Characterization of *Aeromonas hydrophila* complex Isolated from Foods of Animal Origin


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**Abstract**

*Aeromonas* spp. are common contaminants in fish, a variety of raw meat, milk and milk products, and other raw foods. In this study, the incidence and the molecular typing of *A. hydrophila* complex in food of animal origin were determined. Therefore, a total of 300 milk, meat and their products samples were randomly collected from different localities at Ismailia Governorate. The total frequency distribution of *A. hydrophila*, *A. caviae*, *A. sobria* and *A. schubertii* in examined food samples were 40%, 31.7%, 10% and 18.3% respectively. All *Aeromonas* strains were resistant to erythromycin, penicillin, cloxacillin and sulbactam ampicillin. They are found to be sensitive to tobramycin, colistin and nalidixin acid. Using PCR technique, one *Aeromonas* stain was positive for lipase gene while 6 strains were positive for aerolysin gene. Strict hygiene measure should be taken in the slaughter house and during milking to minimize the contamination of food with *Aeromonas*.

**Introduction**

*Aeromonas* organisms are gram negative rod shape, motile by single polar flagellum. *Aeromonas* spp. can be isolated from a variety of food, including meat, poultry, milk, milk products, fish, shell-fish and vegetables (*Melas et al, 1999*). Contamination of meats sold at retail outlets with *Aeromonas* spp. may result from washing of carcasses with water contaminated with *Aeromonas* and manipulation of the meat at the point of sale (*Zeng-Shan et al, 1988*).

The values of microbial contamination of cow’s raw milk are influenced by dairy cow’s health, the hygiene of the environment where dairy cows are housed and milked, methods of udder preparation and milking technique and methods of cleaning and sanitation of milking machines and milk cisterns. (*Bramley and Mckinnon, 1990*). Many *Aeromonas* species show full resistance to ampicillin via chromosomally-mediated inducible β-lactamases (*Walsh et al, 1995*). Quinolones (ciprofloxacin) was the...
most active antibiotic against *Aeromonas* (100% sensitive) followed by nalidixic acid then amikacin, garamicin and cotrimoxazole. (*Wafaa et al*, 2008). Aeromonads produce a number of virulence factors. These include a number of hemolysins including aerolysin, proteases, adhesins, invasins, enterotoxins, phospholipase and lipase (*Alperi and Figueras*, 2010).

The objectives of this study were to determine the incidence of *A. hydrophila* in milk, meat and their products which randomly collected from Ismailia Governorate. Other aim to determine the antibiotic resistance of *A. hydrophila* isolates. Using of Polymerase Chain Reaction technique as reliable tool to detect 2 virulent genes of some *A. hydrophila* isolates (lipase and aerolysin genes).

**Material and Methods**

**Samples Collection:**
A total of 300 samples of raw milk, kareish cheese, pasteurized milk cans, fresh meat, minced meat and frozen sausage (50 samples for each) were randomly collected from supermarkets, street pedlars, dairy shops, butcher shops in different localities at Ismailia Governorate.

**Bacteriological examination of collected samples:**
1. **Cultivation:**
   a. **Enrichment procedure:**
   Ten ml from liquid samples and 10g from solid samples were added individually to 90mL tryptic soy broth containing 10µg ampicillin/mL and blended for 2 minutes then incubated at 28°C for 20-24 hour.

2. **Isolation**
A loopful from previously inoculated tryptic soy broth was streaked on Rimler-Shotts (RS) medium and incubated at 28°C for 48 hour. Suspected yellow colonies of *Aeromonas* species were separately streaked onto tryptone soya agar and incubated at 28°C±1 for 24 hour to ensure purity.

**2. Identification of *A. hydrophila***:
Bacterial colonies were identified morphologically using Gram’s stain and biochemical tests described by *Carnahan et al* (1991).

**Antimicrobial susceptibility testing of 12 *A. hydrophila*, 10 *A. caviae* and 6 *A. sobria* as main pathogens by disc diffusion method:**
The susceptibility to 10 types of antibiotics (Erythromycin, Penicillin, Cloxacillin, Sulbactam ampicillin, Cefadroxil, amoxicillin, tobramycin, trimethoprimsulfamethazole, colistin and nalidixic acid) were tested according to the procedures of *NCCLS (2007)* using disc diffusion technique. The susceptibility of the strains was determined according to the size of inhibition zone.

**PCR detection of lipase (lip) gene and aerolysin (aero) gene of *A. hydrophila***:
Randomly 8 strains were subjected to PCR for detection of lipase (lip) gene, randomly 6 strains were
subjected to PCR for detection of aerolysin \((aero)\) gene. It was applied as the following:

1-Extraction of DNA from \(A.\ hydrophila\) strains Qiagen kit as adopted by Alavandi and Ananthan (2003).

2-Polymerase chain reaction:
DNA samples were tested [in 50 µl. Reaction volume in a 0.2 ml. PCR tube , containing PCR buffer ] ( 50 mM Kcl , 10 mM tris - Hcl , 1mM Mgcl2 ) each dNTPS ( Deoxy nucleotide Triphosphate ) 200 uM each ( dATP , dGTP , dCTP and dTTP ) , [ Two primer pairs each at 50 picomol / reaction ] and 0.5 of taq DNA polymerase . Thermal cycling in a programmable heating block (Coy vorporation, Grasslake, Michan, USA) was done.

PCR Protocol for lipase gene:
Initial Denaturation at 95°C for 5 min, Denaturation at 94°C for 1 min, Annealing at 62°C for 1 minutes, Extension at 72°C for 1.5 min. Cycles repeated for 39 times and proceeded by initial denaturation at 95°C for 5 minutes and followed by final extension at 72°C for 5 min.

PCR Protocol for aerolysin gene:
Initial Denaturation at 94°C for 3 min, Denaturation at 94°C for 45 second, Annealing at 56°C for 45 second, Extension at 72°C for 1 min. Cycles repeated for 34 times and proceeded by initial denaturation at 94°C for 3 min. and followed by final extension at 72°C for 5 min.

3-Screening of PCR products:
Ten µl of amplified PCR product was analyzed by electrophoresis on a 2% agarose gel stained with 0.5 µg of ethedium bromide / ml. Electrophoresis was carried out in 1X TAE buffer at 80 volt for 1 hour. Gels were visualized under UV transilluminator (UVP, UK) and photographed.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Molecular weight (bp)</th>
<th>Annealing temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH-aerAF</td>
<td>5’-GCA GAA CCC ATC TAT CCA G-3’</td>
<td>252</td>
<td>56°C</td>
</tr>
<tr>
<td>AH-aerAR</td>
<td>5’-TTT CTC CGG TAA CAG GAT TG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipase ((lip)) gene forward</td>
<td>5’-AAC CTG GTT CCG CTC AAG CCG TTG-3’</td>
<td>760</td>
<td>62°C</td>
</tr>
<tr>
<td>Lipase ((lip)) gene reverse</td>
<td>5’-TTG CTC GCC TCG GCC CAG CAG CT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results
In the present work the incidence of Aeromonas spp. in milk, meat and their products were revealed in table (2):
Frequency of different Aeromonas spp. in examined milk, kareish cheese, pasteurized milk, meat, minced meat and sausage samples were revealed in table (3). Results of antibiogram of isolated Aeromonas spp. were revealed in table (4).
PCR protocol used for amplification and detection of lipase (lip) gene of A. hydrophila complex isolates to confirm their pathogenicity as the presence of lipase gene is an index of virulence. Eight isolates were subjected to PCR for detection of lip gene and one strain only (12.5%) was positive for lip gene.

PCR protocol used for amplification and detection of aerolysin (aero) gene of A. hydrophila complex isolates to confirm their pathogenicity as the presence of aerolysin gene is an index of virulence. Six isolates were subjected to PCR for detection of aero gene and all were positive (100%) for aerolysin gene.

**Table (2):** incidence of Aeromonas spp. in milk, meat and their products.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Raw milk (50)</th>
<th>Kareish cheese (50)</th>
<th>Pasteurized milk (50)</th>
<th>raw meat (50)</th>
<th>minced meat (50)</th>
<th>Sausage (50)</th>
<th>Total positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total samples No.</td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Rimler-Shotts medium</td>
<td>300</td>
<td>13</td>
<td>26</td>
<td>6</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table (3):** Frequency of different Aeromonas spp. in milk, meat and their products:

<table>
<thead>
<tr>
<th>Samples</th>
<th>Samples No.</th>
<th>A. hydrophila</th>
<th>A. caviae</th>
<th>A. sobria</th>
<th>A. schubertii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Milk</td>
<td>50</td>
<td>13</td>
<td>26</td>
<td>5</td>
<td>38.5</td>
</tr>
<tr>
<td>Kareish cheese</td>
<td>50</td>
<td>6</td>
<td>12</td>
<td>2</td>
<td>33.3</td>
</tr>
<tr>
<td>P. milk</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Meat</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>Minced meat</td>
<td>50</td>
<td>8</td>
<td>16</td>
<td>3</td>
<td>37.5</td>
</tr>
<tr>
<td>Sausage</td>
<td>50</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>300</td>
<td>60</td>
<td>20</td>
<td>24</td>
<td>40</td>
</tr>
</tbody>
</table>
### Table (4) Antibiogram of isolated Aeromonas spp. obtained from samples.

<table>
<thead>
<tr>
<th>Isolates and their numbers</th>
<th>A. hydrophila (12)</th>
<th>A. caviae (10)</th>
<th>A. sobria (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S % R %</td>
<td>S %</td>
<td>R %</td>
</tr>
<tr>
<td>Erythromycin 15µg</td>
<td>0 0 12 100</td>
<td>0 0 10 100</td>
<td>0 0 6 100</td>
</tr>
<tr>
<td>Penicillin 10µg</td>
<td>0 0 12 100</td>
<td>0 0 10 100</td>
<td>0 0 6 100</td>
</tr>
<tr>
<td>Amoxicillin 25µg</td>
<td>0 0 12 100</td>
<td>1 10 9 90</td>
<td>1 16.7 5 83.3</td>
</tr>
<tr>
<td>Tobramycin 10µg</td>
<td>10 83.3 2 16.7</td>
<td>10 100 0 0</td>
<td>6 100 0 0</td>
</tr>
<tr>
<td>Trimethoprim- sulfamethazole 25µg</td>
<td>0 0 12 100</td>
<td>3 30 7 70</td>
<td>5 83.3 1 16.7</td>
</tr>
<tr>
<td>Cloxacillin 10µg</td>
<td>0 0 12 100</td>
<td>0 0 10 100</td>
<td>0 0 6 100</td>
</tr>
<tr>
<td>Cefadroxil 30µg</td>
<td>0 0 12 100</td>
<td>0 0 10 100</td>
<td>1 16.7 5 83.3</td>
</tr>
<tr>
<td>Sulbactam ampicillin 20µg</td>
<td>0 0 12 100</td>
<td>0 0 10 100</td>
<td>0 0 6 100</td>
</tr>
<tr>
<td>Colistin 10µg</td>
<td>11 91.7 1 8.3</td>
<td>10 100 0 0</td>
<td>6 100 0 0</td>
</tr>
<tr>
<td>Negram 30µg (Nalidixic acid)</td>
<td>12 100 0 0</td>
<td>7 70 3 30</td>
<td>6 100 0 0</td>
</tr>
</tbody>
</table>

*S = Sensitive  \quad R = resistant*

### Photo (1): Electrophoretic pattern of lipase gene assay
*Lane 1: (100 bp DNA ladder)*
*Lanes 2, 5 and 6: Showed lipase negative A. caviae strains*
*Lane 7: Showed lipase positive A. caviae strain (760 bp).*
*Lane 4: Showed lipase negative A. sobria strain.*
*Lanes 3, 8 and 9: Showed lipase negative A. hydrophila strains.*
**Photo (2): Electrophoretic pattern of aerolysin gene assay**

Lane 1: (100 bp DNA ladder).

Lanes 2, 5, 6 and 7: Showed aerolysin positive A. caviae strains (252 bp).

Lane 3: Showed aerolysin positive A. hydrophila strain (252 bp).

Lane 4: Showed aerolysin positive A. sobria strain (252 bp).

**Discussion**

*Aeromonas* spp. are free living gram negative rods (*Rowland et al., 1994*). *Aeromonas* spp. are common contaminants in fish, a variety of raw meat, milk and milk products, and other raw foods (*Porteen et al., 2007*).

The obtained results showed that the incidence percentages of *Aeromonas* species in milk, kareish cheese, pasteurized milk, meat, minced meat and sausage by were 26%, 12%, 0%, 50%, 16% and 16% respectively (table 2). The incidence of *Aeromonas* spp. in milk could be attributed to its wide distribution in the environment. The difference in incidence rate of *Aeromonas* spp. in raw milk could be attributed to different localities from which milk samples were randomly collected (*Abeyta and Wekell, 1988*).

*Aeromonas* spp. can invade the udder tissues, multiply, reach significant numbers in mammary tissues and subsequently be discharged in the milk. *A. hydrophila* is commonly present in the farms including feeds, water, faeces, soil and milking equipment, consequently it could contaminate udder and teats of cows and get into the milk (*Abeyta and Wekell, 1988*).

Occurrence of *Aeromonas* spp. in meat and their products is attributed to transmission of Aeromonads to food through using contaminated water during slaughtering and processing of meat and meat products as described by (*Palumbo, 1985*).

Regarding to incidence of *Aeromonas* spp. recovered from all samples it showed that 13 isolates from milk were identified as 5 (38.5%) *A. hydrophila*, 4 (30.8%) *A. caviae*, 1 (7.7%) *A. sobria* and 3 (23.1%) *A. schubertii* (table 3). *A.*
hydrophila was the most predominant species followed by A. caviae. These results come in agreement with those obtained by Kirov et al. (1993). A. hydrophila, A. caviae and A. sobria were recorded in meat samples by 11 (27.5%), 16 (40%) and 1 (2.5%) respectively. Meanwhile, highest incidence rate was obtained by Khalil (1997) who isolated Aeromonas spp. from raw meat and identified as A. hydrophila (26.8%), A. caviae (35.6%) and A. sobria (4.4%).

The obtained data showed that the incidence rate of kareish cheese by Aeromonas species using Rimler-Shotts medium was 12%. Those are biochemically typed into (33.3%) A. hydrophila, (16.7%) A. caviae, (33.3%) A. sobria and (16.7%) A. schubertii. A. hydrophila (42.9%) were the predominant species. This result nearly in agreement with Effat et al. (2000) who isolated Aeromonas species from kareish cheese and that A. hydrophila (42.9%) was the predominant species followed by A. caviae (33.3%) and A. sobria (23.8%). This result nearly in agreement with Khalil (1997) who isolated Aeromonas species from kareish cheese and that A. hydrophila (22.2%) was the predominant species followed by A. caviae (15.6%) and A. sobria (13.3%). And this result disagree with El-Prince (1998) who found that A. hydrophila and A. caviae were the predominant species comprising (39.5%), while A. sobria comprising (21%) of the total isolates.

Regarding to incidence of Aeromonas spp. recovered from minced meat results were (37.5% for A. hydrophila, 50% for A. caviae and 12.5% for A. schubertii) The predominant species in minced meat was A. caviae and this disagree with Gob and Jemmi (1993) who found that the incidence of Aeromonas spp. in minced meat 94.1% (75.7 A. hydrophila, 27% A. caviae and 24.3 A. sobria) and in disagreement with Soltan-Dallal et al. (2012) who found that The predominant species in minced meat was A. hydrophila (59%) followed by A. caviae (20.6%), A. sobria (17%).

Regarding to antibiogram of Aeromonads, all Aeromonas strains were resistant to erythromycin, penicillin, cloxacillin, sulbactam ampicillin (table 4) the obtained results were in agreement with Nagar et al. (2011) and Daoood (2012). Resistance of Aeromonas spp. to penicillin is due to the

The obtained data showed that A. hydrophila and A. caviae were resistant to cefadroxil and this result in agreement with Wafaa et al (2008). Data showed that A. sobria was sensitive to cefadroxil this in agreement with Ko et al (2003) and disagrees with Vila et al (2003) who found that A. sobria was resistant to cefadroxil.

The obtained data showed that Aeromonas strains varied in their sensitivity to antibiotics (trimethoprim-sulphamethoxazole, amoxicillin, tobramycin, colistin and nalidixic acid) which may either be due to resistance of the strains or the character of strains, which need further investigations.

In this work, PCR protocol was used for amplification of 760 bp fragment of lipase gene as shown in Photo (1). Only one A. caviae strain was positive for lipase gene with a percentage of 16.7%, this lower incidence agree with Oliveira-Samira et al (2012) who detect lipase gene with a percentage of 3.51%. This result disagrees with Cascon et al (1996) who detect lipase gene with a percentage of 100%. The lipases and hydrolipases are considered important virulence factors in Aeromonas spp. because they alter the structure of the cytoplasmic membrane of the host and thus exacerbate its pathogenicity, especially if the aerolysin gene is present (Nawaz et al, 2010). The lower incidence of lipase gene may be due to strain variation.

Also PCR was used for amplification of 252 bp fragment of aerolysin gene as shown in Photo (2.) All tested strains were positive for aerolysin gene with a percentage of 100% and this result agree with Yogananth et al (2009) who detect this gene in all examined strains with a percentage of 100%. Also results nearly in agreement with Oliveira-Samira et al (2012), Yousr et al (2007) who detect aerolysin gene with a percentage of 78.95%, 50.5% respectively this indicate virulent strains because aerolysin gene one of the most important virulence factors for A. hydrophila bacteria. According to Heuzenroeder et al (1999), the presence of aerolysin is a strong indication of virulence in pathogenic isolates of Aeromonas spp.

In conclusion, Aeromonas species were found be one of food contaminants in Ismailia Governorate. Four Aeromonas strains were isolated from the examined samples. Strains were highly sensitive to tobramycin, colistin and nalidixic acid. Using PCR revealed that one stain was positive for lipase gene while 6 strains were positive for aerolysin gene.

References


الملخص العربي

يعتبر ميكروب الإيروموناس هيدروفيل من الملوثات الأكثر شيوعا في الأغذية من مصدر حيواني مثل اللحوم والألبان ومنتجاتهما. لذا تم في هذه الدراسة تحديد نسبة تواجد وتوصيف الجيني لميكروب الإيروموناس هيدروفيل في الأغذية ذات الأصل الحيواني. وتم فحص عدد 300 عينة من عينات الألبان واللحوم الطازجة ومنتجاتهما المجمعة عشوائيا من مناطق مختلفة في محافظة الإسماعيلية. وتبين من النتائج أن أجمالي توزيع كل من الإيروموناس هيدروفيل والإيروموناس كافيا والإيروموناس سوبريا والإيروموناس شبرتى في العينات تحت الفحص 30، 1، 3.1، 10، 10.3 و18.3% على التوالي. كانت جميع السلالات مقاومة للمضادات الحيوية الآتية: الإريثروميسين، البنسلين، وكولوكساسيلين وساليكانت الأمبيسين. بينما كانت هناك حساسية من التوبراميسين، كولستين وحمض الناليديكسيك. وباستخدام تقنية اختبار تفاعل عدد البلمرة المتسلسل وجدت عتبر واحدة إيجابية لجين لالليزا بينما كانت ستة عيارات إيجابية لجين الأيدروالاسين. وأوصت الدراسة بضرورة تطبيق الاشتراطات الصحية الجيدة أثناء عملية ذبح وتجهيز اللحوم وكذلك أثناء عملية حلب الابقار لتقليص تلوث الأغذية بМИكروب الإيروموناس.