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Molecular epidemiology and development of vaccines against emerging pathogens for Brazilian fish farming: *Streptococcus dysgalactiae* and *Weissella ceti*

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"…aprendei de mim, que sou manso e humilde de coração."
Jesus (Mateus, 11:29)

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SUMMARY

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ABBREVIATION LIST

bp	base pair
BSFGE	Biased sinusoidal gel electrophoresis
CFU	Colony forming units
d.p.v.	days post-vaccination
ERIC	Enterobacterial repetitive intergenic consensus
g	gram
GCSD	Group C *Streptococcus dysgalactiae*
HDL	High-density lipoprotein
IP	Intraperitoneal
L	Litre
MLST	Multi-locus sequence typing
MRS	Man, Rogosa and Sharpe
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
REP	Repetitive extragenic palindromic
RPS	Relative percent survival
SOF	Serum opacity factor
THA	Todd Hewitt agar
THB	Todd Hewitt broth

ABSTRACT

Streptococcus dysgalactiae and *Weissella ceti* have been considered emergent pathogens for Brazilian Nile tilapia (*Oreochromis niloticus*) and rainbow trout (*Onchorhynchus mykiss*) farming, respectively. Both diseases cause great economic losses in fish farms. Disease prevention by vaccination is a fundamental method for pathogen control in aquaculture. The aims of this study were to evaluate the genetic diversity of *S. dysgalactiae* and *W. ceti* strains isolated from distinct geographic origins and to develop efficient vaccines against these pathogens. Different methods (REP-PCR, ERIC-PCR and PFGE) were used for the genetically characterization of strains of both bacteria, and *sodA* gene sequencing was also tested for genotyping of *S. dysgalactiae* isolates. Aqueous-based whole cell killed bacterin and oil-adjuvanted vaccine were tested. The PFGE was the best genotyping method for *S. dysgalactiae* and detected three different genetic profiles among the evaluated strains. The genetic variability of patterns was clearly associated with the geographic origin of isolates. According to the methods used, Brazilian *W. ceti* isolates was found to be highly homogeneous with a clonal population. Vaccines formulated with whole cell bacterin emulsified in oil adjuvant provided significant protection against *S. dysgalactiae* (RPS = 92,5) and *W. ceti* (RPS = 92,0) infections in Nile tilapia and rainbow trout, respectively. The present results provide scientific information for development of adequate methods to control two emerging infectious diseases that threaten Brazilian fish farming industry.

Keywords: Genotyping; vaccine; aquaculture.

RESUMO

As bactérias *Streptococcus dysgalactiae* e *Weissella ceti* são consideradas patógenos emergentes para a piscicultura nacional, causando perdas significativas nas cadeias produtivas de tilápia do Nilo (*Oreochromis niloticus*) e truta arco-íris (*Onchorhynchus mykiss*), respectivamente. Os objetivos do presente estudo foram avaliar a diversidade genética de amostras de *S. dysgalactiae* e *W. ceti* provenientes de regiões geográficas diversas e desenvolver vacinas eficazes na proteção contra esses patógenos. Diferentes métodos (REP-PCR, ERIC-PCR e PFGE) foram utilizados para caracterização genética das bactérias. A análise do sequenciamento do gene *sodA* foi utilizada somente para a genotipagem de amostras de *S. dysgalactiae*. Duas diferentes vacinas foram testadas: bacterina e bacterina emulsificada com adjuvante Montanide. A técnica de PFGE foi a mais eficaz para a genotipagem dos isolados de *S. dysgalactiae*, detectando três perfis genéticos diferentes. Os padrões genéticos estão associados com a origem geográfica dos isolados. De acordo com os métodos testados, as amostras brasileiras de *W. ceti* apresentam alta similaridade genética, sendo consideradas clonalmente relacionadas. As vacinas formuladas a partir de células inteiras inativadas (bacterina) emulsificadas em adjuvante oleoso conferiram proteção frente as infecções por *S. dysgalactiae* (RPS = 92,5) e *W. ceti* (RPS = 92,0) em tilápia do Nilo e truta arco-íris, respectivamente. Os resultados do presente projeto fornecem informações científicas e ferramentas fundamentais para o controle de duas doenças infecciosas emergentes da piscicultura nacional.

Palavras-chaves: Genotipagem; vacina; aquicultura.

1 – CHAPTER 1

INTRODUCTION

Global aquaculture production has continued to growth over the last years, while the capture production has levelled off. Therefore, the contribution of aquaculture to world food fish production for human consumption is growing each year. In Brazil, the scenario is not different: driven by population growth, rising demand for fish food and a stabilization of production from capture fisheries, the practice of farming aquatic animals becomes an interesting economic sector of the agribusiness.

The rapid growth of aquaculture in Brazil, mainly fish farming, has been associated with culture in high density of fish stocks and animals exposed to environmental stress. The consequence has been the emergence and spread of an increasing number of infectious diseases. In this sense, the Laboratory of Aquatic Animals Diseases (AQUAVET/EV-UFMG) is performing, since 2002, the diagnostic and research of the main infectious pathogens of the Brazilian aquaculture.

In the last years, two emerging diseases have been diagnosed causing economic impact to fish farming in many countries. *Streptococcus dysgalactiae* and *Weissella ceti* are causing substantial losses among farms of Nile tilapia (*Oreochromis niloticus*) and rainbow trout (*Onchorhynchus mykiss*), respectively.

Streptococcal infections have become an increasing problem in cultured fish around the world. In Brazil disease caused by bacteria from the genus *Streptococcus*, mainly *S. agalactiae*, is one of the major threats to Nile tilapia farming, the major fish species produced in the country. *Streptococcus dysgalactiae* was first described as a pathogen of fish during outbreaks in marine fish cultured (amberjack and yellowtail) in Japan. Recently a disease characterized by moderate mortality and presence of abscesses, in musculature of Nile tilapia in Brazil, is attributed to *S. dysgalactiae* infection.

Weissella ceti infections were first described in fish in 2009. Chinese researchers accompanied outbreaks in a rainbow trout farm and the disease was attributed to *W. ceti*. In the same period, our group isolated strains of this pathogen from diseased rainbow trout from farms located in different Brazilian states (São Paulo, Minas Gerais and Rio de Janeiro). Outbreaks were characterized by high mortality rates and the main clinical signs in diseased fish were lethargy, anorexia, melanosis, exophthalmia, ascites, and haemorrhages in the mouth, tongue, eyes, and fins. The disease affected fingerling, juvenile, and adult fish, and occurred during the summer, when water temperature usually is above 17 ºC.

The main method used to control infectious diseases in fish farming is the oral antibiotic therapy. However, this approach is not always effective to control infectious fish diseases because anorexia is present as the first clinical sign of illness. Therefore, disease prevention by vaccination is on economic, environmental and ethical grounds the most adequate method for pathogen control currently available to the aquaculture industry worldwide.

The development of efficient vaccines to prevent *Streptococcus dysgalactiae* and *Weissella ceti* infections in Nile tilapia and rainbow trout, respectively, is one of the main points to control these diseases. To design new vaccines, the identification of molecular features of strains is essential to understand whether a genetic variation implies in an antigenic diversity. Hence, studies on molecular characterization of field isolates of *S. dysgalactiae* and *W. ceti* occurring in Brazil is important to implement efficient control management practices. Furthermore, the genotyping of strains is also essential to understand pathogens distribution and determine if epidemiologically related isolates are genetically related strains pose a clonal structure or not.

In Brazil, there are no licensed vaccines for fish against both pathogens. Herein, the development of vaccines using whole cell antigens from Brazilian strains of *S. dysgalactiae* and *W. ceti* to be tested against

isolates from different national regions are essential to evaluate the effectiveness of this immunoprophylactic method. Furthermore, there are no studies about the relationship among genetic variability, origin of isolates and vaccine protection against different strains for both bacteria.

Objectives

The aims of this study were to evaluate the genetic diversity of *S. dysgalactiae* and *W. ceti* strains isolated from different geographic origins in Brazil; and to develop vaccines to protect against these pathogens infections in Nile tilapia and rainbow trout farms, respectively.

2 – CHAPTER 2

LITERATURE REVIEW

2.1 Brazilian aquaculture

Fish and fishery products are among the most traded food commodities worldwide, with developing countries leading the bulk of world exports. While capture fisheries production remains stable, aquaculture production continues to expand. Aquaculture is one of the fastest-growing animal food-producing sectors and, in the next decade, total production from both capture and aquaculture will exceed that of beef, pork or poultry. In 2011, the total world of fisheries production was 154 million tonnes of which aquaculture contributed with 63,9 million tonnes or about 41,3% of the total world production (FAO, 2012).

Brazil is one of the countries with the highest growth in aquaculture worldwide, and with a further potential to growth due to a coastline of 8,500 km, and 12% of the world's freshwater reserves. Freshwater reservoirs are mainly representing by 10 million hectares of water, which are associated with hydroelectric production (BRASIL, 2012; FAO, 2012).

The average annual growth rate for Brazilian aquaculture production from 2009 to 2011 was 51,2% per cent. The total aquaculture production, in 2011, was 628.704 tonnes of which freshwater fish farming accounts for 86,6% (544.490 tonnes) of total. From 2010 to 2011 the freshwater fish production increased significantly, with a growth rate of 38%. However, the Brazilian domestic consumption of fish increased in the last years, and the national fisheries production is still not enough to supply this market. In 2011, Brazil exported 42 million tonnes (US$ 271 million) and imported 349 million tonnes of fish (US$ 1.262 million), with a trade deficit of US$ 991 million, approximately (BRASIL, 2012).

The main fish species cultured in Brazil is the Nile tilapia (*Oreochromis niloticus*). About a half of freshwater fish production comes from Nile tilapia farms. It is an omnivorous tropical fish species with optimal temperature ranging from 28 to 35ºC. Nile tilapia is considered suitable for culture because the high tolerance to adverse conditions, relative fast growth and the well stablished protocols for breeding (Guerrero, 1985). In 2011, the total production of Nile tilapia in Brazil was 253.824 tonnes (BRASIL, 2012).

Another exotic fish species cultured in Brazil is the rainbow trout (*Onchorhynchus mykiss*). It is a species of salmonid, native to the Pacific drainages of North America, with optimal temperature ranging from 9 to 15ºC. The rainbow trout is a hardy fish that is easy to spawn and fast growing. The species has a high demand of dissolved oxygen in the water, and the lowest concentration survival is 3 ppm (Hardy *et al*., 2000). The rainbow trout production in Brazil is restricted to some regions (cold and hilly areas of South and Southeast), due its requirement for a cold water. The total production of rainbow trout in Brazil was 3.277 tonnes in 2011 (BRASIL, 2012). Despite this small production, rainbow trout has a high market value compared to other cultured fish species.

2.2 Emerging pathogens for fish farming

Emerging disease epizootics frequently cause substantial losses among populations of fish, resulting in large economic losses in commercial aquaculture (USDA, 1991; Ghittino *et al.*, 2003). The increasing rate of

fish diseases has been driven by anthropogenic influences associated with growing of global aquaculture. Farming of aquatic animals commonly involves a stressful environment, the use of artificial feeds, and culture in high stocking densities. This has provided opportunities for exposure to pathogens and conditions that can compromise the fish immune system, and also facilitate pathogen replication and transmission. In addition, the growth in aquaculture and increasing international trade has resulted in the rapid movement of aquatic animals, with associated risks of the transmission of pathogens (Noga, 1996; Walker and Winton, 2010).

Apparent changes in the prevalence and distribution of fish diseases can also be explained by the improved surveillance as a result of the development of more sensitive molecular diagnostic methods and greatly growth in aquatic animal health researches (Eiras, 2008).

By concept an emerging disease can be defined as: a new or previously unknown disease; a known disease appearing for first time in a new species (expanding host range); a known disease in a new location (expanding geographic range); and/or new presentation (different signs) of a known disease due changes in the pathogen, mainly higher virulence (Walker, 2004).

The impacts of emerging pathogens and the extent of diseases spread are often exacerbated by some problems that are typically encountered including: delay in developing tools for the confirmatory diagnosis of disease; poor knowledge of the current or potential host range; inadequate knowledge of the present geographic range; no understanding of critical epidemiological factors; and poor understanding of genetic differences among strains and relationships to the epidemiological factors of disease (Walker and Winton, 2010). Therefore, knowledge of emerging fish diseases is of great relevance, both from a scientific as well as an applied point of view. Detailed knowledge of the emerging pathogens, their interactions with fish as well as the main responses of the host is a pre-requisite for prevention and treatment of fish diseases (Eiras, 2008).

2.2.1 *Streptococcus dysgalactiae*

The taxonomic of the genus Streptococci was first described by Rosenbach, 1884 (Hardie, 1986), and recently reviewed by Facklam (2002). Lancefield (1933) reported specific technique that is used to demonstrate specific carbohydrate "group" antigens.

Streptococcosis was first described as a fish disease during an outbreak in a rainbow trout (*Oncorhynchus mykiss*) farm in Japan, in 1956 (Hoshina *et al*., 1958). Since that, Streptococossis is used as a generic term for some septicaemia infections in fish, caused by Gram-positive cocci. Four genera are associated with diseases in aquatic animals*: Streptococcus, Lactococcus, Vagococcus* and *Carnobacterium* (Mata et al., 2004). In the last years Streptococcosis has increased in importance, with outbreaks occurring in numerous fish species and other aquatic animals (Agnew and Barnes, 2007; Romalde *et al.*, 2008; Netto *et al.,* 2011).

Streptococcosis outbreaks have been considered one of the major barriers to the development of world fish farming (Ghittino *et al.*, 2003; Austin and Austin, 2007). Unofficial data estimate that annual losses arising from Streptococcosis outbreaks exceed the value of US$ 150 million (Romalde *et al*., 2008). In Brazil, three species of *Streptococcus* were described as aetiological agents causing septicaemia in Nile tilapia (*Oreochromis niloticus*): *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus iniae* (Figueiredo *et al*., 2012a; Godoy *et al.,* 2013; Mian *et al*., 2009; Netto *et al*., 2011; Salvador *et al*., 2003, 2005). *Streptococcus agalactiae* infections are probably the most prevalent, and have been reported in farms located in several states of the country (Mian *et al.,* 2009).

Streptococcus dysgalactiae is divided into two subspecies: *S. dysgalactiae* subsp. *equisimilis*, an important human pathogen; and *S. dysgalactiae* susbsp. *dysgalactiae*, a strictly animal pathogen (Suzuki *et al.,*

2011). This division was based on electrophoresis of cell wall proteins and physiological tests proposed by Vandamme *et al.* (1996). Therefore, the name *S. dysgalactiae* subsp. *dysgalactiae* was proposed for strains of animal origin that belong to Lancefield Group C *Streptococcus* (GCS) and Group L (GLS).

Lancefield group C *Streptococcus dysgalactiae* (GCSD) is mainly associated with endometritis, subclinical or clinical mastitis, subcutaneous cellulitis, and toxic shock-like syndrome in bovine (Seno and Azuma, 1983; Aarestrup and Jensen, 1996). The same pathogen is reported as a cause of bacteremia, meningoencephalitis and mastitis in sheep (Scott, 2000; Chenier *et al.,* 2008).

The first report of GCSD in fish occurred in 2002 in Japan. The bacterium was isolated from two cultured marine fish species, amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*), which were displaying necrotic lesions of the caudal peduncle (Nomoto *et al*., 2004). Since that, *S. dysgalactiae* has been associated with severe necrosis lesions in musculature and mortalities of marine fish species as kingfish (*Seriola lalandi*), grey mullet (*Mugil cephalus*), basket mullet (*Liza alata*), soiny mullet (*Liza haematocheila*), amur sturgeon (*Acipenser schrenckii*), and cobia (*Rachycentron canadum*) (Nomoto *et al.,* 2006; Abdelsalam *et al.,* 2009; Yang and Li, 2009; Qi *et al*., 2013).

GCSD has been identified as fish specific pathogen based on 16S rDNA sequence analysis, *sodA* and *tuf* genes sequence analysis, biased sinusoidal field gel electrophoresis and Lancefield grouping. Some biochemical characteristics of the fish isolates were different from those of the typical reference of mammalian strains *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* (Nomoto *et al.*, 2008; Nishiki *et al.,* 2010).

In 2007, Netto *et al.* (2011) investigated a disease outbreak in a Nile tilapia farm located in Ceará State, Brazil. Diseased fish showed clinical signs of septicaemia and subcutaneous abscesses in the caudal region. From diseased Nile tilapia it was successfully isolated *Streptococcus dysgalactiae* strains and it was described the first infection of GCSD in freshwater fish. In the same study, the disease was reproduced in experimental infections using the same host. However, they were unable to reproduce the typical clinical sign of subcutaneous abscesses of *S. dysgalactiae*. In addition, Netto *et al.* (2011) used PFGE to genotype GCSD strains and the analysis showed that isolates belonged to a single pulsotype. This result could be explained by the restricted geographical (a single outbreak in one farm) and contemporary origin of the isolates analyzed.

The search for a competent vaccine against *S. dysgalactiae* is hampered by the short knowledge about its pathogenesis and virulence determinants. The virulence factors of GCSD are mainly based on its cell surface properties such as high hemagglutination and hydrophobic properties, which determine the main adhesion and invasive pathogenic mechanism of the pathogen. This has been confirmed by Abdelsalam *et al.* (2009), who have indicated that GCSD isolates were able to adhere to and invade fish epithelial cell line (EPC: Epithelial papiloma of Carp and CHSE-214: Chinook salmon embryo) cultured *in vitro*. Besides, the pyrogenic, exotoxin G, a superantigen, and streptolysin S genes are regarded as the most important virulence traits, with cultures recovered from diseased fish harboring the streptolysin S structural gene, *sagA* (Abdesalam *et al*., 2010).

Suzuki et al. (2011) demonstrated that some *Streptococcus dysgalactiae* subsp. *dysgalactiae* strains carry a number of prophage proteins that are considered putative virulence genes in *Streptococcus pyogenes*, including hyaluronidase and streptodornase type D, both of which are documented as *Streptococcus* virulence genes (Davies *et al.,* 2007).

Serum opacity factor (SOF) was detected in *S. dysgalactiae* isolated from fish (Nishiki *et al.,* 2011). The SOF obtained from fish isolates, named SOF-FD, consists of a putative signal sequence, a large N-terminal

opacification domain, a putative fibronectin-binding domain, and an LPXTG Gram-positive anchor motif. The structure of SOF-FD is similar to that of the SOF in *Streptococcus pyogenes* (Nishiki *et al.,* 2011; Nishiki *et al.,* 2012). The substrate of SOF is a high-density lipoprotein (HDL), which has anti inflammation activity. The SOF binds to HDL and then disrupts its structure, which may contribute to the virulence of streptococci. *S. dysgalactiae* strains isolated from fish have serum opacification activity and sof-FD genes with highly homologous sequences (Nishiki *et al*., 2011).

2.2.2 *Weissella ceti*

The genera *Weissella*, *Leuconostoc*, and *Oenococcus* together constitute a genetic branch of lactic acid bacteria (Chelo *et al*., 2007). The genus *Weissella* contains species that are Gram-positive, catalase-negative, heterofermentative, non-motile, coccoid or rod-shaped organisms (Collins *et al*., 1993; De Bruyne *et al.,* 2008). Microorganisms of the genus *Weissella* have been isolated from a variety of habitats such as soil, fresh vegetables and fermented foods or meat products (Schillinger and Lucke, 1987; Bjorkroth *et al.,* 2002; Magnusson *et al.,* 2002; De Bruyne *et al.,* 2010; Padonou *et al*., 2010).

Some species of *Weissella* have been identified in intestinal contents of healthy humans, dogs, chickens and ducks (Kurzak *et al.,* 1998; Walter *et al.,* 2001; Beasley *et al.*, 2006; Chelo *et al.,* 2007; Wise and Siragusa, 2007; Sirirat *et al.,* 2008). *Weissella confusa* has been isolated from intestines of healthy fish farmed Asian seabass (*Lates calcarifer*) (Cai *et al.,* 1998; Rengpipat *et al.,* 2008). In addition, *Weissella confusa* have also been associated with cases of bacteremia in humans and primates as well as otitis in dogs (Olano *et al.,* 2001; Bjorkroth *et al.,* 2002; Flaherty *et al.,* 2003; Vela *et al.,* 2003; Svec *et al*., 2007).

Liu et al. (2009) provided the first description of *Weissella* sp. infection in a cultivated fish species. They documented sequential outbreaks of the pathogen in one commercial rainbow trout (*Oncorhynchus mykiss*) facility in China. The diseased fish exhibited hemorrhage in eyes, anal region and intestine, and petechiae in liver. During the epidemic outbreak, a loss of 40% was suffered. *Weissella* sp. strains were isolated from kidney, liver and/or brain of four adult rainbow trout with typical clinical signs (Liu *et al*., 2009).

Figueiredo *et al.* (2012b) described the occurrence of outbreaks of hemorrhagic septicaemia caused by *Weissella* sp. in three commercial rainbow trout farms in Brazil, from 2008 to 2009. During outbreaks was observed that the first clinical sign of disease is anorexia, followed by: exophthalmia, ascites, and hemorrhage in the mouth, tongue, and eyes. High mortality rates (50-80%) occurred during outbreaks and disease affected different age groups as fingerlings, juveniles, adults, and broodstock.

In addition, Figueiredo *et al.* (2012b) investigated the potential routes of infection of *Weissella* sp. in rainbow trout. The disease was successfully reproduced in the laboratory by intraperitoneal injection, immersion, and cohabitation between diseased and healthy fish. These data indicated that bacteria can actively infect healthy fish and are able to proliferate, survive, and be transmitted under normal conditions. Figueiredo et al. (2012b) also evaluated the resistance of the pathogen to five antibiotics. The results showed that all 77 *Weissella* sp. strains tested were resistant to sulfonamide, and based in normalized resistance interepretation (NRI) analysis, one, two, and three isolates were classified as non-wild type (NWT) for erythromycin, oxytetracycline, and norfloxacin, respectively.

The origin of the bacteria *Weissella* sp. associated with these described fish outbreaks is unknown. During an investigation into the microbiota of beaked whales (*Mesoplodon bidens*), Vela *et al.* (2011) isolated nine unidentified Gram-positive staining, rod-shaped or coccoid organisms. Based on the phenotypic, physiological and phylogenetic evidence, it was proposed that the new isolates from whales represented a novel species of the

genius *Weissella*, named *Weissella ceti* sp. nov. (Vela *et al.*, 2011). 16S rRNA sequences analysis of *W. ceti* isolated from whales compared with the sequences of the Brazilian and Chinese isolates showed a high genetic similarity (>99%), suggesting that all strains belonged to the same species.

The third report of a *Weissella ceti* causing infection and loss in rainbow trout farms occurred in the United States (Welch and Good, 2013). The disease signs and bacteria isolated in this outbreak were very similar to those observed in the Brazilian and Chinese cases. Affected fish exhibited as clinical signs: dark skin coloration, bilateral exophthalmia, corneal opacity and ocular hemorrhage.

Elevated temperature is often associated with severe fish disease outbreaks involving mesophillic bacterial pathogens, and this is likely due to higher temperature of water induced increases in microorganism growth rate and/or causes physiological responses to thermal stress in fish. All outbreaks described for *Weissella ceti* in rainbow trout coinciding with seasonal high water temperatures, usually above 17 ºC. This suggests that one of the main predisposing factors associated with *Weissella ceti* infections is the elevation of water temperature.

Ladner *et al.* (2013) presented the first genome sequence of *Weissella ceti*, using a strain from United States. Results of comparative analysis of this genome demonstrated several putative virulence factors, which do not have homologous encoded in any of the other sequenced *Weissella* genomes. These included five collagen adhesins, a platelet-associated adhesion, and a mucus-binding protein (Ladner *et al.,* 2013).

Immunoprophylaxis generally has a positive effect in fish farming by reducing the economic losses caused by disease. In some countries, commercial vaccines are available to protect cultured rainbow trout against some pathogens, including *Lactococcus garviae* and *Yersinia ruckeri* (Vendrell et al., 2006). These vaccines are usually intraperitoneally injected and the requisite anesthetization and handling associated with this process causes significant stress. Thus, it is advantageous, when possible, mix vaccines into multivalent formulations that can be delivered together, minimizing the impact of fish stress by handling, which would be caused by multiple injections. Welch and Good (2013) developed an aqueous-based whole-cell killed bacterin vaccine able to protect rainbow trout from *Weissella ceti* infection in laboratory trials. The authors also proposed a bivalent formulation that conferred a level of protection against *W. ceti* or *Y. ruckeri* challenge that was equivalent to that conferred by vaccination with each component.

The occurrence of several outbreaks of *Weissella ceti* on three continents over a relatively short period of time, suggests that this pathogen is a significant and rapidly emerging concern for the farmed rainbow trout industry.

2.3 Molecular epidemiology

The science of "Molecular epidemiology" has emerged in 1970s when the term was first coined in relation to the study of influenza virus (Kilbourne, 1973). Molecular epidemiology utilizes molecular biology to define the distribution of disease in a population and based on integration of traditional epidemiological approaches with molecular results to identify the etiological determinants of this distribution (Snow, 2011). Understanding pathogen distribution and relatedness is essential for determining the epidemiology of emerging infectious diseases of aquaculture, supporting in design of efficient pathogen control methods (Singh *et al.,* 2006).

Over the years, approaches to the epidemiological analysis of infectious disease have undergone a remarkable evolutionary transition moving from phenotypic to molecular in nature. Since all life is related by common ancestry, bacteria display genetic diversity, which reflects their evolutionary history due to the accumulation and inheritance of mutations when genetic material is copied (Kumar and Filipski, 2008; Goering, 2010). Bacteria are also able

to transfer some genetic elements in a process of acquisition and recombination of foreign DNA. These mobile genetic elements are segments of DNA that encode enzymes and other proteins that mediate the movement of DNA within genomes or among bacterial cells (Wellington *et al.,* 2013). This process is essential to understand some genetic variability during molecular epidemiologic studies.

The search for a clearer comparison of genomic relatedness between bacterial isolates from diseases has involved four generations of molecular approaches. First generation, plasmid analysis, was replaced by a second-generation with the use of restriction enzymes and probes. This was followed by third-generation pulsed field gel electrophoresis (PFGE) and PCR-based methods with movement now to fourth-generation DNA sequence-based approaches (Goering, 1998, 2000). In this context, it is interesting to note that while far from the most current molecular technique, PFGE still has a relative valuable method of genomic analysis and comparison. This is especially true in the clinical setting where for some organisms it can be used for assessing isolate interrelationships (Goering, 2010).

The chromosome is the most fundamental component of identity of the cell and therefore represents a preferred measure for assessing strain interrelatedness. Chromosomal DNA can be digest with restriction enzymes, resulting in a series of fragments of different sizes that form different patterns when analyzed by agarose gel electrophoresis (Singh *et al.,* 2006). Enzymes used to cleave DNA often recognize numerous sites within the bacterial chromosome, resulting in too many band fragments to efficiently and accurately compare. However, the resulting DNA fragments are too large to be separated by conventional agarose gel electrophoresis. A number of alternative methods, generally classified as PFGE, are capable of separating these large DNA fragments (Chang and Chui, 1998). In conventional agarose gel electrophoresis, DNA molecules that are more than 40 to 50 kbp in size fail to migrate efficiently. By periodically changing the direction of the electrical field in which the DNA is separated, PFGE allows the separation of DNA molecules of over 1,000 kbp in length (Carle *et al.,* 1986).

To interpret DNA fragment patterns generated by PFGE and transform them into epidemiologically useful information for typing pathogens, is essential understand how to compare PFGE patterns and how random genetic events can alter these patterns. Ideally, the PFGE isolates representing an outbreak will be indistinguishable from each other and distinctly different from those of epidemiologically unrelated strains. Alternatively, random genetic events can occur, such as point mutations or insertions and deletions of DNA that can alter the restriction profile obtained during the course of an outbreak (Hall, 1994).

Because only a small portion of the organism's genetic component is undergoing analysis, isolates that give identical results are classified as "indistinguishable", not "identical". Guidelines proposed by Tenover *et al.* (1995) are often used for the interpretation of PFGE. With these guidelines, a banding pattern difference of three fragments could have occurred due to a single genetic event and thus these isolates are classified as highly related. Differences of four to six restriction fragments are likely due to two genetic events, and differences of greater than seven restriction fragments are due to three or more genetic events. Isolates that differ by three fragments in PFGE analysis may represent epidemiologically related sub-types of the same strain. Conversely, isolates differing in the positions of more than three restriction fragments may represent lower epidemiologic relation.

Commercially available programs to analysis PFGE and other molecular results have been developed for computer-assisted analysis (CAA) such as BioNumerics and GelCompar (Applied Maths, Sint-Martens-Latem) and Diversity Database Fingerprinting Software (Bio-Rad Laboratories, Hercules, Ca) (Goering, 2010).

In general PFGE is one of most reproducible

and highly discriminatory typing methods available. However, the process of PFGE is time consuming, mainly because of the need to extract DNA without subjecting it to forces that would cause disruption by shearing. This delay can be a serious problem when epidemiological data are needed urgently (Singletone, 2000). A further disadvantage of PFGE, compared with other molecular methods, is the susceptibility to endogenous nucleases, which can degrade target DNA (Marshall *et al.,* 1999).

In this point, rapid and inexpensive PCR-based typing techniques, such as enterobacterial repetitive intergenic consensus - PCR (ERIC-PCR) and repetitive element sequence - PCR (REP-PCR) can be used to screen for genetic relatedness among strains. In many bacterial species there are repetitive DNA sequences spread throughout the genome. PCR primers can be designed for these elements to amplify the genomic DNA between repetitive elements when two of these elements are in relatively close proximity (Versalovic *et al*., 1991).

The regions located between the repeated elements often vary in size due to difference among separate strains, and thus fragments of different sizes will be amplified, creating unique profiles following gel electrophoresis. These different banding patterns are compared one to another to genotype the organisms (Georghiou *et al.,* 1994). Repetitive-element PCR systems have been developed for a variety of pathogens (Hulton *et al.* 1991; Shutt e*t al*., 2005).

In recent years, the rapidly expanding number of sequenced microbial genomes has served as a catalyst for the development of a variety of molecular typing approaches that focus on either single or multiple chromosomal loci. The better example is the multilocus sequence typing (MLST) that are considered as the reference tools for investigating genetic and epidemiological relationships for some pathogens (Haguenoer *et al.* 2011; Nakib *et al.* 2011). Sequence-based molecular epidemiology is attractive in offering the promise of reproducible typing profiles that are highly amenable to standardization, uniform interpretation, and database cataloging, since they are based on simple quaternary data (A, T, G, and C) (Kemp *et al*., 2005).

The development of next generation sequencing technology promises a revolution in the ability to generate sequence data and thus information of potential epidemiological relevance. The process of disease involves many genetic polymorphisms, across the genome, which is now being aggressively explored. Therefore, the analyses performed to choose specific genome regions that can provide assessments consistent with those predicted with full-length genome sequences, would be preferable to serve as phylogenetic markers for molecular epidemiological studies of pathogens (Wellington *et al.*, 2013).

However, descriptive molecular data alone is inadequate in tracing pathogen spread, especially when variation is limited and evolution does not occur in a regular way. The development of methods to systematically record and share such epidemiologically important information thus represents a major challenge for fish health professionals in making the best future use of molecular data in supporting fish health surveillance and disease control. The implementation of the best available molecular epidemiological information would offers great support to surveillance programs in further developing a healthy and sustainable aquaculture industry, which is necessary to satisfy an increasing world demand for cultured fish products (Snow, 2011).

2.4 Fish vaccines

Antibiotic therapy can be used for the treatment of bacterial diseases in aquaculture, however this clearly has some hampers. Repeated use can induce drug resistance in microorganisms or supress the immune system of fish (Rijkers *et al*., 1980). Moreover, harmful residues may be present in fish sold for human consumption. Hence, there is a high interest in protecting fish by vaccination, an effective strategy for disease control (Willem and Nakao, 2013).

The goal of successful vaccination is the capacity to induce the immune system of fish to develop immunological memory. A first contact with an antigen usually induces relatively short-lived effector cell (activated Th, Treg, cytotoxic T cells or plasma cells). However, there are also long-lived memory cells among the progeny of the original non-primed lymphocytes. These memory cells retain the capacity to be stimulated by the same antigen. The height of the secondary response is dependent on the amount of priming antigen (Willem and Nakao, 2013).

Several differences between the secondary responses of mammals and fish have been found. One distinction is that the ratio between the secondary and the primary response is much higher in mammals than in fish. In fish B-cells memory is probably due to an increase in the antigen-sensitive precursor pool without any of the accompanying characteristics observed in mammals (such as a switch in isotype) (Hikima *et al.,* 2011; Willem and Nakao, 2013).

Fish can be immunized by three routes: injection preferably intraperitoneally; immersion, dipping the fish in a diluted vaccine solution; or oral administration of the vaccine. Although these methods have different advantages and disadvantages with respect to the level of protection, side effects, practicality and cost-efficiency, it is widely accepted that injection route give the higher protection to be used as the primary route of fish immunization in commercial production (Taffala *et al*., 2013). There is still a limited understanding of the mechanisms involved in antigen uptake and presentation after immersion and oral vaccination (Nakanishi and Ototake, 1997).

Oral vaccination usually evokes only minimal immune responses in the host. It is not easy to explain this phenomenon. It was showed that the 2nd gut segment plays a key role in antigen transport and antigen processing by macrophages (Rombout and Van Den Berg, 1989). Numerous lymphoid cells are also present in this part of the gut, playing a role in mucosal responses (Rombout *et al*., 1989a). Repeated oral administration of bacterial antigen resulted in presence of antibodies in skin mucus and bile, but not in serum (Rombout et al., 1989b).

For oral vaccination, research has been focused on protecting the antigens from digestion and decomposition during passage through the stomach and anterior part of the gut (Quentel and Vigneulle, 1997). Promising results have been obtained using encapsulation of antigens in alginate or polylactic glycolic acid microparticles (Sinyakov *et al*., 2006). From the economic standpoint, oral vaccination is the ideal route to be employed in a vaccination program, which requires one or more booster immunizations.

Four types of vaccines are tested for a vaccine formulation for fish: bacterin, live attenuated, purified subunits of the pathogen, and DNA vaccines. Most of bacterial vaccines used in aquaculture have been inactivated vaccines obtained from a broth culture of a specific strain(s) subjected to subsequent formalin inactivation (Newman, 1993; Toranzo *et al.,* 1997). The best results are obtained with those bacterins that include both bacterial cells and extracellular products. Whereas with some vaccines acceptable levels of protection are achieved with aqueous formulations administered by injection or immersion. For other bacterins, such as those devised for salmonids against *Aeromonas salmonicida* subsp. *salmonicida,* an acceptable level of protection can only be achieved by immunization with oil-adjuvanted bacterins delivered by injection.

The live attenuated vaccines should potentially have many advantages in aquaculture. In immunization with a live vaccine, if vaccinated fish sheds the vaccine strain, an effective dissemination of the antigen in the population would take place over an extended period of time. However, problems concerning safety, persistence in the fish and in the environment, reversion to virulence, risk of spreading to non-target animals including wild fish, among others, must be resolved before the use of these live attenuated strains can be allowed in the field (Benmansour and Kinkelin, 1997).

DNA vaccines consist of a bacterial plasmid with a strong promoter, the gene of interest, and a polyadenylation/transcriptional termination sequence. This is one of the most promising fish vaccine preparations against viral diseases (delivered intramuscularly). They consisted of naked plasmid DNA that will result in gene expression of viral proteins in the muscle tissue of the vaccinated fish. Overall, the inactivated, subunit or whole virus vaccines in general confer lower level of protection compared to DNA and live, attenuated vaccines (Evensen and Leong, 2013).

A subunit vaccine is produced by MSD Animal Health against infectious pancreatic necrosis (IPN) and is intended for use in salmonids. The vaccine consists of the major IPNV capsid protein (VP2) produced in bacteria (*Escherichia coli*), and is injected intraperitoneally in Atlantic salmon prior to transfer to seawater. There are also subunit, oral vaccines against IPN, infectious salmon anemia (ISA) and salmon rickettsia syndrome (SRS) for use in salmonids in Chile (produced by Centrovet, Chile) (Evensen and Leong, 2013).

Many vaccines based upon recombinant antigens or killed pathogens are not very effective in fish as such. In most cases the use of adjuvants is necessary to stimulate the response of innate and acquired immunity and increase vaccine efficacy. Adjuvants (from Latin adjuvare meaning "to help") have traditionally been defined as helper substances that increase the magnitude of an adaptive response to a vaccine (potency), or ability to prevent infection and death (efficacy). However, nowadays scientists have acknowledged that adjuvants may become more important as a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen (Guy, 2007).

Traditional adjuvants such as mineral oils are routinely used in different commercial bacterial vaccines available for fish; however, important side effects may occur with this type of adjuvants. A search for alternative molecules or certain combinations of them as adjuvants is desirable in order to increase animal welfare without reducing protection levels. Especially, combinations that may target specific cell responses and thus a specific pathogen, with no or minor side effects, should be explored. Despite this, the oil adjuvants currently used are quite friendlier with respect to side effects compared with the oil adjuvants previously used (Taffala *et al.*, 2013).

The development of effective vaccines should be approached by combining the search for protective antigens together with the application of specific, and targeting, adjuvants that increase the immunogenicity with a desired immune response. (Taffala *et al.*, 2013). Many aspects of fish immunology are still unknown and we are far from close to understanding on which immune mechanisms the protection against many of these pathogens resides (Secombes, 2008). Better understanding of the immune system of different fish species is essential to achieve progress in fish vaccinology.

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3 – CHAPTER 3

Genotyping of *Streptococcus dysgalactiae* strains isolated from Nile tilapia, *Oreochromis niloticus* (L.)

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ABSTRACT

Streptococcus dysgalactiae is an emerging fish pathogen that is responsible for outbreaks of disease on fish farms around the world. Recently, this bacterium was associated with an outbreak at a Nile tilapia, *Oreochromis niloticus* (L.), farm in Brazil. The aim of this study was to evaluate the genetic diversity, best genotyping method, and aspects of molecular epidemiology of *S. dysgalactiae* infections in Nile tilapia farms in Brazil. Twenty-one isolates from four farms located in different Brazilian states were characterized genetically using PFGE, ERIC-PCR, REP-PCR, and *sodA* gene sequencing. The discriminatory power of the different typing methods was compared using Simpson's index of diversity. Identical *sodA* gene sequences were obtained from all isolates, and ERIC-PCR and REP-PCR were unable to discriminate among the isolates. PFGE typing detected three different genetic patterns between the 21 strains evaluated; thus, it was the best genotyping method for use with this pathogen. The strains from Ceará State were genetically divergent from those from Alagoas State. The *S. dysgalactiae* isolates analyzed in this study constituted a genetically diverse population with a clear association between geographic origin and genotype.

Keywords: Brazil; molecular epidemiology; pulsed field gel electrophoresis; *Streptococcus dysgalactiae*.

INTRODUCTION

Streptococcus dysgalactiae is an etiologic agent of mastitis in cattle, endocarditis in various domestic animals, and pharyngitis and cardiopulmonary diseases in human beings (Efstratiou *et al.,* 1994; Aarestrup and Jensen 1996; Williams 2003; Nomoto *et al.,* 2004). This pathogen was first associated with fish diseases in 2002 when it was identified as being responsible for disease outbreaks on farms producing amberjack, *Seriola dumerili*, and yellowtail, *Seriola quinqueradiata*, in Japan (Nomoto *et al.,* 2004). *S. dysgalactiae* infections were thought to be restricted to marine fish species, but an outbreak on a farm in Brazil that produces Nile tilapia, *Oreochromis niloticus*, was recently attributed to this pathogen (Netto *et al*., 2011). Currently, data about the genetic diversity and molecular epidemiology of *S. dysgalactiae* infection in tilapia farms are unavailable.

The pulsed field gel electrophoresis (PFGE) technique is considered to be the reference tool for genotyping of *S. dysgalactiae*. Previous studies have used a variation of this technique (biased sinusoidal gel electrophoresis – BSFGE) to determine the genetic diversity of *S. dysgalactiae* strains isolated from marine fish in Asia (Nomoto *et al.,* 2008; Abdelsalam, Chen and Yoshida, 2010; Nishiki *et al.* 2010). The results of these studies suggested that the populations of *S. dysgalactiae* in fish were clonally related and that clonal expansion of the bacterium occurred in that region (Abdelsalam *et al.,* 2010; Nishiki *et al.* 2010). Although PFGE has high discriminatory power, the method is labour intensive and time consuming. Rapid and inexpensive PCR-based typing methods, such enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and repetitive extragenic palindromic-PCR (REP-PCR), have been developed as alternatives for screening the genetic relatedness of bacterial populations (Kidd *et al.,* 2011; Lee *et al.,*

2011). However, the applicability of these approaches to the genotyping of *S. dysgalactiae* has not been evaluated.

The primary aims of this study were to evaluate the genetic patterns of *S. dysgalactiae* strains isolated from diseased Nile tilapia and assess the relationship between these patterns and the geographic origin of the isolates. An additional aim was to evaluate the discriminatory power and congruence of *sodA* gene sequencing, PFGE, ERIC-PCR, and REP-PCR when used for typing of *S. dysgalactiae*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 21 strains of *S. dysgalactiae* from diseased Nile tilapia were evaluated. They were isolated from outbreaks at four Brazilian farms located in two states (Ceará and Alagoas) between 2007 and 2011 (Table 3.1). The farms are geographically distant, and there are no records of animal transport between them. In addition, fingerlings were provided by different suppliers. Lancefield's serotyping was performed using the Slidex Latex Agglutination kit (BioMerieux). Identification of the bacterial species was confirmed by *S. dysgalactiae*-specific PCR according to Hassan *et al.* (2003). The strains were stored at –70 °C until use. The isolates were thawed, streaked onto Todd Hewitt agar (BD), and incubated at 28 °C for 24 h for DNA extraction.

Table 1 *Streptococcus dysgalactiae* strains isolated from Nile tilapia used in this study

Strain[a]	State	Farm	Year of isolation
SD 54	CE	1	2007
SD 56	CE	1	2007
SD 57	CE	1	2007
SD 58	CE	1	2007
SD 61	CE	1	2007
SD 64	CE	1	2007
SD 68	CE	1	2007
SD 92	AL	2	2010
SD 94	AL	2	2010
SD 120	CE	3	2011
SD 121	CE	3	2011
SD 123	CE	3	2011
SD 137	CE	4	2011
SD 138	CE	4	2011
SD 140	CE	4	2011
SD 141	CE	4	2011
SD 142	CE	4	2011
SD 143	CE	4	2011
SD 144	CE	4	2011
SD 145	CE	4	2011
SD 146	CE	4	2011

CE, Ceará; AL, Alagoas.
[a]Aquavet culture collection code.

DNA extraction and *sodA* PCR

Total DNA was extracted using the commercial DNeasy kit (Qiagen) according to the manufacturer's instructions. The amount of DNA extracted was quantified spectrophotometrically using Nanodrop® (Thermo Scientific, GE). The internal fragment constituting 85% of the *sodA* gene was amplified by PCR using the primers d1 (5'-CCITAYICITAYGAYGCIYTIGARCC-3') and d2 (5'-ARRTARTAIGCRTGYTCCCAIACRTC-3') as described by Poyart *et al.* (1998). Thermal cycling was performed in a Veriti 96-Well Thermal Cycler (Life Technologies). The primers used were acquired from Life Technologies.

Sequencing and phylogenetic analysis

The PCR products were purified using a Wizard PCR preps kit (Promega). Sequencing reactions were performed using the Applied Biosystems BigDye terminator cycle sequence kit and run on an ABI 3730XL genetic analyzer (Applied Biosystems).

The *sodA* sequences of the Brazilian strains were aligned in BioEdit using CLUSTALW (Thompson *et al.,* 1994) with sequences of the following bacterial strains and species: six amberjack *S. dysgalactiae* isolates (Nomoto *et al.,* 2008) (Genbank accession numbers AB334725, AB334726, AB334730, AB334732, AB334734, and AB334737); *Streptococcus dysgalactiae* subsp. *dysgalactiae* (Z959000); *Streptococcus dysgalactiae* subsp. *equisimilis* (AJ319591); *S. equi* subsp. *equi* ATCC 33398 (Z95901); *Streptococcus agalactiae* ATCC 12403; and *Streptococcus agalactiae* ATCC 12403 (Z99178). The genetic distance matrix was obtained using Kimura's two-parameter model (Kimura, 1980), and an evolutionary tree was created using the neighbour-joining method (Saitou and Nei, 1987) with Mega4 (Tamura *et al.,* 2007). Bootstrap values from 1000 replicates were displayed as percentages.

Pulsed field gel electrophoresis (PFGE)

PFGE was performed as described previously (Teixeira *et al.,* 1995; Oliveira *et al.,* 2005). *S. dysgalactiae* strains were grown overnight in Todd Hewitt broth at 28 °C. The cells were harvested and washed twice with PIV solution (Tris–HCl 0.01 M, pH 8.0 and NaCl 1 M). The bacterial suspension was mixed 1:1 (v:v) with 2% low-melting-point agarose (Sigma Aldrich) and pipetted into 20 µL plugs. Streptococcal cells in agarose plugs were lysed with 25 U of mutanolysin and 500 mg of lysozyme (both from Sigma Aldrich). Following digestion of the DNA with 12 U of *SmaI* restriction enzyme (Amersham Biosciences), the plugs were submitted to PFGE using the following program: switch time of 1–30 s, 23 h, 120° angle, 11 °C, and a voltage gradient of 6 V cm^{-1} in a CHEF DR III system (Bio-Rad Laboratories). The lambda ladder PFGE marker (New England Biolabs) was used as a DNA size marker. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg mL^{-1} for 30 min), and images were captured using L-Pix EX (Loccus Biotecnologia).

REP-PCR and ERIC-PCR

The REP-PCR was performed as described previously by Ouoba et al. (2010) with some modifications. The reactions were performed using a HotStart Taq Polymerase kit (Qiagen); the mix was composed of 1 × PCR Buffer, 0.5 μmol L^{-1} GTG5 primer (5'-GTGGTGGTGGTGGTG-3'), 0.2 μmol L^{-1} dNTPs, 1.5 mmol L^{-1} $MgCl_2$, 2 U of Taq DNA polymerase, and 35 ng of template DNA. The PCR conditions consisted of an initial step of 95 °C for 15 min followed by 30 cycles of 95 °C for 30 s, 45 °C for 1 min, and 72 °C for 4 min, with a final elongation step of 72 °C for 16 min.

The ERIC-PCR was conducted using the primers ERIC-1 (5'-ATGTAA GCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG - 3') according to Saito *et al.* (2011) with some modifications. The reactions were performed using a HotStart Taq Polymerase kit (Qiagen) in a final volume of 25 µl containing 1 × PCR Buffer, 0.3 μmol L^{-1} of each primer, 0.3 μmol L^{-1} dNTPs, 2 mmol L^{-1} $MgCl_2$, 2 U of Taq DNA polymerase, and 35 ng of template DNA. The PCR conditions consisted of an initial denaturation step at 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 52 °C for 1 min, and 72 °C for 1 min. The final elongation step was at 72 °C for 8 min.

Thermal cycling was performed in a Veriti 96-Well Thermal Cycler (Life Technologies). The primers used were acquired from Life Technologies. The PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide (0.5 μg mL^{-1} for 30 min), and visualized by UV transillumination. Images were captured using an L-Pix EX (Loccus). Ladders of 100 bp and 1 kb (both from

Promega) were used as the molecular size standards for ERIC-PCR and REP-PCR, respectively.

Data analysis and statistics

The PFGE, REP-PCR, and ERIC-PCR gels were analysed using BioNumerics version 6.6 (Applied Maths). The Dice coefficient was used to analyse the similarities of the banding patterns (Dice 1945). Dendrograms were created using the unweighted pair group method with average (UPGMA) approach. Isolates that showed ≥ 80% similarity for PFGE and ≥ 90% similarity for ERIC-PCR and REP-PCR were considered to be clonally related (Tenover *et al.,* 1995; Singh *et al.,* 2006).

The discriminatory power of each typing method was calculated using Simpson's index of diversity (*D*) (Hunter & Gaston 1988). The adjusted Rand index was calculated to quantify the congruence among the different typing methods (Kidd *et al.,* 2011). These analyses were performed using the statistical software R (Chang *et al.,* 2010).

RESULTS

Phylogenetic analysis of the *sodA* gene

Phylogenetic analysis of the *sodA* gene sequences of the *S. dysgalactiae* strains resulted in the neighbour-joining tree shown in Figure 3.1. The sequences of the 21 Brazilian isolates were indistinguishable. They clustered in the same branch as the amberjack strains, showing a bootstrap percentage (based on 1000 replicates) of 99%. In spite of this similarity, all Nile tilapia isolates showed a single deletion at position 421 of the partial sequence of the *sodA* gene. All fish isolates clustered in same branch as the reference strain *S. dysgalactiae* subsp. *dysgalactiae* ATCC 43078.

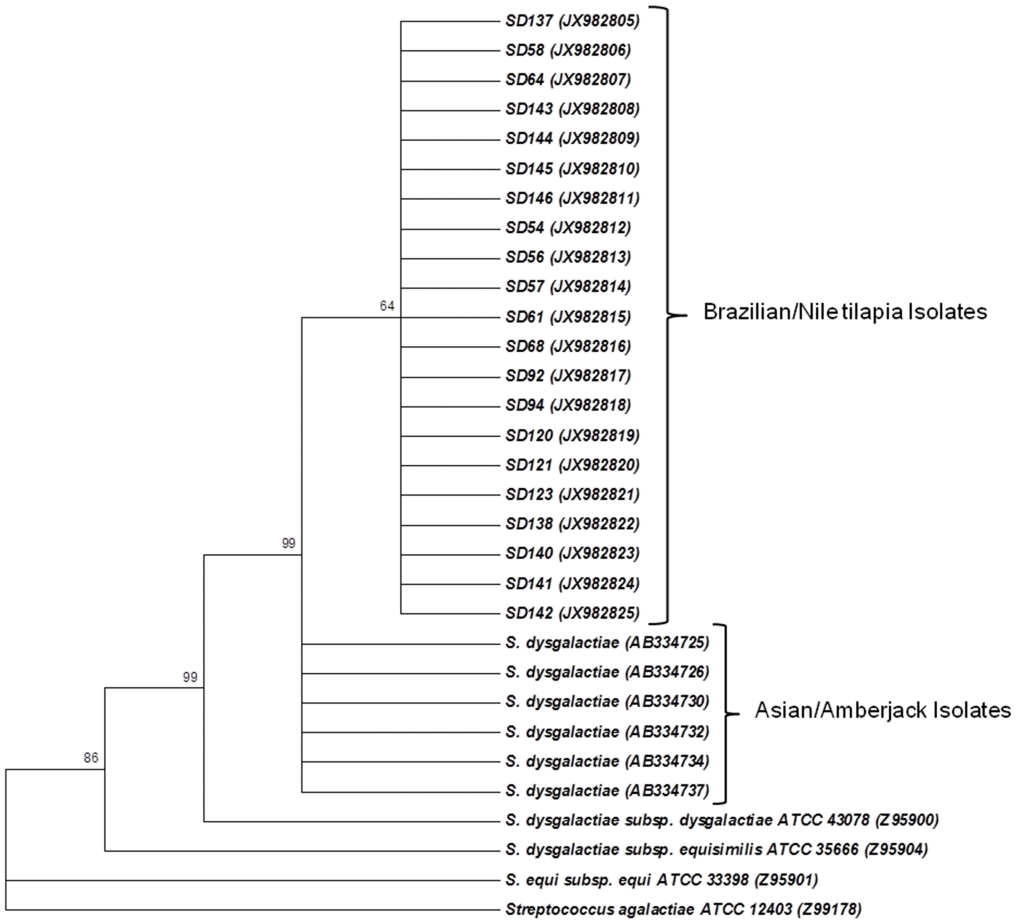

Figure 3.1 Phylogenetic neighbor-joining tree based on *sodA* gene sequences showing the phylogenetic relationships of Brazilian isolates (SD56, SD57, SD58, SD61, SD64, SD68, SD92, SD94, SD120, SD121, SD123, SD137, SD138, SD140, SD141, SD142, SD143, SD144, SD145, and SD146), amberjack isolates (Nomoto et al., 2008), and reference strains of *S. dysgalactiae* subsp. *dysgalactiae* ATCC 43078, *S. dysgalactiae* subsp. *equisimilis* ATCC 35666, *S. equi* subsp. *equi* ATCC 33398, and *Streptococcus agalactiae* ATCC 12403. Bootstrap percentages (based on 1000 replications) are shown at branch points.

PFGE, REP-PCR, and ERIC-PCR profiles

PFGE with *SmaI* digestion revealed 18 different patterns among the 21 *S. dysgalactiae* strains evaluated (Fig. 3.2). These were classified into three PFGE types based on the similarity threshold of 80%. PFGE had a discriminatory power of 0.3428. Approximately 81% (n = 17) of the isolates were pulsetype A.

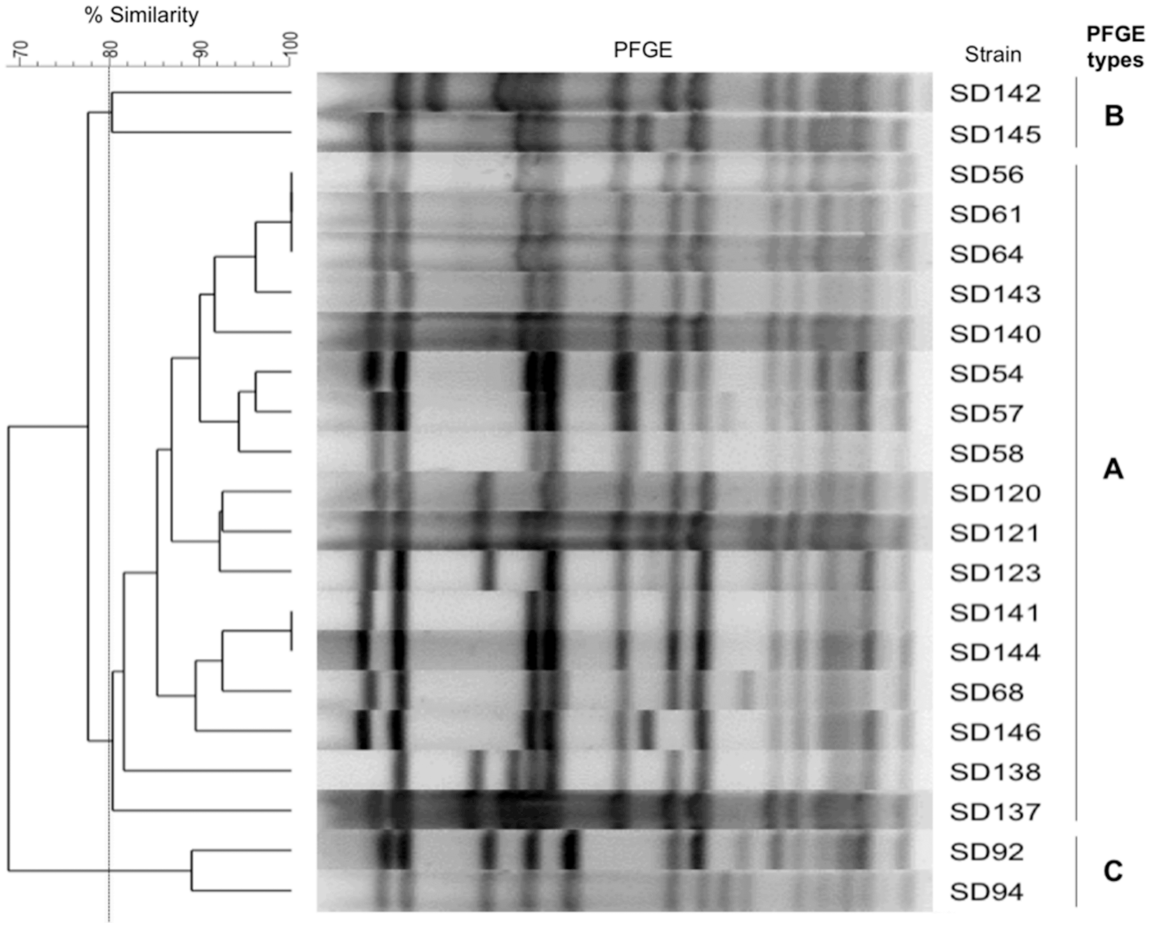

Figure 3.2 PFGE *SmaI* macrorestriction profile analysis of 21 Brazilian strains of *Streptococcus dysgalactiae* isolated from diseased Nile tilapia, using Dice's coefficient and clustered using the UPGMA approach.

REP-PCR of the 21 isolates resulted in a single band of approximately 1200 bp. The strains were indistinguishable by this technique, which had a discriminatory power of 0.0.

ERIC-PCR resulted in the amplification of five bands ranging in size from 400 to 2000 bp. Three different ERIC patterns were detected, and based on the similarity threshold of 90% they belonged to ERIC type I and ERIC type II (Fig. 3.3). ERIC type I contained 95.28% of the isolates (20 strains). Strain 137 was the only isolate of ERIC type II. ERIC-PCR had a discriminatory power of 0.095238. There was no relationship between ERIC type and geographical origin or farm.

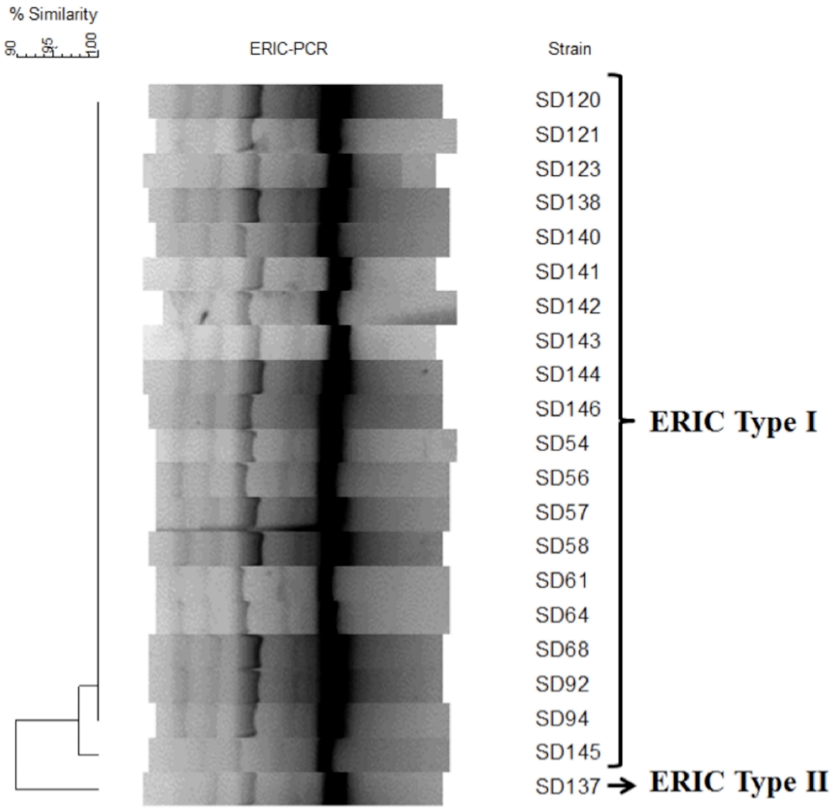

Figure 3.3 Typing results of 21 Brazilian strains of *S. dysgalactiae* obtained using ERIC-PCR. The dendogram was constructed using Dice's coefficient and the UPGMA approach.

Comparison of typing methods

Four typing methods were tested in the present study. *sodA* gene sequencing and REP-PCR were unable to discriminate the *S. dysgalactiae* isolates from Nile tilapia. In contrast, PFGE and ERIC-PCR detected the presence of, respectively, three and two genetically distinct populations among the 21 strains evaluated. Based on Simpson's index of diversity, higher discriminatory power was obtained using PFGE. The congruence between the results of these two techniques was evaluated using the adjusted Rand index (ARI). They showed low concordance, with an ARI value of 0.60. ERIC-PCR results were not a valuable predictor of PFGE results, which was the best typing method to use for isolates of *S. dysgalactiae* from Nile tilapia.

Molecular Epidemiology

Based on PFGE results, the Nile tilapia strains of *S. dysgalactiae* belonged to three different genotypes. All isolates from farms 1 and 3 located in Ceará State belonged to pattern A. Two different types (A and B) were observed on farm 4. The isolates from Alagoas State (SD92 and SD 94; farm 2) were classified as pulsetype C, which was a genetically divergent group. The genotypes showed a relationship with state of origin.

DISCUSSION

First described as an etiologic agent of disease in marine fish, *S. dysgalactiae* recently was associated with infections on farms producing Nile tilapia, a freshwater species (Netto *et al.,* 2011). The present

study evaluated the genetic diversity of *S. dysgalactiae* strains isolated from outbreaks in different Nile tilapia farms in Brazil between 2007 and 2011.

Sequencing of the superoxide dismutase manganese-dependent (*sodA*) gene has been used previously to determine the genetic diversity of *S. dysgalactiae* isolated from mammals and fish (Nomoto *et al.,* 2008; Abdelsalam *et al.,* 2010). Abdelsalam *et al.* (2010) sequenced the *sodA* gene of 30 strains of *S. dysgalactiae* from nine fish species from different Asian countries. They observed 100% sequence identity among the isolates, regardless of geographical origin or fish species. In the present study, the *sodA* sequences of Brazilian strains also showed 100% identity. These sequences were highly homologous to the sequences of Asian isolates, although the Nile tilapia strains had a deletion at position 421 of the partial fragment of the *sodA* gene. This peculiarity, however, did not result in a clear distinction between the strains from Nile tilapia and marine fish. The low polymorphism in *S. dysgalactiae* isolates from fish verified here and previously (Nomoto *et al.,* 2008; Abdelsalam *et al.,* 2010) demonstrates that *sodA* sequencing is not a suitable genotyping method for this pathogen. Moreover, the genetic diversity determined using other techniques was not predicted by *sodA* sequencing, which compromises its usefulness as a typing tool.

The PFGE results after digestion with *SmaI* revealed three different PFGE types among the 21 Brazilian isolates of *Streptococcus dysgalactiae*. Abdelsalam *et al.* (2010) evaluated the use of *ApaI* and *SmaI* for DNA macrorestriction of *S. dysgalactiae* strains isolated from nine fish species. In contrast to the results of the current study, the number of fragments they obtained using *SmaI* digestion did not allow effective discrimination among Asian isolates of *S. dysgalactiae*. However, this enzyme was applied previously to genotyping of fish pathogenic streptococci (Pereira *et al.* 2010; Netto *et al.,* 2011). Different PFGE protocols (e.g., the enzyme concentrations and DNA digestion procedures used) could be responsible for the differences between studies.

Nomoto *et al.* (2006) used BSFGE with *ApaI* digestion to characterize 30 strains of *S. dysgalactiae* isolated from amberjack and yellowtail in Japan. They found eight restriction patterns that were clonally related. Similarly, BSFGE analysis of 284 *S. dysgalactiae* isolates from Japan resulted in 16 different patterns, all of which belonged to a single PFGE type (Nishiki *et al.,* 2010). These authors argued for the possible clonal expansion of this pathogen in Asia. However, results obtained by Abdelsalam *et al.* (2010) contradict this view. They analysed *S. dysgalactiae* strains isolated contemporaneously from nine fish species in different Asian countries. The majority of the isolates belonged to the same PFGE type; however, four other types were detected. The isolates from Malaysia and Indonesia were genetically distant from those from Japan and Taiwan (Abdelsalam *et al.,* 2010). Similar results were found in the present study. The 21 strains from Nile tilapia were classified into three different PFGE types. The isolates from Alagoas State (SD92 and SD94) were genetically divergent from the strains from Ceará. There were no epidemiological relationships between farms from both states. This finding might suggest that *S. dysgalactiae* populations evolved from distinct ancestors.

Based on our results and the data from Abdelsalam *et al.* (2010), fish pathogenic *S. dysgalactiae* seem to have a diverse core population that exhibits meaningful geographically determined genetic diversity that is similar to that found in other streptococci pathogens of fish (Evans *et al.,* 2008; Pereira *et al.,* 2010). Future studies that apply more discriminatory and evolutionary traceable methods, such as

multi-locus sequence typing (MLST), should be performed to provide new insights into the genetic and evolutionary characteristics of this pathogen.

Herein, REP-PCR and ERIC-PCR were used for genotyping analysis of isolates of *S. dysgalactiae* from Nile tilapia. Both techniques had low discriminatory power. The REP-PCR amplified a single band for all isolates and was unable to discriminate among them. With ERIC-PCR, two profiles were verified for the 21 strains. However, the results were incongruent with the data obtained using PFGE, which is the accepted typing tool for this bacterium. Therefore, these techniques were shown to be unsuitable for use as genotyping tools for Nile tilapia pathogenic *S. dysgalactiae*.

In conclusion, isolates of *S. dysgalactiae* from Nile tilapia constitute a genetically diverse population. The PFGE technique showed higher discriminatory power and applicability than PCR-based methods for genotyping this pathogen.

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4 – CHAPTER 4

Efficacy of an oil adjuvanted vaccine against *Streptococcus dysgalactiae* for Nile tilapia, *Oreochromis niloticus* (L.)

ABSTRACT

Lancefield group C *Streptococcus dysgalactiae* has been associated with outbreaks characterized by necrosis of the caudal peduncle and mortalities of marine and freshwater fish species. The disease has a significant impact on Nile tilapia farms in Brazil. The aims of this study were to develop a model of *S. dysgalactiae* challenge able to reproduce the typical lesions of disease in Nile tilapia and to determine the efficacy of an oil adjuvanted vaccine against the illness. Nile tilapia juveniles were experimentally infected with the pathogen by intraperitoneal, intramuscular, immersion and oral routes, associated to different infective doses. Two different *S. dysgalactiae* vaccines were tested: a bacterin vaccine and an oil adjuvanted vaccine. Their efficacy was evaluated in Nile tilapia at 60 and 90 days post-vaccination (d.p.v.). The typical disease was best reproduced by intramuscular route. The pathogen was not able to induce the disease by non-invasive routes (immersion and oral treatment). Oil adjuvanted vaccine exhibited a long-term protection against disease, reaching relative percent survival (RPS = 92,5) significantly ($p < 0,05$) higher in comparison to bacterin (RPS = 57,5) at 90 d.p.v. The oil adjuvanted vaccine provided effective protection against. *S. dysgalactiae* infection in Nile tilapia.

Keywords: Brazil; *Streptococcus dysgalactiae*; challenge model; vaccination.

INTRODUCTION

The Lancefield group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* was first described as an aetiological agent of fish disease in 2002, during outbreaks on farms of amberjack, *Seriola dumerili*, and yellowtail, *Seriola quinqueradiata*, in Japan (Nomoto *et al.,* 2004). Since that, increasing number of outbreaks caused by this pathogen has been reported in many species of cultured marine fish (Nomoto *et al*., 2008; Yang and Li, 2009; Abdelsalam *et al.,* 2010) and Nile tilapia (Netto *et al.*, 2011; Costa *et al*., 2013). *S. dysgalactiae* infection causes high mortality rates in fish farms, with ulceration and/or subcutaneous abscess at the caudal peduncle region.

Antibiotic therapy is the common control measure used to treat disease caused by *Streptococcus dysgalactiae*. However, this approach is not always effective due some abscess characteristics, as the presence of a fibrotic capsule and the low pH that may impairs the activity of many antimicrobials (Brook, 2002). A potential means of controlling the impact of *S. dysgalactiae* in Nile tilapia farms would be the development of an efficient vaccine. Formalin killed *S. dysgalactiae* had been found to be effectively for soiny mullet, *Liza haematocheula* (Qi *et al.,* 2013). Unfortunately, previous study produced an acute disease during experimental infection in Nile tilapia, and failed to produce the clinical signs observed in outbreaks (Netto *et al.,* 2011). For vaccine test efficiency is essential the standardization of a challenge able to reproduce the lesions induced in fish infected with *S. dysgalactiae*, mainly abscesses.

A commercially available *Streptococcus agalactiae* vaccine is frequently used in Brazilian Nile tilapia farms to avoid economic losses. However, the disease caused by *S. dysgalactiae* continues to impact some of these farms. The aim of this study was to test the efficacy of vaccine formulated with a whole-cell inactivated bacterin emulsified in oil adjuvant. In addition, we developed a model for experimental challenge of *S. dysgalactiae* in Nile tilapia.

MATERIALS AND METHODS

Fish

Nile tilapia juveniles (average weight 32 ± 4.3 g) were acquired from a commercial hatchery. Prior to challenge or vaccination, 20 fish were randomly selected and submitted to bacteriological analysis to ensure they were free of bacterial infections. The fish were acclimatized for a minimum period of 20 days and were taken off feed one day prior to challenge or vaccination. Fish were maintained in 120 L aquaria equipped with supplementary aeration system and flow-through water (10 L h^{-1}) at a temperature ranging from 30 to 32 ºC.

Bacterial strain and culture conditions

S. dysgalactiae SD64, a strain isolated from the brain of Nile tilapia during field outbreak in Brazil, was used in this study. This strain was previously tested in experimental infections (Netto *et al.,* 2011). The results of a PFGE analysis demonstrated that SD64 belonged to the pulsotype A, in which 81% of the isolates were classified (Costa et al., 2011). This strain was thawed and streaked onto Todd Hewitt agar (THA) (BD) at 28 ºC for 24 h. A single colony was inoculated in Todd Hewitt broth (BD) and incubated at 28 ºC for 18 h under low agitation (150 rpm). Afterwards, the bacterial culture was centrifuged (3000 x g for 20 min), washed three times, and ressuspended in sterile phosphate buffered saline (PBS) prior to be used in experimental challenges or vaccine production.

Challenge assays

To identify some of the infection routes of *S. dysgalactiae* in Nile tilapia and to develop a model for experimental challenge, four infection routes were tested: intraperitoneal, intramuscular, immersion, and oral. Groups A and B were intraperitoneally infected with 0.2 mL of the bacterial suspension at a final concentration of 10^7 and 10^8 colony forming units (CFU) per fish, respectively. Intramuscular challenges were tested with 0.2 mL of *S. dysgalactiae* suspension at concentration of 10^7 (group C) and 10^8 (group D) CFU $fish^{-1}$. The challenge dose was injected into muscle approximately 2 cm dorsally to the lateral line. Before challenges, fish were anesthetized by immersion in a bath containing 10 mg L^{-1} benzocaine (Sigma-Aldrich).

For immersion trial, 100 mL of bacterial inoculums at 10^9 CFU mL^{-1} were diluted in a 10 L bucket containing 9900 mL of sterile water to reach a final concentration of 10^7 CFU mL^{-1}. Group E was immersed into this solution for 30 min with aeration and then returned to the 120 L aquarium containing uncontaminated water. Oral challenge was carried out with ground commercial feed (Matsuda) mixed with an appropriate volume of the *S. dysgalactiae* suspension at a concentration of 10^9 CFU mL^{-1}. The infected feed was supplied to fish (group F) once time at the rate of 2% body weight. Control groups were submitted to the same experimental procedures applied to all challenge groups: except that sterile PBS was used for intraperitoneal, intramuscular, immersion and oral trials, instead of bacterial inoculums.

Each group was composed by 20 fish and the procedure was performed in duplicate for all challenge assays. The results

presented as average of data from each replicate. Fish were observed for the presence or absence of gross lesions. Records were made in a manner (present or not present) for the presence of subcutaneous abscesses seen as positive. All groups were kept at 32 ºC for a period of 30 days, and dead fish were submitted to bacteriological analyses and macroscopic pathological evaluation.

Vaccination and vaccine test efficiency

To produce the bacterin, bacterial cells were inactivated by the addition of 2% (v/v) formalin (formaldehyde 38% w/v) and kept at 4 ºC for 24 h. The solution was centrifuged (7000 g for 30 min), washed three times, and resuspended in sterile PBS at the final concentration of 1 x 10^9 CFU ml^{-1}. Sterility was confirmed by spreading the bacterin on THA plates and incubating at 28 ºC for 72 h. To produce the non mineral oil adjuvanted vaccine, a ratio of 30:70 of antigen (bacterin) and adjuvant (Montanide ISA 763 A VG; Seppic), respectively, were mixed and emulsified.

Fish were anesthetized by immersion in 10 mg mL^{-1} benzocaine and individually immunized by intraperitoneal (IP) injection of 0.1 ml of vaccine or PBS. Three immunization groups were tested: Group 1 – oil adjuvanted vaccine; Group 2 – bacterin; Group 3 – sterile PBS (negative control). Each experimental group (n = 30) was tested in duplicate.

Groups were challenged with *S. dysgalactiae* at 60 and 90 days post-vaccination (d.p.v.). Fish were challenged by IM injection with 0.2 mL of bacterial inoculum at a concentration of 10^7 CFU per fish (according to protocol described for group C in challenge assays). Samples of brain, kidney, and abscesses were aseptically collected from fish; they were streaked onto THA for bacterial re-isolation. In addition, necropsy examination was conducted for all fish to identify possible vaccine side effects (mainly organ adhesions).

Statistical analysis

The efficacy of the different vaccines (bacterin and oil adjuvanted vaccine) was evaluated by calculating the relative percent survival (RPS) according to Amend (1981) as follows: RPS = 1 – (% mortality in vaccinated fish / % mortality in controls) x 100. The differences in survival and presence of abscess after challenges were compared using the Chi-square test at the significance level of $p < 0.05$. The data were analysed using Statistical Software R (Chang *et al.,* 2010).

RESULTS

Challenge study

The characteristic clinical signs of *Streptococcus dysgalactiae* infection in fish, mainly the presence of subcutaneous abscess, were observed in intramuscular and intraperitoneal challenge groups. The gross lesions were significantly higher (p = 0,0031) reproduced in intramuscular challenge in low dose of bacteria (Table 4.1). In both groups of low (group C) and high dose (group D) of bacteria injected intramuscularly, challenged fish had visible abscesses in the caudal peduncle and base of dorsal fin (Figure 4.1). The intraperitoneal challenges, groups A and B, induced small nodules in the head and ulcerations in dorsal fin in diseased fish. Accumulated mortality ranging from 25% to 60% was verified in fish groups infected intraperitoneally and intramuscularly. Other clinical signs were also observed in those groups, mainly hyporexia, lethargy and skin darkness. Neither mortalities nor clinical signs were observed in groups challenged by immersion and oral (groups E and F), as in the control groups.

The intramuscular challenge using the low

dose (4 x 10^7 CFU $fish^{-1}$) induced abscesses in 65% of fish and a cumulative mortality of 60%. This model was considered the most efficient method to reproduce the lesions of *S. dysgalactiae* in Nile tilapia.

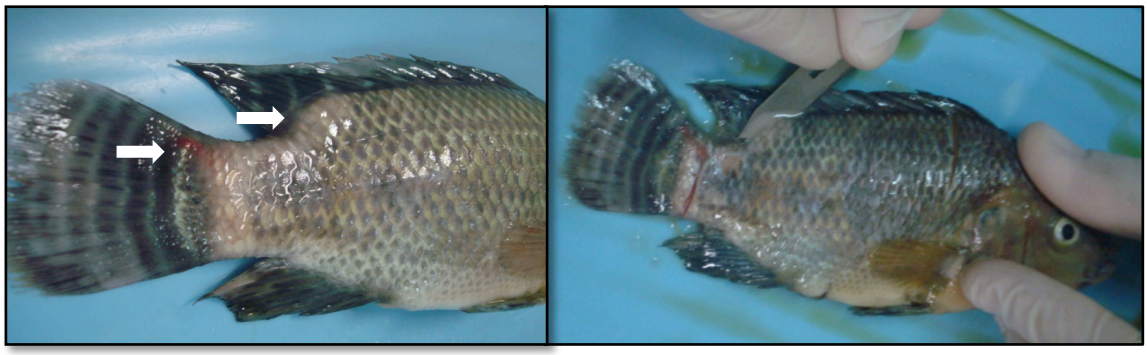

Figure 4.1 Nile tilapia experimentally infected by intramuscular injection with *Streptococcus dysgalactiae* showing subcutaneous abscesses (arrows)**.**

Table 4.1 Results of mortality and presence of lesions observed in different experimentally challenged groups.

Group	Infection route	Challenge (CFU/fish)	Mortality (%)	Fish with abscess (%)
A	Intraperitoneal	4 x 10^{7a}	25*	5**
B	Intraperitoneal	4 x 10^{8a}	35*	10**
C	Intramuscular	4 x 10^{7a}	60*	65*
D	Intramuscular	4 x 10^{8a}	40*	25**
E	Immersion	2 x 10^{7b}	0**	0**
F	Oral treatment	2 x 10^{9b}	0**	0**

[a]cfu $fish^{-1}$

[b]cfu mL^{-1} of water

*Statistically significant ($p < 0,05$) in the same column

Efficacy of *S. dysgalactiae* vaccines

Survival rates for fish inoculated with the different vaccines after challenged with *S. dysgalactiae* at 60 and 90 days d.p.v. are presented in Figure 4.2. Mortalities of 37% and 40% were observed in control groups (PBS) challenged at 60 and 90 d.p.v., respectively. In fish vaccinated with bacterin, mortality was 13% (RPS = 64,8%) at 60 d.p.v.; and the cumulative mortality at 90 d.p.v. was 17% (RPS = 57,5%) (Table 4.2). Diseased fish presented typical signs of illness, including hyporexia, skin darkness, ulceration and/or abscess in the caudal peduncle. The bacterium was re-isolated from all diseased fish.

Mortalities of 7% and 3% were observed in groups immunized with the whole-cell inactivated bacterin emulsified in oil adjuvanted vaccine challenged 60 and 90 d.p.v. and thus had RPS values of 80,5% and 92,5%, respectively. No statistically differences were observed in survival of fish immunized with bacterin and bacterin with oil adjuvanted vaccine challenged at day 60 post vaccination. However, the protection conferred by oil adjuvanted vaccine 90 d.p.v. was statistically (p = 0.0295) superior to that of bacterin. The oil adjuvanted vaccine also avoided the lesions induced in the *S. dysgalactiae* infection and abscesses were only observed in groups immunized with bacterin and PBS. No side effects were observed in vaccinated fish at necropsy examination.

Table 4.2 Mortality, relative percent survival (RPS) and presence of subcutaneous abscess in caudal peduncle of diseased fish after immunization with different *Streptococcus dysgalactiae* vaccines in Nile tilapia.

	Challenge (CFU ml^{-1} per fish)		**Mortality (%)**		**RPS (%)**		**Abscess/fish**	
	Days post vaccination		Days post vaccination		Days post vaccination		Days post vaccination	
Immunization	60	90	60	90	60	90	60	90
Adjuvanted vaccine	4.9 x 10^7	5.6 x 10^7	7	3	80,5*	92,5*	0/30	0/30
Bacterin	4.9 x 10^7	5.6 x 10^7	13	17	64,8*	57,5**	2/30	3/30
Sterile PBS	4.9 x 10^7	5.6 x 10^7	37	40	-	-	10/30	9/30

*Statistically significant (p < 0,05)

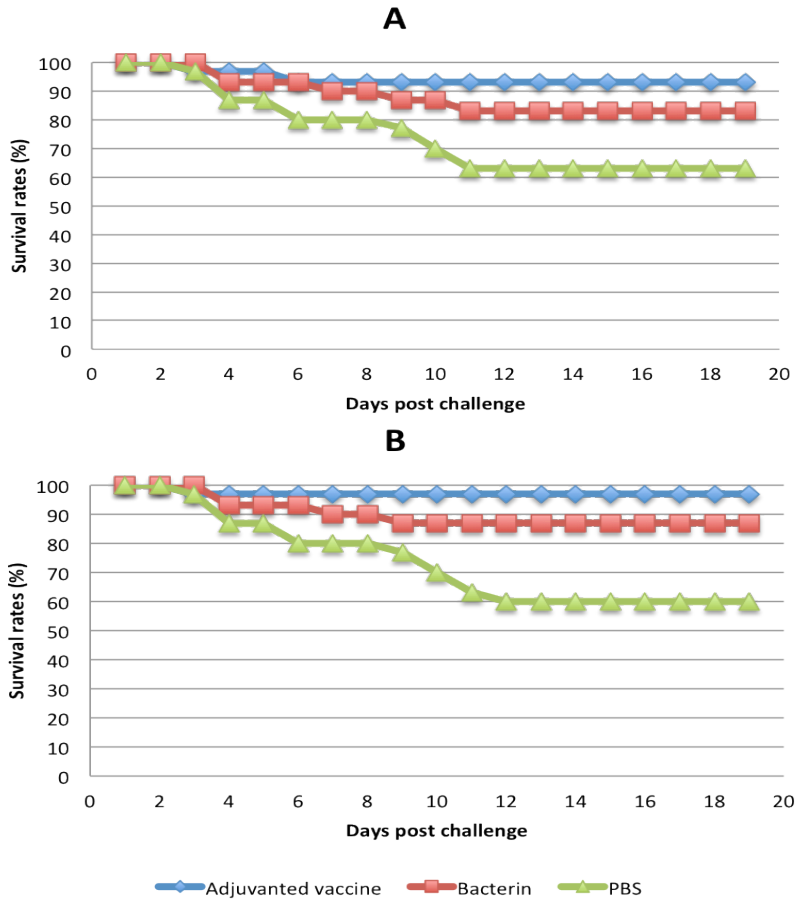

Figure 4.2 Survival rates after intramuscular challenge in different groups of fish vaccinated with solution containing adjuvanted vaccine, bacterin, or PBS. Nile tilapia challenged at 60 d.p.v. **(A)** and 90 d.p.v. **(B)**.

DISCUSSION

The rapid growth of aquaculture over the last decades has been accompanied by some emerging pathogens and comes with the need for effective disease control measures (Evensen and Leong, 2013). Diseases prevention by a safe and efficacious vaccine is the most appropriate method for pathogen control to the fish farming sector (Taffala *et al.,* 2013). However, for vaccine test efficiency is essential to have an experimental model of challenge able to reproduce the disease. In the present study a standard experimental challenge for *Streptococcus dysgalactiae* in Nile tilapia was proposed and the efficiency of different vaccines were tested.

In marine fish species the typical signs of *S. dysgalactiae* disease were successfully reproduced under laboratory conditions by invasive routes: intraperitoneal, intramuscular and intradermal (Hagiwara *et al.*, 2009; Yang and Li, 2009). However, a previous study was not able to induce ulceration and abscess in Nile tilapia infected intraperitoneally and intramuscularly (Netto *et al.,* 2011). In our study the lesions could be better reproduced by intramuscular route, at a low dose of bacteria, and higher mortalities were also

observed in this model of challenge. Instead, the high dose of pathogen used by Netto *et al.* (2011) could explain the acute disease observed by these authors, with high mortality and absence of typical lesions. The virulence of *S. dysgalactiae* was also demonstrated by immersion and oral treatment challenges in *Seriola dumerili* (Hagiwara *et al.,* 2010). However, we could not reproduce the illness by non-invasive routes in Nile tilapia. This could suggest that Brazilian isolate (SD64) are less able to bypass the natural skin and mucosal barriers of the fish, a consequence of some virulence factors absence of our strain and/or high resistance of Nile tilapia to this pathogen. Abdelsalam *et al.* (2013) suggested that some cell surface characteristics such as hemagglutination and hydrophobic properties, which determine the main adhesion and invasive pathogenic mechanism, would be present and more expressed in higher virulent strains. To understand better these relationships among bacterium and host during *S. dysgalactiae* infection, future pathogenesis and virulence studies should be performed.

Vaccines developed by formalin-killed bacteria (bacterin) have been found to be effectively to protect fish against streptococcosis (Evans *et al.,* 2004; Eldar *et al.,* 1997). Recently, Qi *et al.* (2013) tested the efficacy of a formalin inactivated *S. dysgalactiae* for soiny mullet and the mortality in immunized group was significantly lower than that of control group. Herein, we evaluated the efficacy of two vaccines in Nile tilapia juveniles. In our study, the performances of bacterin (RPS = 64,8) and vaccine oil adjuvanted (RPS = 80,5) were not significantly different in groups challenged at 60 d.p.v. However, the efficiency provided by the bacterin (RPS = 57,5) at 90 d.p.v. decreased and the protection of oil adjuvanted vaccine (RPS = 92,5) was significantly ($p < 0,05$) higher compared to bacterin. Therefore, the oil adjuvanted is frequently used in fish vaccines to increase the immunogenicity of an antigen and extend the immune response and protection due the slow release of components (Midtlyng *et al.,* 1996; Taffala *et al.,* 2013). The vaccine prepared with oil adjuvant was also able to avoid the formation of abscesses in all fish after *S. dysgalactiae* challenge at 60 and 90 d.p.v. Instead, in groups immunized with bacterin the lesions were observed in some Nile tilapia (7% and 10% at 30 and 90 d.p.v., respectively). Field trials to test the efficacy of the oil adjuvanted vaccine need to be done at farm conditions.

The present study is to our knowledge the first description of an efficient vaccine for Nile tilapia against *Streptococcus dysgalactiae* infection. The oil adjuvanted vaccine confers successfully protection reducing mortality rates and avoids the lesions induced by this pathogen. In addition, we proposed a model of challenge able to reproduce the typical signs of *S. dysgalactiae* for experimental infection in Nile tilapia.

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5 – CHAPTER 5

Characterization of *Weissella ceti* infections in Brazilian rainbow trout farms and development of an oil adjuvanted vaccine

(Article accepted for publication in Journal of Fish Diseases)

ABSTRACT

Weissella ceti is an emerging bacterial pathogen that affects rainbow trout (*Onchorhynchus mykiss*) farms. The aims of this study were to genotype *W. ceti* strains isolated from distinct geographic origins and to determine the efficacy of an oil adjuvanted vaccine against the disease. Between 2010 and 2012, outbreaks were recorded in five Brazilian farms, and 34 *W. ceti* isolates were genetically characterized by repetitive extragenic palindromic PCR, enterobacterial repetitive intergenic consensus sequences PCR, and pulsed field gel electrophoresis. Two different *W. ceti* vaccines were tested: an aqueous-based whole cell inactivated vaccine (bacterin) and oil-adjuvanted vaccine. Their efficacy was evaluated in rainbow trout at 30 and 60 days post-vaccination (d.p.v.). *W. ceti* was found to be a highly homogeneous population in Brazil, with clonally related genotypes. Oil adjuvanted vaccine exhibited the best ($p < 0.05$) protection against disease, reaching relative percent survival (RPS) values of 92% at 30 and 60 d.p.v. Bacterin resulted in RPS values of 67% and 58% at day 30 and 60, respectively. The oil adjuvanted vaccine provided effective protection against *W. ceti* infection in rainbow trout.

Keywords: Brazil; emerging disease; fish; genotyping; vaccination.

INTRODUCTION

Weissella sp. is a Gram-positive non-motile bacterium that is an emerging pathogen affecting rainbow trout, *Onchorhynchus mykiss*, farms. Outbreaks are characterized by acute haemorrhagic septicaemia and high mortality. The first case of *Weissella* sp. infection in a rainbow trout farm was reported in China in 2009 (Liu *et al.,* 2009). After that, several outbreaks were described in Brazil and the United States (Figueiredo *et al.,* 2012; Welch and Good, 2013). Genome sequencing of an isolate from the United States revealed that this pathogen is supposed to be *Weissella ceti* (Ladner *et al.,* 2013), a recently described bacterial species isolated from beaked whales (Vela *et al.,* 2011).

Liu *et al.* (2009) and Welch and Good (2013) investigated the genetic diversity of *Weissella* sp. isolated from rainbow trout and found no differences among strains. However, isolates from only a few outbreaks were evaluated, which could bias the results. Currently, no data are available about the genetic structure of trout pathogenic *W. ceti* from multiple outbreaks. Moreover, data from South America are lacking.

Antibiotic therapy is the common control measure applied to treat *W. ceti* infection in rainbow trout farms. However, this approach is not always effective because anorexia is present as the first clinical sign of disease. Recently, an experimental vaccine was tested and shown to provide protection 72 days post vaccination against the disease in rainbow trout juveniles (Welch and Good, 2013). However, an aqueous bacterin usually provides short duration of immunity, and it may not enough to protect rainbow trout all the rearing period (Ravelo *et al.,* 2006). A vaccine formulated with bacterin and adjuvant could enhance the potency and/or duration of protection against the *W. ceti* infection in rainbow trout.

In this study, the genetic diversity of *W. ceti* strains isolated from outbreaks in geographically distinct farms in Brazil was evaluated. In addition, the efficacy of a

vaccine containing whole cell bacterin emulsified in oil adjuvant was tested in rainbow trout against the disease.

MATERIALS AND METHODS

Outbreaks

Five disease outbreaks in geographically distant Brazilian rainbow trout farms were reported between 2010 and 2012. The cases did not show epidemiological connection with each other. The farms have different water supplies, and no history of movement or introduction of biological materials, such as embryonated eggs, fry, fingerlings or adults, was reported in the last two years. Diseased fish with clinical signs of haemorrhagic septicaemia (including fingerlings, juveniles, and adults) were sampled, stored at 4 ºC, and taken to the laboratory for bacteriological analysis.

Bacterial isolation and characterization

Samples of brain, eye, kidney, and liver from diseased fish were aseptically taken, streaked on 5% sheep blood agar and de Man, Rogosa and Sharpe (MRS) agar (Sigma-Aldrich), and incubated at 25 ºC for 48 h. Pure colonies from all samples were examined for Gram stain, catalase, and oxidase tests. To confirm the identity of the bacteria, *Weissella* genus-specific PCR was performed for all isolates following Jang *et al.* (2002) with some modifications (Figueiredo *et al.,* 2012). The strains were stored at –80 ºC in MRS broth with 15% glycerol until use.

The isolates used in this study were obtained from five different farms between 2010 and 2012 and from three other Brazilian outbreaks previously described (Figueiredo *et al.,* 2012). A total of 34 strains isolated from these eight farms, at least four per farm (Supplementary table), were selected for molecular analysis. DNA was extracted using the commercial DNeasy kit (Qiagen) according to the manufacturer's instructions. The amount of extracted DNA was quantified spectrophotometrically using Nanodrop® (Thermo Scientific, GE). Amplification and sequencing of the 16S rRNA gene were performed for all 34 isolates. 16S rRNA was amplified by PCR with the universal primers C70 (5'-AGAGTTTGATYMTGGC-3') and B37 (5'-TACGGYTACCTTGTTACGA-3') according to the method described by Fox *et al.* (1995). PCR products were purified using a Wizard PCR Preps kit (Promega) and sequenced using forward and reverse primers. Sequencing reactions were performed using a BigDye™ Terminator Cycle sequencing kit (Applied Biosystems) and run on an ABI 3730XL genetic analyzer (Applied Biosystems). Sequences were then compared to sequences from the NCBI database using the BLASTn algorithm (Madden *et al.,* 1996).

REP-PCR and ERIC-PCR

Repetitive extragenic palindromic (REP) and Enterobacterial repetitive intergenic consensus sequences (ERIC) sequences were searched in the genome of *Weissella ceti* NC36 avaible in the GenBank (NCBI Reference Sequence: NZ_ANCA00000000.1 (data not shown). These sequences were found in the genome of this bacterial species. Therefore, REP-PCR and ERIC-PCR were evaluated as genotyping tool for fish strains of *Weissella ceti*.

Repetitive extragenic palindromic PCR (REP-PCR) was performed as described previously for other Gram-positive bacteria (Ouoba *et al*., 2010), with some modifications. Briefly, the reactions were performed using a HotStart Taq Polymerase kit (Qiagen); the mix was composed of 1 × PCR buffer, 0.5 μmol L^{-1} of GTG5 primer (5'-GTGGTGGTGGTGGTG-3'), 0.3 μmol L^{-1} of dNTPs, 1.5 m mol L^{-1} of $MgCl_2$, 1 U of Taq DNA polymerase, and 40 ng of

template DNA. The PCR conditions consisted of an initial step of 95 °C for 15 min, followed by 30 cycles of 95 °C for 30 s, 45 °C for 1 min, and 72 °C for 4 min, with a final elongation step of 72 °C for 16 min.

Enterobacterial repetitive intergenic consensus sequences PCR (ERIC-PCR) was carried out using the primers ERIC-1 (5'-ATGTAA GCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG -3') according to Saito *et al.* (2011) with some modifications. The reactions were performed using a HotStart Taq Polymerase kit in a final volume of 25 μl containing 1 × PCR buffer, 0.6 μmol L^{-1} of each primer, 0.3 μmol L^{-1} of dNTPs, 2 mmol L^{-1} of $MgCl_2$, 2 U of Taq DNA polymerase, and 35 ng of template DNA. The PCR conditions consisted of an initial denaturation step at 95 °C for 15 min followed by 30 cycles of 90 °C for 30 s, 50 °C for 30 s, 52 °C for 1 min, and 72 °C for 1 min. The final elongation step was at 72 °C for 8 min.

The PCR products were separated by electrophoresis on a 1.5% agarose gel and run for 90 minutes at 120 V. Gel was stained with ethidium bromide (0.5 μg mL^{-1} for 30 min), and visualized by UV transillumination. Images were captured using an L-Pix EX (Loccus). Ladders of 100 bp and 1 kb (both from Promega) were used as molecular size standards for ERIC-PCR and REP-PCR, respectively.

PFGE

Pulsed field gel electrophoresis (PFGE) was performed as described previously (Teixeira *et al.,* 1995; Oliveira *et al.,* 2005). *W. ceti* strains were thawed and grown overnight in MRS broth at 25 °C. The cells were harvested by centrifugation and washed twice with PIV solution (0.01 M Tris-HCl, pH 8.0, and 1 M NaCl). The bacterial suspension was mixed at 1:1 (v:v) with 2% low melting-point agarose (Sigma-Aldrich) and pipetted into 20 μL plugs. The cells fixed in agarose plugs were lysed with 25 U of mutanolysin and 500 mg of lysozyme (both from Sigma-Aldrich). The DNA then was digested with 12 U of *SmaI* restriction enzyme (Amersham Biosciences). The plugs were submitted to PFGE using the following program: switch time of 1–30 s, 120° angle, 11 °C, and a voltage gradient of 6 V cm^{-1} for 28 h in a CHEF DR II System (Bio-Rad). The lambda ladder PFGE marker (New England Biolabs) was used as a DNA size marker. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg mL^{-1} for 30 min) and images were captured using an L-Pix EX (Loccus).

Data analysis

The REP-PCR, ERIC-PCR, and PFGE gels were analysed using the software BioNumerics version 6.6 (Applied Maths). The Dice coefficient was used to determine the similarities of the banding patterns (Dice, 1945). Dendrograms were created using the unweighted pair group method with the average (UPGMA) approach. Isolates that showed similarity ≥ 80% for PFGE and ≥ 90% for ERIC-PCR and REP-PCR were considered to be clonally related (Tenover *et al*., 1995; Singh *et al.,* 2006).

Fish

For the vaccination trials, rainbow trout adults (100 ± 6.28 g) were acquired from a commercial hatchery. Prior to the vaccination, 10 fish were randomly selected and submitted to bacteriological analysis to ensure they were free of bacterial infections. Fish were maintained in 120 L aquaria equipped with a supplementary aeration system and flow-through water (10 L h^{-1}) at a temperature ranging from 16 to 18 ºC. Fish were acclimated for a period of 20 days.

Vaccine and vaccination

W. ceti isolate WS08, which was previously

tested in experimental infections and able to reproduce the disease (Figueiredo *et al.,* 2012), was grown at 25 ºC in MRS broth for 18 h until it reached a final concentration of 1 x 10^9 CFU ml^{-1}. To produce the bacterin vaccine, the bacterial culture was centrifuged (7000 g for 30 min), washed three times and re-suspended in sterile phosphate buffer saline (PBS) pH 7.2. Bacterial cells were inactivated by the addition of 1% (v/v) formalin (formaldehyde 38% w/v) and kept at 4 ºC for 24 h. The solution then was centrifuged (7000 g for 30 min), washed three times, and re-suspended in sterile PBS at the final concentration of 1 x 10^9 CFU ml^{-1}. Sterility was confirmed by spreading the bacterin on MRS agar plates and incubating the plates at 25 ºC for 72 h. To produce the non mineral oil adjuvanted vaccine, a ratio of 30:70 of antigen (bacterin) and adjuvant (Montanide ISA 763 A VG; Seppic), respectively, were mixed and emulsified.

Fish were anesthetized by immersion in 10 mg mL^{-1} benzocaine and individually immunized by intraperitoneal (IP) injection of 0.1 ml of vaccine or PBS. Four immunization groups were tested: Group 1 – bacterin and oil adjuvanted vaccine; Group 2 – bacterin; Group 3 – sterile PBS and oil adjuvant; Group 4 – sterile PBS. Each experimental group (n = 24) was tested in duplicate.

Challenge assays

Groups were challenged with the pathogen at 30 and 60 days post-vaccination (d.p.v.). Strain WS08 was streaked onto MRS agar and incubated at 25 ^oC for 24 h. One colony was inoculated in MRS broth and incubated at 25 ^oC for 18 h under low agitation (150 rpm). Afterwards, the bacterial suspension was centrifuged (7000 g for 30 min), washed three times, and re-suspended in PBS. Fish were challenged by IP injection with 0.1 ml of a bacterial inoculum resulting in a concentration of 10^7 CFU per fish. Challenged fish were kept at 18 ºC, and mortalities were recorded daily for 15 d. Samples of brain, kidney, and liver were aseptically collected from all dead and surviving fish at the end of the experiment; they were streaked onto MRS agar for bacterial reisolation. In addition, necropsy examination was conducted for all fish to identify possible vaccine side effects (mainly organ adhesions).

Statistical analysis

The efficacy of the different vaccines (bacterin and oil adjuvanted vaccine) was evaluated by calculating the relative percent survival (RPS) according to Amend (1981) as follows: RPS = 1 – (% mortality in vaccinated fish / % mortality in controls) x 100. The differences in survival after challenge were compared using the Chi-square test at the significance level of $p < 0.05$. The data were analysed using Statistical Software R (Chang *et al.,* 2010).

RESULTS

Characterization of outbreaks

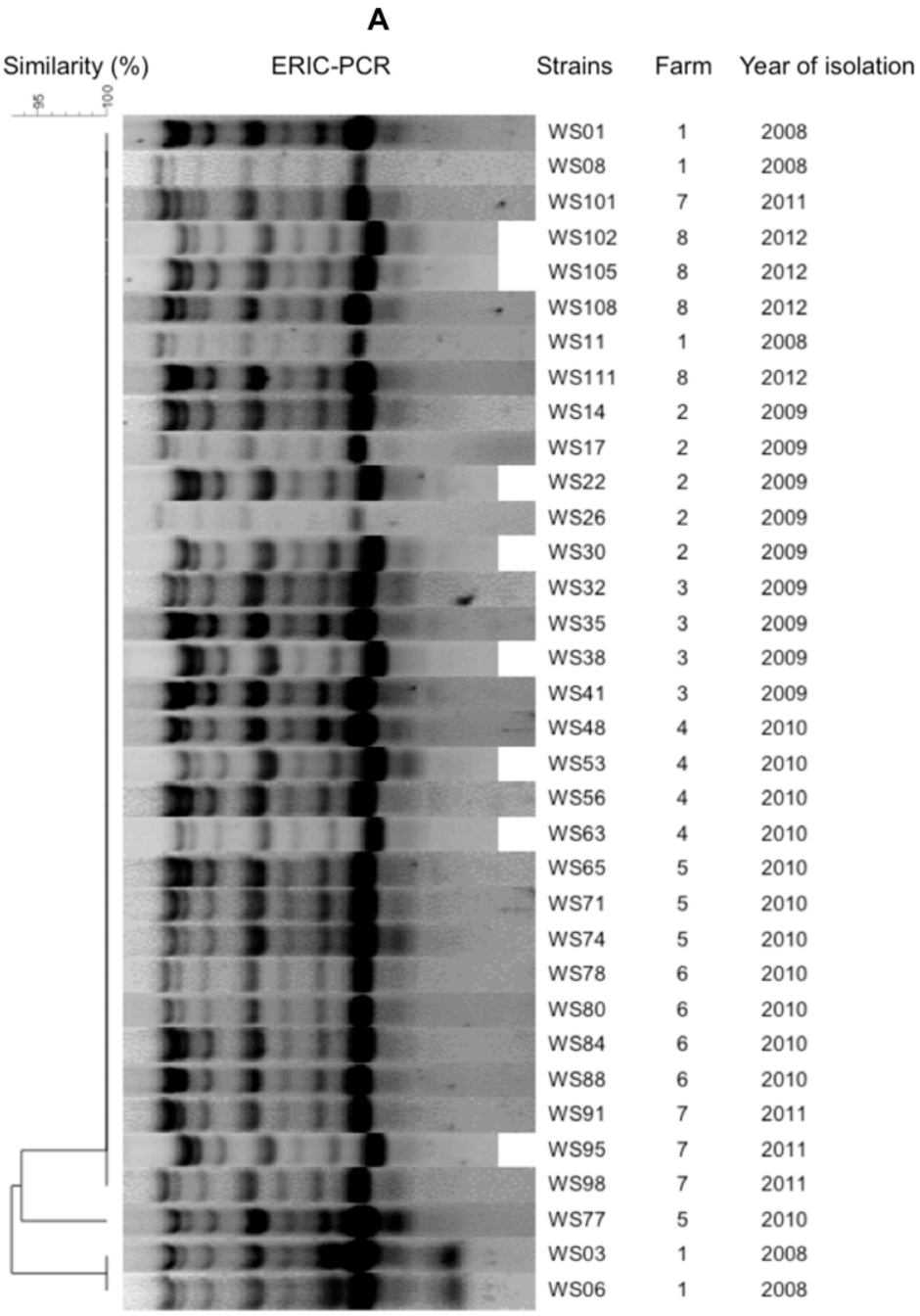
From December 2010 to March 2012, *W. ceti* isolates were obtained from five different rainbow trout farms in Brazil. They were all positive for *Weissella* genus-specific PCR reactions. Amplification of the 16S rRNA fragments yielded a product of approximately 1500bp. Blast analysis of the sequences revealed that all 34 strains showed 99% similarity to a previously reported sequence of the 16S ribosomal RNA gene of a *Weissella ceti* strain (FN813251) isolated from beaked whales (Vela *et al.,* 2011).

The main clinical signs verified in diseased fish were lethargy, anorexia, melanosis, exophthalmia, ascites, and haemorrhages in the mouth, tongue, eyes, and fins. Hypertrophy of kidney and spleen, and petechiae in the cavity coelomic wall were

observed on necropsy. The farms had no recent history of exchange of biological materials. During outbreaks, mortality rates up to 80% were observed, and the disease affected fingerling, juvenile, and adult fish. Increased water temperature (> 17 ºC) during the summer seemed to be the main predisposing factor associated with disease outbreaks.

REP-PCR, ERIC-PCR, and PFGE profiles

REP-PCR amplified nine bands ranging in size from 500 to 4000 bp. All strains have an identical REP profile and presented 100% of similarity among them. ERIC-PCR promoted the amplification of seven to nine bands ranging in size from 300 to 1400 bp. Three different profiles were observed with 93% similarity (Fig. 5.1 A). PFGE with *SmaI* digestion revealed five different patterns among the 34 *W. ceti* strains evaluated (Fig. 5.1 B). The profiles were classified as a single PFGE type based on the similarity threshold of 80%. Overall, the REP-PCR, ERIC-PCR, and PFGE data indicated that the *W. ceti* isolates associated with disease outbreaks in Brazilian farms are clonally related.


A
Similarity (%)
ERIC-PCR
Strains
Farm
Year of isolation
95
100
WS01 1 2008
WS08 1 2008
WS101 7 2011
WS102 8 2012
WS105 8 2012
WS108 8 2012
WS11 1 2008
WS111 8 2012
WS14 2 2009
WS17 2 2009
WS22 2 2009
WS26 2 2009
WS30 2 2009
WS32 3 2009
WS35 3 2009
WS38 3 2009
WS41 3 2009
WS48 4 2010
WS53 4 2010
WS56 4 2010
WS63 4 2010
WS65 5 2010
WS71 5 2010
WS74 5 2010
WS78 6 2010
WS80 6 2010
WS84 6 2010
WS88 6 2010
WS91 7 2011
WS95 7 2011
WS98 7 2011
WS77 5 2010
WS03 1 2008
WS06 1 2008

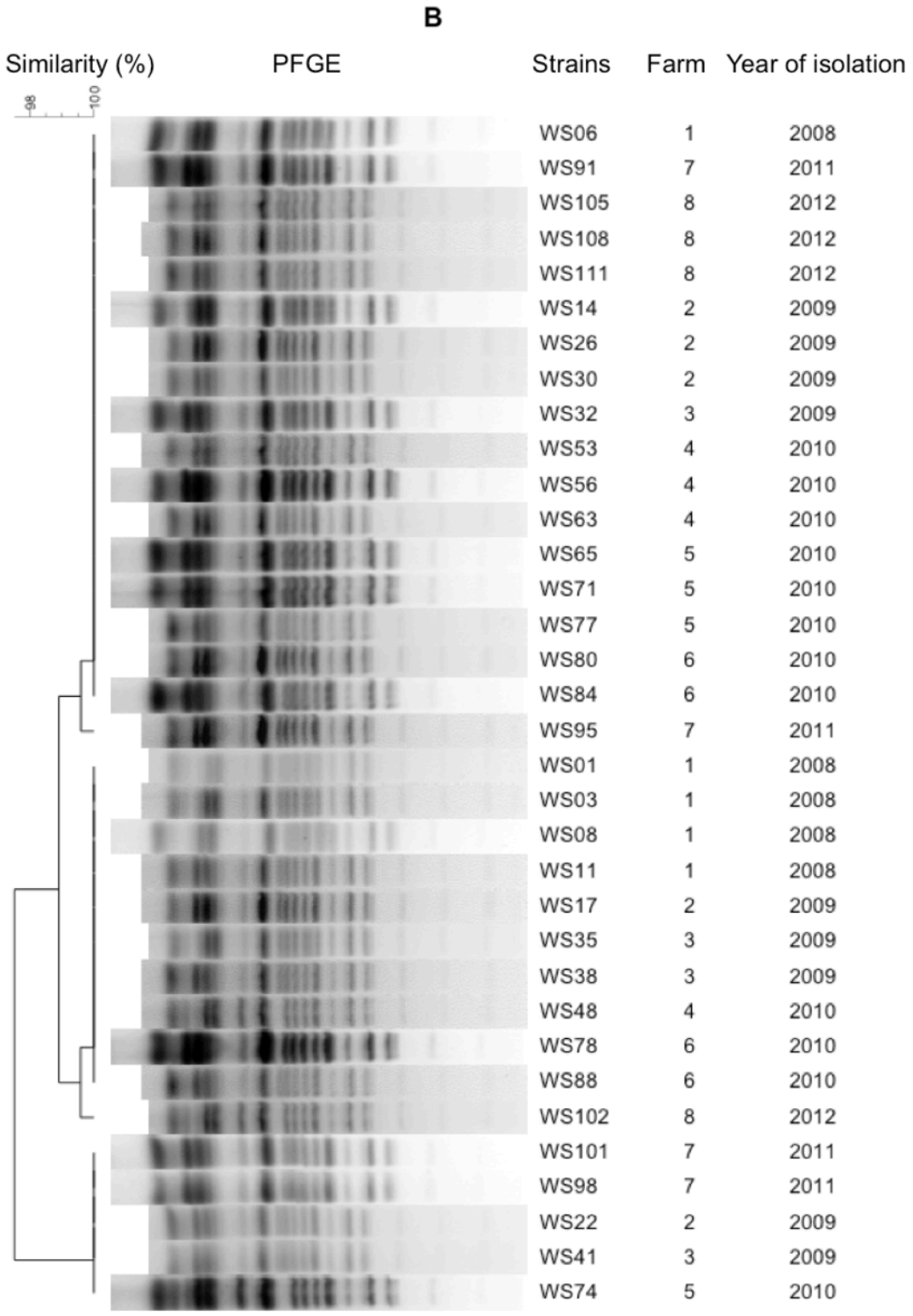


Figure 5.1 Cluster analysis of *Weissella ceti* strains using ERIC-PCR (**A**), and PFGE (**B**). The dendrograms were constructed using Dice's coefficient and the UPGMA approach. *W. ceti* isolates were obtained from eight different Brazilian farms between 2008 and 2012.

Efficacy of *W. ceti* vaccines

Table 5.1 and Figure 3.2 show the mortality results for fish inoculated with the different vaccines. Mortalities of 100% were observed in all control groups (PBS with and without adjuvant). In fish vaccinated with bacterin, mortality was 33% (RPS = 67%) at 30 d.p.v.; at 60 d.p.v., the cumulative mortality was 42% (RPS = 58%). Diseased fish presented typical signs of illness, including anorexia, exophthalmia, ascites, and haemorrhages in the fins. The bacterium was re-isolated from all diseased fish.

Two rainbow trout died (8% of mortality) in each group immunized with the adjuvanted vaccine to 30 and 60 d.p.v. and thus had RPS values of 92%. The protection conferred by adjuvanted vaccines was statistically (p = 0.033) superior to that of bacterin. The bacterium was re-isolated from 18% of surviving fish vaccinated with the adjuvanted vaccine and from 62% and 64% of the bacterin group challenged 30 and 60 d.p.v., respectively (Table 5.1). In all cases the pathogen was isolated only from the kidney. The adjuvanted vaccine reduces significantly (p = 0,014) the condition of carrier state in fish that survived after challenge. No side effects were observed in vaccinated fish at necropsy examination.

Table 5.1 Mortality, RPS and bacterial reisolation from surviving fish after vaccination with different *Weissella ceti* vaccines in rainbow trout.

	Challenge (CFU ml^{-1} per fish)		Mortality (%)		Relative Percent Survival (%)	
	Days post vaccination		Days post vaccination		Days post vaccination	
Immunization	30	60	30	60	30	60
Adjuvanted vaccine	3.4 x 10^7	4.2 x 10^7	8	8	92*	92*
Bacterin	3.4 x 10^7	4.2 x 10^7	33	42	67*	58*
Oil adjuvant	3.4 x 10^7	4.2 x 10^7	100	100	-	-
Sterile PBS	3.4 x 10^7	4.2 x 10^7	100	100	-	-

*Statistically significant (p < 0,05)

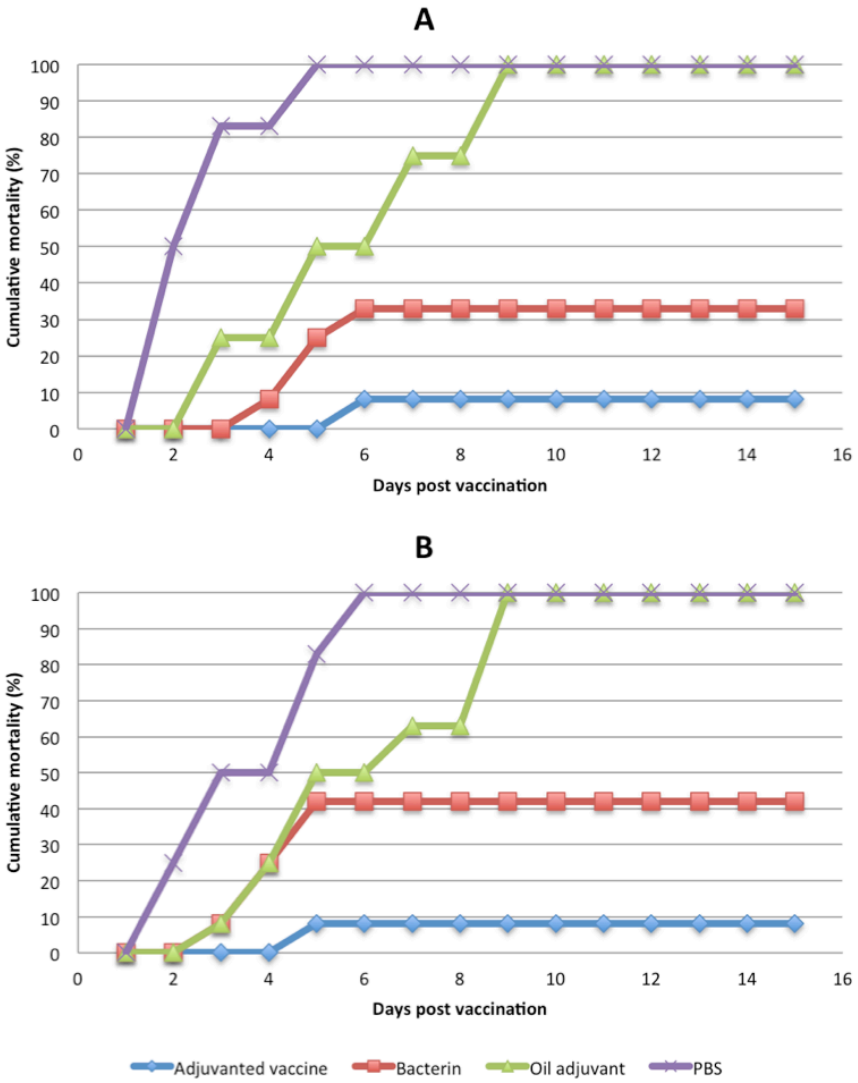


Figure 5.2 Cumulative mortality obtained in different groups of fish vaccinated with solution containing adjuvanted vaccine, bacterin, PBS with oil adjuvant or only PBS. Rainbow trout challenged at 30 d.p.v. **(A)** and 60 d.p.v. **(B)**.

DISCUSSION

Since the first disease outbreak in 2008, *Weissella ceti* infections have been continuously reported in rainbow trout farms located in different Brazilian states. The disease has been contemporaneously described in China, Brazil, and the United States. 16S rRNA sequences from isolates of all three countries are identical each other and very similar (99% in Blast analysis) with sequences of *Weissella ceti*. It suggests that these strains belong to the species *Weissella ceti*. None of these outbreaks showed any clear epidemiological linkage between them, but the clinical signs and evolution of disease were similar in all cases. However, in contrast to reports from China and the United States where clinical signs were observed only in adult rainbow trout (0,5 – 1 kg), the outbreaks in Brazil affected both young (fry and fingerlings)

and adult fish.

The genetic structure of fish pathogenic *W. ceti* is currently poorly understood. Liu *et al.* (2008) and Welch and Good (2013) evaluated the diversity of a few isolates obtained from a limited number of farms within a short period of time. Neither of the studies found differences between the strains analysed, suggesting a clonal composition of this bacterium population in each case.

In Brazil, the genetic diversity of *W. ceti* was unknown prior to the current study. Therefore, we used ERIC-PCR, REP-PCR, and PFGE to study 34 strains from eight farms isolated from disease outbreaks that occurred between 2008 and 2012. Similar to previous reports, these *W. ceti* isolates were found to be clonally related. Future studies need to be performed to address the genetic relationship between Chinese, Brazilian, and American isolates.

Recently, Welch and Good (2013) tested the efficacy of an aqueous-based whole-cell killed bacterin to control Weissellosis. Their vaccine was able to protect juveniles of rainbow trout (mean weight of 35 g), obtaining RPS values of 87.5% and 85% at 38 and 72 d.p.v. Herein, we evaluated the efficacy of two different vaccines in rainbow trout adults. In our study, the performance of bacterin (RPS values of 67% and 58% at 30 and 60 d.p.v.) was lower than that reported by Welch and Good (2013). Differences in the virulence of *W. ceti* strains used in the experiments could explain the different results. Brazilian strain WS08 caused 100% mortality in control groups, whereas the American strain NC36, used by Welch and Good (2013), resulted in 54% mortality. Both trials challenged the fish using the same infection route (IP) and dosages (10^7 CFU $fish^{-1}$). This highly virulent behaviour of the Brazilian isolates might be responsible for the reduced protection provided by bacterin.

Previous study indicated that for Gram-positive bacteria, PFGE could be used as an assisted method for the selection of candidate isolates for vaccine development (Chen *et al.,* 2012). In the present work, the oil adjuvanted vaccine promoted high protection in homologous challenge. The Brazilian isolates of *W. ceti* showed to be clonally related in PFGE, which may suggest their similar antigenicity, and the efficacy of vaccine in heterologous challenges. However, future studies have to be done to evaluate the ability of cross protection of this vaccine.

The use of an oil adjuvanted vaccine can increase and extend the immune response and protection due the slow release of antigen (Midtlyng *et al*., 1996). Emulsions stimulate the presentation of antigen to immunocompetent cells and protect the antigen from rapid degradation by enzymes (Audibert and Lise, 1993). In our study, the protection provided by the oil adjuvanted vaccine was significantly ($P < 0.05$) higher than bacterin at 30 and 60 d.p.v. (RPS of 92% vs. 67% and 58%, respectively). Bastardo *et al*. (2012) observed similar results for an aqueous vaccine (RPS 76.2%) and an adjuvanted vaccine (RPS 90%) against *Lactococcus garviae* at 30 and 90 d.p.v.

Although the adjuvanted vaccine conferred a high protection against *W. ceti*, both vaccines (adjuvanted and bacterin) were not able to eliminate, in the period tested, the presence of the bacterium in some surviving fish. Welch and Good (2013) also re-isolated *W. ceti* from brain and spleen of vaccinated fish that survived the challenge. This suggests a relative ability of *W. ceti* to evade the immune response induced by vaccination and might contribute to the maintenance of the pathogen in the farms. However, field trials are needed to test the efficacy of the oil adjuvanted vaccine at farm conditions.

For some fish pathogens, efficient vaccines have to be composed by whole bacterial cells and extracellular products (ECP) (Mohamed and Soliman, 2013). Based in our results and as previously described by Welch and Good (2013), the immune response against ECP of *Weissella ceti* seems not to be essential to confer protection against the mortalities caused by Weissellosis in rainbow trout. However, the pathogenesis of this disease is poorly understood, and it is possible that virulence factors present in extracellular products may contribute to immune evasion of bacteria. Future studies have to be performed to address the effect of ECP on protection against *W. ceti* infection.

In summary, our results revealed the clonal population structure of pathogenic *W. ceti* in Brazilian rainbow trout farms. The oil adjuvanted vaccine tested herein showed better results than bacterin, as it protected fish against infection for 60 d.p.v.

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Supplementary Table. Stage of fish development, isolation source, geographical origin and year of isolation of 34 *Weissella ceti* strains isolated from diseased rainbow trout (*Oncorhynchus mykiss*).

Strain	Stage of development	Source	Farm	State[a]	Year of isolation
WS01	Adult	Brain	1	RJ	2008
WS03	Adult	Kidney	1	RJ	2008
WS06	Adult	Kidney	1	RJ	2008
WS08	Adult	Brain	1	RJ	2008
WS11	Adult	Brain	1	RJ	2008
WS14	Adult	Brain	2	SP	2009
WS17	Adult	Brain	2	SP	2009
WS22	Adult	Liver	2	SP	2009
WS26	Adult	Eye	2	SP	2009
WS30	Adult	Eye	2	SP	2009
WS32	Fingerling	Brain	3	SP	2009
WS35	Fingerling	Kidney	3	SP	2009
WS38	Fingerling	Brain	3	SP	2009
WS41	Fingerling	Kidney	3	SP	2009
WS48	Juvenile	Brain	4	MG	2010
WS53	Juvenile	Kidney	4	MG	2010
WS56	Juvenile	Brain	4	MG	2010
WS63	Juvenile	Kidney	4	MG	2010
WS65	Adult	Kidney	5	MG	2010
WS71	Adult	Brain	5	MG	2010
WS74	Adult	Brain	5	MG	2010
WS77	Adult	Liver	5	MG	2010
WS78	Adult	Brain	6	MG	2010
WS 80	Adult	Liver	6	MG	2010
WS84	Adult	Brain	6	MG	2010
WS88	Adult	Brain	6	MG	2010
WS91	Adult	Brain	7	RJ	2011
WS95	Adult	Liver	7	RJ	2011
WS98	Adult	Brain	7	RJ	2011
WS101	Adult	Brain	7	RJ	2011
WS102	Adult	Brain	8	MG	2012
WS105	Adult	Brain	8	MG	2012
WS108	Adult	Brain	8	MG	2012
WS111	Adult	Kidney	8	MG	2012

[a]The location of the farms in Brazilian states are presented follow: RJ – Rio de Janeiro (A); SP – São Paulo (B); MG- Minas Gerais (C);

6 – CONCLUSIONS

The present study tested different molecular methods to genotype *Streptococcus dysgalactiae* and *Weissella ceti* Brazilian strains isolated from diseased Nile tilapia and rainbow trout, respectively. Among the techniques used the pulsed field gel electrophoresis (PFGE) demonstrated the highest discriminatory power. Genetic diversity was detected in *S. dysgalactiae* strains from different Brazilian regions, and an association was shown between geographical origin and genetic profiles. According to the genotyping methods used, *W. ceti* strains, from different farms, were classified as indistinguishable and considered clonally related. These data contribute to future researches, using mainly genomic analysis, to understand better the genetic and evolutionary aspects of both pathogens.

The study of genetic variability of pathogens contributed for a vaccine design. In the second part of the study we successfully develop vaccines for Nile tilapia and rainbow trout able to protect against the pathogens *S. dysgalactiae* and *W. ceti*, respectively. However, field trials need to be done to test the real efficacy of these vaccines in farm conditions. Furthermore, there are three PFGE genotypes among *S. dysgalactiae* strains, so the vaccine developed may be tested using "heterologous" challenges. Although *W. ceti* Brazilian strains were presented as a clonal population, suggesting that a vaccine prepared with a single isolate can protect against "homologous" challenges, more discriminatory methods should be performed to confirm this information.

Our studies provide scientific basis for others molecular epidemiology studies of outbreaks caused by *S. dysgalactiae* and *W. ceti*, and offers essential information to use the vaccination as an effective means for controlling these pathogens in fish farming.

APPENDIX

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Genotyping of *Streptococcus dysgalactiae* strains isolated from Nile tilapia, *Oreochromis niloticus* (L.)

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Abstract

Streptococcus dysgalactiae is an emerging fish pathogen that is responsible for outbreaks of disease on fish farms around the world. Recently, this bacterium was associated with an outbreak at a Nile tilapia, *Oreochromis niloticus* (L.), farm in Brazil. The aim of this study was to evaluate the genetic diversity, best genotyping method and aspects of molecular epidemiology of *S. dysgalactiae* infections in Nile tilapia farms in Brazil. Twenty-one isolates from four farms located in different Brazilian states were characterized genetically using pulsed-field gel electrophoresis (PFGE), ERIC-PCR, REP-PCR and *sodA* gene sequencing. The discriminatory power of the different typing methods was compared using Simpson's index of diversity. Identical *sodA* gene sequences were obtained from all isolates, and ERIC-PCR and REP-PCR were unable to discriminate among the isolates. PFGE typing detected three different genetic patterns between the 21 strains evaluated; thus, it was the best genotyping method for use with this pathogen. The strains from Ceará State were genetically divergent from those from Alagoas State. The *S. dysgalactiae* isolates analysed in this study constituted a genetically diverse population with a clear association between geographical origin and genotype.

Keywords: Brazil, molecular epidemiology, pulsed-field gel electrophoresis, *Streptococcus dysgalactiae*.

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Introduction

Streptococcus dysgalactiae is an aetiological agent of mastitis in cattle, endocarditis in various domestic animals, and pharyngitis and cardiopulmonary diseases in human beings (Efstratiou *et al.* 1994; Aarestrup & Jensen 1996; Williams 2003; Nomoto *et al.* 2004). This pathogen was first associated with fish diseases in 2002 when it was identified as being responsible for disease outbreaks on farms producing amberjack, *Seriola dumerili* (Risso), and yellowtail, *Seriola quinqueradiata* Temminck & Schlegel, in Japan (Nomoto *et al.* 2004). *S. dysgalactiae* infections were thought to be restricted to marine fish species, but an outbreak on a farm in Brazil that produces Nile tilapia, *Oreochromis niloticus* (L.), was recently attributed to this pathogen (Netto, Leal & Figueiredo 2011). Currently, data about the genetic diversity and molecular epidemiology of *S. dysgalactiae* infection in tilapia farms are unavailable.

The pulsed-field gel electrophoresis (PFGE) technique is considered to be the reference tool for genotyping of *S. dysgalactiae*. Previous studies have used a variation of this technique (biased sinusoidal gel electrophoresis – BSFGE) to determine the genetic diversity of *S. dysgalactiae* strains isolated from marine fish in Asia (Nomoto, Kagawa & Yoshida 2008; Abdelsalam, Chen & Yoshida 2010; Nishiki *et al.* 2010). The results of these studies suggested that the populations of *S. dysgalactiae* in fish were clonally related and that clonal expansion of the bacterium occurred in that region (Abdelsalam *et al.* 2010; Nishiki *et al.* 2010). Although PFGE has high discriminatory power, the method is labour intensive and time-consuming. Rapid and inexpensive PCR-based typing methods, such enterobacterial

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repetitive intergenic consensus PCR (ERIC-PCR) and repetitive extragenic palindromic-PCR (REP-PCR), have been developed as alternatives for screening the genetic relatedness of bacterial populations (Kidd *et al.* 2011; Lee *et al.* 2011). However, the applicability of these approaches to the genotyping of *S. dysgalactiae* has not been evaluated.

The primary aims of this study were to evaluate the genetic patterns of *S. dysgalactiae* strains isolated from diseased Nile tilapia and assess the relationship between these patterns and the geographical origin of the isolates. An additional aim was to evaluate the discriminatory power and congruence of *sodA* gene sequencing, PFGE, ERIC-PCR and REP-PCR when used for typing of *S. dysgalactiae*.

Materials and methods

Bacterial strains and culture conditions

A total of 21 strains of *S. dysgalactiae* from diseased Nile tilapia were evaluated. They were isolated from outbreaks at four Brazilian farms located in two states (Ceará and Alagoas) between 2007 and 2011 (Table 1). The farms are geographically distant, and there are no records of animal transport between them. In addition, fingerlings were provided by different suppliers. Lancefield's serotyping was performed using the Slidex Latex Agglutination kit (BioMerieux). Identification of the bacterial species was confirmed by *S. dysgalactiae*-specific PCR according to Hassan, Khan & Lammler (2003). The strains were stored at −70 °C until use. The isolates were thawed, streaked onto Todd Hewitt agar (BD) and incubated at 28 °C for 24 h for DNA extraction.

Table 1 *Streptococcus dysgalactiae* strains isolated from Nile tilapia used in this study

Strain[a]	State	Farm	Year of isolation
SD 54	CE	1	2007
SD 56	CE	1	2007
SD 57	CE	1	2007
SD 58	CE	1	2007
SD 61	CE	1	2007
SD 64	CE	1	2007
SD 68	CE	1	2007
SD 92	AL	2	2010
SD 94	AL	2	2010
SD 120	CE	3	2011
SD 121	CE	3	2011
SD 123	CE	3	2011
SD 137	CE	4	2011
SD 138	CE	4	2011
SD 140	CE	4	2011
SD 141	CE	4	2011
SD 142	CE	4	2011
SD 143	CE	4	2011
SD 144	CE	4	2011
SD 145	CE	4	2011
SD 146	CE	4	2011

CE, Ceará; AL, Alagoas.
[a]Aquavet culture collection code.

DNA extraction and *sodA* PCR

Total DNA was extracted using the commercial DNeasy kit (Qiagen) according to the manufacturer's instructions. The amount of DNA extracted was quantified spectrophotometrically using Nanodrop® (Thermo Scientific). The internal fragment constituting 85% of the *sodA* gene was amplified by PCR using the primers d1 (5′-CCITA YICITAYGAYGCIYTIGARCC-3′) and d2 (5′-AR RTARTAIGCRTGYTCCCAIACRTC-3′) as described by Poyart *et al.* (1998). Thermal cycling was performed in a Veriti 96-Well Thermal Cycler (Life Technologies). The primers used were acquired from Life Technologies.

Sequencing and phylogenetic analysis

The PCR products were purified using a Wizard PCR preps kit (Promega). Sequencing reactions were performed using the Applied Biosystems BigDye terminator cycle sequence kit and run on an ABI 3730XL genetic analyzer (Applied Biosystems).

The *sodA* sequences of the Brazilian strains were aligned in BioEdit using CLUSTALW (Thompson, Higgins & Gibson 1994) with sequences of the following bacterial strains and species: six amberjack *S. dysgalactiae* isolates (Nomoto *et al.* 2008) (Genbank accession numbers AB334725, AB334726, AB334730, AB334732, AB334734 and AB334737); *S. dysgalactiae* subsp. *dysgalactiae* (Z959000); *S. dysgalactiae* subsp. *equisimilis* (AJ319591); *S. equi* subsp. *equi* ATCC 33398 (Z95901); *Streptococcus agalactiae* ATCC 12403; and *S. agalactiae* ATCC 12403 (Z99178). The genetic distance matrix was obtained using Kimura's two-parameter model (Kimura 1980), and an evolutionary tree was created using the neighbour-joining method (Saitou & Nei 1987) with Mega4 (Tamura *et al.* 2007). Bootstrap values from 1000 replicates were displayed as percentages.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as described previously (Teixeira *et al.* 1995; Oliveira *et al.* 2005). *S. dysgalactiae* strains were grown overnight in Todd Hewitt broth at 28 °C. The cells were harvested and washed twice with PIV solution (Tris-HCl 0.01 M, pH 8.0 and NaCl 1 M). The bacterial suspension was mixed 1:1 (v:v) with 2% low-melting-point agarose (Sigma Aldrich) and pipetted into 20 μL plugs. Streptococcal cells in agarose plugs were lysed with 25 U of mutanolysin and 500 mg of lysozyme (both from Sigma Aldrich). Following digestion of the DNA with 12 U of *SmaI* restriction enzyme (Amersham Biosciences), the plugs were submitted to PFGE using the following programme: switch time of 1–30 s, 23 h, 120° angle, 11 °C and a voltage gradient of 6 V cm^{-1} in a CHEF DR III system (Bio-Rad Laboratories). The lambda ladder PFGE marker (New England Biolabs) was used as a DNA size marker. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg mL^{-1} for 30 min), and images were captured using L-Pix EX (Loccus Biotecnologia).

REP-PCR and ERIC-PCR

The REP-PCR was performed as described previously by Ouoba *et al.* (2010) with some modifications. The reactions were performed using a HotStart Taq Polymerase kit (Qiagen); the mix was composed of 1 × PCR buffer, 0.5 μM L^{-1} GTG5 primer (5′-GTGGTGGTGGTGGTG-3′), 0.2 μM L^{-1} dNTPs, 1.5 mM L^{-1} $MgCl_2$, 2 U of Taq DNA polymerase and 35 ng of template DNA. The PCR conditions consisted of an initial step of 95 °C for 15 min followed by 30 cycles of 95 °C for 30 s, 45 °C for 1 min and 72 °C for 4 min, with a final elongation step of 72 °C for 16 min.

The ERIC-PCR was conducted using the primers ERIC-1 (5′-ATGTAA GCTCCTGGGGATTC AC-3′) and ERIC-2 (5′-AAGTAAGTGACTGGG GTGAGCG -3′) according to Saito *et al.* (2011) with some modifications. The reactions were performed using a HotStart Taq Polymerase kit (Qiagen) in a final volume of 25 μL containing 1 × PCR buffer, 0.3 μM L^{-1} of each primer, 0.3 μM L^{-1} dNTPs, 2 mM L^{-1} $MgCl_2$, 2 U of Taq DNA polymerase and 35 ng of template DNA. The PCR conditions consisted of an initial denaturation step at 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 52 °C for 1 min and 72 °C for 1 min. The final elongation step was at 72 °C for 8 min.

Thermal cycling was performed in a Veriti 96-Well Thermal Cycler (Life Technologies). The primers used were acquired from Life Technologies. The PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide (0.5 μg mL^{-1} for 30 min) and visualized by UV transillumination. Images were captured using an L-Pix EX (Loccus). Ladders of 100 bp and 1 kb (both from Promega) were used as the molecular size standards for ERIC-PCR and REP-PCR, respectively.

Data analysis and statistics

The PFGE, REP-PCR and ERIC-PCR gels were analysed using BioNumerics version 6.6 (Applied Maths). The Dice coefficient was used to analyse the similarities of the banding patterns (Dice 1945). Dendrograms were created using the unweighted pair group method with average (UPGMA) approach. Isolates that showed $\geq 80\%$ similarity for PFGE and $\geq 90\%$ similarity for ERIC-PCR and REP-PCR were considered to be clonally related (Tenover *et al.* 1995; Singh *et al.* 2006).

The discriminatory power of each typing method was calculated using Simpson's index of diversity (*D*) (Hunter & Gaston 1988). The adjusted Rand index was calculated to quantify the congruence among the different typing methods (Kidd *et al.* 2011). These analyses were performed using the statistical software R (Chang *et al.* 2010).

Results

Phylogenetic analysis of the *sodA* gene

Phylogenetic analysis of the *sodA* gene sequences of the *S. dysgalactiae* strains resulted in the neighbour-joining tree shown in Fig. 1. The sequences of the 21 Brazilian isolates were indistinguishable. They clustered in the same branch as the amberjack strains, showing a bootstrap percentage (based on 1000 replicates) of 99%. In spite of this similarity, all Nile tilapia isolates showed a single deletion at position 421 of the partial sequence of the *sodA* gene. All fish isolates clustered in same branch as the reference strain *S. dysgalactiae* subsp. *dysgalactiae* ATCC 43078.

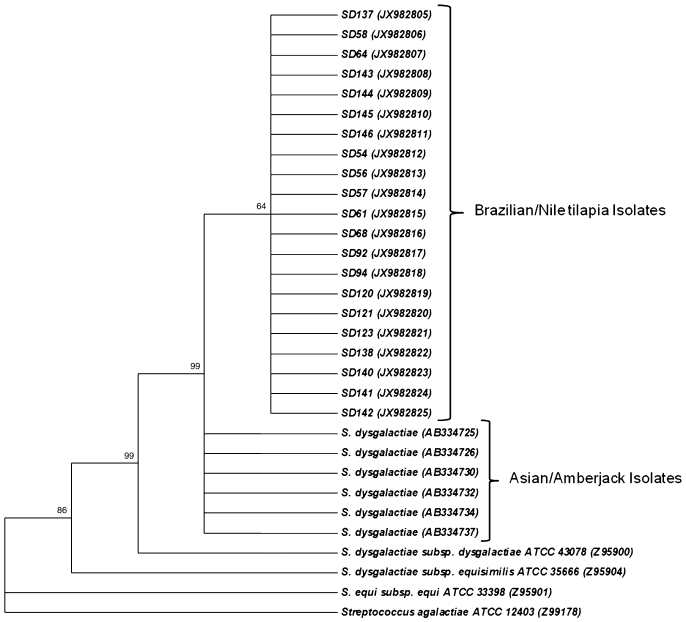

Figure 1 Phylogenetic neighbour-joining tree based on *sodA* gene sequences showing the phylogenetic relationships of Brazilian isolates (SD56, SD57, SD58, SD61, SD64, SD68, SD92, SD94, SD120, SD121, SD123, SD137, SD138, SD140, SD141, SD142, SD143, SD144, SD145 and SD146), amberjack isolates (Nomoto *et al.* 2008) and reference strains of *Streptococcus dysgalactiae* subsp. *dysgalactiae* ATCC 43078, *S. dysgalactiae* subsp. *equisimilis* ATCC 35666, *S. equi* subsp. *equi* ATCC 33398 and *Streptococcus agalactiae* ATCC 12403. Bootstrap percentages (based on 1000 replications) are shown at branch points.

PFGE, REP-PCR and ERIC-PCR profiles

PFGE with *SmaI* digestion revealed 18 different patterns between the 21 *S. dysgalactiae* strains evaluated (Fig. 2). These were classified into three PFGE types based on the similarity threshold of 80%. PFGE had a discriminatory power of 0.3428. Approximately 81% ($n = 17$) of the isolates were pulsetype A.

REP-PCR of the 21 isolates resulted in a single band of approximately 1200 bp. The strains were indistinguishable by this technique, which had a discriminatory power of 0.0.

ERIC-PCR resulted in the amplification of five bands ranging in size from 400 to 2000 bp. Three different ERIC patterns were detected, and based on the similarity threshold of 90%, they belonged to ERIC type I and ERIC type II (Fig. 3). ERIC type I contained 95.28% of the isolates (20 strains). Strain 137 was the only isolate of ERIC type II. ERIC-PCR had a discriminatory power of 0.095238. There was no relationship between ERIC type and geographical origin or farm.

Comparison of typing methods

Four typing methods were tested in this study. *sodA* gene sequencing and REP-PCR were unable to discriminate the *S. dysgalactiae* isolates from Nile tilapia. In contrast, PFGE and ERIC-PCR detected the presence of, respectively, three and two genetically distinct populations among the 21 strains evaluated. Based on Simpson's index of diversity, higher discriminatory power was obtained using PFGE. The congruence between the results of these two techniques was evaluated using the adjusted Rand index (ARI). They showed low concordance, with an ARI value of 0.60. ERIC-PCR results were not a valuable predictor of PFGE results, which was the best typing method to use for isolates of *S. dysgalactiae* from Nile tilapia.

Molecular epidemiology

Based on PFGE results, the Nile tilapia strains of *S. dysgalactiae* belonged to three different genotypes. All isolates from farms 1 and 3 located in Ceará State belonged to pattern A. Two different types (A and B) were observed on farm 4. The isolates from Alagoas State (SD92 and SD 94; farm 2) were classified as pulsetype C, which was a genetically divergent group. The genotypes showed a relationship with state of origin.

Discussion

First described as an aetiological agent of disease in marine fish, *S. dysgalactiae* was recently associated with infections on farms producing Nile tilapia, a freshwater species (Netto *et al.* 2011). The present

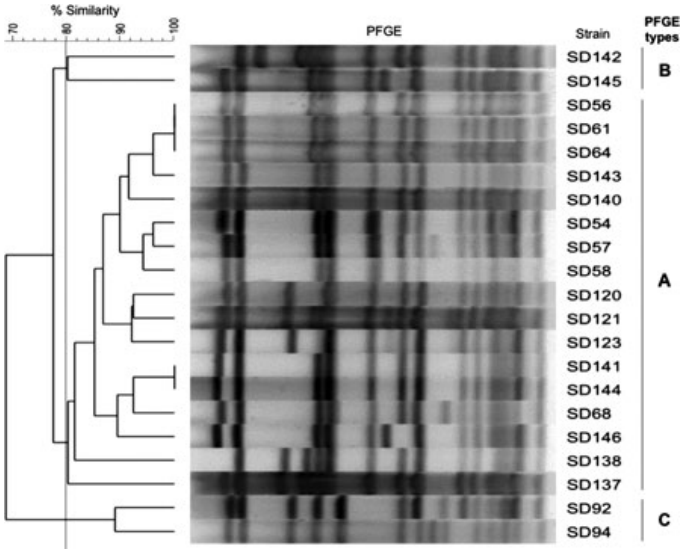

Figure 2 PFGE *SmaI* macrorestriction profile analysis of 21 Brazilian strains of *Streptococcus dysgalactiae* isolated from diseased Nile tilapia, using Dice's coefficient and clustered using the UPGMA approach.

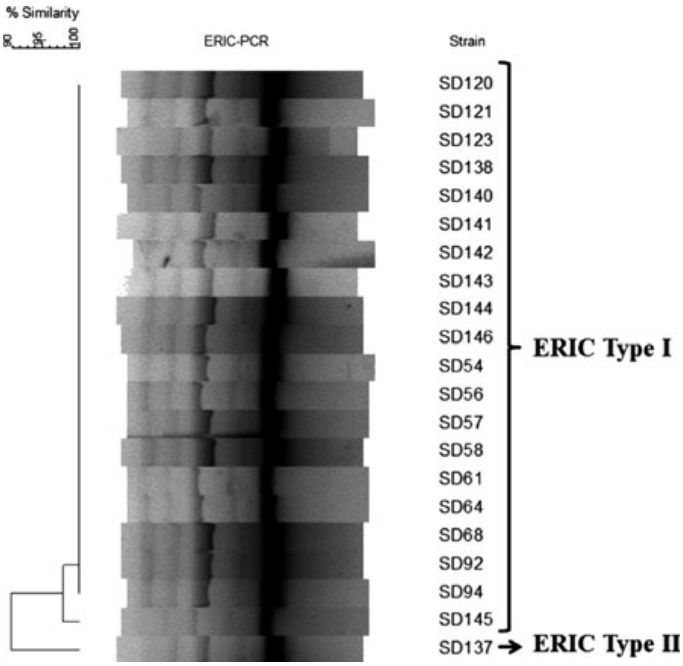

Figure 3 Typing results of 21 Brazilian strains of *Streptococcus dysgalactiae* obtained using ERIC-PCR. The dendrogram was constructed using Dice's coefficient and the UPGMA approach.

study evaluated the genetic diversity of *S. dysgalactiae* strains isolated from outbreaks in different Nile tilapia farms in Brazil between 2007 and 2011.

Sequencing of the superoxide dismutase manganese-dependent (*sodA*) gene has been used previously to determine the genetic diversity of *S. dysgalactiae* isolated from mammals and fish (Nomoto *et al.* 2008; Abdelsalam *et al.* 2010). Abdelsalam *et al.* (2010) sequenced the *sodA* gene of 30 strains of *S. dysgalactiae* from nine fish species from different Asian countries. They observed 100% sequence identity among the isolates, regardless of geographical origin or fish species. In the present study, the *sodA* sequences of Brazilian strains also showed 100% identity. These sequences were highly homologous to the sequences of Asian

isolates, although the Nile tilapia strains had a deletion at position 421 of the partial fragment of the *sodA* gene. This peculiarity, however, did not result in a clear distinction between the strains from Nile tilapia and marine fish. The low polymorphism in *S. dysgalactiae* isolates from fish verified here and previously (Nomoto *et al.* 2008; Abdelsalam *et al.* 2010) demonstrates that *sodA* sequencing is not a suitable genotyping method for this pathogen. Moreover, the genetic diversity determined using other techniques was not predicted by *sodA* sequencing, which compromises its usefulness as a typing tool.

The PFGE results after digestion with *SmaI* revealed three different PFGE types among the 21 Brazilian isolates of *S. dysgalactiae*. Abdelsalam *et al.* (2010) evaluated the use of *ApaI* and *SmaI* for DNA macrorestriction of *S. dysgalactiae* strains isolated from nine fish species. In contrast to the results of the current study, the number of fragments they obtained using *SmaI* digestion did not allow effective discrimination among Asian isolates of *S. dysgalactiae*. However, this enzyme was applied previously to genotyping of fish pathogenic streptococci (Pereira *et al.* 2010; Netto *et al.* 2011). Different PFGE protocols (e.g. the enzyme concentrations and DNA digestion procedures used) could be responsible for the differences between studies.

Nomoto *et al.* (2006) used BSFGE with *ApaI* digestion to characterize 30 strains of *S. dysgalactiae* isolated from amberjack and yellowtail in Japan. They found eight restriction patterns that were clonally related. Similarly, BSFGE analysis of 284 *S. dysgalactiae* isolates from Japan resulted in 16 different patterns, all of which belonged to a single PFGE type (Nishiki *et al.* 2010). These authors argued for the possible clonal expansion of this pathogen in Asia. However, results obtained by Abdelsalam *et al.* (2010) contradict this view. They analysed *S. dysgalactiae* strains isolated contemporaneously from nine fish species in different Asian countries. The majority of the isolates belonged to the same PFGE type; however, four other types were detected. The isolates from Malaysia and Indonesia were genetically distant from those from Japan and Taiwan (Abdelsalam *et al.* 2010). Similar results were found in the present study. The 21 strains from Nile tilapia were classified into three different PFGE types. The isolates from Alagoas State (SD92 and SD94) were genetically divergent from the strains from Ceará. There were no epidemiological relationships between farms from both states. This finding might suggest that *S. dysgalactiae* populations evolved from distinct ancestors.

Based on our results and the data from Abdelsalam *et al.* (2010), fish pathogenic *S. dysgalactiae* seem to have a diverse core population that exhibits meaningful geographically determined genetic diversity that is similar to that found in other streptococci pathogens of fish (Evans *et al.* 2008; Pereira *et al.* 2010). Future studies that apply more discriminatory and evolutionary traceable methods, such as multilocus sequence typing (MLST), should be performed to provide new insights into the genetic and evolutionary characteristics of this pathogen.

Herein, REP-PCR and ERIC-PCR were used for genotyping analysis of isolates of *S. dysgalactiae* from Nile tilapia. Both techniques had low discriminatory power. The REP-PCR amplified a single band for all isolates and was unable to discriminate among them. With ERIC-PCR, two profiles were verified for the 21 strains. However, the results were incongruent with the data obtained using PFGE, which is the accepted typing tool for this bacterium. Therefore, these techniques were shown to be unsuitable for use as genotyping tools for Nile tilapia pathogenic *S. dysgalactiae*.

In conclusion, isolates of *S. dysgalactiae* from Nile tilapia constitute a genetically diverse population. The PFGE technique showed higher discriminatory power and applicability than PCR-based methods for genotyping this pathogen.

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