Virulence Genes and Antibiotic Resistance of *Aeromonas hydrophila* Isolated from Marketed Milk

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Keywords: *Egypt, market buffalo milk, virulence genes, antibiotic resistance, A. hydrophila*

Abstract. *This research aims to assess the existence rate, most dangerous virulence genes (aerolysin (aerA) and hemolysis (ahh1)), antibiotics sensitivity, and resistance pattern of Aeromonas hydrophila strains that were isolated from 100 raw marketed buffalo milk samples, which were gathered from Dakahlia governorate, Egypt. The culturally obtained Aeromonas spp. were evidenced in about 87% of the examined raw market milk samples while the biochemical investigation revealed that 72% of the inspected samples were polluted with Aeromonas species. On the other hand, A. hydrophila was detected in about 30% of the examined market milk samples. In addition, molecular detection of aerA and ahh1 virulence factors of 19 A. hydrophila isolates using multiplex PCR was carried out. Then, it was detected that 8 (42.1%) isolates had aerA gene, while 5 (26.3%) isolates possessed ahhl gene and 4 (21.1%) isolates had both aerA and ahh1 genes. Additionally, 2 (10.5%) isolates were negative for the two inspected genes. All A. hydrophila isolates (19) showed resistance against streptomycin antibiotic; the average multiple antibiotic resistance between A. hydrophila isolates was 0.431, and it reached 1 in one strain (positive for aerA gene) as this strain was resistant to all used antibiotics. In conclusion, this study reveals a high incidence of multiple antibiotic resistance (MAR) of A. hydrophila strains that were isolated from marketed milk samples in Dakahlia governorate. Moreover, it indicates the presence of virulence genes.*

Introduction

It is well known that milk as well as milk products are considered as the principal component of nourishment for all ages. Meanwhile, various human digestive diseases and outbreaks have been reported due to ingestion of raw milk and milk products (Verraes et al., 2015).

It is identified that *Aeromonas* species consist of non-spore pointing gram negative rods which are universal in all aqueous environments. It has been identified that all individuals can be infected with *Aeromonas* spp. which may lead to bacteremia. Diarrhea and wound infections are also considered as consequences of this microbe. Unfortunately, young aged children besides immunocompromised persons, are the most affected persons that show signs of diarrhea (Figueras and Beaz-Hidalgo, 2015). It has been assessed that in advanced states, the portion of people that might be vulnerable to suffer from foodborne illness would be close to 20% (Lund, 2015).

Consequently, foodborne problems are growing and causing excessive threats to milk buyers in Africa. According to WHO (2015), it was identified that diet played an important role in the occurrence of diarrheal sicknesses worldwide, as about 600 million cases of illness and 420 000 deaths were informed in 2010.

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The *A. hydrophila* has been known to produce variable potential virulence toxins. Meanwhile, aerolysins *(aerA)* besides hemolysins *(hlyA)* have been considered as the greatest significant hemolytic poisons resulting in the pathogenesis of the disease following infection (Martin-Carnahan and Joseph, 2015).

There are about 30 identified types of *Aeromonas* spp. (Martínez-Murcia et al., 2016), and in humans there are four subgroups of this *Aeromonas* spp. that are involved in human diseases: *A. hydrophila, A. veronii, A. biovar, A. sobria, A. caviae,* and *A. dhakensis*. Moreover, the severity of chronic digestive illness is linked to *Aeromonas* spp. that is found in diverse water besides food (Teunis and Figueras, 2016). Also, *Aeromonas* spp. reveal a main role in the transmission of antibiotic confrontation, constructing these bacteria as a problematic. They have been employed as intermediaries in the transmission of antibiotic confrontation indicators among hospitals besides ecological strains (Varela et al., 2016).

A. hydrophila is insulated from an extensive range of diets such as fish, eggs, milk, dairy products, seafoods, vegetables, meat and its' products (Kamalpreet, 2017). Commanding *A. hydrophila* contagion is so supreme because this bacterium can threat food safety (Talagrand-Reboul et al., 2017) as it has appeared as a significant food-related microbe universally (Pal, 2018). In addition to food, *Aeromonas* spp. are isolated from medical and ecological samples as

they can grow at low temperatures besides yielding toxins. As a result of this, they raise the danger of foodborne contagion dramatically (Bello-López et al., 2019). *Aeromonas* spp. secrete extracellular proteins, as enzymes (proteases and lipases) and cytotoxins which are correlated to their microbial pathogenicity in addition to showing diverse parts in the infectious procedure (Pessoa et al., 2019 & Fernández-Bravo and Figueras, 2020)

This research aims to assess the existence rate, most dangerous virulence genes (aerolysin *(aerA)* and hemolysis *(ahh1)*), antibiotics sensitivity, and resistance pattern of *A. hydrophila* strains that were isolated from 100 raw market buffalo milk samples gathered from Dakahlia governorate, Egypt.

Materials and Methods

Ethics statement

The gathering of specimens that were used in this research monitored the rules of Mansoura University. The procedures of this research were approved by the Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University (code R/54).

The methods that were useful for sample gathering were directed by the American Public Health Association (A.P.H.A, 1992). One hundred raw market buffalo milk samples (each 250 mL) were haphazardly gathered from different shops and marketplaces in different regions of Mansoura city, Dakahlia governorate, Egypt. The samples were saved in a secure ice box (4 ± 1°C) to be transferred to the laboratory of Food Hygiene and Control Department, Faculty of Veterinary Medicine, Mansoura University for a microbiological check-up. The biosecurity measures during collection of 100 market raw buffalo milk samples from dairy markets were agreed to by OIE (2008). Biosafety measures during sample handing and application of microbiological examination in the laboratory were followed according to the guidelines of the WHO (2004), while the procedures of infection control for research work in laboratories were applied (Burt et al., 2014).

Isolation and identification of *A. hydrophila*

Milk specimens were thoroughly stirred, then we added 25 mL of each specimen to 225 mL of tryptone soya broth, blended well at the time while they were incubated at 37°C for 24 h, and subcultured to the *Aeromonas* selective medium base (Oxoid, CM0833) with supplementary ampicillin for selective quarantine of *Aeromonas* spp. Ampicillin concentration was chosen according to recommendations. The petri plates were incubated at 25°C for 24 h. Five typical opaque green colonies with dark centers were chosen, then purified on nutrient agar slants and kept warm at 37°C for 24 h for more investigation (Palumbo et al., 2001).

Pure cultures of the isolates were morphologically stained by Gram stain (Koneman et al., 1994). Motility and biochemical tests such as: Esculin hydrolysis test, Oxidase test, Arginine hydrolysis, Indole test, Methyl Red Test, Voges Proskauer test, Citrate utilization test, Urease test, Hydrogen sulfide production test, Nitrate reduction test, Gelatin hydrolysis test, Ornithine decarboxylase (ODC), L-lysine decarboxylase (LDC), Arginine decarboxylase (ADH), β- galactosidase (ONPG), and fermentation of sugars (Garrity, 2001). Then 19 isolates from *A. hydrophila* were taken and examined for proteolytic activity (Yucel et al., 2005), lipolytic activity (Collins et al., 1989), and hemolytic activity (Singh and Sanyal, 1997).

Molecular identification of *A. hydrophila*

Genomic DNA extraction of *A. hydrophila*

The DNA was extracted from *A. hydrophila* isolates by genomic DNA extraction kit (Thermo scientific, UK) according to the manufacturer guidelines. Briefly, up to three or five bacterial colonies were taken, homogenized in 200 µL of deionized water, heated at 100°C for 15 min, and centrifuged at 10 000 g for 3 min. The supernatant was then transported to a sterile Eppendorf tube and taken as a DNA template.

Detection of suspected virulence genes in *A. hydrophila* using multiplex PCR

Multiplex PCR protocol was conducted in order to identify *A. hydrophila* species by using specific pairs of primers (Invitrogen, Carlsbad, CA) for detection of aerolysin (*aerA*) and hemolysin (*ahh1*) virulence genetic factors following the methods described by Shah et al., (2009), Meanwhile, the sequence of primers (Pharmacia Biotech) and the PCR product size are illustrated in Table 1. The amplification of virulence genetic factors was achieved following the procedures of Wang et al. (2003) on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany).

PCR amplification was achieved in a 96-well 2720 thermal cycler (Applied Biosystems, Norwalk,

Table 1. Target genes, primers sequences, and amplicon sizes

Target genes	Primers	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Reference
aerA	*AH-aerA (F)*	*5′ CAAGAACAAGTTCAAGTGGCCA '3*	309	Stratev et al. (2016)
	AH-aerA (R)	*5′ ACGAAGGTGTGGTTCCAGT '3*		
ahh1	*AHH1 (F)*	*5′ GCCGAGCGCCCAGAAGGTGAGTT '3*	130	
	AHH1 (R)	*5′ GAGCGGCTGGATGCGGTTGT '3*		

California, USA). PCR mixture was amplified in a total volume of 25 µL containing 12.5 µL of 2X master mix (Thermo scientific), 1 µL of *ahh1* and *aerA* primers, 5 µL of DNA template and the total volume was completed to 25 µL by DNase/RNase-free H_2O. Then, the amplification cyclic conditions of PCR comprised of: initial denaturation at 95°C for 5 min, 50 cycles at 95°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, and final elongation at 72°C for 7 min. The quality of PCR products was tested by electrophoresis on 1.5% agarose gels electrophoresis and imagined via UV transilluminator using a 100-bp DNA ladder (Invitrogen, San Jose, California, USA).

Antimicrobial susceptibility testing and multiple antibiotic resistance (MAR) index value

All the identified *A. hydrophila* isolates were examined via the disc diffusion method; sensitivity discs by adjustable concentrations were practiced to define the susceptibility of the quarantined bacterial isolates (Oxoid Limited, Basingstoke, Hampshire, UK). Pure cultures of the identified *A. hydrophila* were cultured in tryptic soy broth (Oxoid CM0129), incubated at 28°C for 8 h, and then streaked via sterile cotton swabs on nutrient agar petri plates. Then, the antimicrobial discs were put in petri plates, incubated at a suitable temperature (37°C) for 24 h, and finally tested for the development of the microbe near the antimicrobial discs. Concerning the diameters of inhibition zones, the examined isolates were categorized as susceptible, intermediate, or resistant (Clinical & Laboratory Standards Institute, 2016). Thus, the antibacterial discs and their condensation in addition to the widths of the areas of suppression for the examined isolates were established in Table 2.

Multiple antibiotic resistance (MAR) index for each isolate was calculated according to the formula specified by Singh et al. (2010) as below:

MAR index = Number of the resistance (isolates categorized as intermediate were measured sensible for MAR index) (a) / Total No. of examined antibiotics (b). MAR index= a/b.

Statistical Analysis

The data were analyzed as numbers and percentages. Multiple antibiotic resistance (MAR) index for each isolate and the total average were calculated by SPSS (Statistical Package for Social Science) software version 16.

Results

Isolation and identification of *A. hydrophila* in examined raw milk

Culturally *Aeromonas* spp. were detected in 87% of examined raw market milk samples while biochemically 72% of the examined samples were contaminated with *Aeromonas* spp. Identification of confirmed species showed that *A. hydrophila, A. trota, A. janda, A. caviae, A. veronii* and *A. fluvialis* were the main isolates by proportions of 30%, 26%, 15%, 13%, 3%, and 2%, respectively. (There were 17 samples from the positive samples (72) that contained two different species of *Aeromonas* spp.; therefore, they were counted two times (89 – 17 = 72%).

From about 94 confirmed cultures isolated from raw market milk samples, 35 (37.2%) isolates could be identified as *A. hydrophila* followed by 26 (27.7%)

Table 2. Antimicrobial discs, concentration, and interpretation of their action on A. hydrophila isolates

Antimicrobial agent	Sensitivity disc content (ug)	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Cephalothin (CN)	30	14 or less	15–17	18 or more
Ampicillin (AM)	10	13 or less	14–17	18 or more
Nalidixic acid (NA)	30	13 or less	14–18	19 or more
Oxytetracycline (T)	30	14 or less	15–18	19 or more
Meropenem (M)	10	9 or less	10–12	13 or more
Cefepime (FEP)	30	18 or less	19–24	24 or more
Cefazolin (CZ)	30	10 or less	11–14	15 or more
Gentamicin (G)	10	12 or less	13–14	15 or more
Doxycycline (DO)	30	14 or less	15–18	19 or more
Amikacin (AK)	30	12 or less	13–15	16 or more
Ciprofloxacin (CP)	5	15 or less	15–19	20 or more
Cefotaxim (CF)	30	17 or less	18–22	23 or more
Erythromycin (E)	15	13 or less	14–22	23 or more
Streptomycin (S)	10	11 or less	12–14	15 or more
Neomycin (N)	30	12 or less	13–16	17 or more
Sulphamethoxazol (SXT)	25	10 or less	11–15	16 or more

as *A. trota,* 15 (16.0%) as *A. janda*, 13 (13.8%) as *A. caviae,* 3 (3.2%) as *A. veronii* and 2 (2.1%) as *A. fluvialis* (Table 3).

Proteolytic, Lipolytic, and Hemolytic Activities of *A. hydrophila*

A. hydrophila showed proteolytic, lipolytic and hemolytic activities at the ratio of 57.9%, 42.1%, and 21.1%, respectively, while some isolates (5.3%) revealed proteolytic and lipolytic activities at the same time and others (21.1%) had proteolytic and hemolytic activities together also (Table 4).

Molecular identification of *A. hydrophila* using multiplex PCR

Genetic detection of aerolysin (*aerA*) and hemolysin (*ahh1*) genes from 19 *A. hydrophila* isolates were carried out using multiplex PCR technique. There were about 8 (42.1%) isolates that possessed *aerA* gene, while 5 (26.3%) isolates revealed *ahhl* gene and 4 (21.1%) isolates showed *aerA* and *ahh1*genes. However, 2 (10.5%) isolates were negative for the two examined genes (Table 5 and Figure 1, 2).

Antibiotic susceptibility profile of *A. hydrophila* isolates

The antimicrobial drug vulnerability outlines for the 19 *A. hydrophila* isolates that were isolated from raw market milk samples are exposed in Table 6. Higher susceptibility of *A. hydrophila* was reported to be against amikacin (AK) (89.5%) and ciprofloxacin (CP) (78.9%). Also, there was high multiple antibiotic resistance between *A. hydrophila* isolates as shown in Table 7, because the average multiple antibiotic resistance index (MAR) was 0.431, and it reached 1 in one isolate (this isolate is numbered 11 in Figure 2 using multiplex PCR that has a positive result for *aerA* gene), as this isolate was resistant to all the used antibiotics followed by 0.937 (the isolate numbered 1 in multiplex PCR and has positive bands for *aerA* and *ahhl* genes) in the second isolate.

In brief, *A. hydrophila* isolates that are numbered from 1 to 19 in the antimicrobial resistance profile (Table 7) have numbers 11, 1, 2, 3, 12, 13, 14, 4, 15, 5, 16, 17, 6, 7, 8, 9, 18, 19 and 10 when running on agarose gel electrophoresis of multiplex PCR to determine *aerA* (309 bp) and *ahhl* (130 bp) genes (Figures 1 and 2).

Table 3. Frequency distribution and prevalence of *Aeromonas* spp. obtained from examined milk samples

Isolates	Samples		Isolates	
	n	%	n	%
A. hydrophila	30	30	35	37.2
Trota	26	26	26	27.7
Janda	15	15	15	16.0
Caviae	13	13	13	13.8
Veronii	3	3	3	3.2
Fluvialis	2	2	2	2.1

Where total number of examined raw market buffalo milk samples is 100, and total number of *Aeromonas* isolates is 94 isolates.

Table 4. Distribution of proteolytic, lipolytic, and hemolytic activities of 19 *A. hydrophila* isolates isolated from examined milk samples

A. hydrophila	No. of isolates	Proteolytic Activity		Lipolytic activity		Hemolytic activity		Proteolytic +Lipolytic activity		Proteolytic +Hemolytic activity	
	19	n	%	n	%	n	%	n	%	n	%
		11	57.9	8	42.1	4	21.1	1	5.3	4	21.1

Table 5. Incidence of *ahhl,* and *aerA* genes via multiplex PCR of *A. hydrophila* isolates (19) from examined raw milk samples

A. hydrophila	No. of Strains	+ve for *aerA* gene		+ve for *ahhl* gene		+ve for *aerA* and *ahhl* genes		-ve for *aerA* and *ahhl* genes	
	19	n	%	n	%	n	%	n	%
		8	42.1	5	26.3	4	21.1	2	10.5

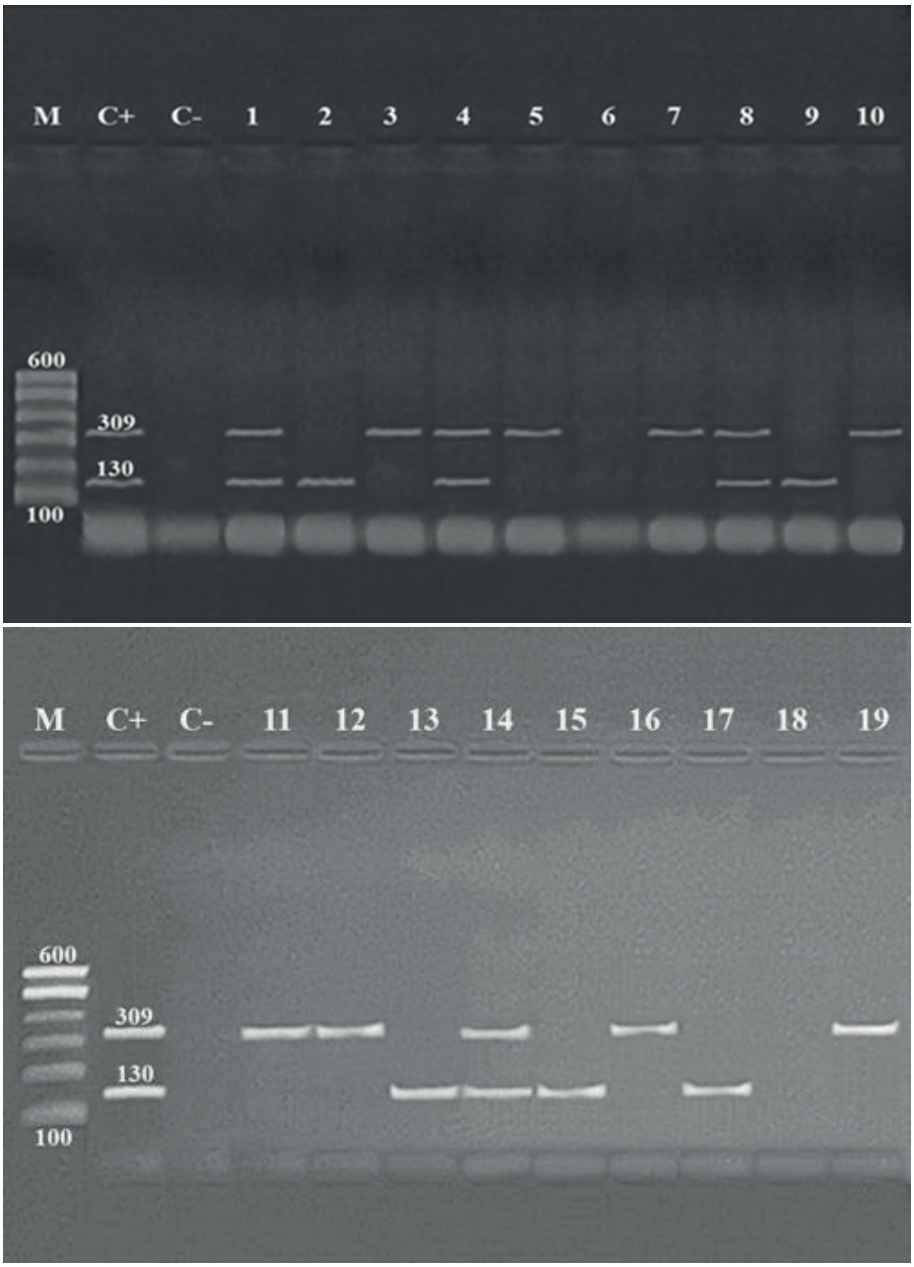
Table 6. Antimicrobial sensitivity of *A. hydrophila* isolates (n = 19)

Drug agent	S		I		R	
	n	%	n	%	n	%
Streptomycin (S)	-	-	-	-	19	100
Erythromycin (E)	-	-	1	5.3	18	94.7
Ampicillin (AM)	1	5.3	2	10.5	16	84.2
Cefazolin (CZ)	3	15.8	1	5.3	15	78.9
Cephalothin (CN)	8	42.1	1	5.3	10	52.6
Doxycycline (DO)	8	42.1	2	10.5	9	47.4
Cefotaxim (CF)	9	47.4	3	15.8	7	36.8
Sulphamethoxazol (SXT)	10	52.6	2	10.5	7	36.8
Neomycin (N)	11	57.9	2	10.5	6	31.6
Gentamicin (G)	12	63.2	1	5.3	6	31.6
Oxytetracycline (T)	12	63.2	3	15.8	4	21.1
Cefepime (FEP)	13	68.4	2	10.5	4	21.1
Nalidixic acid (NA)	14	63.7	1	5.3	4	21.1
Meropenem (M)	14	63.7	2	10.5	3	15.8
Ciprofloxacin (CP)	15	78.9	2	10.5	2	10.5
Amikacin (AK)	17	89.5	1	5.3	1	5.3

Table 7. Antimicrobial resistance profile of *A. hydrophila* strains (n = 19)

No	Antimicrobial resistance profile	MAR index
1	S, E, AM, CZ, CN, DO, CF, SXT, N, G, T, FEP, NA, M, CP, AK	1
2	S, E, AM, CZ, CN, DO, CF, SXT, N, G, T, FEP, NA, M, CP	0.937
3	S, E, AM, CZ, CN, DO, CF, SXT, N, G, T, FEP, NA, M	0.875
4	S, E, AM, CZ, CN, DO, CF, SXT, N, G, T, FEP, NA	0.812
5	S, E, AM, CZ, CN, DO, CF, SXT, N, G	0.625
6	S, E, AM, CZ, CN, DO, CF, SXT, N, G	0.625
7	S, E, AM, CZ, CN, DO, CF, SXT	0.500
8	S, E, AM, CZ, CN, DO	0.375
9	S, E, AM, CZ, CN, DO	0.375
10	S, E, AM, CZ, CN	0.313
11	S, E, AM, CZ	0.250
12	S, E, AM, CZ	0.250
13	S, E, AM, CZ	0.250
14	S, E, AM, CZ	0.250
15	S, E, AM, CZ	0.250
16	S, E, AM	0.187
17	S, E	0.125
18	S, E	0.125
19	S	0.062
	Average 0.431	

S: Streptomycin; E: Erythromycin; AM: Ampicillin; CZ: Cefazolin; CN: Cephalothin; DO: Doxycycline; CF: Cefotaxim; SXT: Sulphamethoxazol; N: Neomycin; G: Gentamicin; T: Oxytetracycline; FEP: Cefepime; NA: Nalidixic acid; M: Meropenem; CP: Ciprofloxacin; AK: Amikacin



Figures (1, 2). Agarose gel electrophoresis of multiplex PCR of *aerA* (309 bp) and *ahhl* (130 bp) genes for a description of *A. hydrophila* (n = 19).

Lane M: 100 bp ladder as a molecular size DNA marker.
Lane C+: Control positive *A. hydrophila* for *aerA* and *ahhl* genes.
Lane C–: Control negative.
Lanes 1, 4, 8 & 14: Positive *A. hydrophila* isolates for *aerA* and *ahhl* genes.
Lanes 2, 9, 13, 15 & 17: Positive *A. hydrophila* strains for *ahhl* gene.
Lanes 3, 5, 7, 10, 11, 12, 16 & 19: Positive *A. hydrophila* strains for *aerA* gene.
Lane 6 & 18: Negative *A. hydrophila* strain for *aerA* and *ahhl* genes.

Discussion

Aeromonas spp. are linked to food poisoning and certain human illnesses such as gastrointestinal disorders in addition to extra-intestinal contagions like skin infections, shocking wound contagions, as well as lower breathing tract/urinary tract contagions (Batra et al., 2016).

In our study, 72 (72%) raw market milk samples contained *Aeromonas* spp. bacteria. But lower results were recorded by Ahmed et al. (2014) (32%), ElBalat et al. (2014) (32%), Sadek et al. (2017) (36%), and Hammad et al. (2018) (25%).

The examination process was focused on the prevalence of *Aeromonas* spp. and the infection with these bacteria occurs more due to ingestion of contaminated diets. In addition, *A. hydrophila* is the most identified *Aeromonas* spp. in raw milk and milk by products (ElBalat et al., 2014).

A higher existence rate of motile *Aeromonads* was identified in raw milk as the bacterium can pollute the udder through the teat, then proliferate in the mammary tissue, and then can be released in milk (EL-Shemawy and Marth, 1990). Thus, higher existence of *Aeromonas* spp. in raw milk samples reflects inappropriate hygienic procedures of milking and allocation.

The prevalence of *A. hydrophila*, *A. trota*, *A. janda*, *A. caviae*, *A. veronii*, and *A. fluvialis* is shown in Table 3. Opposing results with a lower prevalence ratio were reported by ElBalat et al. (2014) (8% of milk samples were contaminated with *A. hydrophila* spp. while 12% of samples revealed *A. trota* and *A. janda* spp. (Zeinhom and Abdel-Latef, 2014), *A. hydrophila* was found in 24% (Alrazakkazal and Abdullah, 2016), *A. hydrophila* was detected in 7% of the examined milk samples as revealed by Tahoun et al. (2016), But *A. hydrophila* was detected in 8% of the examined raw milk samples (Sadek et al., 2017). Also, *A. hydrophila* was found in 16% of the examined milk samples and four isolates of *A. hydrophila* (3.3%) were isolated from raw milk samples as reported by Abdulaal (2019). While the high prevalence ratio of *A. hydrophila* spp. was 40% in the examined food samples as informed by Enany et al. (2013).

Concerning *A. caviae,* similar results were recorded by ElBalat et al. (2014) (10%) and Sadek et al. (2017) (12%), while higher results were reported by Enany et al. (2013) (31.7%). In addition, about 94 strains of *Aeromonas* spp. were taken from raw milk specimens as shown in Table 3. The other prevalence ratios were reported by ElBalat et al. (2014) (25%), while higher outcomes such as 54.3% were documented by Eid et al. (2013). Regarding *A. trota* and *A. janda*, ElBalat et al. (2014) declared other prevalence ratios such as 40% and 25% respectively which were different from our findings. Also, *A. veronii* was detected in 3% of the examined milk samples (Table 3). It had an extensive variety of hosts and may cause diarrhea and sepsis in individuals (Fernandez-Bravo et al., 2020).

Ahmed et al. (2014) informed that *A. hydrophila* showed proteolytic and lipolytic activities at the ratios of 41.7% and 16.7%, respectively. Meanwhile, Al-Oqaili et al, (2016), Simon et al. (2016), and Sadek et al. (2017) proved that all the examined isolates showed 100% proteolytic and lipolytic activities. Also, all the examined *A. hydrophila, A. caviae* and *A. sobria* had 100% β-hemolytic actions.

On the other hand, our results revealed higher proteolytic, lipolytic and hemolytic activities of *A. hydrophila* isolates (Table 4). Proteinases and lipases enzymes from psychotropic bacteria are documented to be the chief spoilage enzymes of milk products (Sorhaug and Stepaniak, 1991).

According to Citterio and Biavasco, (2015), *A. hydrophila* is the most virulent type of *Aeromonas* spp. In addition, these species produce many virulent toxins, contain structural components that are linked to adhesion, cell virulence, and escape from the phagocytosis process. Another factor that helps them in the induction of the poisoning process is the specific extracellular toxins such as aerolysin which cause lysis as well as toxicity of the cells.

Agreeing to our results that are presented in Table 5 and Figures 1 and 2, similar results were reported by Seker et al. (2015) as they demonstrated that about 9 (40.9%) strains of *A. hydrophila* contained *aerA* gene. Also, Tawab et al. (2017) recorded that *A. hydrophila* and *A. caviae* isolates were positive for numerous virulence genetic factors such as *hly, act, ast*, and *aer* , while Seker et al. (2015) revealed contrasting results to ours, as they reported that about 15 (68.2%) isolates of *A. hydrophila* were positive for *hlyA* gene, while 7 (31.8%) isolates of *A. hydrophila* were noticed to have *hlyA* and *aerA* genes together and nothing of these genetic factors remained achieved from 5 (22.7%) isolates. Also, higher occurrences of these genes were documented by Simon et al. (2016). In addition, Sadek et al. (2017) reported the occurrences of *aer*A and *ahh*1 genetic factors in the examined *A. hydrophila* spp. with the percentages of 66.7% and 77.8%, respectively.

Also, Hammad et al. (2018) reported that the prevalence of *aerA* and *ahh1* genes was 34.9% and 20.6%, respectively, and 13 isolates had no hemolysin gene; besides, another 8 hemolytic isolates showed no virulence genetic factor.

So, the foodborne illness created by *Aeromonas* spp. could be resulted from colonization, and intoxication as the bacteria discharge endotoxins as a consequence to their development in foods (Edberg and Browne, 2007).

As presented in Figure 1, it is clear that the isolate of *A. hydrophila* in lane 6 had neither *aer*A nor *ahh*1 genes although it revealed a hemolytic action on sheep blood agar (Table 4). Similar results were reported by Sadek et al. (2017) who illustrated that the hemolytic action of *A. hydrophila* may be reasoned for genes other than *aer*A and *ahh*1 genes.

Biofilm development is the main virulence aspect in pathogenic microbes. It consists mostly of proteins, DNA, and polysaccharides (Singh et al., 2017). This construction provides the antimicrobial confrontation between *Aeromonas* strains (Dias et al., 2018). *Aeromonas* spp. are sensitive to monobactams, aminoglycosides, carbapenems, cephalosporins, and fluoroquinolones (Codjoe and Donkor, 2017).

Antimicrobial drug resistance of 19 *A. hydrophila* strains isolated from raw market milk samples is shown in Tables 6 and 7. The isolated *A. hydrophila* showed variable resistance to different antimicrobial agents. The spreading of antimicrobial confrontation between food borne microbes may be due to the prolonged use of drugs in the animals used for human consumption (Deng et al., 2016). Previous studies on the confrontation of these strains that were obtained from milk and its products are scarce.

Seker et al. (2015) found that *A. hydrophila* revealed a resistance to ampicillin, cefazoline, gentamycin, amikacin, ciprofloxacin, and cefotaxime of 100%, 81.8%, 4.5%, 5.3%, 4.5%, and 4.5%, respectively. Also, Sadek et al. (2017) reported that *A. hydrophila* isolates showed 100% resistance to ampicillin, erythromycin and amoxicillin antibiotics, while they showed sensitivity against kanamycin, ceftriaxone, ciprofloxacin, and trimethoprim-sulfamethoxazole in the ratios of 22.2%, 55.6%, 100%, and 0.0%, respectively. Moreover, Strateva and Odeyemibc (2016) reported that *A. hydrophila* was resistant to commercial antibiotics.

On the other hand, Odeyemi and Ahmad (2017) stated that *Aeromonas* spp. were completely resistant (100%) to ampicillin, trimethoprim, novobiocin, and sulphamethoxazole. However, isolates were susceptible to tetracycline (100%), oxytetracycline (24.5%), kanamycin (5.7%), and gentamicin (5.7%).

The findings of Salem et al. (2020) are similar to ours. They demonstrated that the isolated *A. hydrophila* from Nile tilapia were highly susceptible to amikacin and ciprofloxacin antibiotics. And these results are dissimilar to those of Eid et al. (2013) who stated that 100% of *A. hydrophila* were susceptible to amikacin.

Also, 100% of the isolated *A. hydrophila* were resistant to ampicillin and this result is controversial to our study as only 84.2% were resistant to ampicillin, and Salem et al. (2020) reported that the most resistant *A. hydrophila* spp. which were isolated from Nile tilapia in various localities in Egypt against ampicillin and erythromycin (83.3%). The lowest sensitivity of *A. hydrophila* was reported with erythromycin (5.3%). Our study agreed with the results obtained by Eid et al. (2013) who presented that *A. hydrophila* spp. varied in their susceptibility and resistance to different antibiotics. These results illustrated the uncontrolled use of antibiotics in animals. Also, the environmental differences may play a role in antibiotic resistance. Another theory for antibiotic resistance is the presence of resistant plasmids against the antimicrobial drugs (Seker et al., 2015).

The isolated *A. hydrophila* spp. revealed a multiple antibiotic resistance (MAR) that extended from 0.062 to 1 in one isolate and to 0.937 in the second strain with an average of 0.431 (Table 7). The MAR index is an effective, usable, and low cost method that is used in basis checking of antibiotic resistant bacteria (Sandhu et al., 2016). The MAR index that is greater than 0.2 means the higher contamination risk and higher use of antibiotics in the field (Rotchell and Paul, 2016) as the MAR is of vital importance to public health as the recently developing multi-drug resistance strains do not respond to treatment with the traditional antibiotics leading to severe health problems, as long clinic stay, cure failure, and death.

Conclusion

The present context established the presence of pathogenic multi-drug resistance *A. hydrophila* in some milk samples collected from markets in Dakahlia governorate, Egypt. This pathogen may be considered as a potentially hazardous one for human health conditions as the isolated *A. hydrophila* showed virulence belongings on the foundations of proteolytic, lipolytic, and hemolytic activities in addition to the existence of *aer*A and *ahh*1 genes in most *A. hydrophila* isolates. Also, its resistance to different antibiotics was detected while amikacin was the greatest effective antibiotic against *A. hydrophila*. Pollution is caused through management and processing of milk and its products should be evaded particularly by possession of clean procedures and pasteurization of milk.

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Received 9 November 2021
Accepted 18 May 2022