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Research Article

Prevalence, Virulence Determinants and Antimicrobial-Resistant Profile of *Edwardsiella tarda* Isolated from Nile Tilapia (*Oreochromis niloticus*) in Egypt

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Abstract | *Edwardsiella tarda* (*E. tarda*) is an enterobacterium that causes edwardsiellosis, a fatal disease of farmed fishes that causes severe economic losses in the aquaculture industry. *Edwardsiella tarda* was categorized as a serious food- and waterborne infection that causes a high rate of mortality in people with liver cirrhosis. The goal of this study was to explore the prevalence, virulence-related genes (*edw1, cds1, qseC and pvsA)*, and antibiotic susceptibility profile of *E. tarda* in Nile tilapia collected from Egyptian fish farms. Between December 2019 and March 2020, 250 Nile tilapia were collected from five fish farms located at Dakahlia governorate. Organ tissue samples from liver, kidneys, gills, skin and spleen were examined bacteriologically for the presence of *E. tarda;* and confirmed by PCR targeting *gyr*B1 gene. Disk diffusion was used to test their antimicrobial susceptibility, and PCR was used to screen *E. tarda* isolates for the presence of four virulence-related genes (*cds*1, *edw*I, *qse*C, *pvs*A). In addition, the ability of *E.tarda* to form biofilm was tested by tube test. *E.tarda* was detected in 15 fish (6%) and a total of 40 isolates were recovered from organ tissue samples and confirmed based on phenotypic and molecular characterization. The frequency of *edw1, cds1, qseC and pvsA* genes were 75%, 70%, 42.5% and 2.5% respectively. *E. tarda* isolates displayed high resistance to ampicillin, amoxicillin, clindamycin, cefuroxime, penicillin, and amikacin, while, it is more sensitive to ciprofloxacin. Multi antimicrobial resistance (MAR) was observed in 100% of the tested isolates. In addition, 33 isolates (82.5%) were positive for biofilm production. In conclusion, the presence of virulent -MDR *E.tarda* strains in fish farms constitutes a hazard to aquaculture as well as a significant public health concern, therefore, appropriate sanitary management is required to improve water quality and reduce sickness incidence and economic losses.

Keywords | *E. tarda,* Fish farms, Virulence, Antimicrobial susceptibility, Biofilm.

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INTRODUCTION

Edwardsiellosis is one of the most common bacterial illnesses, inflicting significant economic losses in many countries' fish farms. The disease is caused by *E.tarda* which is a gram-negative, motile, facultative anaerobic, short rod-shaped bacterium (1µm in diameter and 2-3 µm long) (Mohanty and Sahoo, 2007). *E. tarda* has been described as the causative agent of infections in more than 20 fish species (Abbott & Janda 2006; Mohanty & Sahoo

2007). It's also classified as a serious food- and waterborne illness, similar to Aeromonas, Vibrio, and Salmonella (typhoid fever), which cause high mortality in people with severe underlying conditions such liver cirrhosis (Hirai et al. 2015).

The pathogenic strains of *E. tarda* have virulence genes that might be absent in non-pathogenic strains (Srinivasa Rao et al., 2003; Yang et al., 2012; Castro et al., 2016). The infection process of *E. tarda* occurred due to the presence of several potential pathogenic properties, such as AHL-synthase (*edw*I), chondroitinase (*cds*1), sensor protein (*qse*C), and vibrioferrin synthesis (*pvs*A) (Mohanty and Sahoo 2007), which can survive within macrophages and infect a wide range of hosts. Chondroitinase and other enzymes play a key role in the pathogenicity of the pathogenic strains (Tam Harvey & Chan 1982). Detection of virulence-related genes is very important for understanding the pathogenesis of this bacterium.

Antibiotic resistance is one of the most serious threats to aquaculture sustainability and human life nowadays (WHO, 2003). The transfer of R-plasmids is the main cause of this occurrence (Aarestrup, 2005). The rise of multidrug-resistant (MDR) bacterial pathogens is seen as a public health problem, and various prior studies have suggested that multidrug-resistant bacterial pathogens can be transmitted through a variety of sources, posing a hazard to public health.

Previously, cultured fish were not thought to be significant vectors of human infections. This scenario is changing, partially as a result of rising animal numbers as a result of a quickly expanding business, and partly as health care practitioners become more aware of infections in aquatic species that can cause human illness. Concerns about the sector are also addressed, as well as potential remedies. The Nile tilapia (Oreochromis niloticus) is Egypt's most economically important fish species and is consumed widely in Egypt. Although interaction between people and aquatic animals and their pathogens has expanded dramatically in the last several decades, zoonotic diseases from fish particularly *E. tarda*, which is currently considered an emerging gastrointestinal zoonotic pathogen that is acquired from aquatic animals have gotten little attention. As a result, the goal of this work was to determine the prevalence, distribution of virulence-related genes, antibiotic susceptibility profile, and biofilm production capability of *E.tarda* retrieved from Nile tilapia.

MATERIALS AND METHODS

Sampling

A total of 250 apparently healthy fresh fish were aseptically collected from five fish farms located at Dakahlia governorate. Fish samples were collected and packed individually in polythene bags from different sampling areas in an icebox and transported rapidly to the laboratory for bacteriological examination.

Bacteriological Examination

Aseptically, tissue samples from internal organs (kidneys, liver, spleen, spleen , and gills) were inoculated in MacConkey broth and incubated at 37˚C for 24 hrs followed by inoculation on Xylose Lysine Deoxycholate (XLD) agar and incubated at 37˚C for 24 hours. The suspected colonies were carefully selected and subcultured on MacConkey agar plates. All non-lactose fermenting colonies (pale colonies) were purified on Tryptic Soy Agar (TSA) plates for further identification (Lima et al. 2008; Markey et. al., 2013).

DNA extraction

About 3 to 5 colonies of overnight culture were picked up and suspended in 100ml distilled water, the mixture was heated for 10 minutes, and the cell debris was sedimented by centrifugation at 1000g for 10 minutes. Sterile Eppendorf tubes were used to transfer the supernatants containing DNA and stored at -20˚C till molecular examination.

Molecular characterization of *E.* tarda

Suspected *E.tarda* isolates were confirmed by PCR targeting ATPase domain of DNA Gyrase (*gyr*B). A species-specific primer (*gyr*B) was utilized provided by Metabion, Germany. Four sets of primers targeting the *cds*1, *edw*1, *qse*C, and *pvs*A genes were also used to detect virulence-related genes in all verified *E. tarda* isolates, as described in a recent work by Castro et al. (2006). The primers used in the current study for amplification of various genes and sizes of PCR amplicons are illustrated in Table 1. A 25 µl reactions including 12.5 µl of 2X PCR master mix (enzynomics, Korea), 1 µl of each primer (forward and reverse) of 20 pmol concentration, 4.5 µl of PCR grade water, and 6 µl of template DNA were used for PCR amplification, as shown in Table 2. A 96-well Applied Biosystems 2720 thermal cycler was used for the PCR procedure. PCR products were electrophoresed in a 1.5 % agarose gel and seen under UV trans-illumination after being stained with 0.5 g/ml ethidium bromide.

Sequencing reaction

For sequencing, a purified product of the *gyr*B gene from one representative *E. tarda* strain was used. For sequencing, an Applied Biosystems 3130 automated DNA sequencer was used (ABI, 3130, USA). Using a Perkin-Elmer/Applied Biosystems Bigdye Terminator V3.1 cycle sequencing kit (Cat. No. 4336817, Foster City, CA). To demonstrate sequence identity to GenBank accessions, the sequences

Table 1: Oligonucleatide primers sequences

Gene	Predicted function	Sequence	Amplified product	Reference
Edwardsiella tarda gyrB1	*ATPase Domain*	GCATGGAGACCTTCAGCAAT	415 bp	Park et al., 2014
		GCGGAGATTTTGCTCTTCTT		
Cds1	Chondroitinase	TCTCCACCCATAATGCCACG	435 bp	Castro et al., 2016
		CAAACGGCGTCGTGTAGTCG		
EdwI	AHL-synthase	ATCCGCAGCATCGAATGGCT	360 bp	
		GAAGGATAACGATGTGGTGT		
QseC	*Sensor protein*	CAGCAGTAGCAGGATCACCA	260 bp	
		ATGGACGTATGCTGCTCAAC		
PvsA	*Vibrioferrin synthesis*	CTGGAGCAGTACCTCGACGG	313 bp	
		CGATGCTGCGGTAGTTGATC		

Table 2: Cycling conditions for PCRs

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Edwardsiella tarda gyrB1	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 45 sec.	35	72°C 10 min.
Cds1	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	35	72°C 10 min.
edwI	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 40 sec.	35	72°C 10 min.
qseC	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	35	72°C 7 min.
pvsA	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 40 sec.	35	72°C 7 min.

were compared to sequences in a nucleotide database using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of *gyr*B have been deposited in the gene bank under the accession number MW911830.

Phylogenetic analysis

The CLUSTAL W multiple sequence alignment program, version 1.83 of the MegAlign module of Lasergene DNAStar software Pairwise, which was designed by Thompson et al. (1994), was used to compare sequences, and phylogenetic analyses were performed in MEGA6 using maximum likelihood, neighbor joining, and maximum parsimony (Tamura et al., 2013).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of *E. tarda* isolates was tested using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar (Oxoid) plates, as per the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015) against ten different antimicrobials (Oxoid), neomycin (N; 30 µg), ampicillin (AM; 10 µg), penicillin (P; 10 I.U), cefoperazone (CEP; 75 µg), cefuroxime (CXM; 30 µg), amikacin (AK; 30 µg), streptomycin (S; 10 µg), ciprofloxacin (CIP; 5 µg), clindamycin (DA; 2 µg), and amoxicillin were utilized as antimicrobial discs (Oxoid). The selected antimicrobial agents are commonly used for fish farming in Egypt. The results were categorized as susceptible, moderate, or resistant according to clinical and laboratory standards (CLSI 2018). If a single strain was resistant to three or more antibiotic classes, it was classified as multidrug-resistant (MDR) (Waters et al., 2011). Multiple antibiotic resistance index (MARindex) was calculated by dividing the total number of antimicrobial resistances for each isolate by the total number of antimicrobials tested (Krumperman 1983).

Biofilm formation

The tube method was used to examine the ability of E. tarda isolates to generate biofilms. A loopful of each isolate was inoculated separately in 5 mL trypticase soy broth (TSB; Becton Dickinson, Sparks, USA). After a 24-hour incubation period at 28°C, 1 ml of the incubated broth was transferred to a sterilized 4-ml TSB and incubated for another 24 hours at 28°C. A TSB tube that had not been inoculated was used as a control. The previously inoculated broth was carefully eliminated after incubation, and the tubes were stained with 1 percent crystal violet for 15 min-

utes; excess stain was discarded, and the tubes were washed with deionized water. The stained tubes were reversed to allow them to dry correctly.

RESULTS

Phenotypic characterization of *E.tarda* isolates

On the surface of XLD agar, *E. tarda* showed characteristic pale colonies with black centers and white color colonies on macConkey's agar plate (non-lactose fermenter). Indole, methyl red, catalase, glucose fermentation, and nitrate reduction tests were tested positive for *E.tarda*, but lactose and sucrose fermentation, urease, and Voges-Proskauer tests were tested negative. The total prevalence of *E. tarda* among the examined fish was 6% (15/250). The total *E. tarda* isolates retrieved from organs tissue samples was 40 isolates. The distribution of *E.tarda* among the examined fish samples was 12 (4.8%) in gills followed by liver samples 9 (3.6%), kidneys 7 (2.8%), skin and spleen 6 (2.4% each) as illustrated in Table 3. There was no statistically significant difference in the prevalence of *E. tarda* among the investigated fish's internal organs ($P < 0.05$).

Table 3: Distribution of *E.tarda* isolates in fish organs tissue samples

%	No. of isolates	Organ
2.8%	7	Kidneys
3.6%	9	Liver
4.8%	12	Gills
2.4%	6	Skin
2.4%	6	Spleen
16%	40	Total

Molecular characterization of *E.tarda*

E. tarda isolates were confirmed by PCR assay targeting *gyr*B gene which was successfully identified in all isolates (Figure 1). One representative *E. tarda* isolate was selected for sequencing. By comparing the sequence to sequences in a nucleotide database using the National Center for Biotechnology Information's Basic Local Alignment Search Tool, our sequence displayed 100% similarity to the *E.tarda* published sequences and recorded in GeneBank under the accession number of MW911830 (Figure 3).

Distribution of virulence gene in *E.tarda* isolates

By screening *E.tarda* for the presence of four selected virulence-related genes, *edw1* gene was amplified in 30 isolates (75%), *cds1* gene was amplified in 28 isolates (70%), *qseC* was amplified in 17 isolates (42.5%) and *pvsA* gene was harbored by one isolate (2.5%) (Figure 2).

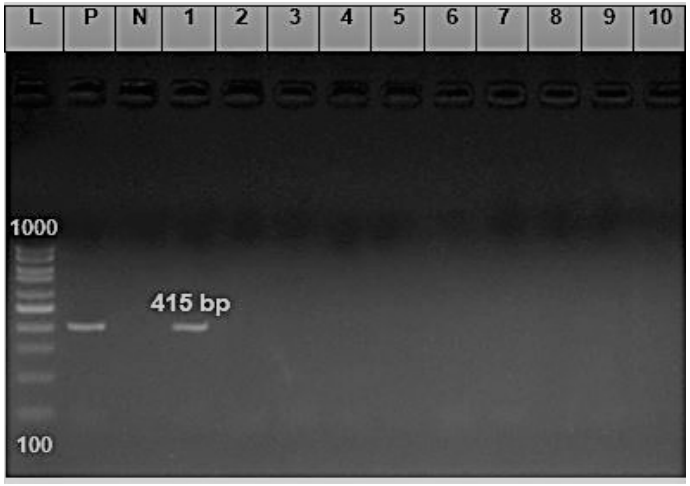

Figure 1: Agarose gel electrophoresis for taxonomic marker *gyrB gene* for *E. tarda* L: 100 bp ladder as molecular size DNA marker; P and N: positive and negative control.

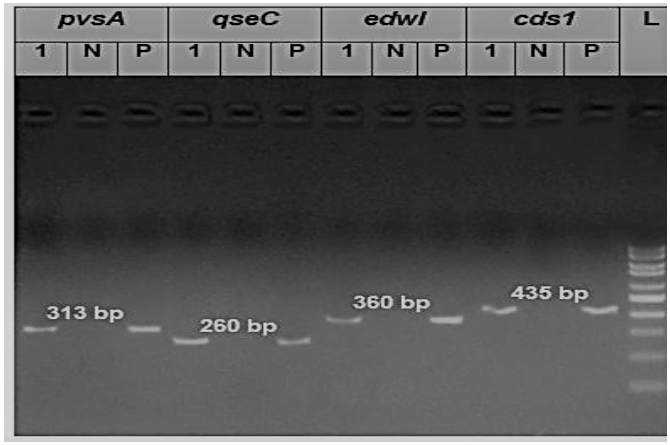

Figure 2: Agarose gel electrophoresis for virulence genes amplification in *E. tarda*. L: 100 bp ladder as molecular size DNA marker; P: positive, N: negative.

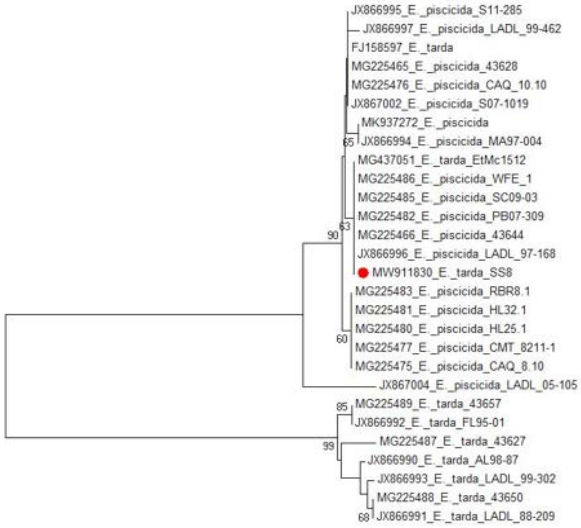

Figure 3: Phylogenetic tree showing the genetic relatedness among *E. tarda* strains based on nucleotide sequence analysis of *gyrB* gene

Table 4: Antibiogram of *Edwardsiella tarda*

Antimicrobial class	Antimicrobial agent	Disc potency	No. & percent(%) of isolates	
			S	R
B – lactams	Penicillin (p)	10 IU	3 (7.5%)	37 (92.5%)
	Ampicillin (Am)	10 µg	0 (0.0%)	100%
	Amoxicillin (AX)	25 µg	0 (0.0%)	100%
Cephalosporin	Cefuroxime (CXM)	30 µg	0 (0.0%)	100%
	Cefoperazone (CEP)	75 µg	24(60%)	16 (40%)
Aminoglycoside	Amikacin (AK)	30 µg	6 (15%)	34 (85%)
	Streptomycin (S)	10 µg	22 (55%)	18 (45%)
	Neomycin (N)	30 µg	24 (60%)	16 (40%)
Li ncosamide	Clindamycin (DA)	2 µg	0 (0.0%)	100%
Fluroquinolone	Ciprofloxacin (CIP)	5 µg	35 (87.5%)	5 (12.5%)

Table 5: Antimicrobial resistance patterns detected in *E. tarda* isolates.

MAR index	NO. of antibiotics resistant to each isolate	Antibiotics Resistance pattern	Isolate NO.
0.5	5	P, AM, CXM, DA, AX	4
0.6	6	P, AK, AM, CXM, DA, AX	5
0.6	6	N, AK, Am, CXM, DA, AX	1
0.6	6	AK, S, AM, CXM, DA, AX	1
0.7	7	CEP, P, S, AM, CXM, DA, AX	1
0.7	7	N, P, S, AM, CXM, DA, AX	1
0.7	7	P, AK, CIP, AM, CXM, DA, AX	1
0.7	7	P, AK, S, Am, CXM, DA, AX	5
0.7	7	N, P, AK, AM, CXM, DA, AX	3
0.7	7	CEP, P, AK, AM, CXM, DA, AX	2
0.8	8	N, P, AK, CIP, AM, CXM, DA, AX	1
0.8	8	CEP, AK, CIP, S, AM, CXM, DA, AX	1
0.8	8	CEP, P, AK, CIP, AM, CXM, DA, AX	1
0.8	8	N, P, AK, S, AM, CXM, DA, AX	1
0.8	8	N, CEP, P, AK, AM, CXM, DA, AX	4
0.8	8	CEP, P, AK, S, AM, CXM, DA, AX	3
0.9	9	N, P, AK, CIP, S, AM, CXM, DA, AX	1
0.9	9	N, CEP, P, AK, S, AM, CXM, DA, AX	3
1	10	N, CEP, P, AK, CIP, S, AM, CXM, DA, AX	1

Antibiotic susceptibility of *E. tarda*

All of the obtained *E. tarda* isolates (n = 40) were tested for their antibiotic susceptibility. The tested isolates exhibited a remarkable resistance to ampicillin, amoxicillin, clindamycin, and cefuroxime (100% each) followed by penicillin (92.5 %), amikacin (85%), streptomycin (45%), neomycin (40%), and cefoperazone (40%). While they were highly sensitive to ciprofloxacin (87.5%) (Table 4). Multiple antimicrobial-resistant (MAR) was displayed by all isolates and MDRindex ranges from 0.5 to 1 and P, AK, AM, CXM, DA, AX was the most common antimicrobial resistant pattern among the tested isolates (Table 5).

Biofilm production by *E. tarda* isolates

Out of 40 *E.tarda* isolates, 33 (82.5%) were positive for biofilm development, with 18 isolates (54.5%) being weak, 6 isolates (18.2%) being moderate, and 9 isolates (27.3%) being strong biofilm producers. While 7 isolates (17.5%) did not develop biofilms.

DISCUSSION

Edwardsiella tarda is a widespread fish pathogen that causes one of the most devastating septicemic illnesses in freshwater fish, resulting in significant financial losses in fish farms throughout the world, including North Ameri-

ca, Japan, Taiwan, Thailand, and Africa. It causes mortality in a variety of fish populations, including carp, tilapia, eel, catfish, mullet, salmon, trout, and flounder. (Bragg, 1991; Durborow et al., 1991; Baya et al., 1997; Galal, 2002). Furthermore, it has the potential to induce gastroenteritis, liver abscesses, meningitis, skin abscesses, and valvular endocarditis in humans. (Mizunoe et al., 2006; Choresca et al., 2011).

A PCR approach based on the use of the *gyr*B gene as a taxonomic marker for the detection of *E. tarda* in diseased fish has been previously developed by.Lan et al. (2008). The ATPase domain of DNA gyrase, an enzyme required for DNA replication, is encoded by the *gyr*B gene, a single-copy gene found in all bacteria (Huang, 1996). In this study, *gyr*B gene was successfully amplified in 40 morphologically and biochemically identified isolates with an overall prevalence of 6% (15/250). A higher prevalence was recorded by Eissa et al. (2016) who detected *E. tarda* in 9.6% among examined marine fish, Abd El-tawab et.al. (2020) detected *E. tarda* in 21% from *O.niloticus* and *C. gariepinus*. Algammal et al. (2022) detected an overall prevalence of 12% in examined fish samples. On the other hand, a lower prevalence was reported by Ali et al. (2008) who detected *E.tarda* in 3.7% in *Tilapia zillii*. The diversity of *E.tarda* prevalence in the different study may contributed to geographical location, temperature and quality of water and stocking density.

Studying the virulence factor of *E.tarda* may be help in the prevention and development of new strategies for treatment as well as enhancing our understanding of the bacterium infection process. The distribution of virulence genes in this study was opposite to Abd El-tawab et al. (2020) who detected *pvs*A gene (2/3, 66.66%) and *edw*1 gene (0/3, 0%) in *E. tarda* isolates. Detection of *edw*1 gene of *E. tarda* is very important virulence marker gene to confirm *E. tarda* pathogenicity (Sakai et al., 2007). The *edw*I and *qse*C genes are quorum sensing sensor proteins that also govern biofilm formation, flagellar motility, and the secretion system of *E. tarda* (Weigel, 2015). Presence of these genes in *E.tarda* isolates confirmed their virulence (Abdeltwab et al., 2021). Similarly, the presence of chondroitinase (*cds*1) in 77.2 % of *E.tarda* isolates could indicate their propensity to colonize and produce biofilms, and therefore disease development (Abdeltwab et al., 2021). Our results demonstrated that *cds1* encoding a chondroitinase enzyme found in 28 isolates (70%) unlike Castero et al. (2016) who recorded this gene in all the European turbot isolates of *E. tarda*, *edwI,* and *qseC* genes which help *E. tarda* to reach to Quorum sensing and biofilm formation was amplified in 30 isolates (75%) and 17 isolates (42.5%) respectively. In a study performed in Egypt by Algammal et al. (2022) *edw*I, and *qse*C genes were identified in 100% of the tested isolates, while, cds1 gene was detected in 77.2%. Vibrioferrin is a type of the siderophores that provide *E. tarda* to iron which essential for growth in host and expressed to its virulence factors that helps in the survival and replication of *E. tarda* (Kokubo et al., 1990). In this study, vibrioferrin (*pvsA*) gene was detected in one isolate (2.5%). Similarly, vibrioferrin detected in *E. tarda by* Castro et al. (2016). While, Algammal et al. (2022) couldn't detected *pvs*A gene in their study.

A biofilm is a clump of microorganisms that can mediate adhesion to a cell surface. E. tarda's ability to form biofilm is important in disease pathogenesis because bacteria living in biofilms are difficult to remove from surfaces, can resist antimicrobial agents and the immune system of the host, and are easy to adhere to host tissues, resulting in relapses of infection, outbreaks of serious diseases, and the production of virulence factors (Oana and Tim, 2011). In the present study, 33 out of 40 isolates were able to form biofilm with different degrees.

Antimicrobial drugs can be used in treatment and disease prevention in aquaculture (Bischoff et al., 2005). Overuse of antimicrobial agents, on the other hand, has the potential to cause antibiotic resistance in harmful bacteria, making them less susceptible to antibiotics. Regarding to antimicrobial susceptibility testing, the *E. tarda* isolates showed a substantial difference in susceptibility to the various antimicrobial drugs tested. The majority of the recovered isolates were susceptible to ciprofloxacin, but were highly resistant to ampicillin, amoxicillin, clindamycin, cefuroxime penicillin, and amikacin. All *E.tarda* were multiple antimicrobial resistant to five or more antimicrobial agents. Similarly, multiple antibiotic resistance was found in 84–87.5 % of *E. tarda* strains recovered from finfish and shellfish in West Bengal and Bihar, India (Kumar et al., 2016). Algammal et al. (2022) also reported MDR in *E. tarda* in Nile tilapia and African catfish. MAR index in this study ranges from 0.5-1 which revealed that cultured freshwater tilapia in Egypt received high-risk exposure to the used antibiotics.

Multiple antimicrobial resistance (MAR) is a global public health threat that has been described as huge global epidemic outbreaks (Crump et al., 2015). As a result, this analysis confirms microbial resistance, demonstrating the potential of resistant bacteria being transferred to humans through the consumption of aquaculture products. (Kikomeko et al., 2016). Antibiotic resistance hazard and associated resultant health effects have been on the increase globally, and while most developing countries are the worse affected; because there are several situations, and human attitudes that support the development and spread of resistant microbes; such as inappropriate drug administration (Tiamiyu et al., 2015).

CONCLUSION

In conclusion, the presence of MDR *E.tarda* in fish farms necessitates the use of antibiotics with caution and the therapeutic use of probiotics and immunostimulants to treat bacterial infections should be used as an alternative to antibiotics.

CONFLICT OF INTEREST

None.

NOVELTY STATEMENT

The frequency of virulence-related genes and Multi antimicrobial resistance (MAR) were observed in high percentages of the tested E.tarda in fish farms constitutes a hazard to aquaculture as well as a significant public health concern in Egypt.

AUTHORS CONTRIBUTION

AS collected samples, performed the experiments and write the original draft. AA and GY designed the study, supervised all experiments, revised and edit the manuscript. All authors approved the final version of the manuscript for submission.

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