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ASSESSMENT OF SINGLE AND COMBINED CO-INFECTION OF *Aeromonas hydrophila* AND *Vibrio parahaemolyticus* ON DISEASE PATHOGENICITY IN *Oreochromis niloticus*

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Abstract

Environmentally transmitted bacterial pathogens might cause both single and combined co-infections in their hosts. Co-infections may lead to increase or decrease virulence of the pathogens, depending on the nature of the interactions between the co-infecting bacterial strains. Four isolated strains of an opportunistic fish pathogens; *Aeromonas hydrophila* and *Vibrio parahaemolyticus*, containing one or two tested virulence genes/each obtained from different fish farms were used to shed light on the effect of single and combined co-infection of these opportunistic bacteria on their virulence and pathogenicity in *Oreochromis niloticus*, recording how different strains with different number of virulence genes affects the disease virulence (measured as mortality and disease signs in *O. niloticus*). The results revealed that the pathogenicity of the disease were significantly influenced by the combination between strains of different species, the number of the co-infecting strains and the number of virulence genes. Infection with two strains of different species containing the four tested virulence genes recorded the highest fish mortality than other infections. Moreover, the co-infection with two strains of different species regardless the number of the contained virulence genes in both strains; even with two virulence genes were more virulent than two strains of same species with three virulence genes, which might clarify the synergistic action between the two pathogens . However, the pathogenicity of the disease in the single and co-infected groups with the same bacterial species was positively correlated with the number of the virulence genes.

Key words: Co-infection virulence genes *Aeromonas* hydrophila *Vibrio* parahaemolyticus.

INTRODUCTION

Aquaculture is the fastest growing sector of agriculture in the world. However, infectious disease is a major obstacle facing aquaculture development and is often the most significant cause of economic loss (Plant and LaPatra, 2011).

The aquaculture production is hindered by diseases caused by various fish pathogens and is constraint to the culture of many aquatic species (Bondad-Reantaso *et al*., 2005).

Nile tilapia (*Oreochromis niloticus*) represents one of the most popular crops in the world. However, species cultivation may present some health problems, which are associated with the presence of pathogenic bacteria (Pech *et al*., 2017).

Bacterial pathogens are the worst causative agents of infectious diseases and responsible for heavy mortalities in both reared and wild fish species; resulting in high economic losses in aquaculture (Meyer, 1991). The list of new pathogenic bacterial species isolated from the fish has been rapidly increasing matching with the rapid growth and intensification of aquaculture, pollution and increased use of water bodies (Harvell *et al*., 1999).

Intensive fish aquaculture and/or inadequate farming practices might lead to stressful conditions increasing infection occurrence and disease outbreak (El-Sayed, 2006).

Naturally, co-infections are very common when fish are infected by two or more genetically different pathogens either by simultaneous or secondary infections where each pathogen has pathogenic affects and causes harm to the fish (Cox, 2001 and Bakaletz, 2004). Co-infections have a basic and serious effect that can alter the course and the severity of different fish diseases. During co-infections episodes, the different pathogens interact with each other resulting in various outcomes; the load of one or both pathogens may be increased, one or both may be suppressed or one may be suppressed and the other increased (Cox, 2001).

Pathogens can compete with each other for target sites inside the same host. Sometimes, one pathogen can impair the host immune response against infections by the other pathogens either by suppressing or promoting the immune system (Telfer *et al*., 2008). Subsequently, there is a change of the host susceptibility to infection and can affect the host-pathogen dynamics, disease severity, duration of infection and host pathology (Telfer *et al*., 2008). Therefore, the interactions between co-infection pathogens can be either synergistic or antagonistic (Cox, 2001).

Synergistic effects, inside the host, can occur when the first pathogen induces immunosuppression and hinders the immune response against subsequent infections, resulting in an increase in the infections severity and the mortality rates (Bradley and Jackson, 2008 and Telfer *et al*., 2008). However, the antagonistic effects can result from competition between different pathogens for nutrients and target sites leading to limit the population size of the infectious agents and, in some cases, alter the site of infection (Andrews *et al*., 1982). Sometimes, the antagonistic effects happen when the first pathogen triggers and modulates the host immune response and hinders the second pathogen (Chen *et al*., 2013).

Up till now, little is known about how the presence of one pathogen can affect the load of other pathogens and how the host mortality rate will be changed during co-infection in comparison with single infection (Bradley and Jackson, 2008). A considerable necessity to investigate the interactions taking place between different bacterial pathogens during mixed infections and the effects of multi-infections on fish disease pathogenesis, and prognosis (Johnson and Hoverman, 2012).

Vibrio sp. is mainly pathogenic to both marine and brackish water fish and occasionally in freshwater fish (Lightner and Redman, 1998). Vibriosis is worldwide major bacterial disease causing considerable economic losses in aquaculture production and processing (Toranzo *et al*., 2005). *Vibrio parahaemolyticus* is widely distributed in the marine environments and considered the main cause of human gastroenteritis disorders after consumption

of contaminated raw or undercooked seafood (Iwamoto *et al*., 2010) and occasionally causes wound infections in humans worldwide (Shimohata and Takahashi, 2010).

Vibrio parahaemolyticus strains have a number of different virulence factors including thermostable direct hemolysin (tdh) and TDH related hemolysin (trh) (Makino *et al*., 2003); that are associated mainly with hemolysis, cytotoxic activity and the pathogenicity in the host cell (Pazhani *et al*., 2014). This means that most of clinical cases of *V. parahaemolyticus* have been associated with *V. parahaemolyticus* strains carrying tdh and/or trh (Pazhani *et al*., 2014).

Aeromonas hydrophila is one of the most important potential freshwater fish pathogens responsible for gill and skin diseases among the fishes and leading to high mortality (Smith, 2006). Virulence in *A. hydrophila* is multifactorial, resulting from the production and/or secretion of virulence factors, such as hemolysins and proteases (Beaz-Hidalgo and Figueras, 2013). Hemolysins (hlyA) are a diverse group of multifunctional enzymes has been suggested as a reliable way for identifying potentially pathogenic *Aeromonas* strains playing a main role in *A. hydrophila* pathogenesis (Heuzenroeder *et al*., 1999). Moreover, Pansare *et al*. (1986) has reported that the protease is the major virulent factor in fish toxicity. Protease virulence gene may have a role in the proteolytic activation of proaerolysin (Howard and Buckley, 1985). Most of these toxic virulence factors have implications in aquaculture Beaz-Hidalgo and Figueras (2013) human and/or animal health (Korkoca *et al*., 2014).

The present work was planned to shed light on the effect of single and combined co-infection of *Aeromonas hydrophila* and *Vibrio parahaemolyticus* as two opportunistic bacteria on their virulence and pathogenicity in *Oreochromis niloticus*, recording how different strains with different number of virulence genes affects the disease virulence. This will deepen the understanding of the disease process and pathogenesis and will prove useful data for disease management.

MATERIALS AND METHODS

Fish:

A- Fish collected for isolation of pathogen:

A total number of 60 naturally diseased *O. niloticus* fish were randomly collected from 10 private affected fish farms in Kafr El-Shiekh Governorate (6 fish /affected fish farm) during the episodes of the seasonal mortalities. The fish were examined externally for any abnormalities e.g. (hemorrhages, ulceration.... ,etc.) according to Austin and Austin (1987) and immediately subjected to post-mortem and bacteriological examination according to Plumb and Browser (1982).

B- Fish used for LD_{50} and pathogenicity experiments:

A total number of 620 apparently healthy *O. niloticus* were kept in a glass aquaria with aeration system and maintenance ration at sakha research unit, Central lab. for Aquaculture Research, for a period of two weeks for acclimation, prior to the lethal dose fifty and pathogenicity experiments (Plumb and Browser, 1982). Random samples were taken for bacteriological examination to ensure that the fish were *A. hydrophila* and *V. parahemoliticus* pathogen free. The fish were supplied with a commercial fish diet containing 25% crude protein, offered twice daily at 3% of biomass (Eurell *et al*., 1978).

Bacteriological examination:

Aseptically, swabs from skin ulcers, liver and kidney of diseased *O. niloticus* were collected and pre-enriched on tryptic soy broth (TSB, Oxoid) and incubated for 24 hrs at 30˚C. A loopful of tryptic soy broth were then streaked on tryptic soy agar (TSA) and incubated for 48 hrs at 30˚C. Each type culture colony was picked up and sub-cultured on selective diagnostic agar media for presumptive identification; Rimler Shotts agar (RS), Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) for *Aeromonas hydrophila* and *Vibrio parahemolyticum*, respectively. The inoculated plates were incubated at 30ºC for 24-48hrs. Well-differentiated single suspected bacterial colonies were

picked up and inoculated into semisolid media (0.5% soft agar) for detection of bacterial motility and/or preservation of the isolates for further investigations. Suspected colonies were stained with Gram's stain and viewed microscopically (APHA, 1992) and the biochemical identification was carried out according to Kreig and Holt (1984) and MacFaddin (2000).

Molecular identification by Polymerase Chain Reaction (PCR):

DNA extraction was performed using QIA amp kit (QIAamp: Qiagen inc., USA) according to Shah *et al*. (2009). The isolates was identified using specific primer to confirm the biochemically identified isolates according to Trakhna *et al*. (2009) and Tarr *et al*. (2007) for *A. hydrophila* and *Vibrio sp*., respectively.

The selected virulence genes of both bacterial pathogens, were screened out by Polymerase Chain Reaction (PCR) using specific primer (Pharmacia, Biotech); Haemolysin (hlyA), and Temperature sensitive protease (eprCAI) genes for *Aeromonas hydrophila* according to (Zheng *et al*. (2012) and Hu *et al*. (2012) and thermolabile hemolysine (trh) and thermostable direct hemolysine (tdh) for *V. parahaemolyticus* according to (Nelapati and Krichnaiah, 2010) and (Bej *et al*., 1999), respectively as shown in Table (1).

Table 1. The primers sequences used for PCR identification of different bacterial pathogens and its associated selected virulence genes.

Target	Primer Name	Oligonucleotide sequence (5′- 3′)	Product size (bp)	Reference
A. hydrophila	16SrRNA-F 16SrRNA-R	GGCCTTGCGCGATTGTATAT GTGGCGGATCATCTTCTCAGA	103 bp	Trakhna *et al*. (2009)
A. hydrophila	eprCAI-F eprCAI-R	GCTCGACGCCCAGCTCACC GGCTCACCGCATTGGATTCG	387bp	Hu *et al.* (2012)
A. hydrophila	hlyA-F hlyA-R	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGGG	592bp	Zheng *et al.* (2012)
Vibrio sp.	V.16SrRNA- F V.16SrRNA- R	CGGTGAAATGCGTAGAGAT TTA CTA GCG ATT CCG AGT TC	663bp	Tarr *et al*. (2007)
V. parahaemolyticum	trh (F) trh (R)	CTCTACTTTGCTTTCAGT AATATTCTGGAGTTTCAT	460bp	Nelapati and Krichnaiah (2010)
V. parahaemolyticum	tdh (F) tdh (R)	TTGGAAACGGTTAAAACGAA GAACCTTCCCATCAAAAACA	270bp	Bej *et al.* (1999)

Experimental infection:

Total bacterial count:

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

Determination of median lethal dose (LD_{50}):

A total number of 400 apparently healthy *O. niloticus* weighting (50±0.5 gm), were divided into four major groups for the four strains (100 fish/ each strain). Each group was subdivided into five subgroups (20 fish/ subgroup). From each subgroup, the fifth group was kept as control. On the

basis of the PCR results; the four strains were taken for the LD50 experiment. 24 hours colony cultures of each *A. hydrophila and V. parahaemolyticus* 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 group was intraperitonially injected with 0.5 ml/fish of each bacterial dilution. The fishes in the control group were injected with 0.5 ml PBS/fish (Phosphate Buffer Saline). All fish were kept 7 days post-inoculation for observations, mortalities were recorded twice daily according to Moustafa *et al*., (2016). Re-isolation and biochemical identification of the pathogens were carried out and the freshly dead fish were moved for P.M examination. The LD_{50} of *A. hydrophila* and *V. parahaemolyticus* strains was calculated according to Reed and Muenchen (1938).

Pathogenicity test:

Experimental infection was performed to investigate the pathogenicity of *A. hydrophila* and *V. parahaemolyticus* strains as either a single or combined infection; using intra-peritoneal route injection according to Ibrahim *et al*. (2011). Pathogenecity test was carried out using 220 apparently healthy *O. niloticus*, weighing (60±0.5 gm). Fish were divided into eleven major groups (20 fish/group).

In groups (1-4); each fish was intraperitoneally injected with 0.2 ml/fish of LD_{50} dose of each single strain (A1, A2, V1 and V2) which was determined previously (*A. hydrophila*: A1; hly- eprcAI+….1.2 x 10^6 cfu, A2; hly+ eprcAI+….. 1.3 x 10^6 cfu, *V. parahaemolyticus*: V1; trh+ tdh-…1.8 x 10^5 cfu, V2; trh+ tdh+…..1.5 x 10^5 cfu, respectively). In groups (5 and 6); each fish was intraperitoneally injected with 0.2 ml/fish of LD_{50} of double strains of the same species (A1A2 and V1V2, respectively). However, groups (7-10); each fish was intraperitoneally injected with 0.2 ml/fish of LD_{50} of double strains of the mixed species (A1V1, A2V1, A1V2 and A2V2, respectively). Group number 11 was kept as control and was intraperitoneally injected with 0.2 ml/fish of PBS (Phosphate Buffer Saline) according to Ibrahem *et al.*, (2011). The injected fishes were observed for 30 days post-injection and the mortalities were

recorded twice daily. Re-isolation and biochemical identification of the pathogens were carried out and the freshly dead fish were moved for P.M examination.

RESULTS AND DISCUSSION

Examination of naturally diseased fish:

Clinical signs and postmortem finding:

The external gross lesions of naturally diseased *O. niloticus* revealed scale detachment, signs of septicemia, petechial haemorrhages all over the body surface and gill cover together with extensive skin, fins and tail hemorrhages, excessive mucus secretion, swollen abdomen, exophthalmia and less to severe cutaneous ulcers. These lesions might be due to septicemia resulting from bacterial toxins or the virulence genes of suspected bacteria involved with disease induction Ahmed and Shoreit (2001). The postmortem findings of naturally infected *O. niloticus* showed ascitic fluid in the abdomen, large congested liver with distended gall bladder, the spleen was enlarged and dark congested together with enlarged dark congested kidney. These results agree with Ahmed and Shoreit (2001)

Bacteriological examination:

Biochemical characterizations have proven to be a valuable method for typing and differentiation of bacterial fish pathogens (Austin *et al*., 1997).

Biochemical analysis suggested the isolated bacteria were *Aeromonas sp*. and *Vibrio sp*.; 12 bacterial isolates of gram negative bacteria; *Aeromonas hydrophila* and *Vibrio parahaemolyticus* (Table 2).

Biochemical characters of the bacterial strains were nearly similar to those reported by Plumb (1994) and Pech *et al*. (2017).

Table 2. Biochemical tests for identification of Gram negative bacteria.

Biochemical Tests	*Aeromonas hydrophila*	*Vibrio parahemolyticus*
Motility	+	-
Gram staining	-	-
Indole	+	+
Methyl Red	-	-
Voges Proskauer	+	+
Citrate utilization	+	+
Catalase	+	+
Triple Sugar Iron	+	-
Urease	-	-
Oxidase	+	+
Lactose	+	-
Glucose	+	-
H_2S Production	+	-

Molecular characterization of some bacterial strains:

The molecular characterization of DNA for four strains *A. hydrophila* from six isolates, using specific primers revealed the presence of common band at 103 bp, Fig. (1). These results were closely similar to that reported by Trakhna *et al*. (2009).

However, the molecular characterization of DNA for three strains *Vibrio sp.* from six isolates, using specific primers revealed the presence of common band at 663 bp, Fig. (2). These results were closely similar to that reported by Tarr *et al*. (2007).

Detection of virulence genes:

Regarding the virulence genes, the molecular characterization of eprCAI virulence gene and hlyA virulence gene of *A. hydrophila* using specific primers revealed the presence of common band at 387 bp (3 isolates) and 592 bp (1 isolate), respectively (Fig. 3 and 4). These results were closely similar to that reported by Zheng *et al*. (2012) and Hu *et al*. (2012).

The molecular characterization of trh virulence gene and tdh virulence gene of *V. parahaemolyticus* using specific primers revealed the presence of common band at 460 bp (2 isolates) and 270 bp (1 isolate) , respectively (Fig. 5

and 6). These results were closely similar to that reported by Bej *et al*. (1999) and Nelapati and Krichnaiah (2010).

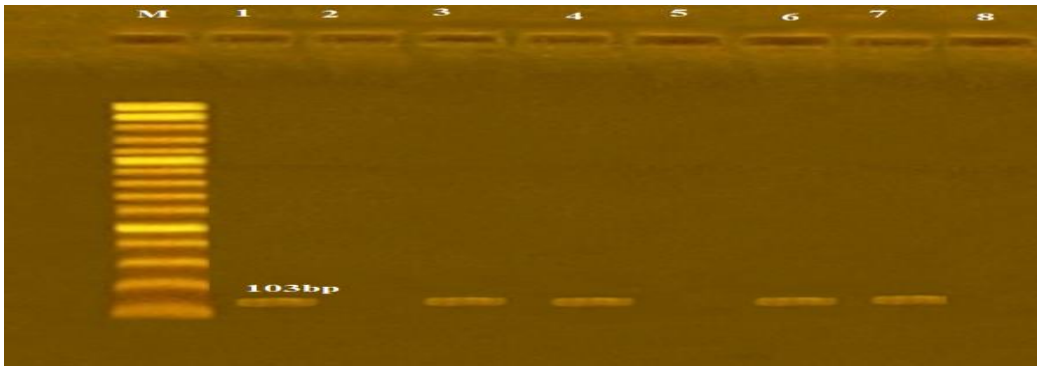

Fig. 1. Agarose gel electrophoresis of PCR of 16S rDNA (103bp) for characterization of *A. hydrophila isolates .*

- Lane M: 100 bp ladder as molecular size DNA marker.
- Lane 1 control positive for *A. hydrophila* .
- Lane 2 control negative for *A. hydrophila* .
- Lane 3,4,6 and 7 positive for *A. hydrophila***.**
- Lane 5and 8 negative for *A. hydrophila* .

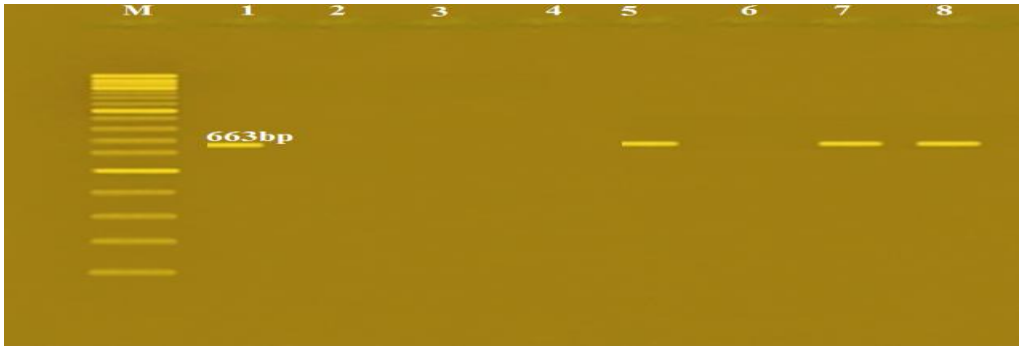

Fig. 2. Agarose gel electrophoresis of PCR of 16S rDNA (663bp) for characterization of *Vibrio sp isolates .*

- Lane M: 100 bp ladder as molecular size DNA marker.
- Lane 1 control positive for *Vibrio sp.*
- Lane 2 control negative for *Vibrio sp.*
- Lane 5,7and8 positive for *Vibrio sp.*
- Lane 3,4and6 negative for *Vibrio sp.*

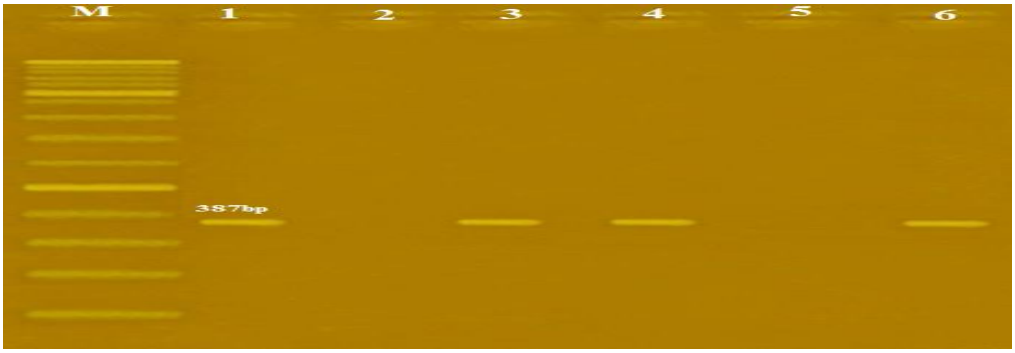

Fig. 3. Agarose gel electrophoresis of PCR of eprCAI gene (387 bp) virulence factor of *A. hydrophila*.

- Lane M: 100 bp ladder as molecular size DNA marker.
- Lane 1 control positive for eprCAI gene.
- Lane 2 control negative for eprCAI gene.
- Lane 3,4 and 6(isolate 1,2and4 respectively) positive for eprCAI gene .
- Lane 5(isolate3) negative for eprCAI gene.

Fig. 4. Agarose gel electrophoresis of PCR of hlyA gene (592 bp) virulence factor of *A. hydrophila* .

- Lane M: 100 bp ladder as molecular size DNA marker.
- Lane 1 control positive for hlyA gene.
- Lane 2 control negative for hlyA gene.
- Lane 5(isolate2) positive for hlyA gene .
- Lane 3,4and6 (isolate4,3 and1 respectively) negative for hlyA gene.

Fig. 5. Agarose gel electrophoresis of PCR of trh gene (460 bp) virulence factor of *Vibrio parahaemolyticus*.

- Lane M: 100 bp ladder as molecular size DNA marker.
- Lane 1 control positive for trh gene.
- Lane 2 control negative for trh gene.
- Lane 4 and 5 positive for trh gene .
- Lane 3 negative for trh gene

Fig. 6. Agarose gel electrophoresis of PCR of tDh gene (270 bp) virulence factor of *Vibrio parahaemolyticus*.

- Lane M: 100 bp ladder as molecular size DNA marker.
- Lane 1 control positive for tDh gene.
- Lane 2 control negative for tDh gene.
- Lane 3 positive for tDh gene .
- Lane 4 negative for trh gene

Strains used in LD_{50}:

On the basis of the PCR and the virulence genes results; two genotypes of *A. hydrophila* and *V. parahaemoliticus* strains were taken for the LD_{50} experiments; A. *hydrophila* positive for the two virulence genes strain: A2 (hyl A+ eprCAI+), positive for one virulence gene strain: A1 (hyl A- eprCAI +), *V. parahaemoliticus* positive for two virulence genes strain: V2 (trh+ tdh+), positive for one virulence gene strain: V1 (trh+ tdh -)

The median lethal dose (LD_{50}) of *A. hydrophila* and *V. parahaemolyticus* strains in *O. niloticus*:

The lethal dose fifty (LD_{50}) of *A. hydrophila* containing one virulence gene (A1) was 1.2 X 10^6 CFU, however, *A. hydrophila* containing two virulence genes (A2) was 1.3 X 10^6 CFU. This results agreed with Pachanawan et al., (2008) who used the LD_{50}; 3.4 x 10^6 CFU for *A. hydrophila*. While, in the strain *V. parahaemolyticus* containing one virulence gene (V1) was 1.8X10^5 CFU and the strain containing two virulence genes (V2) was 1.5 X10^5 CFU.

The differences in the LD_{50} values, between many different authors may be attributed to the number of the virulence genes in the bacterial strains, the pathogen properties, the size and weight of fish species and the environmental conditions such as temperatures difference (Ibrahem *et al*., 2011) .

Pathogenecity test:

Pathogenecity test was carried out to investigate how different strains with different number of virulence genes affects the disease virulence (measured as mortality rates and disease signs in *O. niloticus*).

The gross external signs of experimentally infected *O. niloticus* with *A. hydrophila* and/or *V. parahaemolyticus* strains revealed excessive mucus secretion, scale detachment, haemorrhagic patches on the skin all over the body surface, cutaneous ulcers and tail erosion with congested fins (Plate 1); Present in all groups with variant degrees; A2V2 had a less prominent lesions which may be attributed to the rapid onset of mortality, A1V2, A2V1 and A1V1 had

the sever picture followed by the same species coinfection then the single infection while as the control group showed no clinical changes.

Internally, congested enlarged liver, spleen and internal organs were noticed in the injected fishes (Plate I). Similar clinical signs and postmortem lesions were described by Harikrishnan and Balasundaram (2005) and Pech *et al*. (2017).

Severity of the lesions appeared in the infected *O. niloticus* may be attributed to Temperature sensitive Protease (eprCAI) and hemolysine (hlyA) in *Aeromonas hydrophila* strains and/or thermostable direct hemolysin-related hemolysin (trh) and thermostable direct hemolysin (tdh) of *V. parahemolyticum* strains.

Temperature sensitive protease (eprCAI); a proteolytic enzyme play an important role in the invasiveness and establishment of *A. hydrophila* infections by overcoming initial host defenses and by providing nutrients for microbial cell proliferation (Rivero *et al*., 1990). Proteases degrade fish mucus causing tissue damage, facilitating bacterial invasion into the host cells (Beaz-Hidalgo and Figueras, 2013).

Haemolytic toxins and haemolysin released by *A. hydrophila* may be used as a marker of its pathogenicity (Al-Maleky *et al*., 2011). Hemolysin (hlyA) induces lytic effect and pore formation in the membranes of affected red blood cells, causing anemia (Wang *et al*., 2003). Moreover, Harikrishnan and Balasundaram (2005) recorded that, *A. hydrophila* toxins are cytotoxic, causing acute hemorrhages and necrosis of vital organs (primarily liver and kidney), leading to rapid death due to organs failure.

Detection of the genes encoding the thermostable direct hemolysin (tdh) and the thermostable direct hemolysin-related hemolysin (trh) are strongly correlated with the virulence of *V. parahaemolyticus* (Raghunath, 2014). The presence of these 2 virulent factors (tdh and trh genes) is a useful tool for rapid investigation of suspected pathogenic strains (Gutierrez West *et al*., 2013)

because both are associated with *V. parahaemolyticus* hemolysis and cytotoxicity activity in the host cell (Broberg *et al*., 2011).

TDH is an amyloid toxin having two potential activities; disruption of lipid microdomains and pore-forming toxin, forms pores of ~2 nm in diameter on erythrocyte membrane (Matsuda *et al*., 2010). The fairly large pore size allows both water and ions to flow through the membrane (Honda *et al*., 1992). TRH is a heat labile toxin and immunologically similar to TDH (Honda *et al*., 1988).

Mortality rates of all experimentally infected groups were 95% in A2V2 group, 85% in groups (A1V2 and A2V1), 80% in A1V1 group, 75% in groups (A1A2 and V1V2), 70% in groups (A2 and V2), 60% in groups (A1 and V1) and 0% in the control group, respectively; representing this order A2V2 > A1V2= A2V1 > A1V1> A1A2= V1V2> A2=V2 > A1=V1 in all bacterial injected groups.

The recorded mortality rates revealed the highest mortality with rapid onset in group A2V2 (infected with mixed infection of *A. hydrophila* and *V. parahaemolyticus* strains containing four virulence genes), followed by groups A1V2 and A2V1 (infected with mixed infection of *A. hydrophila* and *V. parahaemolyticus* strains containing three virulence genes). In contrast, group A1V1 (infected with mixed infection of *A. hydrophila* and *V. parahaemolyticus* strains containing two virulence genes) revealed higher mortality rate than groups A1A2, V1V2 (infected with the same species of *A. hydrophila* or *V. parahaemolyticus* strains containing three virulence genes). Moreover, the groups of single infection with higher number of tested virulence genes had higher mortality rate than that of lower number.

The results in the current study revealed that the pathogenicity of the disease were significantly influenced by the combination between strains of different species, the number of the co-infecting strains and the number of virulence genes. Infection with two strains of different species containing four tested virulence genes recorded the highest fish mortality rate than other

infections. Moreover, the co-infection with two strains of different species; regardless the number of the contained virulence genes in both strains; even with two virulence genes were more virulent than two strains of same species with three virulence genes, which might clarify the synergistic action between the two different pathogens. However, the pathogenicity of the disease in the single and co-infected groups with the same bacterial species was positively correlated with the number of the virulence genes. This results are similar to those reported by (Dong *et al*., (2015), however, in contrast to (Johansen *et al.* (2009).

The disease pathogencity in a coinfected host results from complex interactions between the host and the coinfecting strains. The inter-strain interactions are neutral, cooperative and/or competitive depending on the genetic relatedness of the strains (Frank, 1996). Closely related pathogens are likely to cooperate and exploit their hosts economically in order to maximize their transmission, while distantly related pathogens are more likely to compete, leading to increased virulence and decreased transmission due to facilitated host death (Frank, 1996).

The increased virulence in coinfections might be attributed to; one possible cause is that the host immune system has difficulties to cope with a heterogeneous pathogen inoculum compared to a single-strain infection (Davies *et al*., 2002), and the other cause might be that decreasing the relatedness of the coinfecting pathogens (by increasing the number of strains) makes interference competition most beneficial, resulting in higher virulence (Buckling and Brockhurst, 2008). The present study demonstrates that pair wise interactions between coinfecting strains are important for disease virulence; the results are similar to those reported by (Friman and Buckling, 2012).

	
O. niloticus experimentally single infected with *A. hydrophila* containing two virulant gene (A2); showing darkening of the body with hemorrage over the abdomen.	*O. niloticus* experimentally single infected with *A. hydrophila* containing one virulant gene (A1); showing scattered hemorragic patches over the body.
	
O. niloticus experimentally single infected with *V. parahemolyticus* containing two virulant gene (V2); showing scattered hemorragic patches over the pectoral fins and the body.	*O. niloticus* experimentally single infected with *V. parahemolyticus* containing one virulant gene (V1); showing hemorragic patches over the body.
	
O. niloticus experimentally mixed infected with two strains of *V. parahemolyticus* (V1V2: containing three virulant gene); showing hemorragic patches over the body.	*O. niloticus* experimentally mixed infected with two strains of *A. hydrophila* (A1A2: containing three virulant gene); showing benting of the vertebral column together with hemorragic patches over the fins and caudal peduncle.

	
O. niloticus experimentally mixed infected with *A. hydrophila* and *V. parahemolyticus* (V1A1: containing two virulant gene); showing scattered hemorragic patches over the body.	*O. niloticus* experimentally mixed infected with *A. hydrophila* and *V. parahemolyticus* (V2A1: containing three virulant gene); showing abdominal dropsy and hemorrhagic patches over the operculum and abdomen.
	
O. niloticus experimentally mixed infected with *A. hydrophila* and *V. parahemolyticus* (A2v1: containing three virulant gene); showing some hemorragic patches over the abdomen.	*O. niloticus* experimentally mixed infected with *A. hydrophila* and *V. parahemolyticus* (V2A2: containing four virulant gene); showing hemorragic patches over the abdomen together with abdominal dropsy.
	
O. niloticus experimentally mixed infected with *A. hydrophila* and *V. parahemolyticus* (V2A1: containing three virulant gene); showing congested and enlarged liver.	*O. niloticus* experimentally mixed infected with *A. hydrophila* and *V. parahemolyticus* (V2A2: containing four virulant gene); showing severe congestion of all internal organs with enlarged spleen.

Plate 1. Pathogenecity test with *A. hydrophila* and *V. parahemolyticus*

CONCLUSION

Environmentally transmitted bacterial pathogens might cause both single and combined co-infections in their hosts which have major economic impact on aquaculture industry under Egyptian conditions. Experimentally infected *O. niloticus* with different strains of either *Aeromonas hydrophila* or *Vibrio parahaemolyticus* expressed clear septicemia and mortalities. Co-infections; either same or mixed; may lead to increased virulence of the pathogens. The disease pathogenicity was significantly influenced by the combination between strains of different species, the number of the co-infecting strains and the number of virulence genes in both strains. However, the pathogenicity of the disease in the single and co-infected groups with the same bacterial species was positively correlated with the number of the virulence genes. Moreover, the co-infection with two strains of different species regardless the number of the contained virulence genes in both strains; even with two virulence genes were more virulent than two strains of same species with three virulence genes, which might clarify the synergistic action between the two pathogens .

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تقييم العدوى البكتيرية المنفردة والمختلطه من ميكروبى الإيروموناس هيدروفيلا والفيبريو باراهيموليتيكاس على احداث العدوى المرضية فى أسماك البلطى النيلى

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الملخص العربى

مسببات الأمراض البكتيرية المنقولة بيئيا قد تتسبب فى احداث العدوى سواءالمنفردة أو المختلطه في الاسماك. قد تؤدي العدوى المختلطه إلى زيادة أو نقصان ضراوة الميكروب، وهذا يتوقف على طبيعة التداخلات بين السلالات البكتيرية المسببة للعدوى. فى هذه الدراسة تم عزل أربع عترات من مسببات الأمراض الانتهازية فى الأسماك ؛ و هى الإيروموناس هيدروفيلا و الفيبريو باراهايموليتيكاس، و التى يحتوى كل منها على جين أو اثنين من جينات الضراوة ، حيث تم الحصول عليها من مزارع الأسماك المختلفة و ذلك لتسليط الضوء على تأثير العدوى المنفردة والمختلطه من هذه البكتيريا الانتهازية على شدة ضراوة وإمراضية هذه الميكروبات فى أسماك البلطى النيلى (عن طريق تسجيل نسب الوفيات والعلامات الظاهرية للمرض).

و قد أظهرت النتائج أن شدة الاصابة بالمرض تأثرت بشكل كبير بالجمع بين عترات الأنواع البكتيرية المختلفة وعدد عترات البكتيريا المختلفة وعدد جينات الضراوة بهذه العترات. كما قد سجلت العدوى بسلالتين من الأنواع المختلفة التي تحتوي على جينات الضراوة المختبرة الأربع أعلى معدل وفيات بين الأسماك من أنواع العدوى المختلفة الأخرى.علاوة على ذلك، كانت العدوى البكتيرية بسلالتين من الأنواع المختلفة بغض النظر عن عدد جينات الضراوة بهم، حتى مع اثنين من جينات الضراوة هى الأكثر شراسة من استخدام عترتين من نفس النوع مع ثلاثة من جينات الضراوة، والتي قد توضح العمل التآزري بين الايروموناس هيدروفيلا و الفيبريو باراهيموليتيكاس كمسببات للامراض فى البلطى النيلى.ومع ذلك، فإن شدة الاصابة بالمرض فى مجموعات العدوى الفردية والمختلطه تناسبت طرديا مع عدد جينات الضراوة.