


Original Article

Molecular characterization of *Aeromonas hydrophila* isolates from diseased fishes in district Kasur, Punjab, Pakistan

Caracterização molecular de isolados de *Aeromonas hydrophila* de peixes doentes no distrito de Kasur, Punjab, Paquistão

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Abstract

Pakistan is an agricultural country and fisheries play a very important role in the economic development of the country. Different diseases are prevalent in Pakistani fish but information related to the causative agents is not well-known. Keeping in view the significance of bacterial pathogens as the causative agents of multiple fish diseases, the present study was conducted for identification, characterization and analysis of virulence genes of *Aeromonas spp.* isolated from diseased fishes. A total of fifty fish samples having multiple clinical indications were collected from different fish farms of district Kasur, Punjab Pakistan. For isolation of *Aeromonas spp.* samples were enriched and inoculated on *Aeromonas* isolation medium. Isolates were identified and characterized by different biochemical tests, Analytical Profile Index (API) 20E kit and Polymerase Chain Reaction (PCR) assays. All isolates were screened for three putative virulence genes including aerolysin (aer), haemolysin (hyl) and heat labile cytotoxic enterotoxin (alt). Seven isolates of *Aeromonas (A.) hydrophila* were retrieved and identified based on API 20E. These isolates were further confirmed as *A. hydrophila* on the basis of PCR assays. Three isolates were detected positive for the presence of virulence genes (alt and hyl). Whereas aerolysin (aer) gene was not present in any of *A. hydrophila* isolates. The present study confirmed *A. hydrophila* as the causative agent of epizootic ulcerative syndrome and motile *Aeromonas* septicemia in fish farms of district Kasur, Punjab Pakistan. Moreover, detection of two virulence genes (alt and hyl) in *A. hydrophila* isolates is a threat for fish consumers of study area.

Keywords: *Aeromonas hydrophila*, EUS, MAS, virulence genes, fish.

Resumo

O Paquistão é um país agrícola, onde a pesca desempenha um papel muito importante para o desenvolvimento econômico. Diferentes doenças são prevalentes em peixes do Paquistão, mas as informações relacionadas aos agentes causadores não são bem conhecidas. Tendo em vista a importância dos patógenos bacterianos como agentes causadores de múltiplas doenças em peixes, o presente estudo foi conduzido para identificação, caracterização e análise de genes de virulência de isolados de *Aeromonas spp.* de peixes doentes. Foram coletadas 50 amostras de peixes com múltiplas indicações clínicas em diferentes fazendas do distrito de Kasur, Punjab, Paquistão. Para isolar *Aeromonas spp.*, as amostras foram enriquecidas e inoculadas em meio de isolamento. Os isolados foram identificados e caracterizados por diferentes testes bioquímicos, kit Analytical Profile Index (API) 20E, e ensaios de reação em cadeia da polimerase (PCR). Todos os isolados foram selecionados para três genes de virulência putativos, incluindo aerolisina (aer), hemolisina (hyl) e enterotoxina citotônica termolábil (alt). Sete isolados de *Aeromonas hydrophila* foram recuperados e identificados com base no API 20E. Esses isolados foram posteriormente confirmados como *A. hydrophila* de acordo com ensaios de PCR. Três isolados indicaram a presença de genes de virulência (alt e hyl), enquanto o gene aerolisina (aer) não esteve presente em nenhum dos isolados de *A. hydrophila*. O presente estudo confirmou *A. hydrophila* como o agente causador da síndrome ulcerativa epizootica e septicemia móvel por *Aeromonas* em fazendas de peixes, no distrito de Kasur, Punjab, Paquistão. Além disso, a detecção de dois genes de virulência (alt e hyl) em isolados de *A. hydrophila* é uma ameaça para os consumidores de peixes da área de estudo.

Palavras-chave: *Aeromonas hydrophila*, EUS (Síndrome Ulcerativa Epizootica), MAS (septicemia móvel por *Aeromonas*), genes de virulência, peixes.

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Received: July 30, 2021 – Accepted: September 23, 2021



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1. Introduction

As a result of population explosion, satisfying safe, sufficient and nutritious diet have become a global issue. To reduce pressure on food crops, a trend has been observed in utilization other foods such as Fish. Fish is not only a delicious and nutritious part of human diet but of animal feed as well. To meet the growing demand of fish and its products, the freshwater fish culture sector is developing using various modern techniques (Govind et al., 2012; Mert and Bulut, 2014). However, the fisheries sector is confronting numerous difficulties despite its fast development. Among these, substantial financial losses in terms of infections play an essential part. Freshwater fishes are spoiled with various infections of bacterial, viral, fungal and parasitic origins. One of the most critical fish illnesses are bacterial infections, responsible for huge loss in the form of fish mortality (Dias et al., 2012) such as dropsy, tuberculosis, gill spoil, tail decay, fin rot, scale oedema and furunculosis.

Motile *Aeromonas* septicaemia (MAS) and epizootic ulcerative syndrome (EUS) are two of the bacterial diseases of fish associated with considerable financial losses (Jagoda et al., 2014). Many members of *Aeromonas* genus are responsible for MAS (Viji et al., 2012) and EUS. However, EUS is most noticeably caused by *A. hydrophila*, *A. sorbia*, *A. veroni* and *A. caviae* (Oidtmann et al., 2008). While *A. hydrophila* is the most opportunistic, well-known pioneering pathogens of fish, which has been examined as the causative factor for motile *Aeromonas* septicaemia and ulcer disorder (Shao et al., 2004).

Aeromonas hydrophila is a gram-negative, motile and curved edged heterotrophic bacterium (bacilli to coccobacilli shape), which does not form endospores. Generally these are present in temperate climate zones and inhabit natural, estuarine, chlorinated, salt, marine, and un-chlorinated waters (Markov et al., 2007).

Aeromonas hydrophila integrates a range of strains or genotypes having pathogenic potential at one extreme and avirulence at the other conferring little damage to the host. Analysis of pathogenic system related with *A. hydrophila* has revealed harmful factors consolidating poisons which are actually heat labile cytotoxic enterotoxin (Alt), cytotoxic enterotoxin (Act), serine Protease (ahp) and Aerolysin (Aer). However little attention has been devoted towards the relative frequency of virulence genes in *Aeromonas* isolated from unhealthy fish and water bodies (Cascon et al., 2000; Singh et al., 2009). *Aeromonas* species have a tendency to transfer these genes to other microorganisms and environment and vice versa. The detection of these virulence genes in microbes inhabiting diverse hosts can help in risk assessment of cross species transmission and environmental monitoring for contaminating these genes (Roges et al., 2020). The virulent genes associated with pathogenic strains of *A. hydrophila* isolated from fish has been detected in Vietnam (aerA, act), Egypt (aerA, altA) and Tanzania (aer, hly, fla) (Kishk et al., 2020; Mzula et al., 2020; Nhin et al., 2021).

Around the world, information regarding characterizations, PCR based identification and detection virulence gene of bacterial pathogens have been thoroughly

explored, such as in USA, Egypt, India and Spain (Gonzalez-Serrano et al., 2002; Pridgeon and Klesius, 2011 and Sarkar et al., 2013). Unfortunately, Pakistan is standing as an exception to above mentioned countries, with just three reports describing the occurrence of epizootic ulcerative syndrome and haemorrhagic septicaemia in fresh water or dam water (Rab et al., 2001; Shahzad et al., 2014; Ali et al., 2016). Another concern in developing prevention and control strategies of fish diseases in Pakistan is insufficient data relevant to the causative agents of fish diseases. Only two studies provide information about *A. hydrophila* and *A. sorbia* as the causative agents of epizootic ulcerative syndrome and haemorrhagic septicaemia in farmed and wild fish of Pakistan (Shahzad et al., 2014; Ali et al., 2016). Keeping in view the significance of *Aeromonas* species as the causative agent of multiple fish diseases, this study aimed to characterize *Aeromonas* isolates and detection of virulence genes in *Aeromonas* species from disease fish in district Kasur Pakistan.

2. Materials and Methods

2.1. Study area

The present study was conducted in different fish farms of district Kasur, Punjab Pakistan. District Kasur lies from 75°585"E to 73.623"E and 31.363"N to 30.663"N. District Kasur is administratively subdivided into four tehsils i.e. Chunian, Kasur, Kot Radha Kishan and Pattoki (Figure 1). Both government and private sector fish farms are present in these tehsils. Commonly cultured fishes in these farms are rohu (*Labeo rohita*), grass carp (*Ctenopharyngodon idella*), gulfam (*Cyprinus carpio*), mori (*Cirrhinus mrigala*), silver carp (*Hypophthalmichthys molitrix*) and tilapia (*Oreochromis niloticus*). Two types of ponds (mud and cemented) have been observed in district Kasur region but mostly farms have mud ponds. The common water source is tube-well. Organic and inorganic manures are used for fertilization of fish ponds. There are evidences of problems associated with fish health and growth retardation in this area.

2.2. Sample collection and transportation

A total of fifty fish samples having disease symptoms were collected (December 2017 to March 2018) from fish farms of district Kasur, Punjab Pakistan. Ten samples from each fish species were collected including rahu (*Labeo rohita*), grass carp (*Ctenopharyngodon idella*), gulfam (*Cyprinus carpio*), mori (*Cirrhinus mrigala*) and tilapia (*Oreochromis niloticus*), all of which were infected with motile *Aeromonas* septicaemia and epizootic ulcerative syndrome. These samples were then kept into ice box after putting inside zip-lock bags and were transported to laboratory for further processing.

2.3. Isolation and primary characterization of *Aeromonas* isolates

Epidermal muscle, kidney and liver tissues were collected by sterilized forceps from dissected fish, transferred to tryptic soya broth (Himedia Laboratories Pvt.

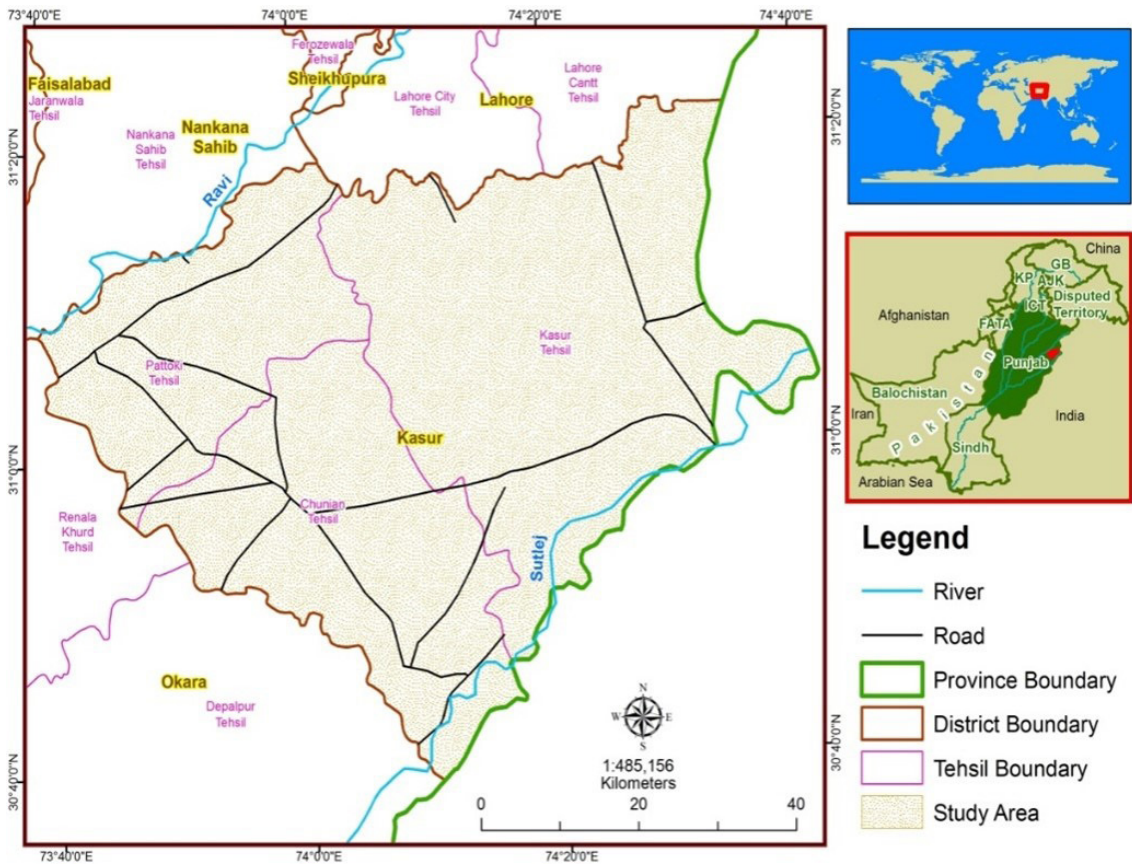


Figure 1. Study area map of district Kasur including tehsil Pattoki, Chunian, Kasur, and Kot Radha Kishan.

Ltd, Mumbai India) and incubated at 37°C for 24 hours for enrichment (Shahzad et al., 2014). After enrichment samples were inoculated on *Aeromonas* isolation medium (Himedia Laboratories Pvt. Ltd, Mumbai India) and incubated at 28 °C to 37 °C for 24 hours. Suspected *Aeromonas* colonies were selected on the basis of green colour and smooth shape, after that gram staining was done according to a reported procedure (Zhou et al., 2013). Morphologically observed colonies were re-inoculated on tryptic soya agar (Himedia Laboratories Pvt. Ltd, Mumbai India) for pure culture and then these isolates were preserved in 50% glycerol till further analysis.

2.4. Biochemical characterization

Initial biochemical identification of isolates was performed using oxidation, coagulation, catalase, voges-proskauer and indole production tests by standard method described by Bergey's manual of systematic bacteriological classification. Moreover, all pure isolates were confirmed by API 20E (Biomérieux, France). Briefly, sample suspension was prepared by dissolving fresh colonies in normal saline and turbidity was adjusted by using 0.5 McFarland standard. All the sections of strip were carefully inoculated according to the manufacturer instructions. To create anaerobic conditions after inoculation, some specific sections were covered with sterile mineral oil. The arranged strip was

enclosed with a lid and incubated for 1-2 days at 24 °C. After incubation, all reactions were logged (Nahar et al., 2016).

2.5. Extraction of DNA

DNA was extracted from pure culture using FavorPerp™ DNA extraction mini kit according to manufacturer instructions (Favorgen Biotech Crop, Taiwan). DNA was preserved at -20 °C for additional studies.

2.6. PCR based confirmation

Aeromonas genus (953 bp) and species specific (for *A. hydrophila* = 103 bp) 16S rRNA PCR assays were done as described in previous published literature (Lee et al., 2002; Trakhna et al., 2009). A 25µl reaction mixture contains 12 µl of 2x Amp Taq master mix (GeneAll cat# 541-005), 1 µl forward and reverse primer (Humanizing Genomics Microgen), 2.5 µl DNA template and 8.5 µl distilled water was used for PCR amplifications.

2.7. Detection of virulence genes

All isolates of *A. hydrophila* were screened against three virulent genes as previously described by Zhou et al. (2013) Table 1. Amplification was performed by initial denaturation at 94 °C for 5 minutes (min), followed by 30 cycles with denaturation at 94 °C for 30 seconds (s),

annealing at 60 °C for 30 s for the *aer* and *hyl* gene or for *aer* gene initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30s, annealing at 55 °C for 30s and extension at 72 °C for 1 min. A final extension step was carried out at 72 °C for 10 min.

2.8. Agarose gel electrophoresis

Visualization of positive amplicons was confirmed in 1.5% Agarose gel by electrophoresis (bioWORLD, bioPLUS Fine Research Chemicals Cat# 40100164-4) after being stained with ethidium bromide. The amplified DNA fragments of specific size were visualized by UV fluorescence and recorded by using a gel imager (BioRad Gel Doc EZ imager).

3. Results

3.1. Bacteriological observation and morphological characteristics of isolates

In the present study a total of seven *A. hydrophila* isolates were recovered from five different diseased fishes

Table 2. Among all seven isolates three (42.8%) were obtained from the kidney and liver of rohu (*Labeo rohita*) and tilapia (*Oreochromis niloticus*) and four (57.1%) were isolated from epidermal muscle of same (rohu (*Labeo rohita*), tilapia (*Oreochromis niloticus*) along with grass carp (*Ctenopharyngodon idella*) as well.

On the basis of conventional biochemical tests and API 20E, all isolates were characterized as *A. hydrophila*. Morphologically isolated colonies were opaque having a darker centre as compared to periphery of *Aeromonas* isolation medium and yellowish opaque, smooth edged, convex, round, and semi-translucent colonies on TSA plates Table 3.

3.2. Biochemical characterization by conventional method

The biochemical characteristics of the isolates performed by conventional methods are summarized in Table 4. All isolates were rod shaped, gram negative and were positive towards oxidase, coagulase, voges-proskauer, catalase and indole. Subsequently, isolates showed positive growth at 37 °C with the optimum time of 24 hours but no growth was observed at 28 °C and 30 °C.

Table 1. Primers used to detect the virulence genes in *Aeromonas hydrophila*.

Target genes	Primer Sequences (5'-3')	bp	References
Aerolysin (<i>aer</i>)	GCTGAACCCATCTATCTCTG TTTCTCCGGTAACAGGATTG	301	Zhou et al. (2013)
Haemolysin (<i>hyl</i>)	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGACGAGACGGG	600	
Cytotoxic enterotoxin (<i>alt</i>)	TGACCCAGTCTGGCACGGC GGTGATCGATCACCACCAGC	442	

Table 2. Detail of culture positive disease fish species and targeted organs.

Sample No.	Sample Type	Fish Species	Disease	<i>Aeromonas</i> Species
1 (a)	Kidney	<i>Labeo rohita</i>	EUS	<i>A. hydrophila</i>
1 (b)	Liver	<i>Labeo rohita</i>	EUS	<i>A. hydrophila</i>
2 (a)	Liver	<i>Oreochromis niloticus</i>	MAS	<i>A. hydrophila</i>
2 (b)	Epidermal muscle	<i>Oreochromis niloticus</i>	MAS	<i>A. hydrophila</i>
3	Epidermal muscle	<i>Ctenopharyngodon idella</i>	MAS	<i>A. hydrophila</i>
4	Epidermal muscle	<i>Labeo rohita</i>	MAS	<i>A. hydrophila</i>
5	Epidermal muscle	<i>Labeo rohita</i>	EUS	<i>A. hydrophila</i>

Table 3. Physiological characterization of *Aeromonas* isolated from diseased fish.

Test	Result	Number (%)
Colony Color on <i>Aeromonas</i> Isolation Medium	Dark Green, Opaque with Dark Centre	7(100)
Colony Color on TSA	Tan/buff-color	7(100)
Colony Shape	Smooth, Convex and Rounded	7(100)

3.3. Identification by API 20E Kit

Twenty tests were performed by using API 20E kit according to manufacturer’s instructions. All positive and negative results are summarized in Table 5. All isolates (n= 7) showed positive results towards ADH, AMY, LDC, IND, AP, GEL, GLU, ONPG, TDA, MAN, SAC, MEL, CIT and

ARA. However, negative reactions were shown for ODC, INO, RHA, SOR, H2S and URE.

3.4. PCR based confirmation and virulence gene detection

All seven isolates were positive for *Aeromonas* genus (953 bp) and species (*A. hydrophila* = 103 bp) (Figure 2).

Table 4. Biochemical characteristics of *A. hydrophila* isolates determined by conventional methods.

Characteristics	Response of <i>Aeromonas</i> isolates						
	A1	A2	A3	A4	A5	A6	A7
Gram Stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Shape				Rod			
Coagulase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Voges Proskauer	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+
Growth at different temperature	28 °C	-	-	-	-	-	-
	30 °C	-	-	-	-	-	-
	37 °C	+	+	+	+	+	+

F: Fermentative; (+): Positive response; (-): Negative response.

Table 5. Analytical profile index (API 20E) of isolates.

Test	Characteristics	Results (%)
API 20E	Beta-galactosidase (ONPG)	+(100)
	Arginine dihydrolase (ADH)	+(100)
	Lysine Decarboxylase (LDC)	+(100)
	Ornithine Decarboxylase (ODC)	-(100)
	Citrate utilisation (CIT)	+(100)
	H2S production (H2S)	-(100)
	Urease production (URE)	-(100)
	Tryptophane deaminase (TDA)	+(100)
	Indole production (IND)	+(100)
	Acetoin production (AP)	+(100)
	Gelatinase (GEL)	+(100)
	Glucose (GLU)	+(100)
	Mannitol (MAN)	+(100)
	Inositol (INO)	-(100)
	Sorbitol (SOR)	-(100)
	Rhamnose (RHA)	-(100)
	Sucrose (SAC)	+(100)
	Melibiose (MEL)	+(100)
	Amygdalin (AMY)	+(100)
	Arabinose (ARA)	+(100)

(+): Positive response; (-): Negative response

Table 6. Virulence genes detection of *Aeromonas hydrophila* isolated from diseased fish.

Sample no	<i>Aeromonas</i> genus	<i>Aeromonas hydrophila</i>	Haemolysin (hyl)	Aerolysin (aer)	Cytotoxic enterotoxin (alt)
A1	+	+	-	-	-
A2	+	+	-	-	-
A3	+	+	+	-	-
A4	+	+	+	-	+
A5	+	+	+	-	+
A6	+	+	-	-	-
A7	+	+	-	-	+

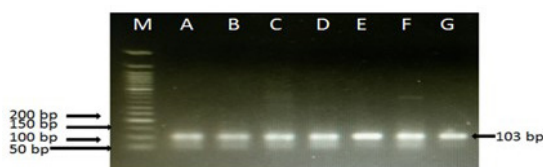


Figure 2. PCR based identification of *Aeromonas hydrophila*, Lane M: (left to right) 50 bp ladder marker (GeneOn), Lane A-G field isolates of *Aeromonas hydrophila* 16S rDNA (103 bp) primer amplification.

The distribution of virulence genes among all *A. hydrophila* isolates is summarized in Table 6. Virulence gene detection revealed that three out of seven (42.8%) isolates were positive for hyl and alt genes Figure 3 and 4. There was no positive isolate for aer gene.

4. Discussion

Despite the fact that the aquaculture sector is facing many challenges with respect to bacterial diseases all around the world and these diseases have a negative impact on the economic affluence of aquaculture fields; still very less determination is observed towards focusing on this aspect of the field (Elshehtawy et al., 2019).

In present study, different fish species were found infected with MAS and EUS. Which indicates that these species are equally susceptible for these bacterial diseases. Similar findings have been reported from Pakistan in previous studies (Rab et al., 2001; Ali et al., 2016). Our findings are in line with Algammal et al. (2020), who found high prevalence of MAS in different farmed fishes of Egypt. Similarly, from Malaysia, India, China, Serbia and Tanzania and Portugal, same results have been documented (Li et al., 2011; Dias et al., 2012; Sarkar et al., 2013; Laith and Najiah, 2014). This particular similarity is a clue of cosmopolitan distribution of these bacterial diseases.

In present study, *A. hydrophila* were obtained from kidney, liver and epidermal muscle of tilapia (*Oreochromis niloticus*), rohu (*Labeo rohita*) and grass carp (*Ctenopharyngodon idella*) during the study, which is also evident from results of previous study in which *Aeromonas spp.* was highlighted as one of the most common disease-causing agents in the aquatic ecosystem (Hassan et al.,

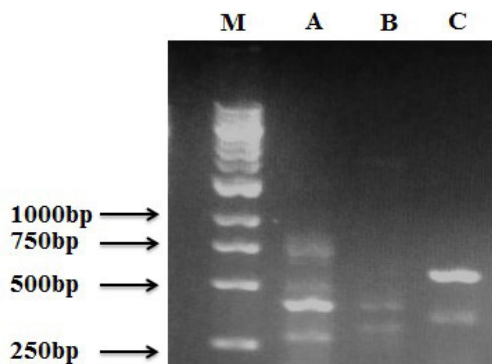


Figure 3. PCR based detection of haemolysin (hyl) gene, Lane M: (left to right) 1kb ladder (GeneOn) Lane C has amplicon (600 bp) of haemolysin (hyl) gene.

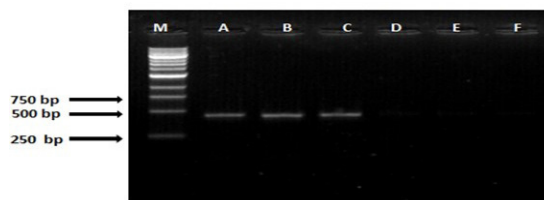


Figure 4. PCR based detection of heat labile cytotoxic enterotoxin (alt) gene, Lane M: (left to right) 1kb ladder (GeneOn), Lane A, B and C has amplicons (442 bp) of heat labile cytotoxic enterotoxin (alt) gene.

2017). In a comparable study *A. hydrophila* was isolated from different diseased wild and farmed fishes in Potohar region Pakistan (Ali et al., 2016).

In another study conducted in Egypt *A. Veronii* was detected from *Oreochromis niloticus* that is an important pathogen for bacterial fish diseases which has been isolated from different organs i.e. liver, spleens, kidney and brain (El Latif et al., 2019). Similar results were also reported from tilapia in Thailand and Vietnam (Peepim et al., 2016; Dong et al., 2017). On the other hand, 33/250 (13.2%) tilapia (*Oreochromis niloticus*) was contaminated with *A. hydrophila* from El-Sharkia Governorate, Egypt. The highest prevalence was recorded in the liver (54.10%) other than all

infected organs (Algammal et al., 2020). Moreover, eleven strains of *A. hydrophila* and three strains of *A. veronii* were isolated from freshwater fish species from a study too place in Ponferrade Leon, Spain (Gonzalez-Serrano et al., 2002).

The colonies of *A. hydrophila* were green, opaque with dark center on *Aeromonas* isolation medium while on TSA medium the isolated colonies color Tan/buff with smooth surface. All isolates were gram negative, rod shaped and pink in color. These results are correlated with the previous studies such as of Dong et al. (2017) and El Latif et al. (2019), who isolated *Aeromonas* species from tilapia with the same morphological and biochemical characteristics as aeromonads. Current study numeric outcomes are also in agreement with previous study of Mansour et al. (2019), in which they isolated *A. hydrophila* from diseased tilapia (*Oreochromis niloticus*) in Egypt and reported the same morphological and biochemical characteristics (Wanja et al., 2019).

API-20E kit was used for confirmation of *A. hydrophila*, which ultimately showed that isolated bacteria had biochemical characters resembling with *A. hydrophila*. Similarly, studies carried out in other parts of world such as in Mymensingh, Bangladesh (Nahar et al., 2016) for identification of isolates of *A. hydrophila* from juvenile farmed *Pangasius (Pangasianodon hypophthalmus)* by using same kit claimed ten isolated bacteria to be positive towards ADH, AMY, LDC, IND, AP, GEL, GLU, ONPG, TDA, MAN, SAC, MEL, CIT and ARA. Negative reactions were shown for ODC, INO, RHA, SOR, H₂S and URE. I. Our results are also coherent with the findings of Dong et al. (2017), who isolated *Aeromonas* from Nile tilapia from Pathum Thani Province, Thailand.

Biochemically identified isolates were further confirmed by genus (*Aeromonas*) and species (*A. hydrophila*) level using 16S rRNA approach by PCR assays. All recovered isolates were positive for genus *Aeromonas* (953 bp). Our results are in accordance with that documented in article by Aboyadak et al. (2015) who observed *Aeromonas* having 953 bp isolated from tilapia in Egypt. Same results were detected by Matter et al. (2018) at Kafr El-Sheikh, Egypt who detect the *Aeromonas* from freshwater fish species (*O. niloticus*, *C. Gariepinus* and *M. cephalus*). All genus amplified isolates were positive for *A. hydrophila* having amplicon size of 103 bp. Our results are similar with those obtained by Aboyadak et al. (2015) who confirmed 12 isolates of *A. hydrophila* at the same band size (103 bp) of DNA fragments isolated from Tilapia at Kafr El-Sheikh north of Egypt. A similar finding was observed in another study that isolated *A. hydrophila* (103 bp) from fish water samples in France, Tunisia and Lebanon the (Trakhna et al., 2009).

In our study, it is evident that the prevalence of the alt and hyl gene is the most common virulence gene of *A. hydrophila*. Similar studies have been conducted in other countries such as Canada, China, India, Malaysia and Spain (Wang et al., 2003; Pridgeon and Klesius, 2011; Zhou et al., 2013). In addition, three strains of *A. hydrophila* had alt and hyl having amplicon size 600 and 442 bp. These figures can be correlated with that of Algammal et al. (2020), who reported alt genes isolation from infected tilapia (*Oreochromis niloticus*). Some recent published data such

as in case of Hossain et al. (2020), reported 80.8% hyl and 48.1% alt gene, which is a fair source of evidence to support our results. On the other side Mansour et al. (2019) found 5/21 hyl genes from diseased fish of Kafr El-Shaikh Egypt. However, a previous study conducted in Spain showed that eleven strains of *A. hydrophila* isolated from freshwater fish were positive for hyl gene (Gonzalez-Serrano et al., 2002).

In contrast, our result revealed that none of *A. hydrophila* strains had harboured aer gene. These results were in disagreement with Eid et al. (2019), who concluded that aer was the most dominant gene in diseases fish with 100% prevalence. 73.1% of *A. hydrophila* isolates from diseased ornamental guppy (*Poecilia reticulata*) were reported with aer gene from Seoul, Korea Hossain et al. (2020).

In conclusion our study indicated that *A. hydrophila* and its virulence genes have a considerable role in imparting diseases to fish. Screening of specific heat labile cytotoxic enterotoxin (alt) and haemolysin (hyl) genes seems to be the most effective way for detecting virulence properties of *A. hydrophila*. It is necessary that fish farms should be monitored regularly to identify *Aeromonas* at broad spectrum for identification of pathogens and management of disease outbreaks in aquaculture. API 20E kit and polymerase chain reaction are the best possible conventional approaches for a further detection and assessment of *Aeromonas* species in the food, environment and to prevent human health risk.

Acknowledgements

This study was supported by the Higher Education Commission (HEC), Pakistan funded project. We also admit the Deanship of Scientific Research at Najran University, Najran, Saudi Arabia for financial support to publish this research work in BJB successfully.

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