility to antibiotics may also be related to the effect of SW on the bacterial cell. Thus, in a study on the adaptive response of *Vibrios* cultured in SW, Abdallah et al. (2009) observed changes in the expression of membrane proteins and suggested these changes could influence susceptibility to certain drugs. According to Carlucci and Pramer (1959), inorganic salts in SW can affect bacterial survival by inducing changes in osmotic condition or ion toxicity.

In the *Vibrios* studied, the chromosomal resistance for Pen and Cet contrasting with results published by Reid and Amyes (1986) who attributed Pen resistance to plasmids encoding for SAR-1, a β-lactamase capable of hydrolyzing and consequently inactivating carbenicillin and Pen – according to the authors, the most common mechanism of resistance to β-lactams.

Three (11.5%) of the 26 *Vibrio* strains cultured in MH+DW were resistant to Cet (Table 1). In a study by Molina-Aja et al. (2002) on the plasmidial profile and resistance to antibiotics of *Vibrio* strains isolated from penaeid hemolymph, the incidence of resistance to Cet (36.1%) was higher than in the present study. The authors attributed this to a 21.226 kbp plasmid encoding for Cet resistance, contrasting with our findings of chromosomal resistance to Cet in all relevant strains.

The effects of SW on the activity of antibacterial agents and their implication for MIC levels (Table 3) are in concordance with the findings of Lunestad and Samuelsen (2001). On the basis of tests using growth media with and without dilution in SW, the authors concluded that differences in susceptibility to antibiotics may be the result of chemical interactions between the components of SW, drug, and growth medium, or the effects of drugs on bacterial physiology, including envelope permeability. The relative importance of each factor for different bacterial species and drugs has not yet been determined.

In this study, the efficiency of Tcy, Pen, Cet, Atm, Amp, and Ipm on *Vibrio* strains was found to be reduced by contact with SW. The use of these agents in marine aquaculture should therefore be reviewed with regard to dosage and route of administration.

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