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Antibiotic Resistance in *Salmonella*: A Risk for Tropical Aquaculture

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1. Introduction

Salmonelas are rod-shaped, non-spore-forming Gram-negative facultative anaerobes measuring 0.7-1.5 by 2-5 μm. With the exception of the serovars Gallinarum and Pullorum, salmonelas are motile organisms. They are classified according to morphology and staining pattern and are divided into serotypes and serovars based on their reaction to somatic (O) and flagellar (H) antigens (Bremer et al., 2003). According to Kumar et al.(2003), the genus *Salmonella* has over 2,000 serovars. Two of these—Saintpaul and Newport—have been isolated from seafood (Ponce et al., 2008).

The prevalence of specific *Salmonella* serovars is related to food type. Thus, the serovars Weltevreden and Rissen are predominant in seafoods, as shown by Kumar et al. (2009) in a study on the distribution and phenotypical characterization of *Salmonella* serovars isolated from samples of fish, crustaceans and mollusks from India.

High incidences of *Salmonella* in seafoods have been reported worldwide (Kumar et al., 2010; Asai et al., 2008) in association with outbreaks of fever, nausea, vomiting and diarrhea (Ling et al., 2002). Since *Salmonella* inhabits the intestinal tract of warm-blooded animals, its presence in aquaculture livestock is most likely due to the introduction of fecal bacteria into culture ponds (Koonse et al., 2005). In fact, in a study on *Salmonella* in shrimp, Shabarinath et al. (2007) concluded this pathogen is generally found in rivers and marine/estuarine sediments exposed to fecal contamination.

The quality of aquaculture products may be compromised by exposure to pathogens and biological or chemical contaminants. The latter include chemical agents commonly used in aquaculture, such as veterinary antibiotics, antiseptics and anesthetics. Few antibiotics have been adapted to or developed specifically for use in aquatic organisms. Thus, in Europe several classes of antibiotics may be used in aquaculture, including sulfonamides, quinolones, macrolides, tetracyclines and emamectin. This, however, poses a considerable risk of release of antimicrobials into the environment and eventually of the development of resistance in pathogenic bacteria (Fauconneau, 2002).

The second half of the 20th century saw two major events in the epidemiology of salmonellosis: the appearance of human infections caused by food-borne *S. enteritidis* and by *Salmonella* strains with multiple resistance (Velge et al., 2005). In fact, Angulo et al. (2000) suggested that the factors determining resistance to multiple antibiotics in strains of *S.* Typhimurium DT104 may first have developed in bacteria in the aquaculture environment, possibly as the result of the regular use of antibiotics in fodder.

The present study is a review of the literature on resistant *Salmonella* strains in aquaculture and an assessment of the risk this represents for human health. In addition, information was collected on the incidence of resistant *Salmonella* strains isolated from shrimp farm environments in Northeastern Brazil.

2. Methods of isolation, identification and evaluation of antibacterial susceptibility in *Salmonella*

2.1 Isolation and identification of *Salmonella*

Salmonella may be detected in samples from aquaculture environments using the traditional method described by Andrews and Hammack (2011). The method includes pre-enrichment of 25-g aliquots in lactose broth, selective enrichment in broth (*eg*, tetrathionate and Rappaport-Vassiliadis or tetrathionate and selenite cystine) and selective plating on MacConkey and Hektoen enteric agar. Typical *Salmonella* colonies grown during the selective enrichment stage are screened biochemically with triple sugar iron agar (TSI), lysine iron agar (LIA) or sulfide indole motility agar (SIM). Serotyping is done with commercially available antisera (Koonse et al., 2005), O:H polyvalent antiserum (Carvalho et al., 2009) or somatic (O), flagellar (H) and capsular (Vi) antisera (Kumar et al, 2009).

In addition, molecular biology techniques may be used for rapid detection of *Salmonella* in foods: TaqMan PCR (Kimura et al., 1999), PCR amplification of a 152-bp segment of the gene *hns* (Kumar et al., 2003), real-time PCR (Malorny et al., 2004), PCR, dot blot hybridization, RAPD and ERIC-PCR (Shabarinath et al., 2007), PCR amplification of the gene *invA* (Upadhyay et al., 2010) and uniplex and multiplex PCR (Raj et al., 2011).

2.2 Antibiogram, MIC and plasmid curing

The phenotypical susceptibility of *Salmonella* to antibiotics may be determined by the method of disk diffusion on Muller-Hinton agar (Kha et al., 2006). When testing salmonellas from aquaculture environments, the selection of antibiotics depends on the origin of the isolates, but usually covers a range of families, including the tetracyclines, sulfonamides, quinolones, macrolides and aminoglycosides (Ponce et al., 2008; Carvalho et al., 2009). The classification of bacteria according to susceptibility or resistance to antibiotics is based on the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2009). The antibacterial resistance index (ARI) may be calculated following the recommendations of Jones et al. (1986). Multiple antibacterial resistance (MAR) may be calculated using the methodology described in Krumperman (1983).

Antibacterial susceptibility may also be estimated by determining the minimum inhibitory concentration (MIC) based on macrodilution of Mueller-Hinton broth (CLSI, 2009).

Salmonella strains with phenotypical profile of antibacterial resistance may be submitted to plasmid curing in Luria-Bertani broth supplemented with acridine orange dye at 100 μg·mL^{-1}. The method makes it possible to determine whether resistance stems from chromosomal or plasmidial elements (Molina-Aja et al., 2002).

2.3 Determination of resistance genes and plasmid profile

Polymerase chain reaction (PCR) has been used to detect genes encoding resistance to tetracycline in *Salmonella* strains from fish farms. Restriction enzymes used in PCR include *Sma*I (for detecting the gene *tetA*), *Sal*I (for *tetC*), *Sph*I (for *tetB*, *tetD* and *tetY*), *Eco*RI (for G) and *Nde*II (for *tetE*, *tetH* and *tetI*) (Furushita et al., 2003).

The extraction of plasmidial DNA from salmonelas is usually done by alkaline lysis, as proposed by Birnboim and Doly (1979), with or without modification, or with acidic phenol, as described by Wang and Rossman (1994). For small plasmids, the extraction product may be submitted to electrophoresis in 1% agarose gel following the protocol of Akiyama et al. (2011). The protocol for electrophoresis of mega-plasmid DNA molecules in 1% agarose gel is described in Ponce et al. (2008).

3. Results

3.1 *Salmonella* in tropical aquaculture

Salmonelas are recognized worldwide as one of the main etiological agents of gastroenteritis in humans. Despite variations in the regulation of microbiological quality of foods around the world, the largest importers of seafoods only buy products completely free from *Salmonella*, based on the claim that salmonelas are not part of the indigenous microbiota of aquatic environments and that, therefore, the presence of salmonelas in aquatic organisms is associated with poor sanitation and inadequate hygiene practices (Dalsgaard, 1998).

Several studies published in the 1990s reported *Salmonella* in shrimp farming environments in tropical countries. Reilly and Twiddy (1992) found *Salmonella* in 16% of their shrimp samples and 22.1% of their pond water and sediment samples collected on farms in Southeast Asia. Weltevreden was the most abundant *Salmonella* serovar identified, followed by Anatum, Wandsworth and Potsdam. According to the authors, the incidence of *Salmonella* was higher in ponds located near urban areas and, not surprisingly, the bacterial load increased during the rainy season. Bhaskar et al. (1995) detected *Salmonella* in 37.5%, 28.6% and 37.4% of shrimp, sediment and water samples, respectively, collected from semi-intensive grow-out ponds in India.

In contrast, despite detecting high indices of thermotolerant and total coliforms, Dalsgaard et al. (1995) found no *Salmonella* in water, sediment and shrimp samples from sixteen different penaeid shrimp farms in Thailand.

Hatha and Rao (1998) reported only one *Salmonella*-positive sample out of 1,264 raw shrimp. They believed the presence of the bacteria was due to pond contamination from different sources, including the use of untreated fertilizer of animal origin. Likewise, Hatha et al. (2003) found the incidence of *Salmonella* to be low in shrimp farm products exported by India.

Koonse et al. (2005) investigated the prevalence of *Salmonella* in six major shrimp-producing countries in Southeast Asia (n=2), Central Asia (n=1), Central America (n=1), North America

(n=1) and the Pacific (n=1). In four of these countries, *Salmonella* was detected in 1.6% of shrimp samples, and two serovars were identified (Paratyphi B var. Java and Weltevreden Z6). The authors highlighted the need to control or eliminate potential sources of fecal matter polluting the water bodies adjacent to the grow-out ponds.

In Brazil, the microbiological quality of shrimp (*Litopenaeus vannamei*) farmed in Ceará was evaluated by Parente et al. (2011) and Carvalho et al. (2009), both of whom detected *Salmonella* in shrimp and water samples (Table 1). The authors associated the presence of salmonelas with discharge of fecal matter into the respective estuaries where the farms are located. The detection of *Salmonella* in estuaries in Ceará is not an isolated finding. Farias et al. (2010) found salmonelas in samples of the bivalve *Tagelus plebeius* collected in the estuary of the Ceará river and identified the serovars Bredeny, London and Muechen. Similar findings were reported by Silva et al. (2003) in a study on *Salmonella* in the oyster *Crassostrea rhizophorae* obtained from natural oyster grounds in the estuary of the Cocó river, on the outskirts of Fortaleza, Ceará.

Country	Sample	N°	Sorovars	Source
Brazil	Water and Shrimp	3	*S.* ser. Saintpaul e *S.* ser. Newport	Parente et al. (2011)
Brazil	Fish	30	*S.* ser. Agona, *S.* ser. Albany, *S.* ser. Anatum, *S.* ser. Brandenburg, *S.* ser. Bredeney, *S.* ser. Cerro, *S.* ser. Enteretidis, *S.* ser. Havana, *S.* ser. Infantis, *S.* ser. Livingstone, *S.* ser.London, *S.* ser. Mbandaka, *S.* ser. Muenchen, *S.* ser. Newport, *S.* ser. Saintpaul, *S.* ser. Thompson, *S.* ser. O4,5:i:-, *S.* ser. O4,5:-:1,7, *S.* O:17	Ribeiro et al., 2010
Brazil	Water, Sediment and Shrimp	23	*S.* ser. Anatum, *S.* ser. Newport, *S.* ser. Soahanina e *S.* ser. Albany	Carvalho et al. (2009)
Vietnam	Shrimp	29	*S.* ser. Bovismorbificans, *S.* ser. Derby, *S.* ser. Dessau, *S.* ser. Lexington, *S.* ser. Schleissheim, *S.* ser. Tennessee, *S.* ser. Thompson, *S.* ser. Virchow, *S.* ser. Weltevreden, *S.* ser. II heilbron	Ogasawara et al. (2008)
India	Shrimp	54	*S.* ser. Bareilly, *S.* ser. Braenderup, *S.* ser. Brancaster, *S.* ser. Derby, *S.* ser. Kottbus, *S.* ser. Lindenburg, *S.* ser. Mbandaka, *S.* ser. Oslo, *S.* ser. Rissen, *S.* ser. Takoradi, *S.* ser. Typhi, *S.* ser. Typhimurium, *S.* ser. Weltevreden, *Salmonella* VI	Kumar et al. (2009)

*Nº: number of positive samples.

Table 1. *Salmonella* in tropical seafood.

Thus, Shabarinath et al. (2007), who also detected *Salmonella* in shrimp, concluded that since salmonelas inhabit the intestinal tract of warm-blooded animals, their presence in rivers and in marine/estuarine sediments exposed to fecal contamination is not surprising.

Tropical fish species may also be infected with salmonelas (Ponce et al., 2008; Heinitz et al., 2000; Ogbondeminu, 1993); in fact, microorganisms of this genus have recently been associated with farmed catfish (McCoy et al., 2011).

3.2 Antimicrobial susceptibility profile of *Salmonella*

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). Thus, Le and Munekage (2005) reported high levels of drug residues (sulfamethoxazole, 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.

According to Seyfried et al. (2010), autochthonous communities in aquatic environments may serve as a reservoir for elements of antibacterial resistance. However, the contribution of anthropic activities to the development of such reserves has not been fully clarified.

Holmström et al. (2003) reported the use, often indiscriminate, of large amounts of antibiotics on shrimp farms in Thailand, and concluded that at a regional scale human health and the environmental balance may be influenced by such practices. Adding to the impact, many of the antibiotics used for prophylaxis in shrimp farming are very persistent and toxic.

Heuer et al. (2009) presented a list of the major antibacterials used in aquaculture and their respective routes of administration: amoxicillin (oral), ampicillin (oral), chloramphenicol (oral, bath, injection), florfenicol (oral), erythromycin (oral, bath, injection), streptomycin (bath), neomycin (bath), furazolidone (oral, bath), nitrofurantoin (oral), oxolinic acid (oral), enrofloxacin (oral, bath), flumequine (oral), oxytetracycline (oral, bath, injection), chlortetracycline (oral, bath, injection), tetracycline (oral, bath, injection) and sulfonamides (oral).

Current aquaculture practices can potentially impact human health in variable, far-reaching and geographically specific ways. On the other hand, the increasing flow of aquaculture products traded on the global market exposes consumers to contaminants, some of which from production areas (Sapkota et al., 2008).

Antibacterial susceptibility in microorganisms associated with aquaculture livestock is an increasingly frequent topic in the specialized literature (Molina-Aja et al., 2002; Peirano et al., 2006; Akinbowale et al., 2006; Costa et al., 2008; Newaja-Fyzul et al., 2008; Dang et al., 2009; Del Cerro et al., 2010; Fernández-Alarcón et al., 2010; Patra et al., 2010; Vieira et al., 2010; Tamminem et al., 2011; Laganà et al., 2011; Millanao et al., 2011; Rebouças et al., 2011; Dang et al., 2011).

In this respect, salmonelas are one of the most extensively investigated groups of intestinal bacteria. Thus, in China salmonelas isolated from fish ponds were resistant to ampicillin

(20%), erythromycin (100%), cotrimoxazole (20%), gentamicin (20%), nalidixic acid (40%), penicillin (100%), streptomycin (20%), sulfanomides (40%), tetracycline (40%) and trimethoprim (20%) (Broughton and Walker, 2009).

Ubeyratne et al. (2008) detected *Salmonella* resistant to erythromycin, amoxicillin and sulfonamides in shrimp (*Penaeus monodon*) farmed in Sri Lanka. Likewise, Ogasawara et al. (2008) found salmonelas resistant to oxytetracycline and chloramphenicol in Vietnamese shrimp samples but concluded ARI values were not as high as in neighboring or developing countries.

Low ARI values were also reported by Boinapally and Jiang (2007) who in a single sample of shrimp imported to the US detected *Salmonella* resistant to ampicillin, ceftriaxone, gentamicin, streptomycin and trimethoprim. This is in accordance with published findings for shrimp in tropical regions, where the major exporters of farmed shrimp are located.

Zhao et al. (2003) evaluated the profile of antibacterial resistance in salmonelas isolated from seafood from different countries and found that most of the resistant bacteria came from Southeast Asia. The authors believe the use of antibiotics in aquaculture, especially in Southeast Asia, favors the selection of resistant *Salmonella* strains which may find their way into the US market of imported foods.

In Brazil, Ribeiro et al. (2010) reported an antibacterial resistance index of 15.1% among salmonelas isolated from an aquaculture system. The *Salmonella* serovars Mbandaka (n=1) and Agona (n=2) were resistant to tetracycline, Albany (n=1) was resistant to sulfamethoxazole-trimethoprim, and London (n=2) was resistant to chloramphenicol. In addition, Carvalho et al. (2009) collected samples from three penaeid shrimp farms in Ceará (Northeastern Brazil) and found *Salmonella* serovars Newport and Anatum to be resistant to tetracycline and nalidixic acid. Water and sediment samples collected in the vicinity of the three farms contained the *Salmonella* serovars Newport, Soahanina, Albany and Anatum, which were likewise resistant to tetracycline and nalidixic acid, suggesting the ponds were contaminated by water drawn from the estuaries.

Bacterial resistance in *Salmonella* may be of either chromosomal or plasmidial nature (Frech e Schwarz, 1999; Mirza et al., 2000; Govender et al., 2009; Tamang et al., 2011; Glenn et al., 2011). In bacteria, the acquisition and diffusion of resistance genes may be influenced by exchanges of DNA mediated by conjugative plasmids and by the integration of resistance genes into specialized genetic elements (Carattoli et al., 2003).

Evidence of plasmidial mediation of antibacterial resistance in *Salmonella* has been available since the 1970s and 1980s (Anderson e Threlfall, 1974; Frost et al., 1982). Thus, Anderson et al. (1977) detected three types of resistance plasmids in *Salmonella* strains from different countries. According to the authors, plasmids of the F_{Ime} type confer resistance to penicillin, ampicillin and streptomycin, whereas, for example, resistance to furazolidone in all *Salmonella* isolates from Israel was considered to be chromosomal. Mohan et al. (1995) determined the plasmid profile of *Salmonella* strains isolated from different regions in India and found a large diversity of small plasmids (2.7 to 8.3 kb) in strains resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole, tetracycline and trimethoprim.

In one study, salmonelas isolated from food animals were found to carry CMY-2, a plasmid-mediated AmpC-like β-lactamase (Winokur et al., 2001). Doublet et al. (2004) found *florR* (a florfenicol resistance gene) and bla_{CMY-2} plasmids to be responsible for resistance to wide-spectrum cephalosporines in salmonelas isolated from clinical samples, animals and foods in the US. The authors added that the use of phenicols in animal farming environments may place a selective pressure on organisms and favor the dissemination of bla_{CMY-2} plasmids. In addition, *florR* is known to confer cross-resistance to chloramphenicol.

Kumar et al. (2010) found evidence that tropical seafood can serve as vehicle for resistant salmonela strains, some of which resistant to as many as four antibiotics (sulfamethizole, carbenicillin, oxytetracycline and nalidixic acid). The authors also identified low-molecular-weight plasmids in the *Salmonella* serovars Braenderup, Lindenburg and Mbandaka.

Six isolates of *Salmonella* serovar Saintpaul from samples of shrimp and fish from India, Vietnam and Saudi Arabia presented one or more resistance plasmids of varying size (2.9 to 86 kb). One of these carried a *Incl1* plasmid (Akiyama et al., 2011).

As discussed above, the indiscriminate use of antibiotics in aquaculture is one of the major causes of the emergence of resistant bacteria in the environment. Several of the mechanisms of resistance in *Salmonella* have been investigated, especially with regard to beta-lactams (Alcaine et al., 2007) and quinolones (Piddock et al., 1998; Piddock, 2002)—two families of antibiotics widely used in aquaculture.

4. Conclusion

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). Human health and environmental balance are further threatened by the emergence of salmonelas resistant to antibiotics employed in farming, in some cases mediated by mobile genetic elements. The elimination of sources of fecal pollution from tropical areas used for aquaculture seems to be the main strategy for minimizing the risk of transference of salmonelas to foods destined for human consumption. As a final consideration, studies should be encouraged on the presence, antibacterial susceptibility and mechanisms of resistance in salmonelas occurring in tropical areas destined for culture of fish, crustaceans and mollusks.

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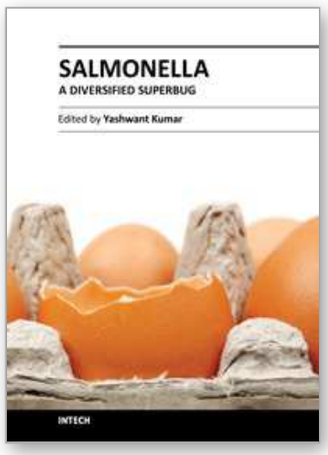

Salmonella - A Diversified Superbug
Edited by Mr. Yashwant Kumar

ISBN 978-953-307-781-9
Hard cover, 576 pages
Publisher InTech
Published online 20, January, 2012
Published in print edition January, 2012

Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Renata Albuquerque Costa, Fátima Cristiane Teles de Carvalho and Regine Helena Silva dos Fernandes Vieira (2012). Antibiotic Resistance in Salmonella: A Risk for Tropical Aquaculture, Salmonella - A Diversified Superbug, Mr. Yashwant Kumar (Ed.), ISBN: 978-953-307-781-9, InTech, Available from: http://www.intechopen.com/books/salmonella-a-diversified-superbug/antibiotic-resistance-in-salmonella-a-risk-for-tropical-aquaculture

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