

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.

Design: Observational study.

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 *thermonuclease (nuc)* gene was 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 MDR *A. 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 μ g/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 Youssr et al. [23] and [24] respectively. The amplification of DNA was achieved using oligonucleotide 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 μ l reaction mixture comprised 12.5 μ l of 2 \times PCR master mix (Promega, Madison, USA), 1 μ l of forward and 1 μ l of reverse primer (Metabion, Germany), 4.5 μ l of PCR-grade water, and 6 μ l of the DNA template under investigation. The PCR-amplified products were separated by electrophoresis in 1.5% agarose gel (Lonza 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 μ g), trimethoprim-sulfamethoxazole (SXT, 25 μ g), gentamicin (CN, 10 μ g), chloramphenicol (C, 30 μ g), and tetracycline (TE, 30 μ g). Additionally, the susceptibility of the *A. hydrophila* isolates was tested against the following antibiotic disks: nitrofurantoin (F, 15 μ g), amoxicillin (AX, 30 μ g), and ceftriaxone (CRO, 30 μ g). Meanwhile, *S. aureus* isolates were tested against penicillin (P, 10 μ g), clarithromycin (CLR, 15 μ g), erythromycin (E, 15 μ g), and amoxicillin-clavulanic acid (AMC, 30 μ g). 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%, 22/178), 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.

Target gene	Primer sequences	Annealing	Amplicon size (bp)	References
<i>hly</i>	5'-CTATGAAAAAATAAAAAATACTG-3' 5'-CAGTATAAGTGGGAAATGGAAAG-3'	55 °C	1500	[23]
<i>aer</i>	5' CACAGCCAATATGTCGGTGAAG3' 5' GTCACCTTCTCGCTCAGGC3'	52 °C	326	[10]
<i>nuc</i>	5' GCGATTGATGGTGATACGGTT3' 5' AGCCAAGCCTTGACGAACTAAAGC3'	55 °C	267	[54]
<i>sea</i>	5' GGTTATCAATGTGCGGGTGG3' 5' CGGCACTTTTTCTCTTCGG3'	57 °C	102	[55]

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

Antibiotic class	Antibiotic disk	<i>A. hydrophila</i>			<i>S. aureus</i>		
		R	I	S	R	I	S
β-Lactams	Amoxicillin	40 (100%)	0	0	-	-	-
	Amoxicillin-clavulanic acid	-	-	-	36 (72%)	0	14 (28%)
	Penicillin	-	-	-	43 (86%)	0	7 (14%)
Cephalosporins	Ceftriaxone	32 (80%)	8 (20%)	0	-	-	-
Phenicol	Chloramphenicol	31 (77.5%)	9 (22.5%)	0	7 (14%)	21 (42%)	22 (44%)
Sulphonamides	Trimethoprim-Sulfamethoxazole	26 (65%)	14 (35%)	0	29 (58%)	0	21 (42%)
	Tetracyclines	Tetracycline	22 (55%)	4 (10%)	14 (35%)	7 (14%)	7 (14%)
Fluoroquinolones	Ciprofloxacin	4 (10%)	22 (55%)	14 (35%)	26 (52%)	0	24 (48%)
Macrolides	Erythromycin	-	-	-	18 (36%)	28 (56%)	4 (8%)
Aminoglycosides	Gentamicin	14 (35%)	4 (10%)	22 (55%)	7 (14%)	0	43 (86%)
	Nitrofurantoin	10 (25%)	24 (60%)	6 (15%)	-	-	-

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

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

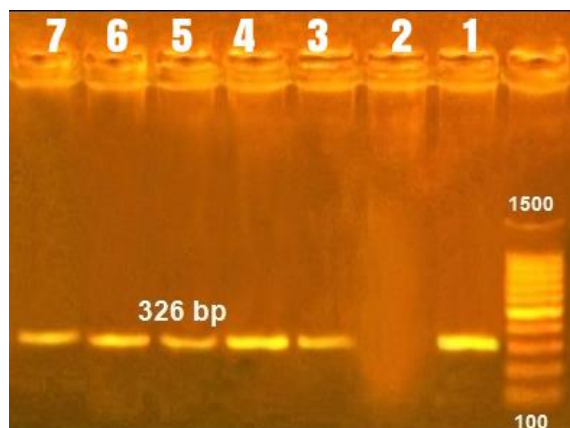


Figure 1. Representative agarose gel electrophoresis of aerolysin gene amplification (326 bp) of *Aeromonas hydrophila* isolates from seafood. Lane (1): control positive. Lane (2): control negative. Lanes (3-7): positive samples.

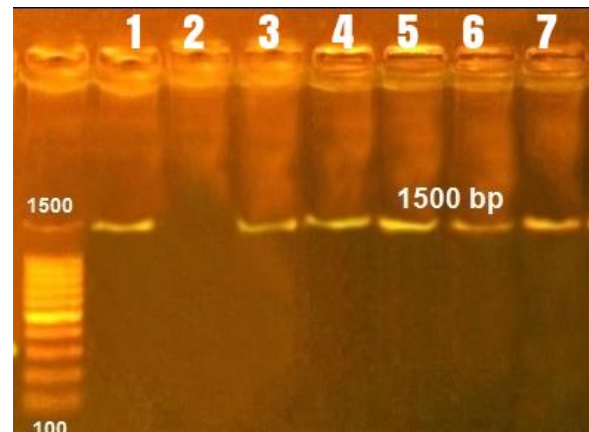


Figure 2. Representative agarose gel electrophoresis of hemolysin gene (1589 bp) of *Aeromonas hydrophila* isolates from seafood. Lane (1): positive control. Lane (2): negative control. Lanes (3-7): positive samples.

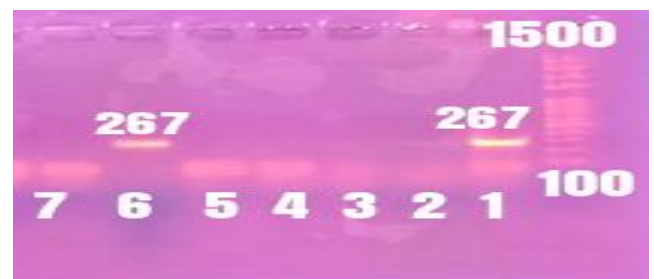


Figure 3. Representative agarose gel electrophoresis of *thermonuclease* gene (267 bp) of *Staphylococcus aureus* isolates from seafood. Lane (1): positive control. Lane (2): negative control. Lanes (3-5,7): negative samples. Lane (6): positive samples.

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).



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.

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 found to be 14.2% with the highest prevalence in oyster samples (22.2%), followed by crab (15.4%), shrimp (12.4%), and squid (11.1%). This result was in line with those previously reported by Ullmann et al. [26] (14.2%) in

Germany, Rahimi et al. [27] (13.5%) in Iran, and Hussain et al. [28] (19%) in India. In contrast, a low prevalence of *A. hydrophila* (5.71%) was reported in Turkey, with the highest occurrence (15%) found in shrimp [29], whereas a high prevalence of 58% was detected by Niamah [30] in Iraq, and 25% was reported by [31] in Egypt. The prevalence of *A. hydrophila* in seafood indicates poor hygienic practices.

S. aureus likewise presents a potential risk to consumers of aquatic food products [9] in this study, the prevalence of *S. aureus* was found to be 17.8 % with the highest prevalence in shrimp samples (20.2%). These results were consistent with [32] (17%) in Spain, Kumar et al. [33] (15.78%) in India, and Othman et al. [34](15%) in Malaysia. However, a higher prevalence was reported by [35] (37.2%) in China with the highest prevalence in freshwater fish (52.1%). Soltan Dallal et al. [36] in Iran also recorded a high prevalence (28%). A lower prevalence for *S. aureus* in seafood was generally found in other recently reported studies. In Switzerland, Boss et al. [37] recorded 9% prevalence with the highest rate in shrimp (18%), and, in Turkey, Mus et al. [38] found a 6% prevalence in seafood with the highest prevalence in shrimp (20%). Additionally, in Libya, Naas et al. [39] detected a 5.3% prevalence in seafood samples. Overall, these results indicated that seafood from retail markets in the study localities presented a potential source of infection for human consumers.

An investigation into virulence-associated genes was performed in order to detect virulent strains isolated from seafood. In this study, *aer* and *hly* genes were detected in all the tested *A. hydrophila* strains (100%). Similarly, Yousr et al. [23] and Niamah [30] identified the *aer* gene in all their *A. hydrophila* isolates from seafood and shrimp. The *hly* gene has also previously been detected in all *A. hydrophila* isolates from fish and shrimp and squid [28]. Moreover, in Egypt, all *A. hydrophila* strains isolated from food samples tested positive for both *aer* and *hly* genes [40].

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. Soltan Dallal 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 aqua-borne 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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