

Detection of *Aeromonas hydrophila* in Raw Milk and Soft Cheese in Baghdad City

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ABSTRACT

The frequency and distribution patterns of *Aeromonas hydrophila* in cow's raw milk and soft cheese were investigated in Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya regions in Baghdad. A total of 120 pooled samples were collected during February until June 2019, in which 60 samples of raw milk pooled from milk containers, 20 from each region (four samples collected monthly per region) and 60 samples of fresh soft cheese made from raw unpasteurized milk were collected from different supermarkets in Baghdad as same as the proportion above. Modified procedures for the isolation and identification were dependent, in which modified tryptone soya yeast extract sheep blood agar supplemented with ampicillin were used. Gram's stain and oxidase reaction aided in the bacterial isolation. Modified Congo red assay was used to detect biofilm, and Kirby-Bauer disc diffusion method was used for determining the sensitivity of isolates to ampicillin (AM 10 µg), cephalexin (CLX, 30 µg), azithromycin (AZM, 15 µg) and vancomycin (VA 30 µg). The results confirmed the recovery of 11 isolates (9.16%) from 120 pooled samples, in which four isolates (3.33%) were obtained from raw milk samples; Two (1.66 %) from Abu-Ghraib and one (0.83 %) from different regions. Seven isolates (5.83 %) were detected from fresh soft cheese samples, in which three (2.5 %) were from Abu-Ghraib and two (1.66%) were from other regions. All of the isolates were Gram-negative rod-shaped, oxidase positive and biofilm producers. Four isolates (36.36%) were resistant to selected antibiotics, in which two (18.18%) from Abu-Ghraib: One (9.09 %) from raw milk and soft cheese; and one (9.09 %) in the other regions in fresh soft cheese only. In conclusion, milk and cheese production in Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya regions in Baghdad are encountered by the problems of bacterial contamination and presence of multidrug resist strain of *A. hydrophila*, which is considered as a risk to public health, may be due both insufficient and misuse treatment with antibiotics or somewhat due to bad quality and/or poor hygiene of processing involved in milk production. Thus, it is recommended to monitor these products for better hygienic status.

Keywords: *Aeromonas hydrophila*, Biofilm, Antibiotics resistance, Raw milk and soft cheese

Introduction

Foodborne diseases impose a burden worldwide, yet much remains unknown about them in both industrialized and developing countries. The Department of Food Safety and Zoonosis (FOS) of the World Health Organization (WHO) takes initiatives to measure the global burden of

mortality and morbidity caused by foodborne diseases, while the Global Foodborne Infections Network (GFN) functions to estimate and mitigate the problem of foodborne diseases (1). *Aeromonas hydrophila* (*A. hydrophila*) is a widespread representative of *Aeromonas* found in water, water habitants, domestic animals and foods (fish, shellfish, poultry and raw meat).

The microorganism has the potential to be a foodborne pathogen associated with clinical cases of illness.

The pathogen produces different virulence factors including hemolysins exotoxins, endotoxins, cytotoxins, etc. As a psychrotroph, *A. hydrophila* grow in foods during refrigeration. The disease spectrum associated with this microorganism includes gastroenteritis, septicemia, and wound

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infections. Multiple resistance to different antibiotics is a fact of high significance. The potential of *A. hydrophila* as a foodborne pathogen is a crucial issue in which, many approaches are progressing for control of the presence of *A. hydrophila* in food chain for human consumption (2, 3). The microbial quality, quantity and safety of raw milk and fresh soft cheese depend mainly on environmental and hygienic status of cows as well as hygienic measurements of milk containers during production and storage of cheese. Thus, contamination of these products with opportunistic, infectious and enterotoxigenic foodborne *A. hydrophila* represents emerging risk. Biofilm productions protect almost strains from harsh conditions and play an important role in the development of resistance to antibiotics.

Transferring of these resistant clones through water and food represents emerging problems. Spoilage and deterioration of refrigerated food with these psychotropic pathogens might indicate another sophisticated brand because of its ability to grow and produce thermo-resistant extracellular enzymes (lipase, protease, amylase and nuclease) which are capable of degrading important milk constituents and thus affect the quality of finished dairy products (4-9). Biofilms are complex architectural and shelf-organized with altered phenotypic and genotypic functions of different microorganisms, including foodborne pathogens. Aeromonads within a biofilm are more resistant to disinfectants than planktonic cells, as shown for *A. hydrophila* strains (1). Biofilm promotes gene exchange and antibiotic resistance. Biofilm structure provides a close cell-to-cell proximity that enhances genetic transfers, mainly conjugation and natural transformation (10-12).

Biofilms enhance stability and protect bacteria against external factors (13, 14). First, bacterial sessile life is associated with an increased persistence and resistance to stressful conditions, including salinity, antimicrobial substances, or oxidative stress, compared to the planktonic lifestyle (15).

Second, biofilms provide cell nutrients in higher concentrations than the surrounding environment via the nutrient-rich solute retained in the interstitial region of the extracellular polymeric matrix. The biofilm is considered part of the pathogenic activity of various bacteria (16).

Due to the ability to attach to visceral organs and

many different material surfaces, biofilms have become major causes of persistent infection and drug resistance (17). After adhering to the matrix surface, sessile bacteria will produce so-called extracellular polymeric substances (EPSs), which are primarily composed of exopolysaccharides, proteins and extracellular DNA (eDNA) (18, 19).

The use of antibiotics is one of the most important factors influencing the emergence of resistance in bacterial pathogens.

Multi-resistant *A. hydrophila* were isolated from different parts of the world and reported to be resistant to penicillin and ampicillin, but sensitive to aminoglycosides, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, quinolones, and second- and third-generation cephalosporin. Plasmids are extra-chromosomal DNA coding virulence determinants. *A. hydrophila* carries stable plasmids playing an important role in microbial resistance and virulence. However, the increase in *A. hydrophila* resistance to antibiotics is a public health concern; therefore, there should be a continuous and concerted effort to monitor the existence of this opportunistic pathogen globally (20).

Regarding the uncontrolled use of antibiotics, which may contribute to the appearance of resistant strains of bacteria for a wide range of antibiotics and possess public health risk, this study was performed in order to highlight this issue.

Materials and Methods

Collection and Processing of Samples

All chemicals and media were purchased from Oxoid. One hundred twenty samples of locally produced cows' raw milk (60 samples) and fresh soft cheese (60 samples) were collected from different dairy shops, farms, and street peddlers in Baghdad province from February until June. All samples were collected aseptically in clean plastic bags and transported to the food laboratory (College of Veterinary Medicine, University of Baghdad) as soon as possible, then processed according to food microbiological procedures with a modification policy, in which they were refrigerated for 48 hours at 4 °C before culturing in order to increase coincidental recovery of these psychotropic pathogens.

Isolation and Identification: all samples were subjected to homogenization before processing. Then, they were divided into two parts (directly and indirectly (21 - 23). Directly, the milk samples were inoculated in tryptone soya yeast extract broth as one part sample (20 ml) to 9 parts (180 ml), then mixed well for two minutes and incubated at 37 °C for 24 - 48 hours for resuscitation of stressed cells, and then streaked on modified tryptone soya yeast extract sheep blood agar supplemented with ampicillin.

Cheese samples were macerated manually with its own whey (original sample), after that processed as above, in which directly one part (25 gm) sample from a whole homogenized cheese lobes (original sample) were diluted decimally with 9 parts (225 ml) in 2% buffered sodium citrate solution. Then, mixed and cultured as above. According to dairy and food microbiological procedures, pure isolated single colonies of *A. hydrophila* showed standard criteria of oxidase positive, Gram's negative, rod shaped features that preserved in slant bottles inside a refrigerator as pure seeds for further identification.

Congo Red Agar Method (CRA)

Freeman et al. (24) had described an alternative method for screening biofilm formation; which required the use of a specially prepared solid medium.

A modification was done by replacing BHI agar with double-strengthen TSA-YE (8 g Tryptone Soya Agar +1 g Yeast Extract/100 ml d. w.) supplemented with 5% sucrose (5 gm/100 ml) and Congo red (10 gm/L) for better results. Congo red was added to the medium directly or prepared as a concentrated aqueous solution and autoclaved at 121 °C for 15 minutes, separately from the other medium constituents, then added when the agar had cooled to 55 °C. As a critical step, the medium had been boiled but not autoclaved.

Plates were inoculated and incubated for 24 to 48 hours at 37 °C. Positive result was indicated by the appearance of black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. Darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an intermediate

result. The experiment performed in triplicate and repeated three times.

Antibiotics Susceptibility Assay

A Kirby-Bauer technique or disk diffusion method was performed according to the instructions of clinical laboratory standards institute (CLSI) or national committee for clinical laboratory standards (NCCLS) by using a Muller-Hinton agar and McFarland opacity tubes (25-26).

The procedure included using ampicillin (AM 10 µg), cephalexin (CLX, 30 µg), azithromycin (AZM, 15 µg) and vancomycin (VA 30 µg).

Selecting well isolated colonies of *A. hydrophila* from freshly inoculated overnight tryptone soya yeast extract agar and transferred to freshly prepared tryptone soya yeast extract broth and incubated for 4 hours at 37 °C was done in order to reach a standard of 0.5 opacity in McFarland tubes or approximately 10^4 - 10^5 cfu/ml in the standard inoculums.

Preparation of freshly agar plate cultures of Muller-Hinton: A sterile cotton swab was soaked into the prepared suspension and rotated several times and pressed firmly on the inside wall of the tube above the fluid level for removing excess inoculums from the swab.

Streaking the surfaces of Muller-Hinton agars four to five times with the rim by a swab was done, and then the plates were left for 10-15 minutes to absorb the inoculums before applying selected antimicrobial disks, which were placed by pressing down to ensure complete contact with the agar surface and distributed evenly.

The plates were inverted and placed in an incubator at 37 °C for 18-24 hours. Then, the plates were read and the results were interpreted (27). The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium.

The zone diameters of each drug were interpreted using the criteria published by CLSI. The results were interpreted as susceptible, intermediate, or resistant isolates. Data were subjected to Chi-square analysis using the Statistical Package for the Social Sciences (SPSS, version 25) (28), and $P \leq 0.05$ was considered statistically significant.

Results and Discussion

The results confirmed the recovery of 11 *A. hydrophila* isolates (9.16%) out of 120 pooled samples, in which four isolates (3.33%) were from raw milk samples: two (1.66 %) from Abu-Ghraib and one (0.83%) from two other regions.

Seven isolates (5.83%) were detected from fresh soft cheese samples, in which three (2.5%) from Abu-Ghraib and two (1.66%) from other regions.

All isolates were Gram-negative, rod-shaped, oxidase positive and biofilm producers. Four isolates (36.36%) were resistant to the selected antibiotics in which two (18.18%) were from Abu-Ghraib: One (9.09 %) from both raw milk and fresh soft cheese; and one (9.09%) from each other regions from fresh soft cheese only. Most isolates recovered from Abu-Ghraib region were during February (Tables, 1, 2, 3 and 4) and photographs 1, 2, and 3.

Table 1. Recovery of *Aeromonas hydrophila* from raw milk samples

Region	No. samples	<i>A. hydrophila</i>	Recovery		
			20%	60 %	120 %
Abu-Ghraib	20	2 ^{A*}	10	3.33	1.66
Al-Fudhaliyah	20	1 ^B	5	1.66	0.83
Al-Sadrya	20	1 ^B	5	1.66	0.83
Total	60	4	20	6.66	3.33

*Indicate highest recovery from Abu-Ghraib. ^{A-B} Indicate significant differences vertically among regions at level $P \leq 0.05$.

Table 1. Recovery of *Aeromonas hydrophila* from fresh soft cheese samples

Region	No. samples	<i>A. hydrophila</i>	Recovery		
			20%	60 %	120 %
Abu-Ghraib	20	3 ^{A*}	15	5	2.5
Al-Fudhaliyah	20	2 ^B	10	3.33	1.66
Al-Sadrya	20	2 ^B	10	3.33	1.66
Total	60	7	35	11.66	5.83

*Indicate highest recovery from Abu-Ghraib. ^{A-B} Indicate significant differences vertically among regions at level $P \leq 0.05$.

Table 3. Recovery season of *A. hydrophila* from all samples

Month	Recovery %
February	5 (4.16 %) ^{A*}
March	3 (2.5 %) ^B
April	1 (0.83 %) ^C
May	1 (0.83 %) ^C
June	1 (0.83 %) ^C

*Indicate highest recovery from Abu-Ghraib. ^{A-B} Indicate significant differences vertically among regions at level $P \leq 0.05$



Figure 1. Heavy growth of *A. hydrophila* on modified ampicillin sheep blood agars



Figure 2. Biofilm feature of *A. hydrophila* on modified Congo red agar

Table 4. Antibiotics sensitivity profile of *A. hydrophila* isolates

Regions	Resistant %	Sensitive %
Abu-Ghraib	2 (18.18 %) ^{Aa*}	3 (27.27 %) ^{Ab}
Al-Fudhaliyah	1 (9.09 %) ^{Ba}	2 (18.18 %) ^{Bb}
Al-Sadrya	1 (9.09 %) ^{Ba}	2 (18.18 %) ^{Bb}
Total	4 (36.36 %) ^a	7 (63.63 %) ^b

*Indicate highest resistant isolates from Abu-Ghraib

^{A-B} Indicate significant differences vertically among regions in resistance at level $P \leq 0.05$.

^{a-b} Indicate significant differences horizontally within region in resistance at level $P \leq 0.05$

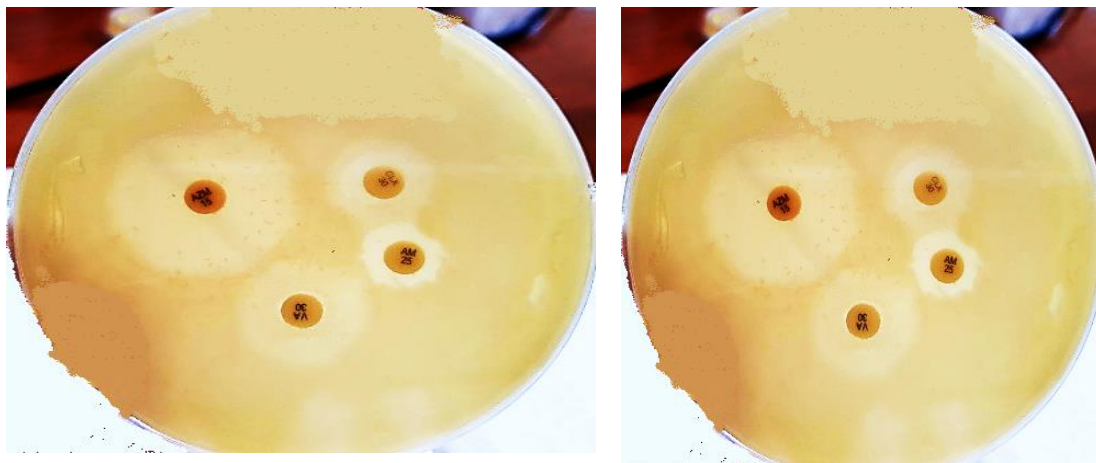


Figure 3. Antibiotics susceptibility profile of isolates. Antibiotics used in this procedure were: ampicillin (AM 10 µg), cephalexin (CLX, 30 µg), azithromycin (AZM, 15 µg) and vancomycin (VA 30 µg)

Microbial contamination of milk and dairy products is a universal problem. Foodborne microbial diseases account for twenty million cases annually in the world. The microbial load and incidence of the bacterial pathogens in foods are indicators of food quality.

Many of the microbiological hazards associated with dairy products such as butter, cheese, and yoghurt are derived from the raw milk. Preventing post process contamination by spoilage microorganisms and retarding the growth of surviving organisms remain challenge to the dairy industry. Therefore, increased emphasis should be placed on the microbiological examination of milk and dairy foods. It is emphasized that milk should be properly pasteurized and adequate hygienic measures should be adopted during the preparation of dairy products. In addition, the education of food handlers about personal hygiene is of pivotal importance from food safety point of view (29).

Foodborne infections have been identified as an important public health and economic problem in developed as well as developing nations. Hence, microbial food safety has emerged as a significant global issue for the consumer, industry, researcher, and regulatory bodies. The microbial

contamination is one of the leading causes of food spoilage worldwide (30). The contamination of food can occur at any stage of the food chain. Spoilage of food involves any change, which renders food unacceptable for human consumption. Microbiological safety of food during storage is related to many factors. Ready-to eat food products are consumed without any treatment between final production step and consumption (31). Contamination of our raw milk and fresh soft cheese by *A. hydrophila* represents new emerging issue in Baghdad environment.

These events might occur due to different reasons such as unclean environment when milk and cheese were produced, contaminated milk containers, infectious biofilm foci, infected and carriers animals and workers, etc. Presence of multidrug resistant clones represent dangerous hazard in daily food and food products. Development of resistance might occur due to biofilm producing clones transferred through food chain as an emerging new style of resistance (32-33). Finally, due to contamination of collected samples with serious food poisoning *A. hydrophila*, therefore, monitoring of these products is recommended for better hygienic status.

Conflict of Interest

The authors declare that there is no conflict of interest.

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التحري عن الإيرومونات هايدروفيلا (الغازية أليفة الماء) في الحليب الخام والجبن الطري في مدينة بغداد

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الخلاصة

أستهدفت هذه الدراسة التحقق من نمط تواتر وتوزيع الجراثيم الغازية أليفة الماء في الحليب الخام للأبقار والجبن الطري المتحرر عنه من مناطق أبو غريب والفضيلية والصدرية في بغداد. إذ تم جمع مائة وعشرين عينة خلال شهر فبراير حتى يونيو (2019)، ومن تم جمع ستين عينة من الحليب الخام المجمع من حاويات الحليب؛ تم جمع عشرين عينة من كل منطقة (أربع عينات يتم جمعها شهرياً لكل منطقة) وستين عينة من الجبن الطري الطازج المصنوع من الحليب غير المبستر (الخام) من محلات السوبر ماركت المختلفة في بغداد كما ذكرات النسبة أعلاه. إذ كانت الإجراءات المعدلة للعزل والتعرف على الغازية أليفة الماء معتمدة على طرائق ميكروبيولوجي الأغذية، إذ تم استخراج خميرة الصويا التربتونية المعدلة بأجار دم الغنم المضاف له المضاد الحيوي الأمبيسلين المستخدم لهذا الغرض. ساعدت تفاعلات الكيموحيوية متمثلة بصبغة الجرام واختبار أوكسيديز في الفصل بين العزلات. وقد تم التحري عن الأغشية الحيوية للعزلات باستخدام الوسط الزراعي المحور مختبرياً لصبغة الكونغو الحمراء وتم الكشف عن مدى حساسية ومقاومة العزلات للمضادات الحيوية بطريقة نشر القرص الحيوي لتقدير حساسيتها للامبيسلين والسيفالكسين والازثرومايسين والفانكوميسين. وقد أكدت النتائج عزل وتشخيص احدى عشرة عزلة (9.16%) من مائة وعشرين عينة مجمعة، منها أربع عزلات (3.33%) من عينات الحليب الخام؛ اثنان (1.66%) من أبو غريب وواحدة (0.83%) من كل المناطق الأخرى. وقد تم الكشف عن سبع عزلات (5.83%) من عينات الجبن الطازج، منها (2.5%) من أبو غريب واثنان (1.66%) من كل منطقة أخرى. كانت جميع العزلات سالبة الغرام على شكل عصويات قصيرة، موجبة لاختبار الاوكسيداز ومنتجة للأغشية الحيوية. كانت أربع عزلات (36.36%) مقاومة لكل المضادات الحيوية المختارة، اثنان منها (18.18%) من أبو غريب، و واحدة (9.09%) من الحليب الخام والجبن الطري؛ واحد (9.09%) من كل المناطق الأخرى في الجبن الطري الطازج فقط. استنتجت الدراسة تلوث عينات الحليب الخام والجبن الطري بالجراثيم الغازية أليفة الماء في مدينة بغداد وبالتالي نوصي بمراقبة هذه المنتجات لتحسين الحالة الصحية.

الكلمات المفتاحية: الغازية أليفة الماء، الغشاء الحيوي، المقاومة للمضادات الحيوية، الحليب الخام، والجبن الطري