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Identification of Bacterial Fish Pathogens in Brazil by Direct Colony PCR and 16S rRNA Gene Sequencing

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Abstract

Intensive fish farming systems in Brazil have increased the disease incidence, mainly of bacterial origin, due to higher stocking density, high organic matter levels and poor quality of the aquatic environment that causes high mortality rates during outbreaks. The identification of pathogenic species using a fast and reliable method of diagnosis is essential for successful epidemiological studies and disease control. The present study evaluated the use of direct colony PCR in combination with 16S rRNA gene sequencing to diagnose fish bacterial diseases, with the goal of reducing the costs and time necessary for bacterial identification. The method was successful for all 178 isolates tested and produced bands with the same intensity as the standard PCR performed using pure DNA. In conclusion, the genetics methods allowed detecting the most common and important pathogens in Aquaculture, including 12 species of occurrence in Brazilian fish farms. The results of the present study constitute an advance in the available diagnostic methods for bacterial pathogens in fish farms.

Keywords

Direct Colony PCR, 16S rRNA Sequencing, Bacterial Fish Pathogens

1. Introduction

Due to its high water availability and favorable climate conditions, Brazil displays high potential for the development of fish farming, which is an activity that has been growing substantially over the last few years. Ac-

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cording to the Food and Agriculture Organization of the United Nations [1], Brazil is the second largest aquaculture producer in Latin America and the Caribbean, and freshwater aquaculture (tilapia, carp, and native fish) represented 87% (545,300 ton) of the total aquaculture production in 2011.

The growing interest in this activity and, consequently, the search for higher profitability, have been leading producers to adopt super-intensive production systems. However, the high density of confined fish, inadequate farming management practices, and water contamination by toxic products cause chronic stress and immunosuppression in farmed animals. These effects lead to the occurrence of diseases and epizootic outbreaks caused by pathogens that would not have high expression in natural environments [2]. Knowledge about the etiological agents, pathogenesis, biochemistry, antigenicity, epizootiology, and inter-relationship of stress and environmental factors of bacterial infections affecting fish is essential to avoid and control diseases. However, these factors have not been well studied, especially because fish farming is a recent activity, with its intensification beginning in the 1990s [3].

Gram-negative bacteria such as *Aeromonas*, *Flavobacterium*, *Pseudomonas*, and *Francisella* and gram-positive bacteria from the genera *Streptococcus* and *Lactococcus* [4]-[11] are some of the pathogens responsible for economic losses in Brazil. They can cause high fish mortality rates up to 72 h after infection [12].

Although the number of studies focusing on the diagnosis of bacterial etiological agents has increased over the last few years, there are still few available alternatives for the control of fish bacterial infections in Brazil. Therefore, quicker and more effective diagnostic alternatives are necessary, which would help control diseases before they lead to irreversible clinical consequences and high mortality rates. Molecular diagnostic methods use reduced volumes of sample material and exhibit high sensitivity, specificity, and accuracy in pathogen detection [13].

Methods that do not require purified DNA extraction, such as direct colony PCR, are quicker and less expensive and may greatly aid in the early detection of fish pathogens [14]. In addition, because not all microorganism sequences are catalogued in current databases, the use of universal and degenerate primers is a wise strategy. For this reason, methods based on 16S rRNA ribosomal gene amplification and sequencing have been widely explored [13].

The use of universal PCR primers is based on the hypothesis that the primers used are complementary to conserved regions of genes in the environment, resulting in amplification; in turn, heterogeneity is found inside of the fragments flanked by the primers, in hypervariable regions [15] [16]. This method has been revolutionizing microbial ecology, from studies of non-cultivable bacteria to the correct identification of pathogens for accurate diagnoses.

The aim of the present study was to evaluate the direct colony PCR combined with 16S rRNA gene sequencing as a faster and less expensive method to identify fish bacterial pathogens, compared to the classic PCR protocol. Moreover, we have used these methods to demonstrate the efficiency of genetic approaches for the practical evaluation of the diagnosis of aquaculture diseases in Brazilian fish farms.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

178 bacterial isolates were obtained between 2010 and 2014 from the following hosts (n = number of assessed fish): tilapia (*Oreochromis niloticus* n = 93), tambaqui (*Colossoma macropomum* n = 10), carp (*Cyprinus carpio* n = 3), cachara (*Pseudoplatystoma reticulatum* n = 34), and pacu (*Piaractus mesopotamicus* n = 8).

The hosts exhibited clinical signs of bacterial diseases, such as skin ulcerative lesions, hemorrhagic septicemia, meningoencephalitis, fin rot, exophthalmia, and were collected at fish farms in different regions of Brazil:Dourados (Mato Grosso do Sul State, 22˚13'16"S, 54˚48'20"W, n = 38), Rio de Janeiro (Rio de Janeiro State, 22˚54'S, 43˚10'W, n = 4), Itambaracá (Paraná State, 23˚0'49"S, 50˚24'7"W, n = 10), Itaju (22˚25'37"S, 45˚27'11"W), Arealva (22˚1'38"S, 48˚54'36"W), Porto Ferreira (21˚51’18’’S, 47˚28'45"W), Guaíra (20˚19'5"S, 48˚18'42"W), Santa Fé do Sul (20˚12'43"S, 50˚55'38"W), Palmital (22˚47'30"S, 50˚12'18"W) and Jaboticabal (21˚15'19"S, 48˚19'21"W—São Paulo State, n = 123).

For the isolation of bacteria, scrapings were performed using sterile swabs on fish kidneys and brain. Gram-negative colonies were plated on TSA (Tryptic Soy Agar-Biolife), and TSB (Tryptic Soy Broth-Biolife) and incubated for 24 h in bacteriological incubator adjusted to 28˚C. While gram-positive colonies were seeded in Columbia blood agar (Difco) incubated for 24 - 72 h at 30˚C and subcultured in BHI (Brain Heart Infusion

Broth, Himedia).

The strains of Palmital (SP) were obtained directly from the Laboratory of Aquatic Animal Disease, APTA, Votuporanga, SP.

2.2. Molecular Identification of Isolates

Two methods of molecular diagnosis were compared in this study aiming to evaluate the efficiency of direct colony PCR (time and cost effectiveness) in relation to the PCR amplification of purified DNA by extraction, both combined with gene sequencing (**Table 1**).

The standard PCR of purified DNA method followed the steps below.

2.2.1. DNA Extraction

One colony of each isolated was transferred to a tube containing appropriate liquid culture medium (TSB for gram-negative and BHI for gram-positive) and incubated at 28˚C until the OD_{600} was between 1 and 1.5. Following incubation, 1.0 mL of the bacteria culture was centrifuged at 12,000×*g* for 1 min, the supernatant was discarded, and the pellet was frozen at −20˚C until DNA extraction. The Axyprep® miniprep kit for bacterial genomic DNA was used according to the manufacturer's instructions (Axygen Biosciences, Union City, CA, USA). DNA was quantified by fluorometry using a Qubit 2.0 fluorometer (Life Technologies, NY, USA).

2.2.2. Standard PCR

PCR was performed in a 25 µL final volume, containing 2.5 µL of 10X buffer (10 mM Tris-HCl, 50 mM KCl), 0.2 µL of 25 mM dNTP, 1.0 µL of 50 mM MgSO4, 0.2 µL of *Taq* High Fidelity (Platinum®Taq DNA Polymerase, Life Technologies, NY, USA), 2.0 µL of each primer (10 pmol), 25 ng of DNA template, and Milli-Q water up to the final volume. The PCR program consisted of 94˚C for 2 min; 35 cycles of 94˚C for 30 seconds, 55˚C for 30 s, and 68˚C for 1.5 min; and final extension at 68˚C for 10 min. We used the primers 8F/907R (**Table 2**), specific for the 16S rRNA bacterial gene [15] [17] [18]. The resulting amplicons of approximately 900 bp (base pair) were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide, according to Sambrook *et at.* [19].

2.2.3. Purification of PCR Products and Gene Sequencing

PCR products were purified using a MinElute Kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. Purified PCR products were quantified using a Qubit 2.0 fluorometer, and gene sequencing was performed using 50 ng/µL per sample. Sequencing was performed according to Sanger [20]. PCR products were amplified using AmpliTaq polymerase and BigDye Terminator (Applied Biosystems) according to the manufacturer's instructions, using the primer 907R. Sequencing was performed using an ABI PRISM 3730 DNA analyzer (Applied Biosystems).

Table 1. Steps of the two methods compared in this study: Standard PCR of purified DNA and direct colony PCR, both combined with the 16S rRNA gene sequencing.

Steps	Standard PCR	Direct colony PCR
Bacterial isolation	X	X
Replication in broth	X	-
DNA extraction	X	-
PCR	X	X
Electrophoresis	X	X
PCR product purification	X	-
16S rRNA sequencing	X	X
Nucleotide analysis	X	X

Table 2. Sequence of primers used for amplification of the 16S rRNA gene.

Primer	Target sequence 5'- 3'
8F	AGA GTT TGA TYM TGG CTC AG
907R	CCG TCA ATT CMT TTR AGT TT

2.3. Direct Colony PCR

This method allows PCR to be performed on colonies isolated from Petri dishes, without the step of DNA extraction. Colonies (1 - 2 mm diameter) were inoculated by placing a sterile tooth pick at the bottom of a PCR tube (0.2 mL) and incubated at −20˚C overnight. The following solution was then added in the PCR tube: 2.0 µL of 10× buffer (10 mM Tris-HCl, 50 mM KCl), 1.2 µL of 50 mM $MgCl_2$, 0.2 µL of 25 mM dNTP, 0.7 µL of each primer 8F/907R (10 pmol/µL), 0.2 µL of *Taq* DNA polymerase (2.5 U), and Milli-Q water up to 20 µL. The PCR program consisted of 95˚C for 5 min; 30 cycles of 95˚C for 1 min, 54˚C for 1.5 min, and 72˚C for 1 min; and final extension at 72˚C for 5 min. The amplified PCR products, at 50 ng/µL mean concentration, were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide [19]. The gels were visualized under UV light, using a ChemiDoc MP imaging system (Bio-Rad). Samples were quantified by fluorometry using a Qubit 2.0 fluorometer and sequenced as described above.

After sequencing, samples of both methods had their nucleotides analyzed.

2.4. Analysis of Nucleotide Sequences

The obtained sequences were visualized using the Bio Edit Sequence Alignment Editor software (v. 7.1.11). Phred quality of sequences was determined. The initial and final portions of the sequences were then removed, keeping only the high-quality fragment.

After trim, sequences were exported in FASTA format and compared with the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the Eztaxon algorithm (http://www.ezbiocloud.net/eztaxon/identify). 100% coverage and identity ≥ 98% were considered for specific identification. Sequences were also submitted to Ribosomal Database Project II (http://rdp.cme.msu.edu) for comparison and identification.

The sequences obtained in the present study were deposited at NCBI GenBank under accession numbers KJ560937 to KJ561113. The complete list of species identified, accession numbers, place of origin, fish species, season and year of collection, and size of amplified PCR products were included as Supplementary Material.

The Brazilian isolates tested were *S. agalactiae* (n = 23), *S. iniae* (n = 4), *Lactococcus lactis* (n = 11), *L. raffinolactis* (n = 2), *L. garvieae* (n = 16), *Enterococcus casseliflavus* (n = 16), *E. durans* (n = 2), *E. faecalis* (n = 11), *Edwardsiella tarda* (n = 5), *Aeromonas hydrophila* (n = 16), *A. jandaei* (n = 2), *A. veronii* (n = 15), *Pseudomonas* sp. (n = 15).

A phylogenetic diagram was constructed for validation of the sequencing data, using the 138 isolates listed above from the 178 of the present study. In addition, we used as reference 16 sequences originated from different countries (**Table 3**), obtained from GenBank database.

The 154 FASTA sequences were aligned using the ClustalW Multiple Alignment tool (BioEdit Sequence Alignment Editor software, v. 7.1.11). The data were then entered in Mega software (v. 5.05) to determine the best substitution model. As a result of the preliminary analysis, a maximum-likelihood phylogenetic diagram was constructed, using the Kimura 2-parameter model, with a gamma-shape parameter with 5 categories, the nearest-neighbor-interchange tree inference option. The stability of internal nodes was assessed by bootstrap analysis with 1000 replicates.

3. Results and Discussion

3.1. The Comparison of the Two Methods: Direct Colony PCR and Classic PCR Protocol

We found no difference in the band size in agarose electrophoresis, nor in the peaks pattern of electropherograms in the two methods evaluated.

Bands resulting from the direct colony PCR exhibited the same intensity as those of the standard PCR of purified DNA, for all 178 isolates tested (**Figure 1**).

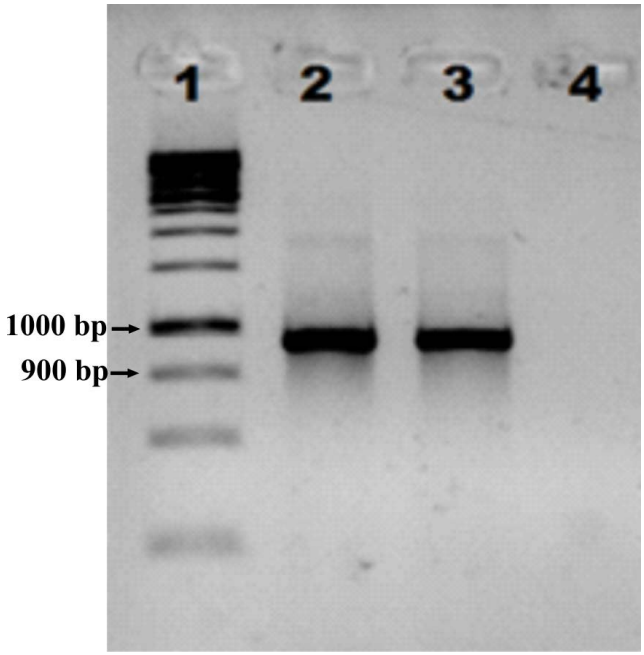

Figure 1. Electropherogram of 1.5% agarose gel stained with ethidium bromide, showing amplification of 16S rRNA gene (primers 8F/907R). Lane 1, Marker 1kb. Lane 2, direct colony PCR. Lane 3, standard PCR. Lane 4, negative control.

Table 3. Reference strains used for the maximum-likelihood phylogenetic analysis and their places of origin.

Genbank access number	Identification	Origin
JX861241	*Aeromonas veronii*	India
ATCC35624	*Aeromonas veronii*	Japan
NR_074841	*Aeromonas hydrophila*	USA
JN644061	*Aeromonas jandaei*	China
ATCC49568	*Aeromonas jandaei*	India
NBRC_105688	*Edwardsiella tarda*	Japan
EU239205	*Pseudomonas fulva*	Korea
KC210866	*Enterococcus casseliflavus*	China
AB530699	*Enterococcus faecalis*	Thailand
NR_036922	*Enterococcus durans*	Germany
KC176716	*Streptococcus agalactiae*	China
NR_027517	*Streptococcus dysgalactiae*	Japan
NR_025148	*Streptococcus iniae*	Israel
NR_044359	*Lactococcus raffinolactis*	South Korea
KC429785	*Lactococcus lactis*	China
ATCC49156	*Lactococcus garvieae*	USA

Electropherograms resulting from the sequencing of both methods exhibited Phred quality scores ≥20. All the isolates had the same results of bacterial identification for both techniques (direct colony PCR and standard PCR of purified DNA). Thus, direct colony PCR was a less expensive and faster diagnostic method, as shown on **Table 4**. There were 51% savings in cost analysis per sample for direct colony PCR compared to Standard PCR of purified DNA. Moreover, direct colony PCR reduces 2 days in time to issue the final report. After the installation of a bacterial outbreak, fish shoals can be decimated by up to 72 hours. Therefore, rapid diagnosis in aquaculture is a critical point in the production chain, which can be assessed by the genetic tools of the present study.

A faster diagnosis is important, since the one based on classical microbiology techniques (isolation, platting and biochemical tests) can exceed the time for treatment in seven to 15 days and, in many cases, ending up inconclusive. The molecular diagnosis, on the other hand, can provide a faster, low cost, conclusive diagnosis, which is essential to determine the best treatment in fish farming (**Table 5**).

Besides, in an attempt to control disease outbreaks, in classical scenery in Brazil, producers use multiple antibiotics indiscriminately, selecting resistant strains, contaminating fish, water and raising the risks to consumer health, endorsing the need for rapid and effective diagnosis [21].

A maximum-likelihood phylogenetic tree was built to validate the sequencing data (**Figure 2**). The bacterial isolates of the same species or phylogenetic related were correctly grouped into a common branch, as expected. The principle of maximum likelihood for phylogenetic inference evaluates the probability of a given model of evolutionary changes explaining the origin of the data observed. In this method, the initial tree is constructed using the neighbor-joining method, and the length of each branch is adjusted to maximize the likelihood that the information will produce the topology of the tree for the desired evolutionary model [22].

These results confirm and validate the direct colony PCR method to be applied as a reliable tool for the identification of bacterial fish pathogens in aquaculture. Although this method has already been used in previous studies for different purposes [14] [23], the present study represents the first practical application for the diagnosis of aquaculture diseases, a field lacking in terms of technological advancement.

Table 4. Cost analysis per sample for bacterial identification, performed in university laboratory already equipped.

	Standard PCR		Direct colony PCR	
Steps	U$	Time	U$	Time
Bacterial isolation on plate	0.31	24 - 72 h	0.31	24 - 72 h
Replication in broth	0.41	24 - 48 h	-	-
DNA extraction	5.6	1 - 3 h	-	-
PCR	2.52	3 h	2.52	3 h
Electrophoresis	0.27	1.5 h	0.27	1.5 h
PCR product purification	2.54	1 h	-	-
16S rRNA sequencing	5.0	24 h	5.0	24 h
Nucleotide analysis	-	1 h	-	1 h
Overall	**16.65**	**4 - 7 days**	**8.1**	**3 - 5 days**

*Isolation times vary depending on the species being cultured.

Table 5. Advantages and disadvantages of each method for aquaculture diagnosis.

	Classical microbiology	Standard PCR	Colony PCR
Advantages	Less technicization	Conclusive diagnosis, faster than the classical Microbiology	Conclusive diagnosis; 51% more economical; 24 - 48 h faster than standard PCR.
Disadvantages	Inconclusive and time consuming diagnosis	More costly; It depends on bacterial culture	1% to 3% can fail; Still depends bacterial culture

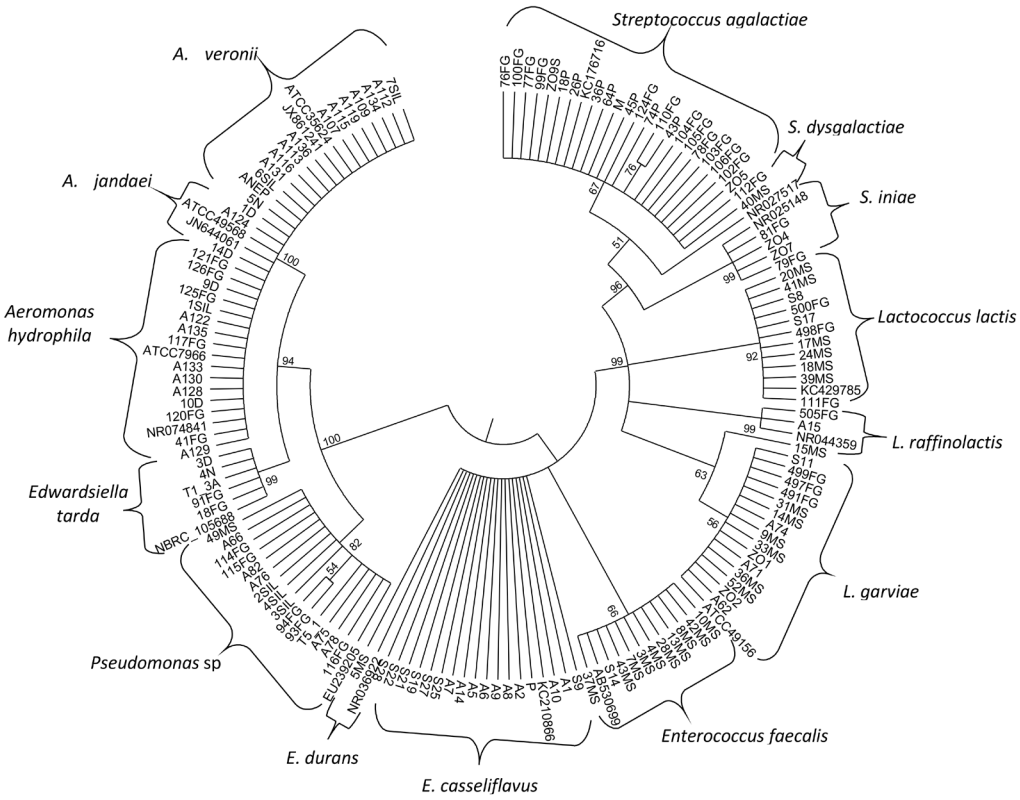

Figure 2. Relationship among different bacteria species using 16S rRNA gene sequences, inferred by maximum-likelihood method. The phylogenetic diagram shows the correct clustering of related fish bacteria isolated in the present study.

3.2. The Analysis of the Common Bacterial Fish Pathogens

Direct colony PCR, combined with gene sequencing, was able to detect the most common and important pathogens in aquaculture, such as *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas jandaei*, *Streptococcus* agalactiae, *Streptococcus iniae*, *Streptococcus dysgalactiae*, *Edwardsiella tarda*, *Pseudomonas* sp., *Lactococcus garvieae*, *Citrobacter freundii*, *Plesiomonas shigelloides*, and *Enterococcus* sp.

As shown in **Figure 3**, genera related to pathogenic bacteria and with higher frequency among 178 bacterial isolates of this study were *Aeromonas* (31%), *Lactococcus* (23%), *Enterococcus* (22%), *Streptococcus* (20%), *Pseudomonas* (11%), *Citrobacter* (6%), *Edwardsiella* (5%), *Acinetobacter* (3%), *Enterobacter* (2%), *Plesiomonas* (1%) and *Weissela* (1%).

Of the 43 *Aeromonas* isolates, 53% were identified as *A. hydrophila* by 16S rRNA gene sequencing. This result is in accordance with previous reports that found this species to be predominant [24]. In turn, *A. veronii* corresponded to 40% of the isolates. The seasonality was also observed in the present study: at higher temperatures (Spring/Summer) there were higher isolation rates of these pathogens [25], which causes hemorrhagic septicemia, characterized by small superficial lesions, focal hemorrhages, ulcers, abscesses, and abdominal distension. Internally, there can be ascitic fluid accumulation, anemia, and lesions in the liver and kidneys [26].

For the genus *Lactococcus*, the emerging species *L. garvieae* corresponded to 52% of the total 29 isolates of this genus, followed by *L. lactis* with 41% incidence in fish originating from the states included in the present study, with higher incidence in *P. reticulatum*. The species *L. garvieae* has been isolated from several fish species worldwide, namely in Japan [27], South Africa [28], Europe [29], and Brazil. Its first outbreak was reported in 2009 [7]. Fish with lactococcal infection exhibit lethargy, anorexia, skin darkening and swim closer to the water surface [30], resulting in considerable economic losses, especially during the summer months when the water temperature increases [4]. Few studies report *L. lactis* as an opportunistic pathogen. However, *L. lactis* subsp. *lactis* has been responsible for a 100% loss of hybrid sturgeons (*Huso huso* × *Acipenser ruthenus*) in a fish farm in Taiwan, China [31].

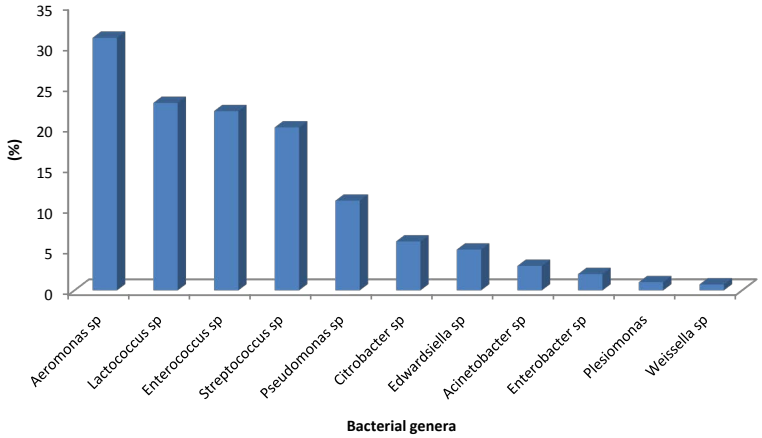

Figure 3. Percentage of bacterial genera identified by 16S rRNA gene of the 178 isolates of this study distributed in the states of Mato Grosso do Sul, São Paulo, Paraná and Rio de Janeiro.

In the present study, 31 *Enterococcus* strains were isolated from skin and kidney samples. Of these, 55% were *E. casseliflavus*, 36% *E. faecalis*, 6% *E.durans*, and 3% *E. sulfureus*. The predominance of *E. casseliflavus* has also been observed among isolates from water and sediment, accounting for 66.7% of a total of 410 *Enterococcus* sp. isolates in Thailand [32].

Of the 27 *Streptococcus* strains originating from the states of Mato Grosso do Sul, Paraná, and São Paulo, 89% corresponded to *S. agalactiae*; this was previously observed by Netto *et al.* [33] and Figueiredo *et al.* [10]. Although infection by *S. agalactiae* is the main cause of losses in tilapia farming worldwide, this pathogen has also been isolated from "cachara" originating from Mato Grosso do Sul. *S. agalactiae* has been identified in several other fish species, such as *Sparus auratus*, *Liza klunzingeri* [34], and *Pampusargenteus* [35]. Infected fish have meningoencephalitis, exophthalmia, erratic swimming, mainly.

The species *P. putida* (27%) and *P. fulva* (20%) were the predominant *Pseudomonas* species observed (n = 15). Eissa *et al.* [36] observed an incidence of 30.83% of *Pseudomonas* species in Nile tilapia in Egypt. Hussain [37] and Zorrilla *et al.* [38] reported 13.5%, and 9.7% incidence, respectively, of *Pseudomonas* species in marine fish, values that are similar to the 11% incidence found in the present study. *P. fluorescens*, *P. angulliseptica*, *P. aeruginosa* and *P. putida* were identified in various species of fish as causative agents of *Pseudomonas* septicemia. The disease is characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascitis and exophthalmia [39].

As the number of isolates from each region was dissimilar and low, it would not be advisable to determine a frequency profile of pathogens by location, neither the prevalence of bacterial genera by fish species, but we emphasize the importance of drawing a regional profile in aquaculture health monitoring programs and preventive management, therefore, in case of disease outbreak, treatment measures are different in each region, since factors such as light, water quality and soil contamination, quantity of parasites, management, etc are also peculiar to each locality.

4. Conclusion

Direct colony PCR combined with 16S rRNA gene sequencing constitutes an efficient alternative for diagnosing bacterial fish diseases, with decreased cost and time compared with the classical methods used in Brazil, such as isolation, biochemical tests, and conventional PCR.

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Supplementary Data

GenBank accession numbers	Sample	Fish	Organ	Molecular identification	Location	Time	Molecular size (bp)
KJ560937	4n	Tilapia	Skin	*Edwardsiella tarda*	Nepean, Jaboticabal sp	Spring 2013	868
KJ560938	T1.3a	Tilapia	Gills	*Edwardsiella tarda*	Porto Ferreira SP	Spring 2013	825
KJ560939	2dp	Tilapia	Kidney	*Edwardsiella tarda*	Porto Ferreira SP	Spring 2013	855
KJ560940	18FG	Tilapia	Skin	*Edwardsiella tarda*	Rio Paranapanema (SP/PR)	Winter 2012	858
KJ560941	91 FG	Tilapia	Skin	*Edwardsiella tarda*	Rio Paranapanema (SP/PR)	Winter 2012	860
KJ560942	8g	Pacu	Kidney	*Edwardsiella tarda*	Caunesp, Jaboticabal, SP	Spring 2013	847
KJ560943	3dp	Tilapia	Kidney	*Edwardsiella tarda*	Porto Ferreira SP	Spring 2013	873
KJ560944	45MS	Cachara	Skin	*Enterobacter asburiae*	MS	Winter 2012	851
KJ560945	47MS	Cachara	Kidney	*Kosakonia cowanii*	MS	Winter 2012	857
KJ560946	48MS	Cachara	Kidney	*Enterobacter ludwigii*	MS	Winter 2012	800
KJ560947	A77	Tilapia	Kidney	*Enterobacter kobei*	Arealva SP	Spring 2011	830
KJ560948	A79	Tilapia	Kidney	*Enterobacter kobei*	Arealva SP	Spring 2011	853
KJ560949	A70	Tilapia	Skin	*Enterobacter ludwigii*	Arealva SP	Spring 2011	855
KJ560950	A1	Tambaqu	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	859
KJ560951	A8	Tambaqui	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	857
KJ560952	A5	Tambaqui	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	869
KJ560953	A9	Tambaqui	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	860
KJ560954	A2	Tambaqui	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	866
KJ560955	A6	Tambaqui	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	841
KJ560956	A10	Tambaqui	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	862
KJ560957	A7	Tambaqui	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	862
KJ560958	A14	Tilapia	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Spring 2011	856
KJ560959	P	Tilapia	Brain	*Enterococcus casseliflavus*	Itambaracá PR	Summer 2010	853
KJ560960	S27	Tilapia	Kidney	*Enterococcus casseliflavus*	Caunesp, Jaboticabal	Winter 2011	879
KJ560961	S22	Tilapia	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Winter 2011	871
KJ560962	S28	Tilapia	Brânquia	*Enterococcus casseliflavus*	Caunesp, Jaboticabal	Winter 2011	876

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KJ560963	S21	Tilapia	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Winter 2011	871
KJ560964	S19	Tilapia	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Winter 2011	867
KJ560965	S25	Tilapia	Skin	*Enterococcus casseliflavus*	Caunesp, Jaboticabal SP	Winter 2011	872
KJ560966	5MS	Cachara	Kidney	*Enterococcus durans*	MS	Spring 2012	840
KJ560967	S9	Tilapia	Skin	*Enterococcus durans*	Arealva SP	Spring 2011	874
KJ560968	3 MS	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	854
KJ560969	4MS	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	840
KJ560970	7MS	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	846
KJ560971	8MS	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	837
KJ560972	10MS	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	852
KJ560973	13ms	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	849
KJ560974	28ms	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	873
KJ560975	42ms	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	838
KJ560976	43ms	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	864
KJ560977	37ms	Cachara	Kidney	*Enterococcus faecalis*	MS	Spring 2012	874
KJ560978	S14	Tilapia	Skin	*Enterococcus faecalis*	Arealva SP	Winter 2011	864
KJ560979	26ms	Cachara	Kidney	*Enterococcus sulfureus*	MS	Spring 2012	850
KJ560980	20b dp	Tilapia	Gills	*Klebsiella pneumoniae*	Porto Ferreira SP	Fall 2014	819
KJ560981	46MS	Cachara	Skin	*Klebsiella pneumoniae*	MS	Spring 2012	855
KJ560982	B1	Tilapia	Skin	*Kurthia gibsonii*	Arealva SP	Spring 2011	837
KJ560983	A71	Tilapia	Skin	*Lactococcus garviae*	Arealva SP	Winter 2011	834
KJ560984	A74	Tilapia	Kidney	*Lactococcus garviae*	Arealva SP	Winter 2011	855
KJ560985	497 FG	Tilapia	Brain	*Lactococcus garviae*	Rio Paranapanema (SP/PR)	Spring 2012	789
KJ560986	491 FG	Tilapia	Brain	*Lactococcus garviae*	Rio Paranapanema (SP/PR)	Spring 2012	843
KJ560987	Zo1	Tilapia	Kidney	*Lactococcus garviae*	Guaíra SP	Fall 2014	865
KJ560988	Zo2	Tilapia	Kidney	*Lactococcus garviae*	Guaíra SP	Fall 2014	866
KJ560989	6n	Tilapia	Skin	*Lactococcus garviae*	Nepean, Jaboticabal SP	Spring 2013	875
KJ560990	A62	Tilapia	Skin	*Lactococcus garviae*	Arealva SP	Winter 2011	845
KJ560991	15ms	Cachara	Kidney	*Lactococcus garviae*	MS	Spring 2012	846
KJ560992	14ms	Cachara	Kidney	*Lactococcus garviae*	MS	Spring 2012	853
KJ560993	31ms	Cachara	Kidney	*Lactococcus garviae*	MS	Spring 2012	866
KJ560994	33ms	Cachara	Kidney	*Lactococcus garviae*	MS	Spring 2012	853
KJ560995	36ms	Cachara	Kidney	*Lactococcus garviae*	MS	Spring 2012	810
KJ560996	52MS	Cachara	Brain	*Lactococcus garviae*	MS	Spring 2012	810
KJ560997	S11	Tilapia	Skin	*Lactococcus garviae*	Arealva SP	Winter 2011	799
KJ560998	9MS	Cachara	Brain	*Lactococcus lactis subsp. cremoris*	MS	Spring 2012	807

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KJ560999	499 FG	Tilapia	Brain	*Lactococcus lactis subsp. cremoris*	Rio Paranapanema (SP/PR)	Spring 2012	799
KJ561000	S17	Tilapia	Skin	*Lactococcus lactis subsp. lactis*	Caunesp, Jaboticabal SP	Winter 2011	869
KJ561001	17ms	Cachara	Kidney	*Lactococcus lactis subsp. lactis*	MS	Spring 2012	852
KJ561002	18ms	Cachara	Kidney	*Lactococcus lactis subsp. Lactis*	MS	Spring 2012	861
KJ561003	20ms	Cachara	Kidney	*Citrobacter freundii*	MS	Spring 2012	842
KJ561004	24ms	Cachara	Kidney	*Lactococcus lactis subsp. Lactis*	MS	Spring 2012	858
KJ561005	500 FG	Tilapia	Brain	*Lactococcus lactis subsp. lactis*	Rio Paranapanema (SP/PR)	Spring 2012	829
KJ561006	39ms	Cachara	Kidney	*Lactococcus lactis subsp. Lactis*	MS	Spring 2012	832
KJ561007	41ms	Cachara	Kidney	*Lactococcus lactis subsp. lactis*	MS	Spring 2012	836
KJ561008	111 FG	Tilapia	Brain	*Lactococcus lactis subsp. lactis*	Rio Paranapanema (SP/PR)	Spring 2012	845
KJ561009	498 FG	Tilapia	Brain	*Lactococcus lactis subsp. lactis*	Rio Paranapanema (SP/PR)	Spring 2012	858
KJ561010	S8	Tambaqui	Skin	*Lactococcus lactis subsp. lactis*	Arealva SP	Winter 2011	862
KJ561011	A15	Tilapia	Brain	*Lactococcus raffinolactis*	Caunesp, Jaboticabal SP	Winter 2011	859
KJ561012	505 FG	Tilapia	Brain	*Lactococcus raffinolactis*	Rio Paranapanema (SP/PR)	Spring 2012	839
KJ561013	A3	Tambaqui	Skin	*Leucobacter aridicollis*	Caunesp, Jaboticabal SP	Winter 2011	830
KJ561014	1sil	Pacu	Kidney	*Aeromonas hydrophila*	Caunesp, Jaboticabal SP	Fall 2014	865
KJ561015	5sil	Pacu	Kidney	*Aeromonas hydrophila*	Caunesp, Jaboticabal SP	Fall 2014	877
KJ561016	10dp	Tilapia	Skin	*Aeromonas hydrophila*	Porto Ferreira SP	Spring 2013	866
KJ561017	9dp	Tilapia	Gills	*Aeromonas hydrophila*	Porto Ferreira SP	Spring 2013	867
KJ561018	A129	Tilapia	Kidney	*Aeromonas hydrophila*	Arealva SP	Winter 2011	858
KJ561019	Atcc7966	Tilapia	Enviroment	*Aeromonas hydrophila*	RJ	Summer 2011	867
KJ561020	A130	Tilapia	Skin	*Aeromonas hydrophila*	Arealva SP	Winter 2011	846
KJ561021	A122	Pintado	Skin	*Aeromonas hydrophila*	Arealva SP	Winter 2011	846
KJ561022	A133	Tilapia	Skin	*Aeromonas hydrophila*	Arealva SP	Winter 2011	851
KJ561023	41FG	Tilapia	Kidney	*Aeromonas hydrophila*	Rio Paranapanema (SP/PR)	Spring 2012	861
KJ561024	A128	Tilapia	Skin	*Aeromonas hydrophila*	Arealva SP	Winter 2011	855
KJ561025	A135	Carpa	Skin	*Aeromonas hydrophila*	RJ	Summer 2011	862
KJ561026	117 FG	Tilapia	Kidney	*Aeromonas hydrophila*	Rio Paranapanema (SP/PR)	Spring 2012	854
KJ561027	120 FG	Tilapia	Kidney	*Aeromonas hydrophila*	Rio Paranapanema (SP/PR)	Spring 2012	821
KJ561028	121 FG	Tilapia	Kidney	*Aeromonas hydrophila*	Rio Paranapanema (SP/PR)	Spring 2012	817
KJ561029	125 FG	Tilapia	Kidney	*Aeromonas hydrophila*	Rio Paranapanema (SP/PR)	Spring 2012	839
KJ561030	126 FG	Tilapia	Kidney	*Aeromonas hydrophila*	Rio Paranapanema (SP/PR)	Spring 2012	694
KJ561031	128 FG	Tilapia	Kidney	*Aeromonas hydrophila*	Rio Paranapanema (SP/PR)	Spring 2012	656
KJ561032	14dp	Tilapia	Kidney	*Aeromonas jandaei*	Porto Ferreira SP	Spring 2013	864
KJ561033	A124	Tilapia	Brain	*Aeromonas jandaei*	Arealva SP	Winter 2011	865
KJ561034	A110	Pintado	Skin	*Aeromonas punctata*	Arealva SP	Winter 2011	843

Continued

KJ561035	7sil	Pacu	Kidney	*Aeromonas veronii*	Caunesp, Jaboticabal SP	Fall 2014	750
KJ561036	an	Tilapia	Kidney	*Aeromonas veronii*	Nepean, Jaboticabal SP	Fall 2014	806
KJ561037	6sil	Pacu	Kidney	*Aeromonas veronii*	Caunesp, Jaboticabal SP	Fall 2014	870
KJ561038	5n	Tilapia	Skin	*Aeromonas veronii*	Nepean, Jaboticabal SP	Spring 2013	880
KJ561039	1dp	Tilapia	Kidney	*Aeromonas veronii*	Porto Ferreira SP	Spring 2013	779
KJ561040	A107	Pintado	Brain	*Aeromonas veronii*	Arealva SP	Winter 2011	859
KJ561041	A115	Pintado	Brain	*Aeromonas veronii*	Arealva SP	Winter 2011	835
KJ561042	A131	Tilapia	Brain	*Aeromonas veronii*	Arealva SP	Winter 2011	854
KJ561043	A119	Pintado	Brain	*Aeromonas veronii*	Arealva SP	Winter 2011	821
KJ561044	A116	Pintado	Brain	*Aeromonas veronii*	Arealva SP	Winter 2011	839
KJ561045	A113	Pintado	Kidney	*Aeromonas veronii*	Arealva SP	Winter 2011	833
KJ561046	A109	Pintado	Brain	*Aeromonas veronii*	Arealva SP	Winter 2011	869
KJ561047	A134	Carpa	Skin	*Aeromonas veronii*	RJ	Summer 2011	855
KJ561048	A136	Carpa	Skin	*Aeromonas veronii*	RJ	Summer 2011	853
KJ561049	A112	Pintado	Muco	*Aeromonas veronii*	Arealva SP	Winter 2011	860
KJ561050	124 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	806
KJ561051	Zo5	Tilapia	Brain	*Streptococcus agalactiae*	Guaíra SP	Fall 2014	850
KJ561052	Zo9s	Tilapia	Brain	*Streptococcus agalactiae*	Guaíra SP	Fall 2014	866
KJ561053	78 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	854
KJ561054	99 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	865
KJ561055	103 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	865
KJ561056	100 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	863
KJ561057	104 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	859
KJ561058	76 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	871
KJ561059	105 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	864
KJ561060	77 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	866
KJ561061	106 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	865
KJ561062	102 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	853
KJ561063	18P	Tilapia	Fígado	*Streptococcus agalactiae*	Itambaracá PR	Fall 2010	840
KJ561064	74P	Tilapia	Brain	*Streptococcus agalactiae*	Itambaracá PR	Fall 2011	789
KJ561065	26P	Tilapia	Kidney	*Streptococcus agalactiae*	Itambaracá PR	Fall 2011	855
KJ561066	45P	Tilapia	Kidney	*Streptococcus agalactiae*	Itambaracá PR	Winter 2011	831
KJ561067	36P	Tilapia	Fígado	*Streptococcus agalactiae*	Itambaracá PR	Summer 2011	853
KJ561068	43P	Tilapia	Brain	*Streptococcus agalactiae*	Itambaracá PR	Winter 2011	603
KJ561069	110 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	422
KJ561070	M	Tilapia	Brain	*Streptococcus agalactiae*	Itambaracá PR	Fall 2011	853

Continued

KJ561071	64P	Tilapia	Brain	*Streptococcus agalactiae*	Itambaracá PR	Winter 2011	681
KJ561072	112 FG	Tilapia	Brain	*Streptococcus agalactiae*	Rio Paranapanema (SP/PR)	Spring 2012	846
KJ561073	Zo4	Tilapia	Brain	*Streptococcus iniae*	Guaíra SP	Fall 2014	839
KJ561074	Zo7	Tilapia	Brain	*Streptococcus iniae*	Guaíra SP	Fall 2014	839
KJ561075	79 FG	Tilapia	Brain	*Streptococcus iniae*	Rio Paranapanema (SP/PR)	Summer 2012	859
KJ561076	81 FG	Tilapia	Brain	*Streptococcus iniae*	Reservatório de Ilha Solteira, rio Paraná SP	Summer 2012	862
KJ561077	40ms	Cachara	Kidney	*Streptococcus dysgalactiae*	MS	Spring 2012	846
KJ561078	52 P	Tilapia	Kidney	*Weissella confusa*	Itambaracá PR	Summer 2011	852
KJ561079	23FG	Tilapia	Skin	*Acinetobacter johnsonii*	Rio Paranapanema (SP/PR)	Spring 2012	843
KJ561080	C1	Carpa	Skin	*Acinetobacter tjernbergiae*	RJ	Summer 2011	819
KJ561081	30 AM FG	Tilapia	Skin	*Acinetobacter radioresistens*	Rio Paranapanema (SP/PR)	Spring 2012	850
KJ561082	84AM FG	Tilapia	Skin	*Acinetobacter ursingii*	Rio Paranapanema (SP/PR)	Spring 2012	849
KJ561083	96AM FG	Tilapia	Skin	*Agrobacterium tumefaciens*	Rio Paranapanema (SP/PR)	Spring 2012	799
KJ561084	69ROSA FG	Tilapia	Skin	*Arthrobacter globiformis*	Rio Paranapanema (SP/PR)	Spring 2012	830
KJ561085	57MS	Cachara	Skin	*Bacillus stratoSPhericus*	MS	Spring 2012	828
KJ561086	22ms	Cachara	Kidney	*Brevibacillus agri*	MS	Spring 2012	833
KJ561087	23ms	Cachara	Kidney	*Brevibacillus agri*	MS	Spring 2012	727
KJ561088	16ms	Cachara	Skin	*Carnobacterium divergens*	MS	Spring 2012	872
KJ561089	11dp	Tilapia	Skin	*Citrobacter freundii*	Porto Ferreira SP	Spring 2013	867
KJ561090	A108	Pintado	Brain	*Citrobacter freundii*	Arealva SP	Winter 2011	856
KJ561091	44 MS	Cachara	Kidney	*Citrobacter freundii*	MS	Spring 2012	850
KJ561092	54MS	Cachara	Kidney	*Citrobacter freundii*	MS	Spring 2012	800
KJ561093	1g	Pacu	Kidney	*Citrobacter freundii*	Caunesp, Jaboticabal SP	Spring 2013	779
KJ561094	A99	Catfish	Baço	*Citrobacter murliniae*	Dourados ms	Winter 2011	860
KJ561095	115 FG	Tilapia	Skin	*Pseudomonas chlororaphis*	Rio Paranapanema (SP/PR)	Spring 2012	752
KJ561096	A78	Tilapia	Skin	*Pseudomonas fulva*	Arealva SP	Winter 2011	845
KJ561097	A75	Tilapia	Kidney	*Pseudomonas fulva*	Arealva SP	Winter 2011	813
KJ561098	116 FG	Tilapia	Skin	*Pseudomonas fulva*	Rio Paranapanema (SP/PR)	Spring 2012	826
KJ561099	114 FG	Tilapia	Skin	*Pseudomonas libanensis*	Rio Paranapanema (SP/PR)	Spring 2012	827
KJ561100	93FG	Tilapia	Kidney	*Pseudomonas monteilii*	Rio Paranapanema (SP/PR)	Spring 2012	846
KJ561101	A76	Tilapia	Skin	*Pseudomonas mosselii*	Arealva SP	Winter 2011	844
KJ561102	A82	Tilapia	Skin	*Pseudomonas mosselii*	Arealva SP	Winter 2011	818
KJ561103	A66	Tambaqui	Skin	*Pseudomonas nitroreducens*	Arealva SP	Winter 2011	821
KJ561104	94FG	Tilapia	Kidney	*Pseudomonas plecoglissida*	Rio Paranapanema (SP/PR)	Spring 2012	854
KJ561105	4sil	Pacu	Gills	*Pseudomonas putida*	Caunesp, Jaboticabal SP	Fall 2014	856
KJ561106	2sil	Pacu	Gills	*Pseudomonas putida*	Caunesp, Jaboticabal SP	Fall 2014	850

Continued

KJ561107	T5-1	Tilapia	Baço	*Pseudomonas putida*	Porto Ferreira SP	Spring 2013	862
KJ561108	3sil	Pacu	Skin	*Pseudomonas putida*	Caunesp, Jaboticabal SP	Fall 2014	690
KJ561109	49MS	Cachara	Skin	*Pseudomonas stutzeri*	MS	Spring 2012	850
KJ561110	49AM FG	Tilapia	Skin	*Stenotrophomonas chelatiphaga*	Rio Paranapanema (SP/PR)	Spring 2012	858
KJ561111	22FG	Tilapia	Skin	*Stenotrophomonas maltophilia*	Rio Paranapanema (SP/PR)	Spring 2012	867
KJ561112	T3.5b	Tilapia	Skin	*Plesiomonas shigelloides*	Porto Ferreira SP	Spring 2013	864
KJ561113	A132	Tilapia	Skin	*Comamonas testosteroni*	Arealva SP	Winter 2011	849