MOLECULAR IDENTIFICATION OF Aeromonas hydrophila STRAINS RECOVERED FROM KAFRELSHEIKH FISH FARMS

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Abstract: The aim of this study was to recover *Aeromonas hydrophila* from Kafrelsheikh tilapia (Oreochromis niloticus) farms and to study its virulence. Adult fish (n = 100) exhibiting the onset of clinical signs were bacteriologically examined using aeromonas isolation base medium. The isolates were identified by PCR using A. hydrophila-specific 16S rRNA primers and the virulence was determined using specific primers for ten virulence genes targeting hidrolipase (Lip), elastase (ahyB), lipase (pla/lip), aerolysin (aer), cytotonic enterotoxin (alt), cytotoxic enterotoxin (act), temperature sensitive protease (eprCAI), serine protease (Ahp), haemolysin (hlyA), and cytotonic heat stable enterotoxin (ast). Molecular screening revealed presence of 35 isolates positive for A. hydrophila-specific 16S rRNA and 4 virulence genes (aer, pla/lip, ast, and hlyA). It also showed that the majority of the examined strains carried one or more virulence genes. These data indicate higher virulence for A. hydrophila in infected O. niloticus in Kafrelsheikh fish farms.

Key words: PCR; A. hydrophila; virulence genes

Introduction

Fish provide cheap healthy protein source to human especially in coast countries, such as Egypt. Therefore, it is not strange to find a fast increase in aquaculture production sector in Egypt during the last two decades. However, this huge aquaculture resulted in elevation of prevalence, pathogenicity, and drug resistance of some bacteria strains. Aeromonas hydrophila, a motile opportunistic aeromonad that normally lives in fish gut, is one of the most common bacteria infected a large variety of fish (including marine and fresh water fish), especially those reared in aquaculture where they subjected to many stressors (inadequate PH, extreme temperature, hypoxia, malnutrition) mainly caused by overcrowding. These stressors depress fish immunity and allow this opportunistic aeromonad to invade fish causing hemorrhagic septicemia or motile aeromonas septicemia (MAS) characterized by high morbidity and mortality thereby leading to severe economic loss to fisheries (1). The typical symptoms of this disease include tail rot, hemorrhage, ulcer and scale desquamation and exophthalmia (2).

To achieve appropriate treatment for bacterial diseases, we should first accurately identify

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not only the bacterial species but also their strains and serotypes. Unlike other animals, there some limitations regarding diagnosis of bacterial pathogens in fish due to lack of rapid, sensitive and accurate means by which fish pathogens can be detected. Although, most of bacteria can be successfully isolated following their culture on specific media, this traditional method of diagnosis failed to accurately differentiate between different strains and serotypes (2, 3). In contrast, molecular based identification can accurately identify different bacterial strains and serotypes quickly, more specifically and in a more sensitive and reliable way. Polymerase chain reaction (PCR) was successfully applied to not only detect bacterial pathogens in fish but also differentiate between different bacterial strains and serotypes (3-7). Therefore, this study was designed to screen for prevalence of A. hydrophila virulent strains in Kafrelsheikh governorate O. niloticus farms.

Materials and methods

Sampling and bacteriological examination

Fish (n = 100) showing clinical signs of skin hemorrhages were aseptically streaked on tryptic soy agar and sub-cultured until obtaining pure colonies then tested on aeromonas isolation base medium to monitor the characteristic bull eye shaped colonies. All bacterial cultures were incubated at 28°C for 24 hours. The biochemical tests were done using commercially available API20NE (Biomerieux[©], France).

DNA extraction and PCR amplification

DNA extraction were done by thermolysis after culturing of bacterial strains on brain heart infusion broth and incubated at 37°C for 12 hours in shaker incubator. PCR amplification of

A. hydrophila specific-16S rRNA gene and virulent genes was performed in thermal cycler using 25μl mixture containing 0.5 μl DNA polymerase (5 U/ml), 2.5 μl 10 X PCR buffer, 2.5 μl dNTP mixture, 1 μl of each primer (20 pmol/μl, Table 1), 5 μl genomic DNA, and 12.5 μl DNAse free water. PCR conditions were done as previously described (4-7).

Results

Bacterial culturing revealed isolation of 35 isolates of A. hydrophila from 100 fish showing typical A. hydrophila clinical signs. The isolated bacterial colonies were creamy-white, circular and convex, and 2-3 mm in diameter on TSA agar plates and resembled bull eye shape with dark green center and light periphery on aeromonas isolation base medium (Fig. 1).

Molecular characterization confirmed detection of 35 isolates positive to 16S rRNA gene with a size of 103 bp (Fig. 2). Among the screened 10 virulence genes; only 4 genes (pla/lip, ast, aer and hlyA) were found in 21 out of 35 isolates and were distributed as follow: pla/lip, ast, aer and hlyA in 5 isolates; pla/lip, aer, and ast in 7 isolates; pla/lip and aer in 6 isolates and pla/lip in 3 isolates. Fourteen strains were free from any virulence genes. The prevalence of A. hydrophila virulence genes among the virulent strains were distributed as follow: 100% (21/21) for pla/lip, 57% (12/21) for ast, 86% (18/21) for aer and 24% (5/21) for hlyA (Tables 2 and 3; Figs. 3-6). Depending on the frequency of the virulence genes in positive A. hydrophila strains, the genotypes carrying virulence genes represent (60.7%), while the genotypes free from virulence genes represent (39.4%).

Table 1: Primers used for detection of *A. hydrophila* virulence genes

Gene	Primers	Size	References
	$5' \rightarrow 3'$	(bp)	
16S rDNA	F: GGCCTTGCGCGATTGTATAT	103	(7)
	R: GTGGCGGATCATCTTCTCAGA		
Hidrolipase (<i>Lip</i>)	:AACCTGGTTCCGCTCAAGCCGTT 65		(6)
	R:TTGCTCGCCTCGGCCCAGCAGCT		
Elastase (<i>ahyB</i>)	F: ACACGGTCAAGGAGATCAAC	540	
	R: CGCTGGTGTTGGCCAGCAGG		
Lipase (pla/lip)	F: ATCTTCTCCGACTGGTTCGG	383 - 389	
	R: CCGTGCCAGGACTGGGTCTT		
Aerolysin (aer)	F: CCTATGGCCTGAGCGAGAAG	431 - 1987	
	R: CCAGTTCCAGTCCCACCACT		
Cytotonic entero-	F: TGACCCAGTCCTGG	442	(5)
toxin (alt)	R: GGTGATCGATCACC		
Cytotoxic entero-	F: GAGAAGGTGACCACCAAGAACA	232	
toxin (act)	R: AACTGACATCGGCCTTGAACTC		
Temperature sensi-	F: GCTCGACGCCCAGCTCACC	387	
tive protease	R: GGCTCACCGCATTGGATTCG		
(eprCAI)			
Serine protease	F: ATTGGATCCCTGCCTA	911	
(Ahp)	R: GCTAAGCTTGCATCCG		
Haemolysin (hlyA)	F: GGCCGGTGGCCCGAAGATACGGG	392	
	R: GGCGGCGCCGGACGAGACGGGG		
Cytotonic heat stable	F: TCTCCATGCTTCCCTTCCACT	331	(4)
enterotoxin (ast)	R: GTGTAGGGATTGAAGAAGCCG		

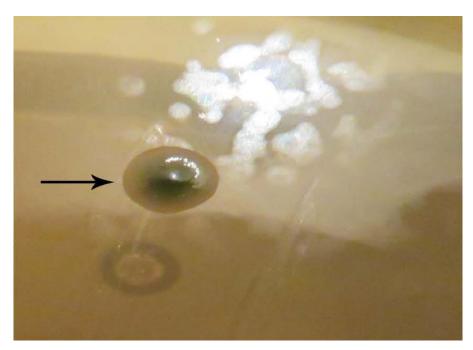


Figure 1: Characteristic bull eye shaped colony on aeromonas isolation base medium showing dark green convex center with light periphery

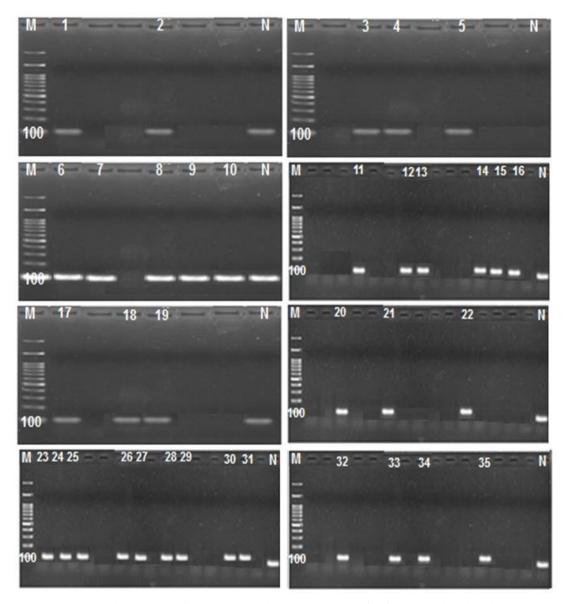


Figure 2: Electrophoretic pattern of PCR production bp specific for A. hydrophila in 2% agarose gel stained with ethidium bromide. Lane M: 100 bp DNA ma Lane N: negative control. Lane 1- 35: positive A. hydrophia

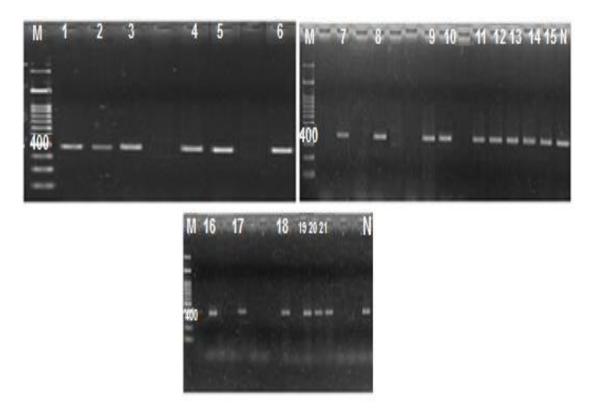


Figure 3: Ethidium bromide stained agarose gel of PCR products representing amplification of 383-389 bp amplicon of the *pla/lip* gene in *A. hydrophila*. Lane M: 100 bp DNA marker, Lane N: negative control, Lane 1-25: positive

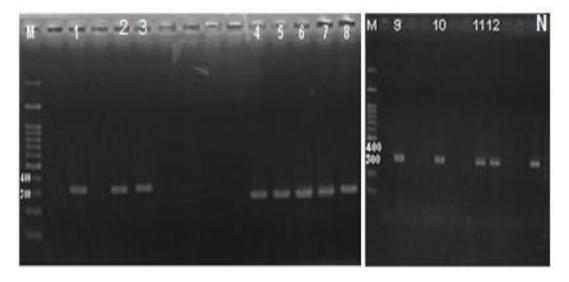


Figure 4: Ethidium bromide stained agarose gel of PCR products representing amplification of 328 bp amplicon of *ast* gene in *A. hydrophila*. Lane M: 100 bp DNA marker, Lane N: negative control, Lane 1-14: positive

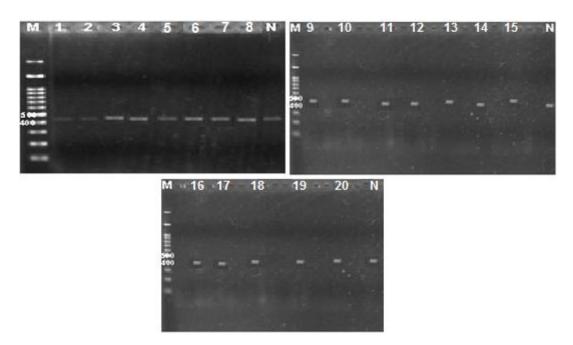


Figure 5: Ethidium bromide stained agarose gel of PCR products representing amplification of 431bp amplicon of *aer* gene in *A. hydrophila*. Lane M: 100 bp DNA marker, Lane N: negative control Lane 1-8: positive

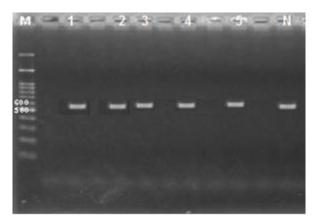


Figure 6: Ethidium bromide stained agarose gel of PCR products representing amplification of 592 bp amplicon of the haemolysin (*hlyA*) gene in *A. hydrophila* .Lane M: 100 bp DNA marker .Lane N: negative control, Lane 1-2: positive

Table 2: List of virulence genes present in the isolated *Aeromonas hydrophila* strains

Gene	Number of	Occurrence	Occurrence
	isolates	(%) in isolated	(%) in viru-
		strains	lent strains
pla/lip	21	60%	100%
ast	12	34%	57%
hlyA	5	14%	24%
aer	18	51%	86%

Gene	Number of	Occurrence	Occurrence
	isolates	(%) in isolated strains	(%) in
			virulent strains
pla/lip, ast, aer, hlyA	5	14%	24%
pla/lip, ast, aer	7	20%	33%
pla/lip, aer	6	17%	29%
pla/lip	3	10%	14%

Table 3: Occurrence and combination of virulence genes in *A. hydrophila* strains

Discussion

In developing coast countries, aquaculture is very important as a good source for animal protein suitable for human consumption (8, 9). This source was threatened by uncontrollable bacterial diseases especially for those caused by drug resistance bacteria and highly virulent bacteria such as *A. hydrophila* (10, 11).

PCR used in diagnosis of bacterial fish diseases, isolated from cultured fish, is a very rapid and accurate method (6, 12, 13). The molecular identification of DNA for 35 *A. hydrophila* isolates, using specific primers revealed the presence of common band at 103 bp. These results were similar to that reported by (7).

Virulence genes act as a key component in determining the potential pathogenicity of the micro-organism, acting multifunctionally and multifactorially and can be used for virulence typing of *A. hydrophila* isolates (2, 6, 14). Herein, we isolated 10 virulence genes from the positive 35 *A. hydrophila* isolates by PCR. The virulence genes in *Aeromonas hydrophila* isolates, were distributed into, fourteen isolates (40%), have no virulence genes while, twenty-one (60%) isolates have at least one or more virulence genes.

From the present work, it was evident that, the lipase (*pla/lip*) was the most frequent and important virulence gene. Lipase has the potential to change the histochemical identity of the cell membrane of the infected cells of target fish tissues thereby allowing *A. hydrophila* colonization which further induce cell necrosis (6, 15). The second more prevalent virulent gene was the aerolysin (*aer*) indicating high RBCs and cellular lysis for this bacteria (16). Presence

of aerolysin in pathogenic *A. hydrophila* infections may help in diagnosis, prevention and control of the disease spreading and mortalities in aquaculture (1, 17). On the other hand, the prevalence of the cytotonic heat stable enterotoxin (ast) was lower than aer but higher than hemolysin. The ast has the ability to increase the vascular permeability of the gut causing detachment of the intestinal mucosa (18). Hemolysin (hlyA) induced lytic activities on red blood cells, causing anemia (1). Haemolytic toxins; haemolysin and aerolysin released by *A. hydrophila* may be used as a marker of pathogenicity of *A. hydrophila* (1)

Conflict of interest

The authors declare that no conflict of interest.

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