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Multiple antibiotic resistant *Aeromonas hydrophila* in Nile tilapia with reference to its public health significance

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ABSTRACT

Nile tilapia aquaculture is a fast-growing industrial sector in Egypt. However, the progress of this industry is hindered by many challenges as poor water quality and associated bacterial infections. *Aeromonas hydrophila* is an important zoonotic waterborne aquatic pathogen responsible for severe outbreaks in tilapia culture so the current study aimed to investigate the prevalence of *Aeromonas hydrophila* in tilapia fish and their aquaculture water, the fish and water samples were collected from three farms located in Assiut and Minia Governorates, Egypt. *Aeromonas hydrophila* was isolated from tilapia five organs including liver, kidney, intestine, spleen and gills with a percentage of 7.1, 6.5, 9.1, 5.1, and 11.7%, receptively, and from water samples with 59.7%. The isolates were molecularly confirmed as *Aeromonas hydrophila* in 70.9 % and 82.4 % of the tested isolates using 16s RNA and *gyr-β* genes, respectively. *Aeromonas hydrophila* isolates revealed a marked resistance for the tested antibiotics; amoxicillin and novobiocin (100%), streptomycin (71.4%), chloramphenicol (57%), doxycycline and trimethoprim/Sulphamethoxazole (50%), colistin (43%), ciprofloxacin and norfloxacin (14%). The isolates showed multiple antibiotic resistance indexes ranging from 0.3 to 1. The present study highlights the *Aeromonas hydrophila* resistance and virulence, tilapia aquaculture health hazard to the human population, so adequate control measures should be applied. Furthermore, there is an essential need to promote an alternative non-antibiotic control in farmed fish.

Introduction

The Egyptian tilapia industry represents the cornerstone of global fish farming, accounting for two-thirds of the continent's production and the third largest tilapia producer globally after China and Indonesia (Ali *et al*., 2020; FAO, 2020). However, the use of farm animal and poultry manure in integrated culturing by some fish farmers in their fish ponds (Minich *et al*., 2018) and the leach of anthropogenic polluted water (Mahmoud *et al*., 2016), could introduce new bacterial isolates and resistant genes to the aquaculture system. These genes may be transferred to aquaculture bacteria (Aly and Albutti, 2014). *Aeromonas* species are a part of the natural microflora of aquatic bodies (El-Wafai *et al*., 2022). *Aeromonas* are opportunistic, secondary pathogens and their infection is linked to stress conditions, resulting in septicemia and severe mortality in both wild and farmed freshwater and marine fish that damage the economics of the aquaculture sector (Beaz-Hidalgo and Figueras, 2013; Hanson *et al*., 2014).

Aeromonas hydrophila, the most prevalent and vigorous aeromonad, had been identified as the main cause of fish Motile *Aeromonas* Septicemia (MAS) (Wang *et al*., 2010). In the Egyptian tilapia culture, *Aeromonas hydrophila* is considered the leading cause of high mortalities and economic losses (Aboyadak, 2015; El-Bahar *et al*., 2019). *Aeromonas hydrophila* had been detected in many tilapia outbreaks around Egypt in summer. *Aeromonas hydrophila* was identified from Aswan Fish Hatchery (Hamouda *et al*., 2019), Kafr El Sheikh Governorate (El Deen *et al*., 2014), Sharkia, El-Abbassa and Abo-Hammad (Ayoub *et al*., 2021), also in Dakahlia Governorate (El-Son *et al*., 2019), and Suez Governorate (Khafagy *et al*., 2021) and caused massive mortalities approached 98% in Behiera Governorate (Elgendy *et al*., 2015), and 80% in Beni-Suef Governorate (Korni *et al*, 2017), all these outbreaks were in summer season. Multiple virulence factors facilitate *Aeromonas* host invasion; importantly, lipopolysaccharides, adhesins, motility, and the mass of exoenzymes such as hemolysin, protease, and lipases (Tomás, 2012; Beaz-Hidalgo and Figueras, 2013). The chief reason behind the *Aeromonas hydrophila* threat is not only due to its virulence but also due to its opportunistic nature, ubiquitous aquatic existence, and can cause latent infection (Cipriano, 1984). Due to disease outbreaks, antibiotics were used to treat such infections, and the misuse of these substances as therapy and prophylactic use resulted in resistance development (Le *et al*., 2018). *Aeromonas* can obtain antibiotic resistance character and has the potential for horizontal gene transfer (Guz *et al*., 2021), so it may be a good candidate for investigating the spread of antibiotic resistance in the water (Usui *et al*., 2016). Moreover, *Aeromonas hydrophila* also, represent a public health significance to human health. As a foodborne pathogen *Aeromonas hydrophila* can grow in foods during refrigeration, and have been isolated from food process line plant (Rossi Júnior *et al*., 2006), ready-to-eat seafood products (Lee *et al*., 2021), raw meat (Alhazmi, 2015), and raw milk and some milk products (Sadek *et al*., 2017). As well as, *Aeromonas* infections have been responsible for water-borne diseases in different parts of the world, especially developing countries as Egypt, due to low personal hygiene, carelessness and lack of quality water (Odeyemi and Ahmad, 2013), which can represent a potential hazard to consumers via food-borne infections and a risk factor for wound infections after handling contaminated fish (Praveen *et al*., 2016, Vasile *et al*., 2017). Increased *Aeromonas hydrophila* antibiotic resistance to multiple antibacterial agents, as well as their virulence have become a serious public health issue (Pessoa *et al*., 2019). So, before implanting preventive measures, it is important to have a precise identification of these organisms. Hence, this study is designed to determine the prevalence of *Aeromonas hydrophila* as well as their virulence

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in aquaculture water and Nile tilapia samples collected from Assiut and Minia Governorates, Egypt. Additionally antibiotic-resistant profiles, and virulence determinants of the identified strains were investigated.

Materials and methods

Ethical approval

Tilapia fish examined in the current research were caught, transported, and examined with permission (Number: 06/2023/0114) of the ethical committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, also, following the National Advisory Committee for Laboratory Animals Research (CCAC, 2005) and NACLAR (2022) for the care and use of fish in research.

Water and fish sampling

Seventy-nine healthy Nile tilapia fish and seventy water samples were collected from three tilapia fish cultured farms, in the routine fishing activity of the farms. The samples were collected through four visits, two visits in Assiut (farm A, farm B) in December 2021, and two visits in Minia (farm C) in Mars and June 2022, Egypt. In visit I, twenty five tilapia fish and ten water samples were taken, while fourteen tilapia fish and twenty water samples were collected in visit II, finally in visits III and IV, twenty tilapia fish and twenty water samples were assembled.

Water Quality Parameters

Four to five water samples of one liter each were collected from different locations of each pond according to Standard methods for the examination of water and wastewater examination (APHA, 2017). Water temperature was recorded in situ by a digital thermometer. Water pH, total dissolved solids, electrical conductivity, and dissolved oxygen were measured using a portable multi-parameter waterproof meter (model HI98192, Hanna Instruments Company, Romania).

Aeromonas hydrophila isolation from aquaculture water and tilapia fish

Tilapia`s body was cleaned from dirt with sterile distilled water, then the scales were removed by a fish peeler. The fish skin was disinfected by alcohol (70%), then fish belly was aseptically opened with a sterile scissors. Swabs were collected aseptically from each fish`s liver, kidney, intestine, spleen, and gills. Sterile loops were inserted through the sterilized area and then the inoculums were transported into trypticase soya enrichment broth. *Aeromonas* were isolated from water samples as previous described (APHA, 2017). The inoculated tubes were incubated aerobically at 30ºC for 24 hours. In the next day, inoculums were streaked into a trypticase soya agar (TSA) plate supplemented with amoxicillin 5mg /L under complete aseptic conditions, and incubation was performed at 30°C for 24 hours. *Aeromonas hydrophila* colonies appeared round, convex, shiny, creamy, and of 2–3 mm diameter (El-sharaby *et al*., 2020; Abdel-Moneam *et al*., 2021). Further identification was performed according to the methods described by Bergey's manual® of Systematic Bacteriology (Garrity *et al*., 2005). Identified isolates were kept at −20°C in 25% glycerol for further identification.

Molecular identification of Aeromonas hydrophila

Bacterial isolates were grown overnight in trypticase soy broth at 30°C, and then DNA extraction was accomplished using the boiling method as described by Queipo-Ortuño *et al*., (2008). One µl of the extracted DNA was used for estimating the purity and concentration using GeneQuant®1300 Spectrophotometer (Bioscience, Sweden). The A260/A280 ratio were calculated and ratios between 1.8 and 2.0 were accepted for DNA purity. A polymerase chain reaction (PCR) assay targeting the 16S rRNA gene was carried out using a universal primer set (27F and 1492R) (Zorriehzahra *et al*., 2020; Bakiyev *et al*., 2022). Further, *Aeromonas hydrophila* isolates were confirmed by a PCR targeting the *gyr-β* gene, and the oligonucleotide sequence of the two primer sets is presented in Table 1 (Yáñez *et al*., 2003). PCR amplifications were performed in a reaction mixture containing 5 µl of Willowfort Master Mix, 1 µl (10 µM) of each forward and reverse primer, 3 µl of DNA template, and DNase-free water up to the total volume of 20 µl. A Veriti thermocycler (Applied biosystem) was used to perform the following PCR reactions: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 35 seconds, annealing according to the primer set and extension at 72°C for 30 seconds then final extension at 72°C for 10 minutes. The amplicons were subjected to electrophoresis in 1% agarose gel.

Antibiotic sensitivity testing (Sicuro et al., 2020)

The antibiotic sensitivity test was conducted by using the agar diffusion method using the following antimicrobial susceptibility discs: chloramphenicol (C) (30 mcg), streptomycin-S (10 mcg), doxycycline–D (30 mcg), colistin-CT (10 mcg), norfloxacin-NOR (10 mcg), amoxicillin-AX (25 mcg), ciprofloxacin-CIP (30 mcg), trimethoprim/sulfamethoxazole-SXT (1.25/23.75mcg), and novobiocin-NV (30 mcg). Inoculation of the tested bacteria into Mueller-Hinton agar dishes was done with a sterile cotton swab. The Petri dishes were dried for 15−20 min at room temperature; the antimicrobial discs were placed onto plates using sterile forceps. After incubation at 30°C for 24 hours, the diameter of the inhibition zone for individual antimicrobial agents was interpreted into susceptible, intermediate or resistant categories according to the interpretative criteria for the tested bacteria using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2016; 2017; 2020).

Aeromonas hydrophila virulence (lipase and protease production)

Aeromonas lipolytic activity was tested (Stepanović *et al*., 2001) with modification using trypticase soya agar enriched with 0.01 % $CaCl_2$ and 1% Tween 80. *Aeromonas* protease action was tested (Nicodème *et al*., 2005) in trypticase soya agar enriched with 1% skim milk.

Statistical analysis

The obtained data were statistically analyzed using the SPSS software, version 22. The statistical analysis for water parameters was done using One-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. P-value of <0.05 was considered significant. The Pearson correlation coefficient was used to determine the association between the water parameters and the prevalence of *Aeromonas* in the water during the four visits. The Kruskall-Wallis test (Chi-square test) was used to com-

Table 1. Nucleotide sequence of the two primer sets used in the study.

Target gene	Primer sequence	Annealing temperature	Amplicon size (pb)	References
16sRNA	27F: AGAGTTTGATCCTGGCTCAG 1492R: GGCTACCTTGTTACGACTT	56ºC for 45 seconds	1500	(Zorriehzahra *et al*. (2020) Bakiyev *et al*. (2022)
Gyr-β	F: TCCGGCGGTCTGCACGGCGT R: TTGTCCGGGTTGTACTCGTC	63ºC for 35 seconds	1100	(Yáñez *et al*. (2003)

pare the variance of the isolated strains among different fish organs and the water isolates and p value < 0.05 was considered to be significant.

Results

Aquaculture water physicochemical parameters

In our study, the water temperature in visits I, II, III, and IV were 14.7, 13.4, 16.8, 28°C, receptively. Moreover, the measured water pH among the examined farms were 7.7, 8.7, 9 and 9.3 in visit I, II, III, and IV, receptively. The water dissolved oxygen, electrical conductivity, and total dissolved solids were presented in Figure 1. Noticeably, during the study period, significant differences were observed in the recorded water parameters among the visited ponds ($p < 0.05$). Correlation analyses of the revealed the presence of strong positive correlations between all physicochemical parameters. For instance, electrical conductivity and total dissolved solids showed a strong significant correlation. At the same time, both of them displayed a positive correlation with water temperature and water pH. While, each of them displayed a negative correlation with the water dissolved oxygen. Moreover, there is a positive relationship between water temperature and pH. In contrast, the water dissolved oxygen displayed a negative correlation with other parameters.

Prevalence of Aeromonas hydrophila in Nile tilapia fish and aquaculture water samples

One hundred and forty-nine motile *Aeromonas* species isolates were obtained from the examined fish and water samples (Table 2). In details, one hundred and three from tilapia fish and forty-six from water samples. From the obtained isolates only seventy-seven *Aeromonas hydrophila* isolates were identified; thirty-one from tilapia fish and forty-six from water samples. The fish *Aeromonas hydrophila* isolates represent 30 % of the fish *Aeromonas* species isolates (31/103). In analysis of variance, there is no significance of difference in the number of isolates among fish organs themselves and to the water isolates. *Aeromonas hydrophila* isolates were Gram-negative, rod-shaped, motile, oxidase and catalase positive, urease test negative, able to hydrolyze esculine, liquefy gelatin and exerted hemolytic activity in blood agar as described by Bergey's manual® of Systematic Bacteriology (Garrity *et al*., 2005). The lowest percentage of the isolates was recorded in the spleen 5.1 %, while the gills displayed the highest number of isolates 11.7 % (Table 3). At visit level *Aeromonas hydrophila* isolated from tilapia in 13 %, 10.4 %, 9.1 %, and 7.8 % of the total *Aeromonas hydrophila* isolates (77) from visits I, visit II, visit III, visit IV, receptively. On contrary, the water isolates were identified in 27.7 %, 16.88 %, 12.98 % and 2.5 % of the total water *Aeromonas hydrophila* isolates (46) in visits I, visit II, visit III, visit IV, receptively. In relation to the

Table 2. Prevalence of biochemically identified *Aeromonas* species in Nile tilapia fish aquaculture.

Visits	Number and percentage of positive *Aeromonas species* isolates in Nile tilapia fish and their aquaculture water														Total isolates	
	Water		liver		Kidney		Intestine		Spleen		Gills		Total Fish isolates			
	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]
I	21	14.1	3	2	5	3.4	5	3.4	8	5.4	5	3.4	26	17.4	47	31.5
II	13	8.7	3	2	2	2	3	2	3	2	6	4	17	11.4	30	20.1
III	10	6.7	3	2	8	5.4	8	5.4	8	5.4	6	4	33	22.1	43	28.9
IV	2	1.3	5	3.4	4	2.6	8	5.4	2	1.3	8	5.4	27	18.1	29	19.5
Total	46	30.8	14	9.4	19	21.8	24	16.1	21	14.1	25	16.8	103		149	

[a]: Data presented as percentage to the total number of *Aeromonas* species isolates (149).

Table 3. Prevalence of biochemically identified Aeromanas hydrophila in Nile tilapia aquaculture.

Visits	Number and percentage of positive *Aeromonas hydrophila* isolates in Nile tilapia and their aquaculture water														Total	
	Water		liver		Kidney		Intestine		Spleen		Gills		Total Fish isolates			
	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]
I	21	27.27	3	3.8	2	2.5	2	2.5	1	1.29	2	2.5	10	13	31	40.2
II	13	16.88	1	1.29	1	1.29	2	2.5	1	1.29	3	3.8	8	10.4	21	27.27
III	10	12.98	1	1.29	1	1.29	2	2.5	1	1.29	2	2.5	7	9.1	17	22.07
IV	2	2.5	1	1.29	1	1.29	1	1.29	1	1.29	2	2.5	6	7.8	8	10.38
Total	46	59.74	6	7.07	5	6.49	7	9.09	4	5.1	9	11.68	31	40.3	77	

[a]: Data presented as percentage to the total *Aeromonas hydrophila* isolates (77).

Table 4. Prevalence of *Aeromonas hydrophila* in tilapia aquaculture depending on biochemical identification.

Visits	Number and percentage of positive *Aeromonas hydrophila* isolates											
	Number of tested tilapia	Liver		Kidney		Intestine		Spleen		Gills		Total	
		No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	No.	%[a]	NO	%[a]
I	25	3	12	2	8	2	8	1	4	2	8	10	40
II	14	1	7.2	1	7.24	2	14.3	1	7.2	3	21.4	8	57
III	20	1	5	1	5	2	10	1	5	2	10	7	35
IV	20	1	5	1	5	1	5	1	5	2	10	6	30
Total	79	6	7.6	5	6.3	7	8.7	4	5	9	2.53	31	

[a] : Data presented as percentage of positive *Aeromonas hydrophila* to the total examined fish in each visits.

number of examined fish (Table 4); *Aeromonas hydrophila* obtained in 7.6, 6.3, 8.7, 5, and 5.2% from liver, kidney, intestine, spleen, and gills, receptively. As a stress factor, water quality parameter alteration is usually associated with pathogen distribution. In our study, there was a significant negative correlation between the recoded water parameters and water *Aeromonas hydrophila* isolates except for dissolved oxygen displayed a positive correlation.

Molecular identification of Aeromonas hydrophila

The data displayed in Table 5, showed the positive molecularly identified *Aeromonas hydrophila* isolates. In our study, a total of seventeen *Aeromonas* isolates (70.8%) were identified to genus level using 16S rRNA *Aeromonas* species specific gene (Figure 2), moreover from the seventeen, fourteen isolates were confirmed as *Aeromonas hydrophila* (82.35 %) by detection of *gyr-β* gene (Figure 3). The highest identified *Aeromonas hydrophila* was detected in the spleen at percentage of 28.6 %, and the lowest identified percentage was in the intestine 7.1%. While in the visits, *Aeromonas hydrophila* was identified with 35.3 and 28.6% in visit I, 35.3 and 35.7% in visit II, 5.9 and 7.1% in visit III, as well as 23.5 and 28.6% in visit IV using 16sRNA and gyr- β genes, receptively.

Aeromonas hydrophila antibiotic resistance

The data of the antibiotic resistance shown in Table 6, revealed that all tested *Aeromonas hydrophila* isolates were antibiotic resistant with 100% resistance to amoxicillin, and novobiocin. Moreover, 71.4% of the isolates were resistant to streptomycin, while near the half of our isolates (57%) were resistant to chloramphenicol. and half of the isolates were resistant to trimethoprim/sulphamethoxazole and doxycycline, and 43% of the isolates were resistant to colistin. For quinolones, including norfloxacin and ciprofloxacin only 14% of the isolates showed resistance. Moreover, our data revealed that the multiple antibiotic resistance indexes of all isolates (100%) were over 0.2, with values of 0.3 to 1.

Table 5. Molecularly positive *Aeromonas hydrophila* in tilapia aquaculture.

Visits	16sRNA			Gyr β		
	Tested	Positive	%[a]	Tested	Positive	%[a]
Visit 1	6	6	35.3	6	4	28.6
Visit 2	6	6	35.3	6	5	35.7
Visit 3	6	1	5.9	1	1	7.1
Visit 4	6	4	23.5	4	4	28.6
Organs	Tested	Positive	%[b]	Tested	Positive	%[b]
Liver	4	2	11.8	2	2	14.3
Kidney	4	2	11.8	2	2	14.3
Intestine	4	3	17.6	3	1	7.1
Spleen	4	4	23.5	4	4	28.6
Gills	4	3	17.6	3	3	21.4
Water	4	3	17.6	3	2	14.3
Total	24	17	70.8	17	14	82.4

[a]: Data is presented as percentage was calculated through dividing the number of positive isolates by the total positive number in each gene.
[b]: Data is presented as percentage was calculated through dividing the positive isolates of each organ by the total positive number in each gene while the total percentage was calculated by dividing the total positive by the total number tested.

Table 6. Antibiotic resistance percentage and multiple antibiotic resistance index of identified *Aeromonas hydrophila* isolates.

Isolate code	Isolate origin	AX	CT	C	D	S	NV	NOR	CIP	SXT	* Multiple antibiotic index
1	liver	r	r	r	r	r	r	s	s	r	0.7
2	kidney	r	r	r	r	r	r	s	s	r	0.7
3	spleen	r	r	r	I	r	r	s	I	r	0.8
4	gills	r	s	I	r	r	r	s	s	s	0.5
5	liver	r	s	r	r	r	r	s	s	s	0.5
6	kidney	r	r	r	r	r	r	r	r	r	1
7	spleen	r	s	r	r	r	r	r	r	s	0.7
8	gills	r	s	I	s	s	r	s	s	r	0.4
9	water	r	r	I	I	r	r	s	s	s	0.6
10	spleen	r	r	I	I	s	r	s	s	r	0.6
11	intestine	r	s	r	s	s	r	s	s	s	0.3
12	spleen	r	s	I	s	s	r	s	s	I	0.4
13	gills	r	s	I	s	r	r	s	s	r	0.5
14	water	r	s	r	r	r	r	s	s	s	0.5
Number of resistant isolates		14	6	8	7	10	14	2	2	7	
**Percentage of resistance		100%	43%	57%	50%	71.40%	100%	14%	14%	50%	

*Multiple antibiotic index was calculated through dividing the number of antibiotics to which the isolate was resistant by the number of tested antibiotics.
**Percentage of resistance to each antibiotic was calculated by dividing the number of resistant strains for each antibiotic by the number of tested isolates.
AX (Amoxicillin), CT (Colistin), C (Chloramphenicol), D (doxycycline), S (Streptomycin), NV (Novobiocin), NOR (norfloxacin), CIP (Ciprofloxacin), and SXT (trimethoprim/sulfamethoxazole).

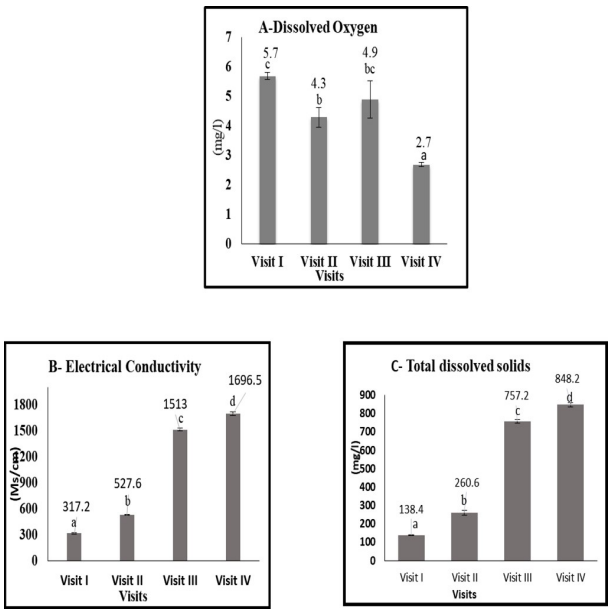

Figure 1. water quality parameters; A, B and C illustrates water dissolved oxygen, the water electrical conductivity, and water total dissolved solids, receptively.

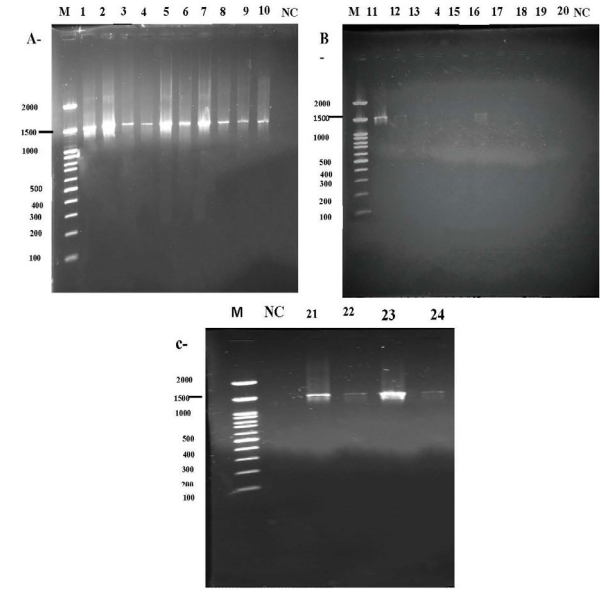

Figure 2. Agarose gel electrophoresis of PCR amplification of 16S RNA (1500 bp) for characterization of *Aeromonas hydrophila*. A; Lane M: 100 bp ladder as molecular size DNA marker. , Lane 1 - 10 positive for *Aeromonas hydrophila*, NC control negative. B; Lane M: 100 bp ladder as molecular size DNA marker. Lane 11, 12, 16 positive for *Aeromonas hydrophila*, lane 13, 14, 15, 17, 18, 19, 20 negative for *Aeromonas hydrophila*, NC negative control. C; Lane M: 100 bp ladder as molecular size DNA marker. Lane 21, 22, 23, 24 positive for *Aeromonas hydrophila*, lane NC for negative control.

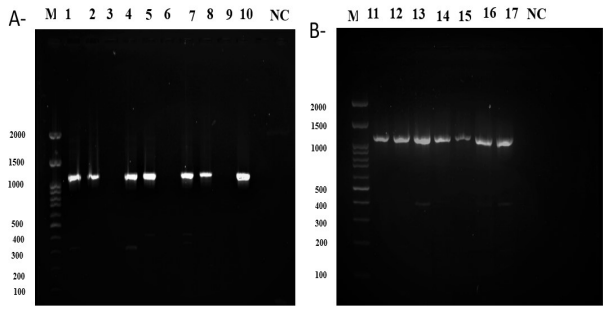

Figure 3. Agarose gel electrophoresis of PCR amplification of *gyr-β* (1100 bp) for characterization of *Aeromonas hydrophila*. A; Lane M: 100 bp ladder as molecular size DNA marker. Lane 1, 2, 4, 5, 7, 8, 10 positive for *Aeromonas hydrophila*, lane 3, 6, 9 negative for *Aeromonas hydrophila*, NC negative control. B; Lane M: 100 bp ladder as molecular size DNA marker, Lane 11,12,13,14,15,16, and 17 positive for *Aeromonas hydrophila*, NC: negative control.

Aeromonas hydrophila virulence (lipase and protease production)

The confirmed resistant *Aeromonas hydrophila* isolates were tested for the production of lipase and protease enzymes as *Aeromonas hydrophila* virulence factors. Lipolytic action was examined using 1% Tween 80 and all of the tested strains have the ability to split Tween 80. Also, all tested *Aeromonas* strains had a protease action on skim milk and were detected by the appearance of a halo zone around the culture.

Discussion

Water quality is the main factor influencing the aquaculture industry and should fit the fish`s physiological requirements for optimal growth of the cultured species (Mercante *et al*., 2018). So, monitoring the microbial and chemical contaminants will help in safeguarding the public from any potential adverse risks. Like many other creatures, the fish have their optimal temperature range where their body functions are maximized. Water temperature above or below the optimum level potentially stresses fish (Malavasi *et al*., 2013; Singh *et al*., 2013). In our survey, water temperatures were 14.7, 13.4, 16.8 and 28°C in visit I, II, III and IV, receptively. Water temperature was below the recommended limits for tilapia culture (28–32°C) (El-Sayed, 2006) in the first three visits, except in visit IV it was at optimum. Since the study was conducted in variable climatic conditions from December (cold season) to June (hot season), there was variability in water temperature. Our recorded data range was higher than that recoded by Elgendy *et al*. (2015), who recorded that, water temperature of Nile tilapia aquaculture at the end of December 2013 was below 8°C and persisted until February 2014, when water temperature increased up to 13°C. Although, our recorded temperature was nearly similar to that recorded by Waruiru *et al*. (2021), who recorded that aquaculture water temperature ranged from 20.50°C to 31.7°C during the period from December 2017 to April 2018 in Kirinyaga County. In the current study, water pH in visit I visit II, visit III and visit IV were 7.7. 8.7, 9 and 9.3. Our recorded water pH range was within the optimum for fish production (6 to 9) (Popma and Masser, 1999) in the first three visits, except in visit IV (9.3) was slightly higher. The variability in pH values may be related to the integration and management practices, as in integration the water organic load will increase with a consequence high ammonia and high basic pH (Amal *et al*., 2015). These results were nearly similar to that recorded by Hassan *et al*. (2017), who recorded a pH range 7.8-8.7 from January 2015 to December 2015 in cultured Nile tilapia at Al-Qatif, Saudi Arabia. Our measured water dissolved oxygen values varied from 5.71 mg/L in visit I to 2.72 mg/L visit IV (figure1A). While dissolved oxygen is below the recommended limits for tilapia culture (≥ 5.0) (El-Sayed, 2006), however, only in visit I dissolved oxygen was suitable for tilapia culture. In intensified system, the fish food was supplemented in large amounts, the decomposition of the feeds use oxygen also high temperature has been known to decrease oxygen content in water as reported by Mustapha, (2017). So it is clear to notice decreased water oxygen between the visits with the lowest value was in visit IV. Besides in visit IV, there was an owner complain of increased algal growth, these algae at night consume the dissolved oxygen in their respiration and this leads to reduced oxygen level (Rahman *et al*., 2020). Our dissolved oxygen reading is nearly similar to the result (3.5 mg/L) recorded by Elgendy *et al*., (2015) in tilapia Barsiq farm, south to Lake Edco, Behiera Governorate during the period between December 2013 to February 2014.The mean value of electrical conductivity in our study varied between 317.2 to 1696.5 µS /cm^2. The lowest mean value was recorded for tilapia visit I while the highest was in visit IV, which was within the optimal range for fish culture (100 – 2,000 µS/cm) (Stone *et al*., 2013).The water electrical conductivity in the current study was close to that recorded by El-Gohary *et al*. (2020) in March from four Nile tilapia fish farms located at Kafr-Elsheikh Governorate (1365-2620 µS/cm^2). Our mean water total dissolved solids were from 138.4, 260.6, 757.3 and 848.25 mg/L in visit I to visit IV. This water total dissolved solids is optimum for tilapia culture (500-1000 mg/L) (PHILMINAQ, 2014). Our water total dissolved solids result is nearly similar to that recorded in March by El-Gohary *et al*. (2020) from four Nile tilapia fish farms located at Kafr-Elsheikh governorate (674.5-1342.1 mg/L) and lower than 1939.2 mg/L that recorded by Elgendy *et al*. (2015). While an appropriate level of ions is necessary to support osmotic and ionic regulation of the fish, if ion levels are too high or too low, fish will have to expend excessive energy to manage tissue hydration necessary for normal physiological function, which will consequently result in reduced growth and fitness. According to Khalefa *et al*. (2021) the water electrical conductivity and total dissolved solids are directly related like in our study. The water's solubility of oxygen decreases with high temperature, so the dissolved oxygen decreases as the water temperature and pH rises (Frisk *et al*., 2012).

According to Sipaúba-Tavares *et al*. (2011) the pond management practices such as the fish stocking density, water change management, and daily feed input, as well as, the fertilizer used as in integrated systems, also, the climatic changes may be related to decreased dissolved oxygen. While water pH directly related with water temperature and this due to increased photosynthesis (Boyd, 2018).

Aeromonas bacteria are ubiquitous in aquaculture representing a part of the normal microflora of fish and water (González-Serrano *et al*., 2002). These bacteria frequently cause devastating disease in fishes (AlYahya *et al*., 2018; Al-Haider *et al*., 2019). We identified 103 and 46 isolates from tilapia fish and water samples, respectively to *Aeromonas* genus in accordance to Bergey's Manual of Systematic Bacteriology (Garrity *et al*., 2005). Motile *Aeromonas* obtained in 9.4, 21.1, 16.1, and 14.1% from liver, kidney, intestine, spleen and gill of healthy tilapia fish and 30.8% from water samples receptively. In analysis of variance, there is no significance of difference in the number of isolates among fish organs themselves and in the total water isolates (Table 2). Our *Aeromonas* species isolate number is nearly similar to that obtained by Salem *et al*. (2020) from the kidney, liver and spleen of infected tilapia fish (128 isolates).

Thirty percent of the tilapia *Aeromonas* isolates were *Aeromonas hydrophila* (31/103), identified on the basis of *Aeromonas hydrophila* prominent virulence characters as ability to move with its flagella, esculine hydrolysis capability, gelatinase and hemolytic activity (Garrity *et al*., 2005). The expression of these virulence factor facilitate *Aeromonas* colonization and invasion of the host (Beaz-Hidalgo and Figueras, 2013). Our result was similar to that obtained by Salem *et al*. (2020) who isolated 48 *Aeromonas hydrophila* isolates from the liver, kidney and spleen of infected Nile tilapia in northern Egypt. Also, we obtained forty-six *Aeromonas hydrophila* isolates from water samples representing 59.7 % of the total *Aeromonas hydrophila*, this percentage was higher than that recorded by El-Gohary *et al*. (2020) who obtained only one isolate with 12.5% percentage from cultured tilapia water of four farms.

Although, most bacteria can be successfully isolated following their culture on specific media, this traditional method of diagnosis failed to accurately *Aeromonas* species due to their phenotypic diversity (Puthucheary *et al*., 2012). In contrast, molecular based identification can accurately identify *Aeromonas* species more specifically and in a more sensitive and reliable way. PCR was successfully applied to *Aeromonas hydrophila* using two genes, the first one was 16s RNA gene which identified the isolates to genus level and the second was the housekeeping gene (*gyr-β*) which confirmed *Aeromonas hydrophila* isolates a conserved gene of *Aeromonas hydrophila* with a specific amplicon size 1100 bp (Algammal *et al*., 2020) and was seen to be better molecular markers than the 16S rRNA gene for the study of taxonomic relationships at the species level (Reshma *et al*., 2015). The PCR amplification using *Aeromonas* species specific gene (16S rRNA) identified seventeen *Aeromonas* strains. This result agreed with those obtained by Zorriehzahra *et al*. (2020) and Bakiyev *et al*. (2022). However, our result was higher than that identified by Zorriehzahra *et al*. (2020) who identified only five *Aeromonas* species isolates using the same 16S rRNA specific gene for *Aeromonas* isolates identification from tilapia fish and aquaculture water. *Gyr-β* gene identified fourteen *Aeromonas hydrophila* isolates, Our result was consistent with those reported by Algammal *et al*. (2020) and El-Gohary *et al*. (2020). However, our isolates number is lower than that identified using the same gene in identifying sixty-five *Aeromonas hydrophila* isolates from tilapia and their water (El-Gohary *et al*., 2020).

The extensive use of antibiotics in human therapy, as well as the huge misapplication in animal, and aquaculture industry has led in the emergence of antibiotic resistance the aquaculture (Marshall and Levy, 2011). *Aeromonas* species are ubiquitous aquatic bacteria, capable of acquiring mobile genetic elements containing antimicrobial resistance determinants (Conte *et al*., 2020). In addition, they may serve as reservoirs of antibiotic resistance genes that can be transferred to other pathogenic bacteria through horizontal gene transfer (Moura *et al*., 2012). Antibiotic susceptibility testing was performed on 14 positive *Aeromonas hydrophila* strains, which was confirmed by PCR. In the present study, resistance of aeromonads to amoxicillin (Table 5) was widespread, as 100% of the isolates showed resistance to amoxicillin, a beta-lactam antibiotic, this may be attributed to the natural aeromonads β-lactamase activity (Sarkar *et al*., 2013; Piotrowska *et al*., 2017). Our findings were consistent with previous studies stating that *Aeromonas* species are resistant to amoxicillin (El-Son *et al*., 2019; Rahman *et al*., 2021). In addition, our *Aeromonas* isolates were resistance to novobiocin (100%), and this resistance may be due to structural changes in the bacteria that render these antibiotics ineffective and the bacteria remain resistant (Kusdarwati *et al*., 2018). *Aeromonas* resistance to novobiocin (100%) is the same as in *Aeromonas hydrophila* isolated from Acipenser baerii (Bakiyev *et al*., 2022). In the current study *Aeromonas* colistin resistance (43%) is due to the *Aeromonas* outer membrane structure of lipopolysaccharide that weakens the binding of colistin with lipopolysaccharide wall (Kusdarwati *et al*., 2018). Our *Aeromonas* resistance to colistin was nearly similar to a previous study which showed only 66 and 33% resistance of *Aeromonas* isolated from freshwater and marine fish, receptively (Dahdouh *et al*., 2016). *Aeromonas hydrophila* resistance to doxycycline (50%), a tetracycline antibiotic, mainly due to the tet (E) gene which has been previously shown to be a dominant tetracycline resistance gene in the fresh fish cultured *Aeromonas* species (Han *et al*., 2012). In the current study *Aeromonas hydrophila* resistance to doxycycline is higher than that recorded by Nhinh *et al*. (2021) who recorded 2.2% of tilapia *Aeromonas hydrophila* resistance to doxycycline. *Aeromonas* resistance to streptomycin resistance (70.4%), this resistance level is slightly lower than that recoded by El-Son *et al*. (2019) who reported 100% resistance of *Aeromonas hydrophila* isolates from Nile tilapia to streptomycin, but higher than that recorded by Rahman *et al*. (2021) who identified 20% streptomycin among *Aeromonas hydrophila* isolated from walking catfish. *Aeromonas* resistance to enrofloxacin and ciprofloxacin, both were synthetic quinolones, was 14% among our isolates. the resistance of aeromonads to quinolones is mainly mediated by specific chromosomal mutations (Chenia, 2016). In our study 43% of *Aeromonas hydrophila* isolates exhibited resistance to chloramphenicol and this is in higher than that recorded by Salem *et al*. (2020) who reported 16% intermediate resistance of their *Aeromonas hydrophila* isolates to chloramphenicol. Furthermore, half (50%) of our isolates showed resistance to trimethoprim/sulfamethoxazole and this result is in higher than that obtained by Abu-Elala *et al*. (2015) and Tartor *et al*. (2021) who reported only 13%, and 17.9% of *Aeromonas hydrophila* isolated from Nile tilapia, Egypt were resistant to trimethoprim/sulfamethoxazole, receptively.

The resistance of our isolates to the tested antibiotics is due to their innate ability to acquire resistance as because their genomes contain integrone and plasmids that facilitate horizontal gene transfer (Piotrowska and Popowska, 2014). *Aeromonas hydrophila*`s high resistance levels to antimicrobials, might be caused by the vast and misuse of antibiotics either as growth promoters or therapeutic agents for various diseases or due to the introduction of antibiotics into tilapia ponds as a result of untreated sewage, industrial waste disposal, and agricultural activities, thereby contaminating the water with resistance genes (Conte *et al*., 2020).

The multiple antibiotic resistance index is calculated as the ratio of the number of antibiotics to which an isolate is resistant to the total number of antibiotics against which the organism is tested (Dhanapala *et al*., 2021). In our study, all fourteen tested isolates (100%) showed multiple antibiotic resistance index values above 0.2, indicating that the antimicrobials originated from commonly used high-risk sources of contamination (Skwor *et al*., 2020). Among those, four isolates showed 0.5 multiple antibiotic resistance index, three isolates showed 0.7 multiple antibiotic resistance index, two isolates exhiptited 0.6 index, and one isolates showed 0.8 multiple antibiotic resistance index. The multiple antibiotic resistance index range in the current study (0.3 –1) is comparatively wider when compared with previous studies. In the same context, multiple antibiotic resistance index ranges of 0.27 and 0.82 (El-Gohary *et al*., 2020) was recorded in *Aeromonas hydrophila* isolated from tilapia and their aquatics. The multiple antibiotic resistance index suggest that the isolate originated from a high-risk source (Skwor *et al*., 2020). *Aeromonas* species resistance to diverse groups of antibiotics is a major concern for human health as fish processing is usually done manually, and resistant bacteria can be transmitted to humans through the food chain or through direct contact from the aquatic environment (Amaravathi *et al*., 2016). All the identified *Aeromonas hydrophila* isolates were found to be resistant more than two classes of the tested antibiotics, making them multidrug-resistant pathogen (Sweeney *et al*., 2018). Multiple antibiotic resistant *Aeromonas hydrophila* presence in ready for marketing tilapia and water is regarded as an occupational risk factor (Praveen *et al*., 2016), because human can acquire infection through contact with fish or water (Igbinosa *et al*., 2012; Fewtrell and Kay, 2015).

The contribution of lipolytic and protease activity to the virulence of *Aeromonas hydrophila* is well documented, as these enzymes dissolve the host epithelium, accelerating their invasion and pathogenicity. *Aeromonas hydrophila* lipase and protease activity their action is due to the presence of genes in their genome (Tomás, 2012). Concerning public health significance, *Aeromonas hydrophila* detection in healthy, ready for marketing tilapia is a warning sign as most of the fish processing task as are usually performed manually (Ahmed *et al*., 2012; Amaravathi *et al*., 2016). *Aeromonas hydrophila* resistance to antibiotics beside their ability to acquire resistance, their several virulence activities enable their destruction activity in-vitro and in-vivo, due to the production of a variety of toxins such as hemolysin, aerolysin, lipase and protease (Pandey *et al*., 2010; De Silva *et al*., 2021). As well as, natural motility of *Aeromonas hydrophila* due to the flagella in their surface structure, that facilitates their adhesion and invasion (Piotrowska and Popowska, 2014). The expression of these virulence factors is due to the presence of virulence genes in their genome (Falcón *et al*., 2008). The production of these toxins has been regarded as

strong evidence of pathogenic potential in aeromonads and the possibility of infection with such virulent pathogen through contacts with water or during the handling of fish (Obi *et al*., 2007).

Conclusion

Aeromonas hydrophila is a zoonotic pathogen that may pose a danger to human health. The findings of the present study are considered an alert to the *Aeromonas hydrophila* contamination level in tilapia and their aquaculture water. Factors including poor sanitation and water quality, stress, overcrowding, and rough handling can make the fish more sensitive to infections and trigger outbreaks of *Aeromonas hydrophila*. All identified *Aeromonas hydrophila* isolates showed multiple antibiotic resistances and exhibited lipolytic and protease enzymatic activity, besides their gelatinase and hemolytic activity, which accelerates their pathogenicity. These results might be a warning to authorities concerned with the aquaculture industry to reduce the spread of resistant *Aeromonas hydrophila* in the culture systems, apply and search for effective disinfectants. As well as, enhance farmer awareness of the appropriate uses of antibiotics in fish farms. Personal protective equipment such as gloves should be worn during fish processing. Also, it is important to encourage scientists to search for an alternative non-antibiotic control strategy for such pathogens and other bacterial infections in farmed fish.

Conflict of interest

The authors declare that they have no conflcit of interest.

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