DETECTION OF ACINETOBACTER SPECIES IN MILK AND SOME DAIRY PRODUCTS

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Received: 16 November 2017; Accepted: 3 December 2017

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 profoundity 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
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 MgCl₂ (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% agarose gel electrophoresis stained with ethidium bromide and visualized and captured on UV transilluminator.

The primers used are shown in the following table:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Oligonucleotide sequence (5’ → 3’)</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-23-like (F)</td>
<td>blaOXA-23-like</td>
<td>5’ GATCGGATTGGAGAACAGA’3 5’ ATTTCTGACCCGATTCCAT’3</td>
<td>501</td>
<td>Woodford et al.</td>
</tr>
<tr>
<td>OXA-23-like (R)</td>
<td></td>
<td></td>
<td>353</td>
<td>(2006)</td>
</tr>
<tr>
<td>OXA-51-like (F)</td>
<td>blaOXA-51-like</td>
<td>5’ TAATGCGTTTGATCGGCTTG’3 5’ TGGATTCGACCTTCATCTGG’3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA-51-like (R)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Int 1 (F)</td>
<td>Class 1 integrase</td>
<td>5’ CAGTGGAATACATAGCCTTTC’3 5’ CCCGAGGCATAGACGTGA’3</td>
<td>160</td>
<td>Koeleman et al.</td>
</tr>
<tr>
<td>Int 1 (R)</td>
<td></td>
<td></td>
<td></td>
<td>(2001)</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Raw milk samples</th>
<th>Positive samples</th>
<th>Acinetobacter species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No./30 %</td>
<td>A. baumannii A. haemolyticus A. calcoaceticus A. junii</td>
</tr>
<tr>
<td>Street vendors</td>
<td>6/20 3.3%</td>
<td>2/6.7% 2/6.7% 1/3.3%</td>
</tr>
<tr>
<td>Dairy shops</td>
<td>4/13.3%</td>
<td>- - - -</td>
</tr>
<tr>
<td>Dairy farms</td>
<td>1/3.3%</td>
<td>- - - -</td>
</tr>
<tr>
<td>Farmers houses</td>
<td>7/23.3%</td>
<td>6/20 1/3.3%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>The examined samples</th>
<th>Positive samples</th>
<th>Acinetobacter species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. %</td>
<td>A. baumannii A. haemolyticus A. calcoaceticus A. junii</td>
</tr>
<tr>
<td>Raw milk</td>
<td>120 18 15</td>
<td>12 10 3 2.5 2 1.7 1 0.8%</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>30 - - - - - -</td>
<td></td>
</tr>
<tr>
<td>Domiati cheese</td>
<td>30 1 3.3 1 3.3 - - - - -</td>
<td></td>
</tr>
<tr>
<td>Kareish cheese</td>
<td>30 4 13.3 3 10 - - 1 3.3 - -</td>
<td></td>
</tr>
<tr>
<td>Cream</td>
<td>30 4 13.3 3 10 1 3.3 - - - -</td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>The isolates source</th>
<th>The tested isolates No.</th>
<th>The lipolytic activity No. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>Domiati cheese</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Kareish cheese</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cream</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>23</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>Positive strains</th>
<th>Identified isolates</th>
<th>A. baumannii</th>
<th>A. haemolyticus</th>
<th>A. calcoaceticus</th>
<th>A. junii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Raw milk</td>
<td>15</td>
<td>9</td>
<td>60</td>
<td>3</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Domiati cheese</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kareish cheese</td>
<td>4</td>
<td>3</td>
<td>75</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Cream</td>
<td>3</td>
<td>2</td>
<td>66.7</td>
<td>1</td>
<td>33.3</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>15</td>
<td>65.2</td>
<td>4</td>
<td>17.4</td>
<td>3</td>
</tr>
</tbody>
</table>

**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. calcoaceticus* (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
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 Rafi 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 activities (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, 29% A. haemolyticus, 13.3% A. calcoaceticus and 6.7% A. junii. Additionally, the only A. baumannii that was isolated from Domiat 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 produceropy 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 microorganism. Also, a precautionary approach is advisable for the reduction or eradication of Acinetobacter from the human food chain should be encouraged.

REFERENCES


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الكشف عن الأسينيتوباكتير في اللبن وبعض منتجات الألبان

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تم جمع مئتان وأربعون عينة عشوانية من اللبن وبعض منتجات الألبان: الجبن الدمياني والجبن القريش والمشوية من مدينة أسوان، مصر. وقد تم فحص العينات لمعرفة مدى تواجد ميكروب الأسينيتوباكتير باستخدام مستنبت ليذ الأسينيتوباكتير المتعدد. وأظهرت النتائج أن الأسينيتوباكتير تم عزلها من 15% من الحليب الخام، و13.3% من الجبن الدمياني، و14% من الجبن القريش، و Acinetobacter baumannii من المشوية، بينما لم يتم عزلها من عينات الجبن المشوية. والتعارضات المعاوزة كانتAcinetobacter junii و Acinetobacter calcoaceticus و Acinetobacter haemolyticus و Acinetobacter baumannii .class 1 integrase genes ، blaOXA-51-like ، blaOXA-23-like. باستخدام جينات Acinetobacter baumannii Acinetobacter baumannii Acinetobacter baumannii Acinetobacter baumannii. وكذلك تم دراسة انتاج الأسينيتوباكتير لإنتاج المحلول للدهون ووجد أن 23 عزلة من أصل 27 تم تشكيل نشاط محلل الدهون. هذا وتم مناقشة خطوة تواجد هذه الميكروبات في اللبن وبعض منتجات الألبان.

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