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DETECTION OF ACINETOBACTER SPECIES IN MILK AND SOME DAIRY PRODUCTS

SAAD, N.M. [1]; AMIN, W.F. [1] and MOSTAFA, S.M. [2]
[1] Department of Food Hygiene, Faculty of Vet. Med., Assiut University
[2] Department of Food Hygiene, Animal Health Institute, Assiut. Corresponding author: wallaa_800@yahoo.com

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ABSTRACT

Two hundred and forty random samples of milk and some dairy products; Domiati, Kareish cheese and cream were collected from Assiut city, Egypt. The collected samples were examined for the incidence of *Acinetobacter species* using Leeds Acinetobacter selective medium. The obtained results revealed that Acinetobacter species was isolated from 15% of the raw milk samples, 3.3% of the Domiati cheese samples, 13.3% of the Kareish cheese samples and 13.3% of the cream samples, while couldn't be detected in the pasteurized milk samples. The isolated species were *Acinetobacter baumannii, Acinetobacter haemolyticus, Acinetobacter calcoaceticus* and *Acinetobacter junii*. *Acinetobacter baumannii* was detected using blaOXA-23-like, blaOXA-51-like, and class 1 integrase genes. The characterization of the *Acinetobacter species* for the production of lipolytic enzyme was studied. Out of the 27 isolated *Acinetobacter species*, 23 possessed lipolytic activity. The public health hazard of this microorganism in milk and some dairy products was discussed.

Key words: *Acinetobacter,* milk, dairy products, lipolytic activity.

INTRODUCTION

The importance of milk in diet is explained by its essential nutritive constituents. However, milk obtained from healthy udder contains numbers of bacteria that may proliferate depending on the handling and processing of milk. *Acinetobacter species* could be one of these bacteria. They are recognized as food borne pathogens and act as a potential human pathogen.

Bacteria of the genus Acinetobacter have gained increasing attention in recent years for their potential to cause severe nosocomial infections (Towner, 2006), their profundity in developing multidrug (MDR) and extreme drug resistance (XDR) (Prashanth and Badrinath, 2005) and for the ability of some strains to produce verotoxins (VA**)** (Grupper *et al.,* 2007). The genus Acinetobacter comprises 38 species which had been recognized (Visca *et al.,* 2011).

Acinetobacter spp. has long been recognized as a part of normal flora of the skin of animals and humans besides being present in soil, water, sewage, food and milk (Wani *et al.,* 2006). The gastrointestinal tract of humans is the most important reservoir of resistant strains. As a result of the food colonization studies, it has been suggested that transmission may be through food. Digestive tract colonization accounts for 41% of *Acinetobacter baumannii* cases (Corbella *et al.,* 1996).

Acinetobacter has emerged as an important microorganism (Peleg *et al.,* 2008). They are often associated with nosocomial infections, community acquired diarrhea outbreaks and pneumonia in tropical regions of the world especially during summer months (Chen *et al.,* 2001). Infrequent manifestations of Acinetobacter are meningitis, bacteremia, urinary tract infection and ophthalmic infections (Jain and Danziger, 2004).

Acinetobacter baumannii is an important emerging nosocomial pathogen worldwide. It is a frequent cause of skin and wound infections, septicemia, peritonitis, cholangitis, osteomyelitis and endocarditis (Dent *et al.,* 2010). *Acinetobacter haemolyticus* is associated with endocarditis and verotoxin production, and hence bloody diarrhea (Grotiuz *et al.,* 2006).

The most common sources of Acinetobacter in milk are residual water in milking machines, milk pipelines or coolers, inadequate cleaning of dairy equipment, dirty udders and teats, transport and storage of milk, and biofilm (Santana *et al.,* 2004).

Acinetobacter show lipolytic activity (Hantsis-Zacharov and Halpern, 2007). As a result of milk fat hydrolysis by bacterial lipases of Acinetobacter, free fatty acids are released causing changes in the

Corresponding author: Dr. AMIN, W.F.
E-mail address: wallaa_800@yahoo.com
Present address: Department of Food Hygiene, Faculty of Vet. Med., Assiut University

product flavor. The lipolytic flavor defects are noticeable in cream, cheese and sterilized milk (Champagne *et al.*, 1994).

Since, *Acinetobacter species* continue to be an important pathogen particularly *A. baumannii*, therefore, this work was planned to estimate the incidence of *Acinetobacter spp.* in milk and some dairy products and to study the lipolytic activity of the isolated strains.

MATERIALS AND METHODS

I. Sampling:

A total of 240 random samples of milk and some milk products were divided as 120 raw milk samples, 30 pasteurized milk samples, 60 Domiati and Kareish cheese (30 each) and 30 cream samples. All the samples were collected from different localities in Assiut city, Egypt. Samples were transferred to the laboratory as soon as possible to be examined for the incidence of *Acinetobacter spp.* Each milk sample supposed to be raw was tested for heat treatment using Storch test according to Lampert (1975) to exclude heat treated milk. The samples were prepared according to the technique recommended by A.P.H.A (1992).

II. Isolation and identification of Acinetobacter species:

One milliliter of each well mixed milk sample or 1 g of each prepared milk product was inoculated into nutrient broth. The broth was incubated at 37°C for 24-48 hours. Aliquots of the broth cultures were streaked onto plates of Leeds Acinetobacter agar medium and incubated at 37°C for 24-48 h. *Acinetobacter spp.* colonies appeared pink with mauve background (Jawad *et al.,* 1994). Gram negative bacilli were identified biochemically according to Procop *et al.* (2017). Acinetobacter isolates were non motile, oxidase negative, catalase positive, failed to decarboxylate lysine. *Acinetobacter species* differentiation was done by biochemical reactions including growth at 42°C, gelatin hydrolysis, citrate utilization and growth at 37°C according to Bouvet and Grimont (1987).

III. Detection of *Acinetobacter baumannii* by multiplex PCR for the identification of blaOXA-23-like, blaOXA-51-like and class 1 integrase genes:

DNA Extraction

Bacterial DNA was extracted following overnight culture on nutrient agar plates using QIAamp kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Amplification of the genes of *Acinetobacter baumannii* according to Turton *et al.* (2006)

The extracted DNA was subjected to PCR amplification in a thermal cycler (Master cycler, Eppendorf, Hamburg, Germany) using 25 µl reaction volumes with 3 µl of extracted DNA, 12.5 pmol of each primer (blaOXA-23-like, blaOXA-51-like & Int 1) (Pharmacia Biotech, USA) and 1.5 U of *Taq* DNA polymerase (Biotools, Madrid, Spain) in 1x PCR buffer containing 1.5 mM $MgCl_2$ (QIAGEN) and 200 µM of each deoxynucleoside triphosphate. The amplification conditions were denaturation 94°C for 3 min, followed by 35 cycles at 94°C for 45 sec, annealing at 57°C for 45 sec, elongation at 72°C for 1 min and extension at 72°C for 5 min. Amplified products were analyzed by 1.5% of agarose gel electrophoresis stained with ethidium bromide and visualized and captured on UV transilluminator.

The primers used are shown in the following table:

Primer	Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
OXA-23-like (F)	**blaOXA-23-like**	**5′** GATCGGATTGGAGAACCAGA **′3**	501	Woodford *et al.* (2006)
OXA-23-like (R)		**5′** ATTTCTGACCGCATTTCCAT **′3**		
OXA-51-like (F)	**blaOXA-51-like**	**5′** TAATGCTTTGATCGGCCTTG **′3**	353	
OXA-51-like (R)		**5′** TGGATTGCACTTCATCTTGG **′3**		
Int 1 (F)	**Class 1 integrase**	**5′** CAGTGGACATAAGCCTGTTC **′3**	160	Koeleman *et al.* (2001)
Int 1 (R)		**5′** CCCGAGGCATAGACTGTA **′3**		

IV. Detection of the lipolytic activity of the isolated *Acinetobacter spp.*

Overnight cultures were streaked onto tributyrin agar and incubated at 30°C for 3 days. The medium appeared opaque, but a clear zone surrounded lipolytic colonies (Harrigan and McCance, 1976).

RESULTS

Table 1: The Incidence of *Acinetobacter species* in the examined raw milk samples.

Raw milk samples	Positive samples		Acinetobacter species							
			A. baumannii		*A. haemolyticus*		*A. calcoaceticus*		*A. junii*	
	No./30	%	No./30	%	No./30	%	No./30	%	No./30	%
Street vendors	6	20	1	3.3	2	6.7	2	6.7	1	3.3
Dairy shops	4	13.3	4	13.3	-	-	-	-	-	-
Dairy farms	1	3.3	1	3.3	-	-	-	-	-	-
Farmers houses	7	23.3	6	20	1	3.3	-	-	-	-

Table 2: The Incidence of *Acinetobacter species* in the examined milk and dairy products samples.

The examined samples	No. of samples	Positive samples		*Acinetobacter species*							
		No.	%	*A. baumannii*		*A. haemolyticus*		*A. calcoaceticus*		*A. junii*	
				No.	%	No.	%	No.	%	No.	%
Raw milk	120	18	15	12	10	3	2.5	2	1.7	1	0.8
Pasteurized milk	30	-	-	-	-	-	-	-	-	-	-
Domiati cheese	30	1	3.3	1	3.3	-	-	-	-	-	-
Kareish cheese	30	4	13.3	3	10	-	-	1	3.3	-	-
Cream	30	4	13.3	3	10	1	3.3	-	-	-	-

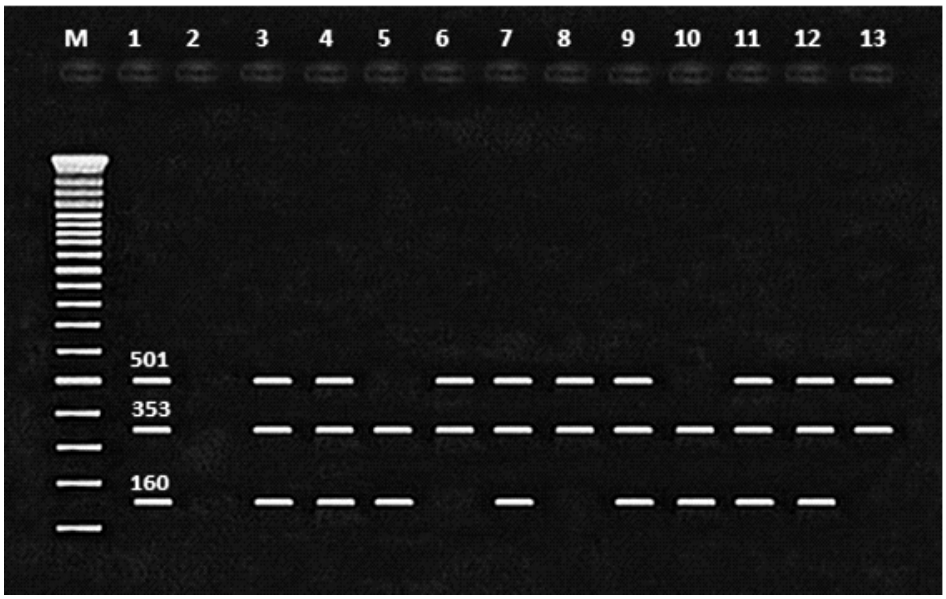

Figure 1. Detection of *Acinetobacter baumannii* by using blaOXA-23-like, blaOXA-51-like and Class 1 integrase (Int 1) genes.

Lane M: 100 bp ladder as molecular size DNA marker.
Lane 1: Control positive *A. baumannii* for blaOXA-23-like gene (501 bp), blaOXA-51-like gene (353 pb) and Int 1 gene (160 bp)
Lane 2: Control negative
Lanes 3- 13: Positive examined *A. baumannii* strains

Table 3: The lipolytic activity of the isolated *Acinetobacter species.*

The isolates source	The tested isolates No.	The lipolytic activity	
		No.	%
Raw milk	18	15	83.3
Domiati cheese	1	1	100
Kareish cheese	4	4	100
Cream	4	3	75
Total	27	23	85.2

Table 4: The lipolytic activity of the identified species of Acinetobacter organisms in the examined samples.

Source of isolates	Positive strains	Identified isolates							
		A. baumannii		*A. haemolyticus*		*A. calcoaceticus*		*A. junii*	
		No.	%	No.	%	No.	%	No.	%
Raw milk	15	9	60	3	20	2	13.3	1	6.7
Domiati cheese	1	1	100	-	-	-	-	-	-
Kareish cheese	4	3	75	-	-	1	25	-	-
Cream	3	2	66.7	1	33.3	-	-	-	-
Total	23	15	65.2	4	17.4	3	13.04	1	4.3

DISCUSSION

Acinetobacter species are of major concern because of their rapid development of resistance to a wide range of antimicrobials and their long persistence in the environment. In addition, their transmission is via person-to-person contact, water and food contamination (Doughari *et al.,* 2011).

This study took in consideration to cover most of the customers' purchasing habits by examining the four types of raw milk sources; street vendors, dairy shops, dairy farms, and farmers' houses.

The recorded results in Table 1 showed that 20% of the examined street vendors' raw milk samples were contaminated with *Acinetobacter spp.;* they included *A. baumannii* and *A. junii* (3.3% each), *A. haemolyticus* and *A. calcoaceticus* (6.7% each). The high percentage obtained in street vendors' raw milk samples may be due to the natural habitats of Acinetobacter in water, soil and environment and may be due to contaminated tanks (Fournier *et al.,* 2006). Moreover, *Acinetobacter species* were detected in 13.3% of dairy shops raw milk samples, only 3.3% of dairy farms milk samples were contaminated with *Acinetobacter spp.* In either type of samples *A. baumannii* was the only detected species. Higher incidence was reported by Nam *et al.* (2009) who isolated *Acinetobacter spp.* in a percentage of 8.2%. Also, *Acinetobacter spp.* was isolated from 23.3% of the examined farmers' houses raw milk samples; out of which, *A. baumannii* was detected in 20% of the samples and *A. haemolyticus* in 3.3%. The obtained results showed that the highest percentage of Acinetobacter was in farmers' houses raw milk samples. This could be explained by Wani *et al.* (2006) who mentioned that *Acinetobacter spp.* is recognized as a normal flora of skin of animals and humans besides being present in soil, water and sewage.

It was clear from Table 2 that *Acinetobacter spp.* was detected in 15% of the raw milk samples; *A. baumannii* (10%), *A. haemolyticus* (2.5%), *A. calcoaseticus* (1.7%) and *A. junii* (0.8%)**.** Other investigators could isolate *Acinetobacter spp.* from raw milk in lower incidences; Uraz and Çitak (1998) could isolate *Acinetobacter spp.* in a percentage of 4.5%, while Gurung *et al.* (2013) isolated *Acinetobacter spp.* (7.7%) and *A. baumannii* (2.5%) from milk samples and Rafei *et al.* (2015) could isolate *A. baumannii* in 2.7% of raw milk samples.

Not surprisingly, *Acinetobacter spp.* was not detected in the examined pasteurized milk samples and it was because of the high pasteurization temperature. This was confirmed by Wang *et al.* (2006) who demonstrated that pasteurization was highly effective against Acinetobacter.

Acinetobacter spp. was isolated from 3.3% of the examined Domiati cheese samples (Table 2) and *A. baumannii* was the only recovered species. The

presence of Acinetobacter in Domiati cheese may be due to the use of raw milk or inefficient heat treated milk for the production of the cheese or contamination after pasteurization during the production and the handling of the cheese. Furthermore, the way of selling of Domiati cheese (not properly packed) could be a cause of contamination in the refrigerator of the groceries where various types of foods are put closely to each other.

The popularity of traditional cheese varieties is increasing progressively all over the world, and some are still being traditionally produced. Kareish cheese is a traditional cheese produced in Egypt from raw milk. Acinetobacter was detected in 13.3% of the examined Kareish cheese samples (Table 2); *A. baumannii* was detected in 10% of the Kareish cheese samples while *A. calcoaceticus* in 3.3%. It is worth mentioning that Rafei *et al.* (2015) isolated *A. baumannii* from raw cheese samples in a percentage of 14.3%. The presence of Acinetobacter in the Kareish cheese samples may be due to the bad handling and cutting during processing in farmers houses and contamination from soil, the primitive way of production and selling and the use of unpasteurized milk.

For the examined cream samples, *Acinetobacter spp.* was isolated (13.3%). *A. baumannii* (10%) and *A. haemolyticus* (3.3%) were identified.

The Multiplex PCR was used to identify the genes of *A. baumannii;* blaOXA-23-like, blaOXA-51-like and class 1 integrase genes (Figure 1). Turton *et al.* (2006) identified blaOXA-51-like gene in each of 141 isolates of *Acinetobacter baumannii*, and they confirmed that blaOXA-51-like is ubiquitous in *A. baumannii*.

Many investigators have reported the role of extracellular enzymes in virulence. Lipases constitute virulence factors by interacting with human leukocytes or by affecting several immune system functions by free fatty acids generated by lipolytic activity (Oliver *et al.*, 1997). Moreover, degeneration of milk components through various enzymatic activities can reduce the shelf life of the processed milk. Lipases hydrolyze tributyrin and milk fat and yield free fatty acids, which produce a range of flavor defects (Shah, 1994).

Out of the 27 tested Acinetobacter strains from the examined milk and dairy products, 23 (85.2%) showed lipolytic activity (Table 3). The results presented in Table 4 showed that out of the 15 strains showing lipolytic activity from the raw milk samples, 60% were *A. baumannii*, 20% *A. haemolyticus*, 13.3% *A. calcoaceticus* and 6.7% *A. junii*. Additionally, the only *A. baumannii* that was isolated from Domiati cheese showed lipolytic activity. The 4 strains obtained from the Kareish cheese samples possessing lipolytic activities were *A. baumannii* (75%) and *A. calcoaceticus* (25%). Only 3 strains out of four from the cream samples showed lipolytic activity; *A. baumannii* (66.7%) and *A. haemolyticus* (33.3%). Gennari *et al.* (1992) could isolate Acinetobacter from dairy sources and reported that only 3.7% of Acinetobacter was able to produce ropy milk due to their lipolytic activity.

Therefore, the aforementioned data proved that attention must be paid to the problems of this pathogen in food. Consequently, more restriction and preventive measures should be taken to improve the quality of milk and dairy products to protect consumers from being infected by the discussed microrganism. Also, a precautionary approach is advisable for the reduction or eradication of Acinetobacter from the human food chain should be encouraged.

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الكشف عن الأسينيتوباكتر في اللبن وبعض منتجات الألبان

نجاح محمد سعد ، ولاء فاروق أمين ، شيماء محمود مصطفى

E-mail: wallaa_800@yahoo.com Assiut University web-site: www.aun.edu.eg

تم جمع مئتان وأربعون عينة عشوائية من اللبن وبعض منتجات الألبان: الجبن الدمياطى والجبن القريش والقشدة من مدينة أسيوط، مصر. وقد تم فحص العينات لمعرفة مدى تواجد ميكروب الأسينيتوباكتر باستخدام مستنبت ليدز أسينيتوباكتر الانتقائي. وأظهرت النتائج أن الأسينيتوباكتر تم عزلها من ١٥٪ من الحليب الخام، و ٣.٣٪ من الجبن الدمياطى ، و١٣.٣٪ من الجبن القريش و ١٣.٣٪ من القشدة ، بينما لم يتم عزلها من عينات الحليب المبستر. والعترات المعزولة كانت *Acinetobacter baumannii* ، *Acinetobacter haemolyticus* ، *Acinetobacter calcoaceticus* و *Acinetobacter junii*. وقد تم الكشف عن *Acinetobacter baumannii* باستخدام جينات blaOXA-23-like ، blaOXA-51-like ، class 1 integrase genes. وكذلك تم دراسة انتاج الأسينيتوباكتر للإنزيم المحلل للدهون ووجد أن ٢٣ عزلة من أصل ٢٧ تمتلك نشاط محلل للدهون. هذا وقد تمت مناقشة خطورة تواجد هذه الميكروبات فى اللبن وبعض منتجات الألبان.

الكلمات المفتاحية: *Acinetobacter* ، اللبن، منتجات الألبان ، النشاط المحلل للدهون.