Slov Vet Res 2018; 55 (Suppl 20): 113–24
DOI 10.26873/SVR-636-2018

Original Research Article

Aeromonas hydrophila IN FISH AND HUMANS; PREVALENCE, VIRULOTYPING AND ANTIMICROBIAL RESISTANCE

Heba A. Ahmed[1]*, Mohamed E.M. Mohamed[1], Maha M. Rezk[1,2], Rasha M. A. Gharieb[1], Shehata A. Abdel-Maksoud[2]

[1]Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511,Sharkia Governorate, [2]Animal Health Research Institute, Damietta branch, Agriculture Researches Center, Egypt

*Corresponding author, E-mail: heba_ahmed@zu.edu.eg

Abstract: This work aimed to investigate the virulence factors and antibiotic resistance of *A. hydrophila* isolated from different sources in Damietta Governorate, Egypt. The samples comprised of tilapia (*Oreochromis niloticus*, n=150), mugil (*Mugil cephalus*, n=144), human stool (n=102) and fish sellers hand swabs (n=85). A total of 25 *A. hydrophila* isolates were recovered and molecularly confirmed, of which, 2.7% were from tilapia muscles, 2% from tilapia viscera, 6.3% from mugil viscera and 8.8% from stool samples. No isolates were recovered from mugil muscles and hand swabs. Phenotypic determination of heamolysis, lipolysis, proteolysis and gelatinase activity revealed that 60%, 56%, 60% and 100% were positive, respectively. Molecular identification of haemolysin (*hly*), aerolysin (*aer*), lipase (*lip*) and cytotoxic enterotoxins (*act*) virulence associated genes showed that 28%, 68%, 28% and 16% of the examined isolates were positive, respectively. Antimicrobial susceptibility of the isolates to 17 antibiotics was determined, the most resistance pattern was observed for cefixime (88%), while, all of the isolates were susceptible to imipenem. This study revealed that *A. hydrophila* isolated from fish and humans showed several virulence factors and exhibit a wide range of antibiotic resistance which is considered a public health hazard as well as.

Key words: *Aeromonas hydrophila*; virulence; vntibiotic resistance; fish

Introduction

Fish production is one of the most important industrial activities in Egypt (1). Damietta is a littoral governorate with an important role in fish production. However, infection of fish with microbial pathogens is considered a risk factor in aquaculture industry resulting in loss of millions of dollars annually (2). *Aeromonas* infection in fish causes world economic problems because of the high number of fish mortalities particularly in China and India (2). Fish can be bacteriologically contaminated either by polluted water or by handling, processing and unhygienic storage conditions (3). Aeromoniasis is a zoonotic disease caused by different species of *Aeromonas*, the disease occurs due to consumption of contaminated fish, sea foods and drinking water, or direct contact with recreational waters (4). This zoonotic pathogen belongs to family *Aeromonadceae*, it is a facultative anaerobic, non-spore forming Gram-negative bacterium, motile, bacilli or coccobacilli (5,6). *A.*

Received: August 2018
Accepted for publication: September 2018

hydrophila is considered the most important zoonotic pathogen of concern. It may cause intestinal and extra-intestinal diseases in humans such as septic arthritis, diarrhea (traveler's diarrhea), gastroenteritis, skin and wound infections, meningitis, and fulminating septicemia (7). *Aeromonas* occurs widely in aquatic environments and belongs to the flora of reptiles, amphibians and fish (8). The principal reservoirs are fish in rivers, estuary waters and salt water, it has also been possible to isolate *Aeromonas* from chlorinated water including water supplies. The United States Environmental Protection Agency has incorporated *Aeromonas* species in the contaminant candidate list of emerging water borne pathogens (9). This is attributed to *Aeromonas* capacity of growing and biofilm formation in chlorinated water distribution systems. *A. hydrophila* has been isolated from retail foods including fish, seafood, raw milk, poultry and red meats (10-12).

The pathogenicity of *Aeromonads* has been related to a number of putative virulence factors such as aerolysin, hemolysin, proteases, lipases and DNases. These toxins play a major role in the development of diseases either in humans or in fish (13). The haemolysins produced by *A. hydrophila* are divided into two major groups, extracellular haemolysin and aerolysin based on immunological studies (14).

Traditional methods for bacterial identification are based on the isolation and biochemical reactions (15). However, molecular methods especially polymerase chain reaction has been proven to be more accurate and rapid for the identification. The 16S rRNA gene is a confirmed method which contributes in signature sequencing for molecular identification of *Aeromonas* species (16). Antibiotics play an important role in the control of diseases affecting animals and humans; however this contributes in bacterial resistance. The ability of bacteria to resist wide range of antibiotics helps in enhancing additional virulence features. Multiple antibiotic resistance (MAR) from *A. hydrophila* is a worldwide problem caused by the mis-use of antibiotics (17,18). It was reported that the resistance of *Aeromonas* species isolated from aquatic environment has been increased due to the increased application of antibiotics in aquaculture treatment (19). The antibiotic resistance may be transmitted from livestock products and fish to humans by infection with pathogenic bacteria (19,20).

This study aimed to investigate the prevalence of *A. hydrophila* in tilapia and mugil samples collected from retail markets in Damietta governorate and in stool and hand swab of humans in the same study area. Moreover, the presence of some virulence factors was investigated by phenotypic and genotypic methods. The antibiotic sensitivity test was also applied to evaluate the antibiotic resistance profile of the isolates.

Material and methods

This study was conducted in Damietta governorate, Egypt, and was approved by the Committee of Animal Welfare and Research Ethics Faculty of Veterinary Medicine, Zagazig University, Egypt.

Samples

A total of 294 fish samples comprised 150 tilapia (*Oreochromis niloticus*) and 144 mugil (*Mugil cephalus*) were collected from marketed fish in Damietta Governorate, Egypt. Fish samples were collected in sterilized poly-ethylene bags and transferred to the lab. Viscera and muscles were sampled separately from each individual fish under complete aseptic conditions; samples were collected from each fish after sterilization of the surface by hot spatula according to the international commissions on microbiological specifications for foods (21).

Human samples; 102 stool and 85 hand swabs were collected and directly immersed in sterile alkaline peptone water (APW, Oxoid CM1028). Stool samples were collected from Endemic Diseases Hospital, while, hand swabs were collected from fish sellers. For each collected sample, demographic data (name, sex, age, occupation, residence) were reported.

Isolation and identification

Each sample (fish viscera, fish muscles, human stool and hand swabs) were pre-

enriched in APW at 37˚C for 24 h, then a loopful of the incubated broth was plated onto *Aeromonas* agar (LAB 167) and the plates were incubated at 37˚C for 24 h under aerobic conditions. Suspected colonies were purified by plating on nutrient agar plates and were then subjected to biochemical identification according to Bergey's Manual of Determinative Bacteriology (22). The colonies were examined for morphological characterizations such as shape, Gram stain and motility test. Biochemical characterization was carried out using oxidase, triple sugar iron, pigment formation, esculin hydrolysis, growth at 42˚C and 4˚C, arginine hydrolysis, indole production, Methyl Red, Voges-Proskauer, citrate utilization, urease, hydrogen sulphide production, nitrate reduction, gelatin liquefaction, ornithine decarboxylase, oxidation-fermentation, L-lysine decarboxylase, arginine decarboxylase, β-galactosidase (ONPG), salt tolerance and sugar fermentation (23).

Molecular identification

The extraction of DNA was done by QIAamp DNA Mini kit instructions (Qiagen, Germany, GmbH, Catalog no. 51304) with some modifications. Briefly, 200 µl of the sample suspension was incubated at 56ºC for 10 min after addition of 10 µl of proteinase K and 200 µl of lysis buffer. Then, 200 µl of 100% ethanol were added to the lysate. Washing and centrifugation of the sample were performed following the manufacturer's recommendations. Then, nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

The reaction contained 25 µl of the following PCR master mix; 12.5 µl of Emerald Amp GT PCR mastermix (2x premix), 4.5 µl PCR grade water, 1 µl forward primer (20 pmol), 1 µl reverse primer (20 pmol), 6 µl template DNA. The amplification of 16S rRNA gene for *Aeromonas* genus was performed using primer pair AerF/AerR; 5'-CTA CTT TTG CCG GCG AGC GG-'3 and 5'-TGA TTC CCG AAG GCA CTC CC-'3 amplifying 953 bp (24). The confirmed *Aeromonas* isolates were subjected to PCR for the identification of *A. hydrophila* using the primer pair AH1/AH2; 5'-GAA AGG TTG ATG CCT AAT ACG TA-'3 and 5'-CGT GCT GGC AAC AAA GGA CAG-'3 of the 16S rRNA gene producing a product of 625 bp (24). The used primers were supplied from Metabion (Germany). The amplification cycles (n=35) were performed under the following conditions; 5 min of primary denaturation at 94ºC, secondary denaturation for 30 sec at 94ºC, annealing for 40 sec at 50ºC and extension at 72ºC for 50 sec in first primer pair and for 45 sec in the second pair. The amplified products were resolved by electrophoresis in 1.5% agarose gel (Applichem, Germany, GmbH). The sizes of the amplified product were determined by a gene ruler 1 Kb plus DNA Ladder (Fermentas, Thermo Scientific, Germany). Photographing was done by gel documentation system (Alpha Innotech, Biomedia) and analysis was performed using computer software.

Phenotypic characterization of A. hydrophila virulence determinants

Virulence determinants including haemolysis, lipase activity, protease activity and gelatin liquefaction of *A. hydrophila* isolates (n=25) were evaluated. Haemolysis activity was tested using blood agar plate containing 5% sheep red blood cells (Eiken, Japan) at 37˚C. The haemolysis was examined after 24 h according to Singh and Sanyal (25). The lipase activity was screened by white zones appearance in a selective media based on the hydrolysis of Tween 80 containing (g/l): pepton, 10; NaCl, 5; $CaCl_2.2H_2O$, 0.1; agar agar, 20 and Tween 80, 10 ml v/v (26). Caseinase production capacity was assayed by growing the cultures on LB (HIMEDIA, M575) medium supplemented with 1% of skim milk at 35ºC for 24 h (27). Clear halo production indicated positive reaction. Gelatinase production was determined using Luria-Bertani agar Miller (HIMEDIA, M1151) containing gelatin (30 g/L), cultivation was done at 30ºC overnight and cooled for 5 h at 4ºC. Positive reaction was described by the appearance of a turbid halo zone around the streaked colonies (28).

Molecular characterization of putative virulence genes

Primer pair used for *hly* gene amplification was 5'-CTA TGA AAA AAC TAA AAA TAA CTG-'3 and 5'-CAG TAT AAG TGG GGA AAT GGA AAG-'3 (29). The amplification conditions were 5 min of primary denaturation at 94ºC, secondary denaturation for 30 sec at 94ºC, annealing 1 min at 55ºC, extension at 72ºC for 1.5 min and final extension at 72ºC for 12 min. In case of *aero* gene, the primer pair used was 5'-CAC AGC CAA TAT GTC GGT GAA G-'3 and 5'-GTC ACC TTC TCG CTC AGG C-'3 (30). The conditions were primary denaturation at 94ºC for 5 min, secondary denaturation for 30 sec at 94ºC, annealing for 40 sec at 52ºC, extension at 72ºC for 40 sec and final extension at 72ºC for 10 min. Moreover, the primers used for *act* gene were 5'-AGA AGG TGA CCA CCA CCA AGA ACA-'3 and 5'-AAC TGA CAT CGG CCT TGA ACT C-'3 (31), the conditions of the amplification were primary denaturation at 94ºC for 5 min, secondary denaturation for 30 sec at 94ºC, annealing 30 sec at 55ºC, extension at 72ºC for 30 sec and final extension at 72ºC for 7 min. *Lip* gene was amplified using the primers 5'-ATC TTC TCC GAC TGG TTC GG-'3 and 5'-CCG TGC CAG GAC TGG GTC TT-'3 as described by Sen and Rodgers (32). The reaction conditions were primary denaturation at 94ºC for 5 min, secondary denaturation for 30 sec at 94ºC, annealing 40 sec at 55ºC, extension at 72ºC for 40 sec and final extension at 72ºC for 10 min. Visualization of the products was carried out as described previously.

Antibiogram analysis

A. hydrophila strains were subjected to antibiotic sensitivity testing using Kirby-Bauer disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) instructions. *A. hydrophila* isolates were inoculated in TSB and incubated at 35ºC for 16-20 h, turbid broth was inoculated in Muller Hinton broth (Oxoid, CM0405), the turbidity was adjusted according to McFarland obesity tube No. 0.5. Isolates were streaked on Muller Hinton agar (Oxoid, CM0337) and disks were placed, incubation was done at 37ºC overnight. The used antibiotics were Amoxicillin (AML, 25 µg), Amoxicilline-Clavulanic acid (AMC, 30 µg), Ceftriaxone (CRO, 30 µg), Cephotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Cefixime (CFM, 5 µg), Imipenem (IPM, 10 µg), Gentamicin (CN, 10 µg), Tobramycin (TOB, 10 µg), Kanamycin (K, 30 µg), Streptomycin (S, 10 µg), Tetracycline (TE, 30 µg), Ciprofloxacin (CIP, 5 µg), Norfloxacin (NOR, 10 µg), Nalidixic acid (NA, 30 µg), Trimethoprim-Sulfamethoxazole (SXT, 25 µg) and Chloramphenicol (C, 30 µg). Choosing antimicrobials was according to the importance and common use in preventing and treating diseases in both fish farms and human clinics. Interpretation was done according to the followings; Amoxicillin/ Clavulanic acid, Ceftriaxone, Cefotaxime, Ceftazidime, Imipenem, Gentamicin, Tetracycline and Chloramphenicol were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (33). Ciprofloxacin was according to The European Committee on Antimicrobial Susceptibility Testing (34). Streptomycin was interpreted according to CLSI (35). In case of Cefixime, Tobramycin, Norfloxacin and Nalidixic acid, the interpretation relied on French Society of Microbiology (36). The interpretation of Trimethoprime/ Sulfamethoxazole was based on the French Society of Microbiology (37). Moreover, Amoxicillin and Kanamycin assessment was according to Comite de l'Antibiogramme de la Societe Francaise de Microbiologie (38). Multiple antibiotic resistances (MAR) index was evaluated according to the equation described by Krumperman (39). Multidrug resistance (MDR) was defined as resistance of an isolate to at least one agent in three or more antibiotic classes (40).

Results

A total of 481 samples collected from Damietta governorate, Egypt, were examined for *A. hydrophila* contamination. Table 1 illustrates that a total of 34 (7.1%) isolates were identified by morphological and biochemical examination. These isolates were confirmed as *Aeromonas* spp. by PCR (Figure 1A).

Molecular amplification of *A. hydrophila* specific 16S rRNA gene revealed that 25 (5.2%) were positive as shown in Figure (1B).

In case of *Orechromis niloticus*, 7 (4.7%) isolates were recovered; 4 (2.7%) from muscles and 3 (2%) from viscera. Meanwhile, *Mugil cephalus* showed 6.3% isolation rate from viscera. In case of human samples, 9 (8.8%) isolates were recovered from stool samples, while hand swabs revealed no *A. hydrophila* contamination.

The phenotypic examination of putative virulence factors revealed that all the 25 isolates (100%) tested positive for gelatin liquefaction test, while 15, 14 and 15 isolates were positive for heamolysis on blood agar, lipase and protease activity with the respective percentages; 60, 56 and 60%.

The analysis of gene profiles based on the presence of 4 virulence-associated genes (*hly*, *lip*, *aer* and *act*) among clinical and environmental *A. hydrophila* isolates revealed that 7 (28%); 7 (28%); 17 (68%) and 4 (16%) of the isolates were positive for the aforementioned genes, respectively (Figure 1C, 1D, Figure 2A and 2B).

The antibiotic susceptibility assay (Tables 2 and 3) performed on 25 confirmed *A. hydrophila* isolates illustrated that all the isolates were susceptible to imipenem. However, the resistance pattern varied among the other tested drugs; the highest resistance was recorded against cefixime with the proportion of 88% followed by amoxicillin/ clavulanic acid and ceftazidime (80%). The lowest resistance (40%) was against gentamycin, streptomycin, norfloxacin and chloramphenicol. Multiple drug resistance was observed in *A. hydrophila* isolates, out of 25 isolates, four isolates were resistant to 2, 5, 12 and 13 drugs (one isolate, each). Moreover, 6 isolates were resistant to 8, 9 and 10 drugs (two isolates, each), 3 isolates were resistant to 6 drugs, 5 isolates were resistant to 4 drugs and 7 isolates were resistant to 15 drugs. The MAR average was 0.489.

Table 1: Occurrence of *A. hydrophila* and virulence factors in the examined samples

Samples		No. of bacterial-ogically suspected isolates (%)	No. of molecularly confirmed *A. hydrophila*	*hly* gene	*lip* gene	*aer* gene	*act* gene	Haemolysis	Lipase activity	Protease	Gelatinase
Tilapia (n=150)	**Muscles**	5 (3.3%)	4 (2.7%)	0	3	0	0	3	2	3	4
	Viscera	5 (3.3%)	3 (2%)	0	0	3	1	1	2	2	3
Mugil (n=144)	**Muscles**	1 (0.7%)	0	0	0	0	0	0	0	0	0
	Viscera	13 (9%)	9 (6.3%)	0	2	5	3	3	3	2	9
Fish Sellers	**Hand Swabs (n=85)**	0	0	0	0	0	0	0	0	0	0
Patients	**Stool (n=102)**	10 (9.8%)	9 (8.8%)	7	2	9	0	8	7	8	9
Total	**481**	34	25 (5.2%)	7 (28%)	7 (28%)	17 (68%)	4 (16%)	15 (60%)	14 (56%)	15 (60%)	25 (100%)

hly: hemolysin gene, *lip*: lipase gene, *aer*: aerolysin gene, *act*: cytotoxic enterotoxin

Table 2: Antimicrobial susceptibility of *Aeromonas hydrophila* isolates to different antibiotics

Antimicrobials (abbreviation)	***Aeromonas hydrophila* isolates (no = 25)**		
	*R	*I	*S
Amoxicillin (AML 25)	17(68%)	2 (8%)	6 (24%)
Amoxicillin/clavulanic acid (AMC 30)	20 (80%)	1 (4%)	4 (16.%)
Ceftriaxone (CRO 30)	11 (44%)	3 (12%)	11 (44%)
Cefotaxime (CTX 30)	14 (56%)	4 (16%)	7 (28%)
Ceftazidime (CAZ 30)	20 (80%)	-	5 (20%)
Cefixime (CFM 5)	22 (88%)	-	3 (12%)
Imipenem (IPM 10)	-	-	25 (100%)
Gentamycin (CN 10)	10 (40%)	1 (4%)	14 (56%)
Tobramycin (TOB 10)	11(44%)	4 (16%)	10 (40%)
Kanamycin (K 30)	14 (56%)	1 (4%)	10 (40%)
Streptomycin (S 10)	10 (40%)	4 (16%)	11(44%)
Tetracycline (TE 30)	18 (72%)	1 (4%)	6 (24%)
Ciprofloxacin (CIP 5)	12 (48%)	1 (4%)	12 (48%)
Norfloxacin (NOR 10)	10 (40%)	5 (20%)	10 (40%)
Nalidixic acid (NA 30)	19 (76%)	-	6 (24%)
Trimethoprim/sulphamethoxazole (SXT 25)	11 (44%)	-	14 (56%)
Chloramphenicol (C 30)	10 (40%)	4 (16%)	11 (44%)

*R: resistant, I: intermediate, S: sensitive. CIP: Ciprofloxacin; CFM: Cefixime; S: Streptomycin; TOB: Tobramycin; IPM: Imipenem; CN: Gentamycin; NOR:Norfloxacin; AML: Amoxicillin; SXT: Trimethoprim-sulphamethoxazole; C: Chloramphenicol; TE: Tetracycline; CRO: Ceftriaxone; CTX: Cefotaxime; K: Kanamycin; NA: Nalidixic acid; CAZ: Ceftazidime and AMC: Amoxicillin-clavulanic acid.

Table 3: Frequency distribution of multidrug resistant *Aeromonas hydrophila* isolates

Resistance pattern	**No. of *Aeromonas hydrophila* isolates**	**Percentage of *Aeromonas hydrophila* isolates**	**MAR index**
Resistant to 2 antibiotics	1	4%	0.11
Resistant to 4 antibiotics	5	20%	0.23
Resistant to 5 antibiotics	1	4%	0.29
Resistant to 6 antibiotics	3	12%	0.35
Resistant to 8 antibiotics	2	8%	0.47
Resistant to 9 antibiotics	2	8%	0.52
Resistant to 10 antibiotics	2	8%	0.58
Resistant to 12 antibiotics	1	4%	0.70
Resistant to 13 antibiotics	1	4%	0.76
Resistant to 15 antibiotics	7	28%	0.88

Average MAR= 0.489.

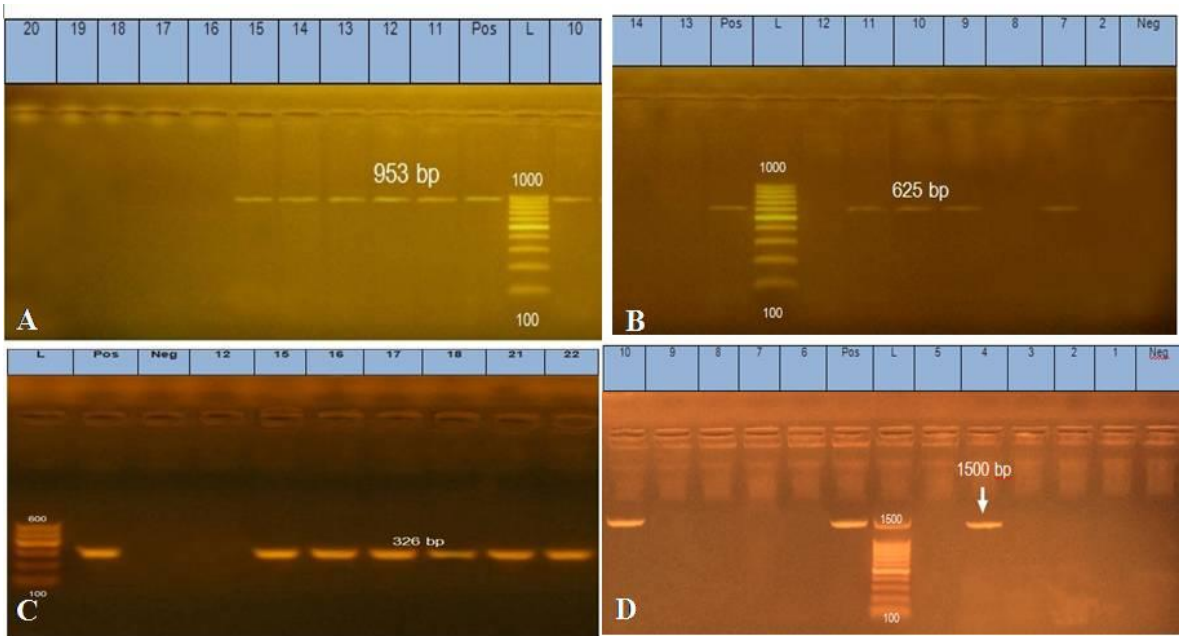

Figure 1: A: garose gel electrophoresis of 16S rRNA gene amplification of *Aeromonas* isolates from different sources with amplicon size of 953bp, L: 100 bp ladder. Lanes (10-15): positive samples. Lanes (16-20): negative samples. Lane Pos:positive control. B: Agarose gel electrophoresis of 16S rRNA gene of *Aeromonas hydrophila* isolates from different sources with amplicon size of 625 bp, L: 100bp ladder. Lanes (7, 9, 10 and 11): positive samples. Lanes (2, 8, 12, 13 and 14): negative samples. Lane Pos: positive control, lane Neg: negative control. C: Examplar of *aero* gene amplification of *Aeromonas hydrophila* isolates. L: 100 bp ladder, Lanes (15, 16, 17, 18, 21 and 22): positive samples. Lane 12: negative sample. Lane Pos: positive control and lane Neg: negative control. D: Examplar of agarose gel of *hly* amplification of *Aeromonas hydrophila* isolates. L: 100 bp ladder. Lanes (4, 10): positive samples, Lanes (1-3, 5, 6-9): negative samples. Lane Pos: positive control and lane Neg: negative control

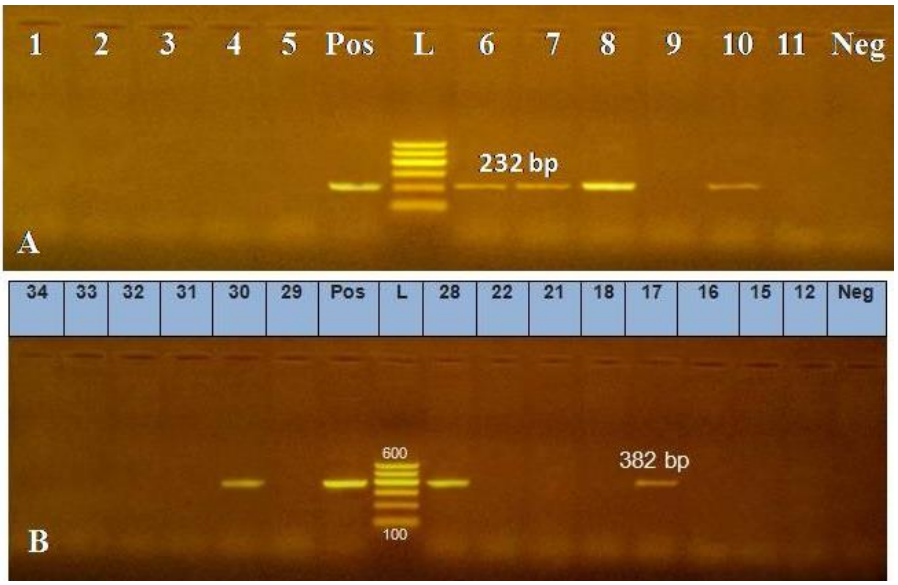

Figure 2: A: Examplar of agarose gel electrophoresis of *act* gene amplification of *Aeromonas hydrophila* isolates. L: 100 bp ladder. Lanes (1-5, 9, 11): negative samples. Lanes (6-8, 10): positive samples. Lane Pos: positive control and lane Neg: negative control. B: Examplar of agarose gel electrophoresis of *lip* gene amplification of *Aeromonas hydrophila* isolates. L: 100 bp ladder. Lanes (17, 28 and 30): positive samples. Lanes (12, 15, 16, 18, 21, 22, 29, 31-34): negative samples. Lane Pos: positive control and lane Neg: negative control

Discussion

A. hydrophila has gained increased attention due to pathogenicity to humans and the ubiquity of the organism in the environment, food and water (41). In this study, the prevalence of *A. hydrophila* in tilapia, mugil, stool and hand swabs was investigated. *A. hydrophila* was isolated from 2.7% tilapia muscles, 2% tilapia viscera, 6.3% mugil viscera indicating that fish in retail in the study area are considered potential source for infection of human consumers.

Ibrahem et al. (42) recorded a prevalence of 3.1% of *A. hydrophila* from the viscera of tilapia in Giza and Sharkia, Egypt. Meanwhile, Ashiru et al. (43) failed to isolate the bacteria from tilapia viscera in Nigeria. Furthermore, tilapia muscles showed isolation rate of 2.7% in our study, this is higher than the results recorded by Castro-Escarpulli et al. (44) who surveyed 250 frozen tilapia samples and reported an isolation rate of 0.8% in Mexico City. Ibrahim et al. (45) reported a higher isolation rate (46.6%) of *A. hydrophila* in fresh tilapia flesh and 53.3% in fresh mugil flesh. Moreover, Ramadan et al. (46) revealed that *Mugil cephalus* rate of infection with *A. hydrophila* was 37% in flesh. This was inconsistent with our study, the inability to isolate the organism from mugil flesh could be attributable to that *Aeromonas* organism is part of intestinal microbiota of either normal or diseased fish and the organism can transport to muscle under stress condition and in case of decrease fish immune state (47), moreover, the difference in individual fish susceptibility is a cause of concern.

The present study showed that *A. hydrophila* was isolated with the percentage of 8.8% from human stool samples, this is nearly similar to the results obtained by Subashkumar et al. (48) who isolated *A. hydrophila* from diarrheic patient stool samples with a rate of 9.7% in Coimbatore, South India. On the other hand, Borchardt et al. (9) reported a lower occurrence of *Aeromonas* species (0.7%) in stool samples in Wisconsin, USA. While, higher isolation rates of 85.7% in the hospitals of India (49) and 18.8% in Sharkia Governorate, Egypt were reported (10). Gastroenteritis caused by *A. hydrophila* is self-limiting watery diarrhea; however, in children and immuno-compromised patients, the disease is more serious (48).

None of the hand swab samples were contaminated with *A. hydrophila* in our study. This might be either because the hand is not suitable for the presence of *A. hydrophila* or due to the traditional habit of fish sellers to frequently wash their hands with water which lead to elimination of *A. hydrophila*. In accordance, another study in Egypt reported that none of the hand swabs from fish handlers were positive (50).

The pathogenesis of *A.* hydrophila is correlated to different factors such as extracellular enzymes, slime production and siderophores (6). *A. hydrophila* can cause cytolytic effect on erythrocytes; this heamolytic activity is a type of enteropathogenicity and may result in diarrhea outbreaks (51). Only 60% of our isolates showed hemolytic activity on blood agar plates. This was lower than other studies that reported high proportions ranging from 72%-95% of *A. hydrophila* isolates with hemolytic activity (42,48,52,53). Meanwhile, Castro-Escarpulli et al. (44) revealed that all the isolates tested positive for hemolytic activity

Lipase activity is another virulence factor which enables *A. hydrophila* to affect the function of the immune system by interaction with human leukocytes (54). Lipase can alter cytoplasmic membrane structure of the host cells and facilitate pathogenicity, the presence of aerolysin gene alleviate this process (31). The lipase activity was observed in 56% of the recovered isolates, while Bhowmik et al. (52) reported a higher prevalence (80.8%). Castro-Escarpulli et al. (44) and Simon et al. (53) reported the lipase activity in 100% of the *A. hydrophila* isolates.

The protease activity has an important role in the pathogenesis of *A. hydrophila* due to the ability of tissue damage and infection establishment by overcoming the host defense (54). Only 60% of our isolates showed protease activity, this is higher than Castro-Escarpulli et al. (44) who reported protease activity in 50% of the isolates, while, Bhowmik et al. (52)

documented 65.4%. Subashkumar et al. (48) reported the protease activity in all the tested isolates (100%). Gelatin liquefaction was observed in 100% of our isolates. This was similar to a study that reported gelatinase activity in 100% of the examined isolates (44).

Aeromonads may express cytotoxic enterotoxins (*act*), which inhibits the phagocytic ability of phagocytes; aerolysin proteins which exhibit enterotoxic properties and hemolysins (*hly*A) which are enterotoxigenic hemolysins (41,54). The haemolysin gene was identified in our study with the percentage of 28%; this is lower than the detection rate of the *hly* gene in different studies with the range from 30-100% (4,10,44,46,52,53).

The aerolysin gene was detected in 68% of the isolates. This is lower than detection rates ranged from 70-100% in different studies (44,46,53,55). While, Tahoun et al. (10) and Yogananth et al. (4) reported lower rates of 66.7% and 50%, respectively.

The detection rate of the lipase gene was 28% in our study. This result is lower than that reported by Younes et al. (56) who reported that 81.8% of *Aeromonas hydrophila* strains isolated from *Oreochromis niloticus* were positive for lipase gene, while, Simon et al. (53) and Castro-Escarpulli et al. (44) reported the gene in all the examined *A. hydrophila* isolates.

The *act* gene is responsible for enterotoxigenicity of *Aeromonas*, 16% of our isolates harbored the gene. This was inconsistent with other studies with higher detection rate of the gene ranging from 23-94% (46,52,53,55,56). The difference in the frequencies of the virulence associated genes between the studies could be attributed to variation in the origin of the samples.

Antibiotic susceptibility profile against 17 antibiotics was performed on 25 *A. hydrophila* isolates. In the present work, 100% of the isolates were susceptible to imipenem. This result is inconsistent with Castro-Escarpulli et al. (44) who reported a resistance of 50% to this drug. The most resistance pattern was recorded against cefixime (88%), followed by amoxicillin/clavulanic acid and ceftazidime (80%). Ramadan et al. (46) reported a resistance of 56% to ceftazidime. The examined isolates showed high resistance rates to both nalidixic acid and tetracycline (76% and 72%, respectively). Tetracycline and nalidixic acid resistance rates of 37.5%, each, were reported by Sarder et al. (57), while 100% of *A. hydrophila* isolates were susceptible to both tetracycline and nalidixic acid as described by Topic Popovic et al. (58) and Castro-Escarpulli et al. (44), respectively. In contrary, Ashiru et al. (43) reported 100% resistance to tetracycline.

Resistance to ceftriaxone, tobramycin and trimethoprim/sulphamethoxazole was 44%, each. Castro-Escarpulli et al. (44) documented 50% resistance against trimethoprim/ sulphamethoxazole, while, Topic Popovic et al. (58) reported 100% susceptibility to the drug.

The resistance of the isolates to cefotaxime and kanamycin was 56%, this is higher than the results of Ramadan et al. (46) who reported 40% and 6% resistance against the two drugs, respectively. Castro-Escarpulli et al. (44) observed 100% susceptibility of *A. hydrophila* isolates to both antibiotics, while, Sarder et al. (57) reported 87.5% susceptibility to kanamycin.

Ciprofloxacin was reported as the most active drug against *Aeromonas* infection (41,59,60). Our isolates showed resistance to ciprofloxacin with the percentage of 48%, this is inconsistent with other studies that reported 100% susceptibility to ciprofloxacin (43,44, 61). Moreover, Sarder et al. (57) recorded a lower resistance rate (6.3%) of the isolates to the drug.

The lowest resistance in our study was reported to chloramphenicol, norfloxacin, streptomycin and gentamicin with the percentage of 40, each. Sensitivity of *A. hydrophila* (100%) to chloramphenicol was reported in different studies (44,53,55). In addi-tion, 100% susceptibility of *A. hydrophila* iso-lates to norfloxacin was reported by Ye et al. (55).

Our isolates expressed 40% resistance to streptomycin this was comparable to 30 and 25% resistance reported by Ramadan et al. (46) and Sarder et al. (57), respectively. While, Ye et al. (55) and Simon et al. (53) reported 100% sensitivity to streptomycin.

The indiscriminate use of antibiotics for therapy in humans and animals and as growth promoters in animal and fish industry resulted in increased resistance and consequently, resistant organisms which can be transmitted to humans and other animals through many routes like food consumption and water contact even contaminated soil, livestock and pets could be considered as sources of resistance transfer (17,19,62).

The multiple antibiotic resistance (MAR) pattern of *A. hydrophila* was calculated and it ranged from 0.11 to 0.88. Krumperman (39) reported that MAR index of more than 0.2 is an evidence of high risk source contamination. Odeyemi and Ahmad (19) reported MAR index range of 0.25: 0.68 in different *Aeromonas* spp isolated from different aquatic sources.

Conclusion

In this study, *A. hydrophila* was isolated from different environmental and clinical samples. Isolation of *A. hydrophila* from fish flesh posed a zoonotic concern to public health. The observed MDR is resulted from the indiscriminate use of antibiotics either in fish farms or in diarrhea treatment. Therefore, a good disease management and control strategy is recommended for both fish farms and treatment assay to deal with antibiotic usage. Further studies are needed on the genetic relatedness among isolates from different sources and the virulence factors of the isolates must be investigated in details.

Conflict of interest

The authors declare no conflict of interest.

References

1. FAO. Food and Agriculture Organization of the United Nations. The state of world fisheries and aquaculture Rome. 2009: 176.

2. Citarasu T, Dhas AK, Velmurugan S, Viji TV, Kumaran T, Babu MM, Selvaraj T. Isolation of *Aeromonas hydrophila* from infected ornamental fish hatchery duriing massive outbreaks. Int J Curr Res. 2011; 2: 37–41.

3. Sarkar A, Saha M, Roy P. Detection of 232bp Virulent Gene of Pathogenic *Aeromonas hydrophila*through PCR Based Technique: (A Rapid Molecular Diagnostic Approach). Adv Microbiol. 2013; 03(01): 83–7.

4. Yogananth N, Bhakyaraj R, Chanthuru a, Anbalagan T, Nila KM. Detection of Virulence Gene in *Aeromonas hydrophila* Isolated from Fish Samples Using PCR Technique. Global J Biotech Biochem. 2009; 4(1): 51–3.

5. Abbott SL, Cheung WK, Janda JM. The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. J Clin Microbiol. 2003; 41(6): 2348–57.

6. Martin-Carnahan A, Joseph SW. Genus I. *Aeromonas*. In: D.J. Brenner NRKaJTS, eds., editor. In Bergey's Manual of Systematic Bacteriology. 2. New York, NY: Springer; 2005.

7. Salunke G, Namshikar V, Gaonkar R, Gaonkar T. A case of *Aeromonas hydrophila* meningitis in septic shock. Trop J Med Res. 2015; 18(1): 54–.

8. Austin B, Allen-Austin D. A Review: Bacterial pathogens of fish. J Appl Bacteriol. 1985; 58(5): 483–506.

9. Borchardt MA, Stemper ME, Standridge JH. *Aeromonas* isolates from human diarrheic stool and groundwater compared by pulsed-field gel electrophoresis. Emerg Infect Dis. 2003; 9(2): 224–8.

10. Tahoun AB, Ahmed HA, Abou Elez RM, El-Gedawy AA, Elsohaby I, Abd El-Ghafar AE. Molecular characterisation, genotyping and survival of *Aeromonas hydrophila* isolated from milk, dairy products and humans in Egypt. Int Dairy J. 2016; 63: 52–8.

11. Sreeremya S. Identification , Characterization, Antibiotic Resistance of *Aeromonas hydrophila* in Chicken Intestine. IRE Journals. 2017; 1(3): 19–22.

12. Wamala SP, Mugimba KK, Mutoloki S, Evensen Ø, Mdegela R, Byarugaba DK, Sørum H. Occurrence and antibiotic susceptibility of fish bacteria isolated from *Oreochromis niloticus* (Nile tilapia) and *Clarias gariepinus* (African catfish) in Uganda. Fish Aquatic Sci. 2018; 21 (1): 6.

13. Umesha D, Srinivasa Rao P, Pani Prasad K, Reddy AK, Srinivas KN. Aerolysin and haemolysin virulence genes of *Aeromonas hydrophila* isolated from diseased ornamental freshwater Oscar fish and goldfish by Polymerase Chain Reaction. Int J Adv Sci Technol. 2011; 3 (1): 82–9.

14. Kozaki S, Asao T, Kamata Y, Sakaguchi G. Characterization of *Aeromonas sobria* hemolysin by use of monoclonal antibodies against *Aeromonas hydrophila* hemolysins. J Clin Microbiol. 1989; 27 (8): 1782–6.

15. Austin DA, McIntosh D, Austin B. Taxonomy of Fish Associated *Aeromonas* spp., with the Description of *Aeromonas salmonicida* subsp. *smithia* subsp. nov. Syst Appl Microbiol. 1989; 11 (3): 277–90.

16. Martinez-Murcia AJ, Esteve C, Garay E, Collins MD. *Aeromonas allosaccharophila* sp. nov., a new mesophilic member of the genus *Aeromonas*. FEMS Microbiol Lett. 1992; 91 (3): 199–205.

17. Odeyemi O, Asmat A, Usup G. Antibiotics resistance and putative virulence factors of *Aeromonas hydrophila* isolated from estuary. J Microbiol Biotechnol Food Sci. 2012; 1 (6): 1339.

18. Sharma I, Rabha D, Das S, Ningombam D. Hemolytic activity and antibiotic resistance of *Aeromonas* sp. isolated from marketed fish. Eur J Pharm Med Res 2015; 2 (4), 304–312.

19. Odeyemi OA, Ahmad A. Antibiotic resistance profiling and phenotyping of *Aeromonas* species isolated from aquatic sources. Saudi J Bio Sci. 2017; 24 (1): 65–70.

20. Didugu H, Krishnaiah N , Rao MT, Ramanipushpa RN . Antibiogram of *Aeromonas* Species Isolated From Livestock Products. Int J Sci Environ Technol. 2016; 5 (2): 475–8.

21. ICMSF. International commission on Microbial specification for foods. Microorganisms in foods. Microbiol Ecology and Food Commodities London, IK: Blackie Academic and Professional. 1998.

22. Austin B, Austin DA. Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. Chichester: Praxis Publishing Ltd; 1999.

23. MacFaddin JF. Biochemical tests for identification medical bacteria. Baltimore, Md. 21202 USA: Warery Press Inc; 2000.

24. Gordon L, Giraud E, Ganière JP, Armand F, Bouju-Albert A, De La Cotte N, Mangion C, Le Bris H. Antimicrobial resistance survey in a river receiving effluents from freshwater fish farms. J Appl Microbiol. 2007; 102 (4): 1167–76.

25. Singh DV, Sanyal SC. Production of haemolysis and its correlation with enterotoxicity in *Aeromonas* spp. J Med Microbiol. 1992; 37 (4): 262–7.

26. Duza MB, Mastan S. Optimization of lipase production from *Bacillus thurngiensis* (Ts11BP), *Achromobacter xylosoxidans* J2 (Ts2 MCN)-isolated from soil sediments near oilseed farm. IOSR j Pharm Biol Sci. 2014; 9: 66–76.

27. Rivero O, Anguita J, Paniagua C, Naharro G. Molecular cloning and characterization of an extracellular protease gene from *Aeromonas hydrophila*. J Bacteriol 1990; 172 (7): 3905–8.

28. Olaniran AO, Nzimande SBT, Mkize NG. Antimicrobial resistance and virulence signatures of *Listeria* and *Aeromonas* species recovered from treated wastewater effluent and receiving surface water in Durban, South Africa. BMC Microbiol 2015; 15 (1): 1–10.

29. Yousr AH, Napis S, Rusul GRA, Son R. Detection of aerolysin and hemolysin genes in *Aeromonas* spp. Isolated from environmental and shellfish sources by polymerase chain reaction. IntFood Res J 2007; 14 (2): 115–22.

30. Singh V, Rathore G, Kapoor D, Mishra BN, Lakra WS. Detection of aerolysin gene in *Aeromonas hydrophila* isolated from fish and pond water. Indian J Microbiol 2008; 48 (4): 453–8.

31. Nawaz M, Khan SA, Khan AA, Sung K, Tran Q, Kerdahi K, Steele R. Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiol 2010; 27 (3): 327–31.

32. Sen K, Rodgers M. Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. J Appl Microbiol 2004; 97 (5): 1077–86.

33. CLSI. Clinical and Laboratory Standards Institute. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; approved guideline M45-P. Proposed Guideline. CLSI document M45-P. 2006; Vol. 25, No. 26.

34. EUCAST. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 8, 2018. http: //www.eucast.org. 2018.

35. CLSI. Clinical and Laboratory Standards Institute. M100 Performance Standards for Antimicrobial Susceptibility Testing 2017. PP: 296.

36. CASFM/EUCAST. *Enterobacteriaceae*. In: French Society of Microbiology Ed; 2018, pp: 36-44. 2018.

37. CASFM/EUCAST. *Aeromonas* sp. In: French Society of Microbiology, Ed; 2018, p: 117. 2018.

38. CASFM. Comite´ de l'Antibiogramme de la Socie´te´ Franc¸aise de Microbiologie; proposer les recommandations 2013. 2013.

39. Krumperman PH. Multiple Antibiotic Resistance Indexing of *Escherichia coli* to Identify High-Risk Sources of Fecal Contamination of Foodst. Appl Environ Microbiol. 1983; 46 (1):165–70.

40. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012; 18 (3): 268–81.

41. Arslan S, Küçüksari R. Phenotypic and Genotypic Virulence Factors and Antimicrobial Resistance of Motile *Aeromonas* spp. from Fish and Ground Beef. J Food Saf. 2015; 35 (4): 551–9.

42. Ibrahem MD, Mostafa MM, Arab RM, Rezk MA, editors. Prevalence of *Aeromonas hydrophila* infection in wild and cultured tilapia nilotica (*O. niloticus*) in Egypt. Proceedings of 8th International Symposium on Tilapia in Aquaculture; 2008; Cairo, Egypt. pp. 1257–71

43. Ashiru AW, Uaboi-Egbeni PO , Oguntowo JE, Idika CN. Isolation and antibiotic profile of *Aeromonas* species from tilapia and catfish fish.pdf. Pak J Nutr 2011; 10 (10): 982–6.

44. Castro-Escarpulli G, Figueras MJ, Aguilera-Arreola G, Soler L, Fernández-Rendón E, Aparicio GO, Guarro J, Chacón MR. Characterisation of *Aeromonas* spp. isolated from frozen fish intended for human consumption in Mexico. Int J Food Microbiol. 2003; 84 (1): 41–9.

45. Ibrahim HM, Amin RA, Shawkey NA, Mohammed HE. Bacteriological Evaluation of Some Fresh and Frozen fish. Benha Vet Med J. 2016; 31 (1): 24–9

46. 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. 2018; 124 (6): 1629–37.

47. Cipriano RC, Bullock GL, Pyle S. *Aeromonas hydrophila* and motile aeromonad septicemias of fish. 1984.

48. Subashkumar R, Thayumanavan T, Vivekanandhan G, Lakshmanaperumalsamy P. Occurrence of *Aeromonas hydrophila* in acute gasteroenteritis among children. Indian J Med Res. 2006; 123 (1): 61–6.

49. Mohana SJ. Sequestration of *Aeromonas hydrophila* for serine protease gene detection from diarrhoeal samples through pcr. Innovative Journal of Medical and Health Science. 2015; 5 (5). 2015. 210–8 p.

50. Mohammed EAM. The role of fish and other invertebrate aquatic organisms in transmitting some bacterial diseases to man: MVSc thesis, Cairo University; 2012.

51. Rahim Z, Sanyal SC, Aziz KM, Huq MI, Chowdhury AA. Isolation of enterotoxigenic, hemolytic, and antibiotic-resistant *Aeromonas hydrophila* strains from infected fish in Bangladesh. Appl Environ Microbiol. 1984; 48 (4): 865–7.

52. Bhowmik P, Bag PK, Hajra TK, De R, Sarkar P, Ramamurthy T. Pathogenic potential of *Aeromonas hydrophila* isolated from surface waters in Kolkata, India. J Med Microbiol. 2009; 58 (12): 1549–58.

53. Simon SS, Lalitha KV, Joseph TC. Virulence properties of *Aeromonas* spp. from modified-atmosphere- and vacuum-packed milk fish (Chanos chanos Forsskal, 1775). Ann Microbiol. 2016; 66 (3): 1109–15.

54. Tom ás JM. The Main *Aeromonas* Pathogenic Factors. ISRN Microbiol. 2012; 2012: 22.

55. Ye YW, Fan TF, Li H, Lu JF, Jiang H, Hu W, Jiang QH. Characterization of *Aeromonas hydrophila* from hemorrhagic diseased freshwater fishes in Anhui Province, China. Int Food Res J 2013; 20 (3): 1449–52.

56. Younes AM, Gaafar AY, Awad ES. Virulence Determinants and Plasmid Profile of *Aeromonas hydrophila* Strains Isolated from *Oreochromis niloticus*. Global Vet 2015; 15 (6): 613–7.

57. Sarder H, Khan T, Saha ML, Punom NJ, Mandal SC, Rahman MS. Prevalence and antibiotic susceptibility of *Aeromonas hydrophila* isolated from freshwater fishes. J Fish 2016; 4 (3): 411–9.

58. TopićPopovic N, Teskeredzić E, Strunjak-Perovic I, Čož-Rakovac R. *Aeromonas hydrophila* isolated from wild freshwater fish in Croatia. Vet Res Commun. 2000; 24 (6): 371–7.

59. Hatha M, Vivekanandhan AA, Joice GJ, Christol. Antibiotic resistance pattern of motile aeromonads from farm raised fresh water fish. Int J Food Microbiol. 2005; 98 (2): 131–4.

60. Sreedharan K, Philip R, Singh ISB. Virulence potential and antibiotic susceptibility pattern of motile aeromonads associated with freshwater ornamental fish culture systems: A possible threat to public health. Braz J Microbiol. 2012; 43 (2): 754–65.

61. Rathore G, Swaminathan TR, Mahanta PC, Kapoor D. Isolation and characterization of motile aeromonads from aquatic environment. IJFAS 2005; 52 (2): 241–8.

62. McDermott PF, Zhao S, Wagner DD, Simjee S, Walker RD, White DG. The food safety perspective of antibiotic resistance. Anim Biotechnol. 2002; 13 (1): 71–84.