Occurrence, Hemolytic Toxins and Antimicrobial Resistance of Aeromonas hydrophila Strains from Dairy Cow and Anatolian Water Buffalo Quarter Milk Samples in Turkey

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ABSTRACT

Background: Motile aeromonads are considered to be one of the most important food-borne pathogens because of potential human health significance. Aerolysin and hemolysin are virulence factors playing a significant role in the pathogenesis of infections. Antimicrobial resistance is an important problem limiting therapeutic options. The main objective of the present study was to isolate motile Aeromonas species from cow and Anatolian buffalo quarter milk samples and determine the antimicrobial resistance and hemolysin (hlyA) and aerolysin (aerA) virulence genes of isolated species by PCR.

Material, Methods & Results: The present study was carried out on apparently healthy 200 dairy cows and 103 Anatolian water buffaloes in different stages of lactation, hand-milked twice a day, held in private farms located in Afyonkarahisar province of Western Turkey. Before milking, 771 and 399 quarter milk samples were collected from cows and Anatolian buffaloes, respectively. For the isolation, APW, SAA and blood agar were used. The certain identification of isolates was made using API 20NE system. The strains were tested for susceptibility to 21 different antibiotics by disc diffusion test. Bacterial DNAs were extracted from all strains using a genomic DNA extraction kit and strains were screened for the presence of hlyA and aerA genes by PCR. While A. hydrophila was isolated from 22 (1.9%) of 1170 quarter milk samples, A. caviae and A. sobria were not detected in the examined milk samples. The isolation rate of A. hydrophila from cow and buffalo milk samples was 2.1% (n = 16) and 1.5% (n = 6), respectively. The highest resistance rate in strains was against ampicillin (100%), followed by ampicillin-sulbactam (95.5%), methicillin (95.5%) and cephalzone (81.8%). The most of buffalo milk samples was 2.1% (n = 16) and 1.5% (n = 6), respectively. The highest resistance rate in strains was against ampicillin (100%), followed by ampicillin-sulbactam (95.5%), methicillin (95.5%) and cephalzone (81.8%). The most of strains were also resistant at least to one of the antibiotics. In DNA samples extracted from 22 A. hydrophila strains, 15 (68.2%) strains were found to be positive for hlyA gene, of these, 8 (36.4%) only harboured hlyA gene alone. The aerA gene was obtained from 9 (40.9%) strains, 2 (9.1%) of these were alone. A total of 7 (31.8%) A. hydrophila strains were detected to harbour both of the hlyA and aerA genes. None of the virulence genes was obtained from 5 (22.7%) strains.

Discussion: This is the first study investigating the hemolysin genes (hlyA and aerA) of A. hydrophila strains isolated from cow and Anatolian buffalo quarter milk samples in Turkey. In this study, the isolation rate of A. hydrophila was lower than other researcher’s isolation rate. The reason of this discrepancy may be related to the difference of sampling methods. Because we tested the quarter milk samples of each animals, while other authors used the bulk tank milk or pasteurized milk samples in their studies. Most of our strains were resistant at least to one of the antibiotics. This finding supports the intensive, prolonged and uncontrolled use of the nonspecific antimicrobials should be prevented in Turkey. We also consider that geographical differences and local selective pressures may be effective on the antibiotic resistance levels. The virulence genes had low isolation rates in our study. The isolates analyzed in this study were obtained from milk samples belong to apparently healthy animals. This may explain, in part, the reason of low prevalence of virulence genes. Nevertheless, it should not be ignored that the hlyA and aerA genes positive A. hydrophila isolates obtained from quarter milk samples in our study may be more virulent than negative isolates for virulence genes and pose a potential hazard for humans.

Keywords: aerolysin, buffalo, cow, hemolysin, milk, motile Aeromonas.
**INTRODUCTION**

Motile aeromonads are considered of potential human health significance because they consist the some zoonotic species such as *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* [8,14]. Investigations related to the prevalence of motile *Aeromonas* spp. in foods show that milk, like other food products, is a potential source in the development of gastroenteritis for humans [17,18,27]. Aerolysin and hemolysin are known to be the most important virulence factors playing a significant role in the pathogenesis of *Aeromonas* infections [3,10,22]. Although β-lactam resistance in the motile *Aeromonas* isolates from clinical and environmental sources was reported [7,10,20], studies associated with antibiotic resistance of these agents isolated from food products, especially milk and milk products, of animal origin are limited [23,28]. In Turkey, generally, researches are focused on the isolation of motile *Aeromonas* spp. from bulk tank milk, pasteurized milk and street milk samples collected from dairy shops, supermarkets and dairy manufacturing factories [1,28,29]. But, there are a limited number of publications on the prevalence of these agents in the mammary quarter milk samples of dairy animals [2].

The aim of the present study was to isolate motile *Aeromonas* species from cow and Anatolian buffalo quarter milk samples and determine the antimicrobial resistance and hemolysin (*hlyA*) and aerolysin (*aerA*) virulence genes of isolated species by PCR. To our knowledge, this is the first study investigating the presence of hemolysin genes (*hlyA*, *aerA*) in the motile aeromonads isolated from quarter milk samples in Turkey.

**MATERIALS AND METHODS**

Sample collection

The present study was carried out on apparently healthy 200 dairy cows and 103 Anatolian water buffaloes in different stages of lactation, hand-milked twice a day, held in private farms located in Afyonkarahisar province of Western Turkey. Before milking, 771 and 399 quarter milk samples were aseptically collected from cows and Anatolian buffaloes, respectively. Firstly, the teat ends were cleaned using 70% alcohol and dried. The first streams of foremilk were discharged, and then 25 mL of milk from each mammary quarter was aseptically collected into sterile bottles. Samples were immediately transported to the laboratory in a cool box on ice.

**Isolation and identification of motile Aeromonas species**

Twenty-five mL milk was added to glass flasks containing 225 mL Alcaline Peptone Water (APW)¹ and incubated under aerobic conditions for 24 h at 28°C. A 10 µL aliquot was taken from pre-enrichment broth and inoculated onto Starch Ampicillin agar (SAA) supplemented with ampicillin² (10 µg/mL) and blood agar³, including 5% of sheep blood. The plates were aerobically incubated at 28°C for 24 h. After the incubation, colonies growing on mediums were examined macroscopically (colony morphology, hemolysis) and microscopically (Gram staining). Then, oxidase, catalase, motility, fermentation in Triple Sugar Iron agar, oxidation/fermentation, Voges-Prouskauer, lysine decarboxylase, DNase activity, growth conditions in 0% and 6% NaCl and resistance to Vibriostatic agent O/129 (2,4-diamino 6,7-diisopropylpteridine) (150 µg/mL) tests were applied to isolates [12,21]. The certain identification of isolates was made using API 20NE system⁴.

**Antimicrobial susceptibility test**

The antimicrobial resistance of strains was determined by using Kirby-Bauer disc diffusion test on Mueller Hinton agar¹ according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [5]. For this purpose, amikacin (30 µg), ampicillin (10 µg), ampicillin-sulbactam (20 µg), aztreonam (30 µg), cefeperezone (30 µg), cefotaxime (30 µg), cefotetan (30µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefazolin (30 µg), ciprofloxacin (5 µg), cefuroxime/sodium (30 µg), gentamicin (10 µg), imipenem (10 µg), pipercillin (30 µg), methicillin (10 µg), tetracycline (10 µg), ticarcillin (75 µg), ticarcillin/clavulanic acid (85 µg), tobramycin (10 µg) and trimethoprim/sulfamethoxazole (25 µg) antibiotic discs¹ were used.

**Detection of hlyA and aerA virulence genes in A. hydrophila strains Extraction of DNA**

DNA was extracted from the positive control strain (*A. hydrophila* ATCC 7966)¹ and the test strains using a genomic DNA extraction kit (Heliosis®)⁴ as described by the manufacturer. Firstly, one bacterial colony grown on tryptone soya agar¹ was inoculated into tryptone soya broth plus 0.6% (w/v) Yeast Ex-
tract (TSBYE) and incubated at 30°C for 18 h. Then, one mL was taken from TSBYE and transferred to sterile DNase and RNase free eppendorf tubes. Eppendorf tubes were centrifuged at 2,900 g for 2 min. Supernatant was discharged and pellet was suspended in 200 µL of sterile deionized water. The extraction was completed by following the steps as indicated in the kit.

**PCR amplification**

The oligonucleotide primers described by Zhu et al. [30] and Heuzenroeder et al. [11] were used for the detection of the hlyA and aerA genes, respectively. For amplification of a 592-bp hlyA gene forward 5’- GGCCGGTGCCCCGAAGATACGGG-3’ and reverse primers 5’- GGCGGCGCCGGACGAGACGGG-3’ were used. Amplification of a 416-bp aerA gene was performed using the forward 5’- GCTCTAGCCGAGAAGGT-3’ and reverse primers 5’- CAGTCCCACCCACTTC -3’. Five microliter of the extracted DNA was used as a template in a 50 µL PCR mixture containing 10 X PCR buffer, 25 mM MgCl2, 10 mM dNTP mix, 20 µM each primers, 2 U of Taq DNA polymerase and deionized water. A. hydrophila ATCC 7966 strain and sterile deionized water were used as positive and negative controls, respectively. The PCR amplification conditions of hlyA gene consisted an initial denaturation step at 95°C for 4 min, and 30 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s and a final step 72°C for 7 min. The conditions for aerA were as follows: initial DNA denaturation at 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s and finally at 72°C for 7 min. All products were analyzed by 1.5% agarose gel electrophoresis and visualized using ethidium bromide under U.V. light. Molecular size markers (100-bp DNA ladder) were included in each agarose gel.

**Statistical analysis**

The differences between the isolation rates of A. hydrophila strains from cows and buffaloes milk were evaluated using Chi-square test. The level of significance was set at \( P < 0.05 \).

**RESULTS**

**Isolation and identification findings**

Of examined 1170 quarter milk samples (n = 771 cows milk, n = 399 buffaloes milk), 22 (1.9%) were positive for A. hydrophila, while A. caviae and A. sobria were not detected in these samples. The isolation rate of A. hydrophila from cows and buffalo quarter milk samples was determined as 2.1% (n = 16) and 1.5% (n = 6), respectively. No statistically significant difference was found in relation to prevalence of A. hydrophila in cow’s and buffalo’s milk (\( P = 0.495 \)). Fourteen (63.6%) of 22 A. hydrophila isolates showed β-hemolysis on blood agar, including of 5% sheep blood.

**Antimicrobial susceptibility**

According to Kirby-Bauer disc diffusion test results, the highest resistance was to ampicillin (100%), followed by ampicillin-sulbactam (95.5%), methicillin (95.5%) and cefazolin (81.8%). None of the 22 isolates was resistant to amikacin, aztreonam, cefoperazone and cefotetan. The antibiotic resistance of A. hydrophila isolates was shown in Table 1.

### Table 1. Resistance rates of Aeromonas hydrophila isolates from dairy cow and Anatolian water buffalo quarter milk samples in Turkey.

<table>
<thead>
<tr>
<th>A. hydrophila (n = 22)</th>
<th>Antibiotic</th>
<th>Resistance n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>22</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>21</td>
<td>95.5</td>
<td></td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Cefotetan</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>3</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>1</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Cefazolin</td>
<td>18</td>
<td>81.8</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime/sodium</td>
<td>2</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>1</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Piperacillin</td>
<td>3</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>21</td>
<td>95.5</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10</td>
<td>45.5</td>
<td></td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>6</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>Ticarcillin/clavulanic acid</td>
<td>1</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>6</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim/sulfamethoxazole</td>
<td>9</td>
<td>40.9</td>
<td></td>
</tr>
</tbody>
</table>
**Distribution of virulence genes**

In DNA samples extracted from 22 strains of *A. hydrophila*, 15 (68.2%) strains were found to be positive for *hlyA* gene, of these, eight (36.4%) only harboured this gene alone. The *aerA* gene was obtained from nine (40.9%) strains, two (9.1%) of these were alone. A total of seven (31.8%) *A. hydrophila* strains were detected to harbour both of the *hlyA* and *aerA* genes. Five (22.7%) of the isolates had none of the virulence genes. Also, all strains composing β-hemolysis phenotypically were determined to be positive for *hlyA* gene. The distribution of *hlyA* and *aerA* virulence genes and PCR products were shown in Table 2 and Figure 1 and Figure 2, respectively.

<table>
<thead>
<tr>
<th>Virulence genes</th>
<th>A. hydrophila (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td><em>hlyA</em>+ (total)</td>
<td>15</td>
</tr>
<tr>
<td><em>aerA</em>+ (total)</td>
<td>9</td>
</tr>
<tr>
<td><em>hlyA</em>+ <em>aerA</em>+</td>
<td>7</td>
</tr>
<tr>
<td><em>hlyA</em>− <em>aerA</em>−</td>
<td>5</td>
</tr>
</tbody>
</table>

**Figure 1.** Detection of *hlyA* gene by PCR. M: 100 bp DNA ladder (Fermentas, Vilnius, Lithuania); -: negative control (sterile deionized water); +: positive control (*A. hydrophila* ATCC 7966); lane 1-5 and 7-9: specific bands of *hlyA* gene (592 bp); lane 6: *hlyA* gene negative isolate.

**Figure 2.** Detection of *aerA* gene by PCR. M: 100 bp DNA ladder (Fermentas, Vilnius, Lithuania); +: positive control (*A. hydrophila* ATCC 7966); -: negative control (sterile deionized water); lane 1-8: specific bands of *aerA* gene (416 bp).
DISCUSSION

The present study investigated the presence and antimicrobial resistance of motile *Aeromonas* spp. in the cow and Anatolian buffalo quarter milk samples and detection of *hlyA* and *aerA* virulence genes in the isolates by PCR.

Motile *Aeromonas* species causing intestinal and extraintestinal infections in humans have appeared among the most important foodborne pathogens [9,10,15]. Recently, the investigations have focused on the prevalence of *Aeromonas* spp. in foods because of an increase in the infection prevalence [6,18,23,27]. Investigations show that milk, like other food products, is a potential source in the development of gastroenteritis [17,18,27]. Also, it is reported that motile aeromonads can grow and continue the synthesis of virulence factors in milk conserved at refrigeration temperature [16]. In the published reports from many countries, *A. hydrophila* is the predominant *Aeromonas* species found in raw milk and dairy products [1,6,13,19,23,29]. In a study, the prevalence of motile aeromonads from 150 raw bovine bulk tank milk samples was reported to be 26.6% [13], while Melas et al. [19] found that 15.9% of 138 raw cow milk samples were contaminated with *A. hydrophila*. ElBalat et al. [6] isolated motile aeromonads from four (16%) of 25 raw cow milk samples, two of these were *A. hydrophila* and two were *A. caviae*. In another investigation, a total of 105 raw milk samples obtained from various milk vendors were analyzed for *A. hydrophila* and 17.14% of the milk samples were found to be contaminated with this agent [23]. Yucel et al. [29] from Turkey found aeromonads in 49.2% of 132 bulk raw milk samples, 40% of 25 raw milk samples sold in the street and 16% of 31 pasteurized milk samples examined and emphasized the most frequently isolated species from these samples was *A. hydrophila* (90.2%). Similarly, Akan et al. [1] detected motile *Aeromonas* spp. in 23 (28.7%) of 80 bulk tank milk samples and reported 15, seven and one of 23 strains were *A. hydrophila*, *A. sobria* and *A. caviae*, respectively. It is seem that in both Turkey and other countries, these investigations connected to the isolation of *Aeromonas* species from milk samples have majored on raw bulk tank milk and/or pasteurized milk samples. The common idea of all these researchers [1,6,13,19,23,29] is to milk may contaminate with *Aeromonas* spp. after the milking (environmental or faecal contamination) because of their intensive presence in the environment, water, animal barns and animal faeces. Although the prevalence/incidence of motile aeromonads in raw bulk tank milk and/or pasteurized milk samples is commonly reported, studies on the presence of aeromonads in quarter milk samples are limited [2]. In a report from Turkey, two and one of 100 mammary quarter milk samples belong to cows were isolated to be *A. hydrophila* and *A. caviae*, respectively [2]. In our study, *A. hydrophila* was isolated from the 22 (1.9%) of 1170 quarter milk samples belong to cows and Anatolian buffaloes, while the isolation of *A. caviae* and *A. sobria* from sampled animals was not achieved. The isolation rate of *A. hydrophila* was 2.1% (16/771) and 1.5% (6/399) in dairy cow and buffalo quarter milk samples, respectively (P = 0.495). While these findings were in agreement with the results of Alişarlı [2], it differed from other authors [1,13,19,23,29] who reported a prevalence that was quite high. The reason of this discrepancy may be related to the difference of sampling methods. Because we tested the quarter milk samples of each animals, while other authors used the bulk tank milk or pasteurized milk samples in their studies. It was also appeared that the hygienic procedures during the milking may dramatically decrease the secretional contamination of milk with *Aeromonas* spp.

Motile *Aeromonas* species are known to be resistant to many β-lactam antibiotics due to the production of multiple inductible chromosomally encoded β-lactamases [25]. Various researchers have emphasized that this problem causes an increase in the risk of treatment failure and cost for antimicrobial therapy and hospitalization, while the range of therapeutic options decrease [10,25]. Although the high resistance rates to ampicillin, ampicillin-sulbactam, methicillin and cefazolin have been reported in the environmental and clinical *A. hydrophila* isolates [7,10,20], reports on the antibiotic resistance of this species isolated from milk and milk products are rare [23,28]. In *A. hydrophila* strains isolated from raw milk samples by Subashkumar et al. [23], resistance to ampicillin, methicillin and cefazolin was found to be 100%, 94.4% and 88.8%, respectively. Yucel & Çıtak [28] from Turkey determined the high resistance to ampicillin and erythromycin in *A. hydrophila* strains obtained from raw milk samples. Similar to other research findings, in our study, the highest resistance rates were to ampicillin (100%), ampicillin-sulbactam (95.5%), methicillin
A. hydrophila strains may carry hlyA and aerA genes playing an important role in the pathogenesis of human infections [4,11]. According to these ideas, the hlyA and aerA genes positive A. hydrophila isolates obtained from quarter milk samples in our study may be more virulent than negative isolates for virulence genes and pose a potential hazard for humans.

**CONCLUSION**

Consequently, the presence of hlyA and aerA genes of A. hydrophila strains isolated from quarter milk samples belong to cow and Anatolian buffalo was reported for the first time in Turkey. It was also shown hygienic precautions during the milking can decrease secretional contamination of milk with Aeromonas spp. Generally, pasteurization is considered as effective for destroying of Aeromonas spp., but, the isolation of motile aeromonads from pasteurized milk has been shown in several studies. Besides, the presence of Aeromonas spp. in milk is of great concern because of their capability of growth and expression of virulence factors at refrigeration temperature. Afyonkarahisar located in Western Turkey has critical position in the breeding of dairy animals, especially water buffaloes, and the milk products obtained from these animals have an important consumer portion in Turkey. To our knowledge, a severe outbreak in associated with Aeromonas spp. has not been reported in Turkey so far. Nevertheless, it should not be ignored that Aeromonas spp. having virulence factors may be more pathogenic for humans and create a potential hazard in terms of public health. Also, the common use of nonspecific antibiotics for the treatment of infections should be restricted because of an increasing resistance problem to antimicrobials all over the world.

**MANUFACTURERS**

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5Fermentas AB. Vilnius, Lithuania.

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**Declaration of Interest.** The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.
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