Summer mortality syndrome bacterial pathogens in farmed Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Background: The high summer mortality in many fish farms, which had detrimental economic and social implications, was a serious challenge that the fish industry had to deal with.

Aim: With an examination of the most effective antibiotic, the ongoing research was intended to shed light on the identification of the main bacterial pathogens associated with the summer mortality syndrome in the diseased farmed Nile tilapia.

Methods: Six hundred dead Nile tilapia samples that had suffered from summer mortality were collected from several fish farms between May and October of 2022. The gathered fish displayed hemorrhagic areas on the skin, scale detachment, fin degeneration, erosions, skin ulcers, and corneal opacity with unilateral and/or bilateral exophthalmia. The most prominent internal appearance was swelling of the internal organs with sanguineous ascetic fluid.

Results: There were 225 bacterial isolates found. Six species were identified through phenotypic and biochemical analysis; they were *Aeromonas*, *Vibrio*, *Streptococcus*, *Pseudomonas*, *Enterococcus*, and *Edwardsiella* spp., in descending percentage, respectively. *Aeromonas* spp., *Vibrio* spp., and *Streptococcus* spp. were the three most frequent isolated bacterial pathogens. The identification of *Aeromonas hydrophila*, *Vibrio* spp., and *Streptococcus iniae*, the three most common bacterial isolates, was confirmed by molecular analysis by polymerase chain reaction. Most of the tested strains were found to be responsive to Ciprofloxacin (CIP), Gentamicin (CN), and Chloramphenicol (C) but resistant to Amoxicillin (AMX), according to an antibiotic sensitivity test.

Conclusion: The three most dangerous common bacterial infections discovered during mass-farmed tilapia summer mortality are *A. hydrophila*, *Vibrio* sp., and *S. iniae*. This makes it clear that high water temperatures may raise the possibility of bacterial infections, which could cause widespread tilapia mortality and substantial financial losses. Therefore, it is crucial to maintain a beneficial fish culture, environment, and husbandry practices to enhance the tilapia-rearing environment and lessen the virulence of the disease. Isolated bacterial strains showed low levels of resistance to AMX but were vulnerable to CIP, CN, and C.

Keywords: *Aeromonas hydrophila*, Nile tilapia, *Streptococcus iniae,* Summer mortality, *Vibrio* sp.

Introduction

Aquaculture is now one of the most crucial methods for supplying the market because natural fisheries have been badly depleted and are no longer a reliable source of protein for humans (Mohamed *et al.*, 2017; Adelekea *et al.*, 2021). According to Reda *et al.* (2021), Egypt is the world's third-biggest tilapia manufacturer and the aquaculture leading supplier in Africa. In addition to serving as an excellent provider of protein, tilapia is regarded as one of the essentials to ensuring the security of food, especially in densely inhabited nations like Egypt (Hussain *et al.*, 2011; Abbas *et al.*, 2017; El-Sayed, 2019). Moreover, many young individuals find employment in this sector as a means of support (Dickson *et al.*, 2016).

Extensive fish farming is unfortunately plagued by plenty of issues, such as diseases, that discourage farmers because of the financial penalties (Eltholth

et al., 2015). Summer high mortality in several aquaculture facilities and the Nile Delta, which has had detrimental economic and social impacts, has been a key concern for this industry since 2013 (Fathi *et al.*, 2017; Ali *et al.*, 2020). The agents responsible for these outbreaks have been traced in several trials. Studies have revealed a number of variables, such as bacterial, fungal, viral, and parasitic causes, that may contribute to tilapia mortality (Yambot, 1998; Ye *et al.*, 2011; Eissa *et al.*, 2015; Fathi *et al.*, 2017; Nicholson *et al.*, 2020; Youssuf *et al.*, 2020).

Under stressed conditions, farmed Nile tilapia is vulnerable to several pathogenic microorganisms (Shaheen *et al.*, 2013; Eissa *et al.*, 2015). The most common cause of the large annual losses in the fish farming industry, which are projected to be worth billions of dollars (Elsheshtawy *et al.*, 2019; Algammal *et al.*, 2020), has been identified as bacterial infections. When water temperatures rise, opportunistic microorganisms in particular represent a serious threat to aquaculture (Pridgeon and Klesius, 2012). The potential for a bacterial infection to result in extensive fish mortality is a complex situation, and many aspects linked to the environment, host, and microbes may collaborate to show the progression of diseases (Harvell *et al.*, 1999). Moreover, disease outbreaks may be sparked by environmental factors including temperature, salinity, oxygen level, and pH, especially throughout intensive aquaculture systems (Eissa *et al.*, 2013).

Due to the serious practices involved in fish farming, infectious illnesses constitute the main issue in aquaculture facilities and have a significant financial impact (Bulfon *et al.*, 2015). *Aeromonas veronii* biovar *sobria* infection was linked to widespread deaths of cultured tilapia in the El-Sharkia governorate (Eissa *et al.*, 2015; Elsheshtawy *et al.*, 2019; Reda *et al.*, 2021). However, Fathi *et al.* (2017) linked these fatalities to tilapia lake virus infection.

According to Fang *et al.* (2004), *Aeromonas* is a dangerous disease that has the potential to spread internationally and produce epidemics in fish farming with extraordinarily high fatality rates. *Aeromonas* spp. are facultatively anaerobic, Gram-negative, oxidase-positive bacteria, according to Stratev and Odeyemi (2016). Aeromonads can be found in several kinds of foods, including fish, prawns, mussels, milk, meat, and vegetables, according to Neyts *et al.* (2000). They are frequently found in the aquatic environment. In tilapia farming, *Aeromonas hydrophila* has been found to be the most abundant species Hassan *et al.* (2017).

Several *Vibrio* species are also widely recognized for their capability to lead to serious fish diseases. The demise of the population occurs when mortality in raised fish happens frequently during the earliest larval stages with rapid incidence (Thompson *et al.*, 2004). The majority of the nonspore-producing, facultative, Gram-negative members of this family, Vibrionaceae, are typically distinguished by comma-shaped rods.

According to Gobarah *et al.* (2023), the most typical heterotrophic microorganisms in the seawater ecosystem are *Vibrio* species. They are found in large numbers in brackish and/or coastal seawaters and have been isolated from fish, shellfish, and saltwater.

One of the bacterial infections in aquaculture that is most frequently reported, streptococci are a nonsporing, facultatively anaerobic, Gram-positive bacterial pathogen that is regarded as a difficult problem in Egypt's tilapia and catfish production (Liamnimitr *et al.*, 2017). Many *Streptococcus* species can cause fish mortality; *Streptococcus iniae, Streptococcus agalactiae,* and *Streptococcus dysgalactiae* (Chen *et al.*, 2007; Abdelsalam *et al.*, 2010 and 2013). The most prevalent fish pathogen with a significant detrimental effect on aquaculture is *S. iniae* (Acar *et al.*, 2015). It has a lot of virulence genes, which allow it to get inside fish and other aquatic creatures, reproduce, and evade their immune systems (Vazirzadeh *et al.*, 2019).

The goal of the ongoing research was to spotlight light on the identification of main bacterial pathogens involved in summer mortality syndrome isolated from diseased farmed *Oreochromis niloticus* throughout the summer season in the province of Kafr El-Sheikh, Egypt, and to investigate the antibiotic sensitivity test for the most predominant ones.

Materials and Methods

This investigation was executed during the summer months of 2022 at the Fish Diseases Laboratory, Veterinary Medicine Faculty at Kafrelsheikh University, Egypt.

Samples collection and gross examination

From May to October 2022, 600 (at a rate of 100 fish/month) clinically decrepit Nile tilapia, *O. niloticus* fish, with varying sizes (30–300 g), were randomly sampled alive or recently dead from multiple tilapia farms in Kafr El-Sheikh governorate. Fish farms, where samples of fish were gathered, suffered from severe summer mortalities with gross lesions of anorexia, surface swimming, hemorrhagic patches over the skin, scale detachment, fin degeneration and erosions, skin ulcers, corneal opacity with unilateral and/or bilateral exophthalmia. Samples of fish were collected, placed in a sterilized, sturdy polyethylene bag with pumped water and compressed oxygen in half of the bag's volume, and immediately transported, while surviving, to the bacteriological section at the Central Diagnostic Laboratory, Fac. Vet. Med. There, the fish samples were kept alive with proper preparation and indefinite aeration using an electronic aerator until the examination (Hamouda *et al.*, 2019). According to Eissa (2016), *O. niloticus* underwent a clinical examination to document any postmortem lesions and evident clinical anomalies.

Isolation of the bacteria responsible for tilapia farms' summer mortality

Skin, liver, kidney, spleen, and intestine tissue specimens from the captured tilapia were aseptically collected for bacteriological examination. Tissue specimens were first pre-enriched over Tryptic Soy Broth (Difco, Detroit, MI) and the incubation process was for 18–24 hours at 28°C using a sterilized loop. Next, they were dispersed out of tryptic soy agar (TSA) and kept for 48 hours at 28°C following preliminary enrichment on tryptic soy broth (TSB) (Austin and Austin, 2012).

Pure isolated colonies over TSA have been then striped on selective diagnostic agar medium, following Markey *et al.* (2013), where Rimler Shotts (RS) agar for *Aeromonas* sp., *Pseudomonas* sp.*,* and *Edwardsiella* sp.; Thiosulphate Citrate Bile salts Sucrose agar (TCBS) (Oxoid, UK) for *Vibrio* spp., *Pseudomonas*-F-agar base media for *Pseudomonas fluorescens*, blood agar, Edwards Medium (modified) (Oxoid, UK) with the addition of 5%–7% bovine blood and colistin sulfate 5 ml/l per manufacturer's instructions and *Streptococcus* selective agar was used to test for *Streptococuss* sp. for 24 hours at 28°C–37°C. In addition, to isolate *Enterococi* bacteria, specimens of tissues were pre-enriched on *Enterococcus* M broth medium and selectively distributed on BBLTM Enterococcosel Agar (EA) before being incubated for a night at 37°C. The classification of colonies of bacteria was done by Reynolds (2011). On the same media, individual colonies of well-differentiated single bacterial colonies were produced, and these were subsequently identified. Gram's stain was applied to the smears from the presumed pure colonies, and they were then examined using an oil immersion lens. A single representative colony was taken from each type of agar media, purified onto it, and then inoculated into brain heart infusion broth (Sigma-Aldrich). This broth included 15% glycerol and was aerobically incubated at 28°C for 24 hours before being conserved at −80°C.

Identification of the bacteria responsible for tilapia farms' summer mortality

The bacterial isolates underwent phenotypic identification as explained previously (Schaperclaus *et al.*, 1992; Bergey's, 1994). It was tested for motility and Gram staining (Cruickshank *et al.*, 1975).

Furthermore, every isolate was biochemically verified using the standards outlined by Kreig and Holt (1984) and MacFaddin (2000). Biochemical examination tests such as catalase, oxidase, voges proskauer, methyl red, indole, triple sugar iron, citrate utilization, gelatin liquefaction, urease, starch hydrolysis, arginine, and lysine decarboxylase as well as sugar utilization and oxidation-fermentation tests were carried out for the recognition as well as bacterial strain distinguishing characters and the outcomes were contrasted with the reference strains for the identified bacteria (obtained from the Fish diseases department, Fac. Vet. Med., Kafrelsheikh University, Egypt) (Quinn *et al.*, 2002; Nicky, 2004 and Markey *et al.*, 2013) (Table 1).

Molecular identification by polymerase chain reaction (PCR)

DNA extraction for *A. hydrophila* and *Vibrio* sp. using QIA amp kit

The QIA amp kit (QIAamp: Qiagen Inc., USA) was used for the DNA extraction process. Correctly, 1 ml of the suspension of the young cell was centrifuged at 10,000 g for ten minutes at 4°C. The pellet of cells was then combined with 600 µl of guanidine hydrochloride buffer (pH 8.0), kept at ambient temperature for 30 minutes, and then centrifuged again at 10,000 g for 10 minutes at 4°C. 500 l of the supernatant was relocated to a different tube, blended with 100% ice-cold ethanol, and centrifuged at 13,000 × g for 20 minutes at 4°C. After washing the pellet with 95% and 90% ethanol, respectively, and discarding the supernatant, it was centrifuged at 10,000 g for 1 10 minutes at 4°C. Before being utilized as a PCR template, the pellet was reconstituted in 50 l of molecular-grade water and kept at −20°C (Haldar *et al.*, 2005).

DNA extraction of *Streptococcus* species

Boiling techniques were used to separate the isolates' genomic DNA from a pure culture. From the slants of the previously retrieved bacteria, three to five characteristic colonies of the same morphological type were extracted and they were enriched for 18 hours at 37°C in a tube containing 2 ml of TSB. A microcentrifuge tube containing 1 ml of the enhanced bacterial culture was centrifugated at 8,000 × g for 2 minutes, after which the sediment was homogenized with nuclease-free water and heated at 95°C for 15 minutes. After centrifuging

Table 1. The primers were used for the amplification of different associated genes for isolated summer mortality bacteria.

Target bacteria	Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
Aeromonas hydrophila	16S rRNA	5′GAAAGGTTGATGCCTAATACGTA ′3 5′ CGTGCTGGCAACAAAGGACAG ′3	625 bp	Gordon *et al.*, 2007
Vibrio spp.	16S rRNA	5′*CGGTGAAATGCGTAGAGAT*′3 5′*TTACTAGCGATTCCGAGTTC*′3	663 bp	Tarr *et al.*, 2007
Streptococcus iniae	Sin1 Sin2	5′GTAGAGTACACATGTACT′3 5′GGATTTTCCACTCCCATTAC ′3	300 bp	Zlotkin *et al.*, 1998

the boiled lysates, the supernatant was employed as a DNA template. To identify *Streptococcus*, all DNA samples were brought to the Central Diagnostic and Research Laboratory.

PCR identification system by using primer sequences
Table 1 introduces the primers that were used to identify the 16srRNA gene in *A. hydrophila,* the *Vibrio* sp., and the Sin gene in several *Streptococcus* species.

PCR protocol for the isolates
According to the instructions on the Emerald Amp GT PCR master mix (Takara, Cat. PR310A) kit, the conditions for cycling for each gene were set up. The process of amplification was carried out on an Eppendorf MasterCyclerR (Eppendorf AG, Hamburg, Germany) in a total reaction volume of 25 µl, which contained 12.5 µl of the EmeraldAmp GT PCR Master Mix, 1.5 µl of each forward and reverse primer, 4.5 µl of molecular biology grade water, and 5 µl of the test DNA.
The amplification conditions for *A. hydrophila* (using primers targeting 625 bp) and *Vibrio* sp. (using primers targeting 663 bp) were; 5 minutes of primary denaturation at 94°C, 35 cycles of secondary denaturation for 30 seconds at 94°C, annealing (16S rRNA PCR 40 seconds at 56°C for *Vibrio* while 40 seconds at 50°C for16S rRNA for *A. hydrophila* and extension at 72°C for 45 seconds (16S rRNA and 1 minutes). A final extension was adjusted for 10 minutes. The amplification conditions for *S. iniae* (using primers targeting 300 bp) were performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). PCR assays were adopted in a 25 µl reaction mixture consisting of 12.5 µl of 2× MasterMix (intron), 1.5 µl of 20 pmol of each oligonucleotide primer, 5 µl of DNA template, and 4.5 µl Milli-Q water. The amplification profile consisted of an initial denaturation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension of 72°C for 10 minutes. Following amplification, the amplified DNA fragments were analyzed by 1.5% agarose gel electrophoresis (Applichem, Germany, GmbH). Lastly, the gel was photographed and seen under a UV transilluminator after being stained with ethidium bromide. To calculate the fragment sizes, a 100 bp plus DNA ladder (Qiagen, Germany, GmbH) was utilized (Moustafa *et al.*, 2021).

Antibiotic susceptibility testing (Antibiogramme)
The retrieved *Aeromonas*, *Vibrio*, and *Streptococcus* strains were subjected to an *in-vitro* antibiotic sensitivity test to determine their susceptibility to various antibiotics utilizing the disc diffusion method (Koneman *et al.*, 1997). The following antimicrobial agents were used: amoxicillin (AMX10 Mg), ciprofloxacin (CIP5 Mg), gentamicin (CN) (GEN10 Mg), and streptomycin (S). At least 4–5 colonies of each isolated strain were seeded into Mueller Hinton broth tubes, which were then cultured for 24 hours at 37°C. One ml of the infected broth was then applied to the Mueller Hinton agar

plates, and they were incubated at 37°C for 24 hours. The data were interpreted by CLSI (2016).

Ethical approval
All procedures, handling of animals, and testing procedures employed in the current research were completed in compliance with the applicable standards and rules issued by Kafrelsheikh University's animal ethics committee.

Results

Clinical and postmortem examination
Anorexia, lethargy, surface swimming, body hemorrhages, scale detachment, fin degeneration and erosions, and corneal opacity with unilateral and bilateral exophthalmia (popeyes) were all symptoms

Fig. 1. Naturally infected *O. niloticus* showed body haemorrhages (Black arrow) and corneal opacity (Red arrow) with unilateral and bilateral exophthalmia (popeyes) (Yellow arrow) in (A–C), scale detachment (Blue arrow) in (D), fin degeneration and erosions (White arrow) in (E), while postmortem examination showed friable enlarged liver with marbling appearance (Yellow arrow), with splenomegaly (Red arrow) and congested gills (White arrow) in (F).

of naturally infected *O. niloticus* (Fig. 1A–E). While this was going on, a postmortem evaluation of the animal revealed an accumulation of the abdominal cavity's sanguineous ascitic fluid, a marbled (mosaic) appearance to the liver, and an enlarged, friable liver with a dilated gall bladder, splenomegaly, and congestion in gills, as shown in Figure 1F.

Bacterial isolation and identification

The naturally infected fish included 225 bacterial isolates, which were isolated. These isolates were phenotypically and biochemically characterized by the generally accepted technique published for bacterial isolation and identification. *Aeromonas* spp., *Vibrio* spp., *Pseudomonas* spp., *Edwardsiella* spp., *Enterococcus* spp., and *Streptococcus* spp. were among the isolates that were detected.

All isolated bacterial strains have been identified phenotypically and biochemically, as shown in Table 2. *Aeromonas hydrophila*, *sobria*, and *veronei* were identified biochemically as separate species. The colonies of *Aeromonas* spp. on TSA medium looked glossy, spherical, creamy, and convex after 24 hours of incubation, but colonies on RS medium were light-yellow or deep creamy with the full edge. The isolated

Table 2. The morphological and biochemical characteristics of isolated bacteria from naturally infected *O. niloticus*.

Identification	Aeromonas hydrophila	Aeromonas veronei.	Aeromonas sobria	Pseudomonas fluorescens	Vibrio alginolyticus	Vibrio harveyi	Vibrio vulnificus	Edwardsiella tarda	Enterococcus faecalis	Streptococcus iniae
Gram-stain	−ve	−ve	−ve	−ve	−ve	−ve	−ve	−ve	+ve	+ve
Motility	+	+	+	+	+	+	+	+	-	-
Shape	Short rod	Short rod	Short rod	Long curved rod	Curved rod	Curved rod	Curved rod	rod	Cocci	Cocci
O/F	F	F	F	O	F	F	F	ND	F	F
Cytochrom oxidase	+	+	+	+	+	+	+	-	-	-
Growth on 0%NaCl	+	+	+	+	+	_	+	+	+	+
6% NaCl	-	-	-	+	+	+	+	-	+	+
3.5% NaCl	+	+	-	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	-	-
Indol	+	+	+	-	+	+	-	+	-	-
H$_2$S (TSI)	+	-	-	-	+	-	-	-	-	-
Citrate utilization	+	+	+	+	-	+	+	-	-	-
Methyl red	+	V	V	-	+	+	+	+	-	+
Gelatin liquefaction	+	+	V	+	V	-	+	ND	V	-
Voges proskauer	+	+	-	-	+	-	-	-	+	-
Starch hydrolysis	-	-	-	-	-	-	-	-	+	+
Urease	-	-	-	+	-	-	-	-	-	-
Arginine dehydrogenase	+	V	-	+	+	-	-	-	+	+
Lysin decarboxylase	+	+	+	-	+	-	-	+	-	-
Ornithine decarboxylase	-	+	-	-	+	-	+	+	-	+
Acid production from										
Arabinose	+	-	-	+	-	-	-	V	-	-
Sucrose	+	+	+	+	+	+	-	-	+	+
Salicin	V	-	-	-	-	-	+	-	+	+
Maltose	+	+	+	-	-	-	-	+	+	+

(+ve): Positive; (-ve): Negative; (V): variable; (ND): Not Done.

Aeromonas species were fermentative, catalase-producing, short rod, oxidase-positive, motile on semisolid medium, and Gram-negative.

Edwardsiella tarda was further recognized biochemically as one of the *Edwardsiella* spp. All isolated *Edwardsiella* spp. emerged as greenish colonies with black centers over RS medium, which serves as a selective diagnostic media for *E. tarda*. The bacteria were determined to be motile, Gram-negative, short rod-shaped, and oxidase-negative organisms.

In addition, *Pseudomonas* spp. colonization on TSA media displayed spindle form and diffusible dim yellow-green fluorescent pigments after 24 hours of incubation, whereas greenish colonies were noticed on the RS medium. On *Pseudomonas*-F-agar medium, however, it showed colonies that were 2–3 mm in diameter, yellowish green in color, and fluoresced after 48 hours of incubation. *Pseudomona fluorescens* was identified biochemically as *Pseudomonas* spp. All isolated *Pseudomonas* species were oxidase-positive, motile, Gram-negative, long, curved rods.

However, additional biochemical identification was done for the *Vibrio* spp.; *Vibrio harveyi*, *Vibrio alginolyticus,* and *Vibrio vulnificus*. Bent rods, fermentative, Gram-negative, and positive for methyl red, catalase, and oxidase were all characteristics of the isolated *Vibrio* species. *Vibrio vulnificus* generated green colonies on TCBS media, whereas *V. alginolyticus* and *V. harveyi* generated yellow colonies.

Colonies of *Enterococcus* sp. emerged on *Streptococcus* selective agar as extremely small, spherical, white colonies with complete borders or creamy rounded, big colonies (2–3 mm) with dew drop-like characteristics. An *Enterococci* displayed dark brown and black colonies on BBLTM EA. According to staining with Gram stain, *Enterococcus faecalis* is Gram-positive and is clustered in couples and occasionally short chains. *Enterococcus faecalis* is nonmotile bacteria and is negative for catalase, oxidase, and lysine decarboxylase.

Biochemical testing identified *S. iniae* as one of the species of *Streptococcus*. It is a nonmotile, Gram-positive cocci that is typically organized in chains and is oxidase and catalase negative. Colonies typically have a greyish to pale color and shine over Edwards agar media. On blood agar, complete hemolysis was present in small, transparent, or pigmented white colonies up to 2 mm in diameter.

Incidence of bacterial isolates in different organs and tissues

According to Table 2, 225 bacterial isolates were discovered in the liver, kidney, spleen, and intestine of naturally ill fish. The total amount of isolated bacteria found in various tissues (liver, kidney, spleen, and intestine) revealed six species; *Aeromonas* spp. (33.33%), *Edwardsiella* spp (4%), *Pseudomonas* spp (6.66%), *Vibrio* spp. (32.46%), *Enterococcus* spp. (4.88%), and *Streptococcus* spp. (13.33%). There were numerous instances of mixed infections in the same fish. *Aeromonas* spp. (75 isolates), *Vibrio* spp. (73 isolates), and *Streptococcus* spp. (30 isolates) were the three most common bacterial species. Prevalent bacterial isolates were chosen at random and tested by PCR. According to Table 3, the liver had the greatest percentage of isolated bacteria (37.7%), followed by the kidney (29.3%), spleen (22.6%), and the intestine (10.2%). In addition, the kidney, liver, and spleen yielded the bulk of the isolates of *Aeromonas* spp., *Vibrio* spp., and *Streptococcus* spp., while the gut had the lowest prevalence rates.

Molecular identification

To confirm the biochemically identified species, conventional PCR was used for molecular characterization. First, for the seven *Aeromonas* isolates chosen, *Aeromonas* spp. isolates identified utilizing the 16S rRNA region unique for *A. hydrophila* produced an amplified product of 625 bp size for five isolates, whereas two isolates were negative (Fig. 2).

Examining seven isolates of *Vibrio* species for the genus gene (16s rRNA gene) allowed researchers to assess the genotypic characteristics of the species. As

Table 3. Prevalence and distribution of isolated bacteria from examined lesion samples.

Isolated bacteria	Liver		Kidney		Spleen		Intestine		Total	
	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Aeromonas sp.	30	13.3	23	10.22	15	6.7	7	3.11	75	33.33
Edwardsiella sp.	3	1.33	1	0.44	3	1.33	2	0.89	9	4
Pseudomonas sp.	7	3.11	2	0.89	3	1.33	3	1.33	15	6.66
Vibrio sp.	27	12	21	9.33	22	9.8	3	1.33	73	32.46
Enterococcu sp.	3	1.33	5	2.22	1	.44	2	0.89	11	4.88
Streptococcus sp.	10	4.44	11	4.89	5	2.22	4	1.78	30	13.33
Other spp	5	2.22	3	1.33	2	2.22	2	0.89	12	5.33
Total	85	37.7	66	29.3	51	22.6	23	10.2	225	100

(%): Percentage in relation to total number of isolated bacteria; (No.): Number.
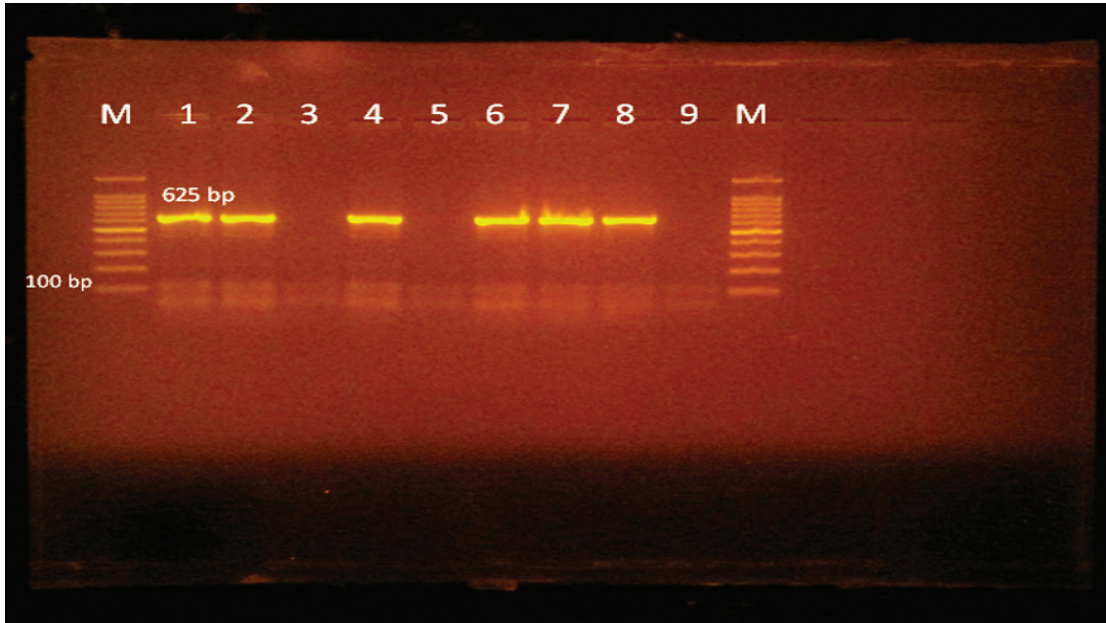
Fig. 2. Agarose gel electrophoresis of PCR products of 16S rRNA (625 bp) as species specific gene for confirmation of *A. hydrophila* isolated from *O. niloticus*. Lane M: 100–1,000 bp. DNA Ladder. Lane (1,2,4,6&7): positive *A. hydrophila* strains. Lane (3&5): negative *A. hydrophila* strains. Lane 8: positive control for *A. hydrophila* (at 625 bp). Lane 9: negative control for *A. hydrophila*.
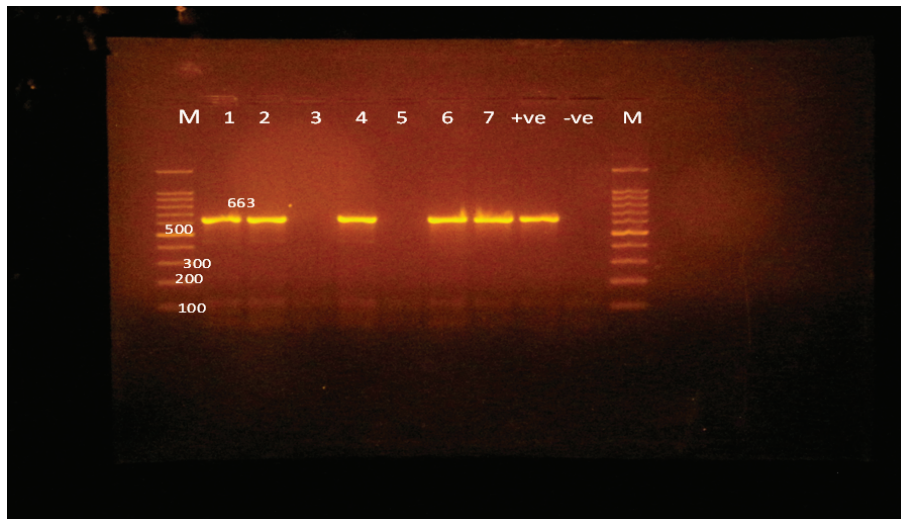

Fig. 3. Agarose gel electrophoresis of PCR products of 16S rRNA (663 bp) as species specific gene for confirmation of *Vibro* spp isolated from *O. niloticus*. Lane M: 100–1,000 bp. DNA Ladder. Lane (1,2,4,6&7): positive *Vibro* spp. Lane (3&5): negative *Vibro* spp. Lane +ve.: positive control for *Vibro* spp (at 663 bp.). Lane -ve .: Negative control or *Vibro* spp.

shown in Figure 3, five out of the seven isolates showed positive outcomes for the 16s rRNA gene, which has a molecular weight of 663 bp.

Streptococcus isolates were identified using (*Sin1, Sin2*) specific genes for *S. iniae*, 16 *Streptococcus* isolates were tested and 15 were positive at 300 bp size, whereas 1 isolate was negative, as shown in Figure 4.

Antimicrobial susceptibility assay of prevalent isolated bacteria
To assess their susceptibility and the incidence of various types of antibiotic resistance among these isolates, the three common species isolates (*A. hydrophila*, *Vibrio* sp., and *S. iniae*) were tested against eight antimicrobial drugs in the current investigation (Table 4). The findings
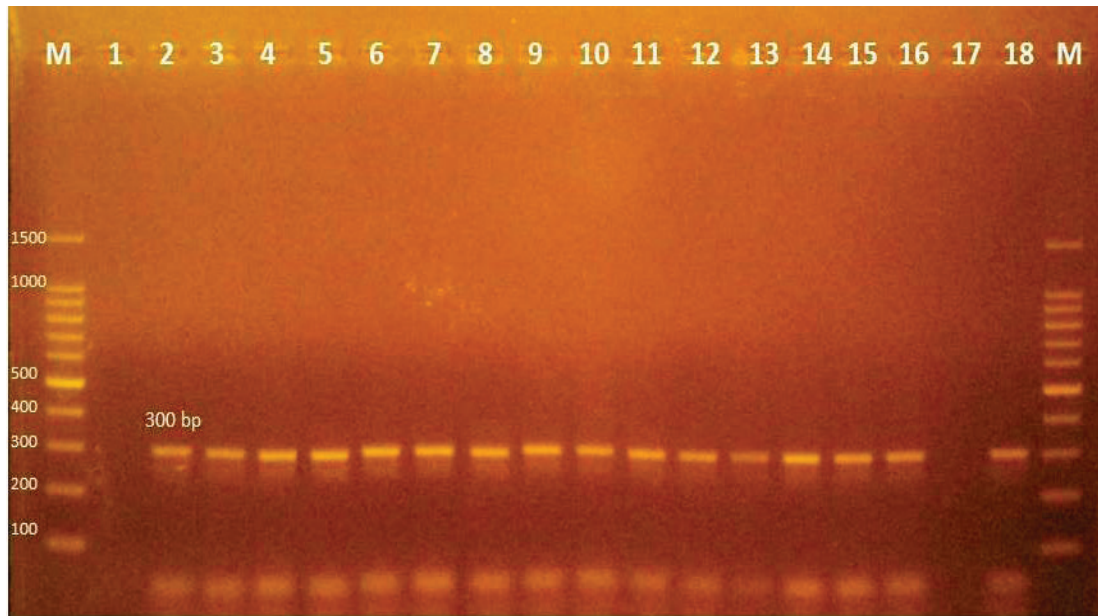
Fig. 4. Agarose gel electrophoresis of PCR products of Sin1, Sin2 (300 bp) virulence genes for characterization of *S. iniae*. Lane M: 100-bp DNA ladder. Lane (2–16): positive *S. iniae*. Lane (1): negative *S. iniae* . Lane (17): negative control *S. iniae*. Lane (18): positive control *S. iniae* (300 bp).

Table 4. Antibiotic sensitivity for *A. hydrophila*, *Vibrio* sp.*,* and *S. iniae*.

Antimicrobial agents	Disk Conc.	*Aeromonas hydrophila*	*Vibrio* spp.	*Streptococcus iniae*
CIP	5 µg	S	S	S
O	30 µg	S	R	R
AMX	10 µg	R	R	R
COT	25 µg	S	R	R
E	15 µg	S	R	R
CN	10 µg	S	S	S
C	30 µg	S	S	S
S	10 µg	S	R	R

(R): Resistant; (S): Sensitive.

demonstrated that most *A. hydrophila* isolates were vulnerable to all antimicrobial substances, apart from AMX, against which they displayed resistance. While some strains of *Vibrio* and *S. iniae* were resistant to Oxytetracycline (O), AMX, CO-Trimethoprim-Sulfamethoxazole (COT), Erthyomycin (E), and S, certain strains of these bacteria were responsive to CIP, CN, and Chloramphenicol (C). All isolates showed high levels of resistance to AMX. However, for all isolated bacteria, CIP, CN, and C were the preferred medications.

Discussion

Even though Egypt's aquaculture industry has had a remarkable development, it continues to face several difficulties due to disease outbreaks (Soliman and Yacout, 2016; ElSheshtawy *et al.*, 2019). The most extensively cultivated species in Egypt is the Nile tilapia. Identical to other types of farmed fish, tilapia is sensitive to a variety of bacterial diseases that are common in freshwater environments, particularly in tough circumstances (Dong *et al.*, 2017).

Bacterial fish infections are a real threat to aquaculture (Bentzon-Tilia *et al.*, 2016), resulting in outbreaks with large mortality rates (Sekar *et al.*, 2008). The currently identified recovered pathogenic bacteria from farmed Nile tilapia constitute proof of the widespread geographic extent of pathogenic bacterial diseases. Current retrieved bacteria were recognized as *Aeromonas* spp. (*Aeromonas sobria, A. hydrophila, and A. veronei*), *Edwardsiella* spp. (*E. tarda*), *Pseudomonas*

spp. (*P. fluorescens*.), *Vibrio* spp. (*V. harveyi, V. alginolyticus,* and *V. vulnificus)*, *Enterococcus* spp. *(Enterococcus faecalis),* and *Streptococcus* spp. (*Streptococcus iniae*).

The fish that were being examined exhibited extensive septicemic symptoms comparable to those mentioned by Austin and Austin (2007) and Matter *et al.* (2018). Such symptoms may be caused by the pathogenic enteric bacteria's virulence components. It is speculated that pathogenic bacteria prove to be more virulent than nonpathogenic bacteria because they possess virulence genes. Both nonpathogenic and pathogenic bacteria encompass virulence genes, but only pathogenic bacteria can employ such genes (Moustafa *et al.*, 2016). The anorexia and frayed and sloughed fins of the diseased fish may have contributed to their lethargy and sluggish movement, which ultimately affected their ability to swim and perform other bodily functions. The source of the hemorrhages throughout the body could be the hemolysin produced by the *Aeromonas* retrieved bacteria, which hemolyzes red blood cells (RBCs), in addition to the elastase enzyme, which significantly contributes to vascular injury because the blood arteries are primarily made up of elastic and collagenous fibers. The clinical symptoms seen in this study almost the same as those identified by previously (Ahmed and Shoreit, 2001; El-Ashram, 2002; Pretto-Giordano *et al.*, 2010; Yardimci and Aydin, 2011; Noor El-Deen *et al.*, 2014; Soliman *et al.*, 2014; Othman *et al.*, 2015; Dar *et al.*, 2016; Omar *et al.*, 2016; Abd El-Tawab *et al.*, 2017; El-Barbary, 2017; Laith *et al.*, 2017; Osman *et al.*, 2017; Pech *et al.*, 2017; Saleh *et al.*, 2017; Amrullah *et al.*, 2018; Khalil and Emeash, 2018; Hamouda *et al.*, 2019; Hassan *et al.*, 2020).

Exophthalmia in the ongoing research may be a result of the *S. iniae* cytotoxic effects on the fish cells or fluid accumulation in the orbits behind the eyeballs (Locke *et al.*, 2007). It is possible that the virulence factor directly causes cytotoxicity to the fish cells, loss of scales, and detachment as well as fins erosion. The virulence factors that *S. iniae* produce, which cause the death of blood cells and result in hemorrhage in many parts of the body, may also be responsible for the hemorrhagic patches over the mouth, operculum, and pectoral fins. *S. iniae* virulence factors may be responsible for eye cloudiness because they make it easier for *Streptococcus* to invade fish eyes, which causes cloudiness from bacterial proliferation (Moustafa *et al.*, 2021).

The postmortem inspection of the body in the current study revealed an accumulation of sanguineous ascitic fluid within the abdominal cavity, a mosaic-like pattern on the liver, and an enlarged, friable liver with a dilated gall bladder, splenomegaly, and clogged gills. The postmortem modifications could be caused by the identified septicemic pathogens and their virulence genes. The postmortem outcomes presented in this study are highly comparable to those investigated by a wide number of investigators in earlier publications

(Pritchard and Lin, 1993; Ahmed and Shoreit, 2001; Khafagy *et al.*, 2009; Musa *et al.*, 2009; Umesha *et al.*, 2011; Soliman *et al.*, 2014; Dahdouh *et al.*, 2016; Moustafa *et al.*, 2016, Omar *et al.*, 2016, 2017; Ortega *et al.*, 2017; Osman *et al.*, 2017; Saleh *et al.*, 2017; Ahmed *et al.*, 2018; El-Gamal *et al.*, 2018; Hardi *et al.*, 2018; Hamouda *et al.*, 2019; Mansour *et al.*, 2019; Hassan *et al.*, 2020; Gobarah *et al.*, 2021; Moustafa *et al.*, 2021).

In the current study, the assumed identification of the retrieved bacteria was conducted phenotypically through colony morphology on a selective differential medium for every suspected isolate; RS agar for *Aeromonas* spp. (Sarkar *et al.*, 2013)., *Pseudomonas* sp. and *Edwardsiella* sp. (Buller, 2004; Acharya *et al.*, 2007; Das *et al.*, 2014); *Pseudomonas*-F-agar base medium for *P. fluorescens* (Omar *et al.*, 2017), TCBS for *Vibrio* spp. (Gobarah *et al.*, 2021); blood agar, Edwards Medium (modified), and *Streptococcus* selective agar for *Streptococcus* spp. (Saleh *et al.*, 2017) and BBLTM EA for the isolation of Enterococci bacteria (Hamouda *et al.*, 2019). Moreover, biochemical analyses seemed to be a useful technique for classifying and differentiating bacterial fish pathogens (Austin *et al.*, 1997; Sarkar *et al.*, 2012).

Gram staining, colonies' shape and biochemical activity are remarkably similar to those of the bacteria previously reported by a large number of prior authors (Ahmed and Shoreit, 2001; Masbouba, 2004; Khafagy *et al.*, 2009; Noga, 2010; Soliman *et al.*, 2014; Dar *et al.*, 2016; Moustafa *et al.*, 2016; Omar *et al.*, 2016; Abd El-Kader and Mousa-Balabel, 2017; Abd El-Tawab *et al.*, 2017; El-Barbary, 2017; Laith *et al.*, 2017; Omar *et al.*, 2017; Osman *et al.*, 2017; Rahmatullah *et al.*, 2017; Saleh *et al.*, 2017; El-Gamal *et al.*, 2018; Hardi *et al.*, 2018; Ortega *et al.*, 2018; Hamouda *et al.*, 2019; Gobarah *et al.*, 2021; Moustafa *et al.*, 2020).

Microbiological analysis of the affected fish showed the isolation of Gram-negative bacteria, with *Aeromonas* spp. having the highest prevalence (33.33%). These results may wholly or partially diverge from those noted by several authors and this may be referred to the abiotic and biotic circumstances of the habitats where the experiments were conducted. The current findings are consistent with some research that claimed *Vibrio* spp., *Aeromonas* spp., and *Pseudomonas* spp. were accountable for the fatal fish outbreak (Daskalov, 2006; Najiah *et al.*, 2012; Youssuf *et al.*, 2020). These findings are also consistent with those made available by El-Gamal *et al.* (2018), who found that the retrieved bacteria from naturally affected Nile tilapia from various fish farms in Kafr El-Sheikh Governorate, Egypt, were 26% *Aeromonas* spp., 23.3% *Pseudomonas* spp. 36.6% of mixed infections are the cause of ulcerative syndrome. This proved that the outbreak was predominantly caused by the same genera as before.

To prevent the continued decline of fish farming, these strains must still be controlled. According to a previous

study by Hardi *et al.* (2018) conducted in Indonesia, Gram-negative bacteria are the most often found bacteria reported in cultured tilapia. It has been discovered that tilapia and catfish have seven different bacterial genera: *Staphylococcus* sp., *Streptococcus* sp., *Pseudomonas* sp., *Aeromonas* sp., *Enterobacter* sp., *Listeria* sp., and *Neisseria* sp. The three most prevalent pathogenic bacteria in the current study were *Aeromonas* spp., members of the *Vibrio* genus, and *Streptococcus* spp. In fish, *Aeromonas* spp. produce motile *Aeromonas* septicemia and epizootic ulcerative syndrome, according to Austin and Austin (2012). *Aeromonas hydrophila* strains that were extremely virulent were found in many cases (Nielsen *et al.*, 2001; Hemstreet, 2010). *Aeromonas* spp. (*A. hydrophila*), which made up around 33.33% of the infected fish farms, was followed by *Vibrio* spp. (32.46%), and *Streptococcus* spp. (13.33%). The same results from the same study region have been obtained (Joh *et al.*, 2013; Walakira *et al.*, 2014). Similar outcomes have been reported by other researchers (Newaj-Fyzul *et al.*, 2008; Ribeiro *et al.*, 2010), and this may be because *Aeromonas* species are common in the aquatic environment, the production systems utilized are comparable, and the fish species employed in the various trials.

The majority of bacterial pathogens found in fish farms are common aquatic dwellers and do not simply arise as a result of an infectious agent being exposed to a host (Wedekind *et al.*, 2010). The identified bacterial disease outbreaks were frequently triggered by environmental modifications (stressors), including *O. niloticus* overwintering, rapid temperature changes, inappropriate handling, injuries sustained during transit, and induced spawning in April and May every year. Fish's homeostatic mechanism is susceptible to environmental stressors, which can lower the fish's resistance to harmful organisms and cause illness outbreaks (Small and Bilodeau, 2005). Because of this, fish raised in aquaculture are more susceptible to a range of diseases (El-Sayed, 2006). Methicillin-resistant *Staphylococcus aureus* and *A. sobria* are two rare bacterial fish diseases that can infect tilapia, but because the aquaculture environment is in close contact with human and/or animal waste, these bacteria thrive there (Soliman *et al.*, 2014; Dar *et al.*, 2016).

In terms of water temperature, the elevated warm temperature present in some fish farms may have a substantial effect and fish are becoming more vulnerable to bacterial diseases. The start and gravity of fish disease caused by Enterobacteriaceae-related bacterial pathogens were predominantly influenced by external circumstances such as contaminated water, high temperature, and excessive amounts of organic matter (Zheng *et al.*, 2004). The stress of high water temperature promotes different virulent factors' expression, speed up the spread of infection, increasing disease prevalence and the size of epidemics (Karvonen *et al.*, 2010; Rodkhum *et al.*, 2011; Kayansamruaj *et al.*, 2014).

According to the current investigation's findings, the liver (37.7%), kidney (29.3%), spleen (22.6%), and intestine (10.2%) were the organs where isolated bacteria were found most frequently. These results align with earlier studies by Eissa *et al.* (2010), who clarified that *Pseudomonas* sp. was most prevalent in the liver (35%) and kidney (30%) of Nile tilapia, followed by the spleen (21.2%) and gills (13.7%), and El-Sayed (2019), where he revealed that naturally infected *O. niloticus* showed an elevated frequency of *V. alginolyticus* in the liver (38.5%), kidney (29.2%), spleen (23%), and heart (9.2%). In the current research, the liver, kidney, and spleen were the primary sources. In contrast, Mahmoud *et al.* (2016) clarified that the liver, spleen, and kidney were the primary locations of the largest distribution of bacterial isolates. According to Elsayed *et al.* (2018), the kidney and liver were used to isolate bacteria at rates of 37.4% and 36.3%, respectively. The spleen (15.9%) and gills (9.85%) were next in line. Furthermore, according to Eissa *et al.* (2016), the prevalence rates of *A. sobria* isolates were highest in the kidneys (25.3%), liver (23.0%), spleen (19.8%), and intestines (15.0%), while gills and skin lesions had the lowest frequency rates at 10.3% and 6.35%, respectively.

Conventional morphological techniques are difficult and long-lasting, and they postpone the execution of control strategies, which results in significant financial losses. Furthermore, many pathogens induce comparable clinical signs in diseased fish and have similar morphological characteristics. As a result, molecular recognition approaches are used (Bader *et al.*, 2003; Altinok, 2011; Tsai *et al.*, 2012). The PCR has shown to be a more precise and efficient way to detect bacterial infections. The highly conserved 16S ribosomal RNA gene, which is found in bacteria, is essential for gene coding. In bacterial phylogenetic studies, it is recognized as a characteristic marker for distinguishing across species (Nagpal *et al.*, 1998).

In the present investigation, *A. hydrophila* was identified using the 16S rRNA gene which gave positive results at a molecular weight of 625 bp size. The outcomes correspond with those of earlier research (Gordon *et al.*, 2007; Abd El-Tawab *et al.*, 2019); however, in contrast to Omar *et al.* (2016), where *A. hydrophila* was identified at a molecular weight 953 bp size and Hamouda *et al.* (2019), where *A. hydrophila* was identified at a molecular weight of 356 bp size.

PCR identified *Vibrio* species, validating the genus (16S rRNA) in all of the isolates at 663 bp. This outcome was consistent with that reported by Gobarah *et al.* (2021). Moreover, it is interesting to note that the sin1 and sin2 virulence genes discovered in the current investigation were exclusive to pathogenic *S. iniae*.

The results obtained agreed with what was mentioned by Baiano and Barnes (2009); Moustafa *et al.* (2021). Research on the susceptibility of fish pathogens to antibiotics is crucial in the development of novel chemotherapeutic medications for bacterial infection treatment in specific populations of cultivated fish. To ascertain the sensitivity and frequency of various antibiotic resistance between the bacterial isolates during the summer mortality of farmed Nile tilapia, eight antimicrobial agents were used. Sensitivity testing clarified that most of the strains tested were susceptible to CIP, CN, and C. Therefore, these antibiotics are effective against these bacteria and can be used to treat affected fish (Drug of choice). However, all isolates displayed high levels of resistance to AMX, indicating that these antibiotics should not be used to treat fish with pathogenic bacterial infections.

The outcomes are remarkably consistent with those of Dahdouh *et al.* (2016), who discovered that freshwater fish isolate *A. hydrophila* was responsive to ofloxacin, enrofloxacin, and CN but resistant to erythromycin. According to El-Barbary (2017), *A. hydrophila* was resistant to Erythromycin and Penicillin but susceptible to Norfloxacin, CIP, Lomefloxacin, Gatifloxacin, Kanamycin, and Gentamycin. According to research by Hamouda *et al.* (2019), *A. hydrophila* isolates were sensitive to the following drugs (in decreasing order): CN, CIP, Amikacin, Nalidixic acid, Cefotaxim, Cephalothin, Kanamycin, Sulfamethoxazole, and Ampicillin and completely resistant to Penicillin and Erythromycin. Similarly to this, El-Barbary and Hal (2016) showed that *P. fluorescens, A. hydrophila, P. putida,* and *Klebsiella*-infected fish may be treated with CIP, Norfloxacin, and Gentamycin. While most isolates of *A. hydrophila* in our investigation displayed resistance to O, Ahmed and Shoreit (2001) demonstrated that *Aeromonas* sp. retrieved from *O. niloticus* from Aswan fish hatcheries was extremely sensitive to the antibiotic. This shows that drug resistance to O in this region has appeared as a consequence of aquaculture's foolish use of antibiotics.

In addition, the current finding regarding *Vibrio* spp. revealed widespread antibiotic resistance found in *Vibrio* spp. retrieved from fish. There was a prevalent resistance to erythromycin. The outcomes are coincided with previous reports (Kitiyodom *et al.*, 2010; Gobarah *et al.*, 2023). This may be attributed to that erythromycin is naturally produced and subsequently scattered around in the environment, this is not surprising (Rosser and Young, 1999; Bani *et al.*, 2007). However, the current investigation disagrees with Gobarah *et al.* (2023), where *Vibrio* spp. revealed sensitivity to Gentamycin.

Streptococcus iniae isolates, on the other hand, revealed that every isolate examined was susceptible to CIP, CN, and C and resistant to O, AMX, Erythromycin, and S. The current investigation agrees with Younes *et al.* (2019) where *S. iniae* was resistant to AMX but, at the same time disagrees with the same author as *S. iniae* is sensitive to O in their study.

Conclusion

According to the current investigations, *A. hydrophila, Vibrio* sp*., and S. iniae* are the most threatening prevalent bacterial pathogens that have been isolated during mass-farmed tilapia summer mortality. This is in line with the hypothesis that high water temperatures may enhance the risk of these bacterial infections, resulting in mass tilapia mortality and significant economic losses. To raise healthy fish, it is therefore essential to maintain a favorable fish culture, environment, and husbandry techniques so that enhancing and improving the tilapia-rearing environment and reducing the pathogen's virulence. The majority of the tested strains were susceptible to CIP, CN, and C; but, displayed high levels of resistance to AMX.

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