Identification of emerging *Acinetobacter johnsonii* virulence and antibiotic resistance genes associated with high mortality in cultured *Oreochromis niloticus*

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ABSTRACT

The present study was planned to investigate the causative agent of *Oreochromis niloticus* summer mortality that resulted in significant economic losses in Port Said Governorate, Egypt during 2017. Many bacterial isolates were identified, among which *Acinetobacter johnsonii* has been selected to be the focus of the current study. *Acinetobacter johnsonii_Egy* was isolated and identified using the analytical profile index (API) 20 E, sequencing 16S ribossomal RNA gene fragment and phylogenetic analysis. A drug resistance evaluation revealed that *A. johnsonii* was resistant to ampicillin, gentamicin, lincomycin, nalidixic acid, tetracycline, and oxytetracycline among the tested antibiotics. Experimental infection was performed and challenged fish revealed similar clinical signs as seen in naturally infected ones. The disease appears to be highly infectious and lethal, causing 100% cumulative mortality during experimental infections. Virulence genes (*fimH*, *traT*, and *iutA*) and antibiotic resistance genes (*qacED1*, *qnrS*, *sul1*, *dfrA*, and *aadA1*) presence was confirmed in the *A. johnsonii_Egy* isolate.

In conclusion, *A. johnsonii* is highly infectious to *O. niloticus* and might have a great influence on transfer of the antibiotic resistance in aquaculture. According to the available knowledge, this study is first to report *A. johnsonii* as emerging opportunistic pathogen in the Egyptian Nile tilapia (in Port Said).

Keywords: *Acinetobacter johnsonii*_Egy; *O. niloticus*; Antibiotic susceptibility; Virulence.

INTRODUCTION

Acinetobacter bacteria have been extensively identified as Gram-negative, non-motile, non-fermentative, catalase positive, and oxidase negative rods. Most strains require incubation temperatures of 20 to 37 ºC. Colony colors vary from translucent, faint yellow, or grayish-white (**Doughari *et al.,* 2011)**.

Acinetobacter are generally distributed in sewage, soil, food, and water (**Huddedar *et al.,* 2002)**, one of the normal digestive tract flora in marine animals (**Chang *et al.,* 2015)** and could be considered as an important emerging nosocomial pathogen (**Vahdani *et al.,* 2011).**

Initially, *Acinetobacter* was isolated from human patients and identified as the causative agent of many types of infections, including meningitis, septicemia, and urinary, genital, respiratory tract, and wound infections (**Singh *et al.,* 2013).** After that, in April 2016, few reports showed up reporting *Acinetobacter johnsonii* infection in the cultured blunt snout bream *Megalobrama amblycephala,* common carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss* (**Pękala-Safińska, 2018)**; even though **there is little information on the role of *Acinetobacter* in fish pathology and disease occurrence**. *A. johnsonii* appears to be highly pathogenic, causing >80% mortality and causing hemorrhaging all over fish skin and fins (**Cao *et al*., 2017).** Significant *A. johnsonii* drug resistance to many antibiotics compounds has previously been reported (**Blossom and Srinivasan, 2008; Nowak *et al.,* 2010)**. This resistance in turn, has caused a decrease in available drug options for treatment (**Park *et al.,* 2009).**

The aim of the current investigation was to identify *Acinetobacter* spp. isolated from diseased *Oreochromis niloticus* in order to evaluate this species' pathogenicity through experimental infection and determine its susceptibility to the most commonly used antimicrobial agents.

MATERIALS AND METHODS

Fish collection and sampling

A total of 80 naturally infected *O. niloticus* with average body weight 200±15 g were collected from different private fish farms in Port Said Governorate, Egypt during the 2017summer infection/mortality season. Clinically diseased and newly deceased fish were immediately transferred cooled in ice boxes and without direct contact with ice to the laboratory of the department of Aquatic Animals Diseases and Management, Benha University. Clinical, postmortem, and bacteriological examinations were carried out according to **Austin and Austin (2007).**

Bacteriological Examination

Under complete aseptic conditions, samples were taken from skin lesions, gills, liver, kidneys, spleen, brain, and intestines of the clinically diseased fish, inoculated onto Brain Heart Infusion broth (BHIB), incubated at 28°C for 24 hrs and then streaked over Brain Heart Infusion agar (BHIA). They were then incubated at 28°C for another 24 hrs **(Tonguthai·et al., 1999).** Separate colonies were later picked up and stored in 15% glycerol (15 ml glycerol in 85 ml tryptic soy broth [TSB] supplement) at –80 °C according to **Hollander and Nell (1954).** Gram staining and motility testing were performed according to **Cruickshank *et al.* (1975).** Isolate was identified biochemically by streaking bacterial colonies over TSA, incubated at 28°C for 24 hrs, and then identified at the genus level using API 20 E strips (Biomerieux, France). Biochemical profiling was done according to catalogue instructions in order to identify the isolated bacteria **(Ottaviani *et al.,* 2006).**

Molecular characterization

Using the QIAamp DNA Mini kit (Qiagen, Germany) according to the company's protocol, DNA was extracted from isolated bacteria that had been previously identified using the API 20 E with a 94% probability rate. DNA concentrations and purity were measured via spectrophotometry and stored at −20 °C until use. Primers used in polymerase chain reaction (PCR) amplifications were obtained from (Metabion, Germany), and reaction conditions are listed in Table 1. The amplification reaction was carried out in an applied biosystem 2720 thermal cycler according to the company's protocol. PCR products were analyzed by electrophoresis and visualized by ultraviolet (UV) transillumination. Sequences were analyzed using CLUSTAL W multiple sequence alignment program, version 1.83, which was designed by **Thompson *et al.* (1984),** and a phylogenetic analysis was done using MEGA6 according to **Tamura *et al.* (2013).**

Antibiogram

The antibiogram profile of *Acinetobacter johnsonii* was constructed according to National Committee for Clinical Laboratory Standard (NCCLS) against eight antibiotic agents (Oxoid, UK) following same procedures of **(El Latif *et al.,* 2019),** namely gentamycin 10 μg, ampicillin 10 μg, nalidixic acid 30 μg, lincomycin 10 μg, tetracycline 30 μg, ofloxacillin 10 μg, oxytetracycline 30 μg, and sulfamethoxazole-trimethoprim 25 μg. The isolate was grown overnight on Muller Hinton broth at 28 °C and then spread on Muller Hinton Agar plates, then incubated at 28 °C for 24 hrs.

Assessment of *Acinetobacter johnsonii* _ Egy virulence

The intensity of *A. johnsonii* virulence was evaluated using a specified protocol. A total of 50 *O. niloticus* fish with average body weight of 30±5.0 g were procured from a private fish farm and divided into five groups of 10 fish each in glass tanks. The first four groups were intraperitoneally injected with 0.2 ml of *A. johnsonii* bacterial cell suspension at different concentrations of 0.3, 0.6, 1.2 and 1.8×10^8 cfu/ml according to **Abbass *et al.* (2010).** The fifth group was used as the control (inoculated with sterile phosphate buffer saline [PBS]). Feeding was done twice daily at a rate of 3% of the fish's body weight and water conditions were kept at (water temperature 25±0.5°C, dissolved oxygen 6±0.2mg/L, ammonia concentration was 0.53±0.07 mg/L and pH was 7±0.4), Clinical signs and mortality rates for all groups were recorded daily for seven days post injection, and Koch's postulates were achieved through re-isolation and identification of bacterial strains as previously described.

Authors followed Benha University's guidelines for the use and care of laboratory animals.

Detection of virulence and antibiotic resistance genes

Primers are presented in Table 1. The reaction was performed as previously described using an Applied biosystem 2720 thermal cycler according to the company's protocol.

RESULTS

Clinical examination

The external clinical signs of diseased *O. niloticus* appeared as loss of appetite, scales loss, darkness, and hemorrhagic patches all over the body. The internal examination revealed congestion of all the internal organs, especially kidneys and liver (Fig.1).

Isolation and identification of the bacteria

The isolated colonies were round, colorless, convex, and 1–2 mm on TSA medium. They were also gram-negative, coccobacilli to rod-shaped and non-motile bacteria.

Biochemical results using the API 20E kit showed positive results for β-galactosidase, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, citrate utilisation, acetoin production, gelatin hydrolysis, and tryptophan deamination. The isolate showed biochemical characteristics resembling *A. johnsonii*.

Table 1 Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	1ry denaturation	Amplification (35 cycles)			Final extension	Reference
				2nd denaturation	Annealing	Extension		
16S rRNA	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	1485	94˚C 5 min.	94˚C 30 sec.	56˚C 1 min.	72˚C 1.2 min.	72˚C 12 min.	Lagacé*et al.*, 2004
Virulence genes								
fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Ghanbarpour and Salehi, 2010
TraT	GATGGCTGAACCGTGGTTATG CACACGGGTCTGGTATTTATGC	307	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec	72˚C 30 sec	72˚C 7 min.	Kaipainen *et al.*, 2002
iutA	GGCTGGACATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300	94˚C 5 min.	94˚C 30 sec.	63˚C 30 sec	72˚C 30 sec	72˚C 7 min.	Yaguchi *et al.*, 2007
papC	TGATATCACGCAGTCAGTAGC CCGGCCATATTCACATAA	501	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Wen-jie*et al.*, 2008
Antibiotic resistance genes								
QacE D1	TAA GCC CTA CAC AAA TTG GGA GAT AT	362	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec	72˚C 40 sec	72˚C 10 min.	Chuanchuen *et al.*, 2007
	GCC TCC GCA GCG ACT TCC ACG							
qnrS	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Randall *et al.* 2004
Sul1	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Ibekwe*et al.*, 2011
dfrA	TGGTAGCTATATCGAAGAATGG AGT	425	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Grape *et al*., 2007
	TATGTTAGAGGCGAAGTCTTGG GTA							
Aada1	TATCAGAGGTAGTTGGCGTCAT	484	94˚C	94˚C	54˚C	72˚C	72˚C	Randall *et al.* 2004

|	GTTCCATAGCGTTAAGGTTTCATT		5 min.	30 sec.	40 sec	45 sec	10 min.	
Kan	GTGTTTATGGCTCTCTTGGTC	621	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Frana*et al.*, 2001
	CCGTGTCGTTCTGTCCACTCC							
blaNDM	GGTTTGGCGATCTGGTTTTC	621	94˚C 5 min.	94˚C 30 sec.	52˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Nordmann*et al.*, 2011
	CGGAATGGCTCATCACGATC							
blaTEM	ATCAGCAATAAACCAGC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Colom *et al.*, 2003
	CCCCGAAGAACGTTTTC							
TetA(A)	GGTTCACTCGAACGACGTCA	576	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Randall *et al.* 2004
	CTGTCCGACAAGTTGCATGA							
aadB	GAGCGAAATCTGCCGCTCTGG	319	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec	72˚C 40 sec	72˚C 10 min.	Frana*et al.*, 2001
	CTGTTACAACGGACTGGCCGC							
floR	TTTGGWCCGCTMTCRGAC	494	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Doublet *et al.*, 2003
	SGAGAARAAGACGAAGAAG							
mphA	GTGAGGAGGAGCTTCGCGAG	403	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec	72˚C 40 sec	72˚C 10 min.	Nguyen *et al.*, 2009
	TGCCGCAGGACTCGGAGGTC							
ermB	CATTTAACGACGAAACTGGC	425	94˚C 5 min.	94˚C 30 sec.	51˚C 40 sec	72˚C 45 sec	72˚C 10 min.	Nguyen *et al.*, 2009
	GGAACATCTGTGGTATGGCG							

Fig. 1. *Oreochromis niloticus* infected with *Aceietobacter johnsonii* showing congestion of all internal organs, especially kidney and liver (asterisk).
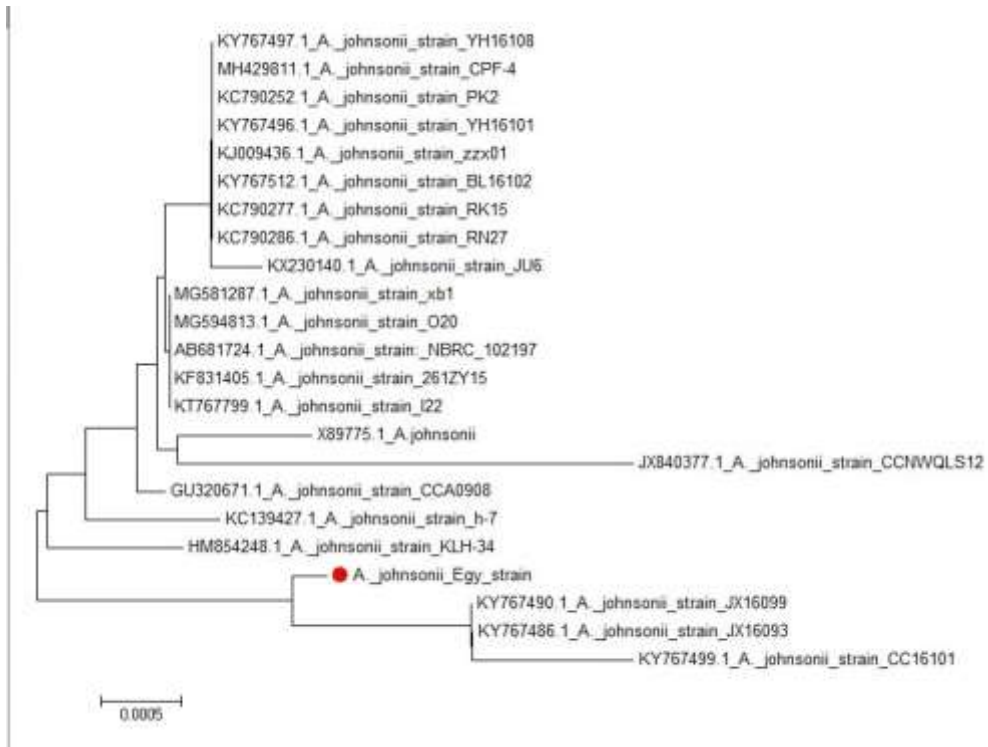
Fig.2. Phylogenetic tree for *16S rRNA* partial sequences that was generated using maximum likelihood, neighbor joining and maximum parsimony in MEGA6. Phylogenetic tree showed clear clustering of the isolated Egyptian strain (*A. johnsonii*_Egy_strain) with different *A. johnsonii* strains uploaded from GenBank.
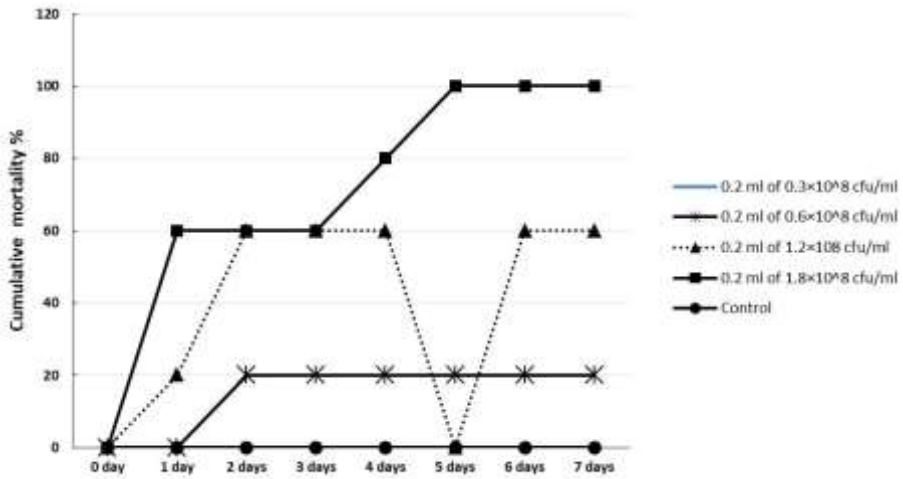
Fig.3. Daily cumulative mortality of experimental intra-peritonealy challenged Nile tilapia fish with 0.2 ml *Acinetobacter johnsonii* at 0.3, 0.8, 1.2 and 1.8×10^8 cfu/ml cells/ml/fish. Number in brackets represents dose of bacterial cells and fish were observed for 7 days.
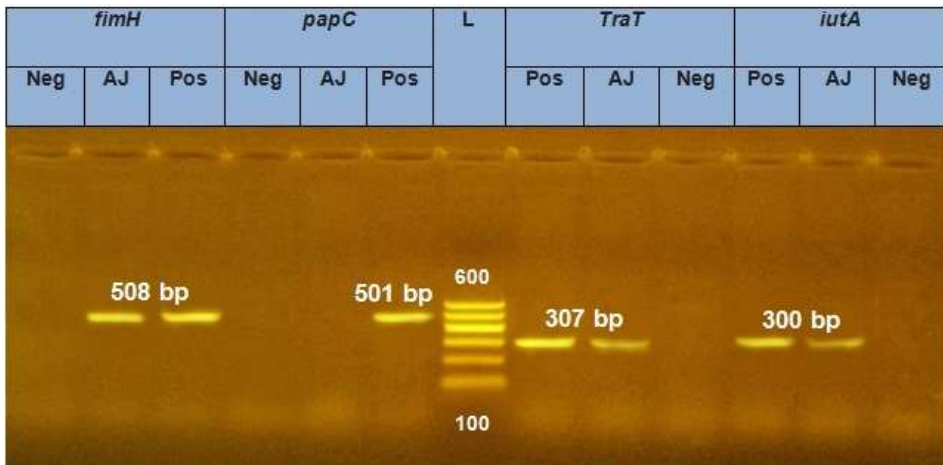

Fig.4. Ethidium bromide staining of a 2% agarose gel with polymerase chain reaction (PCR) products showing virulence genes (*fimH, TraT, and iutA*) +ve samples of 508, 300, and 306 bp PCR products, respectively, from bacterial culture. L represents a 600 bp DNA ladder used as a sizing standard.
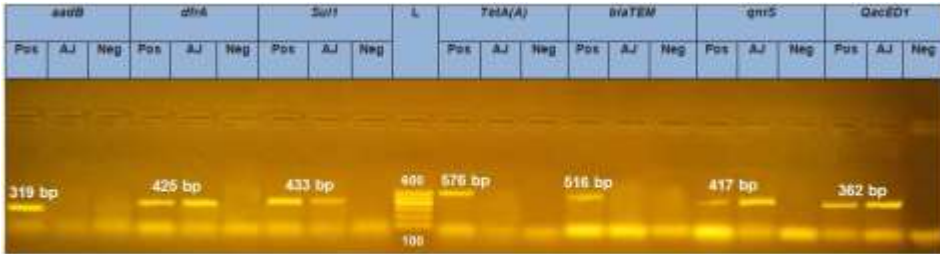
Fig.5. Ethidium bromide staining of a 2% agarose gel with PCR products showing antibiotic resistance genes (*Aada1*) +ve samples of 484 and 494 bp PCR products, respectively, from bacterial culture. L represents a 600 bp DNA ladder used as a sizing standard.
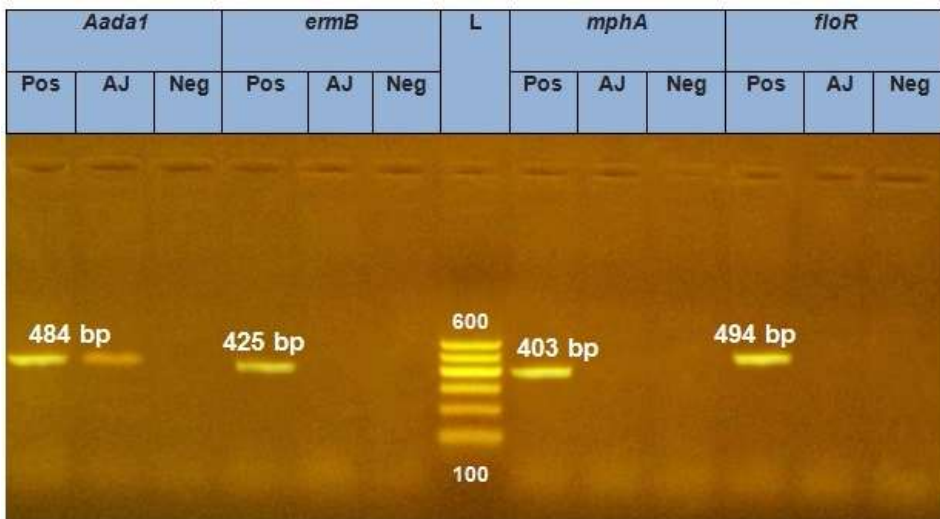
Fig.6. Antibiotic resistance genes (*dfrA, Sul1, qnrS*, and *QacED1*) +ve samples of 425, 433, 417, and 362 bp PCR products, respectively. L represents a 600 bp DNA ladder used as a sizing standard.

Molecular characterization

Sequencing of the PCR product targeting the 16S ribosomal RNA (1485bp) showed an identity of 98.2% to 99.4% homology between the isolated Egyptian strain (*A. johnsonii_Egy*, GenBank accession number MK012190) and different *A. johnsonii* strains uploaded from the gene bank.

Phylogenetic analysis

The phylogenetic tree showed clear clustering of the isolated Egyptian strain with different *A. johnsonii* strains uploaded from the gene bank (Fig.2).

Antibiotic susceptibility

Antibiotic sensitivity test of the isolated *A. johnsonii* revealed that it was resistant to all tested antibiotics (gentamycin, ampicillin, nalidixic acid, lincomycin, tetracycline, ofloxacillin, oxytetracycline, and sulfamethoxazole- trimethoprim) and only sensitive to ofloxacillin and sulfamethoxazole-trimethoprim.

Experimental infection challenge

The tested *A. johnsonii* was pathogenic to tilapia juveniles, and the cumulative mortality rate reached 100% of total injected fishes for 1.8×10^8 cfu/ml dose (Figure 3). Clinically infected fish exhibited similar clinical signs and postmortem changes as recorded in naturally infected ones.

***Acinetobacter johnsonii _ Egy* virulence and antibiotic resistance genes assay**

The specific PCR amplifications of virulence and antibacterial sensitivity genes are shown in Figures 4, 5, and 6. Results demonstrate that the virulence genes (*fimH, TraT, and iutA*) and antibiotic resistance genes (*QacED1*, *qnrS, Sul1, dfrA,* and *Aada1*) existed all in the *A. johnsonii_Egy* isolate, indicating its multidrug resistant property.

DISCUSSION

Acinetobacter are usually perceived as normal saprophytic microorganisms that are present in aquatic environments and colonized in aquatic animals' skin, gills, and digestive tracts. It constitutes a remarkable example of bacterial emergence, evolving from traditionally harmless organisms toward important pathogenic bacteria, probably related to antibacterial resistance transfer in the aquatic environment. *A.johnsonii* was isolated from diseased rainbow trout and this considered the first report concerning its pathogenicity in fish species **(Kozińska *et al.,* 2014).**

The isolated colonies were convex, round, colorless, and 1–2 mm on TSA medium. Also, they were Gram-negative, non-motile coccobacilli to rod-shaped bacteria. Similar results were previously recorded by **(González *et al.,* 2000**; **(Kozińska *et al.,* 2014)**, who reported the same morphological and biochemical characteristics for *Acinetobacter* isolates. In addition, **Gupta *et al.* (2015)** reported that the isolated *Acinetobacter* spp. were gram-negative and non-fermenters organisms.

In the present study, *A. johnsonii* was tentatively identified and confirmed through sequencing of the 16S rDNA gene fragment and phylogenetic analysis. Pathogenicity was proven by experimental *O. niloticus* infection, and disease-related signs were similar to those observed in naturally

infected fish, showing darkness and hemorrhagic patches externally plus organ congestion, especially kidney, liver, and spleen internally.

On a molecular basis, the Egyptian isolate (*A. johnsonii_Egy*, GenBank accession number MK012190) showed 98.2%–99.4% homology with different *A. johnsonii* strains uploaded from GenBank. Similarly, **Cao et al. (2017)** observed 99% and 97% homology in the 16S rRNA gene sequences between the BY3*A. johnsonii* isolate and other *A. johnsonii* isolates from the GenBank database. In addition, **Kozińska et al. (2014)** reported 99.6 % similarity with the isolate K712 to *A. junii* and *A. johnsonii*.

Acinetobacter species are well known to develop resistance to a wide variety of antimicrobial agents, which is currently considered an arising risk to aquaculture. Concerning drug resistance, the current results showed resistance to tetracycline, oxytetracycline, and ampicillin among the tested antibiotics, which are commonly used in aquaculture. At the same time, **Kozińska et al. (2014)** reported that the majority of the tested isolates showed resistance to β-lactams (ampicilin and amoxicillin) and cephalothin and sensitivity to fluoroquinolones (flumequine, enrofloxacin, and norfloxacin) and gentamycin; however, use of those antibiotics can affect antimicrobial resistance levels of natural aquatic microflora and, therefore, careful use should be considered **(Guardabassi et al., 2000)**. Also, **Gupta et al. (2015)** revealed that the isolated *Acinetobacter* spp. were mostly multi-drug resistant.

To our knowledge, the current study is the first report concerning *A. johnsonii* pathogencity and virulence in *O. niloticus*. The results showed that the tested *A. johnsonii* strain was pathogenic to the Nile tilapia, and mortality reached 100% of total injected fishes for a 9×10^8 cells/ml dose, which is in agreement with previous results obtained by **Kozińska et al. (2014)** and **Rauta et al. (2011)**, who studied the pathogenicity of *A. johnsonii* in their respective fishes.

Regarding to the virulence of the isolated bacteria, the amplification of virulence and antibacterial sensitivity genes demonstrated that the virulence genes (*fimH, TraT, and iutA*) and antibiotic-resistance genes (*QacED1*, *qnrS, Sul1*, *dfrA*, and *Aada1*) were present in all of *A. johnsonii_Egy* isolates, indicating that the isolate is multidrug resistant. Similarly, **Montaña et al., (2016)** reported several resistance determinants such as *strA*, *strB*, *ereA* and *sul1* that were previously annotated in the *Enterobacteriaceae* family.

A disease might be considered emerging when it appears with a new presentation or higher virulence than previously seen. Thus, *A. johnsonii* can be regarded as an emerging pathogen for farmed *O. niloticus* in Egypt. Antibiotic use for controlling bacterial fish pathogens and the development of multi-drug resistant microorganisms are currently of great importance, and *Acinetobacter* sp. are known to transmit antibiotic resistance genes. Therefore, they may be incriminated in transmission of gene resistance in aquatic systems, and further studies are needed regarding Egyptian *Acinetobacter* sp. pathogenicity, drug resistance, and virulence.

Conflict of interest
The authors declare no conflict of interests.

Ethical approval
The authors followed all institutional guidelines for the care and use of animals.

Data Availability
There is no shared data available.

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تعريف الميكروب الناشئ Acinetobacter johnsonii وجيناته المقاومة للمضادات الحيوية المرتبطة بوفيات عالية في سمكة البلطي النيلي

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الملخص العربى

تم التخطيط للدراسة الحالية لاستقصاء العامل المسبب للوفيات الصيفية لسمكة البلطي النيلي الذي أدى إلى خسائر اقتصادية كبيرة في محافظة بورسعيد ، مصر خلال عام ٢٠١٧. تم تحديد العديد من العزلات البكتيرية ، من بينها تم اختيار A. johnsonii ليكون محور تركيز الدراسة الحالية. تم عزل Acinetobacter johnsonii_Egy وتم تحديده باستخدام مؤشر التشكيل الجانبي التحليلي (API) 20 E) ، متسلسلًا S١٦ شظية الجين RIBossomal RNA وتحليل النشوء الوراثي. أظهر تقييم مقاومة الأدوية أن A. johnsonii كان مقاومًا للأمبيسيلين ، والجنتاميسين ، والينكوميسين ، وحمض الناليديكسيك ، والتتراسيكلين ، والأوكسيتتراسيكلين بين المضادات الحيوية المختبرة. تم إجراء العدوى التجريبية وكشفت الأسماك التي تم تحديها عن علامات سريرية مماثلة كما رأينا في تلك المصابة بشكل طبيعي. يبدو أن المرض معدي للغاية وقاتل ، ويسبب ١٠٠ ٪ من الوفيات التراكمية أثناء العدوى التجريبية. تم تأكيد وجود جينات حدة الاصابه (fimH و traT و iutA) وجينات مقاومة المضادات الحيوية (qacED1 و qnrS و sul1 و dfrA و aadA1) في عزلة A. johnsonii_Egy.

في الختام ، فإن A. johnsonii معدي بشدة لسمكة البلطي النيلي وقد يكون له تأثير كبير على نقل مقاومة المضادات الحيوية في تربية الأحياء المائية. على حد علمنا ، فإن هذه الدراسة هي الأولى التي تشير إلى A. johnsonii كممرض انتهازي ناشئ في البلطي النيل (في بورسعيد- مصر)