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INTEGRONS AND GENE CASSETTES ENCODING ANTIMICROBIAL RESISTANCE IN VIBRIOS ISOLATED FROM SHRIMP HEMOLYMPH (*LITOPENAEUS VANNAMEI*)

[ver artigo online]

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ABSTRACT

The purpose of the present study was to identify integrons and gene cassettes in vibrios isolated from the hemolymph of shrimp (*Litopenaeus vannamei*) farmed in Ceará (Northeastern Brazil).
Twenty-five vibrio strains were evaluated on PCR for class 1 integrons in chromosomal and plasmidial DNA, using the primers *In, Int*1, *qacE*Δ*1, sul1* and *blaP1*. Eight (32%) isolates yielded integrase (*int*I1) in chromosomal DNA belonging to class 1 integrons, with the gene cassette *blaP1* detected in strains of *V. natriengens* (V10), *V. coralliilyticus* (V11) and *V. parahaemolyticus* (V25). The percentage was 100% in plasmids, with the detection of the gene cassettes *blaP1* and *ereA2* in *V. vulnificus B1* (V4), *V. coralliilyticus* (V7), *V. alginolyticus* (V8), *V. harveyi* (V9), *V. alginolyticus* (V16), *V. harveyi* (V18), *V. parahaemolyticus* (V19), *V. parahaemolyticus* (V21) and *V. mimicus* (V22). The detection of antimicrobial resistance gene cassettes in the isolated strains indicates the possibility of dissemination of these genetic elements to autochthonous bacteria in shrimp culture environment.

Keywords: aquaculture, antibiotic, bacteria, integron

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RESUMO

O objetivo do presente estudo foi identificar integrons e cassetes gênicos em vibrios isolados da hemolinfa de camarão (*Litopenaeus vannamei*) cultivado no Ceará (Nordeste do Brasil). Vinte e cinco linhagens de vibrio foram avaliadas em PCR para integrons classe 1 em DNA cromossômico e plasmidial, utilizando os primers *In*, *Int1*, *qacE*Δ*1*, *sul1* e *blaP*1. Oito (32%) isolados produziram integrase (*intI1*) em DNA cromossômico pertencente a integrons classe 1, com o gene cassete blaP1 detectado em cepas de *V. natriengens* (V10), *V. coralliilyticus* (V11) e *V. parahaemolyticus* (V25). A porcentagem foi de 100% em plasmídeos, com a detecção dos cassetes gênicos blaP1 e ereA2 em *V. vulnificus B1* (V4), V. coralliilyticus (V7), V. alginolyticus (V8), *V. harveyi* (V9), *V. alginolyticus* (V16), *V. harveyi* (V18), *V. parahaemolyticus* (V19), *V. parahaemolyticus* (V21) e *V. mimicus* (V22). A detecção de cassetes de genes de resistência antimicrobiana nas cepas isoladas indica a possibilidade de disseminação desses elementos genéticos para bactérias autóctones em ambiente de cultivo de camarão.

Palavras-chave: aquacultura, antibióticos, bactéria, integron.

INTRODUCTION

The main etiologic agents involved in shrimp culture epizootics are virus and certain bacterial species of the genus *Vibrio* [1]. Some vibrios are known to be pathogenic to aquatic organisms[2] and many shrimp farmers employ antimicrobials to control infectious diseases[3] throughout the production cycle [4]. However, the indiscriminate use of antibiotics in aquaculture favors the selection of resistant strains [5]. Resistance may be encoded by plasmids, transposons or integrons which help disseminate resistance gene cassettes[6]. Integrons are particularly likely to contain gene cassettes, classified according to their integrase sequence—the enzyme responsible for mobilizing the gene cassette inside the integron[7]. The dissemination of integrons among bacteria pertaining to different phyla illustrates the ability of this genetic element to transmit information across a wide range of microorganisms [8].

Vibrios are commonly used as models to study the chromosomal integron/gene cassette system [9] because almost all their genetic elements are mobile [10]. According to Dalsgaard[11], PCR mapping of integrons and DNA sequencing are important tools in epidemiological studies of the evolution and dissemination of antimicrobial resistance in vibrios, especially *V. cholerae*. As shown by Taviani [12], integrons contribute to the development of antimicrobial resistance. The identification of integrons carrying antimicrobial resistance genes is crucial to understanding the potential of a given environment for the development of drug resistance[13]. The purpose of the present study was to identify integrons and gene cassettes in vibrios isolated from the hemolymph of shrimp (*Litopenaeus vannamei*) farmed in Ceará (Northeastern Brazil).

1 MATERIALS AND METHODS

Origin of vibrio strains

The vibrio strains used in this study were isolated from the hemolymph of 80 shrimp collected at four shrimp farms in Ceará. Farms A and B are located west of Fortaleza, in the estuary of the Acaraú/Aracatiaçu river system. Farms C and D are located east of Fortaleza, in the estuary of the Jaguaribe river (Figure 1).

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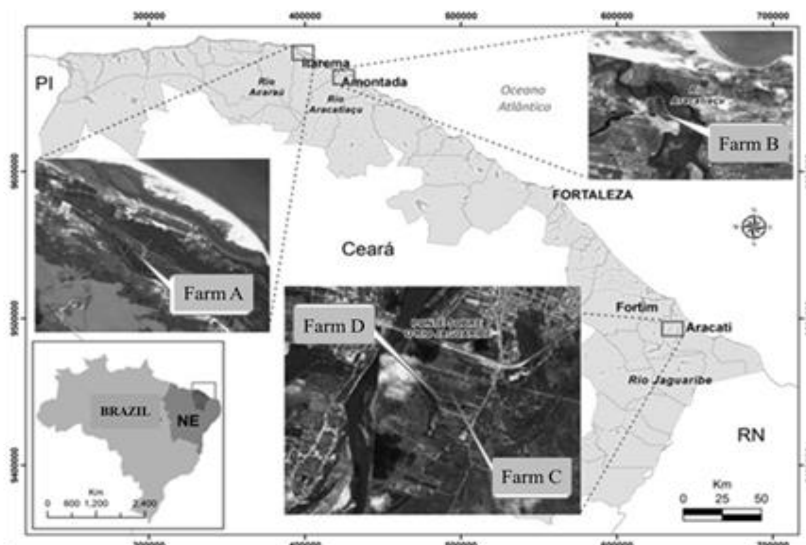


Figure 1 - Location of the farms located in the estuary of the Acaraú River and the estuary of the Rio Jaguaribe.

Selection of vibrio strains

Our samples yielded 122 vibrio strains, of which 25 were selected for the study. The selection was based on criteria of species, origin and antimicrobial resistance profile after plasmid curing (Table 1).

Table 1 - Strains of *Vibrio* spp . selected to detect integrons and gene cassettes on site based resistance , type and lost strength in March after the test " cure plasmid

Code	Origin	Strains	Profile	
			Antes da "cura"	Após a "cura"
V1	Acaraú/FA	*V. alginolyticus*	AMP, CFL	AMP, CFL
V2		*V. harveyi*	AMP, CFL	AMP, CFL
V3		*V. corallilyticus*	AMP, CFL	AMP, CFL
V4		*V. vulnificus* B1	AMP, ERI	AMP, ERI[i]
V5		*V.rumioensis*	AMP, TET, OTC	AMP, TET, OTC
V6		*V. proteolyticus*	AMP, CFL	AMP, CFL
V7		*V. corallilyticus*	AMP, ERI, TET, OTC	AMP, ERI[i] TET, OTC
V8		*V. alginolyticus*	AMP, ERI	AMP, ERI[i]
V9		*V. harveyi*	AMP, ERI	AMP, ERI[i]
V10	Acaraú/FB	*V. natriengens*	AMP, CFL	AMP, CFL
V11		*V. corallilyticus*	AMP, TET, OTC	AMP, TET, OTC
V12		*V. corallilyticus*	AMP, CFL	AMP, CFL

V13		*V. alginolyticus*	AMP, CFL	AMP, CFL
V14		*V. litoralis*	AMP, CFL	AMP, CFL
V15		*V.orientalis*	AMP, TET, OTC	AMP, TET, OTC
V16		*V. alginolyticus*	AMP, ERI	AMP, ERI[i]
V17		*V. corallilyticus*	AMP, CFL	AMP, CFL
V18		*V. harveyi*	AMP, ERI	AMP, ERI[i]
V19		*V. parahaemolyticus*	AMP, CFL*, ERI	AMP, ERI[i]
V20		*V. parahaemolyticus*	AMP, CFL	AMP, CFL[i]
V21		*V. parahaemolyticus*	AMP, ERI	AMP, ERI[i]
V22		*V. mimicus*	AMP, ERI	AMP, ERI[i]
V23		*V. alginolyticus*	AMP, CFL	AMP, CFL
V24	Aracati/FD	*V. mimicus*	AMP, CFL*	AMP
V25		*V. parahaemolyticus*	AMP, CFL*	AMP

AMP - ampicillin ; CFL - cephalothin ; ERI - erythromycin ; OTC - oxytetracycline . i - Intermediate
* Isolated lost resistance to the drug

Polymerase chain reaction (PCR)

DNA was extracted from 25 *Vibrio* strains inoculated in tryptic soy broth (TSB-Difco) containing 1% NaCl, followed by incubation for 24 hours at 35°C. After culture in TSB 1% NaCL, 1-mL aliquots were submitted to DNA extraction with a commercially available kit (DNeasyTissue, Qiagen). The samples of extracted DNA were analyzed in 1% agarose gel (Pronadisa, CONDA). The runs were performed in a horizontal electrophoresis tank (Digel; DGH12/DGH14) at 120 V/300 mA for 90 min using a TBE 1 X buffer solution. The agarose gel was stained with RedGel at the concentration recommended by the manufacturer to make the amplified products visible in the transilluminator (Spectroline-UV) under UV light. The result was photo-documented with a digital camera (Kodac EDAS290). A 1 kb marker (Sigma) was used as standard size for the amplified DNA fragments.

Detection of class 1 integrons and cassettes with the gene blaP1 in chromosomal and plasmidial DNA extracted from vibrio strains

The following primers were used to detect class 1 integrons and gene cassettes in chromosomal DNA extracted from vibrio strains: *in* (variable and conserved regions of the integron), *int1* (class 1 integrons), 3'CS of class 1 integron containing the genes *sul1* (trimethoprim-sulfamethoxazole resistance) and *qacE*Δ*1* (quarternary ammonium resistance), and *blaP1* (β-lacatam resistance). The thermocycling conditions were those recommended by

Aarestrup[14], with modifications. The primers were synthesized by Applied Biosystems (USA) (Table 2)

Table 2- primers and thermal cycling conditions table used in molecular research of *Vibrio* strains of shrimp hemolymph *Litopenaeus vannamei*

<table>
<tr><th>Technique</th><th>Primers</th><th>Sequence</th><th>Cycles</th><th>Conditions of thermal cycling</th><th>Amplicon (bp)*</th><th>Conditions of thermal cycling</th></tr>
<tr><td rowspan="5">PCR</td><td>In</td><td>F: 5´- ggcatccaagcagcaagc-3’
B: 5´- tag tccagttcagacgaa -3’</td><td rowspan="5">35</td><td rowspan="5">94°C/4min
94°C/ 1min
58°C/ 1min
72°C/ 45 seg
72°C/ 10 min</td><td>3381→3415</td><td>COLLIS; HALL, (1992)</td></tr>
<tr><td>Int1</td><td>F: 5´- aaaaccgcc act gcgccg tta-3’
B: 5´- gaagacggctgc act gaa cg-3’</td><td>1163→1200</td><td>FALBO et al.,(1999)</td></tr>
<tr><td>qacEΔ1</td><td>F: 5´- atcgca ata gttggcgaa gt-3’</td><td rowspan="2">1111→1149</td><td rowspan="2">STOKES; HALL (1989), SUNDSTRÖM et al.,(1988)</td></tr>
<tr><td>sul1</td><td>B: 5´- ccgcgcccaaaggcgga acg-3’</td></tr>
<tr><td>blaP1</td><td>F: 5´- cgcttcccgtta aca agt ac-3’
B: 5´- gc gat agactttacttg gtc-3’</td><td>381→419</td><td>ZUHLSDORF; WIEDEMANN (1992)</td></tr>
</table>

* base pair ; F: nucleotide sequence forward; B: nucleotide sequence backward

Detection of the gene cassette ereA2 (erythromycin resistance)

The primer *ereA2* was used to detect resistance gene cassettes in the plasmidial DNA of the nine vibrio strains displaying resistance to erythromycin. Thermocycling followed the protocol of Szczepanowski [15]. The primer was synthesized by Applied Biosystems (USA) (Table 3).

Table 3 - Primer and thermocycling conditions used in molecular studies of erythromycin resistance in strains of *Vibrio* spp . isolated from the hemolymph of shrimp *Litopenaeus vannamei*

Technique	**Primers**	**Sequence**	**C**	**Conditions of thermal cycling**	**Amplicon (bp)***	**Conditions of thermal cycling**
PCR	*ereA2*	F:5-cagcctcaaaagctggagtt-3' B:5-acattccaaaccaatcgcat-3'	30	94°C/4min 94°C/ 1min 58°C/ 1min 72°C/ 1 min 72°C/ 10 min	2038	THUNGAPATHRA *et al.* (2002). Ref. GENBANK AF512546

C * Cycles base pair ; F: nucleotide sequence of forward ; B: sequence of nucleotides backward

RESULTS AND DISCUSSION

Table 4 shows the results of the detection of class 1 integrons and resistance gene cassettes in chromosomal DNA of vibrio strains isolated from the hemolymph of *Litopenaeus vannamei*.

The gene *int*I1 was identified in samples from farms A, B and D, but not in samples from farm C. Of the 25 vibrio strains with multiple resistance profile, 32% (n=8) tested positive for *int*I1: V6 (farm A), V10, V12, V13, V17, V19 (farm B), V24 and V25 (farm D) (Table 4).

Table 4 antimicrobial resistance profile and detection of genes of class 1 integrons and cassettes on the chromosome of strains of *Vibrio* spp. isolated from the hemolymph of marine shrimp *Litopenaeus vannamei*

Code	Origin	Strains	Profile	№ of copies of the gene *int*I1	Size (bp) PCR products amplicons *in*-F e *in*-B	№ of copies of the gene 3'CS	Presence of the gene cassette *blaP1*
V1	Acaraú/ FA	*V. alginolyticus*	AMP, CFL	-	1650, 1000, 400, 300		
V2		*V. harveyi*	AMP, CFL	-	3000, 2500, 1200, 650, 400	2	
V3		*V. corallilyticus*	AMP, CFL	-	1200, 500		
V4		*V. vulnificus* B1	AMP, ERI	-	2000, 1200, 750, 400		
V5		*V. rumioensis*	AMP, TET, OTC	-	2000, 750, 300	2	
V6		*V. proteolyticus*	AMP, CFL	3	2500	1	
V7		*V. corallilyticus*	AMP, ERI, TET, OTC	-	-		
V8		*V. alginolyticus*	AMP, ERI	-	-		
V9		*V. harveyi*	AMP, ERI	-	-		
V10	Acaraú/ FB	*V. natriengens*	AMP, CFL	2	-		+
V11		*V. corallilyticus*	AMP, TET, OTC	-	-		+
V12		*V. corallilyticus*	AMP, CFL	6	-	2	
V13		*V. alginolyticus*	AMP, CFL	2	-		
V14		*V. litoralis*	AMP, CFL	-	-		
V15		*V. orientalis*	AMP, TET, OTC	-	-		
V16		*V. alginolyticus*	AMP, ERI	-	-		
V17		*V.corallilyticus*	AMP, CFL	1	-		
V18		*V. harveyi*	AMP, ERI	-	-		
V19		*V. parahaemolyticus*	AMP, CFL, ERI	2	-		
V20		*V. parahaemolyticus*	AMP, CFL	-	-		
V21		*V. parahaemolyticus*	AMP, ERI	-	-		
V22		*V. mimicus*	AMP, ERI	-	-		
V23		*V. alginolyticus*	AMP, CFL		-		
V24	Aracati/ FD	*V. mimicus*	AMP, CFL	6	-		
V25		*V. parahaemolyticus*	AMP, CFL	6	-	1	+

AMP - ampicillin; CFL - cephalothin; ERI - erythromycin; intI1: gene encoding the integrase class 1; 3 'CS class 1 integron composed of genes sul1 (sulfazotrim resistance) and qacEΔ1 (resistance to quaternary ammonium); blaP1 (resistance to β-lactam).

Importantly, the strains presented similar resistance markers, with the exception of V19 (farm B) which was resistant to erythromycin in addition to ampicillin and cefalotin. According to Fluit and Schmitz [16], integrons may be found in chromosomes, plasmids and transposons. Dalsgaard [17] identified class 1 integrons in *V. cholerae* isolated from both chromosomes and plasmids extracted from clinical samples in South Africa and Mozambique. Similar observations were made by Sá[18] with regard to the significantly greater frequency of *intI*1 in clinical samples than in environmental samples, as opposed to the findings of Mukherjee and Chakraborty [19] and Kitiyodom[20].

Although class 1 integrase is mostly reported in analyses of clinical strains of *V. cholerae*, our study identified a wide range of non-clinical vibrio species carrying this enzyme, supporting the findings of several authors investigating environmental strains from different sources [21, 20, 23, 12] Strains V1-V6 (farm A) yielded amplicons for the genes of the *in-F/in-B* region measuring 300-3,000 base pairs (bp) (Table 4), matching the results of Shi [24] who found the variable region in *V. cholerae* strains to have 700-1,250 bp. In contrast, Dalsgaard [11] observed amplicons with 739-1,237 bp in clinical and environmental *V. cholerae* strains, while [25] reported a range of 400-4,000 bp for *V. fluvialis*.

According to Bennett [26], amplicon size in the conserved region is associated with the number of gene cassettes in the integron. Thus, primer detection of this region in the DNA of micro-organisms makes it possible to identify the location of the antimicrobial resistance [24].

Strains V1, V3 and V4 (farm A) presented variable regions without the genes that make up the structure of the integron (Table 4).

A similar pattern was observed by Lévesque [7] for *Pseudomonas fluorescens*, *P. aeruginosa* and *Escherichia coli* obtained from clinical samples. Also, Koczura [28] evaluated

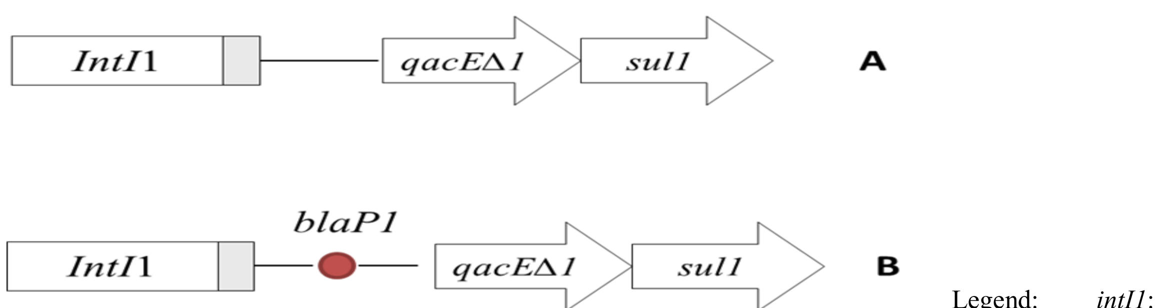
class 1 integrons in *E. coli* isolated from river water and found two strains with empty integrons, indicating the absence of a correlation between the size of the variable region and phylogenetic group, or the presence of genes associated with virulence.

As explained by Di Conza and Gutkind [29], some integrons are not only involved in the acquisition or expulsion of gene cassettes, but have been associated with elements of recruitment (and even expression) of resistance genes in forms other than cassettes.

Strains V10, V11 (farm B) and V25 (farm D) presented class 1 integrons containing the gene cassette *blaP1* (Figure 2). This structure was also identified by Dalsgaard [11] in clinical and environmental strains of *V. cholerae* in Thailand, and by Shi [24] in *V. cholerae* strains from patients with diarrhea in Calcutta (India) between 1992 and 2000.

Figure 2 shows the composition and organization of class 1 integrons in the bacterial chromosome

Figure 2 - Class 1 integron structure of the scheme with the respective genes cassettes detected on chromosome of *Vibrio* spp. isolated from the hemolymph of *Litopenaeus vannamei* shrimp.



Legend: *intI1*: gene encoding the integrase class 1; 3 'CS class 1 integron composed of genes *sul1* (sulfazotrim resistance) and *qacEΔ1* (resistance to quaternary ammonium); *blaP1* (resistance to β-lactam).

The presence of the β-lactamase gene in vibrios isolated from shrimp hemolymph is disturbing because it tends to limit treatment options for vibrio infections. According to Igbinosa and Okoh [30], β-lactam antimicrobials are the most commonly used drugs to treat bacterial infections, but the production of β-lactamases by resistant bacterial strains reduces their efficacy, compromising treatment.

As we have seen, plasmids can carry integrons and play a crucial role in the capture and subsequent expression of genes[26]. Table 5 shows the results of the detection of class 1 integrons and resistance gene cassettes in plasmidial DNA from vibrio strains isolated from the hemolymph of *L. vannamei.*

The strains without gene cassettes observed in this study cannot be classified as empty because only the gene cassette *blaP1* was evaluated.

All nine strains with changes in resistance profile after plasmid curing (especially with regard to erythromycin) presented class 1 integrons. Likewise, Sharma [31] observed class 1 integrons (*intI*1) in 100% of their isolates of *V. vulnificus*, *V. fischeri*, *V. proteolyticus*, *V. mimicus*, *V. cholerae* and *V. parahaemolyticus* from environmental samples (water from the Narmada river, India). On the other hand, Rosser and Young [32] and Sharma and Bora [33] reported class 1 integrons in environmental samples at lower frequencies than in the present study.

A relation may exist between the high percentage of integrons observed and the selective pressure to which the strains are exposed in their habitat. In fact, according to Ghauri [34,] integrons are apparently ubiquitous in nature and may be recovered from clinical and natural environments, especially when exposed to extreme selective pressure. Sharma and Bora [33] believe that horizontal gene transfer by way of mobile elements (such as plasmids and phages) can accelerate the genomic diversification of vibrio species, influencing their physiology, pathogenicity and ecology.

Amplicons related to the primer pair *in*-F and *in*-B in plasmids measured 300-2,000 bp (Table 5).

Table 5 - antimicrobial resistance profile and detection of genes of class 1 integrons and cassettes in plasmid strains of *Vibrio* spp. isolated from the hemolymph of marine shrimp *Litopenaeus vannamei*

Similar results were published by [35]. According to these authors, integrons may be exchanged between micro-organisms of the same or different species, leading to resistance in previously susceptible bacterial strains. On the other hand, Mukherjee and Chakraborty [36] observed amplicons of different size related to the primers *in*-F and *in*-B, pointing out that the difference between the size of the conserved regions depends on the inserted gene cassettes. Strains V9 (farm A), V16, V21 and V22 (farm B) presented a class 1 integron structure (structure A, Figure 3). Although the shrimp was sampled at different farms, the integron structure was the same with regard to the presence of β-lactam (blaP1) resistance gene cassettes. The same structure was reported by 12 for V. cholerae isolated from water from Baía de Maputo (Mozambique).

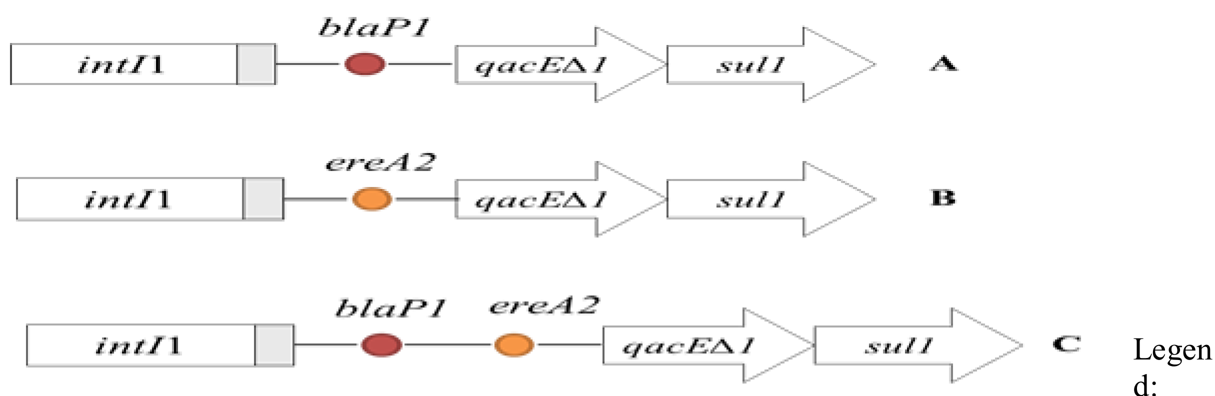
The gene blaP1 was absent from strains V4, V7, V8 (farm A), V18 and V19 (farm B) (Table 5). Thus, the loss or acquisition of gene cassettes is related to the movement of the cassettes into and out of the integrons—a random process like most other genetic rearrangements in micro-organisms. Gene loss is a question of natural selection: if the

Code	Origin	Espécie	Profile	№ of copies of the gene *int*I1	Size (bp) PCR products amplicons *in*-F e *in*-B	№ of copies of the gene 3'CS	Presence of the gene cassette *blaP1*	Presence of the gene cassette *ereA2*
V4		*V. vulnificus B1*	AMP, ERI	5	850, 700, 400	4	-	+
V7	Acaraú/ FA	*V. corallilyticus*	AMP, ERI, TET, OTC	7	2000	7	-	+
V8		*V. alginolyticus*	AMP, ERI	7	600,500,	7	-	+
V9		*V. harveyi*	AMP, ERI	7	1000,600, 500,	4	+	+
V16		*V. alginolyticus*	AMP, ERI	6	600,500,	6	+	+
V18		*V. harveyi*	AMP, ERI	7	-	6	-	+
V19	Acaraú/FB	*V. parahaemolyticus*	AMP, CFL, ERI	4	-	6	-	+
V21		*V. parahaemolyticus*	AMP, ERI	7	2000,850, 300	4	+	+
V22		*V. mimicus*	AMP, ERI	7	300	4	+	+

combination of resistance conferred by an integron is advantageous for the individual, it will harbor and express it, increasing chances of survival in a given environment 37.

Strains V4, V7, V8 (farm A), V18 and V19 (farm B) presented class 1 integron structure with erythromycin resistance (ereA2) gene cassettes (structure B, Figure 3). The same composition was reported by 38 in clinical and environmental non-O1 and non-O139 *V. cholerae* strains from Calcutta (India).

Figure 3 - Class 1 integron structure of the scheme in plasmids with the respective gene cassettes in Vibrio spp. isolated from the hemolymph of shrimp *Litopenaeus vannamei*



Legend:
intI1: gene encoding the integrase class; 3 'CS integron class 1 composed of the genes sul1 (sulfazotrim resistance) and qacEΔ1 (resistance to quaternary ammonium); blaP1 (resistance to β-lactam) and ereA2 (erythromycin resistance).

Other authors have observed the *ereA2* gene cassette in class 1 integrons from different sources [39, 40].The gene evidently circulates with ease among the micro-organisms colonizing humans, animals and the environment, at a frequency mainly dependent on the use of drugs in animal husbandry [41]. Strains V9 (farm A), V16, V21 and V22 (farm B) presented a class 1 integron structure (structure C, Figure 3) containing the gene cassettes *blaP1* and *ereA2*, which confer resistance to β-lactams and erythromycin, respectively.

According to Carattoli [42], the evolution of multiresistance seems to progress relentlessly by way of the acquisition and grouping of resistance genes disseminated by integrons. Controlling this process represents a considerable challenge for public health authorities. The literature has many examples of multiresistance conferred by resistance gene cassettes carried by integrons. In their analysis of class 1 integrons in plasmids from *V. cholerae* and *V. parahaemolyticus*, Ceccarelli [43] found gene cassettes associated with resistance to streptomycin, trimethoprim and β-lactams, while Khan[44] found gene cassettes in *Salmonella* associated with resistance to trimethoprim-sulfamethoxazole and streptomycin. According to these authors, the use of antimicrobials in mariculture should be optimized to prevent the emergence of resistant pathogens and reduce the risk of transmission to humans.

These considerations highlight the usefulness of investigations of integrons and resistance gene cassettes in the surveillance of antimicrobial use on farms [45].

CONCLUSION

The detection of antimicrobial resistance gene cassettes in the strains isolated for this study is an indication of the circulation of these genetic elements in the environment, the possibility of their incorporation into the chromosomal and plasmidial DNA of proximate bacteria by way of integrons, and the risk of their dissemination to autochthonous micro-organisms in shrimp culture. This may be a determinant of the pathogenicity of the bacterial strains currently threatening livestock health and farm productivity.

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