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Molecular detection and genetic diversity of virulent *Aeromonas hydrophila* (Chester, 1901) Stanier, 1943 isolates from raw and processed fish

M Shaikh[a], Anjay*[,a], P Kaushik[a], A Kumar[b], R Kumar[a] & Sanjana[c]

[a]Department of Veterinary Public Health & Epidemiology, Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar – 800 014, India

[b]Department of Veterinary Biochemistry, Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar – 800 014, India

[c]Department of Veterinary Microbiology, Bihar Veterinary College, Bihar Animal Sciences University, Patna, Bihar – 800 014, India

*[E-mail: dranjayvet@gmail.com]

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Aeromonas hydrophila is a mesophilic motile aeromonad present ubiquitously and causes a high number of fish mortalities particularly in India along with various intestinal and extraintestinal diseases in humans. In this study, a total of 88 samples of fish and their processed products were screened by PCR targeting species-specific 16S rRNA gene, and the virulent genes such as *aer*A, *hly* and *ahh*1. The findings of the study showed that a total of 36.36 % of samples harboured *A. hydrophila* of which 51.61 %, 71.87 % and 78.12 % of isolates possessed virulent genes such as *aer*A, *hly*, and *ahh*1, respectively. The phylogenetic analysis of the isolates using AP-PCR assay suggested that five different clusters of organisms were prevalent among *A. hydrophila* with a high degree (> 95.00 %) of lineage. In conclusion, it may be presumed that raw and processed fish harboured the pathogenic *A. hydrophila* which may serve as a reservoir/ source of infection to human beings.

[**Keywords**: *Aeromonas hydrophila*, AP-PCR, Fish, Virulence marker]

Introduction

The Genus *Aeromonas* are gram-negative, facultatively anaerobic, non-spore forming, rod-shaped bacteria belonging to the family *Aeromonadaceae*[1]. *A. hydrophila* is a mesophilic motile aeromonad present ubiquitously and isolated from different aquatic environments and different food and food products of animal origin especially, fishes and seafood, raw and cooked meat, chicken, milk and milk products as well as vegetables which may act as a potential vehicle for human infections[2].

In humans, the motile aeromonads are responsible for various intestinal and extraintestinal diseases such as gastroenteritis, chronic diarrhoea, wound infections, respiratory tract infections, peritonitis, urinary tract infections and septicemia[3]. Among motile aeromonads, *A. hydrophila*, *A. caviae* and *A. veronii* are reported as predominant species for human infection of which *A. hydrophila* has been reported from many countries of the world including India[4]. The presence of this pathogen in foods of animal origin, fish and water, and its isolation from human infections showed its zoonotic perspective[5].

The traditional methods for the identification of bacteria are isolation and biochemical characterization. However, molecular methods especially polymerase chain reaction has been proven to be a more accurate and rapid method for identification. The detection of conserved 16S rRNA gene is considered as an established method that contributes to signature sequencing for molecular identification of *Aeromonas* species[6].

The mesophilic aeromonads comprise a series of virulence factors[7] that imparts in the pathogenicity. The different virulence factors may include aerolysin-related cytotoxic enterotoxin (*Act*), heat-labile cytotonic enterotoxin (*Alt*), heat-stable cytotonic toxins (*Ast*), hemolysin (*hly*A) and aerolysin (*aer*A). In addition to these, type III secretion system, polar flagellum (*fla*), lateral flagella (*laf*), elastase (*Ela*) and lipase (*Lip*) have also been reported to contribute in the pathogenicity of *Aeromonas*[8].

The various methods like Random Amplified Polymorphic DNA-PCR (RAPD), Repetitive Extragenic Palindromic Sequence PCR (REP-PCR), and Enterobacterial Repetitive Intergenic Consensus

sequence PCR (ERIC-PCR) PCR are reported for fingerprinting of bacterial isolates[9]. However, very few epidemiological investigations have been performed to establish a clonal linkage between *A. hydrophila* isolates.

This study is aimed to investigate the distribution of *A. hydrophila* in raw and processed fish samples collected from retail markets in the Patna Municipal Corporation, Bihar, India. Moreover, the presence of some virulence factors was also determined by genotypic methods along with the genetic relatedness of the isolates.

Materials and Methods

The present study was performed for isolation and identification of *A. hydrophila* by molecular means from the raw (gills and flesh) fishes including Rohu (*Labeo rohita*), Catla (*Labeo catla*), Bachwa (*Etropiichthys vacha*) and Tengra (*Mystus tengara*) as well as processed fish (curry) collected from the Patna Municipal Corporation market and the isolates were analyzed for the detection of virulence markers along with phylogenetic analysis.

Samples

A total of 88 samples of 60 raw fish (gills and flesh) and processed fish (curry) (approximately 100 g) were collected in a sterile sample collection bag, to check further environmental contamination of the samples. The samples were placed in the transportation box provided with an ice pack and transported to the laboratory within an hour of collection. Samples were then processed in the laboratory within an hour of collection for isolation and identification of *A. hydrophila*.

Isolation and identification of *A. hydrophila*

Approximately 1 g of samples were homogenized in 10 ml of sterile Alkaline Peptone Water (APW) broth with the help of tissue homogenizer and inoculated for 24 h at 37 °C[10]. A loopful of APW broth grown culture showing turbidity was streaked on the Ampicillin Dextrin Agar (ADA) and incubated at 37 °C for 24 hrs. The characteristic colonies of *A. hydrophila* on ADA *i.e*., typical round, 2 – 3 mm in diameter with a yellow-coloured honey drop like were selected for molecular confirmation.

Molecular identification of *A. hydrophila*

The extraction of genomic DNA was done by Blood and tissue kit (Qiagen, Germany) following the manufacturer's recommendations. PCR assay for identification of *A. hydrophila* was optimized targeting species-specific 16S rRNA gene primer pair[11]. The PCR reaction was prepared as 25 µl reaction mixture to contain 2.5 µl of 10X PCR buffer, 2.5 µl of dNTP (2.5 mM each), 2.0 µl (10 pmol) of forward and reverse primers of 16S rRNA gene, 0.2 µl (1 Unit) *Taq* DNA polymerase, 2 µl of DNA and sterile NFW up to 25 µl.

The cyclic conditions of PCR were optimized at initial denaturation of 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing temperature at 51.5 °C for 30 sec and extension at 72 °C for 30 sec. The final extension was performed at 72 °C for 8 min. The presumptive isolates that showed a specific amplicon size of 103 bp were considered as *A. hydrophila*. The amplicon size was measured with 50 bp DNA molecular weight (Mw) marker (thermo-scientific, USA) in submarine gel electrophoresis containing 1.5 % agarose gel. The agarose gel was visualized under the gel documentation system (Bio-Rad, USA) after staining with ethidium bromide (0.5 µg/ml) and images were stored for analysis.

Molecular identification of aerolysin, hemolysin, and extracellular hemolysin virulence markers

All *A. hydrophila* isolates confirmed by molecular methods were further screened for the presence of virulence markers like Aerolysin (*aer*A gene), hemolysin (*hly* gene) and extracellular hemolysin (*ahh*1 gene) using primer pairs described previously[12,13]. Preparation of PCR reaction mixture and cyclic conditions for amplification of all genes were similar to 16S rRNA PCR except annealing temperature. The annealing temperatures for amplification of *aer*A, *hly*, and *ahh*1 genes were optimized using gradient PCR as 48.1 °C, 62.6 °C, and 59 °C for 30 sec, respectively.

Arbitrarily primed PCR for phylogenetic analysis

Arbitrarily Primed (AP) PCR was performed using an arbitrary primer A1 (5'ACG CGC CCT 3') as per the methods described previously[14]. The reaction mixture (25 µl) was prepared containing 2.5 µl of 10X PCR buffer, 1 µl of dNTP mixture, 2.5 µl of $MgCl_2$, 0.25 µl (20 pmol) of primer, 1-unit *Taq* DNA polymerase and 2.0 µl of genomic DNA (~20 – 40 ng). The PCR cycle conditions were optimized as initial denaturation at 94 °C for 5 min, followed by a middle step of 45 cycles at 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min and a final extension of 72 °C for 7 min. The amplified PCR products were electrophoresed in a 1.5 % agarose gel (Himedia, India), at 80 mV for 85 min with one (1) Kb and one (1) kb plus DNA Mw marker (MBI, Fermentas). The ethidium bromide (0.5 µg/ml) stained electrophoresed

PCR products were visualized and documented under the Gel Documentation system. The DICE coefficient was used to estimate the genetic similarity of *A. hydrophila* isolates and a dendrogram was obtained by the unweighted pair group method with arithmetic mean (UPGMA) with a Bootstrap of 100, using the program PyElph1.4 (Pavel and Vasile)[15]. The discrimination power of the assay was determined with an online discrimination power calculator by adding the number of isolates found within a cluster at 95 % similarity index.

Results

A total of 88 samples including raw fish (n = 60) and processed fish (n = 28) were enriched and selectively plated for isolation of *A. hydrophila.* Among all processed samples 70.45 % (62) samples were having organisms that showed characteristic colonies of *A. hydrophila i.e*., typical round, 2 – 3 mm in diameter with a yellow-colored honey drop like on ADA. 86.67 % (52) and 35.71 % (10) of these presumptive organisms belonged to raw and processed fish. Molecular amplification of the isolate's DNA targeting species specific 16S rRNA gene revealed that only 32 isolates were *A. hydrophila* with a distribution of 30 and 02 in raw and processed fish, respectively.

The PCR amplification of virulence genes among *A. hydrophila* isolates showed that 51.61 % (32/32), 71.87 % (23/32) and 78.12 % (25/32) of isolates were encoded with *aer*A, *hly*, and *ahh*1, respectively (Fig. 1).

The *aer*A gene was found to be distributed among 60 % (18/30) and 50 % (01/02) isolates of raw and processed fish samples, respectively. However, 70 % (21/30) and 100 % (02/02) isolates of raw and processed fish samples, respectively were found to harbour the *hly* gene while 76.66 % (23/30) and 100 % (02/02) for *ahh*1 gene. The virulent genes (*aer*A, *hly*, and *ahh*1) distribution study showed that seven different virulent genotype profiles named G1 to G7 were present in the pathogenic *A. hydrophila* prevalent in this area. Among virulent *A. hydrophila*, 9.38 % (03/32) isolates were encoded with only *aer*A gene (G1), 3.18 % (01/32) with *hly* (G2) and 6.25 % (02/32) with *ahh*1 (G3). Further, 9.38 % (03/32) isolates were found as *aer*A^{+}, *hly*$^{+}$ & *ahh*$^{-}$ (G4); 15.63 % (5/32) as *aer*A^{+}, *ahh*1^{+} & *hly*$^{-}$ (G5); 28.23 % (9/32) as *hly*$^{+}$, *ahh*1^{+} & *aer*A^{-} (G6) and 28.23 % (9/32) as *aer*A^{+}, *hly*$^{+}$ and *ahh*1^{+} (G7).

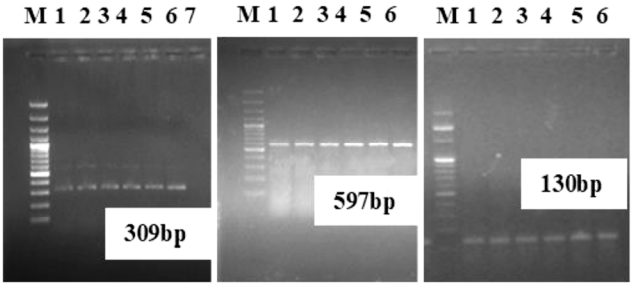

Fig. 1 — PCR detection of *A. hydrophila virulence markers*. M: 100 bp DNA marker; L1-6: Positive amplicon of 309 bp (*aer*A gene), 597 bp (*hly* gene) and 130 bp (*Ahh*1 gene)

AP-PCR amplification using an arbitrary primer A1 (5'ACG CGC CCT 3') of all 32 isolates showed 1 to 11 bands of molecular weight ranging from 300 – 10,000 bp. The banding pattern observed with UPGMA analysis revealed that 5 major clusters naming A to E were formed, illustrating genetic relationships among the isolates. The most frequent clusters were A (56.25 %), B (28.12 %), C (6.25 %), and Clusters E (6.25 %) while Cluster D was formed by only one isolate. The discrimination power of this assay was calculated as 0.6149 (Fig. 2).

Discussion

A. hydrophila is a mesophilic motile aeromonad present ubiquitously and isolated from different aquatic environments and may act as a potential vehicle for human infections. In the present study, it was found that about 36 % of samples including fish (50 %) and fish product (7.14 %) were positive for *A. hydrophila*. The finding of the present study was in concordance with the finding of Thayumanavan *et al*.[16] who reported that 37.3 % of finfish and 35.6 % of prawn samples from coastal South India were contaminated with *A. hydrophila.* Further, the variable rate of distribution of *A. hydrophila* was also reported by different workers from different types of fish samples. The distribution was reported as 40 % in fish samples of the local market, Chennai, Tamil Nadu, India[17], and European fish[18], 22.60 % in freshwater fish[19], and 11.5 % in market fish samples[20]. Different studies were performed in various countries at distinct times showing a variable rate of 19 to 90 % distribution of *A. hydrophila* as from UK, New Zealand, Switzerland, and Taiwan. The finding of the distribution of *A. hydrophila* among processed fish (~7 %) was justifiable with the finding of Abd-El-Malek[21], who reported the presence of 20 % *A. hydrophila* in ready-to-eat fish commonly consumed in Assiut city, Egypt.

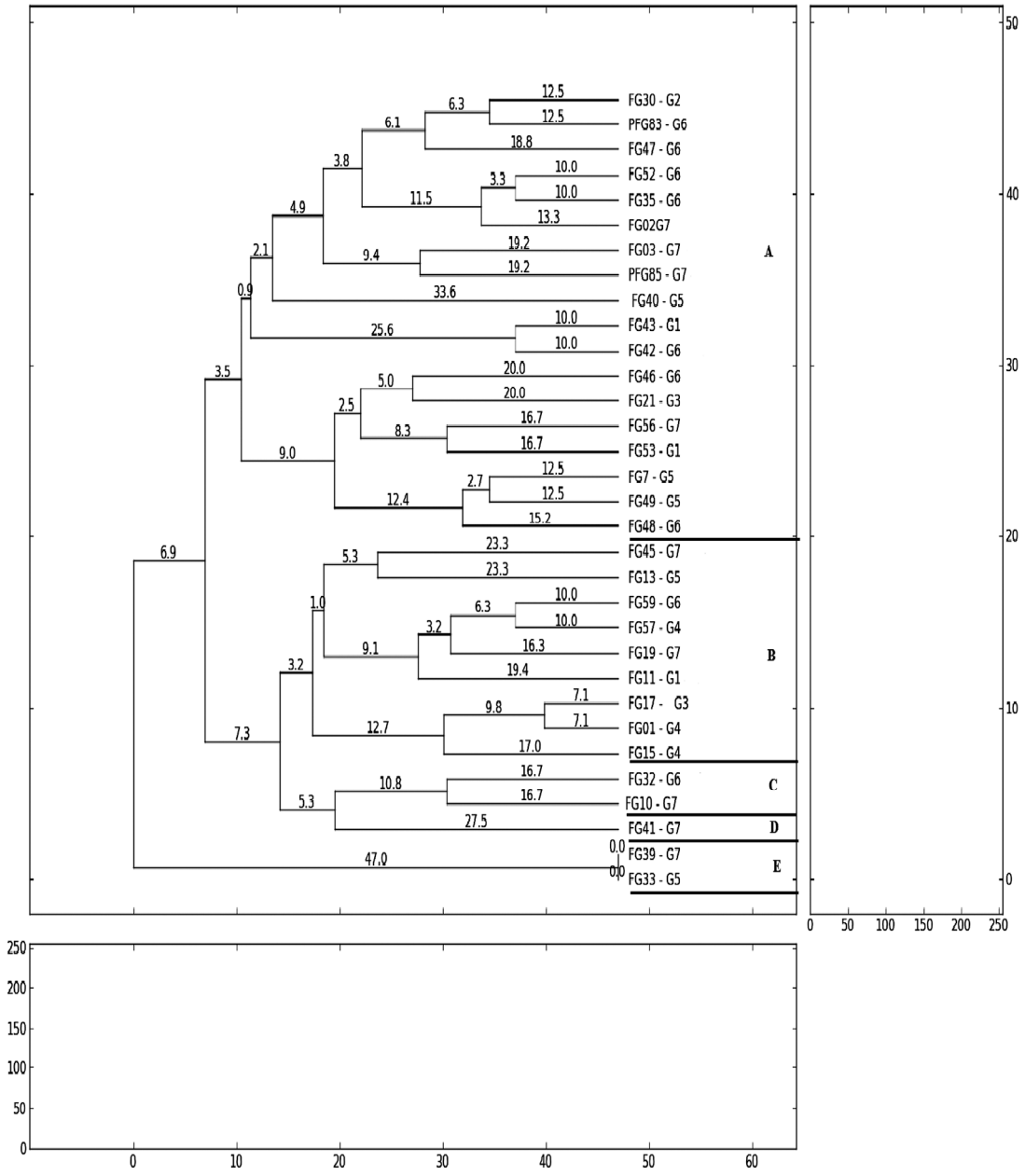

Fig. 2 — Dendrogram showing genetic relatedness of 32 isolates of *Aeromonas hydrophila* determined by analysis of AP-PCR fingerprint patterns using UPGMA cluster method

The *A. hydrophila* isolated in this study showed that about 52 %, 72 %, and 78 % isolates harboured the virulent genes *aer*A, *hly*, and *ahh*1, respectively with seven genotyping profiles. The isolates were also found to be encoded with either of any possible combinations of the screened virulence markers. Similarly, Singh *et al*.[22] reported that 85 % of *A. hydrophila* isolated from fish and pond water were encoded with aerolysin gene while 70 % with *hly* gene. A higher occurrence of 88.88 % of *A. hydrophila* from fresh marine fish were encoded with *aer*A and *hly* gene[23] while 20 % with the aerolysin gene[24]. In contrast to these studies, Pinto *et al*.[25] could not detect *hly*A and *aer*A genes in *Aeromonas* spp. from ready-to-eat seafood products. The finding of such virulent genes highlighted a complex profile belonging to 07 different genotyping profiles. These findings provide a shred of evidence

that different combinations of the virulence genes in *A. hydrophila* isolates indicated their probable role in the pathogenesis of *Aeromonas* infections and represent a risk to human health. From present finding it can be concluded that the presence of virulent *A. hydrophila* encoding *aer*A, *hly*, and *ahh*1 among fish samples including cooked fish available for human consumption represents a risk to the consumer's health. The genetic linkage of *A. hydrophila* isolates of fish and fish product isolates suggested that five different clones with 95 % genetic similarity are circulating in the fish population of which two dominating clones constituted by a major population of 56.25 % and 28.12 % *A. hydrophila*. Further *A. hydrophila* (02) isolated from fish products also belonged (to) one of the largest clones which may be due to post-cooking contamination.

Conclusion

From the finding of the present study, it may be concluded that raw and processed fish harbored the virulent strains of *A. hydrophila* which may serve as a source of human's infection by entering into the food chain.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Statement

This manuscript does not contain any experimental studies performed using live animals.

Author Contributions

SM standardized and performed the designed work; AK, RK & S assisted SM to perform the laboratory work. Anjay and PK conceived and designed the work; Anjay analyzed and interpreted the data and wrote the paper.

References

1 Zhou Y, Yu L, Nan Z, Zhang P, Kan B, *et al*., Taxonomy, virulence genes and antimicrobial resistance of *Aeromonas* isolated from extra-intestinal and intestinal infections, *BMC Infect Dis*, 19 (2019) p. 158.

2 Ghenghesh K S, Ahmed S F, El-Khalek R A, Al-Gendy A & Klena J, *Aeromonas* infections in developing countries, *J Infect Dev Count*, 2 (2008) 81-98.

3 Salunke G, Namshikar V, Gaonkar R & Gaonkar T, A case of *Aeromonas hydrophila* meningitis in septic shock, *Trop J Med Res*, 18 (2015) 54-54.

4 Pal M, Is *Aeromonas hydrophila* a potential pathogen of food safety concern? *J Food Microbiol*, 2 (2018) 1-2.

5 Igbinosa I H, Igumbor E U, Aghdasi F, Tom M & Okoh A I, Emerging *Aeromonas* species infections and their significance in public health, *Scient World J*, (2012) p. 625023.

6 Ahmed H A, Mohamed E M M, Maha M R, Rasha M A G & Shehata A A, *Aeromonas hydrophila* in fish and humans; prevalence, virulotyping and antimicrobial resistance, *Slov Vet Res*, 55 (2018) 113-124.

7 Tomas J M, The main *Aeromonas* pathogenic factors, *ISRN Microbiol*, 25 (2012) p. 6261.

8 Robertson B K, Harden C, Selvaraju S B, Pradhan S & Yadav J S, Molecular detection, quantification, and toxigenicity profiling of *Aeromonas* spp. in source-and drinking-water, *The Open Microbiol J*, 8 (2014) p. 32.

9 Anjay K A, Agarwal R K, Ramees T P, Dubal Z B, Kaushik P, *et al.*, Bi-molecular typing of *Salmonella typhimurium* and *S. enteritidis serovars* from diverse origin by ERIC-PCR, *J Pure Appl Microbiol*, 9 (2015) 2627-2634.

10 Ramadan H, Ibrahim N, Samir M, Abd El-Moaty A & Gad T, *Aeromonas hydrophila* from marketed mullet (*Mugil cephalus*) in Egypt: PCR characterization of β-lactam resistance and virulence genes, *J Appl Microbiol*, 124 (6) (2018) 1629-1637.

11 Trakhna F, Harf-Monteil C, Abdelnour A, Maaroufi A & Gadonna-Widehem P, Rapid *Aeromonas hydrophila* identification by TaqMan PCR assay: comparison with a phenotypic method, *Lett Appl Microbiol*, 49 (2009) 186-190.

12 Wang G, Clark C G, Liu C, Pucknell C, Munro C K, *et al*., Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR, *J Clin Microbiol*, 41 (2003) 1048-1054.

13 Zhou Q L, Wang Y J, Xie J, Ge X P, Xi B W, *et al.*, Distribution and virulence gene comparison of *Aeromonas* strains isolated from diseased fish and water environment, *Pol J Microbiol*, 62 (2013) 299-302.

14 Barrangou R, Yoon S S, Breidt J F, Fleming H P & Klaenhammer T R, Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation, *Appl Environ Microbiol*, 68 (2002) 2877-2884.

15 Pavel A B & Vasile C I, PyElph-a software tool for gel images analysis and phylogenetics, *BMC Bioinfor*, 13 (2012) p. 9.

16 Thayumanavan T, Vivekanandhan G, Savithamani K, Subashkumar R & Lakshmanaperumalsamy P, Incidence of haemolysin-positive and drug-resistant *Aeromonas hydrophila* in freshly caught finfish and prawn collected from major commercial fishes of coastal South India, *FEMS Immunol Med Microbiol*, 36 (2003) 41-45.

17 Yogananth N, Bhakyaraj R, Chanthuru A, Anbalagan T & Nila K M, Detection of virulence gene in *Aeromonas hydrophila* isolated from fish samples using PCR technique, *GJBB*, 4 (2009) 51-53.

18 Davies A R, Cappel C H, Jahanno D, Nychas G J E & Kirby R M, Incidence of foodborne pathogens on European fish, *Food Cont*, 12 (2001) 67–71.

19 Yucel N, Aslim B & Beyatli Y, Prevalence and resistance to antibiotics for *Aeromonas* species isolated from retail fish in Turkey, *J Food Qual*, 28 (2005) 313-324.

20 Radu S, Ahmad N, Ling F H & Reezal A, Prevalence and resistance to antibiotics for *Aeromonas* species from retail fish in Malaysia, *Int J Food Microbiol*, 81 (2003) 261–266.

21 Abd-El-Malek A M, Incidence and virulence characteristics of *Aeromonas* spp. in fish, *Veterinary world*, 10 (2017) p. 34.

22 Singh V, Rathore G, Kapoor D, Mishra B N & Lakra W S, Detection of aerolysin gene in *Aeromonas hydrophila* isolated from fish and pond water, *Indian J Microbiol*, 48 (2008) 453-458.

23 Herrera F C, Santos J A, Otero A & Garcia-Lopez M L, Occurrence of foodborne pathogenic bacteria in retail pre-packaged portions of marine fish in Spain, *J Appl Microbiol*, 100 (2006) 527-536.

24 El Deen A N, Dorgham-Sohad M, Hassan-Azza H M & Hakim A S, Studies on *Aeromonas hydrophila* in cultured *Oreochromis niloticus* at Kafr El Sheikh Governorate, Egypt with reference to histopathological alterations in some vital organs, *World J Fish Mar Sci*, 6 (2014) 233-240.

25 Pinto A D, Terio V, Pinto P D & Tantillo G, Detection of potentially pathogenic *Aeromonas* isolates from ready-to-eat seafood products by PCR analysis, *Int J Food Sci Technol*, 47 (2012) 269-273.