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http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_66.2014.1023, 7 pages

Simultaneous Detection of *Streptococcus* spp. and *Aeromonas* spp. from Diseased Tilapia (*Oreochromis niloticus)* using Multiplex-Polymerase Chain Reaction

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(Received 4.9.2013, Accepted 27.11.2013)

Key words: Multiplex PCR, *Streptococcus* spp., *Aeromonas* spp., Tilapia

Abstract

A multiplex Polymerase Chain Reaction (m-PCR) assay was developed for simultaneous detection of two major pathogens, Streptococcal and Aeromonad bacteria, in farmed tilapia. DNA fragments of *Streptococcus* spp. and *Aeromonas* spp. were amplified using genus specific primers, C1/C2 and AERF/AERR, which produced PCR of 207 bp and 953 bp, respectively. The lowest concentration of each *Streptococcus* and *Aeromonas* spp. extracted genomic DNA from a colony detected by m-PCR was 2 ng. The m-PCR assay was proven applicable for detection of bacterial genomic DNA in tissues (brain and posterior kidney) of infected fish. Specificity of the assay tested with other Gram-positive (*Staphylococcus aureus*) and Gram-negative water borne bacteria (*Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella typhimurium*, *Vibrio harveyi* and *Vibrio parahemolyticus*) showed no amplification. As Streptococcal and Aeromonad infections are common concurrent bacterial diseases, the m-PCR assay established in this study enabled effective simultaneous detection of these two major bacterial infections responsible for current economic losses in tilapia farming in Thailand.

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Introduction

Tilapia (*Oreochromis niloticus*) is one of the most important cultured fish in many countries including, China, Egypt, the Philippines, Indonesia, and Thailand (Fitzsimmons et al., 2011) as well as in Israel. Some have described tilapia as an "aquatic chicken" or "terrestrial tilapia" due to its ability to grow and breed in capture fisheries (Coward and Little, 2001). Tilapia culture has become a sustainable farming industry supplying global production (3.5 million metric tons) and still increasing dramatically (Fitzsimmons et al., 2012). However, massive production has led to increased intensive farming practices which induce stress and increase susceptibility to diseases and/or infections (El-Sayed, 2006).

Streptococcus and *Aeromonas* spp. are common pathogens that cause diseases in tilapia. *Streptococcus* spp., Gram-positive bacteria, are the cause of major losses (80-100%) in tilapia production. In many countries such as United States, Israel, Australia, China, Japan and Thailand there have been outbreaks of *Streptococcus iniae* and *S. agalactiae* (Shoemaker and Klesius, 1997; Agnew and Barnes, 2007; Maisak et al., 2008). *Aeromonas* spp., Gram-negative bacteria, are considered to be opportunistic pathogens resulting in mortality particularly in conditions of stress such as anoxia and poor water quality. *Aeromonas hydrophila, A. caviae, A. sobria* and other *Aeromonas* spp. are widely distributed especially in aquatic environments including, freshwater lakes, rivers, and waste water systems (Faisal et al., 1989; Yambot, 1998). Streptococcal and Aeromonad pathogens cause significant loss of tilapia in aquaculture and affect tilapia production in Thailand.

Generally, diagnosis of fish disease is based on clinical symptoms, pathological findings, and etiological isolation; however, all these methods require experienced technicians and are time-consuming (Noga, 1996). It is particularly concerning when infected fish display nonspecific clinical symptoms such as lethargy, erratic swimming, abdominal distention and exophthalmia, which are common clinical symptoms of many bacterial infections. Molecular diagnostic techniques, such as PCR assays, are increasingly used for genetic characterization and detection of many different bacterial pathogens (Altinok and Kurt, 2003). Conventional PCR was established for detection of single pathogens in each run, on the other hand the multiplex PCR (m-PCR) was developed for the detection of several pathogens in a single run. PCR methods are diagnostically helpful where clinical findings are questionable, and for confirmative diagnosis. The present study demonstrates the use of m-PCR for simultaneous detection of two major bacterial pathogens, *Streptococcus* spp. and *Aeromonas* spp. in diseased tilapia.

Materials and Methods

Bacterial culture and DNA extraction. A list of bacterial reference strains and clinical isolates used in the present study are shown in Table 1.

Table 1. Bacterial strains used in the study.

Strain	*Bacterial species*	*Source*
1	*Streptococcus agalactiae*	Nile tilapia/Field isolate (JW10-SA2)
2	*Streptococcus agalactiae*	Nile tilapia/Field isolate (JW13-SA71)
3	*Streptococcus agalactiae*	Nile tilapia/Field isolate (JW16-SA8)
4	*Streptococcus agalactiae*	Nile tilapia/Field isolate (JW19-SA32)
5	*Streptococcus agalactiae*	Nile tilapia/Field isolate (JW22-SA35)
6	*Streptococcus agalactiae*	Nile tilapia/Field isolate (JW25-SA65)
7	*Streptococcus iniae*	Nile tilapia/Field isolate (JW1-SI1)
8	*Streptococcus iniae*	Nile tilapia/Field isolate (JW3-SI4)
9	*Streptococcus iniae*	Nile tilapia/Field isolate (JW4-SI50)
10	*Streptococcus iniae*	Nile tilapia/Field isolate (JW6-SI52)
11	*Streptococcus iniae*	Nile tilapia/Field isolate (JW7-SI69)
12	*Streptococcus iniae*	Nile tilapia/Field isolate (JW9-SI76)
13	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA1)
14	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA2)
15	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA3)
16	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA4)
17	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA5)
18	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA6)
19	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA7)
20	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA8)
21	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA9)

Strain	*Bacterial species*	*Source*
22	*Aeromonas hydrophila*	Nile tilapia/Field isolate (JWA10)
23	*Streptococcus agalactiae*	ATCC® 13813
24	*Streptococcus iniae*	ATCC® 29178/Amazon freshwater dolphin
25	*Aeromonas hydrophila*	ATCC® 35654/Finished Water
26	*Escherichia coli*	ATCC® 25922/Clinical isolate
27	*Enterococcus faecalis*	ATCC® 29212/Urine
28	*Pseudomonas aeruginosa*	ATCC® 27853/Blood culture
29	*Salmonella* Enteritidis	ATCC® 1076/Digester (thermophilic digester)
30	*Salmonella* Typhimurium	ATCC® 14028/Tissue, animal
31	*Staphylococcus aureus*	ATCC® 6538/Human lesion
32	*Staphylococcus aureus*	ATCC® 29213/Wound
33	*Vibrio harveyi*	PN 9801
34	*Vibrio parahemolyticus*	ATCC® 17802/Shirasu food poisoning, Japan

All clinical isolates were obtained from diseased farmed tilapia in Thailand from 2003 to 2011. All isolates were characterized based on their biochemical properties with API20 system (Biomerieux®, France) and conventional methods as described in Bergey's Manual of Systematic Bacteriology. Reference strains and clinical isolates were stored in 20% glycerol in maintenance broth at -20°C and held in the strain collection of the Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University. Bacteria were grown on Tryptic Soy Agar (TSA; OXOID®, UK) containing 5% sheep blood at 30°C for 18-24 hours. After incubation, genomic DNA was extracted from the pure culture of bacteria using the whole cell boiled lysate technique. The selected colony was suspended in 100 µl sterile distilled water and heated in a dry bath for 15 min. then centrifuged at 12,000 x g for 5 min. after which the supernatant was collected and kept at -20 °C until use.

PCR amplification and analysis of PCR products. The multiplex PCR reactions were performed in a 20 µl reaction mixture consisting of 0.2 µl of Taq DNA polymerase (5U/µl) (iNtRON Biotechnology, USA), 2 µl of PCR buffer (100 mM Tris HCl, pH 8.3), 2 µl of 10 mmol dNTP mix (2.5 mmol each), 10.8 µl of sterile distilled water, 1 µl of each primer (10 µmol each), and 1 µl of DNA template (50 ng). The sequences of primers used in this study are listed in Table 2 (Lee et al., 2002; Meiri-Bendek et al., 2002).

Table 2. Oligonucleotide primers used in the study.

Primer	*Sequence (5´-3´)*	*Size (bp)*	*Targeted gene*	*Reference*
C1	GCG TGC CTA ATA CAT GCA A TAC	207	16S rRNA	Meiri-Bendex et al. (2002)
C2	AAC GCA GGT CCA TCT			
AERF	CTA CTT TTG CCG GCG AGC GG	953	16S rDNA	Lee et al. (2002)
AERR	TGA TTC CCG AAG GCA CTC CC			

PCR amplifications were performed with a T-personal combi thermocycler® (Biometra®, Germany). The PCR thermal cycling protocol included an initial denaturation at 94°C for 2 min, and 30 cycles of denaturation at 94°C for 20 s. The annealing temperatures ranged between 56°C and 64°C for 10 s, DNA extension at 72°C for 30 s, and a final extension at 72°C for 2 min. Genomic DNA of *S. agalactiae* ATCC® 13813, *S. iniae ATCC*® 29178, *A. hydrophila* ATCC® 35654 and sterile distilled water were used as positive and negative controls in each run. After this, 5 µl of PCR products were analyzed with 1.2% agarose gel electrophoresis and stained with 0.5 µg/ml ethidium bromide (Sigma-Aldrich, USA) for 10 min. PCR products were purified using Nucleospin Gel Extraction kit (Nucleospin®, Germany). The purified DNA representative samples were submitted for nucleotide sequencing (1st BASE DNA Sequencing Services, Singapore). The sequencing results were analyzed using NCBI blast search (http://blast.ncbi.nlm.nih.gov/).

Determination of detection sensitivity and specificity. Genomic DNA of *S. agalactiae* ATCC® 13813 and *A. hydro*phila ATCC® 35654 were standardized in sterile distilled water, containing 200 ng/µl of nucleic acid, and then serially diluted down to 2 fg/µl. Duplicate DNA templates containing 1 µl of genomic DNA dilution were used to determine the sensitivity of m-PCR reaction. Analytical specificity of the m-PCR was performed with DNA extracted from pure culture of Gram-positive and Gram-negative bacteria shown in Table 1.

Application of m-PCR on clinical cases. Fish collected immediately after death from farm outbreaks during 2011-2013 were sent, on ice, to the Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University for disease investigation (Table 3). The bacterial pathogens were isolated from the posterior kidney and brain using methods described in *Fish Disease: Diagnosis and Treatment* (Noga, 1996). The biochemical characteristics of bacteria were examined using API20 system (Biomerieux®, France). Approximately 20-50 mg of kidney and brain tissue was collected in absolute ethanol for disease diagnosis using m-PCR technique. For molecular analysis, the genomic DNA from tissue samples was extracted using standard phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with 95% ethanol.

Results

The size of PCR amplicons obtained from primers specific for the genus *Streptococcus* and *Aeromonas* spp. were 207 bp and 953 bp, respectively (Fig. 1).

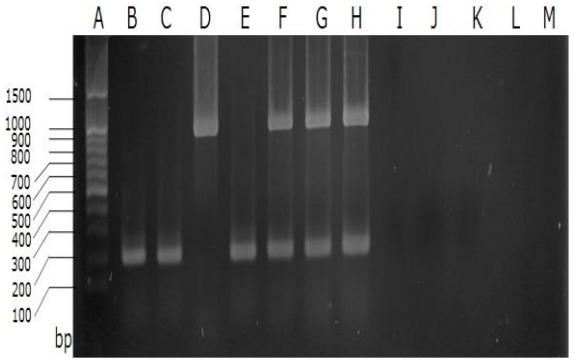

Fig. 1. Chromosomal DNA was amplified using m-PCR and primers specific for the genus of *Streptococcus* spp. and *Aeromonas* spp. The PCR products were 207 bp and 953 bp in size, respectively. Lane A= 100 bp + 1.5 Kb DNA Ladder (SibEnzyme®, Russia), Lane B= template extracted from *S. agalactiae* ATCC® 13813, Lane C= *S. iniae* ATCC® 29178, Lane D= *A. hydrophila* ATCC® 35654, Lane E= mixture of DNA extracted from *S. agalactiae* ATCC® 13813 and *S. iniae* ATCC® 29178, Lane F= *S. agalactiae* ATCC® 13813 and *A. hydrophila* ATCC® 35654, Lane G= *S. iniae* ATCC® 29178 and *A. hydrophila* ATCC® 35654, Lane H= *S. agalactiae* ATCC® 13813, *S. iniae* ATCC® 29178 and *A. hydrophila* ATCC® 35654, Lane I= *S. aureus* ATCC® 29213, Lane J= *E. coli* ATCC® 25922, Lane K= *P. aeruginosa* ATCC® 27853, Lane L= *V. harveyi* PN 9801, Lane M= negative control.

The thermal cycle condition proposed in this study were; 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 56 °C for 10 s, 64°C for 10 s, 72 °C for 30 s, and a final extension at 72 °C for 2 min. suitable for amplification of the DNA templates. The DNA sequence analysis of PCR products revealed that the two primer pairs were specific for their corresponding target organisms. PCR products obtained from m-PCR C1/C2 primers were identical to *Streptococcus* spp. 16s rRNA gene fragments and AERF/AERR primers were identical to *Aeromonas* spp. 16s rDNA gene fragments. The two primer pairs were specific for their corresponding target organisms.

The specificity of the m-PCR method was evaluated with purified nucleic acid templates from 2 Gram-positive and 7 Gram-negative water-habitat bacteria. The m-PCR showed negative results for *Staphylococcus aureus* ATCC® 6538, *S. aureus* ATCC® 29213, *Escherichia coli* ATCC® 25922, *Enterococcus faecalis* ATCC® 29212, *Pseudomonas aeruginosa* ATCC® 27853, *Salmonella* Enteritidis ATCC® 1076, *S.* Typhimurium ATCC® 14028, *Vibrio harveyi* PN 9801, *V. parahemolyticus* ATCC® 17802 (Fig. 1). The sensitivity of the m-PCR was determined using a mixture of DNA extracted from pure cultures of *S. agalactiae* ATCC® 13813 and *A. hydrophila* ATCC® 35654. The lowest amount of each DNA template was visualized at 2 ng (Fig. 2).

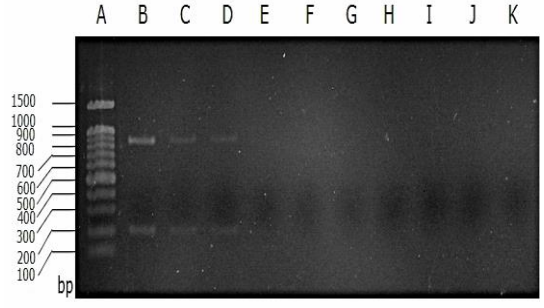

Fig. 2. Sensitivity of m-PCR determined from pure-culture bacterial suspensions. PCR amplification from mixture of DNA extracted from *S. agalactiae* ATCC® 13813 and *A. hydrophila* ATCC® 35654. Lane A= 100 bp + 1.5 Kb DNA Ladder (SibEnzyme®, Russia), Lane B= 200 ng/µl, Lane C= 20 ng/µl, Lane D= 2 ng/µl, Lane E= 200 pg/µl, Lane F= 20 pg/µl, Lane G= 2 pg/µl, Lane H= 200 fg/µl, Lane I= 20 fg/µl, Lane J= 2 fg/µl of each bacteria, Lane K= negative control.

Most samples in this study were of diseased fry and fingerling tilapias. The observed clinical symptoms of diseased fish were non-specific and similar to those of other bacterial infections, (lethargy, loss of scale, skin hemorrhages, abdominal distention and erratic swimming). Mortality rates were over 50%. A representative of diseased fish from each outbreak was detected and identified for the presence of bacterial infection or

targeted gene using bacterial cultivation and m-PCR. Comparison of m-PCR assays and bacterial culture for detecting *Streptococcus* and *Aeromonas* spp. infection in clinical cases are shown in Table 3.

Table 3. Bacteriological culture and m-PCR results of clinical cases from disease outbreaks in farmed tilapia.

No.	*Month/Year*	*Location*	*Bacteriological culture*				*m-PCR*			
			Brain		*Kidney*		*Brain*		*Kidney*	
			S	*A*	*S*	*A*	*S*	*A*	*S*	*A*
1	1/2011	Samut Prakan	+	-	+	-	+	-	+	-
2	2/2011	Petchburi	-	+	-	+	-	+	-	+
3	6/2011	Chachoengsao	-	+	-	+	-	+	-	+
4	6/2011	Petchburi	+	-	+	-	+	-	+	-
5	6/2011	Petchburi	-	+	-	+	-	+	-	+
6	7/2011	Chachoengsao	+	+	+	+	+	+	+	+
7	8/2011	Petchburi	+	-	+	-	+	-	+	-
8	8/2011	Chachoengsao	-	+	-	+	-	+	-	+
9	2/2012	Petchburi	-	-	-	-	+	-	+	-
10	3/2012	Samut Prakan	+	-	+	-	+	-	+	-
11	4/2012	Nakhon Nayok	+	-	+	-	+	-	+	-
12	5/2012	Petchburi	-	-	+	-	+	-	+	-
13	7/2012	Chiang Mai	+	-	+	-	+	-	+	-
14	11/2012	Nakhon Pathom	-	-	-	-	+	-	+	-
15	11/2012	Chachoengsao	+	+	+	+	+	+	+	+
16	1/2013	Chachoengsao	-	+	-	+	-	+	-	+
17	3/2013	Ratchaburi	-	-	-	-	+	-	+	-
18	3/2013	Chachoengsao	+	+	+	+	+	+	+	+
19	6/2013	Petchburi	+	+	+	+	+	+	+	+
20	8/2013	Petchburi	-	+	-	+	-	+	-	+

S= *Streptococcus* spp., A= *Aeromonas* spp., (+)= Found colony or PCR product, (-)= Not found colony or PCR product

Multiplex-PCR exhibited the presence of DNA fragments from both organisms and co-infections were evident in some cases (Case no. 6, 15, 18 and 19). The results of bacterial cultures were comparable to the m-PCR results, particularly for detection of *Aeromonas* spp. Inability of bacterial culture to detect *Streptococcus* spp. is shown in cases no. 9, 14, and 17, while positive results were obtained from m-PCR in case no. 12. *Streptococcus* spp. was evident only from the kidney samples, however the m-PCR result showed the presence of the targeted gene from brain and kidney tissue. From 20 cases studied, more infection of *Streptococcus* spp. than Aeromonas infection was found, and mixed infections were diagnosed in 20% of cases.

Discussion

Intensive farming and improper farming practices led to stressful conditions which increased the chance of infection and disease outbreak (El-Sayed, 2006). Fish are often infected with two pathogens concurrently, and frequently show nonspecific clinical symptoms which are similar to other bacterial infections (Noga, 1996). Where there is a mild degree of infection, fish may exhibit few or no symptoms at all and may survive the infection.

m-PCR assays have been used to diagnose Streptococcosis in different fish species, including tilapia and m-PCR analysis has permitted simultaneous detection of Gram positive cocci species, several species of *Streptococcus,* and *Lactococcus garvieae* (Mata et al., 2004; Itsaro et al., 2012). The present study concentrated on m-PCR for simultaneous detection of *Streptococcus* spp. and *Aeromonas* spp. in diseased tilapia.

The primers used in this study were specific to 16s rRNA gene fragments of *Streptococcus* spp. and 16s rDNA gene fragments of *Aeromonas* spp. All primers were proven to be specific under conditions designed for this m-PCR assay. The 16s rRNA and 16s rDNA gene are widely used for identification and differentiation of bacteria as these genes are well conserved in all bacteria (Van de Peer et al., 1996; Mata et al., 2004; Warsen et al., 2004; Chu and Lu, 2005; Chang et al., 2009).

The m-PCR procedures implemented in this study were highly specific for detection of *Streptococcus* and *Aeromonas* spp. by testing them against other Gram-positive and Gram-negative bacteria respectively, and comparing the specificity of PCR products by the DNA sequencing. The m-PCR assay appeared to be highly sensitive, with a low detection limit of 2 ng for each *Streptococcus* and *Aeromonas* spp. nucleic acid. The detection limits of the molecular techniques targeting genes of *Aeromonas* spp. shown in previous studies were 0.89 pg (Cascon et al., 1996), 0.5 pg to 50 ng (Balcazar et al.,

2007), 20 pg (Panangala et al., 2007), and 70 fg (Onuk et al., 2010). For *Streptococcus* spp., the limit of detection of *S. mutans* and *S. sobrinus* in previous reports were 100 pg and 100 fg, respectively (Sato et al., 2003). These results indicate that each primer used in this procedure did not interfere with the other, and the adjusted PCR conditions were suitable for individual targeted gene amplification.

The results for *Streptococcus* and *Aeromonas* spp. infection in fish from m-PCR assay were compared with bacteriological cultures. The m-PCR was able to confirm concomitant infections with *Streptococcus* and *Aeromonas* spp. while in some cases the bacterial culture failed to discover *Streptococcus* spp. This finding suggests that false negative results may occur when bacterial culture is performed. This may be due to the prior use of antimicrobials for treatment which may inhibit normal bacterial growth in culture systems. The suppression of bacterial growth in cultures was also found in mixed infections when an out-competition of one bacterium on others occurred (Panangala et al., 2007). As a result, bacterial culture systems may not always be suitable for the detection of bacterial infections, especially in cases of multiple infections.

In the present study we found that m-PCR can be employed as a diagnostic procedure for detection of *Streptococcus spp.* and *Aeromonas* spp. infections in tilapia. The method offers a rapid and reliable procedure for detection of the infection and it is applicable to diagnostic testing schemes in different developmental stages of tilapia; eggs, fry, and broodstock. The use of m-PCR allows accurate identification of the infected fish or carrier fish, particularly when cultures fail to detect the bacterial pathogens. Our study justifies the use of m-PCR as a diagnostic assay for specific detection of *Streptococcus spp.* and *Aeromonas* spp. in tilapia as well as a disease control measure to be implemented in tilapia farming.

Acknowledgements

This work was supported by a Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumbol Adulyadej and the Thailand Research Fund through the Royal Golden Jubilee Ph.D. program.

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