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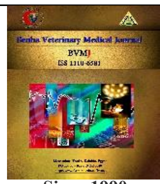

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The occurrence of *Aeromonas* species in farmed fishes at Kafr El-Sheikh Governorate

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ABSTRACT

The objective of this study was to examine the occurrence of *Aeromonas* species in farmed fishes, specifically *Oreochromis niloticus*, Common carp, and *Mugil cephalus*, obtained from Kafr El-Sheikh Governorate. The incidence of *Aeromonas spp*. in examined fish (30 samples of each) was 36.7% in *O. niloticus*, 23.3% in Common carp and 20% in *M. cephalus*. Serotyping of positive samples revealed multiple strains with varying distribution rates. Evaluation of fish quality contaminated with pathogenic *A. hydrophila* revealed that acceptable samples constitute 83.3% of *O. niloticus*, 90% of Common carp and 93.3% of *M. cephalus*, while rejection rates were 16.7%, 10%, and 6.7%, respectively. Antimicrobial susceptibility of *A. hydrophila* isolates exhibited greater susceptibility to gentamicin, imipenem and amikacin, but showed resistance to streptomycin, erythromycin, cloxacillin, cefotaxime, nalidixic acid and cephalothin. A laboratory experiment was conducted to assess the antibacterial activity of lemon oil against *Aeromonas hydrophila* and to evaluate its effect on the sensory properties of fish fillets. Results demonstrated that lemon oil treatment, particularly at the 1.5% concentration. So the study underscores the importance of continuous monitoring of bacterial pathogens in aquaculture, rationalizing the use of antibiotics to limit resistance, and exploring natural preservatives like lemon oil as sustainable solutions. The integration of such natural agents into aquaculture practices could help reduce the health risks posed by pathogenic bacteria, improve the quality of fish products, and minimize economic losses

1. INTRODUCTION

Fish constitutes a critical component of the human diet, serving as a high-quality source of animal protein, essential vitamins, minerals, and beneficial lipids (Steffen and Wirth 2005; EUMOFA, 2014). Egypt contributes approximately 73.8% of Africa's total farmed fish production and ranks eighth globally, with an output of around 919,585 tons of cultured fish that represents 1.54% of the total farmed fish production globally (FAO, 2012). Bacterial infections represent a major challenge in aquaculture, with profound economic and social implications, especially in developing regions (Defoirdt et et.2005; FAO 2012).

There is a critical bacterial genus in aquaculture which is Aeromonas, that includes both psychrophilic (*A. salmonicida*) and mesophilic motile species (*A. hydrophila, A. caviae, A. sobria)* (Ayoub et et.2024). These bacteria are ubiquitous in freshwater, brackish environments and have been linked to infections across a wide variety of hosts, such as fish, amphibians, reptiles, birds, and mammals. (Hayes, 2003). Human infections linked to Aeromonas include gastrointestinal illness and extra-intestinal manifestations including septicemia, endocarditis, meningitis and infections of the skin and soft tissues, particularly in individuals with weakened immune systems (Batra et et.2016).

In recent years, the food industry has shown growing interest in antimicrobial packaging, fueled by increasing Consumer preference leans toward foods that are minimally processed and contain no artificial preservatives. (Sand, 2024).

Essential oils (EOs) are naturally occurring compounds produced by plants, playing a role in defense against pathogens, environmental challenges, and stress conditions. They are known for their diverse biological effects, including antimicrobial, antioxidant, anti-inflammatory, and anticancer activities (Miguel, 2010; Yu et et.2011; Swamy et et.2016; Araújo Couto et et.2019; Ozogul and Kulawik, 2021). Because of these properties, assessing the antimicrobial potential of citrus-based essential oils, such as lemon oil, is important for controlling microbial growth in refrigerated fish and helping preserve its quality during storage. So, the specific objectives of the current study are detection the contamination of fish with *aeromonas* spp., detection of antibiotic resistance of the isolated pathogens (*A.hydrophila* strains (Antibiogramme) and study the antibacterial activity of lemon oil on viability of *A. hydrophila* inoculated into fish fillets.

2. MATERIAL AND METHODS

This study was approved by the Ethical Committee, Faculty of Veterinary Medicine, Benha University, with approval number BUFVTM 38-09-25.

1- Collection of samples:

A total of 90 randomly selected samples of farmed fishes—comprising 30 each of *Oreochromis niloticus* with average

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weight (150-200gm), Common carp with average weight (150-250gm), and *Mugil cephalus* with average weight (200-250gm), were obtained from various fish farms in Kafr El-Sheikh Governorate. Each sample was individually sealed in a plastic bag and transported to the laboratory in an insulated ice box, following stringent aseptic procedures. Bacteriological examinations were conducted promptly to identify the presence of *Aeromonas* species and evaluate the samples' safety for human consumption.

2. *Isolation of Aeromonas species*:

According to Ashiru et al. (2011), Five grams from the fish muscle of each sample were weighted and macerated in sterile mortar and pestle then transferred into separate conical flasks. 45ml of alkaline peptone water were added into each sample and then incubated at 37ºC for 24hrs, then streaking on *Aeromonas* agar media to which *Aeromonas* selective supplement (AES80004) was added. The plates were incubated at 37ºC for 24 hrs. The green colonies with opaque center were selected.

2. *Identification of Aeromonas species:*

According to Garrity (2001), Pure cultures of the isolates were morphologically and biochemically identified following the criteria proposed by those described in the Bergey's Manual of Determinative Bacteriology

3. *Antibiotic Resistance of the isolated pathogens (Antibiogram):*

The susceptibility of *A. hydrophila* isolates to various antimicrobials was assessed using the disc diffusion technique, as described by Stratev et al. (2010). The procedure adhered to the guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS, 2001). Furthermore, the Multiple Antibiotic Resistance (MAR) index for each isolate was determined using the calculation method outlined by Singh et al. (2010).

4. *Experimental Part: to Study the antibacterial activity of lemon oil on A. hydrophila inoculated into fish fillets.*

4.1. *Preparation of fish fillets:*

Accurately, 100g of each prepared fish fillet (*Oreochromis niloticus*) were exposed to ultraviolet (UV) light inside a cabinet for about 20 minutes to minimize the microbial load present on their surface.

4.2. *Preparation of inoculation* according to Tassue, et al. (1995).

4.3. *Inoculation of fish fillet with the tested bacteria:* According to Nithin (2024).

For inoculation of the fish fillet, 1 ml of the dense suspension of *A. hydrophila* was separately inoculated into the fillet to limit of 2×10^6 /g.

The inoculated fish fillet samples were categorized into four groups: the first served as the untreated control. The second group was treated with 0.5% lemon oil for 15 minutes, the third with 1% lemon oil, and the fourth with 1.5% lemon oil for the same duration. Following treatment, all samples were stored in a refrigerator at a temperature of 2 ± 1°C. All groups underwent bacteriological and sensory evaluations at specific time intervals: initially on day zero (within two hours post-treatment), and subsequently at 8, 16, and 24 hours.The lemon oil obtained from Food Analysis Center, Faculty of Veterinary Medicine, Benha University.

4.5. *Bacteriological examination*:

Actually, detection of *A. hydrophila* was determined in the control and inoculated samples as recorded by Garrity (2001).

4.6. *Sensory evaluation:*

Sensory evaluation was carried out using a scoring method based on the approach proposed by Klinic and Caki (2004).

5. *Statistical Analysis:*

The collected data were analyzed using Analysis of Variance (ANOVA) to determine statistical significance according to Feldman et al. (2003).

3. RESULTS

The data presented in table 1 reflects the incidence of *Aeromonas* species isolated from the examined fish samples.

Table (1): Incidence of Aeromonas species in the examined fish samples at Kafr el-shiekh governorate (n=90).

Fish species	No. of positive samples	%
Oreochromis niloticus	11	36.7
Common carp	7	23.3
Mugil cephalus	6	20
Total (90)	24	26.7

* The percentages were calculated according to number of samples. (30 of each type)

The distribution of identified *Aeromonas* species across the examined fish samples is summarized in table 2.

Table (2): Incidence of identified *Aeromonas* species isolated from the examined fish samples at Kafr el-sheikh governorate (n=90).

Fish species / *Aeromonas spp.*	*Oreochromis niloticus*		*Common Carp*		*Mugil cephalus*	
	No	%	No	%	No	%
A. caviae	9	30	5	16.7	3	10
A. hydrophila	5	16.7	3	10	2	6.7
A. punctata	1	3.3	0	0	0	0
A. sorbia	12	40	7	23.3	4	13.3
A. veronii	4	13.3	2	6.7	2	6.7

Table 3 illustrates the assessment of fish sample fitness in relation to *Aeromonas hydrophila* contamination. The evaluation of fish quality parameters (color, odor, and consistency) was carried out responsibly and ethically, following standardized guidelines.

Table (3): Fitness of the examined fish samples based on their contamination with *Aeromonas hydrophila* (n=90).

Fish species	Accepted samples		Unaccepted samples	
	No.	%	No.	%
Oreochromis niloticus	25	83.3	5	16.7
Common carp	27	90	3	10
Mugil cephalus	28	93.3	2	6.7
Total (90)	80	88.9	10	11.1

*Egyptian Organization for Standardization "ES" (2005)
*The criteria 0f fitness includes (color –odour- consistency).

Table 4 demonstrates the percentage of antimicrobial susceptibility patterns of *Aeromonas hydrophila* strains isolated from examined fish samples. The antimicrobial testing was conducted in accordance with internationally accepted microbiological and biosafety standards to ensure the reliability and validity of results.

The antimicrobial resistance profile of *Aeromonas hydrophila* strains, as presented in table 5, highlights the multiple antibiotic resistances (MAR) index of the isolates. All laboratory experiments, including bacterial isolation, identification, and antimicrobial resistance profiling, were performed under strict biosafety and ethical guidelines.

Table 6 presents the antibacterial effect of lemon oil on the viability of *Aeromonas hydrophila* artificially inoculated into fish fillets at different concentrations and storage times. The experiment was performed under carefully controlled laboratory conditions to ensure accuracy and reproducibility. All handling of fish fillets and bacterial inoculation procedures followed institutional ethical standards for food safety research.

The sensory evaluation of control and lemon oil–treated fish fillets is shown in table 7, highlighting the effects of lemon oil concentrations on appearance, texture, odor, flavor, and overall acceptability during different storage intervals. All sensory analysis procedures were carried out by trained panelists in accordance with ethical guidelines ensuring safety, transparency, and scientific integrity.

Table (4): Percentage of Antimicrobial susceptibility of *Aeromonas hydrophila* strains isolated from examined fish samples (n=10).

Antimicrobial agent	sensitive NO	sensitive %	intermediate NO	intermediate %	resistant NO	resistant %
Erythromycin (E)	-	-	-	-	10	100
Streptomycin (S)	-	-	1	12.5	10	87.5
Cloxacillin (CL)	-	-	3	37.5	8	62.5
Cefotaxim (CF)	1	12.5	2	25.0	7	62.5
Nalidixic acid (NA)	3	37.5	-	-	6	62.5
Cephalothin (CN)	3	37.5	3	37.5	5	50.0
Ampicillin (AM)	4	50.0	-	-	5	50.0
Sulphamethoxazol (SXT)	2	25.0	3	37.5	4	37.5
Tetracycline (T)	4	50.0	1	12.5	3	37.5
Ciprofloxacin (CP)	4	50.0	1	12.5	3	37.5
Amikacin (AK)	5	62.5	-	-	3	37.5
Meropenem (M)	4	50.0	2	25.0	2	25.0
Ipipenem (IPM)	6	75.0	1	12.5	2	12.5
Gentamicin (G)	7	87.5	-	-	1	12.5

Table (5): Antimicrobial resistance profile of *Aeromonas hydrophila* strains isolated from examined fish samples (n=10).

NO	Strain	Antimicrobial resistance profile	MAR index
1	*A. hydrophila*	G, S, CL, CF, AK , CN, AM, SXT, T, CP, NA M, IPM, E	1
2	*A. hydrophila*	E, S, AK, CF,T, CN, AM, SXT, NA,CP,CL, M, IPM	0.928
3	*A. hydrophila*	E, S, CL, CF, CN, AM, SXT, NA, T, CP, AK	0.685
4	*A. hydrophila*	E, S, CL, CF, CN, AM, SXT, NA	0.571
5	*A. hydrophila*	E, S, NA, CF,CL , CN, AM	0.500
6	*A. hydrophila*	E, S, CL, CF, NA	0.357
7	*A. hydrophila*	E, S, CL, CF	0.286
8	*A. hydrophila*	E, S, CL	0.214
9	*A. hydrophila*	E, S	0.143
10	*A. hydrophila*	E, S	0.143
Average	0.421		

S: Streptomyci E: Erythromycin CL: Cloxacillin NA: Nalidixic acid AK: Amikacin CN: Cephalothin CP: Ciprofloxacin SXT: Sulphamethoxazol T: Tetracycline CF: Cefotaxim AM:Ampicillin G: Gentamicin IPM: Ipipenem M: Meropenem

Table (6): Antibacterial activity of lemon oil on viability of *Aeromonas hydrophila* inoculated into fish fillets by intensity 2×10^6 (n=5).

Treatment / Storage time	Control Count	Control R %*	0.5 % Lemon oil Count	0.5 % Lemon oil R %	1 % Lemon oil Count	1 % Lemon oil R %*	1.5 % Lemon oil Count	1.5 % Lemon oil R %
Zero time	$2.0\times10^6\pm 0.21\times10^6$	----	$2.0\times10^6\pm 0.21\times10^6$	------	$1.99\times10^6\pm 0.22\times10^6$	------	$1.99\times10^6\pm 0.22\times10^6$	------
8 hours	$1.97\times10^6\pm 0.16\times10^{6\,a}$	1.5	$1.72\times10^6\pm 0.15\times10^{6\,b}$	14	$1.48\times10^6\pm 0.16\times10^{6\,c}$	25.5	$1.20\times10^6\pm 0.13\times10^{6\,d}$	39.7
16 hours	$1.89\times10^6\pm 0.17\times10^{6\,a}$	5.5	$1.39\times10^6\pm 0.14\times10^{6\,b}$	30.5	$1.05\times10^6\pm 0.12\times10^{6\,c}$	47.2	$8.70\times10^5\pm 1.09\times10^{5\,d}$	56.3
24 hours	$1.82\times10^6\pm 0.15\times10^{6\,a}$	9	$9.50\times10^5\pm 1.52\times10^{5\,b}$	52.5	$7.73\times10^5\pm 0.91\times10^{5\,c}$	61.1	$3.10\times10^5\pm 0.27\times10^{5\,d}$	74.4

R %*= Reduction %

** Mean values within the same row that bear different superscript letters indicate a statistically significant difference at the level of ($P<0.05$)..

Table (7): Sensory characteristics of control and lemon oil treated fish fillets (n=5).

Character / Treatment	Appearance (5)	Texture (5)	Odor (5)	Flavor (5)	Overall (20)	Grade
1. <u>Control:</u>						
Zero time	5	5	5	5	20	Excellent
8 hours	4.6	3.8	4.2	4.2	16.8	Good
16 hours	3.8	3.0	3.4	3.4	13.6	Middle
24 hours	3.0	2.6	2.8	2.8	12.2	Middle
2. <u>0.5% Lemon oil:</u>						
Zero time	5	5	5	5	20	Excellent
8 hours	4.4	4.2	4.0	4.4	17	Good
16 hours	4.2	4.0	3.8	4.2	16	Good
24 hours	4.0	3.6	3.6	3.8	15.0	Middle
3. <u>1% Lemon oil:</u>						
Zero time	5	5	5	5	20	Excellent
8 hours	4.6	4.2	4.4	4.4	17.6	Good
16 hours	4.4	4.0	4.2	4.2	16.8	Good
24 hours	4.4	3.8	4.0	4.0	16.2	Good
4. <u>1.5% Lemon oil:</u>						
Zero time	5	5	5	5	20	Excellent
8 hours	4.8	4.4	4.4	4.6	18.2	Very good
16 hours	4.6	4.2	4.2	4.4	17.4	Good
24 hours	4.4	4.2	4.2	4.2	17.0	Good

4. DISCUSSION

Bacterial infections represent a major challenge in aquaculture, often resulting in substantial economic losses (Gophen, 2017). Several species of *Aeromonas* are recognized as pathogens affecting aquatic organisms. (Chenia and Duma, 2017), the results in Table (1&2) revealed the presence of *Aeromonas* species in the analyzed samples of *Oreochromis niloticus*, Common carp, and *Mugil cephalus* were 36.7%, 23.3% and 20%, respectively, and serotyping for the positive samples of *Oreochromis niloticus*, was *A. caviae* (30%), *A. hydrophila* (16.7%), *A. punctate*(3.3%). *A. sorbia*(40) and *A. veronii* (13.3%). The results also revealed that *A.caviae* was higher than those recorded by El-Tawab et al. *(*2021) who detected it by1.4%, while *A.hydrophila* were lower than that recorded by the same aauthor,36.1%. In Common Carp, the serotyping for the positive samples showed that that *A.caviae* detected at (16.7%), *A.hydrophila* (10%), *A.punctate*(0%). *A.sorbia* (23.3) and *A.veronii* (6.7%). These results were lower than those recorded by Abdelsalam et al. (2021). Furthermore these serotyping of *Aeromonas spp*.in examined *Mugil cephalus* samples were *A. caviae* (10%), *A. hydrophila* (6.7%), *A. punctate* not detected, *A. sorbia* (13.3%) and *A. veronii* (6.7%), the results of *A. caviae* were higher than that recorded by El-Tawab et al. (2021) who detected it (8.3%), while the results of *A. hydrophila* were lower than that recorded by El-Tawab et al. (2021) (54.2%). So, the lower results of this study may be due to differences in the geographical origin, season and living habits of fish, while higher results may be due to environmental stressors or illnesses (Elgendy et al. 2015), bacterial load due to severity environmental pollution, and immune defense system of fish all influence disease progression (Elgendy et al. 2015) and (Elgendy et al. 2017).

Moreover in the results in table (3) measured the fitness of the examined fish samples based on their contamination with *Aeromonas hydrophila*, the percent of accepted samples were 83.3, 90 and 93.3 in *Oreochromis niloticus*, Common carp and *Mugil cephalus*, and the percent of rejected samples were 16.7, 10 and 6.7, respectively (Egyptian Standards "ES" 2005).

Antimicrobial agents are widely employed to treat infectious diseases in humans and are also used in aquaculture for both treatment and prevention. However, the overuse of these medications has led to the emergence of antimicrobial-resistant pathogens. (Cheng et et.2016). The antibiotic susceptibility for *Aeromonas hydrophila* strains recorded in Tables (4 & 5) were performed on 10 samples against 14 antibiotics. All isolates were found to resist Erythromycin (100%), while 87.5% were resistant to Streptomycine, these results agree with Kirkan, et et.(2006) and Forhad, et al. (2013) and Nehad, et al. (2019) and disagree with Alperi, et al. *(*2008).

However, susceptibilities to gentamicine (87.5%), ipipenem (75%) were observed and that agree with Alperi, et al. (2008) and Sharma,et al. (2009), while the tested strains showed medium susceptibility to Amikacin, Ampicillin, Ciprofloxacin and tetracycline from (62.5-50%) which agree with Yucel, et al. (2005); Akinbowale, et al. (2006) ; Alperi, et al. (2008) and Nagar, et al. (2011) and disagree with Guz and Kozinska, (2004); Saavedra, et al. (2005);Yucel et al. (2005); Akinbowale, et al. (2006); Awan, et al. (2009); Awaad, et al. (2011) and Nagar, et al. (2011) Multiple drug resistance of *Aeromonas hydrophila* isolates were recorded in Table (5) and Variations in MAR indices may be attributed to differences in the origin and source of the samples, as well as the methodologies used for testing. The widespread occurrence of antimicrobial-resistant bacteria is largely linked to the frequent and prolonged use of antibiotics. This raises significant concerns for both human and animal health, as it indicates the trends and practices associated with antibiotic usage (Xie et et.2015) and (Letchumanan et et.2015).

Natural antimicrobial agents have attracted growing interest from researchers because of their safety and non-toxic properties. Lemon oil, derived from *Citrus limonum*, is a highly volatile essential oil recognized for its therapeutic potential, particularly due to its antioxidant properties, antibacterial, and anti-cancerous merits (Hsouna et et.2017) and (Kaur et et.2020) .The results in Table (6) showed antibacterial activity of lemon oil on viability of *Aeromonas hydrophila* inoculated into fish fillets by intensity of 2×10^6 cfu/ml. and revealed that the bacterial count in group (1) control with count $2\times10^6 \pm 0.18\times10^6$ at zero time decreased to $1.82\times10^6 \pm 0.15\times10^6$ at 24 hours with reduction 9%.While in group (2) with 0.5% lemon oil the bacterial count decreased from count $2\times10^6 \pm 0.21\times10^6$ at zero time to $9.50\times10^5 \pm 1.52\times10^5$ at 24 hours with reduction 52.5%.In group (3) with 1% lemon oil the bacterial count decreased from count $1.99\times10^6 \pm 0.22\times10^6$ at zero time then decreased to$7.73\times10^5 \pm 0.91\times10^5$ at 24 hours with reduction 61.1% and in the last group (4) with 1.5% lemon oil the bacterial count decreased from count $1.99\times10^6 \pm 0.22\times10^6$ at zero time to $3.10\times10^5 \pm 0.27\times10^5$ at 24 hours with reduction74.4%,So the group(4) which treated by lemon oil(1.5%) had the best reduction percentage in the examined fish fillet samples. These results agree with Mancuso et al. *(*2019); Hussain et al. (2021).

Sensory evaluation offers a rapid, straightforward, and effective approach to assessing the acceptability and overall quality of a product. It relies on organoleptic attributes such as appearance, aroma, texture, taste, color, and overall consumer appeal. (Fisher and Phillips, 2006). The results in Table (7) revealed that the sensory evaluation of the examined fish fillet samples had the best overall acceptability in group (4) which treated by lemon oil 1.5% which gave a good score through 24 hours and had acceptable appearance, odor, texture and flavor followed by group (3) treated by lemon oil 1.% and finally group (2) treated by lemon oil 0.5%. The results indicated that incorporating 1.5% lemon oil can prolong the shelf life of fish meat while maintaining its sensory qualities unchanged. The results obtained agree with the view of Oladosu et al. *(*2020); Hao et al. (2021) and Jackson et al. *(2023*).

Lemon oil contributed positively to preserving the physical properties of fish throughout storage. This suggests that lemon essential oil may serve effectively as a natural preservative, as demonstrated by the enhanced nutrient retention in treated samples over extended storage durations. Finally, The use of natural preservatives in fish represents a significant advancement in encouraging the intake of well-preserved and health-conscious fish products.

5. CONCLUSIONS

In conclusion, the study confirmed that Aeromonas spp., particularly *A. hydrophila*, are prevalent in farmed fishes from Kafr El-Sheikh, with the highest incidence observed in *Oreochromis niloticus*. Although most fish samples were of acceptable quality, a notable percentage required rejection due to contamination. Antimicrobial testing revealed susceptibility to gentamicin, imipenem, and amikacin, but resistance to several commonly used antibiotics, highlighting the need for cautious antimicrobial use. Laboratory trials demonstrated that lemon oil, especially at 1.5% concentration, was effective against *A. hydrophila* and maintained favorable sensory properties of fish fillets, suggesting its potential as a natural alternative to synthetic preservatives in aquaculture and fish processing.

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