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Ecotypes and virulence factors of *Salmonella* spp. detected in shrimp farms in State of Ceara, Brazil

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ABSTRACT. Our objective was to group in ecotypes 12 serovars of *Salmonella* isolated from shrimp farming environments in the State of Ceara (Northeast Brazil). Grouping was done based on genotypic virulence factors. Two groups based on the similarity of the Box-PCR were identified: a group consisting of three strains (01 *S*. ser. Madelia serovar and 02 *S*. ser. *enterica* subs. *houtenae*) and another group consisting of nine isolates (02 *S*. ser. Saintpaul serovars; 03 *S*. ser. Infantis; 02 *S*. ser. Panama; 01 *S*. *enterica* subs. *enterica*; and 01 *S*. *enterica* subs. *houtenae*). Distribution pattern of the serovars was not influenced by the origin matrices (water and sediment). Plasmid virulence genes *pefA* and *invA* were detected, unrelated to the serovar and environmental origin of the isolates. The presence of virulence genes in the isolates underlines the potential to trigger salmonellosis events via shrimp consumption. Biomonitoring of these sources of contamination should be encouraged as a protective measure, minimizing health risks and economic losses for the industry.

Keywords: sediment, water, enteric bacteria.

Ecotipos e fatores de virulência de *Salmonella* spp. detectadas em fazendas de carcinicultura do Ceará, Brasil

RESUMO. Nosso objetivo foi agrupar em ecotipos 12 sorovares de *Salmonella* isolados em ambientes de carcinicultura no Estado do Ceará. O agrupamento foi feito a partir da pesquisa de fatores genotípicos de virulência. Constatou-se a formação de dois grupos baseados na similaridade do Box-PCR: um grupo com três estirpes (01 sorovar *S*. ser. Madelia e 02 sorovares *S*. *enterica* subs. *houtenae*) e outro constituído por nove isolados (02 sorovares *S*. ser. Saintpaul, 03 sorovares *S*. ser. Infantis, 02 sorovares *S*. ser. Panama, 01 sorovar *S*. *enterica* subs. *enterica* e 01 sorovar *S*. *enterica* subs. *houtenae*). O padrão de distribuição dos sorovares não sofreu influência das matrizes de origem (água e sedimento). Os genes de virulência plasmidial *pef*A e *inv*A foram detectados independente do sorovar e da origem ambiental dos isolados. A presença desses genes de virulência nos isolados de carcinicultura evidencia o potencial para desencadear eventos de salmonelose relacionados ao consumo de camarão. O biomonitoramento dessas fontes de contaminação deve ser incentivado como medida protetiva, minimizando os riscos do ponto de vista sanitário e das perdas econômicas para o setor da carcinicultura.

Palavras-chave: sedmento, água, bactéria entérica.

Introduction

Salmonellosis is one of the most problematic zoonoses in public health, owing to the large number of animals that may act as infection sources, and therefore be able to transmit the bacteria. Although the majority of contamination cases related to the bacteria are caused by eggs and fowl meat, a large variety of food, including meat and fish, were already connected to the disease (Duffy et al., 1999; Hofer, Zamora, Lopes, & Moura, 2000).

The growing incidence of *Salmonella* in tropical aquaculture environments is a worldwide concern which may have local impacts (in the culture area) or global impacts (considering the dynamics of the international seafood market). In order to avoid the sources of fecal pollution from aquiculture areas, it will be important to minimize the risk of transference of salmonelas to foods destined for human consumption (Costa, Carvalho, & Vieira, 2011).

Different studies have been realized about the bacteriological quality in shrimp culture farms

environment of *Litopenaeus vannamei* in Ceará State, Brazil. *Salmonella* bacteria were detected in water, sediment and shrimp samples. These results indicated contamination by anthropogenic activity and fecal pollution by animals in culture environment farm (Figueredo et al., 2015; Carvalho, Sousa, Carvalho, Hofer, & Vieira, 2013; Parente et al., 2011; Carvalho, Barreto, Reis, Hofer, & Vieira, 2009).

Salmonella is included in RDC 12 (Brasil, 2001) as an epidemic bacteria, not tolerated in any seafood product. Classic phenotypic methods were used in the detection and characterization in genus, species, subspecies, serogroup and serotypes. As for the grouping of the strains in ecotypes and the detection of virulence genes, we resort to molecular biology processes (Caffer & Terragno, 2001; Carvalho, et al., 2013; Oludairo, Kwaga, Dzikwi, & Kabir 2013).

The goal of this study was to group in ecotypes previously identified *Salmonella* serovars isolated from water and sediment of two shrimp farms in Ceará and also research the genes: a) *pef A* (plasmid virulence encoded by fimbriae); b) *invA* (pathogenicity) and *spvC* (plasmid virulence).

Material and methods

Salmonella strains

Samples were collected at two farms rearing freshwater-acclimated *Litopenaeus vannamei,* with salinity approximately 0*,* located in the estuary of the Jaguaribe River (Jaguaruana City, State of Ceara, Brazil). A total of hundred and twenty samples were collected from shrimp farms (60 from ponds and 60 from inlet canals) in two time periods: from June to December 2007 and from June to September 2008.

In this study, 20 strains of *Salmonella* were used. They were isolated from two shrimp farms (*Litopenaeus vannamei*, adapted to fresh water). Strains were previously identified in the Enterobacteria Laboratory of *Fundação Oswaldo Cruz, Rio de Janeiro*. The farms (A and B), located in Jaguaruana - Ceara, Brazil, have the Jaguaribe River as their main water source, provided samples in which the following serovars and FIOCRUZ subspecies were identified: *S.* ser. Saintpaul (n=2), *S*. ser. Infantis (n= 3), *S*. ser. Panama (n= 2), *S*. ser. Madelia (n=5), *S*. ser. Braenderup (n=2), *S. enterica* subsp. *houtenae* (n=4) and *S*. *enterica* subsp. *enterica* (n=2).

Extraction of cromossomal DNA from *Salmonella* strains

Strains (n=20) were inoculated in brain heart infusion broth (BHI broth; Difco) and incubated at 35ºC for 24 hours. Parts of 1 mL were taken from the culture and processed for DNA extraction, using the commercial kit DNeasy Tissue (Qiagen).

Samples containing the amplified DNA were analyzed in agarose gel 1% (Pronadisa; CONDA). The run was performed in a horizontal electrophoresis tray (DIGEL; DGH12/DGH14) at 120V/500 mA during one hour in the buffer solution TBE 1 X. Agarose gel was dyed with "RedGel" (in the concentration indicated by the manufacturer) to make the amplified products visible in ultraviolet transilluminator (Espectroline-UV). Gel was documented in a Kodac EDAS290 digital photodocumentation system. A marker with molecular size of 1kb (Sigma) was used as standard molecular gene size.

Detection of *Salmonella* virulence genes

Primers used were *pefA* (plasmid virulence encoded by fimbria), *invA* (pathogenicity) and *spvC* (plasmid virulence). They were manufactured by INVITROGEN-BRASIL (Table 1).

The strain used as control in all amplifications was *Salmonella* ser. Enteritidis ATCC 13076.Total DNA extracted was amplified by Multiplex PCR in a themocycler - Techne.

Box-PCR Conditions

Total DNA of the cultures was extracted in accordance to the protocol established by Sambroock, Fritsch, and Maniatis (1989). Amplification of the 16S gene fragments of total DNA (PCR) was made with a specific primer BoxA1R (Versalovic, Schneider, Bruijn, & Lupski, 1994), synthesized by INVITROGEN - Brazil. Primer sequence and amplification parameters are indicated on Table 2. Reference strain used as control in all amplifications was *Salmonella* ser. Enteritidis ATCC 13076.

Tabela 1. Primers and thermocycling conditions used in the PCR for identification (*invA*) and pathogenicity (*pefA* and *spvC*) genes in *Salmonella* environmental isolates.

Genes	Primer sequence (5'- 3')	Amplicons (bp)[d]	Cycle	Thermocycling conditions	Source
pefA	F: gcgccgctcagccgaaccag R: gcagcagaagcccaggaaacagtg	157	1	94°C 3 min.$^{-1}$ 94°C 1 min.$^{-1}$	Trafny, Kozlowska, and Szpakowska (2006)
invA	F: acagtgctcgtttacgacctgaat R: agacgactggtactgatcgataat	244	30	58°C 1 min.$^{-1}$ 72°C 1 min.$^{-1}$	
spvC	F: actccttgcacaaccaaatgcgga R: tgtcttctgcatttcgccaccatca	571	1	72°C 5 min.$^{-1}$	

*bp:base pairs.

Table 2. Primers and themocycling conditions used in the BOX-PCR reaction.

Genes	Primers sequence (5'- 3')	Amplicons (bp)	Cycle	Thermocycling conditions	Source
BoxIAR	ctacggcaaggcgacgctgacg	400 to 2500	1	95°C 2 s^{-1}	Versalovic et al., (1994)
			30	94°C 3 s^{-1} 92°C 30 s^{-1} 50°C 1 $min.^{-1}$ 65°C 8 $min.^{-1}$	Albufera, Bhugaloo-Vial, Issack, & Jaufeerally-Fakim, (2009)
			1	65°C 8 $min.^{-1}$	

*bp:base pairs.

Samples containing the amplified DNA were analyzed in agarose gel 2% (Pronadisa; CONDA). The run was performed in a horizontal electrophoresis tray (DIGEL; DGH12/DGH14) at 120V/500 mA for five hours in the buffer solution TBE 1 X. Agarose gel was dyed with "RedGel" (in the concentration indicated by the manufacturer) in order to make the amplified products visible in the ultraviolet transilluminator (Espectroline-UV). Gel was documented in a Kodac EDAS290 digital photodocumentation system. A marker with molecular size of 2kb (Sigma) was used as the standard molecular gene size.

Similarity analysis

BOX-PCR profiles were analyzed with the help of the BioDiversity Professional Beta statistics software (Mcaleece, Gage, Lambshead, & Patterson, 1997), using the Jaccard similarity coefficient and UPGMA grouping algorithm.

Results and discussion

An analysis of the dendrogram (Figure 2) shows the formation of two groups. One of them contains three strains (n=1 serovar *S.* ser. Madelia and n=2 serovars *S.* ser. *enterica* subs. *houtenae*) isolated from the Jaguaribe River waters in farm A. The other comprises nine isolates from the Jaguaribe River water and from sediment on farm B, represented by *S.* ser. Saintpaul (n=2), *S.* ser. Infantis (n=3), *S.* ser. Panama (n=2), *S. enterica* subs. *enterica* (n =1) and *S.* ser. *enterica* subs. *houtenae* (n=1).

No correlation between the phenotipically identified serovars and the sampling site was verified. Environmental characteristics, such as sample source (water or sediment), were also not relevant in the establishment of a distribution model for *Salmonella* serovars. The group with the highest similarity index was composed by different serovars, shown in Table 4 and Figure 2, represented by strains (10, 4, 9, 16, 15, 14, 12 and 1), which share the same sample source (water samples).

In this large group, it is possible to verify subgroups with even higher similarity indexes: two subspecies of *S. enterica* subsp. *houtenae* (27, 28), isolated from the sediment on farm B, showed a 100% similarity profile. Likewise, serovars Madelia (14, 15 and 16) isolated from the same area presented comparable degrees of similarity. However, another Madelia serovar (13), isolated in the same conditions (water samples from farm B) showed a similarity coefficient little higher than 50% in relation to the isolates 14, 15 and 16. The similarity was independent of the source location and may indicate the presence of different clones.

In the observations of Albufera et al. (2009), from 18 *Salmonella* isolates from human and non-human sources (food), identified only as serogroups, the genomic profile of the majority of human isolates was different than those of non-human isolates. Wheeler, Cann, and Mackie (2011), analyzing the genomic fingerprinting and serotypes of *Salmonella* isolated from marine and land iguanas of two islands (Santa Fe and Plaza Sur) located in Galapagos, observed a low similarity in the ecotypes of *Salmonella* present in the islands. In relation to *S.* ser. Panama, the authors found a low similarity in the isolates of marine and land iguanas in the Plaza Sur island. They also highlight the low similarity in *Salmonella* ecotypes.

Virulence genes *pefA* and *invA* were detected in 10 and 12 serovars, respectively, while *spvC* gene was not found in any of the samples tested (Table 5).

Salmonella virulence is related to a combination of chromossomes and plasmidial factors (Oliveira et al., 2003). For Oliveira, Sola, Feistel, Moreira, and Oliveira (2013), pathogenicity in *Salmonella* sp. is complex and multifactorial. It may include genes that encode virulence factors, which are necessary in order to invade, colonize, survive, and multiply within the host to cause diseases.

Table 4. Serovar groups of *Salmonella* (A and B) isolated from water and sediment from two farms which have the Jaguaribe River as main water source, located in Jaguaruana, Fortaleza, Ceara State. Data was used to compose a similarity analysis dendrogram.

Origin	Sample	Strains	Groups	Identified serovars
Farm B	Sediment	19	Group A	*S*. ser. Braenderup
Farm A	Water	3	Group A	S.ser. Madelia
Farm B	Sediment	18	Group A	*S*. ser. Braenderup
Farm B	Sediment	22	Group A	*S. enterica* subsp. *enterica*
Farm B	Sediment	13	Group B	*S*.ser. Madelia
Farm B	Sediment	28	Group B	*S. enterica* subsp. *houtenae*
Farm B	Sediment	27	Group B	*S. enterica* subsp. *houtenae*
Farm A	Water	5	Group B	*S. enterica* subsp. *houtenae*
Farm A	Water	2	Group B	*S*. ser. Saintpaul
Farm B	Sediment	24	Group B	*S*. ser. Infantis
Farm B	Water	8	Group B	*S*. ser. Infantis
Farm B	Sediment	23	Group B	*S. enterica* subsp. *enterica*
Farm B	Water	10	Group B	*S*. ser. Infantis
Farm A	Water	4	Group B	*S. enterica* subsp. *houtenae*
Farm B	Water	9	Group B	*S*. ser. Panama
Farm B	Water	16	Group B	*S*. ser. Madelia
Farm B	Water	15	Group B	*S*. ser. Madelia
Farm B	Water	14	Group B	*S*. ser. Madelia
Farm B	Water	12	Group B	*S*. ser. Panama
Farm A	Water	1	Group B	*S*. ser. Saintpaul

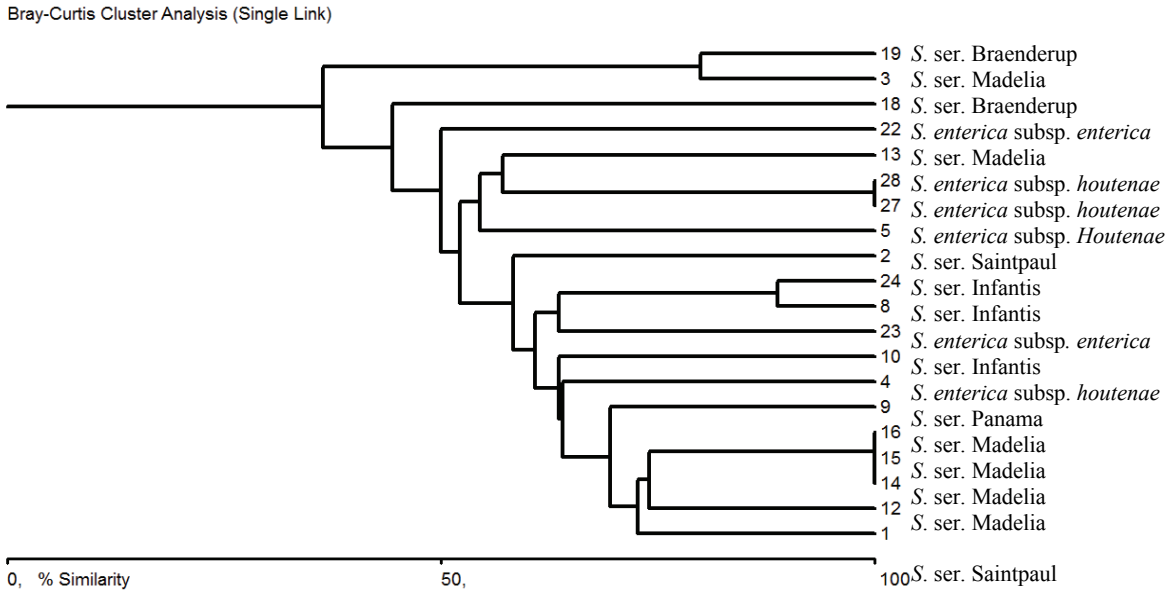

Figure 2. Dendrogram of *Salmonella* strains isolated from water samples from Jaguaribe River and sediment from two shrimp farms, A and B, located in Jaguaruana, Ceara State.

Table 5. Results of the Multiplex-PCR for the detection of *pefA*, *invA* and *spvC* virulence genes in *Salmonella* isolated from water samples from Jaguaribe River and sediment samples from two shrimp farms, A and B, located in Jaguaruana, Ceará State.

	Virulence genes detected in the PCR			
Isolates	Serovars/subspecies	*pefA*	*invA*	*spvC*
1; 2	*S*. ser. Saintpaul			
14; 15; 16	*S*. ser. Madelia			
24	*S*. ser. Infantis	-	+	-
27	*S. enterica* subsp. *houtenae*			
3	*S*. ser. Madelia			
5	*S. enterica* subsp. *houtenae*			
10	*S*. ser. Infantis	+	-	-
18; 19	*S*. ser. Braenderup			
4	*S. enterica* subsp. *houtenae*			
8	*S*. ser. Infantis			
9; 12	*S*. ser. Panama	+	+	-
22;23	*S. enterica* subsp. *enterica*			
13	*S*. ser. Madelia			
28	*S. enterica* subsp. *houtenae*	-	-	-

+-gene presence; - gene absence.

Turki, Ouzari, Mehri, Benaissa, and Hassen (2012), when determining the presence of virulence genes *invA* and *spvC* in strains of *S*. ser. Kentucky collected from environmental samples (residual water), animals, food and humans in Tunisia, found very similar results for *spvC* compared to those reported in the present study.

On other hand, Smith et al. (2010) in a research on the susceptibility profile to antimicrobials and genotypes of *S. enterica* isolated in clinical cases of salmonellosis in New Mexico in 2008, found gene *invA* in all isolates and *pefA* in only three. *Salmonella* can present genes related to invasion and virulence (*invA* and *spvC*), but are not always capable of expressing them. According to Craciunas, Keu, Flonta, and Cristea (2012) the gene *invA,* typical of

Salmonella, is conserved in the species and serovars and considered a direction mark when detecting *Salmonella*.

Oludairo, Kwaga, Dzikwi, and Kabir (2013), in a research about the detection of *invA* virulence genes by polymerase chain reaction (PCR) in *Salmonella* spp. isolated from wild animals in captivity, saw that the *invA* gene is not always present in strains of *Salmonella* spp. meaning that those in which the *invA* gene is absent are not virulent, unable to invade or cause infection.

Conclusion

This study was conducted with a limited number of isolates, however, different environmental sources and *Salmonella* serovars were represented. Our results suggest that the selective pressure exerted in different environmental compartments influences the genotypical profile of serovars, and the technique of Box-PCR provides more efficient results in differentiation of bacterial ecotypes.

Detection of genes related to virulence in environmental isolates represents the first step for the physiopathology in a case of salmonellosis in its spread through food (shrimp). Certainly, the biomonitoring process of these vehicles is a fundamental point to be adopted as a prevention measure, as well as for the minimization of economic losses in shrimp farming.

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