# Detection of Hemolysin gene and Antibiogramme of *Aeromonas veronii biovar sobria* Isolated From Mass Mortalities in Cultured Nile Tilapia in El-Sharkia governorate, Egypt

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**Abstract:** In this study, a well identified *Aeromonas veronii biovar sobria* (identified by Vitek 2 system and sequencing and previously checkd for hemolysis on blood agar) isolated from cultured Nile tilapia (*Oreochromis niloticus*) during an outbreak of mass mortality was investigated for the virulence of bacteria by detection of hemolysin gene (asa1) and 16S rRNA (for confirmation of species) using multiplex PCR. Both ofhemolysin gene (asa1) and 16S rRNA was detected in the selected strain. The antibiogramme of the bacteria was investigated using biodisc diffusion test and Vitek 2 system and was found to be sensitive to Tigecycline, Trimethoprim + Sulphamethoxazol and Ciprofloxacin while it has intermediate sensitivity to Ceftriaxone. On the other side it was resistant to Cefazolin and Ampicillin/Sulbactam.

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**Key words:** *Aeromonas sobria*, *Aeromonas veronii biovar sobria*, Vitek 2 system, Hemolysin gene (asa1), Antibiogramme.

## 1. Introduction

Aeromonas septicemia is a fatal infectious disease of cold-blooded animals like fish, reptiles, amphibians and in human often caused by the motile Aeromonas, particularly *A. hydrphila*, *A. sobria* and *A. caviae* ***(Das et al., 2013)***.

The pathogenesis of Aeromonas infections is a multifactorial complex, as aeromonads produce a wide variety of potential virulence factors, including hemolysins, cytotonic and cytotoxic enterotoxins, proteases, lipases, and leucocidins. Moreover, cell-surface components such as the outer membrane proteins, lipopolysaccharide (LPS), the S-layer, polar flagella, and pili have been identified as putative virulence factor ***(Turska-Szewczuk et al., 2014)***.The presence of virulence factors, particularly those related to extracellular products, play an important role in the translocation of Aeromonas spp. in the epithelium, thus being broadly associated with bacterial virulence ***(Jutfelt et al., 2008)***. Furthermore, ***Erdem et al. (2010)*** found that *Aeromonas hydrophila* and *Aeromonas veronii biovar sobria* strains possess a strong haemolytic activity.

PCR-based method is rapid, sensitive, and specific for the detection of virulence factors of Aeromonas spp. It overcomes the handicap of time-consuming biochemical and other DNA-based methods.

Intensive and semi intensive *Oreochromis niloticus* farming is associated with risk for the incidence and spread of infectious diseases commonly associated with therapeutic and prophylactic use of antibiotics and so, thetilapia producers tend to administer antibiotics in a non-systematic and uncontrolled manner, thus making the selection and spread of antibiotic-resistant bacteria possible. The importance of antibiotic resistance in Aeromonads, that these bacteria are interconnected with the water ecosystem, colonize fish, and can cause various infectious processes in them. Also, they have been proven to cause human infections (***Čížek et al., 2010)***. Beside Aeromonads ranked as the single most common pathogen identified in Tsunami survivors with skin or soft tissue infections in Thailand in 2004 ***(Hiransuthikul et al., 2005).*** Aeromonads can produce various b-lactamases which confer resistance to a broad spectrum of b-lactams, and therefore in vitro susceptibility testing must be used to guide antimicrobial therapy ***(Chen et al., 2012).*** Like enteric Gram-negative bacteria, the emergence of resistance among aeromonads will be accelerated by the clinical use of antibiotics (***Chaudhury et al., 1996***).

The present study was aimed for, Detection of hemolysin gene (asa1) and 16S rRNA (for confirmation of species) using multiplex PCR and studying the antibiogramme of *Aeromonas veronii biovar sobria*.

## 2. Materials and Methods

**Bacterial isolates:**

A well identified *Aeromonas veronii biovar sobria* (identified by Vitek 2 system and sequencing). It was chosen according to degree of hemolysis it produced on blood agar.

**Antibiograme using antimicrobial sensitivity discs:**

The sensitivity of *A. veronii biovar sobria* to different antibiograms (Ampicllin, Ciprofloxacin, Gentamycin, lincomycin, novobiocin 30, erythromycin and Trimethoprim + sulpha methoxazol) was estimated. The culture of *A. veronii biovar sobria* was cultivated in nutrient broth for 24hrs\ 37˚C then flooded on the surface of Muller Hinton agar and the excess was removed. The biodiscs were used and gently pressed on the surface of agar using sterile forceps. Then the plates were incubated at 37 ±1˚C for 24 hrs. The results were interpreted according to the criteria given by***Finegold and Martin, (1982)***

**Antibiograme using vitek 2 system:**

The test organisms from colonies grown on 5% horse blood agar after 18 hrs incubation were suspended in sterilized physiological saline to 0.5 McFarland standards. Approximately 2 ml of this suspension was automatically loaded into the VITEK 2 antimicrobial susceptibility testing (AST) GN71 cards.

**Nucleic acid isolation**:

Genomic DNA was extracted by heating at 100 C° for 4 min then centrifuged at 4000 rpm for 4 min at 4C° and take the supernatant.

**Oligonucleotide primers and PCR conditions *(Wang et al., 2003):***

The primer pairs used for PCR amplification was shown in Table (1)**.**

The PCR mixture according to Biotek corporation company was 10µl of master mix with 1µl of each primers (F&R) and 4µl distilled water then add 2µl DNA template except the control negative 2 µl distilled water added instead of the DNA reaching a total volume 20µl. PCR amplification was performed using PCR master cycler (Eppendorf AG Germany) under the following conditions: initial denaturation at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 0.5 min, annealing of the primers at 59°C for 0.5 min, and primer extension at 72°C for 0.5 min. A final extension at 72°C for 7 min. Ten microliters of the reaction mixture was then analyzed by agarose gel electrophoresis in 1.5% agarose at 100 volt for 30 minutes, and the reaction products were visualized with UV light after staining with ethidium bromide. The identities of the amplicons were confirmed by comparison of the amplicon sizes with the predicted sizes, as indicated in Table 1

## 3. Results

**Antibiograme sensitivity discs:**

The results of in-vitro sensitivity test of *A.veronii biova rsobria* was shown in table (2). It was revealed that the it was sensitive to Ciprofloxacin, Trimethoprim + Sulphamethoxazol and Gentamycin and resistant to lincomycine, Ampicillin,Novobiocin and Erthromycin.

**Antibiograme using Vitek 2 system**

The antibiograme of *A. veronii biovar sobria* using Vitek 2 system were shown in table (3) revealed that as itwas sensitive to Meropenem, Tigecycline, Trimethoprim + Sulphamethoxazol, Ciprofloxacin, Moxifloxacin, Gentamycin, Tobramycin, Cefepime, Imipenem, Amikacin while it has intermediate sensitivity to Ceftriaxone. On the other side it was resistant to Aztreonam, Nitrofurantion, Cefazolin, Ampicillin, Ampicillin/ Sulbactam.

**Table (1): Showing primer pairs used for PCR amplification including the primers position, sequences and product length**

| Primer | Position | *Sequence ( 5`-3` )* | PCR product length (bp) |
|---|---|---|---|
| **Asa1 F** | 863–883 | TAAAGGGAAATAATGACGGCG | 249 |
| **Asa1 R** | 1111–1091 | GGCTGTAGGTATCGGTTTTCG | |
| **A16S F** | 1020–1041 | GGGAGTGCCTTCGGGAATCAGA | 356 |
| **A16S R** | 1375–1355 | TCACCGCAACATTCTGATTTG | |

**Table (2) Showing the antibigram of *A. veronii biovar sobria***

| Antimicrobial agent | Symbol | Concentration (mcg) | Susceptible zone (mm) | inhibition zone (mm) | Interpretation |
|---|---|---|---|---|---|
| Ampicllin | AM | 10 | ≥17 | 11 | R |
| Ciprofloxacin | CIP | 5 | ≥21 | 34 | S |
| Erthromycin | E | 15 | ≥23 | 20 | R |
| Gentamycin | CN | 10 | ≥15 | 18 | S |
| Lincomycin | L | 2 | ≥21 | 0 | R |
| Trimethoprim /sulphamethoxazol | Sxt | 1.25 / 23.75 | ≥16 | 24 | S |
| Novobiocin | NV | 30 | ≥19 | 14 | R |

R: Resistent S: Sensitive

**Table (3) Showing *A. veronii biovar sobria* Antibiogram using Vitek 2 system**

| Antimocrobial agent | Concentration ranges µg/ml | CLSI breakpoint µg/ml S | R | MIC µg/ml | Interpretation |
|---|---|---|---|---|---|
| Ampicillin | 2 to 32 | ≤8 | ≥32 | ≥ 32 | R |
| Ampicillin/Sulbactam(2/1) | 2 to 32 | ≤8 | ≥32 | ≥ 32 | R |
| Cefazolin | 4 to 64 | ≤8 | ≥32 | ≥ 64 | R |
| Ceftriaxone | 1 to 64 | ≤8 | ≥32 | 16 | I |
| Cefepime | 1 to 64 | ≤8 | ≥32 | 4 | S |
| Aztreonam | 1 to 64 | ≤8 | ≥32 | ≥ 64 | R |
| Imipenem | 0.25 to 16 | ≤4 | ≥16 | 4 | S |
| Meropenem | 0.25to 16 | ≤1 | ≥4 | ≤ 0.25 | S |
| Amikacin | 2 to 64 | ≤8 | ≥32 | 8 | S |
| Gentamicin | 1 to 16 | ≤4 | ≥16 | 2 | S |
| Tobramycin | 1 to 16 | ≤4 | ≥16 | 2 | S |
| Ciprofloxacin | 0.25 to 4 | ≤1 | ≥4 | 0.5 | S |
| Moxifloxacin | 0.25 to8 | ≤2 | ≥8 | 1 | S |
| Tigecycline | 0.5 to 8 | ≤4 | ≥16 | 1 | S |
| Nitrofurantion | 16 to 512 | ≤32 | ≥128 | 128 | R |
| Trimethoprim/Sulfamethoxazole | 20 to 320 | ≤40 | ≥160 | ≤ 20 | S |

CLSI: Clinical and Laboratory Standards Institute; MIC: Minimum Inhibitory Concentration; R: Resistant; I: Intermediate; S: Sensitive

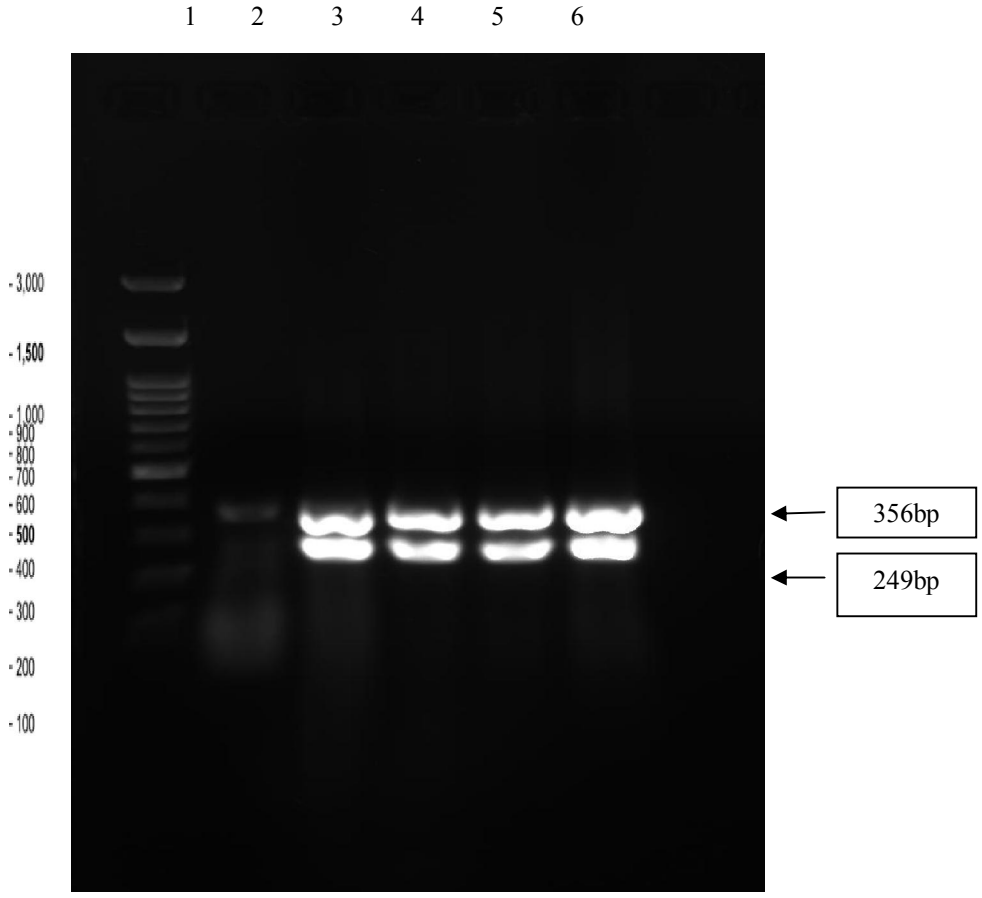


**Fig. (1) Detection and identification of *Aeromonas veronii biovar sobria* hemolysin (249bp) and 16S rRNA (356bp) genes by amplification of fragments in the multiplex PCR assay. Lanes 1: 100bp ladder (Genedirex); lane 2: negative control. lane 3-6: *A. veronii biovar sobria* showing the hemolysin and 16S rRNA genes (249 and 356 bp fragments, respectively).**

**Results of identification of hemolysin and 16S rRNA genes**:

The sizes of the amplification products obtained by the multiplex PCR were identical to those predicted from the design of the primers. No bands appeared in any position on the gel except for the predicted bands (249 and 356 bp).

The results observed in fig.(1) revealed that the amplification products of the hemolysin gene was produced by the four isolates of *A. veronii biovar sobria* at 249 bp. The four isolates also showed bands with the 16S rRNA gene at 356bp.

## 4. Discussion

Several extracellular enzymes and toxins including hemolysins, proteases and cytotoxins have been reported as virulence factors of motile Aeromonas but the role of each single factor in relation to pathogenesis *varies* ***(Allan & Stevenson 1981; Thune et al., 1982; Rodriguez et al., 1992*** and ***Kozinska, 2007).*** In our study, hemolysin gene was detected by PCR, which was a pathogenic factor in isolated *Aeromonas veronii biovar sobria*. The hemolysin gene was detected in the four chosen isolates. The hemolysin gene (asa1) PCR amplified product size was 249 bp. No bands appeared in any position on the gel except for the predicted bands. Similarly ***Das et al. (2013)*** found that 76.78% of *A.sobria* isolates were positive for *asa*1 gene of 249bp fragment. Also, a majority of the *A. veronii bv. sobria* isolates (67%) carried hemolysin *(asa1)* genes ***(Wang et al., 2003)***.

Concerning the results of the antibiogram sensitivity, it was revealed that *A. veronii biovar sobria* was sensitive to Carbapenems (Meropenem, Imipenem), Glycylcyclines (Tigecycline), Trimethoprim + Sulphamethoxazol, quinolones (Ciprofloxacin, Moxifloxacin), Aminoglycosides (Gentamycin, Tobramycin and Amikacin) and 4th generation cephalosporins (Cefepime) while it has intermediate sensitivity to 3rd generation cephalosporins (Ceftriaxone). On the other side, it was resistant to Monobactams (Aztreonam), Nitro furantoin, 1st generation cephalosporins (Cefazolin), Lincomycine, Ampicillin, Ampicillin/ Sulbactam, Novobiocin and Erythromycin. These results come in agreement with ***Wu et al. (2007), Lamy et al. (2009) and Quiroga and Vergara (2011)*** except Ceftriaxone was sensitive,***Aravena-Román et al. (2012)*** except Ceftriaxone, Nitrofurantoin and Aztreonam were sensitive, ***Ramalivhana et al. (2009)*** except Nitrofurantoin and Aztreonam were sensitive***, Eissa et al. (2011) and LI Xu et al.(2014).*** On the other side***, Majtán et al. (2012)*** found that the isolated *A. sobria* was only resistant to ampicillin but was sensitive to 1st, 3rd and 4thgeneration cephalosporins.

Antimicrobial resistance in these organisms is usually chromosomally mediated, but β -lactamases produced by aeromonads may occasionally be encoded by plasmids or integrons ***(Aravena-Román et al., 2012).*** In this study, our strain was resistant to 8 of 19 tested antibiotic with one intermediate. This may be due to that A. veroniibv.sobria can produce 3 classes of β-lactamases a class C cephalosporinase, class D penicillinase and class B metallo β –lactamase (MBL) ***(Janda and Abbott, 2010).*** The most commonly mentioned MBL in Aeromonas species is CphA, which being active on penems and carbapenems only, but not on penicillins and cephalosporins ***(Segatore et al., 1993)*** AmpC b-lactamases can hydrolyze many b-lactam antibiotics, including cephamycins and third generation cephalosporins, and are resistant to b-lactamase inhibitors, such as clavulanic acid, tazobactam, and sulbactam. However, 4thgeneration cephalosporins are not recognized by AmpC b-lactamases ***(Chen et al., 2012).*** This comes in agreement with our results that it showed resistance to all β-Lactam antibiotics including the penicillins, cephalosporins, clavams, carbapenems, and monobactams except Cefepime while it has intermediate sensitivity to Ceftriaxone but it showed sensitivity to carbapenems which may be due to ***Chen et al. (2012)*** who mentioned that Aeromonads with AmpC genes do not always express AmpC b-lactamases and may display cefotaxime susceptibility. The mechanisms involved in the expression of AmpC b-lactamases include inducible b-lactamase production in the presence of suitable inducers (cefoxitin or imipenem) ***(Walsh et al., 1995).*** Also, may be false susceptibility to carbapenems that the resistance to imipenem and meropenem may not be detectable and so Aeromonas species should be reported as resistant to carbapenems with the exception of A. caviae since strains of this species do not possess a carbapenemase ***(Bush et al., 1995).*** Finally, the CphA MBL production is not easily detected by conventional in vitro susceptibility tests with EDTA-based combination disk diffusion, E-test, or agar dilution methods with standard inocula, unless large inocula is adopted ***(Wu et al., 2012).***

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