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Macrogyrodactylus spp. and bacterial co-infection in the farmed African catfish *Clarias gariepinus*

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ABSTRACT

Monopisthocotylean gyrodactylids and *Edwardsiella tarda* are potential piscine pathogens mainly associated with fish mortality and significant economic losses. In this study, a total of 100 African catfish, *Clarias gariepinus* were collected from an earthen ponds-based farm that experienced considerable mortalities with obvious septicaemic signs. The collected catfish were subjected to clinical, parasitological and microbiological investigations. Gyrodactylids were detected on the gill filaments of the examined fish specimens and were identified as *Macrogyrodactylus* spp. based on their morphological characteristics. The prevalence and intensity of *Macrogyrodactylus* spp. were 65% and 7.76 ± 0.20 / fish, respectively. On the other hand, *E. tarda* has been concurrently isolated from 70% of investigated catfish. *E. tarda* was phenotypically and molecularly characterized, and their antibiotic-resistant profile was investigated. The existence of pathogenic *E. tarda* in the same catfish infested with gyrodactylids probably augmented the clinical signs accompanied with the expansion of fish mortality. The experimental challenge with *E. tarda* proved that the current bacterial isolate was pathogenic to catfish based on the severity of the clinical signs and mortality rate. These findings suggested the existence of a possible synergism between *E. tarda* and *Macrogyrodactylus* spp. infections.

INTRODUCTION

African catfishes belonging to the genus *Clarias* have widely inhabited the African freshwater streams, rivers, pools and swamps, while *C. gariepinus* is considerably the

most farmed catfish species in Africa owing to its profitable growth **(Attia *et al.*, 2021a)**. *Clarias gariepinus* is commonly stocked in polyculture with some other fish species, such as the Nile tilapia to overcome its overpopulation problem and expand the final output of fish farm productivity **(Pouomogne, 2008)**. The farmed *C. gariepinus* is more tolerant to microbial pathogens than other fish species; however, *C. gariepinus* under adverse environmental stressors is more susceptible to numerous pathogens (**Attia *et al.*, 2021a**). Waste food and chicken viscera could be applied to feed farmed *C. gariepinus* due to their low cost compared to the formulated diet. These unacceptable practices compromise the health status of catfish, increasing the risk of severe epidemic occurrence **(Abu-Elala *et al.*, 2015; EI-Jakee *et al.*, 2020)**. African catfishes are among the main freshwater fishes parasitized by various monogenean parasites **(Oniye *et al.*, 2004; Arafa *et al.*, 2009; Barson *et al.*, 2010)**. *C. gariepinus* fries and fingerlings infected with parasites may be vulnerable to severe losses **(Mgbemena *et al.*, 2020)**. *C. gariepinus* could be infected by different trematodes and bacterial pathogens **(Zhokhov *et al.*, 2017)**. Parasitic infestations have been overwhelming the health status and the immunity of farmed fish **(Abdelsalam *et al.*, 2016, 2020; Attia *et al.*, 2021a, 2021b, 2021c; Salem *et al.*, 2021; Mahdy *et al.*, 2021)**, causing an increase in the fish vulnerability to opportunistic bacterial pathogens and acting as vectors for several infections **(Eissa *et al.*, 2015, 2021; Mahmoud *et al.*, 2016, 2021; Sherif et al., 2021a)**. Parasites belonging to monogenia such as *Gyrodactylus* sp. cause severe fish morbidity and mortality **(Malmberg, 1993)**. They attach themselves to fish gills, skin, and fins feeding on the epithelial cells, mucus and blood **(Bakke *et al.*, 2007)**, creating portals of entry for secondary bacterial and viral infections **(Levya *et al.*, 2015)**. Gyrodactylids including *Macrogyrodactylus* spp. have been previously identified in *C. gariepinus* in Egypt by studying the morphological characteristics of this parasite **(El-Naggar *et al.*, 2001)**. On the other hand, edwardsiellosis is a septicaemic disease caused by *Edwardsiella tarda* that causes significant financial losses in several farmed fish species, including channel catfish, tilapia, mullet, carp, eels, Chinook salmon and flounder **(Park *et al.*, 2012; Abraham *et al.*, 2015; Sherif *et al.*, 2021b, 2021c)**. *Edwardsiela tarda* causes emphysematous putrefactive disease in catfish **(Darwish *et al.*, 2000)**. Reptiles, mammals, and humans are also at risk. Gastroenteritis, meningitis, liver abscesses, and endocarditis are relevant infections in infected patients with acquired immune deficiency syndrome **(Kerie *et al.*, 2019)**. Edwardsiellosis outbreaks are multifactorial, triggered in farmed fish grown under unfavorable aquatic circumstances, such as extreme temperature and excessive organic loads **(Park *et al.*, 2012)**. *Edwardsiela tarda* invades and survives within-host phagocytic cells **(Rao *et al.*, 2003)**. It produces a variety of extracellular and intracellular products, including hemolysins, dermatotoxins and exoenzymes, which contribute to its pathogenicity **(Adikesavalu *et al.*, 2016)**. The present study aimed to investigate the multiple factorial causes associated with high mortalities among farmed *C. gariepinus* cultured in earthen ponds-based farms using different morphological and molecular

assays. Interestingly, this paper also shed light on the potential synergy between *E. tarda* and *Macrogyrodactylus* spp. naturally coinfected *C. gariepinus*.

MATERIALS AND METHODS

Specimens collection

Mortalities in earthen ponds-reared *C. gariepinus* occurr frequently in a private fish farm located at Manzala Lake, Damietta, Egypt. Accordingly, specimens were collected and examined to determine the incriminated etiological agents. The investigated farm has a case history of recurrent high mortalities a year ago, and *E. tarda* was identified from the dead fish. Unfavorable managemental practices were noticed in the farm, including the earthen ponds that were rarely drained with continuous production cycles without dryness or disinfection. The farm owner did not treat freshly stocked *C. gariepinus* fries and fingerlings with antiparasitic drugs. The average recorded values for water quality measures in the farm were 4 ± 0.15 (mg/L), 7.4 ± 1.03, and 0.4 ± 0.02 (mg/L) for dissolved oxygen, pH and unionized ammonia (NH_3), respectively. Upon the request of a fish farmer, a total number of 100 *C. gariepinus* specimens, with lengths ranging from 12-19 cm and weights from 90-120 g, were randomly collected from the earthen pond of the fish farm. An increase was noticed in the rates of fish mortality, accompanied with septicaemic clinical signs. Fish specimens were transported alive to the laboratory within the minimum time of delay. Fish were maintained in aeration-controlled covered glass aquaria till inspection. Samples clinically examined, and abnormal signs were recorded.

Parasitological examination

Fish were killed by pithing and severing the spinal cord. The gills were removed and placed in Petri dishes with filtered water. Gills were examined for parasitic infestation using a dissecting microscope (Olympus; Japan). Some living specimens (5) were also compressed between a glass slide and a coverslip following the method of **El-Naggar and Serag (1987)**. Living specimens were examined using a light microscopy (Olympus). In addition, muscle, liver, and kidney were examined by compression between two slides to detect any encysted metacercaria **(Younis *et al.*, 2020; Attia *et al.*, 2021b)**.

Bacteriological examination

Swabs from pool samples of fish tissues (liver, spleen and kidneys) were streaked into tryptic soy broth and incubated for 24hr at 28°C. Then, they were inoculated onto Salmonella-Shigella agar (Oxoid, UK) and incubated for 48hr at 28°C, stepping the procedures of **Sherif *et al.* (2021b)**. The biochemical profile of the isolate (in triplicates) using the API20 E (BioMerieux, Marcy l'Etoile, France) was performed following the manufacturer's instructions. Phenotypic characterization of the bacterial isolates was confirmed according to the study of **Abo El-Yazeed and Ibrahem (2009)**.

DNA extraction

The bacterial isolates were stored in vials containing glycerol and kept in the refrigerator at –80°C, following the procedures of **Sherif *et al.* (2021c)**. The frozen bacterial isolates were revived by inoculating on tryptic soy broth and incubated for 24hr at 28°C. The DNA of bacterial isolates was extracted using PathoGene-spin™ DNA Extraction Kit, following the instructions of the commercial kit. The extracted DNA was then rinsed in 100 mL elution buffer and stored at −20°C for the sequencing method.

Sequencing of 16S rRNA gene

The 16S rRNA gene was amplified by PCR following the procedure illustrated by **Xiao *et al*. (2008)**, using the universal primer pairs of 16S rRNA; forward primer 16S-F (5′-AGAGTTTGATCATGGCTCAG-3′) and reverse primer 16S-R (5′-GGTTACCTTGTTACGACTT-3′). PCR technique was performed in a 25 µL total volume reaction containing 12.5 µL Maxima® Hot Start PCR Master Mix (Thermo Fisher Scientific, USA), 1.0 µL of each primer (with 20 pmol concentrations), 5 µL extracted DNA and 5.5 µL RNase-free water. The fragment of 16S rRNA was amplified under the following condition: the initial denaturation at 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, extension at 72°C for 2 min, and the final extension was performed at 72°C for 7 min. The amplicon was purified using the Purification Kit of GeneJET™ PCR (Thermo Fisher Scientific, USA). The purified amplicon was directly submitted to the Sigma Scientific Services Laboratory (Cairo, Egypt) to be sequenced in both directions by ABI 3730xl DNA sequencer (Applied Biosystems™, USA), using the same primer of PCR reaction. The sequenced amplicon was carefully edited, assembled, and checked for any abnormalities using Bio Edit **(Hall, 1999)**. The assembled sequence was aligned against other related gene sequences deposited in the database of GenBank, using BLASTN search (National Center for Biotechnology Information, NCBI). Finally, the assembled sequence was submitted to GenBank to get the accession number of this isolate.

Molecular identification

The principles of bacterial identification, based on the sequence-based assay of the 16S rRNA gene, were characterized following the descriptions of **Drancourt *et al*. (2000)**. The investigated bacterial strains were recognized to the species level when the similarity values are equal to ≥99% to the related 16S rRNA sequences of specific bacteria in GenBank. However, the identification of bacterial strain was only achieved to the genus level when the similarity touches ≥97%. Finally, this assay represented a new genus and new species of tested bacterial isolate when the similarity score was < 97%.

Phylogenetic tree

The Neighbor-Joining phylogenetic tree was created using MEGA X with 1000 bootstrap replicates (**Kumar *et al.*, 2018)**. The subsequent factors were used: rate of variation among sites: uniform, substitutions: transversions and transitions, and pattern among lineages: homogeneous. Different types of complex phylogenetic trees usually exist, but this method is satisfactory in locating the current Gram-negative bacteria at branch terminals. *Streptococcus iniae* was chosen as the out-group.

Antibiogram of *E. tarda*

Isolates of *E. tarda* were revived in Tryptic Soy Broth TSB (Oxoid) and incubated at 26°C for 24 hr. Pure colonies were subcultured in Mueller Hinton Agar (MHA) plates (Oxoid). Results were recorded after incubation at 26°C for 24 hr by disc diffusion including florfenicol (KF 10 µg), erythromycin (E 15 µg), ampicillin (AMP 10 µg), ciprofloxacin (CIP 5 µg), amoxicillin AML (10 µg), cefotaxime (CTX 30 µg), streptomycin (S 10 µg), tetracycline (TE) (30 µg), sulpamethazol + trimethoprim (SXT 25 µg) (Oxoid, UK) following the method of **Finegold and Martin (1982)**. Furthermore, *E. tarda* was categorized into sensitive, intermediate and resistant **(NCCLS, 1999).**

Challenge test

In this study, the challenge experiment employed the *E. tarda* strain that was isolated and identified from infected *C. gariepinus*. In this experiment, sixty specimens of *C. garipinus* (free from *Macrogyrodactylus* spp. and *E. tarda*) were collected from another area (Tolompate 7, Kafrelsheikh, Egypt), with an average total length of 21.3 ± 1.2 cm (mean ± SD) and an average body weight of 115± 2.8 g. Upon arrival at the lab, samples were kept in 100 × 30 × 50 cm glass aquaria, filled with dechlorinated tap water and aerated with an electric air pump (Rena, Italy). All aquaria were covered with nylon netting for protection according to the work of **Abo El-Yazeed and Ibrahem (2009)**. Two fish groups in triplicate were used in the experimental challenge with *E. tarda*. The first fish group was injected I/P with 0.2 mL of sterile phosphate-buffered saline. The second was experimentally challenged by injection of a 0.2 mL dose of 10^4 CFU/mL bacterial suspension of *E. tarda*, using the I/P route as described in the studies of **Ibrahem *et al.* (2011)** and **Algammal *et al.* (2022)**. Fish samples were observed daily for 14 days; the number of deaths, signs of illness, and behavioral abnormalities were documented. Loops from the gills, liver, kidneys, and intestines were aseptically taken from dead fish and cultured onto agar media (sheep blood agar) to confirm the cause of death as described previously in the studies of **Abo El-Yazeed and Ibrahem (2009)**.

RESULTS

Clinical examination

Infected fish showed anorexia, darkness, tail rot, excessive mucus, skin erosions, extensive skin ulcers and hemorrhagic vents. Moreover, several infected fish showed signs of septicaemic syndrome, manifested by the existence of petechial hemorrhages distributed along the fish body and at the base of fins. Paleness of gills with over secretions of gill mucus was commonly noticed in infected fish. The main post-mortem signs were ascites, congested and enlargement of internal organs, including kidney, spleen, and liver with the distended gall bladder.

Parasitological examination

Macrogyrodactylus spp. was detected in 65% of farmed *C. gariepinus*. The intensity of parasites was 7.76 ± 0.20 / fish. *Macrogyrodactylus* spp. was detected in the gills and skin of infected *C. gariepinus.* Its translucent bodies are characterized by the presence of a large uterus in the center that included embryos. *Macrogyrodactylus* spp. appeared as a large, relatively elongated punch with an average body length of 1-2 mm (1.5±0.46). The prohaptor was lobed with a pair of head organs, and the cirrus sac was elliptical. The opisthaptor consisted of one pair of large anchors (Fig.1).

Bacteriological examination

The collected catfish samples were bacteriologically investigated for the presence of bacterial infection. Approximately, 70% of the investigated *C. gariepinus* were infected with pathogenic bacteria. The suspected bacterial colonies on Salmonella-Shigella agar appeared small with a black center. The retrieved bacterial isolates were motile and Gram-negative rods. The recovered isolates produced H_2S; they were positive for

catalase, nitrate reduction tests, methyl red, indole and fermented glucose. The same isolates were negative for oxidase, Voges-Proskauer and urease tests, and could not ferment lactose and sucrose. The retrieved bacterial isolates were identified as *E. tarda* by using the API20 E system (4,144,000); further identification was conducted with sequencing technique.

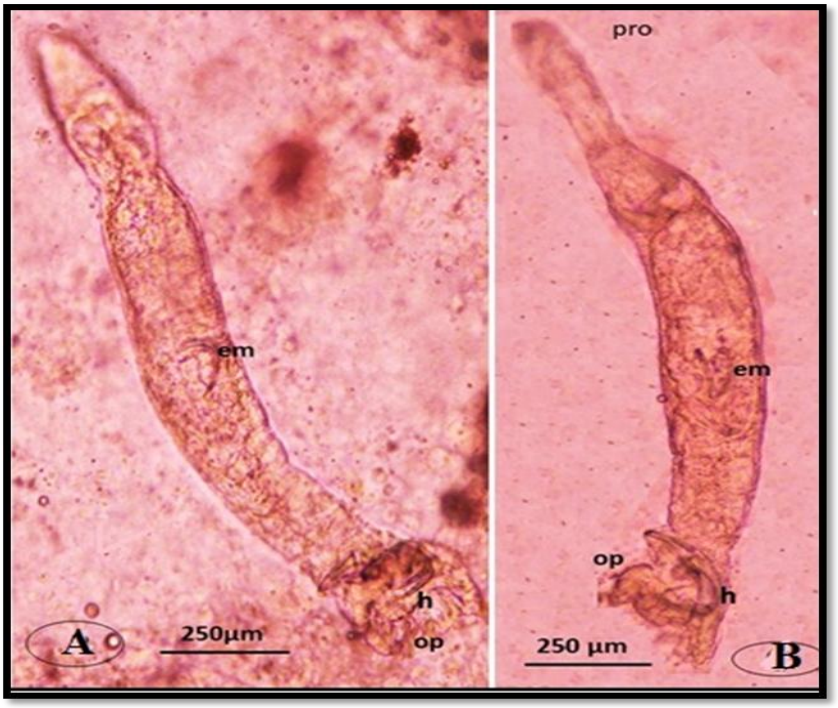

Fig. 1. Light micrograph showing some morphological features of *Macrogyrodactylus clarii* **(A-B)**. Fresh specimens showed embryo (em) in the middle region of the body; Posterior region showing opisthaptor (op), provided with marginal hooks (h) on the posteriorly projecting flap.

Phylogenetic analysis

The 16S rRNA gene sequencing is considered a golden technique for the bacterial identification and rebuilding the phylogenetic relationships to position the bacteria at the terminal branch. The BLAST analysis of 16S rRNA sequences confirmed that, the bacterial isolate belonging to *Edwardseilla* sp. was identified as *E. tarda* and submitted to the database of GenBank under the accession numbers (OK033932). The GenBank accession no. (OK033932) was 1426-bp and exhibited 99.72% similarity with *E. tarda* (LC504014.1 in Japan; CP023706.1 in Korea; KC570942 in China), 99.65% similarity with *E. tarda* (LC504020 in Japan; FJ405297 in Korea, MK312670 in China), and 99.58% similarity with *E. tarda* (LC504022-LC504023 in Japan, KU879058-KU860461 in Thailand).

The phylogenetic analysis displayed two major clades. The first lineage was further divided into two subclades with strong nodal support and 100% bootstrap value.

The two subclades comprised of *E. tarda* isolates that separated from *Citrobacter fruendii* and *Enterobacter cloacae* isolates. The current isolate of *E. tarda* is embedded among other *E. tarda* isolates and separated from *C. fruendii*, *S. iniae* and *E. cloacae* isolates. The neighbor-joining phylogenetic tree of the sequenced 16S rRNA genes of *E. tarda* is grouped with other *E. tarda* sequences and separated from other bacterial species as exhibited in Fig. (2).

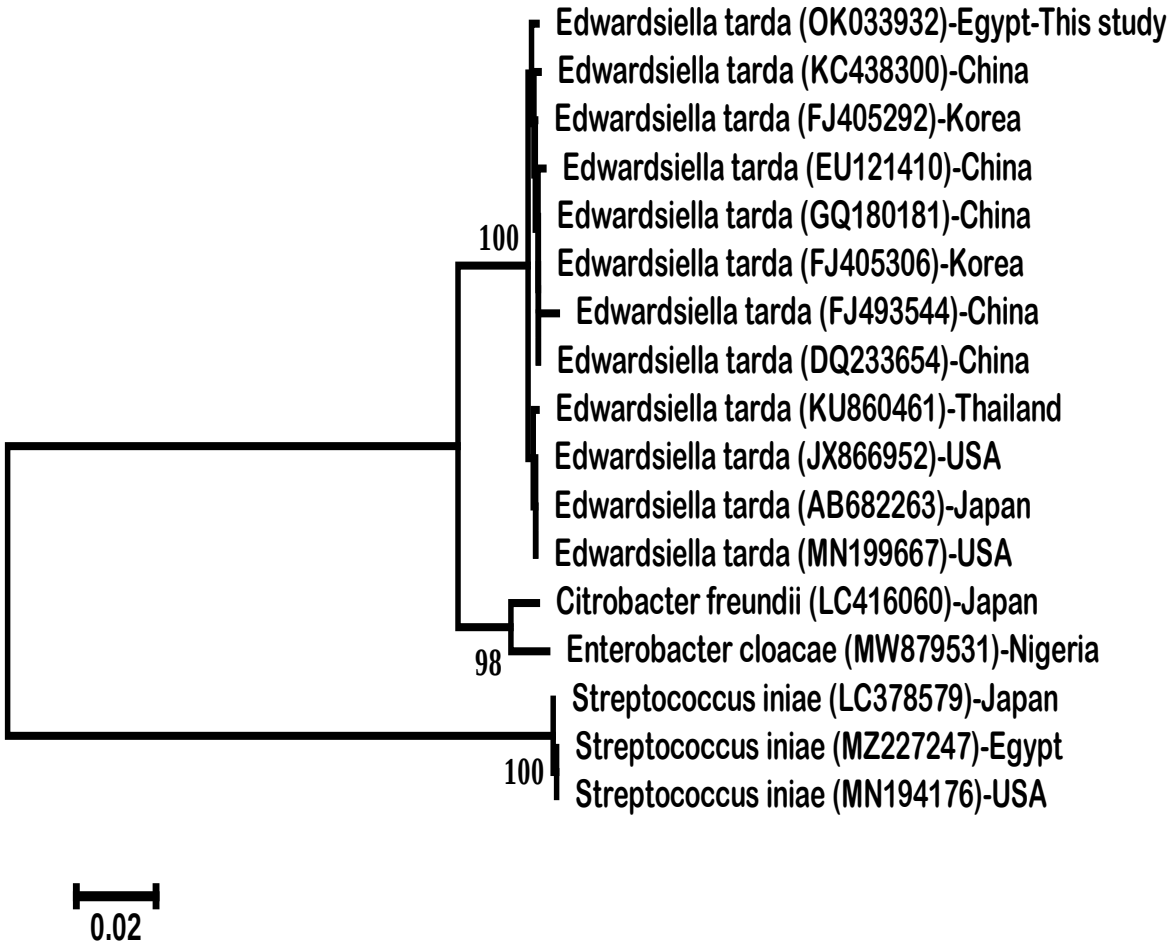

Fig. 2. Phylogenetic tree showing the comparative analysis of the 16S rRNA gene sequence of *E. tarda* infecting *C. gariepinus* and other related bacterial sequences

Antibiogram of *E. tarda*

The result of the antibiogram susceptibility for the recovered *E. tarda* isolates has revealed that isolates were sensitive to erythromycin and florfenicol, and mildly sensitive to ciprofloxacin. While, they were resistant to amoxicillin, ampicillin, tetracycline and trimethoprim-sulphamethoxazole.

The experimental infection with *E. tarda*

Fish experimentally infected with *E. tarda* showed clinical signs of septicemia, including petechial hemorrhages on the skin, fins and the operculum. Anorexia, excess mucus production in the skin and gills were commonly noticed. Internally, the liver, spleen, and kidney were congested. Blood-tinged fluid was deteced in the abdominal cavity. Mortalities started after 5 days post-exposure to bacteria. The experimentally infected *C. gariepinus* exhibited 30% of mortality rate after the challenge with *E. tarda*. Nevertheless, no mortality (0%) was observed in *C. gariepinus* in the control group. *E. tarda* was re-isolated in pure culture from all moribund and dead fish. Bacterial isolates were further confirmed using standard methods, including plating on culture media, API20 E system and molecular characteristics. *E. tarda* was not isolated from the internal organs of healthy catfish.

DISCUSSION

Poor water quality measures in the studied farm (low dissolved oxygen, high un-ionized ammonia) and the presence of various contaminants in the farm's supply of water might explain the presence of parasitic and co-bacterial infections in the investigated catfish **(Attia *et al.*, 2021a)**. Catfish raised in polluted water are known to have compromised immune system, making them more vulnerable to parasitic and bacterial infections **(Attia *et al.*, 2021b)**. Large amounts of untreated domestic, agricultural and industrial effluents, with an annual estimate of 7500 million cubic meters thrown into the lake Manzala from different sources, deteriorated the lake water quality measures **(Attia *et al.*, 2021b)**. The largest source of these effluents is centered in the the drain of Bahr El-Baqar **(Mahmoud *et al.*, 2016; Attia *et al.*, 2021b)**. Due to a scarcity of fresh water supplies in Egypt, the reuse of runoff water in aquaculture has become a viable solution to alleviate the shortages. The present findings are consistent with those of **Afolabi *et al.* (2020)** who reported parasitic infestations in wild and captive *C. gariepinus*. Environmental deteriorations, such as high temperatures, overcrowding, and high organic load in water can trigger parasitic or bacterial outbreaks in farmed fish. This explanation coincides with the finding of **Park *et al.* (2012)**. On the other hand, catfishes are scavengers that consume detritus, invertebrates in the water column, and mud, causing them to be at risk of diseases. Parasitic or bacterial infections in wild catfish are more likely due to the variety of food sources available in natural fisheries and the diverse feeding habits of *C. gariepinus* **(Attia *et al.*, 2021a)**. Similarly, the gyrodactylids and edwardsiellosis infections are rapidly increased and disseminated among stressed fishes **(Buchmann & Lindenstrøm, 2002)**.

Gyrodactylids are dangerous parasites that cause significant harm and destruction to fish species. Their damaging effects depend on their size, mobility and mode of attachment **(Borucinska & Caira, 1993)**. *Macrogyrodactylus* spp. morphological characteristics concur with those of **El-Naggar *et al.* (2016)**. Results showed that 65% of

the farmed *C. gariepinus* was infected with *Macrogyrodactylus* spp. The high prevalence of *Macrogyrodactylus* spp. in farmed *C. gariepinus* may be relevant to the farm's inadequate management control measurements, since the earthen ponds were seldomly drained, dried or disinfected, providing a perfect habitat for *Macrogyrodactylus* spp. to flourish. Infected wild fish that may escape into the farm may spread diseases among the farmed fish **(Matejusová *et al.*, 2001; Johansen & Mikkelsen, 2011)**. In addition, *C. gariepinus* was stocked in the earthen ponds without prior anti-parasitic treatments. Newly stocked fish from untrustworthy outlets should be quarantined in sanitary ponds and treated with sufficient anthelmintics to avoid parasite infestations **(Attia *et al.*, 2021a)**. Gyrodactylids attach themselves firmly to the epithelial tissues by a set of hooks on the opisthaptor, causing widespread injuries and irritations. Mucus oversecretion disturbs the defence mechanism of fish skin **(Harms, 1996)**. These small injuries are the assumed gateways for bacterial infections such as *E. tarda* to invade the fish. In line with earlier reports, fish parasites may increase fish susceptibility to microbial infections by destroying the protective mucous layers covering the gills and skin, acting as important barriers against secondary infections **(Dash *et al.*, 2018)**. Extensive injuries and open wounds caused by parasite feeding activities expose the underlying tissues resulting in lowering osmoregulatory failure and respiratory dysfunction, which increases fish mortalities **(Eissa *et al.*, 2021)**. Fish with heavy parasite infestations have a poorer physiological state and a reduced immunocompetence, which increase the chance of opportunistic diseases **(Johansen & Mikkelsen, 2011).**

E. tarda was detected in 70% of the investigated catfish. The identity of *E. tarda* was confirmed by phenotypic, biochemical characterizations, and sequencing of the 16S rDNA gene. *E. tarda* was isolated from the intestinal tract of infected fish indicating that *E. tarda* infections in natural aquatic environments may arise through the water-borne route and fish intestinal tract is one of its target organs. The current findings matches with that deduced in the study of **Abo El-Yazeed and Ibrahem (2009)**. Fish are remarkably infected by *E. tarda* that probably exist in the aquatic environment due to several carrier species, including fishes, amphibians and reptiles **(Xiao *et al.*, 2008)**. The challenge experiment demonstrated that the current isolate of *E. tarda* was pathogenic, causing a mortality percentage of 30 among experimentally infected fish, forming a result that agrees with that of a previous study **(Abo El-Yazeed & Ibrahem, 2009)**. Similarly, ectoparasite infestations increase bacterial invasions in fish and lead to substantially greater mortality **(Eissa *et al.*, 2021)**. The present findings support earlier reports stating the mortality rate of the tilapia, simultaneously challenged with Ichthyophthirius and *Streptococcus iniae*, was considerably greater than that of the fish infected solely with *S. iniae* **(Xu *et al.*, 2009)**.The finding of experimental infection revealed that, the *E. tarda* is a pathogen capable of infecting fish with or without parasite infestations, as shown by the deaths of experimentally infected *C. gariepinus* that were free of *Macrogyrodactylus* spp. The present results are in line with those of **Abo El-Yazeed and Ibrahim (2009)** who

reported that *E. tarda* is a primary fish pathogen, and mechanical damages to fish skin are not required to infect fish. The current study indicates a possible connection between the endemic parasite infestations with *Macrogyrodactylus* spp., poor management practices, undesirable water quality measurements, and the recurring outbreaks of *E. tarda* in the investigated farm.

CONCLUSION

Gyrodactylids are dangerous parasites that cause significant harm and destruction to both wild and captive fish species. *Macrogyrodactylus* spp. adhere to fish gills, impairing the respiratory functions, and making fish more susceptible to *E. tarda.* Endemic *Macrogyrodactylus* spp. and unfavorable management methods are suspected in the occurrence of *E. tarda* outbreaks. In conclusion, a notable link is recognized between parasitism and the increased susceptibility of fish to *E. tarda.*

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