JJBS

Volume 15, Number 2,June 2022
ISSN 1995-6673
Pages 239 – 247
https://doi.org/10.54319/jjbs/150211

Jordan Journal of Biological Sciences

Prevalence of Some Pathogenic Bacteria in Caged- Nile Tilapia *(Oreochromis Niloticus)* and their Possible Treatment

Ahmed Hammad Sherif [1,*] and Rasmia Hanafy AbuLeila[2]

[1] *Fish Disease Department, Animal Health Research Institute AHRI, Agriculture Research Center ARC, Kafrelsheikh, Egypt,* [2] *Fish Diseases Department, Animal Health Research Institute AHRI Agriculture Research Center ARC, Egypt*

Received: February 25, 2021; Revised: June 5, 2021; Accepted: June 26, 2021

Abstract

Bacteria are the primary cause of fatal disease outbreaks in aquaculture. Nine fish cages located at three different sites (3 cages/site) in the north Rosetta branch of the Nile River have exhibited high mortality rates. A total of 220 moribund *Oreochromis niloticus* and fish feed and water samples were examined for pathogenic bacteria in this study. Fish infected with *Vibrio parahaemolyticus* were located at only site 1 (62.5% infection rate), and *Streptococcus agalactiae* was isolated from fish at sites 1 and 3 (25% and 37.5% infection rates, respectively). Fish infected with *V. parahaemolyticus* or *S. agalactiae* were coinfected with *Aeromonas hydrophila*. Further investigation revealed that *V. parahaemolyticus* infection at site 1 may occur via a fish feed that was contaminated with *V. parahaemolyticus* (the fish feed was containing improperly manufactured marine fish meal). The median lethal dose (LD_{50}) 96h of *A. hydrophila, V. parahaemolyticus,* and *S. agalactiae* was 2.4×10^5, 1.9×10^5, and 5.2×10^3 colony-forming unit / ml, respectively for *O. niloticus* (50 ± 2.5 g b.w.) at a water temperature of 25.1 °C ± 1.5 °C. In an indoor experiment, *O. niloticus* were injected with the LD_{50} of the isolated bacteria. Florfenicol was found to be superior to ciprofloxacin in treating *A. hydrophila* and *V. parahaemolyticus* infection (mortality 13.3 % and 16.7 %, respectively), and ciprofloxacin was found to be more efficient in treating *S. agalactiae* infection (mortality 13.3%). In conclusion, inappropriately manufactured marine fishmeal was the source of *V. parahaemolyticus* infection in caged fish. *V. parahaemolyticus* or *S. agalactiae* infection co-occurred with *A. hydrophila* in fish cages containing low-quality water (high unionized ammonia content).

Keywords: Aeromonas hydrophila; Vibrio parahaemolyticus; Streptococcus agalactiae; fish cages; Oreochromis niloticus.

1. Introduction

Tilapia species come after carp species as the second major cultured fish around the world. In 2018, the production of *O. niloticus* in Egypt was 1.2 million tonnes, which, formed 65.15%, of total production which was 1.5 million tonnes (FAO, 2020). High market demands have led to intensive fish culture, wherein fish are exposed to infectious diseases, which have been considered one of the main obstacles facing aquaculture industries due to severe economic losses (Plant and LaPatra, 2011; Hamidan and Shobrak, 2019). In recent years in Egypt, the high mortality and morbidity rates recorded in freshwater fish farms were due to the prevalence of bacterial diseases that are concomitant with water temperature in summer (Enany *et al.,* 2019). In Egypt, Osman *et al.* (2021) reported that *Pseudomonas* sp. Isolated from Nile tilapia farmed or wild captured (River Nile) were carried antibiotic resistance, Quorum sensing, and virulence genes. In Bangladesh, Hamom *et al.* (2020) found that farmed diseased tilapia harboured *Edwardsiella tarda Streptococcus agalactiae,* and *Flavobacterium columnare* while live fish carried *Streptococcus iniae* and *Aeromonas salmonicida*. They also added that *columnare* and *E. tarda* caused a coinfection status in tilapia. *Aeromonas* is considered the most important pathogen in the aquatic environment that results in significant economic losses. This bacterium could be considered as a specific primary pathogen in freshwater, and it also acts as a secondary opportunistic pathogen attacking immunocompromised or stressed freshwater fish where it normally inhabits in the gut of *O. niloticus* (Sherif *et al.,* 2020). *Vibrio* spp. are bacteria that are ubiquitous in marine and brackish waters and are typical examples of opportunistic bacteria that cause diseases. These bacteria cause disease not only in fish but also in humans and shrimp. Vibriosis, a disease caused by Vibrio spp., is highly dependent on water quality deterioration, which results in severe immunosuppression and initiates bacterial infection outbreaks (Amal *et al.,* 2015). *Streptococcus* spp. are gram-positive bacteria that normally present in the aquatic environment causing a haemorrhagic disease in fish (Chang and Plumb, 1996). They caused a condition called by streptocococcosis predominantly in *O. niloticus*. This disease is considered one of the worst diseases in *O. niloticus* worldwide (Yang et al., 2018).

Several fish cages in the Nile River exhibit severe mortalities due to common bacteria that infect freshwater fish, and no uncommon species have been observed through classic bacterial investigation. Coinfection in fish is common, as indicated by the fact that a single fish could

* Corresponding author e-mail: ahsherif77@yahoo.com.

harbour more than one species of bacteria; such coinfection could be explained by the kind of the fish diet, sampling sites, human activities and water parameters at the fish culture sites (Abdelsalam *et al.,* 2017). Comparison of 16S rRNA sequences is a reliable approach to distinguish between the different species of pathogenic bacteria (Woo and Bruno, 2014).

One of the common ways to control bacterial infections in fish culture is through the use of antibiotics. A bacterial outbreak is considered as a major threat for farming, due to which a large number of antibiotics are used not only for treatment but also for prophylaxis (Noga, 2010). In the Vietnam aquaculture sector, 82% of lobster farmers and 28% of fish farmers used antibiotics at an average rate of 5 and 0.6 kg per produced ton of lobster and fish respectively (Hedberg *et al.,* 2018). In the United States of America (USA), fish treatment with florfenicol and ciprofloxacin was recommended by the food and drug administration (FDA) under the veterinary feed directive and the investigational new animal drug (INAD) to combat fish diseases in aquaculture (Noga, 2010). Some antibiotics were approved for aquacultures such as florfenicol, ciprofloxacin, enrofloxacin, difloxacin, sarafloxacin, chlortetracycline, oxytetracycline, doxycycline, and erythromycin by the authority of veterinarian pharmaceuticals in highly producing countries (Noga, 2010).

Therefore, the aim of this investigation was to highlight the possible treatment and to enhance the understanding of the circumstances of the massive mortality of fish reared in cages. Therefore, we conducted this study to investigate the bacteria associated with caged fish mortality outbreaks in the northern Nile River and the treatment prospects.

2. Materials and Methods

2.1. The sites of the investigated Cages

This study focused on nine fish cages located at three different sites (3 cages/site) north of the Edfina Barrage in the Rosetta branch of the Nile River, Egypt. The fish cages (3 × 2 × 2 m) were stocked with the freshwater fish *O. niloticus*. A total of 220 moribund *O. niloticus* (80, 60, and 80 fish at sites 1–3, respectively) were collected along with fish feed and water samples. The moribund *O. niloticus* were immediately transported alive to the Animal Health Research Institute (AHRI), Fish Diseases Department, Kafrelsheikh Provincial Laboratory, Egypt, in the summer of 2017. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

2.2. Bacterial Analyses

2.2.1. Primary bacterial isolation

The *O. niloticus*, feed, and water samples were examined for the presence of bacteria. Bacterial culture was attempted from hepatopancreas, spleen and kidney tissues according to previously described methods (Woo and Bruno, 2014). Water samples (3 replicate/site) were aseptically collected at 0.5 m depth in glass containers. Samples of fish feed (3 replicate/site) were randomly and aseptically collected in sterile plastic bags at different cage sites, each sample of 1 g aseptically dissolved in 9 ml distilled water. The tubes containing tryptic soy broth (TSB) were inoculated with samples of fish tissue, water, and dissolved fish feed then the tubes were incubated for 24 h at 26 ± 1 °C. In addition, samples were inoculated into TSB with 1.5% NaCl then incubated for 24 h at 26 °C ± 1 °C.

2.2.2. Biochemical profiles

Phenotypic characterization of the bacterial isolates was demonstrated according to (Madigan and Martinko, 2005). Biochemical analyses (in triplicates) were conducted using API20 E following guidelines of (BioMerieux, Marcy l' Etoile, France).

2.2.3. Selective isolation of bacterial strains

For *Aeromonas hydrophila* isolation, the inoculum was spread onto Rimler-Shotts agar then incubated for 24 h at 26 °C ± 1 °C. For *Streptococcus agalactiae* isolation, the inoculum was streaked onto tryptic soy agar (TSA) with 5% sterile sheep blood then incubated for 72 h at 26 °C ± 1 °C, according to previously described methods (Facklam and Carey, 1985), and then spread onto brain heart infusion (BHI) agar then incubated for 24 h at 26 °C ± 1 °C. For *Vibrio parahaemolyticus* isolation, inoculates were streaked onto thiosulfate citrate bile salt (TCBS) agar (to produce green colonies) and incubated at 26 °C ± 1 °C for 24 h.

2.2.4. Determination of bacterial strains and their virulence genes

Further identification of the recovered bacteria was done using the technique of polymerase chain reaction (PCR), the bacterial DNA was extracted by means of a QIAamp DNA Mini Kits (Qiagen GmbH, Germany) following to the manufacturer's guidelines, then the products of PCR were analyzed using gel electrophoresis (AppliChem GmbH, Germany) and a documentation system (Alpha Innotech, Biometra), and then the results were evaluated using the Chip PCR computer software (Rodiger and Burdukiewicz, 2013).

Molecular identification (sequencing) of the isolated strains was performed using a universal primer specific for 16S rRNA (F: AGA GTT TGA TCC TGG CTC AG and R: GGT TAC CTT GTT ACG ACT T) with a PCR product size of 1500 bp (Weisburg *et al.,* 1991). The sequencing process was conducted using ABI 3730xl DNA sequencer. To identify the bacterial strains, the obtained sequences were matched with the other related ones that were registered in GenBank by using the Blastn program.

In Table 1, the primers of virulence genes were cytotoxic enterotoxin (act and alt) for *A. hydrophila*; regulatory gene of toxin (toxR) and haemolysin genes (tdh and trh) for *V. parahaemolyticus*; and a CAMP factor (cfb) that enhances the haemolysis processes and C-b protein (bac), a protein serving as an IgA-binding protein for *S. agalactiae*. All primers were manufactured by Metabion, Germany.

Table 1. The sequences of primers, virulence genes, sizes and annealing temperature.

Gene name	Sequences 5'-3'	Size (bp)	Annealing temperature	References
A.hydrophila				
act	F:AGAAGGTGACCACCACCAAGAACA R:AACTGACATCGGCCTTGAACTC	232	55°C	Nawaz *et al.,* 2010
alt	F:TGACCCAGTCCTGGCACGGC R:GGTGATCGATCACCACCAGC	442	55°C	Nawaz *et al.,* 2010
V. parahaemolyticus		368	57 °C	Kim *et al.,* 1999
toxR	F:GTCTTCTGACGCAATCGTTG R:ATACGAGTGGTTGCTGT CATG			
tdh	F:CCATTCTGGCAAAGTTATT R:TTCATATGCTTCTACATTAAC	534	48	Cai *et al.,* 2007
trh	F:TTGGCTTCGATATTTTCAGTATCT R:CATAACAAACATATGCCCATTTCCG	500	52	Cai *et al.,* 2007
S. agalactiae				
cfb	F:GGATTCAACTGAACTCCAAC R:GACAACTCCACAAGTGGTAA	600	72°C	Kannika *et al.,* 2017
bac	F:CTCCAAGCTCTCACTCATAG R:GAAACATCTGCCACTGATAC	750	47°C	Kannika *et al.,* 2017

2.3. Antimicrobial Sensitivity Analyses

The activity of different antimicrobial drugs against the isolated bacteria was analyzed following the procedures described by Finegold and Martin (1982). Pure cultures of the strains were cultivated in TSB (Oxoid) then incubated for 24 h at 26 °C ± 1 °C. Subcultures were spread with a sterile cotton stick onto Mueller–Hinton agar plates (Oxoid). Results were recorded after incubation at 26 °C ± 1 °C for 24 h, by disc diffusion including florfenicol (KF 10 µg), ciprofloxacin (CIP 5 µg), clindamycin (DA 2 µg), amoxy+clavulinic AMC (30 µg), amoxicillin AML (10 µg), doxycycline (DO 30 µg), sterptomicin (S 10 µg), spiramycin (SP 100 µg), sulpamethazol +trimethoprim (SXT 25 µg), lincomycin (MY 10 µg), cefotaxime (CTX 30 µg), and cepharadin (CE 30 µg) manufactured by Oxoid, Waltham, MA, USA. According to the standards provided by the manufacturer and guidelines of NCCLS (1999), the isolated bacteria could be classified into three categories: resistant, intermediate, and sensitive depending on the diameters of inhibition zones.

2.4. Antibiotics Treatment Trial

Florfenicol: Floricol® 100 mg/g reg. No. 2533/2015 (Pharma Swede Company, Egypt) and ciprofloxacin: Ciprofar® (tablet) 500 mg/g reg. No. 21515/2012 (Pharco Pharmaceutical Company, Egypt) were used. Antibiotics were coated onto the surface of the pellets using capelin oil to prevent antibiotic dissociation, heat oil to 40 °C and antibiotics were added then mixtures of oil-antibiotic were evenly spread on the fish feed. The dosages of antibiotics were 10 mg/kg b.w./day for 10 successive days and capelin oil was 20 g/kg fish feed. The dosages and application methods of the antibiotics were implemented according to the methods described by (Noga, 2010).

2.5. Examination of Water Parameters

The water samples were analyzed at cages sites for temperature and salinity, (model YSI environmental, EC300) as well as dissolved oxygen (DO) (Aqualytic, OX 24) and pH (Thermo Orion, model 420A). Samples of 1 L were placed in a polyethylene bottle and transferred on ice to the laboratory to analyze the total ammonia nitrogen (TAN), unionized ammonia (NH_3), nitrite (NO_2), and nitrate (NO_3) using a UV/Visible spectrophotometer (Thermo-Spectronic 300) as described by Rice and Bridgewater (2012).

2.6. Median Lethal Dose (LD50)

The LD_{50} values of *A. hydrophila, V. parahaemolyticus,* and *S. agalactiae* in *O. niloticus* (mean body weight = 50±2.5 g) were estimated following the method described by (Reed and Muench, 1938). Fish were acclimated in indoor tanks for 2 weeks at a water temperature of 25°C ±1.5 °C. Serial 10-fold dilutions were made of the bacteria cultured in BHI broth for 24 h at 30 °C and then adjusted to 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6 or 1×10^7 (CFU/ml) in normal saline. Then, 100 µl of the bacterial suspension was intraperitoneally injected into duplicate groups of five *O. niloticus*. Each bacterial dose (CFU/ml) was based on a standard curve generated by performing plate counts. Mortality rates were recorded for 96 h; however, accidental mortalities occurring in the first 24 h were excluded. All bacterial strains were re-isolated from the dead fish (liver, spleen, and kidneys) and confirmed by PCR using specific primers (Table 1).

2.7. Treatment Trial with Antibiotics

A total of 360 healthy *O. niloticus* fish with a mean body weight of 40 ±0.5 g were collected from a local fish farm and acclimated for 2 weeks at a water temperature of 25°C ±1.5 °C. *O. niloticus* were subdivided into four groups G1-4 (90 fish/ group) and then infected with *A. hydrophila* (G1), *V. parahaemolyticus* (G2), and *S. agalactiae* (G3), whereas un-challenged (G4) fish were considered as the control negative group. Each group was subdivided into three treatments, viz., T1–3, each consisting of three replicates (10 fish/replicate) as follows: control-untreated (T1), ciprofloxacin-treated (T2), and florfenicol -treated (T3). The antibiotics were applied for 10 days before and after bacterial infection. *O. niloticus* were injected i.p. with the LD_{50} of bacteria as described by Alcaide *et al.,* (1999). *A. hydrophila,* and *S. agalactiae* isolates were grown overnight on TSA and TSA

containing 3% NaCl for *V. parahaemolyticus* at 30 °C, one of each resulted colonies was subcultured TSB for another 16 h. The mortality rate (MR %) was calculated as follows:

$$MR\ \% = \frac{\text{number of dead fish at the end}}{\text{number of fish in the same group at the start}} \times 100$$

2.8. Statistical Analyses

Data analyses were performed by determining the variance (ANOVA) using the SPSS software for windows, SPSS Inc., Chicago, IL, USA (SPSS, 2004). The obtained data are presented as mean ± SE (standard error). The significant difference among treatments is determined at a level of 0.05 using Duncan's multiple range test (Duncan, 1955).

3. Results

3.1. Clinical Signs and Gross Lesions

Moribund *O. niloticus* exhibited a lack of appetite, lethargy, skin petechiae, detached scales and exophthalmia (Figure. 1). Postmortem examination of the fish revealed intestinal inflammation, visceral adhesion, hepatomegaly, splenomegaly, and gall bladder distension (Figure. 2).

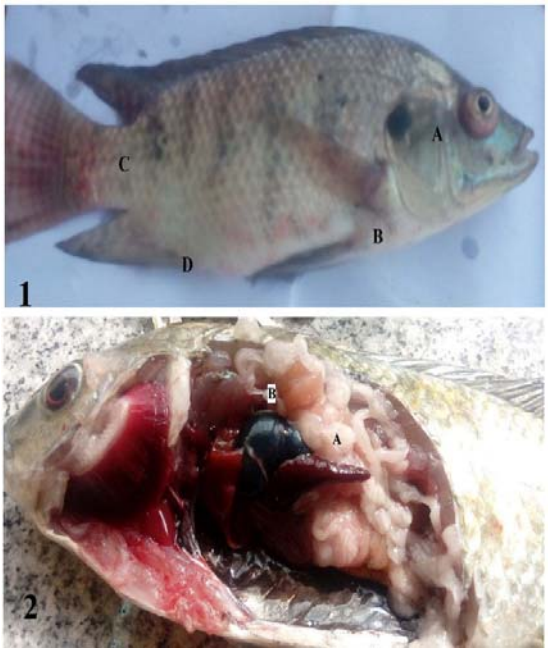

Figure 1. (1) *O. niloticus* collected from fish cages suffered from exophthalmia (A), haemorrhages on base of pectoral fine (B) and tail (C), and slightly distended abdomen. (2) *O. niloticus* collected from fish cages suffered from splenomegaly (A) and distended gallbladder (B).

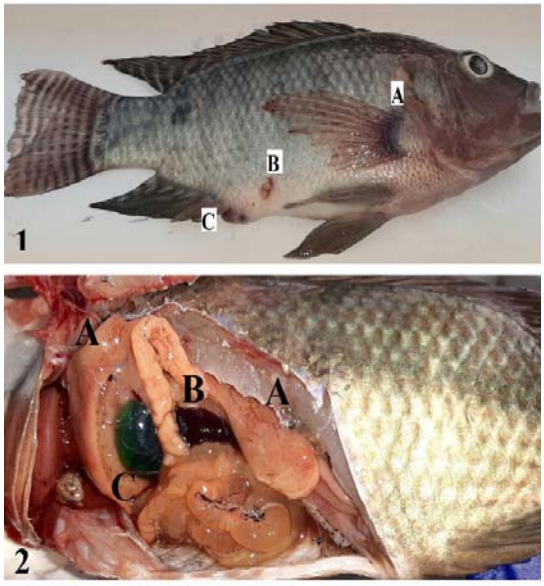

Figure 2. (1) *O. niloticus* experimentally infected with *Aeromonas hydrophila* suffered from pectoral haemorrhages (A), site of injection (B), and protruded inflamed-intestine (C). (2) *O. niloticus* experimentally infected with *A. hydrophila* with haemorrhages on hepatopancreatic tissue (A), splenomegaly (B), distended gall bladder (C).

3.2. Microbiological Examination

Bacteriological analyses (Table 2) using classical methods and PCR technique showed that the moribund *O. niloticus* were mostly infected with virulent strains of *Aeromonas hydrophila* (78.18%), followed by *Vibrio parahaemolyticus,* and *S. agalactiae* (22.73% each). The identification numbers obtained with API20 E were 107126, 4046107, and 1463410 for *A. hydrophila*, *V. parahaemolyticus,* and *S. agalactiae*, respectively. The blast results of the obtained isolates revealed 100% homology with *A. hydrophila*, *V. parahaemolyticus*, and *S.* agalactiae, in the GenBank database. The isolated strains (*A. hydrophila* AHRAS2, *V. parahaemolyticus* AHRAS44, and *S. agalactiae* AHRAS33) were deposited to the GenBank under the accession numbers of MW092007, MW092008, and MW092091, respectively. Phylogenetic trees were generated for the three strains (Figures. 3, 4, and 5). No other bacterial species were isolated from the collected samples.

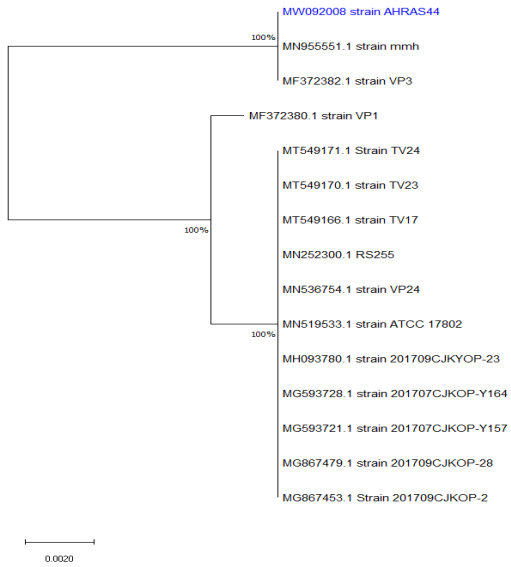

Figure 3. Phylogenetic analyses of *Aeromonas hydrophila;*

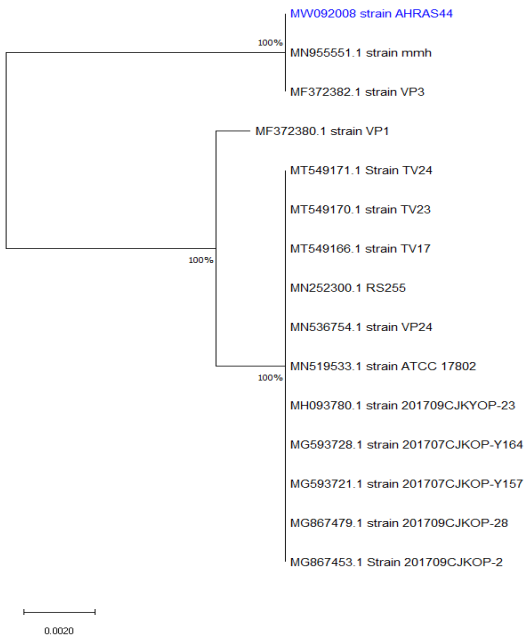

Figure 4. Phylogenetic analyses of *Vibrio parahaemolyticus;*

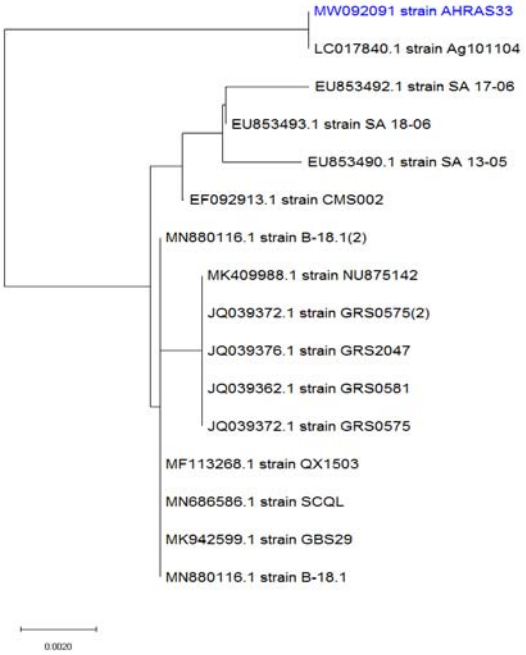

Figure 5. Phylogenetic analyses of *Streptococcus agalactiae*

At site 1, only *V. parahaemolyticus* infection occurred concurrently with *A. hydrophila* infection, with an infection rate of 62.5% (Table 2), whereas at site 3, all the *S. agalactiae* isolates were concurrently present with *A. hydrophila* and *V. parahaemolyticus.* Although *V. parahaemolyticus* is a classic pathogen of marine and brackish water fish, it was isolated from caged *O. niloticus* at site 1. *V. parahaemolyticus* was isolated only from caged fish that were fed a diet formulated with locally produced fish meal. The caged *O. niloticus* exhibited obvious infection through contaminated feed as *V. parahaemolyticus* was isolated from water and feed in site 1. The infection rates of *S. agalactiae* in *O. niloticus* 25% and 37.5% at sites 1 and 3, respectively, and the infection occurred concurrently only with *A. hydrophila* infection. *A. hydrophila* and *S. agalactiae* were isolated from water samples at (all three sites, and sites 1 and 3, respectively, and both organisms were not isolated from fish feed.

Table 2. Infection rates by bacterial isolate in *O. niloticus* collected from fish cages.

Items	No.	*A. hydrophila*		*V. parahaemolyticus*		*S. agalactiae*		Co-infection	
		No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Site 1	80	70	**87.5**	50	**62.5**	20	**25**	50	**62.5**
Site 2	60	52	**86.7**	0	**0**	0	**0**	0	**0**
Site 3	80	50	**62.5**	0	**0**	30	**37.5**	30	**37.5**
Overall	220	172	**78.18**	50	**22.73**	50	**22.73**	100	**45.45**

No.= number of fish.

3.3. Water Parameters of the Examined Cages

The physicochemical parameters of the water (Table 3), including temperature, DO, salinity, pH, TAN, NH_3, NO_2 and NO_3, were insignificantly differed in the three sites. The water parameters were suitable range for *O. niloticus* culture; however, NH_3 content was high at 0.2 mg/l, resulting in stress conditions.

Table 3. Physicochemical parameters and ammonia compounds of the water samples.

Item*	S 1	S 2	S 3
Temperature (°C)	27±0.5	28±1.5	28±1.5
DO (mg/l), mid-day	5.4±0.5	5.3±0.7	5.13±0.3
Salinity (ppt)	1.5±0.05	1.6±0.1	1.5±0.2
pH	7.9±0.2	8±0.2	8.2±0.1
TAN (mg/l)	2.1±0.5	2.4±0.25	2.5±0.4
NH_3 (mg/l)	0.3±0.1	0.28±0.12	0.27±0.1
NO_2 (mg/l)	0.01±0.0	0.01±0.0	0.01±0.0
NO_3 (mg/l)	1.65±0.2	1.6±0.1	1.7±0.2

S, cage site; water DO, dissolved oxygen; pH, hydrogen ions; TAN, total ammonia nitrogen; NH_3; unionized ammonia; NO_2, nitrite; NO_3, nitrate. * Significant difference ($P \leq 0.05$) indicates by different letters in the same row.

3.4. LD50 of the Isolated Bacteria

The LD_{50} 96h of values *A. hydrophila, V. parahaemolyticus,* and *S. agalactiae* were 2.4 $\times 10^5$, 1.9 $\times 10^5$, and 5.2 $\times 10^3$ CFU/ml, respectively, for *O. niloticus* with a body weight of 50 ±2.5 g at a water temperature of 25°C ±1.5 °C.

3.5. Antibacterial Profile

The bacterial isolates were highly sensitive to ciprofloxacin and florfenicol, confirming that these antibiotics would be suitable for treatment, whereas the bacteria exhibited intermediate resistance to clindamycin, amoxicillin clavulanate, and sulfamethoxazole-trimethoprim and full resistance to amoxicillin, lincomycin, cefotaxime, streptomycin, doxycycline, spiramycin, and cephradin.

3.6. Treatment Trial

The three groups of *O. niloticus* injected with the LD_{50} of the isolated bacteria exhibited mortality, and gross lesions were eye opacity, haemorrhages at the base of the pectoral fin, inflamed intestines, haemorrhages on hepatopancrease, splenomegaly and gall-bladder distension (Figure. 5). The two different treatment regimens decreased the mortality compared with the control regimen. The florfenicol -treated (T2) group had the lowest mortality rates (13.3 % and 16.7 %) among groups challenged with *A. hydrophila* (G1) and *V. parahaemolyticus* (G2), respectively, whereas the ciprofloxacin-treated (T3) group showed 13.3 % mortality among fish challenged with *S. agalactiae* (G3) compared with the control positive treatment (T1) group (Figure. 6). The antibiotic treatments had no effect on the mortality rate (3.33 %) in the unchallenged *O. niloticus* (G4) group.

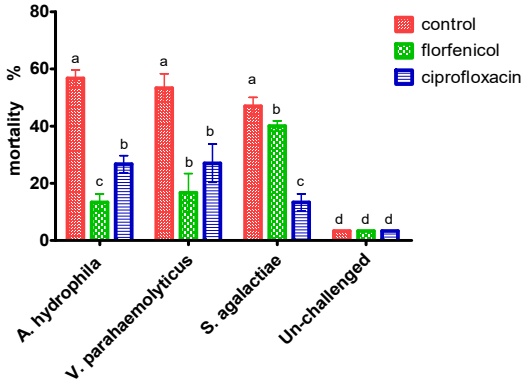

Figure 6. Mortality percentages of the different groups of *O. niloticus* experimentally infected with the three bacterial isolates and unchallenged group. Significant difference ($P \leq 0.05$) indicates by different letters.

4. Discussion

Aeromonas hydrophila, Vibrio parahaemolyticus, and *Streptococcus agalactiae* produced common symptoms of bacterial diseases. Similar to the clinical sings observed in this study, previous researchers have also reported the most common clinical and postmortem signs of *V. parahaemolyticus* infection as external haemorrhages, white nodular skin lesions, necrotic eyes, sudden death with haemorrhages in the skeletal muscles, deep ulcers, liver haemorrhage, pale kidneys and splenomegaly in studies on *Dicentrarchus labrax* (Rahman *et al.,* 2010), Iberian toothcarp (*Aphanius iberus*) (Alcaide *et al.,* 1999), and *Amphiprion sebae* (Marudhupandi *et al.,* 2017). Similarly, *Streptococcus* infection in *Oreochromis niloticus* was manifested by behavioural disorders, pop eye, and haemorrhagic dots on the body surface and bases of the fins in naturally infected tilapias (Figueiredo *et al.,* 2006). Consistently, the post mortem investigation of *O. niloticus,* experimentally infected with *A. hydrophila,* showed hepatomegaly and splenomegaly (Sherif *et al.,* 2015) and *S. agalactiae* in red tilapia (Abdelsalam *et al.,* 2017).

To confirm the identification of *Aeromonas* spp., some genes such as gyrB, rpoD, dnaJ, gyrA, dnaX, recA, and atpD are commonly used (Zhou *et al.,* 2019). Moreover, *V. parahaemolyticus* strains have been previously identified through PCR-based methods similar to those in our study (Kim *et al.,* 1999). The 16S rRNA sequencing approach (Figures. 3, 4, and 5) along with virulence genes (Table 1) were used to confirm the accuracy of bacterial identification of the isolated bacteria. Several researchers have identified bacterial species using PCR (16S-23S rDNA intergenic spacers) such as *Aeromonas* spp., and *Streptococcus* spp. (Sebastiao *et al.,* 2015).

In the examined cages, *A. hydrophila* was the most prevalent bacterium at a rate of 78.18% irrespective of the site. The most important diseases affecting fish in Egypt are *A. hydrophila* infection, Saprolegniasis, Aflatoxicosis, *Icthyophonus* infection, *Trichodina* infestation, Costiasis, and *A. hydrophila* and *Saprolegnia* coinfection (Aly, 2013). In the Nile River, *A. hydrophila* and *Pseudomonas fluorescens* have been isolated from both *O. niloticus* and *Clarias gariepinus* (Mohamed *et al.,* 2006). *V. parahaemolyticus* is a classic pathogen of marine and brackish water fish; however, it was isolated from caged *O. niloticus* (62.5% infection rate). At site 1, *V. parahaemolyticus* infection co-occurred with *Aeromonas* spp infection in caged *O. niloticus* (infection rate: 62.5%) and the fish feed and water samples were contaminated with *V. parahaemolyticus*. Supporting the obtained findings, *V. parahaemolyticus* has been isolated from tilapia cultured in freshwater cages (Amal *et al.,* 2010) and fish cultured in low-salinity water (5~30 ppt NaCl) (Iwamoto *et al.,* 2010). Moreover, infection rates of 52.11%, 29.5%, and 18.4% have been recorded in the Terengganu River, Pedu Lake, and Kenyir Lake, respectively (Ismail *et al.,* 2016). *S. agalactiae* was concurrently isolated from caged-*O. niloticus* with *A. hydrophila* at sites 1 and 3 with infection rates of 25% and 37.5%, respectively. *S. agalactiae* has been reported to cause large-scale outbreaks in cultured tilapia Thailand and Latin America (Marcusso *et al.,* 2015). Furthermore, similar to the obtained findings regarding coinfection, Abdelsalam *et al.,* (2017) reported that *S. agalactiae* concurrently infected red hybrid tilapia reared in cement ponds in north coast, Egypt.

Conversely, *Aeromonas* spp., *Streptococcus* spp., *Vibrio* spp., and *Flavobacterium* spp., coinfected cultured *O. niloticus*, water, and sediment (Al-Harbi and Uddin, 2006). Moreover, *A. hydrophila, A. sobria, P. fluorescens* and *P. aeruginosa* were concurrently isolated from *O. niloticus* (Sherif *et al.,* 2015). In our findings, *A. hydrophila, V. parahaemolyticus,* and *S. agalactiae* were isolated from water samples. Supporting this finding, it has been reported that infected fish released bacteria through feces, which survive in water and spread infection (Apun *et al.,* 1999).

The suitable water parameters are an important part of aquaculture systems. The parameters of water samples (temperature, DO, salinity, and pH) in the fish cages were suitable for fish culture (Table.3). Maintaining optimal water quality conditions or parameters is a vital part of fish for optimal performance (FAO, 2020). Caged-*O. niloticus* suffer from stressful conditions that predispose them to bacterial infection. Although most of the water parameters in this study were suitable for fish culture, high levels of ammonia were observed with the levels of TAN and NH_3 being 2.1, 2.4 and 2.5; 0.3, 0.28, and 0.27 mg/l, respectively. Accordingly, Noga (2010) mentioned that NH_3 level > 1.00 mg/l are lethal whereas those > 0.05 mg/l are sublethal concentrations for freshwater fish. The outbreaks of *Aeromonas* infection in fish farms were due to the limited knowledge and awareness of fish farmers regarding appropriate management (Sherif *et al.,* 2015; Mzula *et al.,* 2019).

Based on antibiogram findings, ciprofloxacin (despite being illegal for veterinary use in Egypt, some farmers and paramedics use ciprofloxacin unintentionally and erratically for treating bacterial fish diseases) and florfenicol are the optimal antibacterial substances for bacterial isolates. As shown in Figure. 6, the mortality rates of fish infected with *A. hydrophila* (G1) and *V. parahaemolyticus* (G2) and then treated with florfenicol were significantly lower (13.3% and 16.7, respectively) than those of control fish (56.7 and 53.3 %, respectively). In *S. agalactiae* infection (G3), ciprofloxacin-treatment (T3) resulted in significantly lowest mortality (13.3%) compared with florfenicol-treatment (T2) (40%) and control (47 %). Similarly, Ashiru *et al.,* (2011) found that pefloxacin, ofloxacin, and ciprofloxacin are suitable drugs for controlling *Aeromonas* infection, although, oxytetracycline, nitrofurans, potentiated sulfonamides, and oxolinic acid have been successfully used, bacteria, especially *V. anguillarum* and *V. salmonicida*, can exhibit resistance to these drugs. Despite the alleviated mortality (14.4%) resulting from ciprofloxacin treatment, immunosuppression has been detected *in A. sebae* infected with *V. parahaemolyticus* (Marudhupandi *et al.,* 2017), whereas *S. agalactia,* which was isolated from cultured yellowtail (*Seriola quinqueradiata*) in Japan, was resistant to these antibiotics (Kitao and Aoki, 1979).

5. Conclusion

This study highlighted the presence of unusual pathogens that cause mortality in caged fish in the north Rosetta branch of the Nile River. *V. parahaemolyticus* or *S. agalactiae* infection co-occurred with *A. hydrophila* infection. The source of *V. parahaemolyticus* infection in cage-cultured *O. niloticus* would be fish feeds containing inappropriately manufactured marine fishmeal so that this classical marine bacterial pathogen causes fish mortality in the freshwater environment. The infections caused by the bacteria *A. hydrophila, S. agalactiae*, and *V. parahaemolyticus* correlated with high unionized ammonia content in cage-water. In such cases, florfenicol was the most effective antibacterial agent along with the maintenance of water quality.

Conflicts of interest

None of the authors has any conflict of interests to declare.

References

Abdelsalam M, Shaalan M and Moustafa M. 2017. Rapid identification of pathogenic streptococci isolated from moribund red tilapia (Oreochromis spp.). *Acta Vet Hun,* **65**: 50-59.

Alcaide E, Amaro C, Todoli R and Oltra R. 1999. Isolation and characterization of *Vibrio parahaemolyticus* causing infection in Iberian tooth carp *Aphanius iberus*. *Dis Aquat Org,* **35**: 77-80.

Al-Harbi AH and Uddin MN. 2006. Seasonal changes in bacterial flora of fish pond sediments in Saudi Arabia. *J Appl aquac,* **18**: 35-45.

Aly SM. 2013. A Review of fish diseases in the Egyptian aquaculture sector. Working report. Research program on livestock and fish. CGIAR, pp. 1-41.

Amal MNA, Zamri-Saad M, Siti-Zahrah S and Zulkafli AR. 2015. Water quality influences the presence of *Streptococcus agalactiae* in cage cultured red hybrid tilapia, *Oreochromis niloticus* × *Oreochromis mossambicus*. *Aquac Res,* **46**: 313-323.

Amal, MNA, Zamri-Saad, M., Siti-Zahrah, A, Zulkafli, R, Misri, S, Nur-Nazifah, M and Shahidan, H. 2010. Prevalence of *Streptococcus agalactiae* in tilapia kept indifferent water bodies. Online *J Vet Res,* **14**: 153-162.

Apun K, Yusof AM and Jugang K. 1999. Distribution of bacteria in tropical freshwater fish and ponds. *Int J Environ Health Res,* **9**: 285-292. https://doi.org/10.1080/09603129973083

Ashiru AW, Uaboi-Egbeni PO, Oguntowo JE and Idika CN. 2011. Isolation and antibiotic profile of *Aeromonas* species from tilapia fish (Tilapia nilotica) and catfish (*Clarias betrachus*). *Pak J Nutr,* **10**: 982-986.

Cai SH, Wu ZH, Jian JC and Lu YS. 2007. Cloning and expression of the gene encoding an extracellular alkaline serine protease from *Vibrio alginolyticus* strain hy9901, the causative agent of Vibriosis in *Lutjanus erythopterus* (Bloch). *J Fish Dis,* **30**: 493–500. https://doi.org/10.1111/j.1365-2761.2007.00835.x

Chang PA and Plumb JA. 1996. Histopathology of experimental *Streptococcus* sp. infection in tilapia, *Orochromis niloticus* (L.), and channel catfish, *Ictafurus punctatus* (Ratinesque). *J Fish Dis,* **19**: 235-241. https://doi.org/10.1111/j.1365-2761.1996.tb00130.x

Duncan DB. 1955. Multiple ranges and multiple "F" test. Biometrics, pp10–11

Enany M, Eidaroos N and Eltamimy N. 2019. Microbial causes of summer mortality in farmed fish in Egypt. *SCVMJ,* **24**: 45-56. https://doi.org/10.21608/scvmj.2019.59198.

Facklam RR and Carey RB. 1985. *Streptococci* and *Aerococi*. In **"Manual of clinical microbiology"** 4th Ed. Lennette, Hauser, Shadomy (Eds). American Society for Microbiology, Washington DC, pp. 154-175.

FAO. (2020). Fishery and aquaculture statistics. Global production by production source 1950–2018 (FishstatJ). Retrieved from FAO Fisheries and Aquaculture Department [online] website: www.fao.org/fishery/statistics/software/fishstatj/en.

Figueiredo HC, Carneiro DO, Faria FC and Costa GM. 2006. *Streptococcus agalactiae* associado à meningoencefalite e infecção sistêmica em tilápia-do-nilo (*Oreochromis niloticus*) no Brasil. *Arq Bras Med Vet Zoo,* **58**: 678-680. https://doi.org/10.1590/S0102-09352006000400036

Finegold, SM and Martin WJ. 1982. Bailey and Scott's Diagnostic microbiology. (Eds.) Mosby, Philadelphia.

Hamidan NAF and Shobrak M. 2019. An Update on Freshwater Fishes of Saudi Arabia. *Jordan J Biol Sci,* **12**: 495-502.

Hamom A, Alam MM, Iqbal MM, Khalil SM, Parven M, Sumon TA and Mamun MA. 2020. Identification of pathogenic bacteria from diseased Nile tilapia Oreochromis niloticus with their sensitivity to antibiotics. *Int J Curr Microbiol Appl Sci,* **9**: 716–1738. https://doi.org/10.20546/ijcmas.2020.903.200

Hedberg N, Stenson I, Nitz Pettersson M, Warshan D, Nguyen-Kim H, Tedengren M and Kautsky N. 2018. Antibiotic use in Vietnamese fish and lobster sea cage farms; implications for coral reefs and human health. *Aquaculture*, **495**: 366-375.

Ismail NIA, Amal MNA, Shohaimi S, Saad MZ and Abdullah SZ. 2016. Associations of water quality and bacteria presence in cage cultured red hybrid tilapia, *Oreochromis niloticus* × *O. mossambicus. Aquacult Rep,* **8**: 39-44. https://doi.org/10.1016/j.aqrep.2016.06.004

Iwamoto M, Ayers T, Mahon BE and Swerdlow DL. 2010. Epidemiology of seafood-associated infections in the United States. *Clin Microbiol Rev,* **23**: 399-411.

Kannika K, Pisuttharachai D, Srisapoome P, Wongtavatchai J, Kondo H, Hirono I, Unajak S and Areechon N. 2017. Molecular serotyping, virulence gene profiling and pathogenicity of *Streptococcus agalactiae* isolated from tilapia farms in Thailand by multiplex PCR. *J Appl Microbiol,* **122**: 1497-1507. https://doi.org/10.1111/jam.13447

Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S and Nishibuchi M. 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the toxR gene. *J Clin Microbiol.,* **37**:1173-1177.

Kitao T and Aoki T. 1979. Therapeutic studies of doxycycline of Streptococcosis of cultured yellow tail (*Seriola Quinqueradiata*). *Bull Fac Agric Univ Miyazaki,* **26**: 357-63.

Madigan MT and Martinko J. 2005. Brock Biology of Microorganisms, 11th Ed. Prentice Hall.

Marcusso PF, Eto SF, Claudiano GD, Vieira FC, Salvador R, Moraes JR and Moraes FR. 2015. Isolamento de *Streptococcus agalactiae* em diferentes órgãos de tilápias do nilo (*Oreochromis niloticus*) criadas em tanques-rede. *Biosci J,* 549-554.

Marudhupandi T, Kumar TTA, Prakash S, Balamurugan J and Dhayanithi NB. 2017. *Vibrio parahaemolyticus* a causative bacterium for tail rot disease in ornamental fish, *Amphiprion sebae. Aquacult Rep,* **8**: 39-44. https://doi.org/10.1016/j.aqrep.2017.09.004

Mohamed LA, Osman KM, El-Seedy S and Soliman W. 2006. Isolation and characterization of *Aeromonas* species and *Pseudomonas fluorescens* from freshwater fishes. International Conference, Research Division, *NRC,* 219-229.

Mzula A, Wambura PN, Mdegela RH and Shirima GM. 2019. Phenotypic and molecular detection of *Aeromonads* infection in farmed Nile tilapia in Southern highland and Northern Tanzania. *Heliyon*, **5**: e02220. https://doi.org/10.1016/j.heliyon.2019.e02220

Nawaz M, Khan SA, Khan AA, Sung, K, Tran Q, Kerdahi K and Steele R. 2010. Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. *Food Microbiol,* **27**: 327-331. https://doi.org/10.1016/j.fm.2009.11.007

NCCLS (National Committee for Clinical Laboratory Standards). 1999. Performance standard for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved Standard M 31A19 (11). NCCLS, Wayne, Pennsylvania.

Noga EJ. 2010. Fish diseases: Diagnosis and treatment 2nd Ed, Wiley-Blackwell

Osman KM, da Silva Pires Á, Franco OL, Saad A, Hamed M, Naim H, Ali AH and Elbehiry A. 2021. Nile tilapia (Oreochromis niloticus) as an aquatic vector for Pseudomonas species of medical importance: Antibiotic Resistance Association with Biofilm Formation, Quorum Sensing and Virulence. *Aquaculture,* **532**:736068. https://doi.org/10.1016/j.aquaculture.2020.736068

Plant KP and LaPatra SE. 2011. Advances in fish vaccine delivery. *Dev Comp Immunol,* **35**: 1256-1262. https://doi.org/10.1016/j.dci.2011.03.007

Rahman MM, Ferdowsy H, Kashem MA and Foysal MJ. 2010. Tail and fin rot disease of Indian major carp and climbing perch in Bangladesh. *J Biol Sci,* **10**: 800-804. https://scialert.net/abstract/?doi=jbs.2010.800.804

Reed LJ and Muench H. 1938. A simple method of estimating fifty percent endpoints. *Am J Epidemiol,* **27**: 493-497. https://doi.org/10.1093/oxfordjournals.aje.a118408

Rice EW and Bridgewater L. 2012. A.P.H. Association, standard methods for the examination of water and wastewater, American public health association, Washington, DC.

Rodiger S and Burdukiewicz M. 2013. ChipPCR: toolkit of helper functions to pre-process amplification data Available from: https://github.com/michbur/chipPCR

Sebastiao FD, Lemos EG and Pilarski F. 2015. Validation of absolute quantitative real-time PCR for the diagnosis of

Streptococcus agalactiae in fish. *J Microbiol Methods,* **119**: 168-175. https://doi.org/10.1016/j.mimet.2015.10.021

Sherif AH, Elgamel A and Amin HA. 2015. Investigation of mass mortality of some fresh water fish in late summer in some fish farms at Kafr El Shiekh governorate. *J Arabaqs,* **10**: 33-44. https://doi.org/ 10.12816/0026660

Sherif AH, Gouda MY, Naena NA and Ali AH. 2020. Alternate weekly exchanges of feeding regime affect the diversity of intestinal microbiota and immune status of Nile tilapia *Oreochromis niloticus*. *Aquac Res,* **51**: 4327-4339. https://doi.org/10.1111/are.14778

SPSS 2004. "Statistical and package for social science, SPSS for windows release14.0.0, 19 June, 2004." Standard version, copyright SPSS Inc., 1989- 2004.

Weisburg WG, Barns SM, Pelleitier DA and Lane DJ. 1991. 16S Ribosomal DNA Amplification for Phylogenetic study. *J Bacteriol*, **173**: 697-703.

Woo P and Bruno D. 2014. Diseases and Disorders of Finfish in Cage Culture. 2nd Ed. CABI Pub, pp. 159-160.

Yang Q, Liu JX, Wang KY, Liu T, Zhu L, He SY, Geng Y, Chen DF, Huang XL, and Ouyang P. 2018. Evaluation of immunogenicity and protective efficacy of the elongation factor Tu against *Streptococcus agalactiae* in tilapia. *Aquaculture*, **492**: 184-189. https://doi.org/10.1016/j.aquaculture.2018.03.056

Zhou Y, Yu L, Nan Z, Zhang P, Kan B, Yan D and Su J. 2019. Taxonomy, virulence genes and antimicrobial resistance of *Aeromonas* isolated from extra-intestinal and intestinal infections. *BMC Infect Dis,* **19**: 158.