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Isolation and Molecular Characterization of Some Bacteria Implicated in the Seasonal Summer Mortalities of Farm-raised *Oreochromis niloticus* at Kafr El-Sheikh and Dakahlia Governorates

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Abstract

In the last years, there were some bacterial outbreaks occurred during the summer season of farm-raised *Oreochromis niloticus* at Kafr El-Sheikh and Dakahlia Governorates resulting in high fish mortalities and severe economic losses in these farms. So, this study aimed to detect the causes of the summer fish mortalities in these governorates to facilitate the diagnosis methods of study pathogens. Thirty fish farms were surveyed (20 in Kafr El-Sheikh and 10 in Dakahlia). 150 examined diseased fish (five from each farm) were succumbed for bacteriological isolation and biochemical and molecular identification. LD_{50} experiments were done to detect the infectivity and pathogenicity of some strains of bacterial isolates. The obtained results cleared that *O. niloticus* summer mortalities were caused by mixed infections with different bacterial species. The isolated bacterial species were 9 types of bacteria; 7 gram negative bacteria; 78 (30.5%) *Aeromonas hydrophila*, 52 (20.3%) *Pseudomonas aeruginosa*, 40 (15.6%) *Vibrio anguillarum*, 20 (7.8%) *Edwardsiella tarda*, 12 (4.7%) *Edwardsiella ictulari*, 10 (3.9%) *Pseudomonas fluorescens*, 7 (2.7%) *Flavobacterium columnare* and 2 gram positive bacteria; 30 (11.7%) *Streptococcus agalactia* and 7 (2.8) *Enterococcus feacalis* which were the most prevalent bacteria affecting fish farms and implicated in the *O. niloticus* seasonal summer mortalities and LD_{50} experiments confirmed the pathogenicity of some isolated bacteria. From the obtained results, it could be concluded that, the main cause of summer fish mortalities in studied farms was 81.82% gram negative bacteria and 18.18% was gram positive bacteria.

1. INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is the most commonly cultured species among tilapia in many countries around the world Zaki *et al.*, (2011) and Salem (2015). It has been contributing to the world aquaculture since the ancient Egyptian days and remains a major freshwater fish species to be cultured, Amal and Zamri Saad, (2011). Recently, there has been a parallel rise in the incidence of the seasonal mortalities with the expansion of *O. niloticus* culturing.

Bacteria are the most common pathogens of cultured warm water fish, implicated in the huge losses to the aquaculture industry elsewhere. Many of bacteria, are considered to be saprophytic in nature normal commensally associated the host or live free in the environment These bacteria able to cause diseases when fishes are immune-compromised by some form of stressors that allow opportunistic bacterial infections to progress Briede, (2010) and Noga (2010).

Aeromonas hydrophila, Streptococcus iniae, Pseudomonas fluorescens , Pseudomonas aeruginosa, Vibrio alginolyticus, Vibrio anguillarum, Streptococcus agalactia, Edwardsiella tarda, Yersinia ruckeri, Flexibacter columnaris, Renibacterium salmoninarum and Flavobacterium species, are considered the most common bacteria affecting fish farms Khalil *et al.*, (2001) and Robert and Moeller, (2014). Zaky and Ibrahim (2017) has screened the bacterial and fungal infections of Nile tilapia in Lake Manzala and they

isolated enteropathogenic bacterial pathogens, *Klebsiella pneumoniae, E. coli, Proteus sp. and Citrobacter freundii.* Nofal and Abd El-Latif (2017) studied the effect of Ectoparasites and bacterial co-infections during summer 2016 from pond cultured fishes at Al-Manzala fish farms.

PCR provides faster and more sensitive direct detection of bacteria in clinical samples without the need for previous culturing Gonzalez *et al*., (2002) detecting fish pathogens from asymptomatically fish, thereby disease outbreak and creation of antibiotic resistant bacteria may be limited Altinok and Kurt, (2003). The study aimed to detect the causes of the summer fish mortalities of *O. niloticus* to facilitate the diagnosis methods of study pathogens; through isolation of different pathogenic bacteria from cultured *O. niloticus* specimens, biochemical identification of these isolates, confirmed by PCR using specific primers and the pathogenicity of some isolated bacterial strains were investigated through the LD_{50} experiments.

2.MATERIALS AND METHODS

2. 1. Examined fish

A total number of 150 naturally diseased *O. niloticus* fish randomly collected from private fish farms in Kafr El-Sheikh and Dakahlia Governorates (20 and 10 affected farms respectively); 5 fish /affected fish farm among the 2016 summer season during the seasonal mortalities. The fish were examined for external lesions e.g. (hemorrhages, ulceration...., etc.) according to Austin and Austin (1987) and immediately subjected to clinical, post-mortem according to Plumb and Browser (1982) and bacteriological examination.

2. 2. Fish for LD_{50} experiments

A total number of 200 apparently healthy *O. niloticus* kept in a glass aquarium with aeration system and maintenance ration at Sakha Research Unit, Central lab. of Aquaculture Research.

2. 3. Bacteriological examination

Aseptically, inoculum from liver, kidney and skin ulcers of diseased fish were taken and inoculated in tryptic soy broth and incubated for 24 hrs at 30˚C. A loopful of tryptic soy broth were then spread on TSA and incubated for 48hrs at 30˚C. Each type culture colony was picked up and sub-cultured on a selective diagnostic agar media for 18-72 hrs at 30ºC for 24-48 hrs. Suspected bacterial colonies are picked up and inoculated into semisolid (0.5% soft agar) for detection of bacterial motility and for preservation of the isolates, for further investigations. Gram's stain were carried out according to APHA, (1992) and the biochemical identification were carried out according to Kreig and Holt, (1984) and MacFaddin, (2000).

2. 4. Polymerase Chain Reaction (PCR)

After DNA extraction according to Shah *et al.,* (2009), the isolates undergoes identification using specific primer to confirm the biochemically identified isolates Table (1).

Table (1). Primer sequences used for PCR identification system

Target	Primer Name	Oligonucleotide sequence (5′- 3′)	Product size (bp)	Reference
Aeromonas hydrophila	16SrRNA-F 16SrRNA-R	GGCCTTGCGCGATTGTATAT GTGGCGGATCATCTTCTCAGA	103 bp	**Trakhna *et al.* (2009)**
Pseudomonas aeruginosa,	PA-SS-F PA-SS-R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	958 bp	**Spilker *et al.* (2004)**
Vibrio anguillarum	Van-ami8 Van-ami417	ACATCATCCATTTGTTAC CCTTATCACTATCCAAATTG	429 bp	**Gyeong-Eun *et al.* (2007)**
Streptococcus agalactia	F1 IMOD	GAGTTTGATCATGGCTCAG ACCAACATGTGTTAATTACTC	220 bp	**Qasem *et al.*, (2009)**
Edwardsiella tarda	16S rDNA -F 16S rDNA-R	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA	450bp	**Castro *et al.* (2014)**

2. 5. Total bacterial count

Using the pour Plate method for estimation of the number of *Aeromonas hydrophila* and *Streptococcus agalactia* strains per 1 ml that will be used in demonstration of the inoculum dose for experimental studies, according to Cruickshank *et al.,* (1975).

2. 6. Determination of median lethal dose (LD_{50}) of *Aeromonas hydrophila* and *Streptococcus agalactia*

A total number of 200 apparently healthy *O. niloticus*, were divided into two major groups for the two strains, one hundred fish per each. Each group was subdivided into five subgroups, twenty fish per each, from each subgroup the fifth group

was kept as control. On the bases of the PCR results; the two strains were taken for the LD_{50} experiment. 24 hours colony cultures of each *A. hydrophila* and *S. agalactia* strains on TSA were used; the colonies were picked up and suspended in sterile saline in a tenfold serial dilution, only the dilutions (10^3-10^6) were used. Each fish group was intraperitonially injected with 0.5 ml of each bacterial dilution and kept 14 days post-inoculation for observations, mortalities were recorded daily according to Moustafa *et al*., (2016), re-isolation and biochemical identification of the pathogen were carried out and the freshly dead fish were moved for P.M examination. The LD_{50} of *A. hydrophila* and *S. agalactia* strains were calculated according to Reed and Muenchen, (1938).

3.RESULTS AND DISCUSSION

Ethical approval

Animal ethics committee, faculty of veterinary medicine, Kafr El-Sheikh University, Egypt, approved the protocol and conducting of the study.

3. 1. Examination of naturally diseased fish

External gross lesions of naturally diseased *O. niloticus* showed scale detachment, signs of septicemia, extensive skin, fins and tail hemorrhages, petechial haemorrhages all over the body surface, gill cover, excessive mucus, swollen abdomen, protruded haemorrhagic anus, exophthalmia and less to severe cutaneous ulcers as shown in Fig. (1&2) which may be due to septicemia resulting from bacterial virulence genes and toxins responsible for pathogenesis of the disease Marzouk and Nawal, (1991). These results agree with Ahmed and Shoreit, (2001); lbrahem *et al., (*2008); Enany *et al*., (2009); Oliveira *et al*., (2012); Sarkar and Rashid, (2012) and El-Son, (2016).

Postmortem findings of naturally infected *O. niloticus* showed ascitic fluids, congested liver with distended gall bladder. The spleen was enlarged, dark and congested. Enlarged and dark congested kidney (Fig. 3&4). These results agree with Amal and Zamri, (2011)**;** Oliveira *et al.*, (2012) and EL-Son, (2016).

3. 2. Bacteriological examination

Bacterial isolation and biochemical identifications revealed isolation of 256 bacterial isolates representing 9 types of bacteria; 7 gram negative bacteria; 78 (30.5%) *Aeromonas hydrophila*, 52 (20.3%) *Pseudomonas aeruginosa*, 40 (15.6%) *Vibrio anguillarum*, 20 (7.8%) *Edwardsiella tarda*, 12 (4.7%) *Edwardsiella ictulari*, 10 (3.9%) *Pseudomonas fluorescens*, 7 (2.7%) *Flavobacterium columnare* and 2 gram positive bacteria 30 (11.7%) *Streptococcus agalactia* and 7 (2.7) *Enterococcus feacalis* (Fig. 5) and Table (2-4). However, Nofal and Abd El-Latif, (2017) retrieved that the bacterial isolates responsible for summer mortality were *Vibrio alginolyticus, V. harveyii, Aeromonas hydrophilia, Enterococcus faecalis,* and *Edwardsiella tarda*.

Morphological, culture and biochemical characters of the 9 bacterial strains were nearly similar to those reported by Ansaruzzaman *et al*., (2005); Shaaban, (2007); lbrahem *et al.,* (2008); Enany *et al*., (2009); Suanyuk *et al*., (2010); Park *et al.*, (2012); Sarkar *et al.*, (2013); Salem, (2015) and El-Son, (2016).

3. 3. Molecular characterization of some bacterial strains:

The molecular characterization of DNA for 8/10 *A. hydrophila* isolates, using specific primers revealed the presence of common band at 103 bp (Fig. 6). These results were closely similar to that reported by Tarakhan *et al*., (2009).

The molecular identification of DNA for 9/10 *Ps. aeruginosa* using specific primers revealed the presence of common band at 958 bp (Fig. 7), which agree with that reported by Spilker *et al*., (2004).

The molecular characterization of DNA for 8/10 *V. anguillarum* revealed the presence of common band at 429 bp (Fig. 8), these results were in agreement with the results of Gyeong *et al*., (2007).

The molecular characterization of DNA for 9/10 *E. tarda* revealed the presence of common band at 450 bp (Fig. 9); these results were nearly similar to that of Castro *et al.*, (2014).

The molecular characterization of DNA for 6/10 *S. agalactiae* revealed the presence of common band at 220 bp (Fig. 10); these results were nearly similar to that of Itsaro *et al.*, (2012).

3. 4. Determination of median lethal dose (LD_{50}) of *A. hydrophila* and *S. agalactia*

Mortality, clinical signs also, the external and internal signs of clear septicemia appeared on the experimentally infected fish in the LD_{50} experiments confirmed the pathogenicity of *A. hydrophila* and *S. agalactia* (Fig. 11&12); these results were in agreement with that of Ibrahem *et al* ., (2008) and Liu *et al.*, (2014).

Figure 1. Naturally diseased *O. niloticus* showing scale detachment, signs of septicemia, extensive skin and fins Petechial hemorrhages and cutaneous ulcers.

Figure 2. Naturally diseased *O. niloticus* showing scale detachment, skin and fins Petechial hemorrhages and severe cutaneous ulcers.

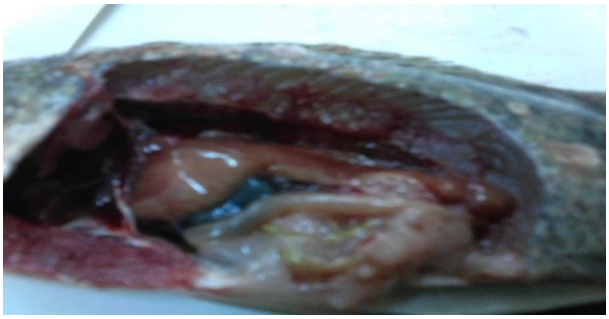

Figure 3. Naturally diseased *O. niloticus* showing enlarged congested liver with distended gall bladder, dark congested enlarged spleen and coagulated fibrinous ascitic fluids.

Figure 4. Naturally diseased *O. niloticus* showing enlarged liver dark, congested enlarged spleen and ascitic fluids.

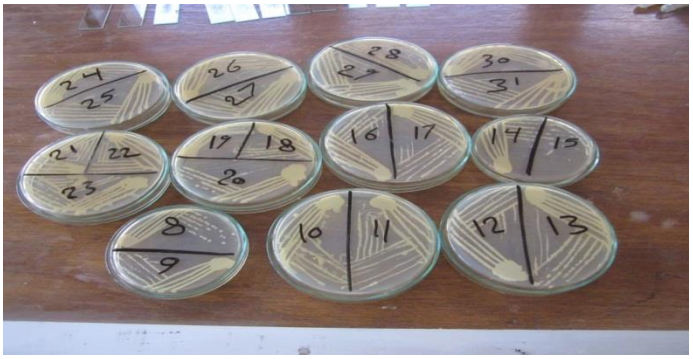

Figure 5. Primary isolation of different bacteria on TSA media**.**

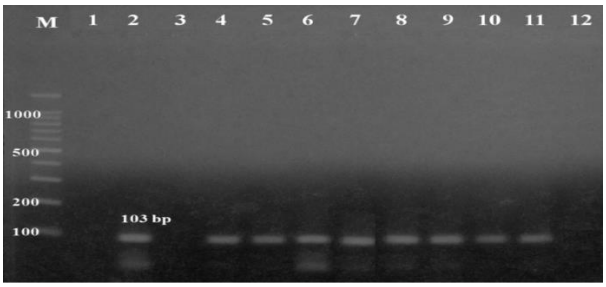

Figure 6. Agarose gel electrophoresis of PCR amplification of (450 bp) for characterization of *A. hydrophila* Lane M: 100 bp ladder as molecular size DNA marker. , Lane 1 control negative for *A. hydrophila*, , Lane 2 control positive for *A. hydrophila*,, Lane 4-11 positive for *A. hydrophila*,, Lane 3,12 negative for *A. hydrophila*.

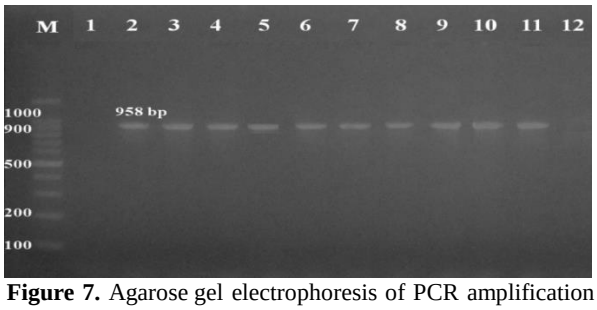

Figure 7. Agarose gel electrophoresis of PCR amplification of (450 bp) for characterization of *P. aeruginosa*, Lane M: 100 bp ladder as molecular size DNA marker, Lane 1 control negative for *P. aeruginosa*, Lane 2 control positive for *P. aeruginosa*, Lane 3-11 positive for *P. aeruginosa*, Lane 12 negative for *P. aeruginosa*.

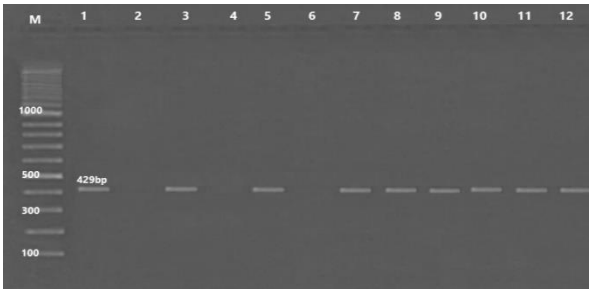

Figure 8. Agarose gel electrophoresis of PCR amplification of (450 bp) for characterization of *V. anguillarum***,** Lane M: 100 bp ladder as molecular size DNA marker.**,** Lane 1 control positive for *V. anguillarum* **,** Lane 2 control negative for *V. anguillarum***,** Lane3,5,7,8-12 positive for *V. anguillarum***,** Lane 4,6 negative for *V. anguillarum*.

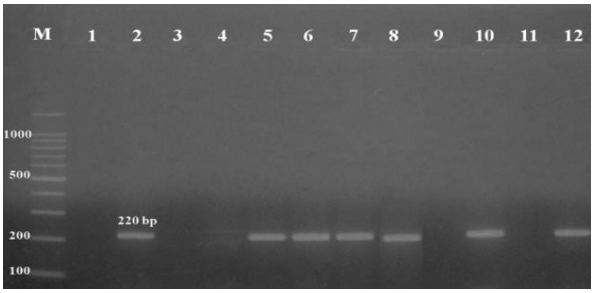

Figure 9. Agarose gel electrophoresis of PCR amplification of (220 bp) for characterization of *S. agalactia***,** Lane M: 100 bp ladder as molecular size DNA marker, Lane 1 control negative for *S. agalactia* **,** Lane 2 control positive for *S. agalactia***,** Lane 5-8,10,12 positive for *S. agalactia***,** Lane 3,4,9,11 negative for *S. agalactia*

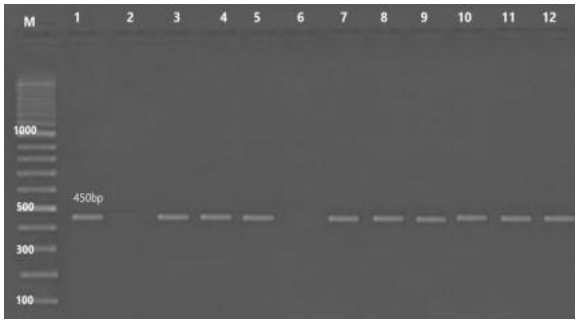

Figure 10. Agarose gel electrophoresis of PCR amplification of (450 bp) for characterization of *E. tarda* , Lane M: 100 bp ladder as molecular size DNA marker. Lane 1 control positive for *E. tarda*, Lane 2 control negative for *E. tarda*, Lane 3-5,7-12 positive for *E. tarda*, Lane 6 negative for *E. tarda*

Figure 11. *O. niloticus* experimentally infected with strains in LD_{50} experiments showed mild haemorrhages on the body, loss of pigmentation and exophthalmia.

Figure 12. *O. niloticus* experimentally infected with strains in LD_{50} experiments showed congested liver dark, congested enlarged spleen and ascitic fluids.

Table 2. Occurrence of bacterial isolates.

Total No. of examined fish	*Aeromonas hydrophila*	*Pseudomonas aeruginosa*	*Vibrio anguillarum*	*Streptococcus*	*Edwardsiella tarda*	*Edwardsiella ictulari*	*Pseudomonas fluorescens*	*Flavobacterium columnare*	*Enterococcus feacalis*	Total No. of isolates
150	78 (30.5%)	52 (20.3%)	40 (15.6%)	30 (11.7%)	20 (7.8%)	12 (4.7%)	10 (3.9%)	7 (2.7%)	7 (2.7%)	256

Table 3. Biochemical tests for identification of Gram negative bacteria.

	Aeromonas hydrophila	*Pseudomonas aeruginosa*	*Vibrio anguillarum*	*Edwardsiella tarda*	*Edwardsiella ictulari*	*Pseudomonas fluorescens*	*Flavobacterium columnare*
Gram stain	-	-	-	-	-	-	-
Motility	+	+	-	+	+	+	-
Urease	-	V	-	-	-	V	-
H_2S production	+	-	-	+	+	-	+
Indole	+	-	+	+	+	-	+
MR	V	-	-	+	+	-	V
VP	V	-	+	-	-	-	V
Citrate utilization	v	+	+	-	-	+	+

+ = Positive, - =Negative, v = Variable

Table 4. Biochemical tests for identification of Gram positive bacteria

Test	*S.agalactiae*	*Enterococcus feacalis*
Gram stain	+	+
Catalase	-	-
Sugar fermentation Mannitol	-	+

+ = Positive, - = Negative

4.CONCLUSION

The obtained results cleared that, bacterial infections led to great economic losses in fish farms raised *O. niloticus*. *A. hydrophila, P. aeruginosa, V. anguillarum, E. tarda, E. ictulari, P. fluorescens, F. columnare, S. Agalactia, E. feacalis* were the most prevalent bacteria affecting fish farms and implicated in the *O. niloticus* seasonal summer mortalities. The isolated strains give clear septicemia in experimentally infected fish, confirming the strains pathogenicity. The early detection of bacterial pathogen using PCR technique is considered a great help in fish farms before the onset of the clinical picture allowing rapid interference and treatment.

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