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Incidence of *aac(6')-Ib-cr* Mediated by Class 1 Integrons in *Aeromonas* Species Isolated from Diseased *Oreochromis niloticus*

Abdelgayed M. Younes[1*], Alkhateib Y. Gaafar[1], Elham S. Awad[1], Waleed S. Soliman[1], Marwa B. Salman[2], Mohamed F. Eida[3] and Laila A. Mohamed[1]

[1] *Hydrobiology Department, Veterinary Division, National Research Centre, Dokki, Cairo, 12622, Egypt.*

[2] *Department of zoonotic diseases, Veterinary Division, National Research Centre, Dokki, Cairo, 12622, Egypt.*

[3] *Microbiology Department, Agriculture Division, National Research Centre, Dokki, Cairo, 12622, Egypt.*

THE virulent clonal populations of *Aeromonas* spp. are recognized as the etiological agent of motile *Aeromonas* septicemia (MAS) outbreaks in aquaculture. This study aimed to identify *Aeromonas* spp. isolated from diseased *Oreochromis niloticus*, evaluate their virulence factors, and assess their resistance to antimicrobial agents. Species identification was conducted through analysis of the *gyrB* genes. Antibiotic resistance phenotypes and genotypes (bla_{TEM}, bla_{CTX-M}, *aac*(*6'*)*-Ib-cr*, and *intI1*) were determined. The results indicated that 44,4% of the isolates belonged to *A. veronii*, 38.9% to *A. sobria*, and 16.7% to *A. hydrophila*. PCR analysis revealed the presence of bla_{TEM}, *aac*(*6'*)*-Ib-cr*, and *intI1* genes among 100%, 77.8%, and 83.3% of the isolates, respectively, while bla_{CTX-M} was not detected. In conclusion, the detection of the *aac(6')-Ib-cr* gene in aquatic environments can serve as a significant indicator of multi-drug resistance (MDR). The presence of MDR, particularly mediated by integrons in *Aeromonas*, raises serious concerns regarding potential health risks. Moreover, the emergence of MDR complicates the efficacy of therapeutic treatments for infections. Therefore, adopting a comprehensive One Health approach becomes imperative to effectively control the spread of MDR and mitigate its impact on public health.

Keywords: *Aeromonas* spp., *Oreochromis niloticus,* Virulence genes, Antimicrobial resistance genes.

Introduction

Aeromonas species are widespread facultatively anaerobic, motile, Gram-negative, non-spore-forming, catalase and oxidase positive bacilli that cause a variety of host infections in humans and animals [1]. These water-borne organisms have been linked to freshwater and marine fish diseases, including Motile *Aeromonas* Septicemia (MAS) and Epizootic Ulcerative Syndrome [2,3].

Motile *Aeromonas* Septicemia has been associated with several *Aeromonas* spp., including *A. hydrophila*, *A. veronii*, *A. sobria*, and *A. caviae*, affecting various commercial fish, shrimp, reptiles, and other aquatic species [4]. *Aeromonas hydrophila*, in particular, has been a major concern in global commercial aquaculture for a long time. It poses a significant threat to freshwater fish aquaculture in Egypt, leading to irreversible problems [5]. Moreover, *A. hydrophila*, known as one of the most robust *Aeromonas* spp., possesses zoonotic significance due to its diverse antimicrobial resistance against multiple antibiotic classes [6].

Motile aeromonads produce a variety of toxins and extracellular hydrolytic enzymes include esterases, elastases, amylases, arylamidases, chitinases, deoxyribonucleases, peptidases, cytotonic heat-stable and heat-labile enterotoxins, cytotoxic heat-labile enterotoxin, elastase, lipase, aerolysin, flagella, serine protease, ADP ribosyl transferase toxin, Dnases, amylases, chitinases, and nucleases. Other virulence factors include iron acquisition, secretion systems, quorum systems, adhesins, and β-lactamases [2,7-8]. Naturally occurring plasmids

*Corresponding author: Abdelgayed M. Younes, E-mail: tahoon176@yahoo.com, Tel.: +2 01064477278
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encoding different virulence factors and antibiotic resistance genes have been identified in the *Aeromonas* genus, considered a public health hazard since they may be transmitted from animals to humans [2,9].

The overuse of antibiotics, coupled with the presence of antimicrobial resistance enzymes, contributes to the development of resistance in various bacterial species [10]. *Aeromonas* spp. are capable of acquiring β-lactamase resistance, which is mediated by three types of β-lactamases: penicillinase, cephalosporinase, and metallo β-lactamase [4]. Studies have demonstrated their ability to develop and disseminate antibiotic resistance in both clinical and environmental settings. Integrons, primarily classified as class 1 integrons, have been identified in *Aeromonas* spp. These integrons carry a broad range of antibiotic resistance gene cassettes. Notably, resistance genes associated with aminoglycosides, such as *aadA1* and *aadA2*, as well as the trimethoprim resistance gene *dfrA1*, are frequently found within these resistance gene cassettes [11].

Quinolones are broad-spectrum antibacterial drugs that are extensively used in human, veterinary, and aquaculture. Quinolone resistance can arise through two mechanisms: chromosomal mutations affecting the DNA gyrase and topoisomerase IV genes (*gyrA* and *parC*) or via plasmid-mediated quinolone resistance (PMQR) genes. PMQR determinants include *qnr* genes, *aac(6')-Ib-cr*, and efflux pumps [12]. The *aac(6')-Ib-cr* gene, a plasmid-mediated quinolone resistance gene, is commonly integrated within an integron [13]. *Aeromonas* isolates carrying PMQR genes on IncU plasmids have been detected in fish populations, river water, and lake water [14].

To better understand the transmission and prevalence of drug-resistant pathogens and determinants in aquaculture, we isolated *Aeromonas* spp. from diseased *O. niloticus* and analyzed for the presence of virulence genes, antimicrobial resistance genes (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *aac*(*6'*)*-Ib-cr), and the class 1 integrons (intI1*) gene.

Material and Methods

Bacterial isolation

A total of 18 *Aeromonas* strains were used in this study, which was isolated from the *O. niloticus* freshwater fish farms at KafrElsheikh governorate in September 2021. For purity, the isolates were streaked on Trypticase soy agar (TSA) (Oxoid, Hampshire, UK). Enriched samples were inoculated onto *Aeromonas* agar base (LabM, UK) at 25°C overnight to produce a typical green colony. After conducting Gram staining, motility, catalase, and oxidase tests, the biochemical properties of Aeromonas spp. were assessed using the API 20NE kit (bioMerieux, France) following the guidelines provided by the manufacturer.

Molecular confirmation of *Aeromonas* spp.

For genomic DNA extraction, the Purelink™ Genomic DNA Purification Kit (Invitrogen, USA) was employed following the instructions provided by the manufacturer. To identify the presence of *Aeromonas* spp., a PCR assay targeting the *gyrB* gene was performed for species identification [15]. PCR amplification was carried out in a thermal cycler gradient (Bio-Rad, USA) with a final PCR volume of 25 µl, consisting of 12.5 µl Go Taq® Green Master Mix (ThermoScientific, USA). The thermal cycling conditions were set as follows: an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 58°C for 60 seconds, extension at 72°C for 1.5 minutes, and a final extension step at 72°C for 3 minutes. Subsequently, the amplified products were separated by electrophoresis on 1.5% agarose gels, visualized, and captured using GelDoc (Bio-Rad). Finally, the purified PCR products were sent for sequencing.

Determination of antibiotic susceptibility

The isolates were tested for antibiotics sensitivity using the disc diffusion method for: amoxicillin (25 µg), ampicillin (10 µg), gentamicin (10 µg), erythromycin (15 µg), streptomycin (10 µg), kanamycin (30 µg), oxytetracycline (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), chloramphenicol (30 µg), novobiocin (30 µg), and cefotaxime (30 µg). Inhibition zone results were subsequently interpreted based on the CLSI standards [16].

Virulence genes genotypic characterization

To analyze the genetic characterization of virulence genes, a PCR amplification technique was employed to identify the existence of six specific genes: *Aeromonas* cytotoxic enterotoxin (*Act*), cytotonic heat-stable enterotoxins (*Ast*), cytotonic heat-labile enterotoxins (*Alt*), elastase (*Ela*), lipase (*Lip*), flagella A and B (*Fla*). The PCR amplification process for these virulence genes used previously established PCR primers and conditions [7].

Detection of drug resistance genes

To characterize the isolates' resistance genes, extended spectrum β-lactamase (ESBL) genes (*bla*$_{TEM}$, *bla*$_{CTX}$), and quinolone plasmid-mediated resistance gene (*aac(6')-Ib*) were screened by PCR amplification using the different primers listed in Table (1). The PCR products were run on 1.5% agarose gel and was then visualized with Gel Doc (Bio-Rad). Using BLAST, the sequences were aligned with known sequences in the NCBI database.

TABLE 1. List of oligonucleotides sequences of primers used in this study

Gene	Nucleotide sequence	Product size (bp)	References
gyrB	F 5´- TCCGGCGGTCTGCACGGCGT-3´ R 5´-TTGTCCGGGTTGTACTCGTC-3	1100	[15]
TEM	F 5′-ATCAGCAATAAACCAGC 3′ R 5′-CCCCGAAGAACGTTTTC 3′	516	[17]
CTX-M	F 5′-TTTGCGATGTGCAGTACCAGTAA-3′ R 5′-CGATATCGTTGGTGGTGCCAT A-3′	540	[17]
Intl1	F 5′-CCTTCGAATGCTGTAACCGC-3′ R 5′-ACGCCCTTGAGCGGAAGTATC-3′	248	[17]
aac(6')-Ib	F 5′-TTGCGATGCTCTATGAGTGGCTA-3′ R 5′-CTCGAATGCCTGGCGTGTTT-3′	482	[18]
Act	F 5′-AGAAGGTGACCACCAAGAACA3′ R 5′- AACTGACATCGGCCTTGAACTC 3′	232	[7]
Fla	F 5′-TCCAACCGTYTGACCTC-3′ R 5′-GMYTGGTTGCGRATGGT-3′	608	[7]
Ast	F 5′-TCTCCATGCTTCCCTTCCACT-3′ R 5′-GTGTAGGGATTGAAGAAGCCG-3′	331	[7]
Lip	F 5′-ATCTTCTCCGACTGGTTCGG–3′ R 5′-CCGTGCCAGGACTGGGTCTT-3′	382	[7]
Alt	F 5′-TGA CCC AGT CCT GGC ACG GC-3′ R 5′-GGT GAT CGA TCA CCA CCA GC-3′	442	[7]
Ela	F 5′-ACACGGTCAAGGAGATCAAC-3′ R 5′-CGCTGGTGTTGGCCAGCAGG-3′	513	[7]

Detection of class 1 integrons

The presence of Class 1 integrons was identified using specific primers and conditions that were previously documented [16].

Results

Bacterial identification

The colonies formed by *Aeromonas* spp. on TSA agar exhibited a creamy, round, and convex appearance. The isolates of *Aeromonas* spp. demonstrated Gram-negative staining, and they appeared as rod-shaped, motile bacteria. These isolates also exhibited fermentative metabolism, tested positive for oxidase and catalase, but tested negative for indole production.

The confirmed identification of *Aeromonas* spp. was done using the PCR assay. Isolates that tested positive for the *gyrB* gene were then sequenced. Three types of *Aeromonas* spp. were obtained in this study, with the majority of *A. veronii* (no: 8, 44,4%), followed by *A. sobria* (no: 7, 38.9%), then *A. hydrophila* (no: 3, 16.7%).

Antibiotic susceptibility

The findings from the antibiotic susceptibility testing revealed diverse patterns of resistance among the *Aeromonas* isolates. All isolates were ampicillin, amoxicillin and cefotaxime-resistant (100%), followed by ciprofloxacin (94%), kanamycin (80%), nalidixic acid (80%), streptomycin (59.0%), and oxytetracycline (30%). However, all *Aeromonas* isolates were sensitive to chloramphenicol.

Detection of virulence genes

The results in this study showed a variety in the distribution of virulence factors in the *Aeromonas* isolates. All isolates (100%) harbored two or more virulence gene. But none isolate harbored all genes. *Act* gene was the most frequent virulence gene and detected in (88.9%) of strains. While, *Ast* gene was the lowest detected in three isolates (16.7%) (Fig. 1).

Detection of drug resistance genes

Amplification of bla_{TEM} and bla_{CTX-M} genes

The existence of antibiotic-resistance genes in Aeromonas isolates was detected by PCR amplification. The β-lactam resistance gene bla_{TEM} was found in all (100%) of the *Aeromonas* isolates, whereas the bla_{CTX-M} gene was not found in any isolates (0%). The selected partially sequenced 486 bp bla_{TEM} from this study was found to be 100% homology with both $bla_{TEM-116}$ and $bla_{TEM-229}$ genes. The bla_{TEM} gene sequence from this study was deposited in GeneBank under accession number (OR418118).

Amplification of the *aac(6')-Ib-cr* gene

The 446 bp *aac(6')-Ib-cr* gene (associated with aminoglycosides and fluoroquinolone resistance) was obtained from 14 isolates (77.8 %).

After sequencing of some selected isolates, these isolates were found to lack the BstF5I restriction site (5'...G G A T G↑N N↓...3'), with mutations at 102 codon (Trp-Arg) and 179 codon (Asp-Tyr) suggesting that these were cr-variants. The *aac(6')-Ib-cr* gene sequence from this study was deposited in GeneBank under accession number (OR372637).

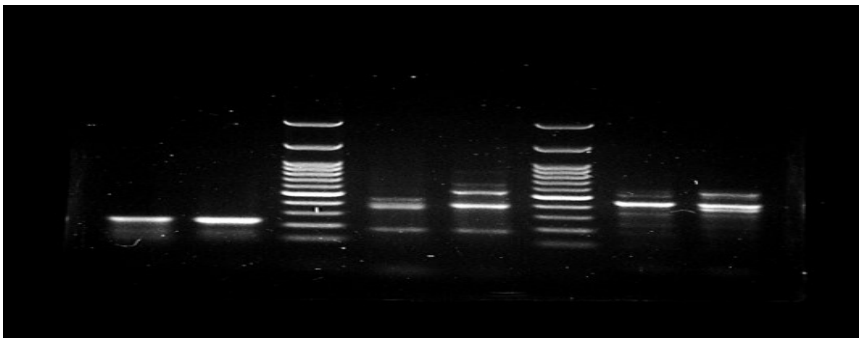

Fig. 1. Agarose gel showing amplification of virulence genes in *Aeromonas* spp.

Lanes 2 and 3: Act gene (232 bp). Lane 5: Lip gene (382 bp). Lane 6: Lip and Ela genes (382 and 513 bp). Lanes 8 and 9: Alt gene (442 bp). Lanes 4 and 7: 100 bp DNA ladder.

Detection of Class 1 Integrons

From tested *Aeromonas* isolates examined for the presence of class 1 integrons using PCR primers targeting conserved regions of integron-encoded integrase *intI1* gene, fifteen (83.3%) of these isolates carried a class 1 integrons.

Discussion

The fish gut serves as a natural reservoir for *Aeromonas* spp., which may be involved in the cellulose degradation in omnivorous freshwater fish that feed plant detritus. However, the pathogenic impact is only evident when the host fish is subjected to stress [19]. Motile aeromonad infections are prevalent in Egyptian freshwater fish and are considered the most common bacterial infections affecting them. These infections can occur in fish of all sizes and lead to significant mortality rates and losses in aquaculture [2,5]. Motile aeromonads are known to be potential carriers of antimicrobial resistance genes due to their ability to rapidly acquire and exchange such genes. The high intrinsic resistance to β-lactam antibiotics observed in motile aeromonad infections is further enhanced by mechanisms such as active efflux, external membrane impermeability, and secondary resistance mechanisms like β-lactamases [1,4]. Multidrug resistance in *Aeromonas* spp. has been identified as a significant public health concern. These bacteria can cause various illnesses in humans, including gastroenteritis, septicemia, and skin infections. The transmission of these infections to humans can occur through skin lesions or the consumption of contaminated food and water [6].

In this study, the identification of *Aeromonas* species was carried out by sequencing the *gyrB* gene, which codes for the B subunit of DNA gyrase. The selection of this gene was based on its significance as the primary housekeeping gene utilized in the phylogenetic analysis of aeromonads, ensuring precise species determination [15]. The *Aeromonas* isolates obtained in this study were categorized into three distinct species. Among the isolates, *A. veronii* exhibited the highest prevalence.

Consistence with these findings, previous studies have indicated that *A. veronii* is the predominant species of *Aeromonas* in cultured *O. niloticus* [3]. Additionally, *A. veronii*, *A. sobria*, *A. hydrophila*, and *A. caviae* were found to be predominant in market fish samples [20]. Nevertheless, alternative investigations have indicated *A. hydrophila* as the most prevalent isolate, accounting for 77% of cases [21]. In a more recent study conducted by Sicuro et al. [22], it was discovered that *A. sobria* (37%) and *A. hydrophila* (18%) were the species most frequently identified in kidney samples taken from 134 ornamental fish that were imported into Italy.

Six virulence genes were identified in *Aeromonas* strains isolated from diseased *O. niloticus*, indicating their presence in all isolates. These findings are consistent with prior studies that indicate motile *Aeromonas* isolates may possess one or more virulence factors that play a crucial role in the process of pathogenesis [2,8].

During this study, the presence of resistance genes was identified in multiple isolates. These included genes conferring ampicillin resistance (bla_{TEM}) and plasmid-mediated quinolone resistance (*aac(6')-Ib-cr*). These findings align with existing literature, which consistently reports the presence of bla_{TEM} in all *Aeromonas* isolates (100%) [23,24]. A separate study conducted in Egypt revealed that the bla_{TEM} gene was detected in all *A. hydrophila* strains (100%) isolated from Nile tilapia and catfish samples, while 83.33% of *A. caviae* strains carried the bla_{TEM} gene [25]. Interestingly, a previous study found no detection of the bla_{TEM} gene in *Aeromonas* strains obtained from rainbow trout, despite the presence of a β-lactam resistance phenotype [21].

Previous studies have demonstrated that the *Aeromonas* genus is capable of producing various types of β-lactamases. Three main classes of β-lactamases have been identified in *Aeromonas* spp., namely penicillinase Class D, cephalosporinase Class C, and Metallo-β-lactamase Class B [26]. Moreover, the presence of CphA and AmpC β-lactamases has been detected, conferring resistance to cephamycins, extended-spectrum cephalosporins, and penicillinases. These enzymes are also capable of deactivating β-lactamase inhibitor compounds [1].

In this study, the *aac(6')-Ib-cr* gene was obtained from 77.8% of total isolates. The *aac(6')-Ib-cr* gene exhibited distinct characteristics that set it apart from all other known variants. Notably, it possessed a unique sequence of twelve base pairs at its 5′ end, along with specific mutations at codons 102 (Trp-Arg) and 179 (Asp-Tyr) [27]. This gene encodes an aminoglycoside acetyltransferase enzyme that confers resistance to aminoglycosides (such as kanamycin, amikacin, and tobramycin) as well as fluoroquinolones (including ciprofloxacin) simultaneously [27].

The *aac(6')-Ib-cr* gene, which has been observed in various Gram-negative bacteria, particularly *Enterobacteriaceae*, has been detected in different geographical regions and genetic environments [12]. Typically, it is situated within a gene cassette found in class 1 integrons. Furthermore, the *aac(6')-Ib-cr* gene cassette tends to occupy the first position within class 1 integrons [13]. It is frequently encountered as a gene cassette within various integrons and is commonly associated with quinolone resistance genes such as *qepA*, *qnrA1*, *qnrS1*, *qnrS2*, *qnrB2*, *qnrB4*, *qnrB6*, and *qnrB10*, as well as β-lactamase genes like $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-24}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{DHA-1}}$ [12].

Previous studies have reported varying observations regarding the presence of the *aac(6')-Ib* gene and its variants in *Aeromonas* spp. Varela et al. [28] found that 59% of *Aeromonas* isolates from wastewater, which were resistant to nalidixic acid, contained the *aac(6')-Ib-cr* gene. Interestingly, they also observed the presence of a variant called *aac(6')-Ib* in 13% of the isolates. This variant did not have the two mutations associated with resistance to quinolone antibiotics. Specifically, they detected this variant in five isolates of *A. caviae*/*punctata* and ten isolates of *A. hydrophila*. In contrast, Chenia [29] found that the *aac(6')-Ib* gene was present in 29% of *Aeromonas* spp. isolated from freshwater fish in South Africa, but they did not find any cr-variants capable of deactivating ciprofloxacin. Similarly, Arias et al. [30] found that although two clinical isolates of *Aeromonas* spp. tested positive for the *aac(6')-Ib* gene, no -cr variants were identified.

Integrons play a pivotal role in the emergence of multidrug resistance by acquiring resistance gene cassettes [31]. These integrons, which contain gene cassettes, are predominantly present in gram-negative bacteria of the *Enterobacteriaceae* family. They are often integrated into conjugative plasmids or transposons, enabling horizontal transfer among various pathogens [32].

In the present study, it was observed that 83.3% of the isolates (n = 15) carried the integrase 1 gene, indicating the presence of class 1 integrons. These findings highlight the prevalence and significance of integrons, particularly class 1 integrons, in conferring multidrug resistance among fish-pathogenic *Aeromonas* strains. The presence of integrons on mobile genetic elements further emphasizes the potential for the horizontal transfer of resistance genes among different pathogens. Previous studies have also documented the presence of integrons harboring inserted gene cassettes in *Aeromonas* isolates that are pathogenic to fish. Lukkana et al. [33] examined the presence of class 1-3 integrons in 50 *A. hydrophila* strains obtained from tilapia. They identified class 1 integrons in 23 (46%) of the *A. hydrophila* strains, while class 2 and 3 integrons were not detected. Furthermore, Deng et al. [34] discovered that 19.6% of *Aeromonas* isolates derived from freshwater animals raised in aquaculture carried the class 1 integrons. Nawaz et al. [35] also detected (48%) integrons in *A. veronii* from catfish. Moreover, class 1 integrons have been reported in 64.62% [36], 31% [21], 28.3% [37], 24% [38], and 21.7% [39] from ornamental fish borne bacteria.

Conclusion

Drug resistance has emerged as a significant public health concern. This study highlights the identification of multiple antibiotic-resistant *Aeromonas* spp. strains carrying various virulence genes. Of particular interest is the presence of the *aac(6')-Ib-cr* gene, which may serve as an indicator of multidrug resistance (MDR) in aquatic environments. The presence of integrons mediating multidrug resistance in *Aeromonas* spp. has the potential to complicate future antibiotic treatments in aquaculture and facilitate the transfer of resistance genes. These findings underscore the importance of monitoring and addressing antibiotic resistance in order to mitigate its impact on both human health and aquaculture practices and demonstrating the necessity for immediate infection control measures.

Conflict of interest

None to report

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Author`s contributions

Authors contribute equally in this work.

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انتشار جين aac(6′)-Ib-cr بوساطة الانتيجرون فى ميكروبات الايروموناس المعزولة من أسماك بلطى نيلى مصابة

عبدالجيد يونس[1]، الخطيب جعفر[1]، الهام عواد[1]، وليد سليمان[1]، مروة سلمان[2]، محمد عيده[3]، ليلى على محمد[1]

[1]- قسم بحوث الاحياء المائية- معهد البحوث البيطرية - المركز القومي للبحوث - الدقى- الجيزة- 12622- مصر.

[2] - قسم الامراض المشتركة- معهد البحوث البيطرية - المركز القومي للبحوث - الدقى- الجيزة- 12622- مصر.

[3] - قسم الميكروبيولوجيا الزراعية - معهد البحوث الزراعية - المركز القومي للبحوث - الدقى- الجيزة- 12622- مصر

تُعرف السلالات العنيفة لبكتيريا الايروموناس بأنها العامل الرئيسى المسبب لتفشي امراض التسمم بواسطة بكتيريا الأيروموناس المتحركة (MAS) في تربية الأحياء المائية. وهدفت هذه الدراسة إلى تحديد أنواع بكتيريا الايروموناس المعزولة من سمك البلطى النيلى المصاب، وتقييم عوامل الضراوة، وكذا تقييم مقاومتها للمضادات الحيوية. تم إجراء تحديد الأنواع البكتيرية من خلال استخدام تحليل جينات *gyrB* وتم تحديد الجينات المقاومة للمضادات الحيوية مثل (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *aac*(*6′*)*-Ib-cr*, *intI1*). أظهرت النتائج أن 44.4٪ من العزلات تنتمي إلى نوع *A. veronii*، و 38.9٪ إلى نوع *A. sobria*، و 16.7٪ إلى نوع *A. hydrophila*. كشف تحليل PCR عن وجود جينات *bla*$_{TEM}$ و*aac(6′-Ib-cr)* و*intI1* في 100٪ و 77.8٪ و 83.3٪ من العزلات على التوالي، في حين لم يتم اكتشاف جين *bla*$_{CTX-M}$. في الختام، فإن اكتشاف جين *aac(6′-Ib-cr)* في البيئات المائية يمكن أن يكون مؤشرًا مهمًا على المقاومة المتعددة للأدوية. إن وجود المقاومة المتعددة للأدوية، وخاصةً بوساطة الأجسام المتكاملة (الانتيجرون) في بكتيريا الايروموناس، يثير قلقًا جديًا بشأن المخاطر الصحية المحتملة. علاوة على ذلك، تعقد ظهور المقاومة المتعددة للأدوية فعالية العلاجات للعدوى البكتيرية. ولذلك، يصبح من الضروري اعتماد نهج شامل للصحة العامة واحد للسيطرة بفعالية على انتشار المقاومة المتعددة للمضادات الحيوية والحد من تأثيرها على الصحة العامة.

الكلمات الدالة: ميكروبات الايروموناس، أسماك البلطى النيلى، جينات الضراوة، جينات مقاومة المضدات الحيوية.