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IDENTIFICATION OF COMMON FISH BACTERIAL PATHOGENS IN KAFR EL SHEIKH GOVERNORATE EGYPT USING PCR

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ABSTRACT

As the Egyptian aquaculture industry is ranked the first in the area of Africa and Mediterranean Sea countries, the diagnosis of bacterial fish diseases is a challenging process since the bacterial pathogens threaten this industry. During the current study polymerase chain reaction (PCR) as a powerful molecular technique was validated for the identification and the diagnosis of bacterial fish pathogens in Kafr El Sheikh governorate. The present study was conducted on 121 diseased and apparently healthy Nile tilapia fish (*Oreochromis niloticus n*=105), and Catfish (*Clarias gariepinus n=16*). The examined *Oreochromis niloticus* were positive for *V. mimicus*, *V.* alginolyticus, *V. fluvialis*, *V. parahaemolyticus*, *V. vulnificus*, for *A. hydrophila* and, for *Providencia rettgeri*. The most isolated bacteria from the examined *Clarias gariepinus* fishes were *Pseudomonas fluorescens* and *Proteus vulgaris*. PCR allowed the successful detection and discrimination of three species: *V. fluvialis*, *Pseudomonas fluorescens* and *A. hydrophila*.

Keywords*: Clarias gariepinus, Oreochromis niloticus, V. fluvialis, Pseudomonas fluorescens, A. hydrophila* and PCR

INTRODUCTION

Egypt's aquaculture production were estimated over 705,490 tonnes in 2009 as the largest of any African country and 11th in the global production and the importance of this sector as it is providing a cheap source of protein for the Egyptian people **(Macfadyen *et al.*, 2011; Sadek, 2013).**

There are seven finfish which are important in the Egyptian aquaculture production (tilapia, Mullet spp, Carp spp., Catfish, Bayad, Sea bream and sea bass, besides three crustacean species, Macrobrachium rosenbergii, Paneus semisulcatus and P. japonicus **(Sadek *et al.*, 2006).**

Tilapia has chaired 55.3 percent of the total aquaculture production, followed by Mullet spp., Carp spp., African catfish and other species (Gilthead seabream, European seabass, etc.), 29.7 percent, 10.5 percent, 2.5 percent and 2 percent respectively during 2009 (**Sadek, 2013)**. Moreover, he stated that Kafr-El- Sheik and Beheira governorates are the most important sites for the tilapia cage projects.

Outbreaks of bacterial diseases are largely responsible for the high mortality of wild and farm-cultured fish causes severe economic losses to fish farms (**Olsson *et al.*, 1998; Ramkumar *et al.*, 2014).** Species of genus *Aeromonas*, *Vibrio*, *Edwardsiella* a nd *Streptococcus* are the most common fish pathogenic bacteria that cause economically devastating losses in aquaculture **(Zhang *et al*., 2014).**

Recently, vibriosis as an important pathogenic cause for outbreaks in Egyptian aquaculture industry was recorded in many studies **(Abd El-Galil and Mohamed, 2012; Abd-Elghany and Sallam, 2013; Abdel-Aziz *et al.*, 2013; Eissa *et al.*, 2013** ; **El-Hady et al., 2015).**

Concerning, Pseudomonas incidence in Egypt, in the last decade there also some studies such as **Khalil *et al.* (2010) and El-Hady and Samy, (2011).** Moreover, **Khairul Afizi *et al.* (2013); El-Hady and Ahmed (2014) and Noor El Deen *at al.* (2014)** isolated *Aeromonas* species from Egypt.

Such a combination of biochemical and physiological characteristics assays typically require long assays times and are not practical for high throughput analysis. Alternately, the use molecular techniques such as

polymerase chain reaction (PCR) can preclude the use of culture-based and biochemical-based techniques **(Lyon, 2001; Panicker *et al.*, 2004; Raghunath *et al.*, 2007).**

The present study was undertaken to study the prevalence of bacterial pathogens in Nile tilapia fish and Catfish in Kafr Elsheikh governorate by developing a PCR assay for the identification of the most commonly pathogenic bacteria.

MATERIALS AND METHODS

Fishes:

During the period from January to December 2014, 121 diseased and apparently healthy fishes were collected from different localities in Kafr El Sheikh Governorate, Egypt for the current study; 105 Nile tilapia fish *(Oreochromis niloticus)*, and 16 Catfish *(Clarias gariepinus)*. Directly after the collection, fishes were labeled and transferred alive from fish farms to the laboratory of Microbiology Department, Animal Health Research Institute, Kafr El Sheikh for the bacteriological examination.

Clinical examination and post-mortem examination of fish:

Fish were examined clinically for any abnormal lesions according to **Noga (1996) and Austin and Austin (2007)**. After euthanasia and evisceration, samples of liver, spleen, heart and kidney were aseptically transferred to culture medium for the bacteriological examination.

Isolation and identification of different bacterial spp.:

Samples of the liver, spleen, heart and kidney from the examined fishes were streaked directly onto different media such as nutrient agar, Trypticase soy agar (TSA), MacConkey agar, CIN, Aeromonas agar, Thiosulfate-citrate-bile salts-sucrose agar (TCBS) and Pseudomonas agar medium plates then incubated at 28ºC for 24-48 hr. Identification of all isolates was done by cultural, morphological and biochemical characters according to **Quinn *et al.* (2002), Austin and Austin (2007), Panangala *et al.* (2007)** and through examination using API-20E and API-20NE (Biomérieux, France) for the biochemical confirmation.

Experimental infection of Nile tilapia fish *(Oreochromis niloticus),* with *Pseudomonas fluorescens; Aeromonas hydrophila and Vibrio fluvialis:*

A total number of 40 apparently healthy Oreochromis niloticus of 60 ± 5 g

body weight were held in aerated freshwater aquaria supplied with dechlorinated tap water at 25˚ C for ten days prior to challenge. Tilapias under the experiment were divided into four groups (Ten fishes for each group). The first group injected intra-peritoneal with 0.2 ml of trypticase soya broth and acts as a control group while the other groups injected intra peritoneal with 0.2 ml of trypticase soya broth containing $3x10^7$ CFU /ml of *Pseudomonas fluorescens* ***(Eissa et al., 2010)***; 0.1ml of *Aeromonas hydrophila* suspension of ($1x10^8$ CFU) ***(Aly et al., 2008);*** and 0.5 ml of bacterial suspension of *Vibrio fluvialis* at the dose 1x108 cell/ml ***(Al-Sunaiher et al., 2010)*** and acts as experimentally infected group**.** After the inoculation, the fish were observed over a period of two week; during which the clinical signs, morbidity and mortality percent were recorded and re-isolation of challenged bacteria was carried out from different organs such as liver, spleen, kidney and heart.

Molecular characterization of *Pseudomonas fluorescens; Aeromonas hydrophila and Vibrio fluvialis:*

Single PCR were conducted on randomly selected positively and biochemically identified isolates for the molecular characterization of different species of the bacterial cultures and different organs samples of positive isolates for *Pseudomonas fluorescens; Aeromonas hydrophila and Vibrio fluvialis* spp. DNA extraction, PCR amplification, DNA gel electrophoresis, DNA Staining and DNA gel documentation were conducted according to **Swaminathan *et al*. (2004), Scarpellini et al. (2004), Kim and Bang (2008).** Application of PCR for identification and characterization of the different three was applied using different primers (Pharmacia Biotech) as shown in Table 1.

Table 1: Primers were used for different three species:

	Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
V.fluvialis	vflu (F)	5′ TATACTATCCGACGACTGGC ′3	250	Kim and Bang (2008)
	vflu (R)	5′ TTCGTAGATATTCGGAGGAA ′3		
*P.*fluorescens	16SPSER (F)	5′ TGCATTCAAAACTGACTG ′3	800	Scarpellini et al. (2004)
	16SPSER (R)	5′ AATCACACCGTGGTAACCG ′3		
Aeromonas Hydrophila	lip (F)	5'-AACCTGGTTCCGCTCAAGCCGTTG-3'	760	Swaminathan et al. (2004)
	lip (R)	5'-TTGCCTCGCCTCGGCCCAGCAGCT-3'		

RESULTS

Clinical and Post-mortem examination of the examined fishes:

Hemorrhages all over the fish body especially at the base of fins, tail, anal opening, mouth and fins rot; darkness of skin, increased in mucous secretion are the common clinical signs were recorded during the clinical examination of examined fishes. Concerning, the postmortem findings were pale anemic liver in some cases and in other cases the livers were hemorrhagic and congested. Kidneys and spleen were enlarged and congested. Intestine was inflamed, hemorrhagic.

Isolation and Identification of bacterial fish pathogens:

The results indicated that 68 out of 121 (56.2%) collected fishes were infected with different types of bacteria. Among the examined Oreochromis niloticus 58 out of 105 (55.24%) were positive, while 10 out of 16 (62.5%) Clarias gariepinus fish were positive. The bacteriological examination of apparently health and naturally diseased fishes are shown in Fig. 1.

Figure 2 illustrated that 18.97 % of the examined Oreochromis niloticus were positive for V. mimicus, 10.34% for V. alginolyticus, 8.62% for V. fluvialis, 6.89% for V. parahaemolyticus, 5.17% for V. vulnificus and 10.34% for A. hydrophila. Providencia rettgeri was the most isolated bacterial spp. (39.66%) recovered from Oreochromis niloticus. The most isolated bacterial spp. from examined Clarias gariepinus fishes were 80% Pseudomonas fluorescens and 20% Proteus vulgaris (Fig. 3).

Results of re-isolation of the pathogenic bacteria from the experimentally infected fish:

Re-isolation of different pathogenic bacteria was obtained from the different organs (liver, kidney, heart and spleen) of freshly dead and sacrificed experimentally infected fish to confirm the rapid identification of pathogenic bacteria affected fishes. Experimentally infected Oreochromis niloticus with Pseudomonas fluorescens showing ulceration, congestion of gills, congested enlarged liver, hemorrhages on belly and tail. In the contrary, the control group remained clinically health and showed neither pathological lesions nor bacterial isolation and none of the control group died.

Molecular characterization of Pseudomonas fluorescens; Aeromonas

hydrophila and Vibrio fluvialis:

The PCR was performed with simultaneous use of the three pairs of primers targeting 16 SPSER, 16S rRNA, and vflu genes specific for Pseudomonas fluorescens; Aeromonas hydrophila and Vibrio fluvialis respectively, as shown in Fig. 4, 5, and 6.

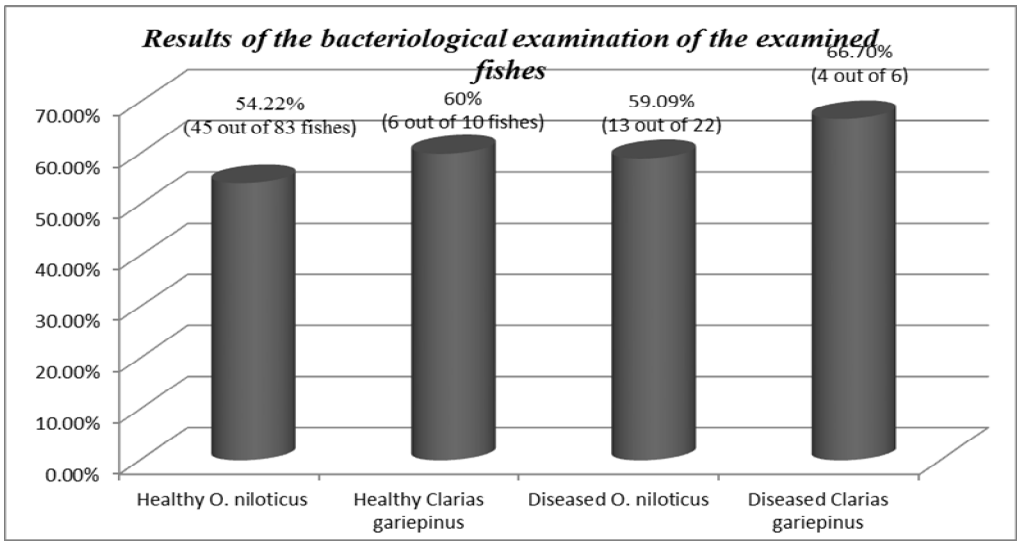

Fig. 1: Results of the bacteriological examination of the examined *Oreochromis niloticus* and *Clarias gariepinus* fishes

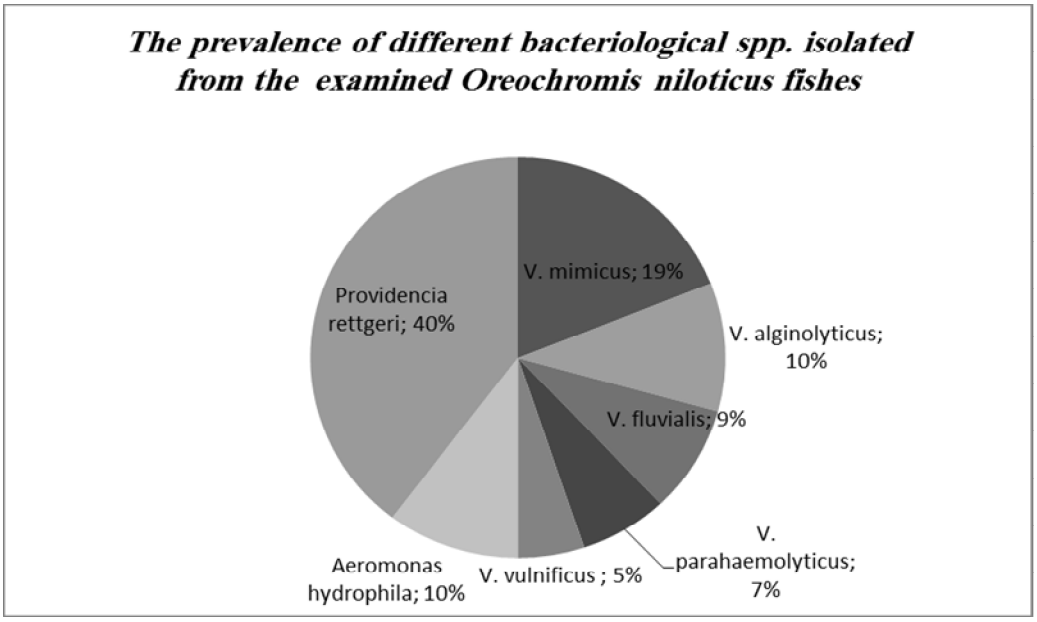

Fig. 2: prevalence of different bacteriological spp. isolated from the examined *Oreochromis niloticus*

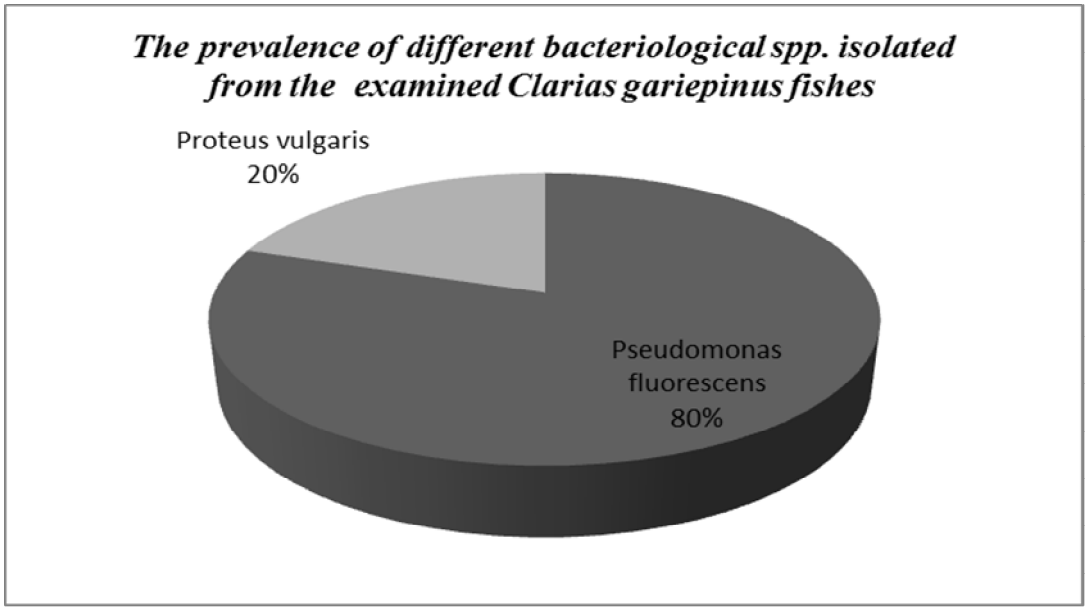

Fig. 3: prevalence of different bacteriological spp. isolated from the examined *Clarias gariepinus*.

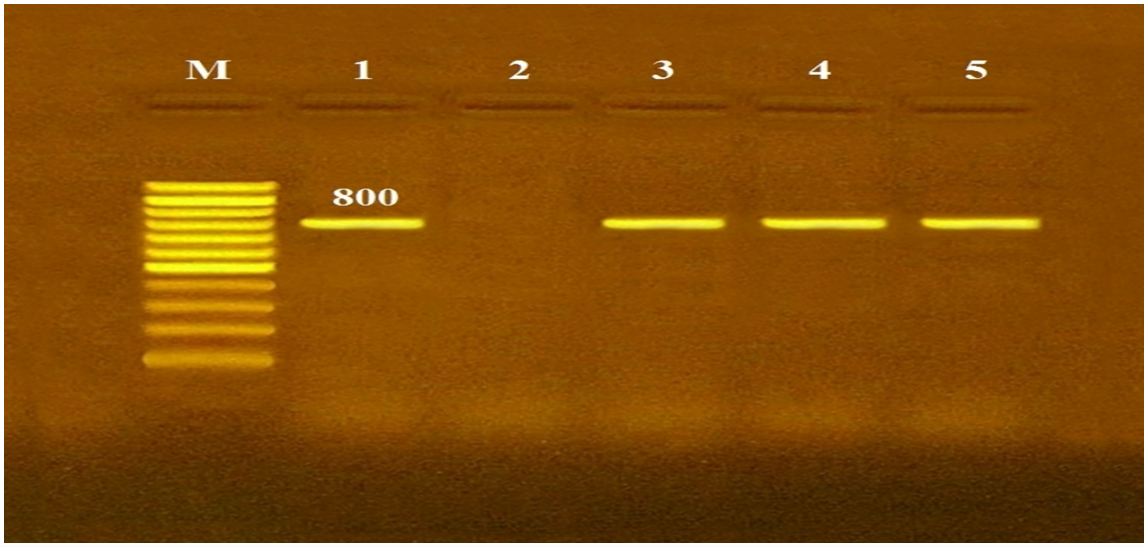

Figure 4: ***Agarose gel electrophoresis of PCR of 16 SPSER gene (800 bp) for characterization of Pseudomonas*** **fluorescens.**
Lane M: 100 bp ladder as molecular size DNA marker.
Lane 1: Control positive for 16SPSER gene.
Lane 2: Control negative.
Lane 3 (L), 4 (K) & 5 (S): Positive samples for Pseudomonas *fluorescens*.

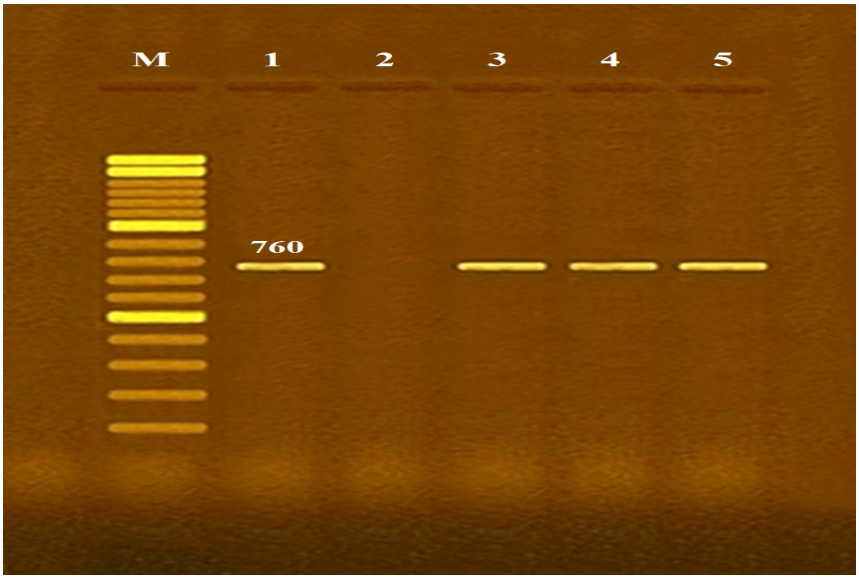

Figure 5: Agarose gel electrophoresis of PCR of lip gene (760 bp) for characterization of Aeromonas hydrophila.
Lane M: 100 bp ladder as molecular size DNA marker.
Lane 1: Control positive for lip gene.
Lane 2: Control negative.
Lane 3 (L), 4 (K) & 5 (S): Positive samples for *Aeromonas hydrophila*.

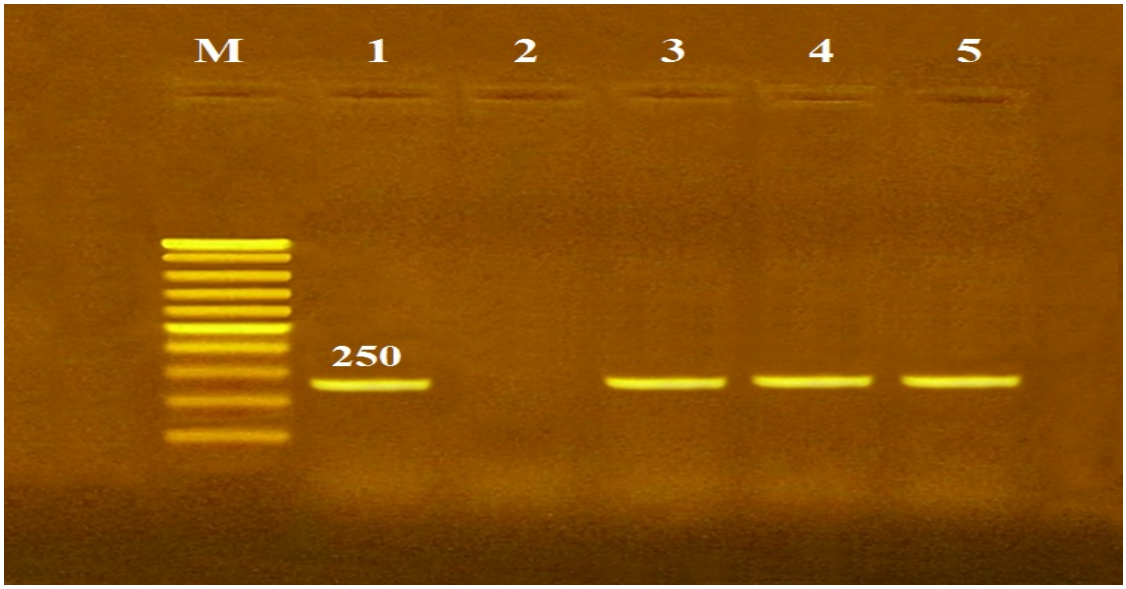

Figure 6: Agarose gel electrophoresis of PCR of vflu gene (250 bp) for characterization of Vibrio fluvialis.
Lane M: 100 bp ladder as molecular size DNA marker.
Lane 1: Control positive for vflu gene.
Lane 2: Control negative.
Lane 3 (L), 4 (K) & 5 (S): Positive samples for *Vibrio fluvialis*.

DISCUSSION

Since the fishes are the cheapest source of protein for the Egyptian peoples which their numbers in ongoing increase, the application of advanced molecular techniques in the rapid identification of the pathogenic microorganisms affecting fish is very important.

During the current study we tried to uncover the role of PCR as advanced tools for the rapid diagnosis of bacterial pathogens affecting farm-cultured fishes. Where, the bacterial diseases are largely responsible for high mortalities in aquacultured fish **(Ramkumar *et al*., 2014).**

We decided to choose Kafr Elsheikh governorate as it is sharing with huge production in the Egyptian fish industry. Other aim of this study was to elaborate the prevalence of the most pathogenic bacteria in Kafr Elsheikh governorate.

The positive fishes for the isolation of these different pathogenic bacteria showed clinical signs similar to those in septicemic diseases affecting fish. These results are in agreement with those of **El-Hady and Samy (2011), Ruwandeepika *et al*. (2012).**

The prevalence results of this study revealed that Providencia rettgeri was the most isolated bacterial spp. from Oreochromis niloticus followed by V. mimicus, V. alginolyticus, A. hydrophila, V. vulnificus, V. parahaemolyticus and V. fluvialis. Pseudomonas fluorescens. Concerning Clarias gariepinus, Proteus vulgaris were also isolated from the examined fishes. These results were in the agreement with previous studies of **El-Hady and El-Katib (2008), Khalil et al. (2010), El-Hady and Samy (2011).**

Morphological and biochemical methods as conventional methods for the diagnosing of bacterial infections in fish are complex and time-consuming for reaching a definitive diagnosis. Consequently, causing delay in the implementation of control measures causes massive economic losses due to potential spreading of diseases. Moreover, many pathogens share common morphological characteristics and cause similar clinical signs in diseased fish. Molecular identification techniques were used as alternative diagnostic technique **(Bader et al. 2003, Bilodeau et al. 2003, Altinok 2011, Tsai et al. 2012).**

The experimental inoculations of tilapia fish with isolated bacteria validate the use of PCR for the rapid identification of pathogenic bacteria affecting fish. Since, the experimental studies evoked the natural disease where these experimental studies are supporting evidence of pathogenicity of these bacteria **(Fawzy *et al*. 2014).**

In conclusion, the present study validate the use of PCR as a valuable molecular method for detection and identification of fish bacterial pathogens in fish farms of Kafrelsheikh governorate.

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