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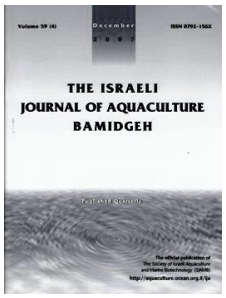

Establishment of a Multiplex PCR Assay to Detect Five Major Freshwater Bacteria

Weihua Gao[1,2,Δ], Kete Ai[1,Δ], Kai Luo[1], Tinghua Huang[1], Min Yao[1], Wei Hu[1], Liu Fang[1], Zhitao Qi[1], Qiaoqing Xu[1,2]*

[1] *Engineering Research Center of Ecology and Agricultural Use of Wetland, Ministry of Education, Yangtze University, Jingzhou 434024, China*
[2] *School of Animal Science, Yangtze University, Jingzhou, 434020, China*
Δ The two authors contributed equally.

Keywords: multiplex PCR; freshwater bacteria; detection

Abstract

A multiplex polymerase chain reaction (mPCR) method for simultaneous detection of *Aeromonas hydrophila*, *Streptococcus agalactiae*, *Klebsiella pneumoniae*, *Edwardsiella tarda,* and *E. ictalur* was developed to rapidly and accurately identify the five most common bacteria that infect aquatic animals. The expected amplicons for *ahe2* gene of *A. hydrophila*, *cpsE* gene of *S. agalactiae*, *khe* gene of *K. pneumoniae*, *mukF* gene of *E.tarda*, and the *serC* gene of *E. ictaluri* were 853 bp, 685 bp, 428 bp, 356 bp, and 124 bp, respectively. In the single PCR assays, the minimum detectable DNA contents were 13.2 pg for *A. hydrophila*, 27.4 pg for *S. agalactiae*, 1.95 pg for *K. pneumoniae*, 1.63 pg for *E. tarda*, 1.02 pg for *E. ictalur.* The detection limits of the multiplex PCR were 0.66 ng, 1.91 ng, 0.68 ng, 0.41 ng, 0.71 ng for *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E. tarda* and *E. ictalur*, respectively. The established multiplex PCR is significant for the rapid detection of common pathogenic bacteria of aquatic animals and provides the basis for the diagnosis of fish diseases.

* Corresponding author. Tel.: +86 716 8066289; fax: +86 716 8066289.
Email: xuqiaoqing@yangtzeu.edu.cn

Introduction

Bacterial diseases are common worldwide and result in significant economic losses in the fish farming industry. For example, *Aeromonas hydrophila* is a ubiquitous aquatic microorganism that has been associated with hemorrhagic septicemia. Infections by *A. hydrophila* have been reported in *Carassius auratus*, *Parabramis pekinensis*, *Cirrhinus molitorella*, *Cyprinus carpio, Hypophthalmichehys molitrix*, *Aristichthys nobilis, Ctenopharyngodon idellus, Plecoglossus altivelis, Lateolabrax japonicas, Salmo gairdneri, Tilapia nilotica, Ictalurus punctatus*, *Monopterus albus* and *Channa striata* (Lu, 1992; Duc *et al*., 2013). *Streptococcus agalactiae* can cause septicemia and meningoencephalitis, which occurs in *Oreochromis spp, Salmo gairdneri, Cyprinus acutidorsalis, Mugil cephalus* and *Pagrosomus major* (Evans *et al*., 2002). *Klebsiella pneumoniae*, which causes rot disease, has been reported in Shrimp*, Eriocheir sinensis, Hypophthalmichehys molitrix, Anguilla japonica* and *Trionyx sinensis* (Singh and Kulshreshtha, 1992; Tang *et al*., 2007; Deng *et al*., 2009; Xu and Shu, 2002). *Edwardsiella tarda* leads to serious systemic septicemia and affects a wide range of host fish such as *Scophthalmus maximus*, *Paralichthys olivaceus*, *Ictalurus punctatus*, *Anguilla anguilla*, *Pelteobagrus fulvidraco*, *Astronotus ocellatus*, *Paralichthys lethostigma*, *Tilapia nilotica*, *Hypophthalmichthys molitrix*, *Scortum barcoo*, etc. (Xu and Zhang, 2014; Mohanty and Sahoo, 2007; Park, Aoki, and Jung, 2012). *E. ictalur* usually produces enteric septicemia, which mainly appears in *Ictalurus punctatus* and has also been reported in *Micropterus salmoniodes, Aristichthys nobilis, Plecoglossus altivelis* and *Trionyx sinensis* (Nagai and Nakai, 2014; Chen, 2004).

Accompanying the rapid development of aquaculture and the expansion of scale breeding, highly intensive aquaculture conditions create serious risks for the spread of contagious diseases in fish populations. Therefore, detection of pathogens from farmed fish is essential for effective fish disease control. Polymerase chain reaction (PCR) is an effective tool for diagnostics. PCR assays have been developed for the rapid detection and identification of microorganisms (Yogananth *et al*., 2009; Lan *et al*., 2008; Panangala *et al*., 2007). However, a multiplex PCR assay is necessary if single primer sets are used on a large number of clinical samples. The objective of the present study was to develop a multiplex PCR (mPCR) assay to detect five major fish pathogens: *A. hydrophila, S. agalactiae, K. pneumoniae, E. tarda* and *E. ictalur* that cause disease in fish.

Materials and Methods

Source of the bacteria. Strains of *A. hydrophila* B11, *S. agalactiae* SF-96-5508, *K. pneumoniae* B12, NTUH-K2044, *E. tarda* B79 *E. ictalur* ETV, *Flavobacterium cloumnare* EU395799 and *Escherichia coli* Top10 were kindly supplied by the Institute of Hydrobiology, Chinese Academy of Sciences. Strains of *A. hydrophila* 1.927, 1.2017, 1.1816, 1.1814 and *S. agalactiae* 1.1481 were purchased from China general microbiological culture collection center. Strains of *E. tarda* CCTCC AB 2010161, CCTCC AB 206580 and CCTCC AB206591 were purchased from China center for type culture collection. Strains of *E. ictalur* GXEi0501, GXEi0602 were purchased from Guangxi Veterinary Research Institute. A strain of *S. agalactiae* 32420 was purchased from the National center for medical culture collections. A strain of *S. agalactiae* 10465 was purchased from China center of industrial culture collection. *K. pneumoniae* B0094, *Aeromonas sobria* LD081008A, *Pseudomonas fluorescens* B0115 and *Staphylococcus aureus* B0125 were purchased from the national aquatic pathogen library, Shanghai Ocean University.

Genomic DNA extraction. Bacteria were inoculated into tryptic soy broth (TSB). *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E. tarda*, *E. coli*, *A. sobria*, *P. fluorescens* and *S. aureus* were incubated at 37°C for 24 h, *E. ictalur* was incubated at 28°C for 48 h. *F. cloumnare* was incubated in Shieh at 25°C for 48 h. All the cultures were incubated and shaken at 200 rpm in an orbital incubator. The bacteria were harvested by centrifugation at 12000 g for 1 min, and the supernatant was discarded. DNA was extracted from the cell pellets of all samples following the instructions of the bacteria genomic DNA extraction kit (TIANGEN, China).

Primer design. Target sequences of the *ahe2* gene of *A. hydrophila and* the *cpsE* gene of *S. agalactiae* were downloaded from the National Centre for Biotechnology Information (NCBI). Specific primers corresponding to each bacterium were designed with primer premier 5.0. Primers for the *khe* gene of *K. pneumoniae*, the *mukF* gene of *E. tarda*, the *serC* gene of *E. ictalur* described by He *et al*., 2012; Jiang *et al*., 2008; Liu *et al*., 2013 (Table 1).

Table 1. Specific primer and their target genes for the bacteria used in this study

Bacteria	*Primers*	*Target gene*	*Size (bp)*
Aeromonas hydrophila	F:GGGCAATGACCTCAACCTC R:CGAGGAGGTGCCGTTCAT	Serine protease (*ahe2*)	853
Streptococcus agalactiae	F:TTATATCGCTGTCTGTATCTTGGACT R:GCGTGTTCCTACTAAACTCATATCACC	Capsule (*cpsE*)	685
Klebsiella pneumoniae	F: TGATTGCATTCGCCACTGG R: GGTCAACCCAACGATCCTG	haemolysin (*khe*)	428
Edwardsiella tarda	F:TTGCTGGCTATCGCTACCCTT R:AACTCATCGCCGCCCTCTTCT	*mukF*	356
Edwardsiella ictalur	F: CATGATAATACCCGGTGTTGG R: GTATTGCTGGGGAACAACTC	Phosphoserine transaminase (*serC*)	124

Reaction conditions. The optimized PCR was set up in a 25 µL reaction mixture comprising 2.5 µL of 10× Taq Buffer, 2 µL of $MgCl_2$ (25 mM), 2 µL of dNTP (2.5 mM), 1 µL (10 µM) each of forward and reverse primers, 0.25µL of rTaq DNA polymerase (5 U/µL, NovoGene, China), 1 µL of DNA and nuclease free water to 25µL.

In the single PCR assays, the cycling conditions consisted of an initial denaturation (95°C for 3 min), followed by 35 cycles of denaturation (95°C, 30 s), primer annealing (59°C for the *ahe2* gene of *A. hydrophila* and the *cpsE* gene of *S. agalactiae*, 57°C for the *khe* gene of *K. pneumoniae* and the *serC* gene of *E. ictalur*, 60°C for the *mukF* gene of *E. tarda*, 30 s, respectively.), and extension (72°C, 1 min). After a final extension (72°C, 10 min), the PCR products were held at 4°C. The reaction products were checked by 2% agarose gel electrophoresis.

In the mPCR assay, the primer annealing was set at 59°C, 30 s, the other cycling conditions were same as the single PCR assay.

Specificity analysis. The five primer sets were used with the specific DNA or with mixed DNAs from other bacteria to test their specificity.

Sensitivity analysis. The genomic DNA contents were serially diluted by 5 or 10 times and subjected to PCR amplification. The minimum concentrations of DNAs were considered as the sensitivity of m-PCR detection.

Fish tissue analysis. Genomic DNA of liver, spleen, and muscle tissue from *Cyprinus carpio* was extracted and mixed with bacterial DNA of the five bacterial pathogens as simulated diseased samples. Following m-PCR amplification and detection, the mixed DNAs were evaluated in practice.

Results

Primer specificity. Based on the designed primer sets, the specificities of each set against bacterial DNAs obtained from *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E. tarda*, *E. ictalur* were examined. For all primer pairs, only a band for the specific bacteria appeared (Fig. 1a-b). When DNA from other bacteria strains was evaluated, no bands were visible on gel electrophoresis (Fig. 1c).

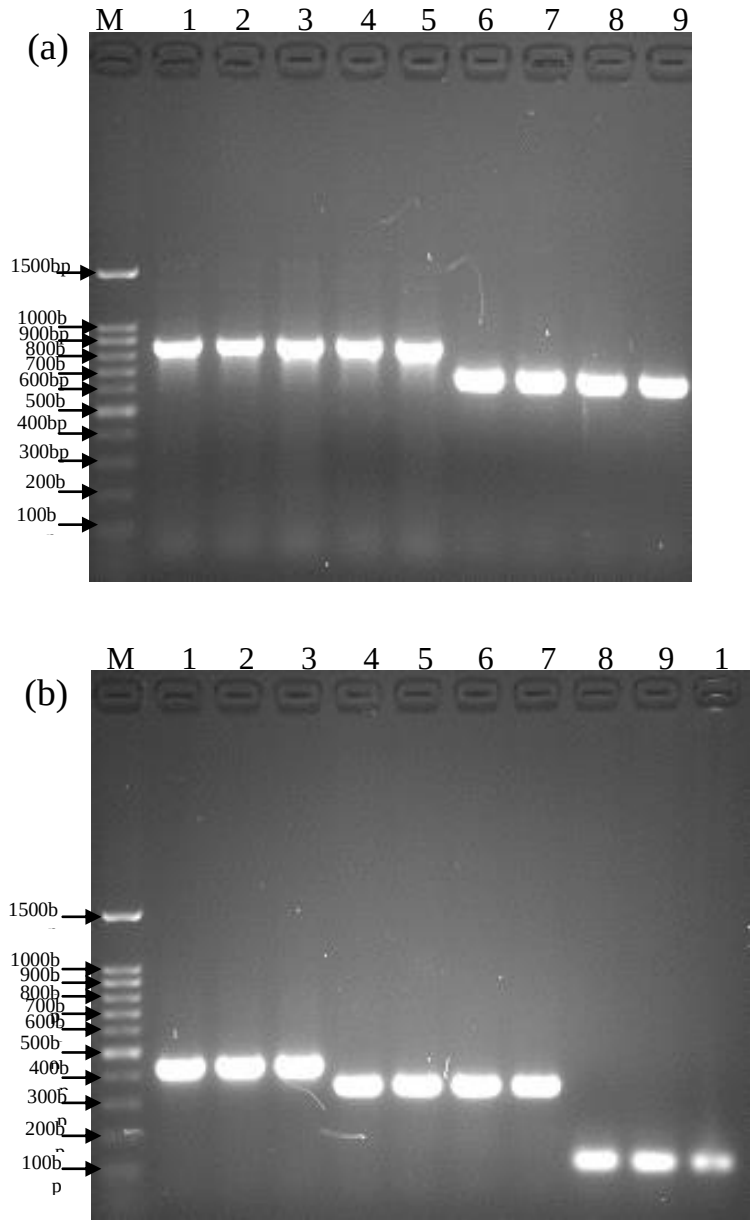

Fig. 1 Specificity of single PCR of DNA from *Aeromonas hydrophila*, *Streptococcus agalactiae*, *Klebsiella pneumoniae*, *Edwardsiella tarda* and *E. ictalur*. (a): Lane M, 100 bp DNA marker; lane 1-5=*A. hydrophila* B11, 1.927, 1.2017, 1.1816 and 1.1814 (853 bp) respectively; lane 6-9=*S. agalactiae* SF-96-5508, 1.1481, 32420 and 10465 (685 bp) respectively; (b): lane 1-3=*K. pneumoniae* B12, NTUH-K2044 and B0094 (428 bp) respectively; lane 4-7=*E. tarda* B79, CCTCC AB 2010161, CCTCC AB 206580 and CCTCC AB206591 (356 bp) respectively; lane 8-10=*E. ictalur* ETV, GXEi0501 and GXEi0602 (124 bp) respectively; (c): Lane M, 2000 bp DNA marker; lane 1, *Flavobacterium cloumnare*; lane 2, *Escherichia colI*; lane 3, *Aeromonas sobria*; lane 4, *Pseudomonas fluorescens*; lane 5, *Staphylococcus aureus*.

Detection sensitivity. After diluting the target DNA by 5 or 10 times, the minimum detectable DNA contents were determined as 13.2 pg for *A. hydrophila* (Fig. 2a), 27.4 pg for *S. agalactiae* (Fig. 2a), 1.95 pg for *K. pneumoniae* (Fig. 2b), 1.63 pg for *E. tarda* (Fig. 2c), 1.02 pg for *E. ictalur* (Fig. 2d) in the single PCR reactions. The detection limits of the multiplex PCR was in the range of 0.66 ng, 1.91 ng, 0.68 ng, 0.41 ng, 0.71 ng for *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E. tarda* and *E. ictalur* (Fig. 2d).

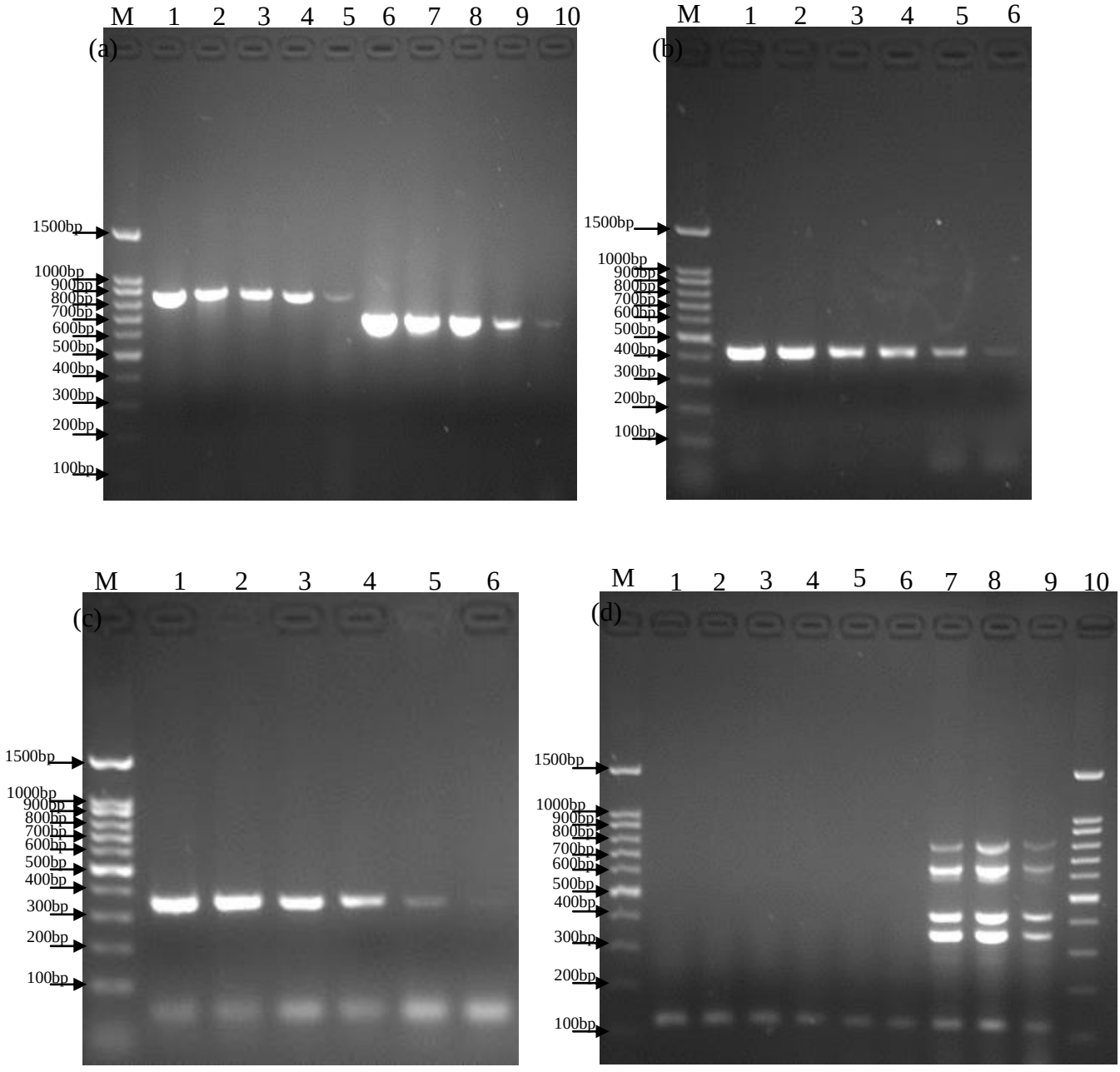

Fig. 2 Sensitivity of the detection of *A. hydrophila*, *S. agalactiae*, *K. pneumoniae* , *E. tarda* and *E. ictalur* by single PCR and mPCR. (a): Lane M, 100 bp DNA marker; lanes 1-4=1 to 10^{-3} dilutions of *A. hydrophila*, respectively; lane 5, the 2×10^{-4} dilution of *A. hydrophila*; lanes 6-10=1 to 10^{-4} dilutions of *S. agalactiae,* respectively; (b): Lane M, 100 bp DNA marker; lanes 1-5=1 to 10^{-4} dilutions of *K. pneumoniae*; lane 6, the 2×10^{-5} dilution of *K. pneumoniae ;* (c): Lane M, 100 bp DNA marker; lanes 1-5=1 to 10^{-4} dilutions of *E. tarda,* respectively; lane 6, the 2×10^{-5} dilution of *E. tarda*; (d): Lane M, 100 bp DNA marker; lanes 1-6=1 to 10^{-5} dilutions of *E. ictalur,* respectively; lanes 7-9=1 to 10^{-2} dilutions of *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E. tarda* and *E. ictalur*, respectively.

mPCR. The genomic DNAs of the five bacterial pathogens were mixed to see whether single or multiple species of pathogen were effectively amplified. The results demonstrated that all combinations of the pathogenic DNA could be detected successfully *(Fig. 3).*

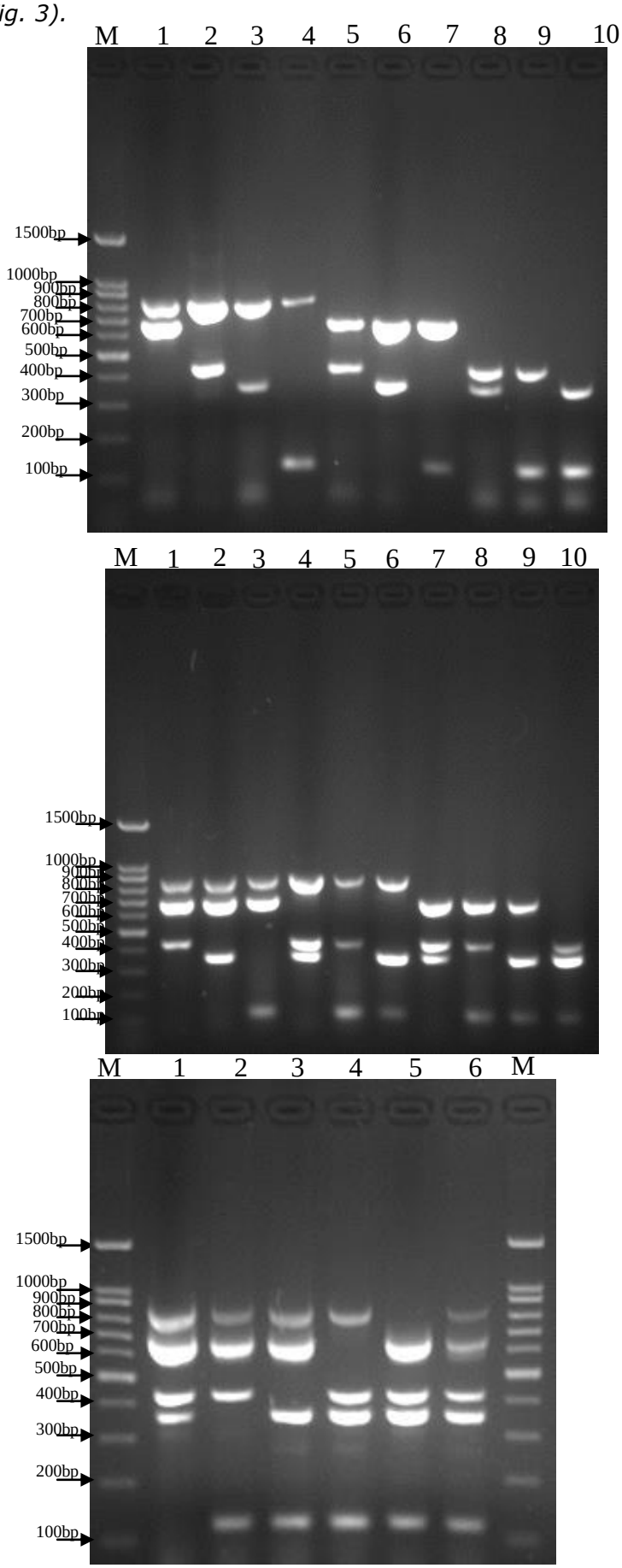

Fig. 3 The results of mPCR amplification of genomic DNA from two, three, four or five bacteria with different combinations of target PCR templates. (a): Lane M, 100 bp DNA marker; lane 1, *A. hydrophila* and *S. agalactiae*; lane 2, *A. hydrophila* and *K. pneumoniae*; lane 3, *A. hydrophila* and *E. tarda*; lane 4, *A. hydrophila* and *E. ictalur*; lane 5, *S. agalactiae* and *K. pneumoniae*; lane 6, *S. agalactiae* and *E. tarda*; lane 7, *S. agalactiae* and *E. ictalur*; lane 8, *K. pneumoniae* and *E. tarda*; lane 9, *K. pneumoniae* and *E. ictalur*; lane 10, *E. tarda* and *E. ictalur*; (b): Lane M, 100 bp DNA marker; lane 1, *A. hydrophila*, *S. agalactiae* and *K. pneumoniae*; lane 2, *A. hydrophila, S. agalactiae* and *E. tarda*; lane 3, *A. hydrophila, S. agalactiae* and *E. ictalur*; lane 4, *A. hydrophila, K. pneumoniae* and *E. tarda*; lane 5, *A. hydrophila, K. pneumoniae* and *E. ictalur*; lane 6, *A. hydrophila, E. tarda* and *E. ictalur*; lane 7, *S. agalactiae* , *K. pneumoniae* and *E. tarda*; lane 8, *S. agalactiae* , *K. pneumoniae* and *E. ictalur*; lane 9, *S. agalactiae* , *E. tarda* and *E. ictalur*; lane 10, *K. pneumoniae*, *E. tarda* and *E. ictalur*; (c): Lane M, 100 bp DNA marker; lane 1, *A. hydrophila*, *S. agalactiae*, *K. pneumoniae* and *E. tarda*; lane 2, *A. hydrophila*, *S. agalactiae*, *K. pneumoniae* and *E. ictalur*; lane 3, *A. hydrophila*, *S. agalactiae*, *E. tarda* and *E. ictalur*; lane 4, *A. hydrophila*, *K. pneumoniae*, *E. tarda* and *E. ictalur*; lane 5, *S. agalactiae*, *K. pneumoniae*, *E. tarda* and *E. ictalur*; lane 6, *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E. tarda* and *E. ictalur*.

Fish tissue. Following mPCR amplification and detection with the mixed DNAs, the diagnostic efficiency was maintained with predicted amplicons, which suggests that no interfering effects occurred (Fig. 4).

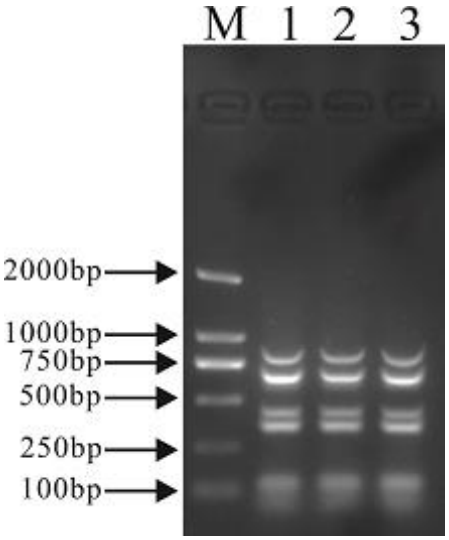

Fig. 4 The results of mPCR amplification of genomic DNA from five bacteria in combination with genomic DNA from *Cyprinus carpio* tissue. Lane M, 2000 bp DNA marker; lane 1, mixture of genomic DNA from the target bacteria and *Cyprinus carpio* liver ; lane 1, mixture of genomic DNA from the target bacteria and *Cyprinus carpio* spleen; lane 1, mixture of genomic DNA from the target bacteria and *Cyprinus carpio* muscle.

Discussion

The aim of this study was to develop a simple and sensitive novel PCR method for simultaneous identification of common pathogens that cause disease in fish. Primers were designed to uncover different length fragments of 853 bp, 685 bp, 428 bp, 356 bp, and 124 bp from *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E.tarda*, and *E. ictaluri*. The primer specificities of each set were examined. The size of PCR products was as expected with no additional fragments from target pathogens (Fig. 1). Detection sensitivity was important to the efficiency of PCR amplification. After serially diluting the target DNA by 5 or 10 times, the minimum detectable DNA contents were determined as 0.66 ng for *A. hydrophila*, 1.91 ng for *S. agalactiae*, 0.68 ng for *K. pneumoniae*, 0.41 ng for *E. tarda*, 0.71 ng for *E. ictalur* in the multiplex PCR (Fig. 2). These results suggest that the established multiplex PCR is sensitive to the rapid detection common pathogenic bacteria of aquatic animals.

Traditional methods of diagnosis are carried out by culturing bacteria on agar plates followed by biochemical characterization (Zhang *et al*., 2010). Some disadvantages of these techniques are: the length of time taken, which could delay effective treatment of fish diseases; and compared with PCR assay, it is not accurate to detect pathogenic bacteria by colonies morphology and bacterial characteristics, etc.

The molecular assay using real-time quantitative PCR could be a rapid and sensitive diagnostic tool to detect pathogens (Bilodeau *et al*., 2003; Griffin *et al*., 2013) however, this requires high quality equipment, high costs, and complex operation, which makes it non-optimal for the detection of bacteria pathogens. The specific, easy to use, low-cost mPCR system developed in this study could simultaneously detect *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E. tarda* and *E. ictalur.*

PCR has been widely applied to detect various viruses and bacteria (Pollard *et al*., 1990; Lan *et al*., 2008; Sakai *et al*., 2007). Bacterial diseases may be diverse and depend on the farmed species, and culture area; therefore it is difficult to assess disease outbreaks using a universal diagnostic measure or procedure, especially when newly emerging diseases are probable. And it would be undetected if two or several pathogenic bacteria affected the fish. To avoid wasting time using a single PCR, an mPCR assay was

developed for simultaneous detection of *A. hydrophila*, *S. agalactiae*, *K. pneumoniae*, *E. tarda* and *E. ictalur.* This new mPCR assay is important for the diagnosis of fish diseases.

Identification of virulence genes is useful for the diagnosis of fish disease. Pathogenic bacteria may have virulence genes that are absent in nonpathogenic bacteria. Virulence genes may also be present in both pathogenic and nonpathogenic bacteria, but may be functional only in pathogenic ones (Srinivasa Rao *et al*., 2003). The *ahe2* gene of *A. hydrophila,* the *cpsE* gene of *S. agalactiae*, the *mukF* gene of *E. tarda*, the *serC* gene of *E. ictalur* are virulence genes (Li *et al*., 2011; Schaffner *et al*., 2014; Srinivasa Rao *et al*., 2003; Rodrigues *et al*., 2006). The *mukF* gene of *E. tarda* was present only in virulent and not in avirulent strains, indicating that they are specific to pathogenic *E. tarda* (Srinivasa Rao *et al*., 2003). However the *ahe2* gene of *A. hydrophila,* the *cpsE* gene of *S. agalactiae* and the *serC* gene of *E. ictalur* are associated with their toxicity (Yu *et al*., 2005; Rodrigues *et al*., 2006). If using avirulence genes to diagnose diseased fish that were infected with nonpathogenic bacteria, the diagnosis would be incorrect. Detection of these virulence genes will be useful for diagnosing the cause of fish diseases as they emerge.

Acknowledgments

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