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Original Research Article

Multiplex PCR for simultaneous detection of 3 major fish pathogens incriminated in bacterial septicemic syndrome

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ABSTRACT

Fish with bacterial septicemic syndrome (BSS) exhibit very similar clinical signs regardless of the etiological agents. Members of Aeromonas, Pseudomonas, Vibrio, Edwardsiella, Streptococcus and Lactococcus species are considered the most reported bacterial pathogens incriminated in such syndrome. Aeromonas hydrophila, Edwardsiella tarda and Streptococcus iniae are 3 major pathogens share in the BSS associated losses in aquaculture and considered problematic for growth of tilapia and catfish production in Egypt. Therefore, rapid and accurate diagnosis is highly needed for controlling their disease outbreaks, particularly, in mixed infections. In an attempt to elucidate the main causative pathogen, a novel multiplex PCR (m-PCR) was newly designed in this study. The developed m-PCR involves amplifying the three multiple genes in single reaction based upon primers deduced from the regions carrying 16S rRNA, etfA and 16S RNA genes of A. hydrophila (Aeromonas spp.), E. tarda and S. iniae, respectively. Prior to perform m-PCR, individual PCR assays were carried out to adapt suitable laboratory and m-PCR assays conditions. The specificity of the developed m-PCR was confirmed by the fact that only specific fragments were amplified equivalent for 953, 415 and 300 bp corresponding to A.hydrophila, E. tarda and S. iniae, respectively, and that was evident with both extracted DNAs and the bacterial cells. More specifically, these specific bands were obtained also when either the extracted DNAs or the bacterial cells of the three pathogens mixed together in the reaction. The developed m-PCR is accurate, sensitive, fast and simple technique for the simultaneous detection of A. hydrophila (Aeromonas spp.), E.tarda, and S. iniae, three major bacterial pathogens involved in BSS incidence in Egypt.

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1. Introduction

Freshwater aquaculture, particularly, Nile tilapia (*Oreochromis niloticus*) and sharp toothed catfish, (*Clarias gariepinus*), as well as their related species are of great importance among Egyptian farmers because of its high economic value for local consumers (FAO, 2016). The growing interests in this activity and, consequently, the search for higher profitability, have been driving producers to adopt intensive production systems. However, as a result of their ponds high stocking density, numerous obstacles are hindering the growth of this industry. Most of these obstacles are closely tied to the disease outbreaks associated with high morbidities and mass mortalities (Brum et al. 2017). A part of fish diseases, bacterial septicemic infections are considered the most influential problems.

Bacterial septicemic syndrome (BSS), from clinical point of view, is a generic term could be used to designate diseases with similar signs of different bacterial etiologies, in which any member (s) of Gram negative or positive bacteria is involved (Chang et al., 2012; Hussein et al., 2013). Fish with BSS exhibit very similar signs and clinical signs regardless of the etiological agents. Members of Aeromonads, Pseudomonads, Vibrios, Edwardsiella spp., Streptococci and Lactocci are considered the most reported bacterial pathogens incriminated in such syndrome. (Janda and Abbott, 2010; Rad et al., 2010; Liamnimitr et al., 2017; Kusdarwati et al., 2017). Infections associated with these micro-organisms are commonly characterized by anorexia, septicemia, ascitis, exophthalmia, hemorrhages, skin ulceration together with high morbidities and mortalities (Austin & Austin, 2012).

In Egypt, motile *Aeromonads* septicemia (MAS), edwardsiellosis and streptococcosis are considered the most prevalent BSS with economically devastating losses in cultured tilapias and catfish. The genus of *Aeromonas* has at least 25 species, a number of which (*A. hydrophila, A. veronii, A. sobria* and *A. caviae*) are common fish pathogens (Zaky et al. 2011; Younes et al., 2015; Aboyadak et al., 2015) that usually cause motile aeromonas septicemia (MAS). The genus *Edwardsiella, E. tarda* is recognized as an important member that causes edwardsiellosis in various commercial fishes, such as eel, flounder, catfish, carp, turbot and tilapia (Moustafa et al., 2016; Kumar et al., 2016). Different from the previous Gram negative genera, *Streptococci* and *Lactococci* are the Gram-positive ones that have been isolated from Nile tilapia, sturgeon, spotted silver scat, yellow tail, amberjack and others suffered from signs of BSS (Mata et al., 2004; Osman et al. 2017). Among streptococci, *S. iniae, S. agalactia, S. dysgalactia, S. ubris, S. paraubris* and *L. gareviae* are the most important Gram positive pathogens that incriminated in fish streptococcosis, however, *S. iniae* and *S. agalactia* are the main ones reported from diseased tilapias with streptococcosis (Al-Harbi, 2016; Hardi et al., 2018).

Traditional methods in microbiology for diagnosis of bacterial infections in fish involve culture, isolation and identification, which are time consuming to complete, complex and the delayed implementation of control measures leading to massive economic losses. Moreover, many pathogens share common morphological characteristics and cause similar clinical signs in diseased fish, particularly, those of BSS. Therefore, quicker, sensitive, accurate, specific and more effective diagnostic alternatives are necessary to overcome the clinical consequences and high mortality rates. Molecular methods based on genetic properties have evolved to fulfill such needs. Polymerase chain reaction (PCR) assays based identification methods are more sensitive, accurate, specific, effective diagnosis and fast than traditional microbiological approaches, since the entire process of DNA extraction, amplification, gel electrophoresis and visualization of PCR products can be performed in a workday (Gonzalez et al., 2004). Additionally, the

molecular approaches are more satisfactory, particularly, within microorganisms that showed biochemical diversity of their various isolates (Abdel Azeem et al., 2016). Moreover, 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 (Sebastião et al., 2015). Regular PCR assay is only detecting a single species of pathogen, which would be a relatively costly and labor process especially when single primer sets are used on a large number of clinical samples. To cope with the limitation of the individual PCR assay, multiplex PCR (m-PCR) as another PCR approach was developed for simultaneous detection of several pathogens at the same time, with different primer sets and different PCR protocols. This approach has successfully been applied to detect fish and shellfish pathogens (Tsai et al., 2012; pinto et al., 2017).

The aim of the present study was to develop a m-PCR approach for the concurrent detection of Aeromonas spp., E. tarda and S. iniae the most prevalent fish pathogens incriminated in BSS incidence in Egypt, from the extracted DNA as well as pure cultures.

2. Materials and methods

2. 1. Bacterial strains

The selected bacterial strains (n=3) namely, A. hydrophila (represent Aeromonas spp. and/or their biotrophs), E. tarda and S. iniae were isolated from diseased fish showed characteristics signs of BSS over the period from February, 2014 to February, 2015. All selected strains were inoculated into 10 mL Brain heart infusion broth (BHIB) then incubated at 25° C for 24 hours. The inoculated broth was centrifuged in a cooling centrifuge at 5000 rpm for 10 min at 10° C. Subsequently, the obtained pellets were diluted in sterile phosphate buffered saline (PBS) to obtain 3 $\times 10^8$ CFU/mL and then serially diluted to the desired dilutions in respect of the applied PCR approach (Hussein & Hatai, 2006).

2. 2. Extraction of DNA, target genes and primer sets

The chromosomal DNAs (cDNAs) from bacterial cells were extracted using DNA extraction kits (GeneJET Genomic DNA extraction Kit, Thermo Scientific®, Lithuania) following the manufacturer's protocol. The concentration and purity of the DNA that had been extracted were determined by estimating the optical density at a wave length of 260 and 280nm using the spectrophotometer. The developed m-PCR involves amplifying the three multiple genes in single reaction based upon primers deduced from the regions carrying etfA of E. tarda (Sakai et al., 2007), 16S RNA of S. iniae (Zlotkin et al., 1998) and 16S rRNA of Aeromonas spp. (Lee et al., 2002). All primers used were synthesized by Invitrogen® and Biomatik®, Lithuania. The primers oligonucleotides sequences and their specific target genes are illustrated in (Table 1).

2. 3. PCR amplification protocols

2. 3.1. Uniplex PCR (u-PCR)

Prior to perform m-PCR, individual PCR (u-PCR) assays were carried out for the three selected pathogens to adapt the suitable annealing temperature according to our laboratory conditions (Department of Fish Diseases & Management, Faculty of Veterinary Medicine, Beni-Suef University). The u-PCR amplifications were performed in 25-µL reaction tubes mixtures by adding 12.5 µl PCR Master Mix (Thermo Scientific®, Lithuania), 20 pg (equal to 1 µL) of each primer and 1µL of template (extracted DNA) and 9.5 µL purified DNA/RNA free water (Thermo Scientific®, Lithuania). Samples were subjected to designated regime of amplification (Table 2) in PCR gradient thermal cycler (Multi GENE Gradient®, USA) with little modification. The modification targeted the annealing temperatures of the primer sets of E. tarda and A. hydrophila, to be 59° C for both bacteria species instead of 55° C. Control negative contains PCR mixture without template was also included.

2. 3. Development of m-PCR assay

Two sets (A&B) of 25-µL PCR reaction tubes (4 tubes in each) were prepared. Set A, was designated for m-PCR with extracted DNA templates while set B for m-PCR with pure bacterial culture. The two m-PCR approaches were used in a comparative way to evaluate the efficacy of using the bacterial cells at concentrations of $3x10^2$ CFU/mL directly as templates in the reaction with amplification of purified extracted DNAs. The reaction mixture contained 12.5 µL PCR Master Mix (Thermo Scientific®, Lithuania), 10 pg (equal to 0.5 µL) of each primer (the six primers), 3 µL of the template (extracted DNA or bacterial cells) and 6.5 µL purified DNA/RNA free water. Then, m-PCR were run in PCR gradient thermal cycler (Multi GENE Gradient®, USA) with initial denaturation at 94º C for 3 min, 35 serial cycles of denaturation at 94º C for 1 min, annealing at 56º C for 1 min (the annealing temperature decreased by 0.2° C per cycle, from 63 to 56° C), extension at 72º C for 1 min and final extension at 72º C for 5 min (Table 2). At all level tested, negative controls were included in each patch of PCR amplification reactions that contained the PCR mixture without template.

2. 4. Visualization of both u-PCR and m-PCR products by electrophoresis

PCR-generated products were simultaneously analyzed by electrophoresis of 5-µL of each amplification mixture in 1% agarose gel in Tris-acetic acid EDTA (TAE) buffer at (100 V, 20 min) gels containing 0.001% ethidum bromide (Sambrook and Russell, 2001). A100-1500 bp DNA ladder were included as a molecular weight standard on left side of the gel. Then, gel was photographed under UV-light transillumination.

Table 1. Oligonucleotides sequences of primers sets used in PCR assays and the expected amplicon sizes

Gene	Primer sequence	Bacterial pathogen	Amplicon Size (bp)	Annealing temp (°C)	Reference
*etf*A	F: $^{5'}$CGGTAAAGTTGAGTTTACGGGTG$^{3'}$ R: $^{5'}$ TGTAACCGTGTTGGCGTAAG$^{3'}$	*Edwardsiella tarda*	415 bp	55	Sakai et al., 2007
16S RNA	F: $^{5'}$ CTAGAGTACACATGTACTAAG$^{3'}$ R: $^{5'}$ GGATTTCCACTCCCATTAC$^{3'}$	*Streptococcus iniae*	300 bp	55	Zlotkin et al., 1998
16S rRNA	F: $^{5'}$CTACTTTTGCCGGCGAGCGG$^{3'}$ R: $^{5'}$ TGATTCCCGAAGGCACTCCC$^{3'}$	*Aeromonas spp*	953 bp	ranging from 55 to 63	Lee et al., 2002

Table 2. PCR protocols employed in u-PCR and m-PCR for the concurrent detection of selected pathogens isolated from diseased fish

PCR assay type	Bacterial pathogen and target gene (s)		PCR Protocols			
			Initial denaturation	94 ºC for 3 min		
			Amplification (35 cycles of)			
			Denaturation	Annealing	Extension	Final extension
Uniplex	E. tarda	etfA	94 ºC 1 min	**59**ºC 1min	72ºC 1min	72ºC 5 min
	S. iniae	16SRNA	94 ºC 1 min	**55**ºC 1min	72ºC 1min	72ºC 5 min
	Aeromonas spp.	16S rRNA	94 ºC 1 min	**59**ºC 1min	72ºC 1min	72ºC 5 min
Multiplex	E. tarda S. iniae Aeromonas Spp.	etfA 16SRNA 16S rRNA	94 ºC 1min	**56**ºC 1min (63 to 56ºC)	72ºC 1min	72ºC 5 min

3. Results

In the present study, u-PCR was performed to equalize and establish the protocol for amplification of the target genes of the corresponding selected pathogens. After modification of annealing temperature, the generated results revealed that the selected oligonucleotide primer sets (Table 1) amplified the 16S rRNA, fimbrial gene type 1 etfA and 16S rRNA specific target genes of Aeromonas spp. (A. hydrophila), E. tarda and S. iniae with production of specific amplicons sizes of 953, 415 and 300 bp, respectively, (Fig. 1). The specificity of the m-PCR assay was evaluated by testing the three primer sets with the pure DNA templates extracted and direct bacterial cells ($3x10^2$ CFU/mL) of Aeromonas spp. (A. hydrophila), S. iniae, E. tarda. As a result, electrophoresis analysis of both DNA templates and bacterial cells amplified by the designed m-PCR assay produced a single specific fragment bands for each pathogen with predictable sizes of 953, 415 and 300bp, respectively, (Fig. 2). At all level tested, u-PCR and designated m-PCR assays neither produce non-specific amplification products of used samples nor false positive ones.

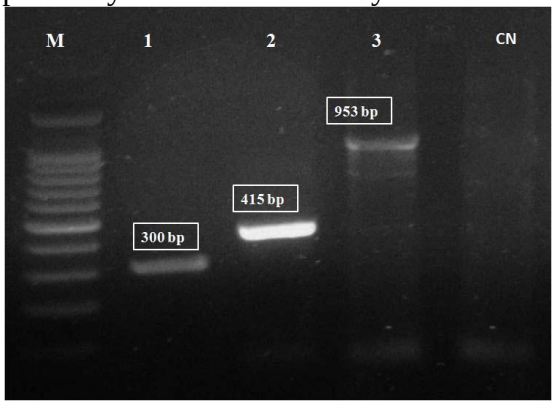

Fig. 1

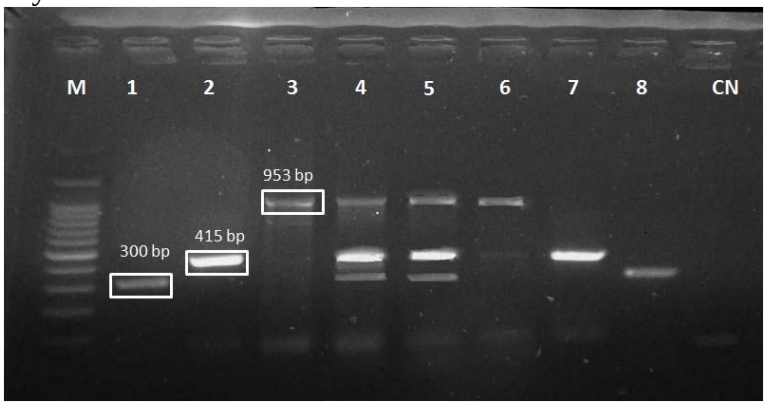

Fig. 2

Figure legends

Fig. 1. Uniplex PCR assay for detection of selected bacterial pathogens isolated from diseased cultured *O. niloticus* and *C. gariepinus.* Lane M, DNA marker 100 bp; lane 1, *S. iniae* 300 bp; lane 2, *E. tarda* 416 bp; lane 3, *A. hydrophila* 953 bp; CN, Control negative.

Fig. 2. Multiplex PCR (m-PCR) assay for the concurrent detection of *A. hydrophila*, *E. tarda* and *S. iniae*. Lane M, DNA marker 100 bp; Lanes 1&8, *S. iniae*, 300 bp; Lane 2&7, *E. tarda*, 415 bp; Lane 3&6, *A. hydrophila*, 953 bp; Lanes 4&5, multiplex for the three pathogen; Lanes 1,2,3&4 refer to extracted DNAs templates; Lanes 5,6,7&8 refer to bacterial cells templates.

4. Discussion

The current study addressed simultaneous detection of 3 major pathogens share in the BSS the most important bacterial infections causing losses in fish farm stocks and considered problematic in growth of tilapia and catfish production in Egypt. MAS caused by different species of motile Aeromonads, streptococcosis that caused mainly by S. iniae and edwardsiellosis caused by E. tarda could affect rearing of tilapia and catfish in different aquaculture systems, at any size and time during the production cycle, from nursery to marketable size (Jimenez el al. 2011; Amanu et al., 2017; Dong et al., 2017). Besides their incrimination in serious fish disease outbreaks, the three pathogens can also cause infections for other living creatures including humans (Keirstead et al., 2014; Abu-Elala et al., 2015; Suezawa et al., 2016). Ultimately, rapid, sensitive, accurate and reliable diagnosis method for these pathogens would be appreciated and highly desired for controlling their diseases outbreaks. Previous studies were concerned with the usefulness in phenotypic identification of bacterial species by conventional biochemical tests, commercial biochemical test kits and antimicrobial susceptibility profiles. However, they lack the resolution of molecular techniques in differentiating between largely similar, phenotypically ambiguous bacterial strains (Chou et al., 2014).

The u-PCR analysis within the present study highlighted the specificity of the used primer sets in detection of the target genes of A. hydrophila (Aeromonas spp.), E. tarda and S. iniae with production of specific amplicons sizes of 953, 415 and 300 bp, respectively, (Fig. 1). In addition, a new amplification protocols were elucidated according to our laboratory conditions. The established new protocols were illustrated in (Table 2). Meanwhile, the aforementioned amplification results obtained only after adapting the annealing temperatures of the primer sets of E. tarda and A. hydrophila, to be 59° C which was differ from that reported by (Sakai et al., 2007) and (Pinto et al., 2012), respectively, (Table 1). The alterations in annealing temperatures used in our u-PCR and those previously reported by aforementioned authors may probably due to the differences in laboratory and reaction conditions. Moreover, the carried out modification is in agreement with the fundamentals of molecular biology that clarified changing of annealing temperatures around the melting temperatures (Tm) of designated primer by 3 to 4º C formerly or latterly may enhance the amplification process (Sambrook and Russell, 2001). Although u-PCR validated the specificity of each pair of chosen specific primers, the assays lack to save the time and costs as well.

In this work, a m-PCR was developed for simultaneous detection of A. hydrophila (Aeromonas spp.), E. tarda and S. iniae using extracted DNA and the bacteria itself. The specificity of the developed m-PCR was confirmed by the fact that only specific fragments were amplified equivalent for 953, 415 and 300 bp which are characteristic for A. hydrophila (Aeromonas spp.), E. tarda and S. iniae, respectively, and that was evident with both extracted DNA and the bacterial cells (Fig. 2). More specifically, these specific bands were obtained also when either the extracted DNAs or the bacterial cells of the three pathogens mixed together in the reaction. These results are comparable with those of Pinto et al., (2012) for

Aeromonas spp. and Al-Harbi, (2011) for S.iniae and Castro et al., (2010) for E. tarda, they used the same primers for detection the three pathogen but by using u-PCR. Interestingly, the developed m-PCR within this study could detect the three pathogens as low as a concentration of $3x10^2$ CFU/mL indicating its specificity and sensitivity as well. Similarly, previous studies by Hussein & Hatai, (2006) reported efficient sensitive detection of S. iniae in bacterial mixture of Lactococcus garvieae and S. dysgalactiae at a concentration of $3x10^2$ CFU/mL by using m-PCR. One the other hand, Castro et al., (2010) noted that the minimal E. tarda can be detected in a mixed bacterial culture by PCR was 10^5 CFU/mL, this concentration is higher than that detected by our m-PCR.

In this study, A. hydrophila used as a model species for Aeromonads and could be detected by the developed m-PCR through amplification of the 16S RNA gene, however, this gene is genus-specific for the genus Aeromonas and almost represented in all Aeromonas species (Pinto et al., 2012). Thus, detection A. hydrophila with the developed m-PCR reflects its capability in detection of other Aeromonas species but only at genus level. In addition, presence of obvious characteristic clinical signs of BSS together with detection of Aeromonas spp. will be enough to applying the control and therapeutic measures. Zhang et al., (2014), developed m-PCR using genus-specific primers for differentiating between 4 genera of fish pathogenic bacteria including the genus Aeromonas. They confirmed that their m-PCR identified Aeromonas species involved in clinically diseased fish samples at genus-level with no amplification failure, while differentiation at species-level were successfully confirmed through biochemical tests together with characteristic clinical signs. Thus, it is also important to indicate that the identification of a pathogen alone doesn't demonstrate its involvement in the disease process (Xue et al., 2018). Therefore, additional information as clinical signs, and past history of infections in the fish farm are also needed to be considered into account.

From data generated within this study, it can be concluded that the proposed m-PCR assay can be useful as a diagnostic tool. Moreover, the developed m-PCR is an accurate, sensitive, fast and simple technique that can be accomplished within 4 hours for simultaneous detection of A. hydrophila (Aeromonas spp.), E.tarda, and S. iniae, three major bacterial pathogens involved in BSS incidence, particularly, in Egypt.

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