Prevalence and antibiotic resistance of *Aeromonas hydrophila* and *Staphylococcus aureus* isolated from seafood in Egypt

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ABSTRACT

**Objective:** To investigate prevalence of *Aeromonas hydrophila* and *Staphylococcus aureus* in seafood, and to detect the consistent virulence genes as well as to assess the antimicrobial susceptibility.

**Samples:** 280 marketed seafood samples (178 shrimp, 54 oysters, 26 crabs, 18 squid, and 4 octopuses).

**Procedures:** Isolation and identification of *Aeromonas hydrophila* and *Staphylococcus aureus* were performed using conventional methods. The identified isolates were examined for virulence genes (*aer* and *hly* genes for *A. hydrophila* as well as *nuc* and *sea* genes for *S. aureus*) as well as for antimicrobial susceptibility.

**Results:** *A. hydrophila* was isolated from 40 of the 280 seafood samples (14.3%), with the highest prevalence (22.2%) in oyster samples, whereas *S. aureus* occurred in 50 samples (17.9%) with the highest prevalence (20.2%) in shrimp samples. Moreover, *aer* and *hly* genes were detected in all isolates of *A. hydrophila*, and *nuc* and *sea* genes were detected in all tested *S. aureus* strains, whereas *staphylococcal enterotoxin A* (*sea*) gene was found in only 44% of *S. aureus* strains. *A. hydrophila* strains were absolutely resistant to amoxicillin (100%), followed by ceftriaxone (80%), chloramphenicol (77.5%), trimethoprim-sulfamethoxazole (65%), and tetracycline (55%), whereas *S. aureus* strains showed high resistance to penicillin (86%), followed by amoxicillin-clavulanic acid (72%), and trimethoprim-sulfamethoxazole (58%). Multidrug resistance (MDR) to more than two classes of antibiotics was found in 77.5% (31/40) of *A. hydrophila* strains and 66% (28/50) of *S. aureus* isolates.

**Conclusion and clinical relevance:** Our data highlights the importance of awareness of virulent strains of *Aeromonas hydrophila* and *S. aureus* strains of seafood samples in Egypt. Consequently, the continuous surveillance of these bacteria in seafood with a strong focus on their antibiotic resistance characteristic should be considered in further studies.

**Keywords:** *Aeromonas hydrophila*; Antibiotic resistance; Seafood; *Staphylococcus aureus*.

1. INTRODUCTION

Seafood is an essential component of community nutrition, however the consumption of contaminated seafood often causes gastrointestinal diseases in humans[1] and leads to a significant number of outbreaks in many countries[2]. Aquaculture farms and seafood are important reservoirs of many of the pathogenic bacteria, seafood hosts bacteria due to its flesh texture and their habitat which is loaded by microbes[3].

*Aeromonas* is considered a universal and causative agent of outbreaks primarily in aquaculture, and *A. hydrophila* and *A. salmonicida* in particular are common causative agents of ulcerative and hemorrhagic skin ulcers. Conditions such as stress, poor sanitation, and nutritional deficiencies favor these infections[4]. The distribution of *A. hydrophila* is worldwide and it has been isolated from various sources including meat, fish, and shellfish. It can lead to infections in humans ranging from gastroenteritis to grave septicemia[5]. *A. hydrophila* can tolerate a wide range of temperatures from freezing to boiling[6].

*S. aureus* ranks third among the leading etiological agents of reported food-borne illnesses worldwide[7]. The frequent occurrence of staphylococcal food poisoning is attributed to human carriers, due to unhygienic food handling. Hence, the control of *S. aureus* depends on the implementation of proper hygiene measures and protocols[8]. *S. aureus* has been detected in aquatic food products in many countries and presents a potential risk factor for consumers[9].

The pathogenicity of *A. hydrophila* comprises various virulence factors, including cytotoxic, hemolytic, and enterotoxic activities[10]. Aerolysin, produced by some *A. hydrophila* strains is an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties and leading to diarrhea in humans[11]. Aerolysin binds to specific glycoprotein receptors on the surface of eukaryotic cells before inserting into the lipid bilayer and forming holes. The hole-forming aerolysin toxin crosses the inner bacterial membrane as a preprotoxin containing a signal peptide which is removed cotranslationally. Hemolysins produced by *A. hydrophila* have a linear relationship with gastroenteritis in humans[12]. Detection of virulence genes, particularly aerolysin (*aer*) and hemolysin (*hly*) genes, by polymerase chain reaction (PCR) assay is an accepted approach to identify pathogenic strains of *A. hydrophila*[13].
S. aureus produces thermonuclease, many types of staphylococcal enterotoxins (SEs) and SE-like toxins. Thermonuclease is an exoenzyme catalyzing the hydrolysis of DNA and RNA. The recognition of nuc gene encoding thermonuclease was useful to improve S. aureus detection from different food sources[14]. In some areas of the world, food poisoning outbreaks are frequently associated with SEs [15]. SEA is the most frequently isolated enterotoxin among SFD outbreaks in France, Japan and UK[16].

Antibiotics are widely used in aquaculture to systemically deal with diseased fish as well as to promote growth and enhance production by reducing mortalities[17] Multidrug-resistant (MDR) strains of A. hydrophila, (mainly resistant to β-lactam antibiotics) have emerged and been isolated in a number of different regions across the world[4].

S. aureus strains have a high capacity to develop antibiotic resistance, by different routes as genetic mutations and horizontal gene transfer from an external source [18]. The most abundant enzyme produced by S. aureus after exposure to β-lactam antibiotics is β-lactamase[19].

The present study aimed to investigate the prevalence of virulent and multidrug-resistant strains of A. hydrophila and S. aureus that may be considered emerging pathogens in seafood in Egypt.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 280 marketed seafood samples [178 shrimp (caridea), 54 oysters (Ostrea edulis), 26 crabs (brachyuran), 18 squid (teuthida), 4 octopuses (octopoda)] were randomly and aseptically collected from 10 different retail markets in Mansoura city, Egypt, during the period from March to September 2018. The samples were packed in an ice box and transported to the laboratory for analysis of pathogenic bacteria.

2.2. Bacteriological analysis

For the isolation of A. hydrophila, samples were cut by sterilized scalpel, dipped into screw cap bottles containing alkaline peptone water and incubated at 35°C for 18 h and then a loopful was streaked onto sheep blood agar with 10 μg/ml ampicillin (ASBA) and incubated for 18–24 h at 35°C[20].

For the isolation of S. aureus, samples were cut by sterilized scalpel, dipped into screw cap bottles containing tryptic soy broth (TSB, Oxoid, Basingstoke, UK) and incubated at 37°C for 24 h. The overnight-inoculated broth was streaked on Baird-Parker agar (BPA, Oxoid, Basingstoke, UK) with egg yolk tellurite (Oxoid, Basingstoke, UK). The presumptive colonies of A. hydrophila and S. aureus were morphologically and biochemically identified [21, 22].

2.3. Molecular determination of virulence-associated genes in A. hydrophila and S. aureus isolates

Conventional PCR was performed to investigate the virulence determinants using oligonucleotide primers including the genes encoding aer and hly in A. hydrophila isolates as well as the genes encoding thermonuclease (nuc) and staphylococcal enterotoxin A (sea) in S. aureus isolates. Briefly, DNA of A. hydrophila and S. aureus was extracted by boiling according to Yousr et al. [23] and [24] respectively. The amplification of DNA was achieved using oligonucleotides primers and specific cyclic conditions as illustrated in Table 1. The PCR assay was conducted using an Applied Biosystem, 2720 Thermal Cycler (USA). The 25 μl reaction mixture comprised 12.5 μl of 2×PCR master mix (Promega, Madison, USA), 1 μl of forward and 1 μl of reverse primer (Metabion, Germany), 4.5 μl of PCR-grade water, and 6 μl of the DNA template under investigation. The PCR-amplified products were separated by electrophoresis in 1.5% agarose gel (Lanza Rockland, ME, USA). Gels were visualized and photographed using a gel documentation system (Cleaver Scientific Ltd., USA).

2.4. Antibiotic susceptibility testing

Bacterial isolates of either A. hydrophila or S. aureus were examined for their susceptibility to 12 different antibiotics (Oxoid, Basingstoke, UK) on Mueller-Hinton agar (Oxoid, Basingstoke, UK) using the disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI) (2014). The susceptibility of all isolates was tested against ciprofloxacin (CIP, 5 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), gentamicin (CN, 10 μg), chloramphenicol (C, 30 μg), and tetracycline (TE, 30 μg). Additionally, the susceptibility of the A. hydrophila isolates was tested against the following antibiotic disks: nitrofurantoin (F, 15 μg), amoxicillin (AX, 30 μg), and ceftriaxone (CRO, 30 μg). Meanwhile, S. aureus isolates were tested against penicillin (P, 10 μg), clarithromycin (CLR, 15 μg), erythromycin (E, 15 μg), and amoxicillin-clavulanic acid (AMC, 30 μg). The tested antibiotics were selected according to their clinical use in both human and veterinary medicine. The diameter of the inhibition zone was measured and interpreted in accordance with the guidelines of the CLSI (2014). Strains resistant to more than two classes of antibiotics were considered multidrug-resistant (MDR) strains.

3. RESULTS

3.1. Occurrence of A. hydrophila and S. aureus in seafood

A. hydrophila was isolated from 40 of the 280 seafood samples (14.3%), whereas S. aureus was isolated from 50 samples (17.9%) (Table 2). Both A. hydrophila and S. aureus isolates were identified by morphological and biochemical examinations. The highest prevalence of A. hydrophila strains was detected in oyster samples (22.2%, 12/54) followed by crab (15.4%, 4/26), shrimp (12.4%, 22/187), squid (11.1%, 2/18) and octopus (0/4, 0%), whereas the highest prevalence of S. aureus strains was found in shrimp samples (20.2%, 36/178) followed by crab (15.4%, 4/26), oyster (14.8%, 8/54), squid (11.1%, 2/18) and octopus (0/4, 0%).
Table 1. Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences</th>
<th>Annealing</th>
<th>Amplicon size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>hly</td>
<td>5'-CTATGAAAAAACATAAAAAATCTG-3' 5'-CAGTATAAGTGCGGAAAAATGGAAG-3'</td>
<td>55 °C</td>
<td>1500</td>
<td>[23]</td>
</tr>
<tr>
<td>aer</td>
<td>5'-CACAGCCAATATGTCGGTGAAG-3' 5'-GTCACTCTTCTCGTCAGGGC-3'</td>
<td>52 °C</td>
<td>326</td>
<td>[10]</td>
</tr>
<tr>
<td>nuc</td>
<td>5'-GGATTTGATGGTGATACGGTT-3' 5'-AGCCAAGCCTTGACGAACTAAGCG-3'</td>
<td>55 °C</td>
<td>267</td>
<td>[54]</td>
</tr>
<tr>
<td>sea</td>
<td>5'-GTATGATGGTGATACGGTT-3' 5'-CGCACTTTTTTCTCGTCAGGGC-3'</td>
<td>57 °C</td>
<td>102</td>
<td>[55]</td>
</tr>
</tbody>
</table>

Table 3. Antibiotic resistant strains of Aeromonas hydrophila (n=40) and Staphylococcus aureus (n=50) isolated from seafood.

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Antibiotic disk</th>
<th>A. hydrophila</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>I</td>
<td>S</td>
</tr>
<tr>
<td>β-Lactams</td>
<td>Amoxicillin</td>
<td>40 (100%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Amoxicillin-clavulanic acid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Ceftriaxone</td>
<td>32 (80%)</td>
<td>8 (20%)</td>
</tr>
<tr>
<td>Phenicols</td>
<td>Chloramphenicol</td>
<td>31 (77.5%)</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>Sulphonamides</td>
<td>Trimethoprim-Sulfamethoxazole</td>
<td>22 (65%)</td>
<td>14 (35%)</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Tetracycline</td>
<td>22 (55%)</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Ciprofloxacin</td>
<td>4 (10%)</td>
<td>22 (55%)</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Gentamicin</td>
<td>14 (35%)</td>
<td>4 (10%)</td>
</tr>
<tr>
<td></td>
<td>Nitrofurantoin</td>
<td>10 (25%)</td>
<td>24 (60%)</td>
</tr>
</tbody>
</table>

Table 2. Occurrence of Aeromonas hydrophila and Staphylococcus aureus strains in seafood.

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of samples</th>
<th>No. of A. hydrophila isolates (%)</th>
<th>No. of S. aureus isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shrimp</td>
<td>178</td>
<td>22 (12.4)</td>
<td>36 (20.2)</td>
</tr>
<tr>
<td>Oysters</td>
<td>54</td>
<td>12 (22.2)</td>
<td>8 (14.8)</td>
</tr>
<tr>
<td>Crabs</td>
<td>26</td>
<td>4 (15.4)</td>
<td>4 (15.4)</td>
</tr>
<tr>
<td>Squid</td>
<td>18</td>
<td>2 (11.1)</td>
<td>2 (11.1)</td>
</tr>
<tr>
<td>Octopus</td>
<td>4</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>280</td>
<td>40 (14.3)</td>
<td>50 (17.9)</td>
</tr>
</tbody>
</table>

**Figure 1.** Representative agarose gel electrophoresis of aerolysin gene amplification (326 bp) of Aeromonas hydrophila isolates from seafood. Lane (1): control positive. Lane (2): control negative. Lanes (3-7): positive samples.

**Figure 2.** Representative agarose gel electrophoresis of hemolysin gene (1589 bp) of Aeromonas hydrophila isolates from seafood. Lane (1): positive control. Lane (2): negative control. Lanes (3-7): positive samples.

**Figure 3.** Representative agarose gel electrophoresis of thermonuclease gene (267 bp) of Staphylococcus aureus isolates from seafood. Lane (1): positive control. Lane (2): negative control. Lanes (3-5,7): negative samples. Lane (6): positive samples.
3.2. Determination of virulence-associated genes in A. hydrophila and S. aureus isolates

A. hydrophila isolates were screened for the existence of virulence genes (aer and hly). Both aer and hly genes were detected in all tested strains (100 %, 40/40) (Figure 1, 2). Additionally, S. aureus strains were tested for the presence of virulence genes (nuc and sea). The nuc gene was found in all tested strains; whereas the sea gene was found in only 44% (22/50) of S. aureus strains (Figure 3, 4).

3.3. Antibiotic susceptibility results

Antibiotic susceptibility testing was performed on A. hydrophila and S. aureus strains isolated from seafood. A. hydrophila isolates were absolutely resistant to AX (100%) and highly resistant to CRO (80%), C (77.5%), SXT (65%), TE (55%), and CN (35%). The lowest resistance was noted for CIP (10%) and F (25%).

S. aureus strains showed high resistance to P (86%), followed by AMC (72%), SXT (58%), and CIP (52%). Additionally, S. aureus strains showed high sensitivity to CN (86%), followed by TE (72%), CLR (58%), and C (44%) (Table 3). MDR to more than two classes of antibiotics was determined in 77.5% of A. hydrophila strains (31/40) and 66% of S. aureus isolates (28/50).

4. DISCUSSION

Seafood is a rich source of proteins, unsaturated fatty acids, and vitamins. However, due to the texture of its flesh and its microbe-filled habitat, seafood also represents a highly significant host for many pathogenic bacteria [3]. Pathogenic bacteria may be present in high numbers in food without producing noticeable changes in odor, taste, or features[25]. Therefore, detection of these bacteria in seafood is vital, particularly A. hydrophila and S. aureus which are associated with gastrointestinal infections in humans. In this study, the prevalence of A. hydrophila in seafood was determined in 77.5% of A. hydrophila strains (31/40) and 66% of S. aureus isolates (28/50).

Figure 4. Representative agarose gel electrophoresis of staphylococcal enterotoxin A gene (102 bp) of Staphylococcus aureus isolates from seafood. Lane (1): negative control. Lane (2): positive sample. Lanes: (3-6): negative samples.

The pathogenicity of S. aureus in cases of food poisoning is associated with the ability of some strains to produce enterotoxins [41]. Thus, the present study aimed to detect one of the most common staphylococcal enterotoxins in aquaculture, the sea gene, by PCR assay. In the current investigation, the sea gene was detected in 44% of S. aureus strains. This result was compatible with the 45.2 % that Arfatahery et al. [42] detected in S. aureus strains isolated from fish and shrimp samples. SoltanDallal et al. [36] and Rong et al. [35], however, detected lower prevalence of sea gene at 39.3 % and 22.7%, respectively.

Although the use of antibiotics might promote growth and increase productivity in aquacultures, there is an emergent concern surrounding antibiotic resistance selection as a result of the wide use of antibiotics, especially when used inadequately or in overdose [43]. In this investigation, the isolates were tested against different classes of antibiotics to evaluate the antibiotic efficacy in the treatment of aquaborne microbes A. hydrophila and S. aureus. The results showed that A. hydrophila strains were completely resistant...
to AX (100%) and highly resistant to CRO (80%), C (77.5%), SXT (65%), TE (55%), and CN (35%). These results confirm the previously reported high resistance of *A. hydrophila* to a wide range of the β-lactams antibiotics family, as well as SXT, and TE [44-46]. In contrast, Stratev et al. [47] reported no observed resistance to TE and SXT in *A. hydrophila* strains. Contradictory to our findings, high sensitivity to C was observed in a previous study [46, 48], whereas Stratev et al. [47] showed absolute resistance to C. Findings by Saavedra et al. [49] supported the existence of CN resistant strains, with a frequency of 31%.

*S. aureus* is notorious for its MDR and, in the present study; *S. aureus* isolates showed resistance to P, Ox (86% each), AMC (72%), SXT (58%), CIP (52%), and E (36%). The antibiotic resistance of *S. aureus* to extended-spectrum P, AMC, SXT, and E support the findings of other recent studies [35, 42, 50]. However, [39] reported a high sensitivity in *S. aureus* strains isolated from shrimp in Libya to SXT and AMC as well as to CIP and C.

MDR bacteria complicate the treatment of human and animal infections. Unfortunately, our results revealed MDR against three or more antibiotics in 77.5% of *A. hydrophila* isolates. This result was compatible with Kaskhedikar and Chhabra [51] who found 100% of *A. hydrophila* isolated from fish to have MDR. Moreover, the current study found that 66% of *S. aureus* strains displayed MDR. In China, Rong et al. [35] detected MDR in 90.6% of *S. aureus* strains.

The development of multidrug-resistant strains is associated with the over-exposure to either bactericidal or bacteriostatic antibiotics used for the prevention and treatment of infections in aquaculture farming [52, 53]. The higher MDR strains observed in the present study might be attributed to the widespread use of antibiotics in the study locality in Egypt, as well as the indiscriminate use of antibiotics either at recommended doses or at sub-therapeutic doses as feed additives to promote growth in developing countries. These findings have significant implications for public health.

**Conclusion**

In this research, the prevalence of virulent and MDR strains of *A. hydrophila* and *S. aureus* in seafood represents an important aspect of food safety and poses a zoonotic concern to public health in Egypt. Current data indicates poor hygienic practices, stress conditions during farming, contaminated additives, cross-contamination between seafood, and the indiscriminate use of antibiotics in fish farms have led to an increased prevalence of MDR strains worldwide. Consequently, this suggests that a suitable disease management and control strategy must be endorsed for fish farms, and treatment assays implemented to deal with antibiotic usage. Also, the continuous surveillance of these bacteria in seafood with a strong focus on their antibiotic resistance characteristics should be considered in further studies.

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**Conflict of interest statement**

No conflict of interest.

**Research Ethics Committee Permission**

The current research work is permitted to be executed according to standards of Research Ethics committee, Faculty of Veterinary Medicine, Mansoura University.

**Authors’ contribution**

Gamal A. Younis designed the experiment and revised the manuscript. Gamal A. Younis, Rasha M. Elkenany supervised in carrying out the practical part. Rasha M. Elkenany shared in writing the paper and took the responsibility of correspondence to the journal. Basma M. Elkamouny collected samples and carried out the practical part. All authors approved the final version of the manuscript for publication.

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