Zagazig Veterinary Journal, ©Faculty of Veterinary Medicine,
Zagazig University, 44511, Egypt.
Volume 47, Number 4, p. 388-397, December 2019
DOI: 10.21608/zvjz.2019.14730.1059

RESEARCH ARTICLE

Coliforms Contamination in Raw Milk and Some Dairy Products with Special Reference to Comparative Identification of *Enterobacter* spp.

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Article History: Received: 11/07/2019 Received in revised form: 01/08/2019 Accepted: 18/08/2019

Abstract

The current study was undertaken to examine 200 sample of raw milk and some dairy products (Kariesh cheese, plain yoghurt, milk powder and infant formula) for contamination with Coliform group especially *Enterobacter* spp. Coliforms were detected in; 42/50 (84%) raw milk samples from farmers' houses, 25/30 (83.33%) kariesh cheese samples and 23/30 (76.67%) plain yoghurt samples, however, they could not be detected in any of raw milk samples from dairy shops, milk powder and infant formula samples. The mean values of coliforms in the examined samples were $2.80x10^6 \pm 0.73x10^5$, $2.30\times10^6 \pm 0.75\times10^4$ and $1.08\times10^6 \pm 1.50\times10^4$ cfu /ml or gm in raw milk from farmers' houses, kariesh cheese and plain yoghurt samples, respectively. The biochemically identified coliforms were *E. aerogenes, E. agglomerans, E. cloacae, C. diversus, C. freundii, E. coli, K. oxytoca* and *K. pneumonia* with respective percentages of; 1.19, 1.19, 2.38, 25, 15.48, 26.19, 13.09 and 15.48, in raw milk from farmers' houses, 6, 0, 2, 28, 10, 22, 0, and 32 in kariesh cheese, 0, 0, 2.12, 23.91, 0, 36.96, 26.09, 10.87 in plain yoghurt. Comparative identification of isolated *Enterobacter* spp. by standard biochemical methods and Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry revealed that the total conformity of identification of *Enterobacter* strains between standard biochemical technique and MALDI-TOF MS technique was 66.6%, where, it ranged from 50% to 100 for *E. aerogenes* and *E. cloaca*, respectively. However, the only identified *E. agglomerans* isolates from raw milk could not be confirmed by MALDI-TOF MS technique. It has been shown through these results that the presence of coliform and Enterobacter bacteria is an evidence of the lack of health requirements and thermal treatments of raw milk and some of its products (kariesh cheese and yoghurt).

Keywords: Milk, Dairy products, Coliforms, *Enterobacter*, MALDI-TOF MS.

Introduction

Good nutrition is the key of the good health which protects human life against diseases. In general, dairy foods are nutritious and typically balanced food stuffs, which become important sources of a healthy food [1]. Nowadays, it's known that "Milk is the most nearly perfect food" [2]. Raw milk is considered as a very good medium for micro-organisms growth due to its high nutrient content [3]. Coliforms is a group of Gram-negative bacteria which are used as indicators for sanitary quality of foods [4], also some of them are responsible for the development of objectionable taints in raw milk and unpasteurized dairy products [5]. Coliforms count above 500 cell / mL in milk indicates poor hygiene [6]. These bacteria cause occasionally food-borne illness [7].

Enterobacter spp. is one of coliform group found in the natural environment in variable habitats such as water, sewage, vegetables and soil [8]. They are known to act as opportunistic pathogens, which can cause numerous infections, including eye and skin

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infections, meningitis, bacteremia, pneumonia, urinary tract infections, wound, intestinal infections and surgical site infections [9].

Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was used as a comparative identification technique. It is an ionization technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation. It has been applied for the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) [10]. Therefore, the present study was conducted to investigate the public health importance of coliform group through isolation of coliforms from raw milk and some dairy products then biochemical and MALDI-TOF MS identification of the isolated coliforms with special reference to *Enterobacter* spp.

Therefore, the present study was conducted to investigate the public health importance of coliform group through isolation of coliforms from raw milk and some dairy products then biochemical and MALDI-TOF MS identification of the isolated coliforms with special reference to *Enterobacter* spp.

Materials and Methods

Collection of samples

A total of two hundred samples of raw milk and dairy products were examined in this study including 80 raw milk samples (30 from dairy shops and 50 from farmers' houses) and 30 each of kariesh cheese, plain yoghurt, milk powder and infant formula. Samples were collected randomly from Mansoura city, Dakahlia Governorate, Egypt during the period from February to July 2018. All samples were aseptically collected in their original containers or in sterilized capped bottles and transported in an ice box to Animal Health Research Institute laboratory (Mansoura) and analyzed immediately with a minimum of delay.

Preparation of samples

Preparation of serial dilution

Raw Milk

Samples were thoroughly mixed under aseptic condition. A total of 11 mL of well mixed samples were aseptically transferred into sterile bottle containing 99 mL of sterile phosphate buffer saline (PBS) to yield a final dilution of 1:10, from which decimal dilutions were prepared [11].

Kariesh cheese

Each sample was thoroughly mashed in a sterile morter before being emulsified in a diluent solution. Eleven grams of each prepared sample were transferred into a sterile blender warmed to 40- 45°C, followed by addition of 99 mL of 2% of Sodium citrate phosphate buffer warmed to 40-45°C and then blending for 2 min. at a speed sufficient to emulsify the sample thoroughly to make a dilution of 1:10, from which decimal dilutions were prepared [11].

Yoghurt samples, milk powder and infant formula samples

Yoghurt samples were thoroughly mixed under aseptic condition, however, milk powder and infant formula samples were reconstituted by following reconstitution instruction found on its original packages. Eleven grams of previously prepared sample were transferred into sterile, wide mouth container, containing 99 mL of sterile dilution buffer (40°C to 45°C) and thoroughly mixed until a homogeneous 1:10 solution obtained, from which decimal dilutions were prepared [11].

Enumeration of coliforms

One milliliter of each previously prepared dilutions was transferred into an empty sterile plate to which, 10 to 15 mL of Violet Red Bile Agar (VRBA, Hi Media) tempered to 44-46 °C were added. The mixture was allowed to solidify on a level surface, then an additional 3 to 4 mL of plating medium were distributed as an overlay, completely covered the surface of the solidified medium. The plates were inverted and incubated for 24 ± 2 hr at 32 ±1°C. Dark red colonies measuring 0.5 mm or more in diameter on un-crowded plates were counted (15-150 coliforms colonies) and the results were recorded [11].

Confirmed test with colonies from a solid medium

Typical and/or atypical colonies were transferred to tubes of Brilliant green bile

(BGB, HiMedia) broth and incubated for 48 ± 3 hr at 35 ±1 ºC. The presence of gas in the inverted Durham tube or effervescence after gentle agitation indicates a positive confirmed test. Failure of gas production within 48 hours indicates the absence of coliforms. The confirmed isolates were identified biochemically by Indole test, Methyl Red test, Voges – Proskauer test and Citrate utilization test [12].

Fish production is one of the most important industrial activities in Egypt [1]. Damietta is a littoral Governorate with an important role in fish production. However, infection of fish with microbial pathogens is considered a risk factor in aquaculture industry resulting in a dramatic loss in economy [2]. *Aeromonas* infection in fish causes world economic problems because of high number of fish mortalities in different countries [2]. Fish can be contaminated with *Aeromonas* spp. either by polluted water or by handling, processing and bad storage conditions [3]. *A. hydrophila* is considered the most important zoonotic pathogen of concern. It may cause intestinal and extra-intestinal diseases in humans such as septic arthritis, diarrhea (traveler's diarrhea), gastroenteritis, skin and wound infections, meningitis, and fulminating septicemia [4-8]. Numerous case reports have described the isolation of *Aeromonas* from patients with acute diarrhea, but the bacterium can also be isolated from stool of healthy persons [9]. Random Amplification of Polymorphic DNA technique (RAPD) is used to clarify the genetic relatedness among different strains and is considered an accurate method in classifying microorganisms for epidemiological studies [10, 11]. Moreover, RAPD could be utilized as species specific indicator and bacteriological diagnostic marker [3]. This technique has a role in differentiation among different subgenera, which helps in organizing the variance pattern of their genetics [12].

Proteomic identification of isolated Enterobacter spp. based on MALDI-TOF MS

Nine biochemically confirmed isolates of coliforms (4 *E. aerogenes*, 1 *E. agglomerans* and 4 *E.* cloaca) were subjected to MALDI-TOF MS (MALDI- TOF /TOF ultra flextreme bruker daltonics Germany, flex control, Biotyper RTC) in the Clinical Laboratories, Faculty of Medicine, Alexandria University to calculate the agreement of identification between the biochemical methods and MALDI-TOF MS, according to the following technique

Preparation of Bacterial Extracts for MALDI-TOF MS

For the MALDI-TOF MS analysis, low-molecular-weight soluble proteins were extracted from intact bacterial cells using the simple and fast method [13].

Spectra Acquisition

The analyte was embedded in a very large excess of a matrix compound deposited on a solid surface called a target, usually made of a conducting metal and having spots for several different samples to be applied. After a very brief laser pulse, the irradiated spot was rapidly heated and became vibrationally excited. The matrix molecules energetically ablated from the surface of the sample absorbed the laser energy and carried the analyte molecules into the gas phase as well. During the ablation process, the analyte molecules were ionized by being protonated or deprotonated with the nearby matrix molecules because the most common MALDI ionization format is for analyte molecules to carry a single positive charge.

Results and Discussion

Raw milk is strongly implicated in human food poisoning (FP) and transmission of various human pathogens [14].

Table 1: Statistical analytical results of total coliforms count (cfu /ml or gm.) in raw milk and some milk products in Dakhlia Governorate, from February to July 2018

Samples	No. of examined samples.	Positive samples		Count		
		No	%	Min.	Max.	Mean ± S.E
Raw milk (dairy shops)	30	0	0.00	0.00	0.00	0.00
Raw milk (farmers)	50	42	84.00	6.00×10^5	8.09×10^6	$2.80 \times 10^6 \pm 0.73 \times 10^5$
Kariesh cheese	30	25	83.33	2.00×10^5	4.04×10^6	$2.30 \times 10^6 \pm 0.75 \times 10^4$
Plain yoghurt	30	23	76.67	1.87×10^5	2.26×10^6	$1.08 \times 10^6 \pm 1.50 \times 10^4$
Milk powder	30	0	0.00	0.00	0.00	0.00
Infant formula	30	0	0.00	0.00	0.00	0.00

S.E = Standard error of mean
Min: Minimum
Max: Maximum
cfu: Colony forming unit

In the current study, a total of 200 milk and dairy products samples were analyzed for the presence of coliforms. The results illustrated in Table (1) reveal that 42 (84%) out of 50 raw milk samples from farmers' houses, 25 (83.33%) out of 30 kariesh cheese samples and 23 (76.6%) out of 30 plain yoghurt samples were contaminated with coliforms. Coliforms failed to be detected in raw milk samples from dairy shops, while, they ranged from 6.00×10^5 to 8.09×10^6 with a mean value of $2.80 \times 10^6 \pm 0.73 \times 10^5$ in raw milk samples collected from dairy farmers' houses. Lower findings obtained by Sobeih *et al.* [16] who found that, the overall average count of coliforms was $2.5 \times 10^6 \pm 3.9 \times 10^5$ cfu/ml and Ibrahim *et al.* [17] who found that, the logarithmic average of colifrms was 2.5×10^3 cfu/ml. On the other hand, higher count was obtained by Chye *et al.* [18]. This result may be attributed to machines, manual milking, and handling as well as inferior quality water.

While, coliforms count in kariesh was ranged from 2.00×10^5 to 4.04×10^6 with Mean value of $2.30 \times 10^6 \pm 0.75 \times 10^3$. Nearly similar results were obtained by Abd El-Latif [19].

Many sources lead to microbial contamination of kariesh cheese as beginning with using inferior quality of raw milk, processing under unorganized environments and selection of the unfitting starter culture for the fermentation [7].

In plain yoghurt coliforms were ranged from 1.87×10^5 to 2.26×10^6 with Mean value of $1.08 \times 10^6 \pm 1.50 \times 10^4$. This result is nearly similar to El-Diasty *et al.* [20] and lower than El-Biaa [21]. On the other side, coliforms were absent in the examined yoghurt samples [22]. The presence of coliforms in milk and milk products is an indication of unsanitary production and or improper handling of either milk or milk utensils [23].

Table 2: Biofilm production in *Aeromonas hydrophila* species isolated from fish tissues and human stool at 4°C, 25°C and 35°C

Temperature	Non-producer	Degree of biofilm production (%, average OD± SD)			Overall biofilm producers
		Weak	Moderate	Strong	
4˚C	12 (48%, 0.029± 0.008)	5 (20%, 0.130± 0.011)	8 (32%, 0.279± 0.014)	0	13 (52%, 0.222± 0.013)
25˚C	4 (16%, 0.051± 0.009)	6 (24%, 0.171± 0.010)	7 (28%, 0.334± 0.020)	8 (32%, 0.785± 0.009)	21 (84%, 0.459± 0.013)
35˚C	1 (4%, 0.072± 0.002)	0	8 (32%, 0.297 ± 0.020)	16 (64%, 0.714± 0.018)	24 (96%, 0.575± 0.019)

OD: Optical Density
SD: Standard Deviation

The prevalence of *E. aerogenes, E. agglomerans, E. cloacae, C. diversus, C. freundii, E. coli, K. oxytoca* and *K. pneumoniae* in raw milk collected from farmers' houses were 1.19%, 1.19%, 2.38%, 25%, 15.48%, 26.19%, 13.09% and 15.48 %, respectively(Table 2). These results differ from those recorded by Bahout and Moustafa [24] who found that the prevalence of *E. aerogenes, E. agglomerans, E. cloacae, C. diversus, C. freundii, E. coli, K. oxytoca* and *K. pneumoniae* were 38.89%, 34.34%, 28.77%, 31.34%, 47.77%, 18.89%, 36.78% and 20.00%, respectively. *Enterobacter* is a member of the coliform group of bacteria. There are two clinically important species from this genus, *E. aerogenes* and *E. cloacae* [25]. On the other hand the prevalence of *E. aerogenes, E. cloacae, C. diversus, C. freundii, E. coli,* and *K. pneumoniae* in kariesh cheese isolates were 6.00%, 2.00%, 28.00%, 10.00%, 22.00%, and 32.00%, respectively, but *E. agglomerans* and *K. oxytoca* failed to be detected in these samples. These results were less than those reported by El-Bagory *et al.* [26]. The high prevalence of coliforms in kariesh cheese samples indicates the bad quality and neglected sanitary measures of these products. Detection of coliforms often reflects faecal contamination [27]. *Enterobacter* species are known to act as opportunistic pathogens; they can cause numerous infections, including eye and skin infections, meningitis, bacteremia, pneumonia, urinary tract infections, wound, intestinal infections and surgical site infections [28]. The occurrence of *E. cloacae, C. diversus, E. coli, K. oxytoca* and *K. pneumoniae* in plain yoghurt samples were 2.17%, 23.19%, 36.96%, 26.09% and 10.87%, respectively, while *C. freundii, E. aerogenes* and *E. agglomerans* could not be detected in these isolates (Table 2). The prevalence of *E. freundii, E. aerogenes, E. cloacae, E. coli,* and *K. pneumoniae* in yoghurt samples was 2 (6.89%), 3 (10.34%), 3 (10.34%), 4(13.79%) and 13 (44.82%), respectively [29].

E. aerogenes have taken on clinical significance as opportunistic bacteria and have been emerged as nosocomial pathogen from intensive care patients, especially to those who are on mechanical ventilation [30]. *K. pneumoniae* constitutes a part of the pneumonia-causing microorganisms worldwide [31]. *K. oxytoca* causes meningitis, severe neurological complications such as hydrocephaly, empyema, and brain abscesses [32].

Table 3: Prevalence of MALDI-TOF MS identified *Enterobacter* species in examined milk and milk products' samples from Dakhlia Governorate

Enterobacter spp.	Raw milk		Kariesh		Yoghurt	
	No.	%	No.	%	No.	%
E. aerogenes	1	1/1	3	1/3	0	0/0
E. agglomerans	1	0/1	0	0/0	0	0/0
E. cloacae	2	2/2	1	1/1	1	1/1

The results in Table (3) revealed that *E. aerogenes* was confirmed by MALDI-TOF MS technique in 1 (100%) and 1 (33.3%) isolates out 1 and 3 of biochemically identified *E. aerogenes* isolates from raw milk and kariesh cheese, respectively. On the other hand, *E. cloacae* was confirmed in 2/2, 1/1 and 1/1 of the biochemically identified *E. cloacae* isolates from raw milk, kariesh cheese and plain yoghurt, respectively. However, *E. agglomerans* could not be confirmed by MALDI-TOF MS technique in the single biochemically identified isolate from raw milk. *E. agglomerans* is known today as *Pantoea aagglomerans*, occasionally reported to be an opportunistic pathogen in immunocompromised patients, causing wound, blood, and urinary-tract infections. Infections are typically acquired from infected vegetation parts penetrating the skin. Contaminated intravenous fluids or blood products are rarely the causative agent [33].

Blood stream infection can lead to disseminated disease and end-organ infection, mainly septic arthritis, endophthalmitis, periostitis, endocarditis and osteomyelitis in humans [34].

Table 4: Comparative identification between standard biochemical methods and MALDI-TOF MS for coliform isolates of milk and milk products from Dakhlia Governorate

Isolates	No. of isolates identified by biochemical methods	No. of identical isolates Identified by MALDI-TOF MS	AI * %
E. aerogenes	4	2	50
E. agglomerans	1	0	0
E. cloaca	4	4	100
Total conformity of identification			66.66

AI*: agreement of identification (No. of identical isolates identified by MALDI-TOF MS ÷ No. of isolates identified by biochemical methods)

The stated findings in Table (4) showed a total conformity of identification of *Enterobacter* strains between standard biochemical technique and MALDI-TOF MS technique was 66.6%, where, it ranged from 50% to 100 for *E. aerogenes* and *E. cloaca*, respectively. However, the only identified *E. agglomerans* isolates from raw milk could not be confirmed by MALDI-TOF MS technique.

This result was lower than that reposted by Van Veen *et al.* [35] who found by MALDI-TOF MS that correct species identification was observed in 97.7%. Meanwhile, it differs from Cherkaoui *et al.* [36] who evaluated his finding by both MALDI-TOF MS system (Bruker MS) and conventional biochemical test system, which gave 99.1%, while Rodrigues *et al.* [37] found matching in identification of *Enterobacter spp.* biochemically and by MALDI-TOF MS by 92.9%. Also Singhal *et al.* [38] compared between the burker MALDI-TOF MS and conventional phenotypic and found an agreement with a percentage of 95.4.

MALDI-TOF MS is a rapid diagnostics technique with low costs and accurate method of identification. It may be considered as an alternative technique for conventional biochemical methods for correct bacterial identification. MALDI-TOF MS has been successfully and extensively applied for the identification and typing of microorganisms implicated in clinical sector [39] and recently in food sector [40], proving higher identification and discrimination potentials, less-costiveness, rapidity and labor-saving compared to traditional tools [41].

In microbiology, MALDI-TOF MS allows the identification of microorganisms such as yeasts and bacteria. This identification is based on the analysis of the peptidic spectra (also called protein fingerprint signature) which is specific of each species and family [38]. Nowadays, MALDI-TOF MS can be used as a sensitive, reliable and rapid procedure for identification of various clinical bacterial isolates such as Enterobacteriaceae [42].

MALDI-TOF MS involves an ionization of the sample covered of an excess of matrix by using a laser which form protonated molecules, an acceleration of molecules by an electric field until a detector trough a vacuum flight tube, and a mass spectrum obtained from data analysis [43].

Conclusion

The current results allowed to assume that the sanitary measures adopted during production, handling and processing of raw milk from farmers' houses, kariesh cheese and plain yoghurt were neglected and this represent a public health hazard for humans. This was contrary to what the results showed for dairy shops raw milk, milk powder and infant formula. MALDI-TOF MS has a high discrimination and identification potentials, rapidity, less- costiveness and labor-saving

compared to traditional methods for bacterial identification.

Conflict of interest

There is no conflict of interest.

Acknowledgment

I wish to express our great thanks to all staff members of Food Control Department, Faculty of Vet. Med., Zagazig University, for their help and encouragement during this work. Also our gratitude for all members of Animal Health Research Institute – Mansoura Provincial Lab.

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الملخص العربي

مدي تواجد القولونيات فى اللبن الخام وبعض منتجاته مع إشارة الى التصنيف المقارن للانتيروباكتر

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أجريت الدراسة الحالية لفحص ٢٠٠ عينة من الحليب الخام وبعض منتجات الألبان (الجبن قريش ، زبادي عادي ، مسحوق الحليب وحليب الأطفال) للتلوث بجموعة الكوليفورم خاصة الانتيروباكتر. تم اكتشاف مجموعة الكوليفورم فى ٥٠/٤٢ (٨٤٪) من عينات الحليب الخام من منازل المزارعين ، ٣٠/٢٥ من عينات الجبن القريش (٨٣.٣٣٪) و٣٠/٢٣ من عينات اللبن الزبادي (٧٦.٦٧٪) ، فى حين لم يتم اكتشافها في أي من عينات الحليب الخام من محلات الألبان ومسحوق الحليب وعينات حليب الأطفال. أظهرت النتائج ان معدل تواجد بكتريا الكوليفورم فى اللبن الخام والجبن القريش ومنتجات الزبادى هى ٨٤ ٪و٨٣.٣٣ ٪و ٧٦.٦٧٪ على التوالى بمتوسط ٢.٨±٠.٧٣ , ٢.٣ ± ٠.٧٥ , ١.٠٨ ± ١. تم تصنيف العزلات بالأختبارات البيوكيميائية الى السيتروباكتر دايفرساس والإنتيروباكتر ايروجيينز و والانتيروباكتراجلوميرانز و الإنتيروباكتر كولواكي و الكليبسيلا اوكسى توكا و الكليبسيلا نيمونى و الايشيريشيا كولاى و الستروباكتر فروندای بنسب مختلفة من اللبن الخام والجبن القريش و الزبادي. تم تصنيف ميكروبات الأنتيروباكتر بأستخدام تقنية المالدى توف ومقارنتها بنتائج الأختبارات البيوكيميائية واسفرت النتائج عن وجود توافق بين نتائج الأختبارات بنسبة ٦٦.٦ ٪ حيث تراوحت هذه النسبة بين ٥٠٪ لعزلات الانتيروباكتر أيروجينز و ١٠٠٪ لعزلان الانتيروباكتر كلواكا. وقد اتضح من خلال هذه النتائج ان وجود ميكروبات الكوليفورم وخاصهة الأنتيروباكتر دليل على نقص الاشتراطات الصحية والمعاملات الحرارية للبن الخام وبعض منتجاته من الجبن القريش والزبادى السادة.