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Insights into *Staphylococcus epidermidis* in Farmed Nile Tilapia in Egypt: Molecular Characterization and Antibiotic Resistance

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Abstract

Staphylococcus epidermidis is one of the causative agents of mass mortality in farmed Nile tilapia (*Oreochromis niloticus*) in Kafr Elsheikh Governorate, Egypt. Diseased farmed Nile tilapia were collected randomly during the fall of 2023 from farms suffering from mass mortalities. Clinical examination revealed severe lesions in the hepatopancreas and eye of the collected diseased fish. Based on morphological and biochemical analyses, the isolated bacteria were suggested to be *Staphylococcus* sp. Five of the nine isolates were selected for 16S rRNA sequencing based on their consistent phenotypic characterization criteria. One isolate was found to be *Bacillus rugosus* based on the 16S rRNA sequencing, which is non-pathogenic and out of the scope of this study. The four remaining isolates were found to be *S. epidermidis*, which have similarities to other *S. epidermidis* isolates. All isolated *S. epidermidis* were resistant to ampicillin, erythromycin, chloramphenicol, streptomycin, and amoxicillin; however, they were moderately susceptible to tetracycline and were sensitive to gentamicin and vancomycin.

Keywords: Nile tilapia, *S. epidermidis*, 16S rRNA, Phylogenetic analysis, Antibiogram.

Introduction

Fish are widely recognized as a valuable source of food due to their nutritional composition, high palatability, and digestibility. However, diseases significantly impact the fish populations within their ecosystems [1]. The World Bank estimated that diseases cause an annual economic loss of 6 billion USD in aquaculture [2]. The prevalence of diseases in aquatic ecosystems is influenced by various environmental factors, including infectious organisms and stressors, which contribute to the susceptibility of fish to diseases [3].

Egypt is a leading producer of Nile tilapia accounting for more than 84% of the total production in Africa [4]. Kafr Elsheikh Governorate is a major contributor (324,479 tons) of the country's farmed fish production, accounting for 55% of the total production. For cultured tilapia, it also produces 44% adding 259,583 tons to the overall production, highlighting its significant role in Egypt's aquaculture industry [5]. In Egypt, fish producers have experienced substantial losses as a result of "summer mortality" in tilapia which is caused by several factors [6].

Ecological studies have revealed that *S. epidermidis* is present in the aquatic environment [7]. Therefore, monitoring for bacterial pathogens is crucial to protect aquaculture economy, and for early detection of potential contamination threats. *S. epidermidis* has previously been recorded as one of the bacterial pathogens that cause disease in freshwater fish and marine environments [8].

It can be challenging to determine which fish pathogen is responsible for specific infection

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symptoms, as many bacterial pathogens can cause similar clinical signs [9]. For example, while infections caused by *S. epidermidis*, *Streptococcus agalactiae*, and *Aeromonas veronii* are commonly associated with exophthalmos and *A. hydrophila* or *A. veronii* cause congestion to the hepatopancreas, it is important to note that these pathogens can potentially cause overlapping lesions, as supported by previous records [10, 11]. To address those challenges, 16S rRNA sequencing has become a cornerstone in reliable and accurate identification for fast detection and diagnosis of bacterial diseases [12].

Due to the intensification of modern technologies in aquaculture, disease prevalence has increased, and new pathogenic bacteria have emerged that are detrimental to fish health. This situation previously led to the extensive use of antibiotics, particularly in developing countries, for the prophylaxis or management of bacterial infections in fish; however, this practice has significantly declined in Egypt in recent years [13].

This work aimed to assess the prevalence of *S. epidermidis* in diseased *O. niloticus* collected from cultured freshwater farms. It also aimed to construct a phylogenetic tree based on the 16S rRNA sequencing of the four isolates and test their antibiotic susceptibility.

Material and Methods

Collection of fish samples

Fifty samples diseased O. niloticus, averaging 150–200 g in weight, were randomly collected between September and November 2023 from ten different fish farms in Kafr Elsheikh Governorate that experienced mass mortalities and placed in sterile bags [14]. All samples were transferred within 6 hours of collection in an icebox with crushed ice to the Biotechnology lab, Basic and Applied Sciences Institute, Egypt-Japan University of Science and Technology (E-JUST).

Clinical and postmortem examination

A clinical examination was applied to the fish samples, both external signs and post-mortem examination on the diseased fish following the methods described by Heil [15].

Bacterial isolation

Bacterial isolation was performed according to the methods described by Metin et al. [16]. A sterile normal saline solution was used to rinse the external surface of the fish, followed by spraying with 70% ethyl alcohol. Under complete aseptic conditions, fish were dissected, and then a loopful of tissues from the kidney, spleen, and hepatopancreas were separately streaked onto tryptic soy agar (TSA), (HiMedia, India). Inoculated agar plates were incubated at 25 °C for 24-48 hours. Single colonies were selected for purification and further characterization.

Phenotypic characterization

Morphological characterizations, such as shape, size, Gram staining, and motility tests, were performed on suspected pure colonies of the isolates [17]. Biochemical identification was conducted for confirmation of isolates using the following tests: coagulase, catalase, oxidase, Methyl Red (MR), Vogues Proskauer (VR), citrate, urease, and nitrate reduction [18]. The hemolytic activity of bacterial isolates was assessed by inoculating an aliquot of an overnight bacterial suspension onto blood agar plates containing 5% sterile defibrinated sheep blood, according to the described method by Ruaro et al. [19]. The plates were then incubated for 24 hours at 25 °C.

Identification of the isolates using 16S rRNA sequencing

DNA extraction and purification

The genomic DNA was extracted using the described procedure by Monir et al. [20]. The isolated bacteria were sub-cultured onto TSA plates to produce a fresh overnight culture. A single colony from the subculture was inoculated into 10 mL of tryptic soy broth (TSB) and incubated at 25 °C for 24 hours. The fresh culture was centrifuged at 5000 rpm for ten minutes. Next, 400 µl of solution I (50 mM Tris.HCl pH-8.0, 50 mM EDTA pH-8.0, 25% sucrose, 1 mg lysozyme) were added to the washed cell pellet, thoroughly mixed, and incubated at 37 °C for 15 minutes. The cells were treated with 400 µl of solution II (10 mM Tris. HCl pH 8.0, 5 mM EDTA pH 8.0, 1% SDS, 40 µg Proteinase K) and incubated at 55 °C for 3 hours. The suspension was then centrifuged at 6,000 rpm for 10 minutes. The aqueous layer on top was carefully removed to avoid protein debris and transferred to a new microfuge tube. To precipitate the DNA, ice-cooled ethanol was added to the aqueous phase twice. The DNA was pelletized by centrifugation at 12000 rpm for 10 minutes. The pellet was rinsed with 70% ethanol,

dried, and dissolved in 100 µl of TE buffer (pH 7.6). The quality (A260/A280) of purified PCR products was measured using a Nanodrop spectrophotometer.

Sequencing of 16S rRNA

Five of the nine isolates were selected for 16S rRNA sequencing based on their consistent phenotypic characterization criteria. The 16S rRNA sequencing was performed by FASMAC (Kanagawa, Japan). Briefly, the 16S rRNA gene fragment was amplified with PCR using the 27F/1492R primer. BLASTn was used for the sequence analysis by putting the sequences as queries to Genbank to confirm the identities and closest relatives of the samples [21].

Phylogenetic analysis of isolates

Multiple sequences were aligned using ClustalW in Mega 11.0. Then, the sequences of S. epidermidis and the most closely related sequences of bacteria in GenBank were compared (Table 1). The Maximum Likelihood approach was implemented in Mega 11.0 to infer phylogenetic relationships [22]. The confidence level in the Maximum Likelihood method trees was determined by analyzing 1,000 bootstrap repetitions using the Mega software.

Antimicrobial susceptibility testing

An antibiotic susceptibility test was conducted on isolates of S. epidermidis using the disc diffusion Kirby-Bauer technique on Mueller-Hinton agar [23]. The following antibiotics were tested: Ampicillin (10µg), Tetracycline (30µg), Erythromycin (20µg), Chloramphenicol (30µg), Gentamycin (10µg), Vancomycin (30µg), Streptomycin (30µg), and Amoxicillin (10µg). The discs were firmly placed on the agar plates previously streaked with the test organism (0.5 McFarland) using sterile forceps and then incubated at 37 °C for 18-24 hours. Susceptibility or resistance of the isolates to different antibiotics was indicated by the appearance or non-appearance of clear zones of inhibition, which were measured to the nearest millimeter using a vernier ruler. The diameters of the zone of inhibition were compared with the cut-off points, and the interpretations of the results were performed according to the recommendations of the Clinical and Laboratory Standards Institute [24].

Results

Clinical pathology

Clinical signs of infected fish were recorded, including exophthalmia (pop-eye), hemorrhage, and abdominal distension. The post-mortem examination showed congestion of the hepatopancreas and spleen, and distended gall bladder (Fig. 1).

Bacterial isolation and identification

The cultural characteristics of suspected *Staphylococcus* spp. were examined, and they appeared to be white-raised, cohesive colonies on TSA.

Prevalence of S. epidermidis

A total of 8 out of 50 examined fishes were infected by *S. epidermidis* (The overall prevalence was 16%).

Characteristics of isolated Staphylococcus spp.

Table 2 illustrates the phenotypic characteristics and biochemical identification of *Staphylococci* spp. recovered from internal organs of diseased Nile tilapia. The isolates were Gram-positive, cocci, and non-motile. They were positive for the catalase, urease, and nitrate reduction tests. The isolated bacteria were coagulase negative and showed no hemolysis on sheep blood agar.

Confirmation of isolates by 16S rRNA sequencing

Nine bacteria were isolated from 50 diseased Nile tilapia samples cultivated on TSA and subsequently identified through biochemical and phenotypic assays. Based on consistent phenotypic characterization criteria, five isolates were selected for 16S rRNA sequencing.

The results of the 16S rRNA sequencing revealed that one isolate was identified as *Bacillus rugosus*, which is non-pathogenic and irrelevant to this study. Its nucleotide sequence has been deposited in the GenBank database under the accession number PQ865795.

The remaining four sequenced isolates were identified as *S. epidermidis*, and their 16S rRNA gene nucleotide sequences have been submitted to the GenBank database under the following accession numbers: PP781962.1, PP781976.1, PP781979.1, and PP781988.1, respectively.

Phylogenetic analysis of the four sequenced S. epidermidis compared to the bacterial isolates archived in the NCBI

The phylogenetic tree (Fig. 2) was generated by aligning the sequences of our isolates with those

recorded in Table 1. It illustrates the strains of *S. epidermidis* that have been isolated from a variety of sources, such as fish farms, natural fisheries, and fish products.

The first isolate, *S. epidermidis* strain AS1 (PP781962.1), has a nucleotide sequence that is 99.63% the same as the reference isolate, *S. epidermidis* strain B7, which was found in Shidal, India and has the GenBank entry number KP979596. The second strain of *S. epidermidis* we found, AS2 (PP781976.1), is 99.77% similar to the reference strain of *S. epidermidis*, LGC 305, which was found in the gut of a freshwater Indian loach (GenBank accession number OQ271316.1). Two of our *S. epidermidis* isolates, AS3 (PP781979.1) and AS4 (PP781988.1), are 99.86% and 99.93% identical with the reference strain found in Chinese fermented fish (GenBank entry number MH491958.1).

Antimicrobial susceptibility testing

All eight isolated *S. epidermidis* samples were subjected to an antibiogram test utilizing the disc diffusion method to determine their antimicrobial susceptibility profiles. The results in Figure 3 show that all *S. epidermidis* isolates were sensitive to gentamycin and vancomycin while being moderately susceptible to tetracyclines. Additionally, all isolates were resistant to ampicillin, erythromycin, chloramphenicol, streptomycin, and amoxicillin. (GenBank entry number MH491958.1).

Discussion

Nile tilapia is the most widely farmed fish in Egypt, valued for its ability to tolerate fluctuations in water physicochemical parameters and its diet of phytoplankton. Nile tilapia has a tremendous commercial effect and high nutritional value among fish species [25]. Intensive farming practices have increased fish susceptibility to various pathogens, such as bacteria, fungi, parasites, and viruses.

Staphylococcus spp. indisputably leads to high mortality rates in freshwater aquaculture under stressful conditions [26]. *S. epidermidis* bacterium colonizes mucous membranes and skin of humans, but when transferred into fish, it infects the spleen, kidney, and eye, causing uni or bilateral exophthalmia [27]. Diseased Nile tilapia of exophthalmia is lined with symptoms first recorded by Kusuda and Sugiyama [28] who isolated this bacterium from the eyes of cultured *Seriola quinqueradiata* and *Pagrus major*.

Based on morphological and biochemical characteristics data, 8 bacterial isolates were identified as of *Staphylococcus* sp. Illumina-based 16S rRNA sequencing effectively confirmed the identification of the four isolates as *S. epidermidis*. The data presented in table 2 align with established literature, confirming the prevalence of *S. epidermidis* in the freshwater aquatic environment [29].

The phylogenetic tree of the four *S. epidermidis* reveals high evolutionary similarities, despite their isolation from different fish farms in Kafr Elsheikh Governorate. That similarity indicates the incidence of *S. epidermidis* in Nile tilapia's fish farms. Most farms were irrigated with water from the same canal used for wastewater drainage, which facilitates reinfection and exacerbates the spread of *S. epidermidis* in the farms. Contamination from livestock feces and feed wastes deteriorates irrigation water quality, making it difficult to decrease the risk of water contamination with microbes [30]. Fish hatcheries can be sources of infection for fish farms, mainly because they produce and distribute fries to various aquaculture facilities. Without strict biosecurity measures, pathogens like bacteria, viruses, fungi, and parasites can be introduced and spread through contaminated water, equipment, or fish stocks [31]. *S. epidermidis* was one of the common bacterial causes of mortality in tilapia fries collected from hatcheries in Kafr Elsheikh Governorate [32].

The phylogenetic tree (Figure 2) illustrates that the four representative bacterial strains isolated from Nile tilapia in this study show a significant degree of similarity to 33 GenBank *S. epidermidis* strains associated with fish, their habitats, and fish products from various countries.

S. epidermidis is an opportunistic pathogen that contributes to the mass mortality of farmed tilapia, impacting the economic, nutritional, and health aspects of freshwater farms. Although we did not perform a bacterial challenge test using the isolates, a previous study had performed this test on sea bream, and they found that a high mortality rate (63.3%) as a result of intraperitoneal injection of *S. epidermidis* [33]. It is also reported that *S. epidermidis* caused infections in cultured *Oncorhynchus mykiss* [34].

Data from Table 3 show different prevalence rates of bacteria isolated from fresh and marine fish species. The current study is nearly lined with a previous survey that recorded the most common bacterial pathogens of the Nile tilapia fries in Kafr Elsheikh Governorate, Egypt. The researchers isolated *S. epidermidis* by 20% of 6000 tilapia fries [32]. In a study conducted in Taiwan, *S. epidermidis* was recorded as the most dominant pathogenic species isolated from moribund tilapia with a percentage of 10% (16 cases out of 159 moribund tilapia) [35]. This percentage is slightly lower than that of current study. Our study is matched with a previous report in Pakistan in which the authors found out the prevalence of pathogenic bacteria in the collected fresh fish samples is 17.85% (15

positive samples out of 84 collected fish) [36]. S. epidermidis was reported as the primary causative agent of mass mortality in diseased sea bass, with a high percentage incidence of 80% (four fish were infected out of five diseased) [37].

In the current study, all isolates of *S. epidermidis* were resistant to ampicillin, erythromycin, chloramphenicol, streptomycin, and amoxicillin. Our isolates of *S. epidermidis* were sensitive to gentamycin and vancomycin, and they recorded an intermediate susceptibility with tetracycline. Our finding agrees with Eladli et al. [38], in which isolates were sensitive to gentamycin and vancomycin. Bacteria have evolved many defense mechanisms against antimicrobial agents, and drug-resistant pathogens are rising. Genome studies of *S. epidermidis* have shown that various genes offer resistance to the bacteria against severe environmental conditions. *S. epidermidis* can form biofilm by producing extracellular polysaccharides, proteins, and DNA to enhance its resistance mechanism [39]. The bacteria develop resistance factors through genetic recombination and acquiring new genes. Some antibiotics can accumulate in sediments and the environment, causing bacteria to develop resistance to many effective antibiotics. Other factors contribute to this resistance, such as the intensive farming of various fish species, agricultural and industrial waste entering water systems, and insufficient government regulations on the excessive use of antibiotics in aquaculture [40].

This study did not investigate the pathogenicity of the isolates or conduct experimental challenge tests. Future research will focus on assessing the pathogenicity of *S. epidermidis*, performing histopathological examinations, and applying Koch's postulates to establish a definitive causal relationship between the isolates and the observed disease.

Conclusion

Accurate identification of the causative agents of fish pathogens is crucial for effective farm management. This identification is challenging because many bacterial pathogens can cause similar clinical signs. Combining clinical and biochemical identifications and 16S rRNA sequencing is essential for the correct diagnosis of S. epidermidis and crucial to avoid misleading diagnosis. Phylogenetic analysis revealed a high degree of evolutionary similarity between the isolates and other reference strains, suggesting that shared environmental factors. An antibiogram test should be performed before using an antibiotic in aquaculture to ensure effective treatment and minimize the development of antibiotic resistance mechanisms.

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Conflict of interest statement

The authors have disclosed that they do not hold any conflicts of interest related to the publication of this article.

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Ethical of approval

Not applicable.

Fig.1. Unilateral exophthalmia in infected farmed Nile tilapia observed in a suspected *S. epidermidis* case

TABLE 1. Bacterial species used for constructing the phylogenetic tree

Nature of Sample	Source (host/country)	Accession number
Hatcheries of tilapia	Tilapia fries / Egypt	MT293804.1
Fish-tank	Plankton / United Kingdom	AJ491709.1
Product (Filleted)	Sea catfish (*Arius heudeloti*) / Senegal	EU128487.1
Estuarine	Estuarine cat fish (*Mystus gulio*) /India	JX625992.1
Ocean water fish	Puffer fish (*Sphoeroides annulatus*) / Mexico	HM584018.1
Fish processing plant	Fish processing wastewater / India	KC161905.1
Ocean and Coastal fish	Marine fish /India	KJ459012
Fish gut	Fish gut/India	KR006922.1
Ngari (fermented fish)	*Puntius sophore* fish /India	KJ699166.1
Fish sauce fermentation	Fish sauce/ Thailand	KU132366.1
Fish kills	Fish kill / Korea	KP115686.1
Fish processing plant	Fish processing wastewater / India	KT387371.1
Fermented fish	Large yellow croaker/ China	KX267887.1
Shidal (Fermented fish product)	*Puntius sophore* /India	KP979596.1
Fermented fish	Large yellow croaker/ China	KX237940.1
Salted fish	Salted fish / United Arab Emirates	MF067464.1
Fermented fish	Fermented fish / China	MH491958.1
Fish sausage	Fish sausage/ China	MH915445.1
Fish Product	Stinky mandarin fish / China	MN867689.1
Tributaries of the Pacific Ocean	Rainbow trout (*Oncorhynchus mykiss*) / Turkey	MN923048.1
Fish pond	*Sciaenops ocellatus* / China	MT071633.1
Fish pond	*Sciaenops ocellatus* / China	MT071657.1
Farmed bivalve	*Perna viridis* /India	MT491104.1
Refrigerated Fillets	Sturgeon (*Acipenser*) / China	OK103766.1
Gut of Extensively cultured Shrimp	Whiteleg shrimp (*Litopenaeus vannamei*)/ India	OK244469.1
Fish gut from estuary	Esturine fish / India	ON332486.1
Fish pond	Fish / India	ON387515.1
Farmed Fish	Gilt-head bream (*Sparus aurata*) / Saudi Arabia	OP704017.1
Freshwater fish	Gut of Loach / India	OQ271316.1
Pond Shing fish	Shing fish gut / Bangladesh	OR871836.1
Freshwater fish	Blood of *Cyprinus carpio* koi / India	MN515417.1
Farmed Nile tilapia	Spleen of *Oreochromis niloticus* / Egypt	MN153038.2
Estuarine fish	Gut of *Mugil jerdoni* / India	KJ623584.1

TABLE 2. Morpho-chemical characteristics of *S. epidermidis*

Characters	Result
Gram stain	+
Motility	-
Shape	Cocci
Coagulase	-
Catalase	+
Oxidase	-
Methyl Red (MR)	-
Voges Proskauer (VR)	-
Citrate	-
Urease	+
Nitrate Reduction	+
Hemolysis	-

(+) Positive reaction, **(-):** Negative reaction

TABLE 3. Summary of the prevalence of *S. epidermidis* in some previous studies compared to the current study

Fish Name	Percentage of Prevalence (%)	Reference
Cultured Nile tilapia	16	This study
Cultured Nile tilapia fries	11.1	[32]
Cultured Tilapia	10	[35]
Cultured Silver carp	17.85	[36]
Cultured European sea bass	80	[37]

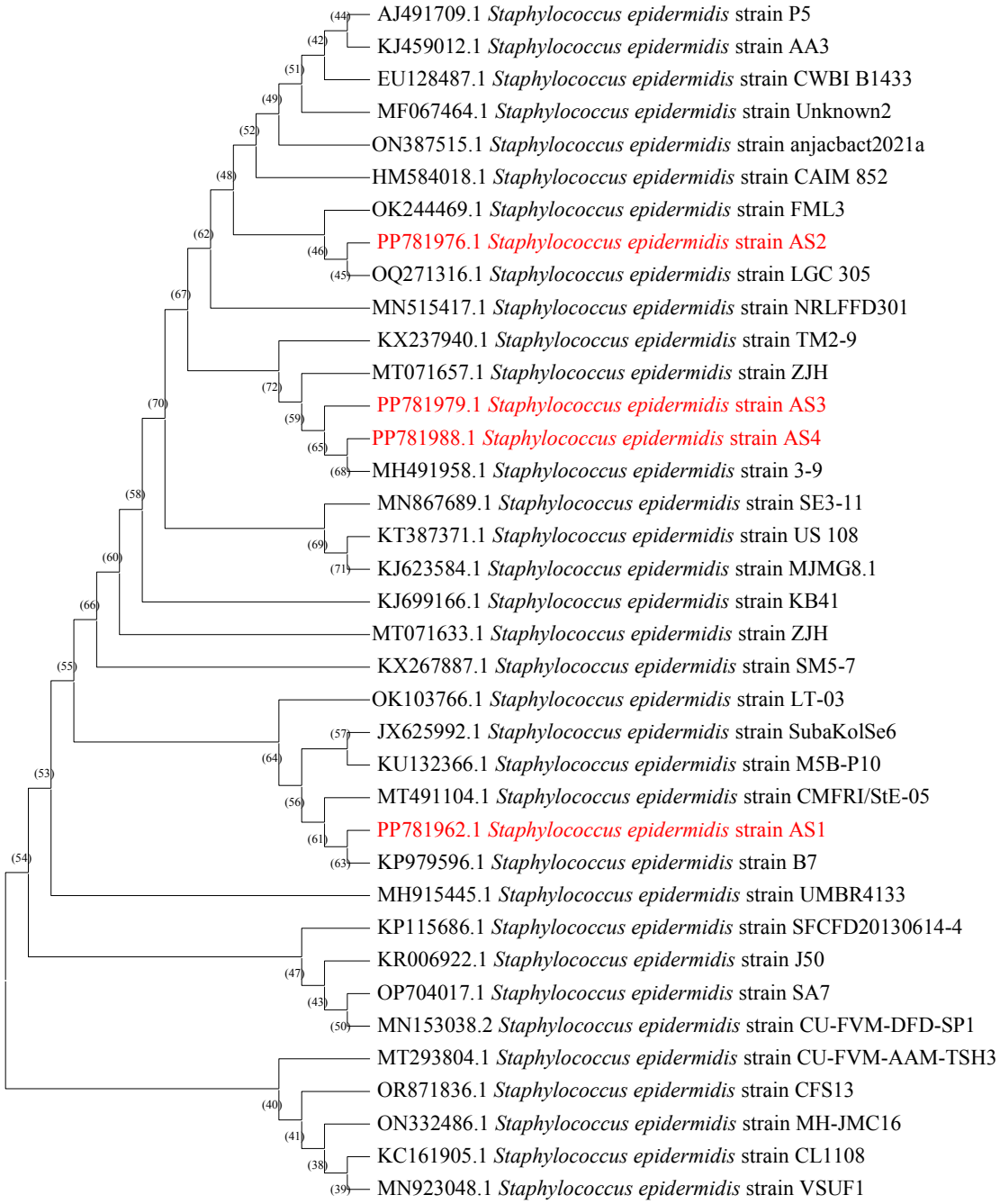

Fig.2. Phylogenetic tree of isolates compared to recorded isolates showing Evolutionary analysis

Phylogenetic tree of chosen members of *S. epidermidis* based on 16S rRNA sequences. The GenBank accession numbers for every sequence that constitutes the analysis are provided before the taxon names. The red color shows the sequences found in this study. The evolutionary history was inferred using the Maximum Likelihood method and Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-7703.26) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.9541)). This analysis involved 37 nucleotide sequences. There were a total of 1996 positions in the final dataset.

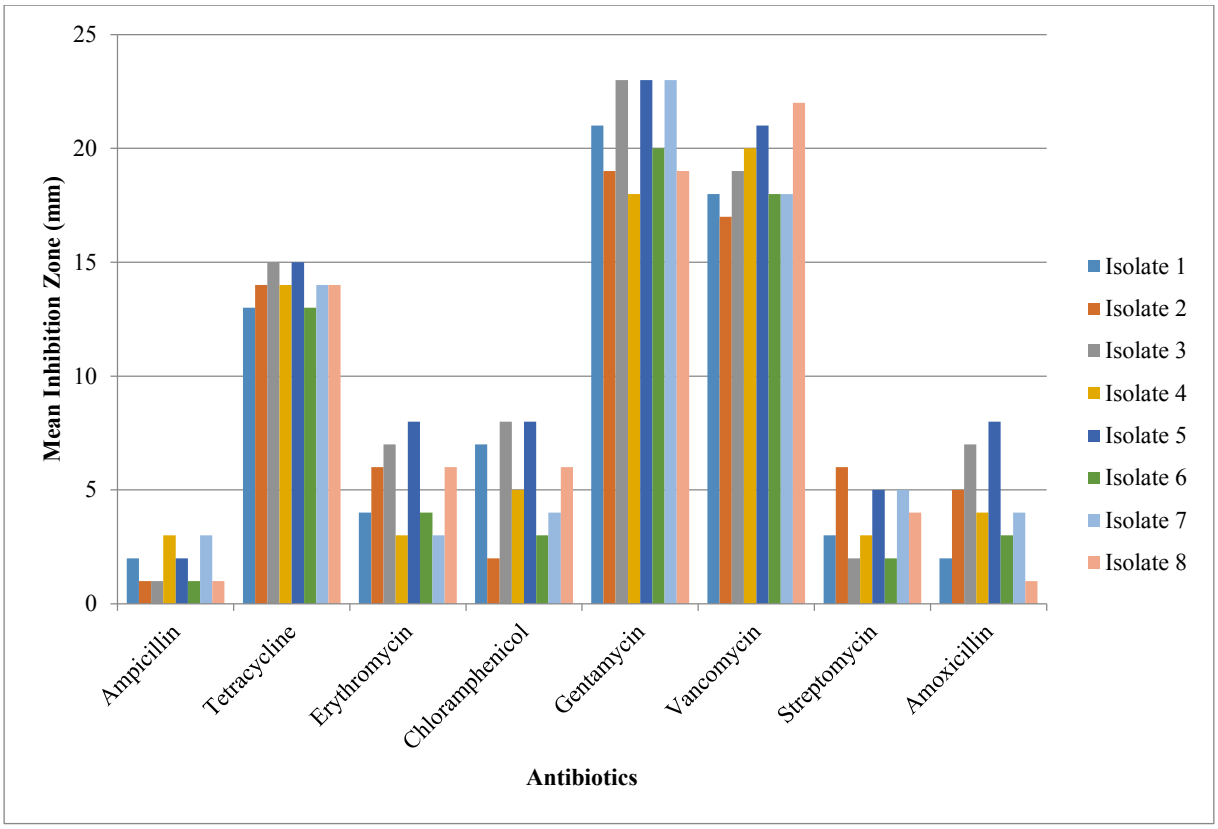

Fig.3. Antibiogram test for *S. epidermidis* against all the tested antibiotics under study
R: Resistant (0-12 mm), **I:** Intermediate (13- 17 mm), **S:** Susceptible (≥17 mm)

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رؤى حول بكتيريا *Staphylococcus epidermidis* في أسماك البلطي النيلي المستزرعه في مصر: التوصيف الجزيئي ومقاومة المضادات الحيوية

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الملخص

تعَدُّ *بكتيريا Staphylococcus epidermidis* أحد العوامل المسببة للنفوق الجماعي في أسماك البلطي النيلي المستزرعه في محافظة كفر الشيخ، مصر. تم جمع عينات عشوائية فى خريف 2023 من أسماك البلطي النيلي المصابه من المزارع التي تعاني من حالات نفوق جماعي ، حيث كشفت الفحوصات الإكلينيكية عن وجود إصابات شديدة في كبد وعين الأسماك المريضه التى تم جمعها، وباستخدام الفحوصات المورفولوجيه الظاهريه والبيوكيميائية تبين أن البكتيريا المعزولة تنتمى إلى المكورات العنقودية ،

تم اختيار خمسة من بين تسعة عزلات بناء على تشابه الفحوصات المورفولوجيه الظاهريه والبيوكيميائية لعمل 16S rRNA sequencing، وأظهرت النتائج أن إحدى العزلات *Bacillus rugosus* وهي بكتيريا غير ممرضة وخارج نطاق هذه الدراسة، بينما العزلات الأربعه المتبقية فقد تم تعريفهم كبكتيريا *S. epidermidis* طبقا للتشابه الجينى مع العزلات المرجعية المستخدمة.

أظهرت جميع السلالات المعزولة لبكتيريا *S. epidermidis* مقاومة للأمبيسلين، الإريثروميسين، الكلورامفينيكول، الستربتومايسين، والأموكسيسيلين، وأظهروا حساسيه متوسطه للتتراسيكلين ، بينما كانت البكتيريا أكثر حساسيه للجنتاميسين والفانكومايسين.

الكلمات الدالة: البلطي النيلي ، العنقوديه البشرويه، 16S rRNA ، تحليل التطور ، حساسية المضادات الحيويه.