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Biochemical and Molecular Characterization of *Aeromonas* Species Isolated from Fish

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ABSTRACT

In this study, a total of 150 *Oreochromis niloticus* (Nile tilapia) (90) and *Ictalurus punctatus* (Channel cat fish) (60) fish were collected randomly during an outbreak of disease mass mortalities from different fish farms in El Sharkia and El Ismailia governorate. Clinical signs and postmortem were recorded. *Aeromonas veronii* were isolated and presumptively identified using API20E system with recovery rate of 36.66% from total number of fish. The identification was confirmed for *Aeromonas veronii* biovar *sobria* by PCR and sequence analysis of 16s rRNA gene. The experimental infection was carried out by *Aeromonas veronii* biovar *sobria* isolate with 60 % of mortality rate. By antibiotic sensitivity test, *Aeromonas veronii* biovar *sobria* isolate was sensitive to Trimethoprim + Sulphamethoxazole and Erythromycin but it was resistant to Oxytetracyclin.

1. INTRODUCTION

Aeromonas species are gram-negative rods, non-spore forming, oxidase positive, ferment glucose and facultative anaerobic bacteria, widely distributed in freshwater, estuarine, and marine environments (Holmes et al., 1996 and Martin-Carnahanand Joseph, 2005). The organisms grow at a wide range of temperatures. *Aeromonas* species cause a wide spectrum of disease syndromes among warm- and cold-blooded animals, including fish, reptiles, amphibians, mammals, and humans (Roberts, 1993; Gosling, 1996 and Janda and Abbott, 2010). It is common in warm water aquaculture in Egypt and cause most serious infectious disease known as Motile *Aeromonas Septicemia* (MAS), associated with high mortality rates without symptoms in per acute phase but in acute phase, skin and fin ulcers appear on the external surface of fish with ascites in the abdomen and exophthalmia (Bondad et al., 2005 and Austin and Austin, 2012). The disease can produce significant losses in aquaculture industry because of reduced growth in survived cases (Pachanawan et al., 2008). *Aeromonas sobria* was responsible for outbreak of septicemia and red mouth disease with mass mortality in private fish farm at Sahl El-Housinia, Sharkia Governorate (El-Bouhy et al., 2015). Also, *Aeromonas veronii* biovar *sobria* was isolated and molecular identified from outbreak of disease mass mortalities in different fish farms at the areas of Bahr El-Baqar (Eissa et al., 2015). Nucleotide sequence analysis only *for identification of Aeromonas veronii* biovar *sobria* provides a rapid and reliable diagnostic techniques (Hassan et al., 2017).

2. MATERIALS AND METHODS

2.1. Fish samples:

Ninety in addition to sixty of naturally infected *Oreochromis niloticus* and *Ictalurus punctatus* respectively with variable size were freshly collected aseptically and examined from private aquaculture fish farms at El Sharkia and El Ismailia Governorate, Egypt in September, 2015. Then immediately transported in a cleaning bag in ice box with cooled ice bags to the laboratory and processed within 3hrs.

2.2. Bacteriological examination:

Swabs were collected from the surfaces of ulcers, erosion, tail, fin, gills, muscles, liver, spleen, kidney, eye and ascetic fluids which were firstly sterilized with heated spatula; sterile swab was inserted through the sterilized area and then inoculated into trypticase soya broth. The inoculated tubes were

incubated at 24-25ºC for 24 hrs. A loopfull from the broth was streaked onto tryptic soya agar, Aeromonas selective agar with Ampicillin supplement media and Rimler's – Shotts medium (R-S medium) then incubated at 24-25ºC for 24-48 hrs. The pure colony was picked up to be subcultured on blood agar medium and incubated at 25 C° for 24 hours for detection the hemolytic activity. A loop full of pure colony inoculated on nutrient agar slant for further identification. Another loop full inoculated on semisolid nutrient agar for testing the motility and preservation. Bacterial isolates were identified according to Schaperclaus et al., (1992) and Bergey (1994) using conventional methods as morphological characters and biochemical testing using analytical profile index of (API 20 E system (BioMérieux).

2.3. Molecular identification and sequencing:

The DNA was extracted from a representative one isolate of the same biochemical result by Gene jet genomic DNA purification kit (Thermo Fisher Scientific Inc., USA) according to manufacturer's instructions. The extracted DNA was subjected to PCR for the amplification of 16S rRNA of *Aeromonas* species (Forward primer: 5′-AGA GTT TGA TCA TGG CTC AG -3′ and Reverse primer: 5′-TACGGT TAG CTT GTT ACG ACT T-3′) with product size at 1500bp. (Borrell et al., 1997) The PCR was performed in 50-µl volume consisting of 25-µl Maxima Hot Start Master Mix (2X) (Thermo Fisher Scientific Inc., USA), 1 µl (20 uM) for each forward and reverse primers, 5 µl Template DNA and PCR grade water up to 50 µl. The PCR cycling conditions were performed in Bio-Rad programmable thermal cycler which consist of initial denaturation at 93 C for 3 min followed by 35 cycles of denaturation at 94 C for 1 min., annealing of the primers at 56 C for 1 min. and extension at 72 C for 2 min. A final extension at 72 _C for 10 min was done following the last cycle. After that 4 ul the PCR product was loaded and run on 1% agarose gel stained with Ethidium bromide (10 mg/ml) in Tris Acetate EDTA buffer (TAE) agaist 1Kb plus ladder (Fermentas) by electrophoresis and visualized with UV Transilluminator. The sequence resulted were aligned against those in database of the GenBank (National Center for Biotechnology Information ‘‘NCBI”, Bethesda, MD, USA) using BLAST**.**

2.4. Experimental infection designe for studing the pathogenicity of *Aeromonas veronii* to *Oreochromis niloticus*:

Ninety apparently healthy live *Oreochromis. niloticus* with an average body weight of 50 ± 9 g were obtained from Ismailia fish hatchery, Egypt for experimental challenge according to Schaperclaus et al., (1992). After two weeks of adaptation, apparently healthy fish were distributed into 3 groups with (30 fish / group) stocked in 9 glass aquaria each aquarium (80 X 40 X 30 cm) was stocked with 10 fish each. One group was injected with an *Aeromonas veronii* strain isolated (18-h culture) I/P with 1.2 x 10^8 cfu/mL, and another group was I/P injected by sterile normal saline as a sham control, and the last group was kept as a control without injection. During the experiment, fish were adapted on feeding of fish diet twice daily at 3% of their body weight. Water was changed every week to maintain good water quality. The aquaria were supplied with sufficient chlorine free tap water; aeration was carried by electric aerator. The temperature and pH were adjusted at 25 ± 2 C and 7.4 respectively.

2.5. Antibiogram testing:

The antibiotic susceptibility was carried out using disc diffusion method on Muller-Hinton Agar (Oxoid) and standards guidelines were used for result evaluation (CLSI, 2006). Six antibiotic discs namely Trimethoprim + Sulphamethoxazole (SXT, 1.25/23.75 ug), Amoxicillin (AX, 25 ug), Ampicillin (AM, 10 ug), Streptomycin (S, 10 ug), Oxytetracycline (Ox, 30 ug) and Erythromycin (E, 15 ug) (Oxoid). After 24 h incubation, the zone of inhibition were measured and compared according to the manufacturer’s instruction.

3. RESULTS AND DISCUSSION

Aeromonas species are Gram-negative bacteria that belong to the family Aeromonadaceae (Aberoum and Jooyandeh 2010). These facultative anaerobic bacteria are known as psychrophilic and mesophilic microorganisms that have a broad host spectrum with either both cold and warm blooded animals, including humans. *Aeromonas* species cause hemorrhagic septicemia disease in aquaculture and characterized by high mortality rates (Bondad et al., 2005 and Lio-Po et al., 1983). Unlike phenotypic and biochemical identification, which can be modified by the variability of expression of characters, the 16S rDNA sequencing provides unambiguous data even for rare isolates, which are reproducible in and between laboratories (Hossain, 2008). Moreover, confirmation of *Aeromonas* species may be obtained by sequencing the16S RNA gene (Sreedharan et al., 2011). The present work was performed to isolate and identify the causative agent that infecting the cultured *Oreochromis niloticus* and *Ictalurus punctatus*cat (channel cat fish) in Egypt, then determination the best antibacterial agents that

effective against it in vitro. Infected fish were randomly collected from different fish farms at El Sharkia and El Ismalia governorate in Egypt. The clinical signs of naturally infected *Oreochromis niloticus* (nile tilapia) and *Ictalurus punctatus*cat (channel cat fish) and post mortem examination revealed that fish showed dark body coloration, exophthalmia with corneal opacity and hemorrhage in the eyes, loss of balance, frayed and torn tail and fins, scale detachment and skin discoloration with scattered hemorrhages all over the body surface with slight ascites, petechial hemorrhages were seen on the ventral abdominal wall and the base of the fins and deep ulcer. Photos (1-3).

On the other hand the postmortem examination of naturally infected fish tilapia revealed congestion of spleen, kidney, ovaries and liver with distended gall bladder, intestine were hemorrhagic and inflamedPhoto.

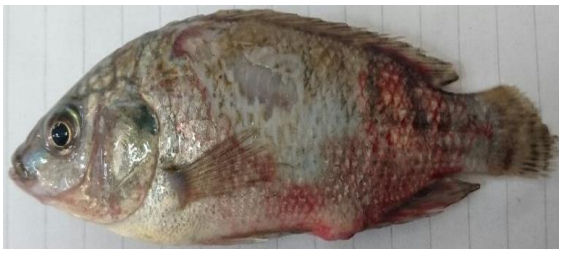

Photo. (1) Naturally infected fish show deep ulcer and haemorrhages all over the external body surface.

Photo. (2) Naturally infected tilapia showing hemorrhages on the vent and anal prolapsed

Photo (3) Naturally infected tilapia showing detachment of scales and haemorrhage

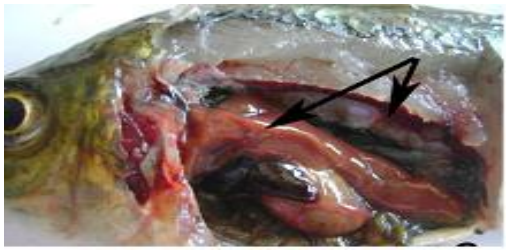

Photo. (4) Naturally infected tilapia showing enlarged, inflamed and congestion of liver, kidney and spleen.

The clinical signs and post mortum examination were the same as described by Schaperclaus et al., (1992) who determine the clinical alteration due to *Aeromonas* species infection which include skin alterations as body color, presence of opaque films, exophthalmia, raised and detached scales, eroded opercula, redness, ulcers, body swellings, clubbed and abraded gills, fins and tail. The collected fish were then transported in ice box with ice bags immediately to bacteriological lab of animal health department, Desert Research Center on September, 2015. Another 90 apparently healthy *Oreochromis niloticus* were obtained from private fish farm at EL Ismailia governorate, Egypt with an average body weight of 50 grams for application of pathogenicity test. About bacteriological examination; bacterial swabs obtained from different fish organs (Kidney, Liver, Intestine, Gills) revealed several bacterial isolates. The full phenotyping, biochemical and molecular identification with selected presumptively biochemical characters revealed that 55 isolates of 150 fish samples, 36.66 % were *Aeromonas veronii* (40 isolates of 90 *Oreochromis niloticus* fish, 44.44% and 15 isolates of 60 *Ictalurus punctatus* fish, 25%) which agree with Beaz-Hidalgo and Figueras (2012) and Hussin et al., (2017). Table (1), (2) and Fig. (1).

The results of bacteriological examination were positive for *Aeromonas* infection. Also, the lesions which appeared on infected fish as hemorrhage at base of fins or on the skin, distended abdomens, protruded eyes which are the same lesions of with hemorrhagic septicemia disease caused by *Aeromonas* spp. The post mortem examination in this study revealed that the naturally infected *Oreochromis niloticus* and *Ictalurus punctatus* showed degenerative change, inflammatory reaction and congestion especially in haemobiotic organs (liver, spleen, and kidney) and gill. Skin and muscles could be attributed to bacterial pathogenesis and virulence factors. These factors have effect on pathogenicity are extracellular toxins (enterotoxin, hemolysin and protease), structural features (pilli, S layer, lipopolysaccharide), adhesion and invasion. This symptoms nearly similar observation was recorded by Eissa et al., (2015). This may be due to the bacterial multiplication inside the intestine causing a hemorrhagic mucous disquamative catarrh.

Table (1): Phenotyping , biochemical and molecular identification of most isolated bacterium

Parameter	Presumptively identified *Aeromonas veronii* (55 isolates to 150 total number of fish (36.66%)
Colony characters	-White small convex colonies on TSA. -Yellow colonies on R-S medium 18-24hr, 25°c. -haemolysis on the blood agar. -Cannot grow at 4°c.
Gram´s stain	Gram-negative short bacilli
Motility	Motile
Biochemical identification	Oxidase + Catalase+
Molecular identification	Specific polymerase chain reaction-16S ribosomal RNA
Size of amplified product	(*Aeromonas veronii*)B565 strain B565 16S ribosomal RNA, complete sequence).

Table (2): Biochemical reactions of presumptively identified *Aeromonas veronii* isolates by API E kit:

Test	Reaction	Test	Reaction
Motility	+	Nitrate reduction	+
Gram staining	-	Citrate utilization	+
Gelatin liquefaction	+	Arginin hydrolysis	+
Oxidase	+	Fermentation of sugar	
O / F	F	Glucose	+
Growth on 5% Na Cl	-	Sucrose	+
Indol	+	Lactose	-
V.P	+	Maltose	+
Methyl red	+	Galactose	+
H2s production	-	Fructose	+
Catalase	+	Trehalose	+

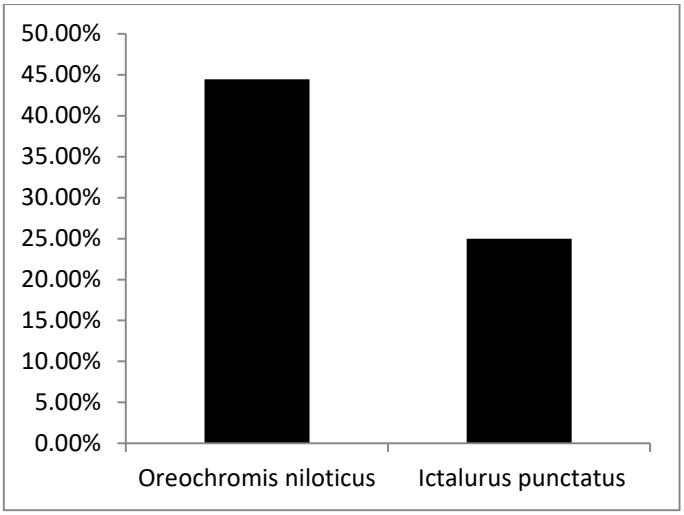

Fig. (1): Recovery rate of *Aeromonas veronii* isolates according to number of *Oreochromis niloticus* and *Ictalurus punctatus*

Toxic metabolites of *Aeromonas* species are absorbed from the intestine and induce toxemia. Capillary hemorrhage occurs in the dermis of fins and trunk and in the sub mucosa of stomach. The morphological cultural and biochemical characters of the isolates identified as *Aeromonas* species as they were characterized by circular convex, white colonies with entire edge, 2 mm in diameter on TSA agar white convex, colonies on specific Aeromonas agar base medium. While on Rimeler's – Shotts medium (R-S. medium) gave yellow colonies and on the blood agar medium made beta hemolysis, the results also supported by Eissa et al., (2015). These isolates were gram negative rods, motile facultative anaerobic. They were oxidase and catalase positive. API 20E results were positive for ONPG, ADH, INO, Gel and VP and negative for LDC, ODC, CIT, H2S, URE and TDA. They fermented glucose and sucrose. They didn't ferment inositol, rhamnose, mannitol and sorbitol which indicate *Aeromonas veronii* (Table 2). These were consistence with results obtained by many authors (Martin- Carnahanand and Joseph, 2005; Austin and Austin, 2012 and Eissa et al., 2015).

The molecular identification is the most accurate identification tool of the fish pathogens even in early infection phases. The rapid, confirmatory and accurate microbial identification in recent years are the molecular diagnosis (Buller, 2004). The 16 S rRNA gene is essential to bacteria and has been used as specific molecular marker for their identification (Alperi et al., 2008). PCR confirmed that the representative isolated strain is *Aeromonas* species and size of amplified product is about 1500 bp and by sequencing, the similarity was about 94 % with *Aeromonas veronii* B565 strain B565 by 16S ribosomal rRNA, complete sequences analysis (Photo (5), (6) and Table 3).

The pathogenicity test which carried out on *Oriochromis niloticus* groups with *Aeromonas veronii* biovar *sobria* revealed that the examined isolate was highly pathogenic to examined fish with (1.2×10^8 cfu/ml) that caused mortality of 60% (Table, (4)) after I/P injection without mortality in control groups. The clinical signs and post mortem of experimental infected fish in this study are similar to those described during the naturel infection as hemorrhage all over the body surface and ulcers with muscular necrosis in addition to macroscopic lesions in internal organs as congestion in gas bladder, anterior and posterior kidneys, liver, spleen, gall bladder, heart, brain and intestine. *A. veronii biovar sobria* was re-isolated from the internal organs of moribund and freshly dead fish. Phenotypically and biochemical confirmations for re-isolated bacteria were performed. These results were nearly similar to Eissa et al., (2011), Iqbal et al., (1999). Eissa et al., (2015) who found that the mortality in the experimental infection was 70%. The mortality was caused by expression of virulence factors as proteolytic enzymes, acetyl cholinesterase, haemolysin, enterotoxins, cytotoxin, dermonecrotic factors, gelatinase, elastase and staphylotoxin in addition to toxic metabolites (Austin and Austin, (2012), and Pachanawan et al., (2008).

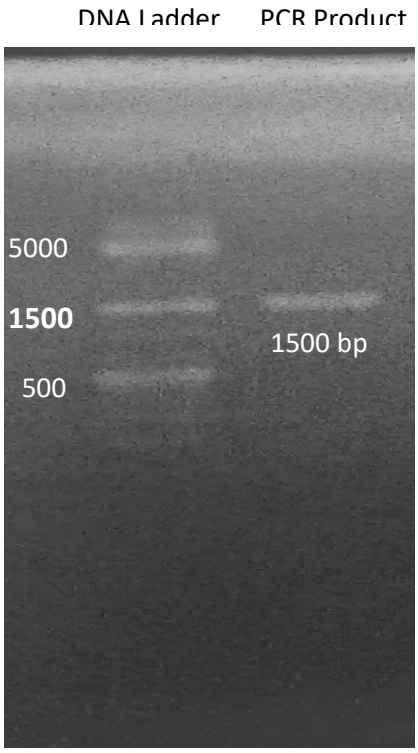

Photo (5): Molecular Identification of *Areomonase veronii* (*Aeromonas veronii biovar sobria* B565 strain B565 16S ribosomal RNA, complete sequence)

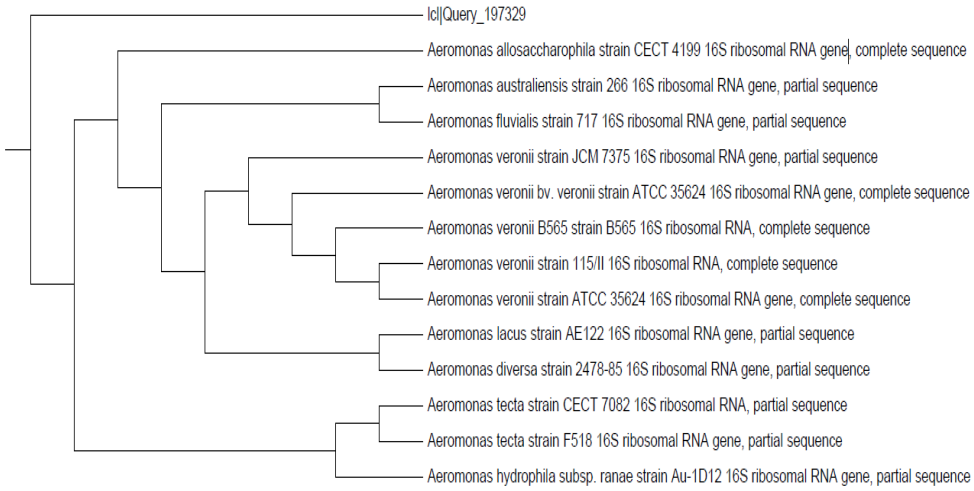

Photo (6): Dandogram of the sequences producing significant alignments for Aeromonas isolate revealed 13 sequences but the maximum score was for *Aeromonas veronii* B565 strain B565.

Concerning to antibiogram sensitivity test, it was found that *Aeromonas veronii* biovar *sobria* was sensitive to Trimethoprim + Sulphamethoxazole, Erythromycin and Streptomycin which agree with Hassan et al., (2017) and was resistant to Ampicillin and Amoxicillin, as shown in Table (5). Adanir and Turutoglu, (2007), Guz and Kozinska, (2004) and Hassan et al., (2017) confirmed the resistance of *Aeromonas sobria* to Ampicillin (AM 10) andother B-lactam antibiotics such as amoxicillin. The resistance to Oxytetracycline in this study may be attributed to *tet* genes which recently discovered as responsible for oxytetracycline resistance in *Aeromonas veronii* (Nawaz, et al., 2006).

Table (3): Sequences producing significant alignments for *Aeromonas* isolate

No	Description	Max score	Indent	Accession
1	*Aeromonas veronii* B565 16s ribosomal RNA,complete sequence	1182	94%	NR 102789,1
2	*Aeromonas veronii* strainJCM7375 16 s ribosomal RNA gene ,partial sequence.	1182	94%	NR 112838.1
3	*Aeromonas veronii* by veronii strain ATCC 3562416s ribosomal RNA,complete sequence	1182	94%	NR 118947,1
4	*Aeromonas veronii* strain ATCC 3562416s ribosomal RNA,complete sequence	1182	94%	NR 119045,1
5	*Aeromonas austrilienses* strain 26616s ribosomal RNA, partiale sequence	1177	94%	NR 108872,1
6	*Aeromonas fluvialis* strain 71716s ribosomal RNA, partiale sequence	1177	94%	NR 116586,1
7	*Aeromonas veronii* strain115/11 16s ribosomal RNA complete sequence	1175	94%	NR 044845,1
8	*Aeromonas lacus* strain AE122 16s ribosomal RNA gene , partiale sequence	1171	94%	NR 136831,1
9	*Aeromonas allosaccharophila* strain CECT 419916s ribosomal RNA genepo, complete sequence	1166	94%	NR 025945.2
10	*Aeromonas tecta* strain 708216s ribosomal RNA gene , partiale sequence	1155	93%	NR 118043,1
11	*Aeromonas diversa* strain 2478-8516s ribosomal RNA gene , partiale sequence	1155	93%	NR 117303,1
12	*Aeromonas hydrophila* subspranae AU-1D12 16s ribosomal RNA gene , partiale sequence	1155	93%	NR 042518,1
13	*Areomonas tecta* F51816s ribosomal RNA gene , partiale sequence	1155	93%	NR 114868,1

Table, (4): Mortality percent of *Oreochromis niloticus* challenged with *Aeromonas veronii*.

Fish group	I/P injection with 1.2 x 108 cfu / ml of *Aeromonas veronii.*												
	No of fish	1st day	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	Mortality rate	Survivability
Aeromonas inejected group	30	0	6	9	0	3	0	0	0	0	0	60%	40%
Saline injected group	30	0	1	0	0	0	0	0	0	0	0	3.33%	96.67%
Control	30	0	0	0	0	0	0	0	0	0	0	0%	100%

Table (5): In vitro susceptibility of *Aeromonas veronii* to Six Antimicrobial Agents.

Antibiotic Sensitivity of *Aeromonas veronii*	Sensitivity of *Aeromonas veronii*
Trimethoprim + Sulfamethoxazol	S
Amoxicillin	R
Ampicillin	R
Streptomycin	M
Oxytetracyclin	R
Erythromycin	S

S = susceptible M = moderately susceptible R = resistant.

4. CONCLUSION

In this *Aeromonas veronii* which is considered as fish and human pathogen, it could be isolated from *Oreochromis niloticus* (nile tilapia) and *Ictalurus punctatus* (channel cat fish) during an outbreak of disease mass mortalities from different fish farms in

El Sharkia and El Ismailia governorate. In addition to public health hazards of the human handling such fish. Nucleotide sequence analysis is only considered as a reliable diagnostic techniques in *Aeromonas veronii* identification.

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