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INCIDENCE OF PATHOGENIC *Aeromonas* SPP. IN MILK AND CERTAIN DAIRY PRODUCTS COLLECTED FROM DAKAHLIA GOVERNORATE

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ABSTRACT

Two hundred samples of raw milk and dairy products were examined for enumeration, isolation and identification of *Aeromonas* species. 10 ml/gm of each analyzed samples were added to 90 ml sterilized trypticase soya broth ampicillin, incubated at 28°C for 24h or 6 ±2°C for 7d to enrich *Aeromonas* spp. Plating on two media starch ampicillin agar (SAA) and bile salts irgasan brilliant green agar (BSIBG), incubated for 24 h at 28°C. Positive samples were enumerated, comparing cold with warm enrichment and between (SAA) and (BSIBG), then isolates were identified. Viability of *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* in the presence of lactic acid bacteria were studied too. (BSIBG) agar and warm enrichment were better than (SAA) and cold enrichment in isolation of *Aeromonas* spp. Kareish cheese has the highest contamination rate (30%) by *Aeromonas* spp., followed by raw cow's milk samples (28.6%). Raw goat's milk samples were the lowest contamination among all of the raw milk samples. The contamination rate of ice-cream samples was also of the lowest among all of the analyzed samples. (6.4×10^4) and (0.02×10^3) CFU/ml were the maximum and minimum detected populations of *Aeromonas* spp in raw cow's milk and ice-cream samples on (BSIBG) agar and (SAA), respectively. *A. hydrophila*, *A. caviae* and *A. sobria* were detected in all contaminated samples, but at varying degrees. Studying the presence of *Aeromonas* spp in raw milks and some dairy products then identification of the isolates in Dakahlia Governorate were the aim of this work.

Keywords: Isolation, identification, *Aeromonas* spp., milk and dairy products.

INTRODUCTION

The genera *Aeromonas* and *Plesiomonas* are included in the family Vibrionaceae and are primarily aquatic. They are Gram-negative rods, usually motile by a single polar flagellum, but peritrichous flagella may be found on solid media in young cultures or nonmotile, (Holt *et al*., 1994) Aeromonas is now classified within the family Aeromonadaceae, which can be divided into a psychrotrophic group and a mesophilic group. The psychrotrophic group contains the fish pathogen *A. salmonicida*, whereas the *Aeromonas* spp. regarded as potential human pathogens belonging to the motile mesophilic group (Fernandez- Escartın and Garcıa, 2001). Aeromonads are facultatively anaerobic, oxidase and catalase positive, reduce nitrate to nitrite, and ferment D-glucose with or without production of gas. (Holt *et al.;* 1994) and (Altwegg, 1999). *Aeromonas* spp. grow at the temperature range 0 to 42°C for example, *A. hydrophila* grow optimally around 28°C. However, of concern for microbial food safety, many strains grow at refrigeration temperatures (sometimes as low as 0.1°C). *Aeromonas*

spp. have the ability to grow anaerobically and are non-spore-forming bacteria (Daskalov, 2006; Fernandez-Escartın and Garcıa, 2001). Most microbiological laboratories dealing with food, water or clinical samples still report Aeromonas isolates as *A. hydrophila, A. sobria,* or *A. caviae* because it is difficult to differentiate the new *Aeromonas* species from the better known species. This is now being done with the understanding that these phenospecies are a collection of several genetically distinct groups that are biochemically similar, but could not unambiguously be separated from one another by phenotypic methods (Abbott *et al.,* 1992).The fatality rate of patients infected with *Aeromonas* species is up to 61% (Davis *et al*. 1978)

The major infections caused by *Aeromonas* spp. in humans can be classified in two major groups: septicemia, a general infection caused mainly by *A. veronii* subsp. *sobria* and *A. hydrophila*; and gastroenteritis, which is due primarily to *A. hydrophila* and *A. veronii* (Daskalov, 2006; Fernandez-Escartın and Garcıa, 2001). *Aeromonas* spp. may play a significant role in "summer diarrhea," a worldwide seasonal problem affecting children under 5 years old, the elderly, and travelers particularly. Acute self-limited diarrhea is more frequent in young children, whereas in older patients, chronic enterocolitis may also be observed. Fever, vomiting, and fecal leukocytes or erythrocytes (colitis) may be present in these types of infections. Furthermore, *Aeromonas* spp. have been responsible for extra intestinal infections, including meningitis and pulmonary and wound infections, and have been linked to cases of hemolytic uremic syndrome (Fernandez-Escartın and Garcıa, 2001).

Mesophilic aeromonads have been found in a wide variety of aquatic environments, including drinking water, sewage, groundwater, and streams and rivers. These pathogens have also been isolated from many foodstuffs, including green vegetables, raw milk, ice cream, beef, lamb, chicken, fish, and seafood (Daskalov, 2006; Fernandez-Escartın and Garcıa, 2001).

MATERIALS AND METHODS

A total of 45 raw cow's, 35 raw buffalo's, 20 raw goat's, 12 raw ewe's milk samples;10 pasteurized milk samples and 20 each of yoghurt, Domiati and kareish cheese samples, were collected from livestock farms, milk-collecting centers , local markets and street peddlers in Dakahlia Governorate. All samples were collected under aseptic precautions and transferred to the laboratory as soon as possible to examine bacteriologically for the presence of *Aeromonas* spp.

Milk samples were tested for heat treated and shaken thoroughly, while yoghurt, kareish and domiati cheese were grind well in sterile mortar Richardson (1985) For enrichment, ten ml of raw and pasteurized milk or 10 gram of kareish, domiati cheese, yoghurt and ice cream were homogenized in 90 ml sterilized trypticase soya broth containing 10 µg ampicillin/ml and blended for 2 minutes, for each sample, two flasks were prepared one of

them incubated at 6±2°C for while7 days the another at 28°C for 24 hours Villari *et al*, (2000)

For the isolation of the examined species, starch ampicillin agar (SAA) containing 10 µg ampicillin/ml Palumbo *et al*., (1985) and Jeppesen (1995) was prepared, autoclaved at 121°C for 15 min and Bile Salts Irgasan Brilliant Green agar (BSIBG) containing 10 µg ampicillin/ml Hunt *et al* (1987) ingredients, including 10 ml of Irgasan solution (50 mg/100 ml in ethanol), were added to the water, gently bring to the boil to dissolve completely and holded for one minute at boiling point. Both (SAA) and (BSIBG) were tempered to 50°C, ampicillin was added in a very small quantity of distilled water to achieve a concentration of 10 µg /ml in the two media.

1 ml of enrichment cultures was dilated and plated on duplicate sterile Petri plates, prepared media (SAA) and (BSIBG) were poured in the plates. Incubated at 28°C for 24 hours. After incubation, plates of (SAA) are flooded with Lugol iodine solution (ca.5 ml), and amylase-positive colonies (those having a clear zone surrounding the colony) are considered *A. hydrophila* The numbers of isolated Aeromonas were enumerated, and typical colonies were picked into triple sugar iron (TSI) and nutrient agar slants for biochemical identification(EL-prince,1998)

For the identification of motile *Aeromonas* spp., the colonies were examined for the esculin hydrolysis, growth on KCN broth, H_2S formation from cystein, gas formation from d-glycose, acid formation from arabinose, d-mannitol and salisin fermentation, methyl red -voges proskauer and indol tests (Palumbo *et al,* 1992). The biochemical reactions of motile *Aeromonas species* were given in Table 1.

Table (1): Identification tests applied for motile *Aeromonas* species.

Biochemical test	***A. hydrophila***	***A. caviae***	***A. sobria***
Esculin hydrolysis	+	+	–
Growth in KCN broth	+	+	–
H_2S from cysteine	+	–	+
L-arabinose utilization	+	+	–
Fermentation of salicin	+	+	–
Fermentation of mannitol	+	+	+
Gas from D-glucose	+	–	+
Methyl Metil red test	+	+	–
Voges-proskauer test	+	–	V
Indol production	+	+	+

V: Variable

Lyophilized culture of *Lactobacillus acidophilus* (type 145) and *Bifidobacterium* spp.420 were obtained from Laboratorium Wiesby, Niebull, Germany and yoghurt, was obtained from Chr. Hansen's Lab, Denmark, these cultures were activated in selective media.

Spray dried skim milk powder, low heat, of France origin was used during this work Viability of pathogenic *Aeromonas* spp. in the presence of lactic acid bacteria.

Three flasks of sterilized skim (50ml) were artificially infected with activated *A. hydrophila,* then inoculated by 2% active cultures of Yoghurt culture *Bifidobacterium* spp., or *L. acidophilus* the same work was done with *A. caviae, and A. sorbia*, after coagulation the flasks were stored at 6 ±2 °C, viable pathogenic bacteria were counted at zero, 1 and after 3days on (BSIBG) agar at 28°C for 24h.

RESULTS AND DISCUSSION

Concerning isolation of *Aeromonas* ssp., BSIBG as an isolation plating medium, was considered better than SAA, likewise warm enrichment at 28°C for 24 h was superior cold enrichment at 6 ±2°C for 7d in trypticase soya broth (Table 2). BSIBG realized higher isolation percentages (I.P.) achieved, compared with SAA in all of the examined samples, which agree with Gobat and Jemmi, 1995) who confirmed that BSIBG agar has been shown to be superior to that of ampicillin-containing media for the isolation of *Aeromonas* spp. from foods. Mattick and Donovan (1998) also found that BSIBG agar was of good selectivity more than SAA. BSIBG agar and SAA yielded the same (I.P.) (10%) in pasteurized milk samples. Warm enrichment surpassed cold enrichment, with the exception of only four cases in Table (2). Cold enrichment is important to these bacteria Palumbo *et al.* (1985) reported that *A. hydrophila* was not detected in two raw milk samples, at the time of purchase; however after 7 days of refrigerated storage at (5°C), levels reached to 10^3 - 10^4/ml., Callister and Agger (1987) found that *A. hydrophila* grew at 12 ºC slower than 22- 35 °C, but growth at 5 °C was considerably slower, their findings confirmed what we have reached. Villari *et al*, (2000) confirmed that 28°C is the best incubation temperature to enrich *Aeromonas* spp. *A. hydrophila* grows optimally around 28 °C. With regard to microbial food safety, many strains grow at refrigeration temperatures (sometimes as low as 0.1°C) (Daskalov, 2006).

Table (2): counts of contaminated samples with *Aeromans* spp. on different selective media with different enrichment conditions

Type Of Sample	NO. S.	SAA agar				BSIBG agar			
		Cold Enrichment at 6±2°C		Warm Enrichment at 28°C		Cold Enrichment at 6±2°C		Warm Enrichment at 28°C	
		p. s.	P .s.%	p. s.	p. s.%	p. s.	p. s.%	p. s.	p. s.%
Raw Cow's Milk	**42**	**8**	**19**	**10**	**23.8**	**10**	**23.8**	**12**	**28.6**
Raw buffalo's milk	33	4	12.1	5	15.1	5	15.1	6	18.2
Raw ewe's milk	20	2	10	2	10.0	3	15.0	4	20.0
Raw goat's milk	12	1	8.3	1	8.3	1	8.3	2	16.6
Pasteurized milk	10	0	0.0	1	10.0	1	10.0	1	10.0
kareish cheese	20	4	20.0	5	25.0	5	25.0	6	30.0
Dominate cheese	20	2	10.0	3	15.0	3	15.0	4	20.0
Yoghurt	20	1	5.0	2	10.0	2	10.0	3	15.0
Ice cream	20	0	0.0	1	5.0	1	5.0	1	5.0

No. S. = number of samples p. s. = positive samples
p. s. % = % of positive sample
These results the average of duplicate.

Table (2) revealed that raw cow's milk has the highest contamination rate by the mesophilic *Aeromonas* species (28.6%) among all analyzed raw milks, followed by raw ewe's (20%) milk, raw buffalo's milk (18.2%), then raw goat's milk (16.6%). kareish cheese has the highest contamination rate by the mesophilic *Aeromonas* species among all samples, and the manufactured analyzed products (30%), positive domiati cheese samples, were (20%). The positive yoghurt samples were (15%). It could also be appeared that pasteurized milk and ice cream samples have the lowest contamination rate 10, 5%, respectively. These results corresponded with Shweizer *et al* (1995), who detected 14.9% in raw milk, unlike findings (FDA, 1985) in a survey in the U. S. A. was made by the Food and Drug Administration and 50% of the raw milk samples tested contaminated with *A. hydrophila*. More recently, Korashy (2006), found very high contamination rate of raw milk was reached (86.7%), 76.7% in yoghurt, 70% in Kareish cheese and 16.7 in Pasteurized milk.

Generality, the higher incidence of Aeromonas in raw milk could be attributed to its wide distribution in the nature; Aeromonas microorganism is commonly present in farms, in feeding stuff, water, faeces and soil El-Shenawy and Marth (1990). The organisms can invade the udder tissues; multiply in mammary tissues and subsequently discharge in milk. Also the contaminated water used for washing milking equipments is considered as a significant source of contamination. So presence of mesophilic Aeromonas in a high level in raw milk samples is indicative to bad hygienic measures of milk production and distribution.

It's of interesting observation, that raw cow's milk has the highest contamination rates with *Aeromonas* spp. that back to that mentioned by El-Shenawy and Marth (1990), It might be Bedding and silage are also an important source of contamination by *Listeria* spp. and other potential human pathogens, such as *Yersinia enterocolitica* and *A. hydrophila*. (Griffiths, 2004). Early Neilson, (1978) found large numbers of these bacteria in sludge. The process of pasteurization was done at 71-75 °C for 15 second. *Aeromonas* organisms are sensitive to temperature above 48°C (Palumbo *et al,* 1987), and the strain of *Aeromonas* should have been killed during this process. However; in this study some pasteurized samples were found contaminated with it. So, the presence of mesophilic *Aeromonas* species in the pasteurized milk might be due to inefficient pasteurization or post pasteurization contamination during packaging of pasteurized milk. The contamination of the pasteurized milk might be due to subsequent handling of the milk. It should not be ignored even if the population is extremely small, since the pathogen can grow at refrigeration temperature and attain numbers which can cause illness (Palumbo *et al*, 1985; and Abeyta and Wekell, 1988) However, raw goat's milk is of the lowest contaminated samples with the *Aeromonas* spp among the analyzed raw milks; this might be due to a distinct antimicrobial impact of goat’s milk. Slacanac *et al*. (2004) attributed this to their specific composition, which might result in the increased antimicrobial compounds.

The high contaminated rate of Kareish cheese samples (30%) may be due to non-observance of sanitary conditions in production and skip pasteurization step, which are important and necessary.

The counts of *Aeromonas* ssp. ranged from 1.5 × 10^3 to 3.1×10^4 and from 2.5×10^3 to 6.4×10^4 on SAA and BSIBG, respectively in the raw cow's milk positive samples. It could also be noticed that 6.4×10^4 was the highest number in the all analyzed samples. Hence it could be concluded that raw cow's milk were of the highest positive samples, and contained the highest numbers of *Aeromonas* ssp. (Table 3) On the contrary, raw goats' milk positive samples recorded the lowest numbers (1.1 ×10^3 –1.5×10^4) and (1.7 ×10^3 -3.2 ×10^4) on SAA and BSIBG, respectively. On the other hand, positive pasteurized milk samples attained 5.1 ×10^4 on BSIBG, despite it was of low contamination rate samples. This indicates that post contamination is perilous, because of possibility sovereignty contaminator microbe.

Table (3): Counts of *Aeromaas* spp. on different solid selective media

Samples	SAA agar		BSIBG agar	
	Min× 10^3	Max×10^4	Min×10^3	Max ×10^4
Raw cow's milk	1.5	3.1	2.5	6.4
Raw buffalo's milk	3.1	2.8	2.5	5.9
Raw ewe's milk	1.5	2.5	2.1	3.7
Raw goat's milk	1.1	1.8	1.7	3.2
Pasteurized milk	0.5	0.1	1.1	5.1
kareish cheese	1.8	4.9	2.3	5.7
Dominate cheese	2.0	3.1	2.2	4.2
Yoghurt	0.7	1	1.1	3.1
Ice cream	0.02	0.07	0.5	1

Min = minimum counts Max = maximum counts

Respecting positive cheese samples,BSIBG agar yielded 2.3×10^3 – 5.7×10^4 and 2.2×10^3- 4.2 ×10^4 colonies of *Aeromonas* ssp. in kareish and Domiati cheese, respectively. Perhaps this is back to kareish cheese manufacture negligence in the countryside. Counts of *Aeromonas* ssp in positive yoghurt samples ranged between 0.7× 10^3 – 1 ×10^4 and 1.1× 10^3 – 3.1×10^4 on SAA and BSIBG, respectively, Role of lactic acid bacteria was clear in decreasing the contamination rate and numbers (CFU/ml) of *Aeromonas* ssp in positive yoghurt samples. Colonies corresponding *Aeromonas* spp. in positive ice cream samples were (1×10^4) per gram. Korashy, (2006) found the mean count value of 9.2 x10^6, 5.2 x 10^4, 5.1 x10^6 and 8.3 x10^5 in positive raw, pasteurized milk, yoghurt and kareish cheese samples, respectively.

Data given in table (4) show that 37 isolates were detected in raw cow's milk samples, 23 (62.2%) were identified as *A. hydrophila*, 12 (32.4%) *A. sorbia*, and *A. caviae* not detected, two isolates(5.4%) were not identified and were considered *Aeromonas* spp.; from 32 isolates in raw buffalo's milk, 18 (56.3%) were identified as *A. hydrophila*, 7(21.9%), as *A. sorbia* 3(9.4%) as *A. caviae* and 4(12.5%) as *Aeromonas* spp., from 16 isolates in Raw

ewe's milk, 7(43.8%) were identified as *A. hydrophila*, each of the *A. caviae* , *A. sorbia* and *Aeromonas* spp. were 3(18.8%). Raw goat's milk has 7(46.6%) of *A. sorbia* and 2(20%) of *A. hydrophila* and *A. caviae*. One isolate of both *A. hydrophila* and A. caviae were identified from two isolate's pasteurized milk. The highest isolates in kariesh cheese were *A. hydrophila* 11(39.3%), but *A. sorbia* was 8(28.6%) and *A. caviae* was 5(17.9%), undefined isolates were 4(14.3%). Domiate cheese isolates were 3(42.9%) *A. hydrophila*, 2(28.6%) *A. caviae* and 2(28.6%) *A. sorbia,* In Yoghurt A. caviae was the highest Isolates followed by both of *A. hydrophila* and *A. sorbia*. At the same time, ice cream was of the greatest contamination by *A. caviae*.

Table (4): Incidence of pathogenic Aeromonas isolates recovered from different samples of milk and certain dairy products.

Type Of Sample	NO.S.	T.I.	*A. hydrophila*		*A. sobria*		*A. caviae*		*Aeromonas spp.*	
			N.I.	%	N.I.	%	N.I.	%	N.I.	%
Raw cow's milk	42	37	23	62.2	12	32.4	0	0	2	5.4
Raw buffalo's milk	33	32	18	56.3	7	21.9	3	9.4	4	12.5
Raw ewe's milk	20	16	7	43.8	3	18.8	3	18.8	3	18.8
Raw goat's milk	12	15	3	20	7	46.6	3	20	2	13.3
Pasteurized milk	10	2	1	50	-	-	1	50	-	-
kareish cheese	20	28	11	39.3	8	28.6	5	17.9	4	14.3
Dominate cheese	20	7	3	42.9	2	28.6	2	28.6	-	-
Yoghurt	20	4	1	25	1	25	2	50	-	-
Ice cream	20	3	1	33.3	-	-	2	66.6	-	-

No.s. = number of samples **T.I. = total of isolates**
These results the average of duplicate. **N.I. =number of isolates**

The antagonistic effect of lactic acid bacteria on the pathogenic isolates of*A. hydrophila, A. sorbia,* and *A. caviae,* was clear in Table (5). From these data It could be seen that a great number of these bacterial cells was destructed by *Bifidobacterium* spp., whereas percentage of destruction reached 91.2, 96.9 and 96.5 for *A. hydrophila*, *A. caviae* and *A. sorbia* after 3 days at 6 ±2 °C, respectively. *L. acidophilus* was the second in its destructive effect on the pathogenic bacteria under study. Yoghurt culture was less effective which realized a lower destruction percentage of isolates. These results might be due to acetic, formic or lactic acids produced by *Bifidobacterium* spp. from fermentable sugars. Acetic acid is much of inhibitory toward G-negative bacteria than is lactic acid. Furthermore, bifidobacteria produce H_2O_2 , bacteriocins, short fatty acids, some organic acids and unidentified antibacterial substances (Hassan *et al*., 1994) Thus, bifidobacteria might possess an advantage over *L.* acidophilus and yoghurt culture in some cases in inhibiting the growth of Gram- negative pathogens, the present results agree with those of Ozbas and Aylac (1995) and Ananthaya and Mongkol (2009) who reported that *Bacillus subtilis* P33 and 72 (as Probiotic strains) were found to have high inhibition activities against the growth of *A. hydrophila* by two assay methods: paper disc and well diffusion. On the other hand *L. acidophilus* produces several antibiotics – like substances such as acidolin, acidophilin, and lactocidin (shahani *et al*, 1977).

Table (5): survival of *Aeromonas* spp. in cold stored fermented milk with *Bifidobacterium* spp., *L. acidophilus* or yoghurt starter

Isolates of *Aeromonas* and their counts	Time of storage	Fermented milk with Lactic acid bacteria					
		Yoghurt starter	P.D.	*Bifidobacterium* spp.	P.D.	*L. acidophilus*	P.D.
A. hydrophila $12.5x10^4$	Zero	13.5		12.2	2.4	12.4	0.8
	Day	6.5	48	6.1	51.2	6.5	48
	3d	2.3	81.6	1.1	91.2	1.3	87.6
A. caviae $10.5x10^4$	Zero	11.1		10.2	2.9	10.3	1.9
	Day	5.6	46.7	4.2	60	4.5	57
	3d	2.9	72.4	0.32	96.9	1.5	85.7
A. sorbia $17.3x10^4$	Zero	18 .1		17.1	1.2	17.0	1.7
	Day	7.2	58.4	6.6	61.8	6.8	60.7
	3d	2.2	87.3	0.9	96.5	1.6	90.8

P.D. = Percentage of destruction **all numbers multiplied by 10^4**
These results the average of duplicate.

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وجود أنواع من البكتريا الممرضة أيروموناس باللبن وبعض المنتجات اللبنية في محافظة الدقهلية

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جُمعت مائتان من عينات اللبن الخام وبعض منتجات الالبان من محافظة الدقهلية ، أُخضعت للكشف عن وجود بكتريا *Aeromonas* species، أستخدمت بيئة Trypticase soya broth ampicillin, كبيئة تعزيزه لنمو الميكروب حيث تم التحضين على درجة 6 ±2°C لمدة 7 يوم وعلى 28°C لمدة 24 ساعة ، أُستخدمت بيئتان للعزل Starch ampicillin agar (SAA) و Bile Salts Irgasan Brilliant Green agar (BSIBG) حيث تم التحضين على 28 °C لمدة 24 ساعة .

تم مقارنة العينات الموجبة لوجود الميكروب على أساس درجة التعزيز البارد والدافىء، وكذلك على نوعى بيئة العزل، تم تعريف العزلات المتطابقة وتحديد نسبة كل من *A. hydrophila*, *A. caviae* and *A. sobria* و دراسة حيويتها في وجود بكتريا حامض اللاكتيك.

تفوق التعزيز الدافئ على البارد فى العزل وكذلك أظهرت بيئة ((BSIBG تفوقا على SAA)، كانت عينات اللبن البقرى الخام أكثر تلوثا (28.6%) لكن كانت عينات الجبن القريش الاكثر تلوثا بين العينات الخام والمصنعة (30%) على العكس كانت عينات الآيس كريم الأقل تلوثًا كان (6.4×10^4) أكبر عدد تم ضبطة بالنسبة للعينات وكان فى اللبن البقرى الخام ، كذلك (0.02×10^3) كان أقل عدد وتم رصده فى الآيس كريم .تأثرت العزلات الممرضة فى وجود بكتريا اللاكتيك بشدة.

قام بتحكيم البحث

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