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PREVALENCE OF ENTEROBACTERIACEAE IN RAW MILK

By

Amira, O. Farhat [1], Nagah, M. Hafiz[2], Halawa, M.A.[2], Saad, M.F. [2]

[1]Veterinary Quarantine, Cairo Airport

[2]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Cairo University, Giza, P.O Box 12211, Egypt

ABSTRACT

Fifty samples of raw milk were randomly collected from different dairy shops and supermarkets in Cairo and Giza Governorates,Egypt.Collected samples were microbiologically examined for enumeration, isolation and identification of Enterobacteriaceae. The results showed that, the mean value of Total Enterobacteriaceae Count was $2.56\times10^7 \pm 0.05\times10^7$ cfu/ml. *Escherichia coli* could be found in 14% of the examined samples with a mean value of $5.65\times10^5 \pm 0.11\times10^5$ MPN/ml. *E.coli* serovars O146 and O27 could be detected in a percentage of 11.11 for each, while the majority of isolates was *Klebsiella* spp. with a percentage of 38.55. *Salmonella* could not be detected. It could be concluded that raw milk may harbor serious health risk in the study areas.

Keywords:

Enterobacteriaceae, raw milk, *Escherichia coli, Klebsiella* spp.

INTRODUCTION

Milk is considered a nutritious food; for the new-born mammals, human beings and it is considered as an excellent medium for microorganisms **(Espie and Madden, 1997; Rajagopal *et al.*, 2005 and LeJeune and Rajala-Schultz, 2009).** The microbiological quality of milk is influenced by the initial raw milk flora, conditions of processing and post heat treatment contamination **(Richter *et al.*, 1992)**. Enterobacteriaceae have medical and economic importance; their presence in large number indicates faecal contamination, unqualified processing and post processing contamination of food. Enterobacteriaceae spp. has been implicated in many food poisoning outbreaks **(Koneman *et al.*, 1994)**. The presence of Enterobacteriaceae organisms in milk is considered objectionable as they render the product of low quality and unsafe for consumption. This family includes a number of remarkable foodborne pathogens such as *Salmonella*, pathogenic *E. coli* and *Shigella* spp.

Other family members are regarded as opportunistic pathogens in clinical settings **(*Klebsiella* spp. and *Citrobacter* spp.)**. *Erwinia* spp**.** is associated with food spoilage and cause a great economic loss **(Baylis *et al.,* 2011).** In many developed countries, *Salmonella* is the most common cause of bacterial foodborne illness. *Salmonella* is widely distributed with several host species including mammals, birds, fish, reptiles and many recognized animal reservoirs. Also it is an important zoonotic pathogen and causes two distinct disease forms; non-typhoid salmonellosis and typhoid disease **(Baylis *et al.,* 2011).** *E.coli* is found in gastrointestinal tract of humans and a broad range of other animals **(Nataro and Kaper, 1998 and Baylis *et al.,* 2006).** It is a major organism in the microbiology of food, involved in foodborne gastroenteritis and an important indicator of probable fecal dairy products contamination **(El-Bagoury and mosaad, 2002)**. *Citrobacter* spp. is considered as environmental contaminants or harmless bacteria in intestinal tract of animals and man. These bacteria are distributed in soil, sewage, water, food and associated with high antimicrobial agents' resistance **(Lin *et al.,* 1987, El -Harrif-Heraud *et al.,* 1997 and Nada *et al.,* 2004).** *Klebsiella* spp. has been identified as colonizing hospital patients, where spread is associated with the handling of patients. Patients at highest risk are those with low immune systems, such as the elderly or very young. *K. pneumoniae* and *K. oxytoca* may cause destructive lungs inflammation **(Bartram *et al.,* 2003 and Ainsworth, 2004)**.

MATERIAL AND METHODS

1. Collection of samples:

Fifty samples of raw milk were randomly collected under complete sanitary conditions from different dairy shops and supermarkets in Cairo and Giza Governorates, Egypt. Collected samples were transferred to laboratory in an insulated ice-box with minimum of delay to be examined.

2. Guaiac test according to (Schonberg, 1956):

Each sample of raw milk was subjected to Guaiac test for elimination of that samples proved to be heat-treated. Five ml of sample were placed in a test tube to which 0.5 ml of freshly Guaiac reagent was added. The contents were thoroughly mixed for 3 second before being left to stand for 3 minutes, after which the developed color was matched with standard color scale.

3. Bacteriological examination:

3.1 Preparation of decimal serial dilutions according to (ISO, 2001).

One ml of milk sample was aseptically transferred with a sterile pipette to 9 ml of diluent 0.1% peptone water (Lab M, 104). The primary dilution was shaken for 10 second using mechanical agitator to obtain a 1/10 dilution, then transfer one ml of primary dilution to nine ml of sterilized diluents to obtain decimal serial dilutions.

3.2 Enumeration of Total Enterobacteriaceae Count according to (ISO, 2004).

One ml of each dilution was transferred into each of appropriately marked duplicate dishes, then approximately 10 ml of the violet red bile glucose medium (Oxoid, CM0485) was poured into each dish. After solidification of the mixture, a covering layer off approximately 15 ml of the violet red bile glucose medium was added to prevent spreading growth and to achieve semi-anaerobic conditions, and then the prepared dishes were incubated at 37˚C/24 hours. Characteristic colonies were purple or pink to red (with or without precipitation haloes). The dishes containing < 150 characteristic colonies were selected and counted. Five such colonies were randomly selected for subculturing for the confirmatory tests (glucose fermentation and oxidase test) and then Total Enterobacteriaceae Count was calculated CFU/ml. **3.3 Enumeration and isolation of *Escherichia coli* (MPN/ml) according to (BAM, 2002).** One ml from each previously prepared decimal dilution was inoculated into a series of 3 fermentation tubes containing Lauryl Sulphate Tryphosa (LST) broth (Oxoid, CM0451) supplemented with inverted Durham's tubes for collection of gas. Inoculated tubes, as well as, the control were incubated at 35ºC for 48 hours and examined for gas production. From each gassing LST tube (presumptive test), a loopful of each suspension was transferred to *Escherichia coli* (EC) broth tube (Oxoid, CM0853), EC tubes were incubated for 48 hours at 45.5 °C and examined for gas production. From the results obtained, MPN/ml of *E.coli* was calculated. Loopful from positive tube was streaked on the surface of petri dish containing Levine Eosin Methylene Blue Agar (Oxoid, CM0069) before being incubated at 35º C for 48 hours. The plates were examined for suspected colonies of *E. coli*.

3.4 Isolation of *Salmonella* according to (ISO, 2002).

Twenty-five ml of milk sample were aseptically added to two hundred and twenty-five ml buffered peptone water and incubated at 37˚C for 18 hours. 0.1 ml of the previous culture was transferred to 10 ml of Rapport-Vassiliadis (RVS) medium (Oxoid, 0669) tube**.**The inoculated

RVS broth was incubated at 41.5˚ C for 24 hours, then loopful of the culture obtained in the RVS broth was streaked on the surface of one large size petri dish containing Xylose Lysine Deoxycholate (XLD) agar (Oxoid, 0469). The dishes were inverted and placed in the incubator set at 37˚ C for 24 hours. After incubation, the plates were examined for typical and atypical colonies.

3.5 Identification of Enterobacteriaceae.

3.5.1 Morphological and Biochemical examination according to (Bergey's manual, 2005).

3.5.2 Biotyping of some *Escherichia coli* isolates using VITEK 2 compact system according to (Pincus, 2005). Examined at the Animal Health Research Institute, El Dokki, and Giza. The VITEK is an automated microbiology system utilizing growth-based technology, which accommodates the colorimetric reagent cards that are incubated and interpreted automatically. The reagent cards have sixty-four wells that can each contain an individual test substrate. Substrates measure various metabolic activities such as acidification, enzyme hydrolysis and growth in inhibitory substances presence. A pre-inserted transfer tube was used for inoculation for each card. Cards have bar codes that contain information on type of product, date of expiry and a specific identifier that can be joined to the sample either before or after loading the card onto the system. Under sterile condition, swab was used to transfer an enough number of colonies of a pure culture and to suspend microorganism in three ml of sterile saline. The turbidity was adjusted and measured using a turbidity meter called the DensiChekTM. Identification cards were inoculated with microorganism suspensions using an integrated vacuum device. The microorganism suspension in test tube was placed into a special rack (cassette) and the identification card was placed in the adjacent slot, while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 15 tests. The filled cassette was transported automatically into vacuum chamber station. After vacuum was applied and air was reinserted into the station, the organism suspension was obliged through the transfer tube into micro-channels that plug all test wells. Inoculated cards were passed by a technique, which cuts off the transfer tube and sealed the card before loading into the carousel incubator. The carousel incubator can adjust up to 60 cards. All card types were incubated on-line at 35.5 ºC. Each card was removed from the carousel incubator once every fifteen minutes, transported to optical system for reaction readings, then returned to incubator until the next reading. Data were collected at

15-minute intervals during the whole incubation period. Transmittance optical system permitted interpretation of test reactions by different wavelengths in the visible spectrum.

3.5.3 Serological identification of the isolated *Escherichia coli* according to (BAM, 2011). Slide agglutination technique, at the Animal Health Research Institute, El-Dokki, Giza, was adopted for serotyping of the same isolates of *E. coli*, which identified by VITEK 2 compact system, using available coli antisera of BEHRING WEKE AG, MARBURG W., Germany.

RESULTS

Table(1): Statistical analytical results of Total Enterobacteriaceae Count (CFU/ml) and *E.coli* Count (MPN/ml) of the examined raw milk samples.

Parameter	Total no. of samples	Positive samples		Min.	Max.	Mean	±S.E.M
		No.	%				
Total Enterobacteriaceae Count	**50**	**50**	**100**	**9.0×10**	**2.8×10^{8}**	**2.56×10^{7}**	**0.05×10^{7}**
***E. coli* Count**	**50**	**7**	**14**	**1.30×10^{3}**	**3.22×10^{6}**	**5.65×10^{5}**	**0.11×10^{5}**

Table (2): Frequency distribution of Total Enterobacteriaceae Count in the examined samples.

Intervals	no. of samples	%
$< 10^{2}$	**2**	**4.00**
10^{2}- $< 10^{4}$	**7**	**14.00**
10^{4}- $< 10^{6}$	**27**	**54.00**
10^{6}- $\leq 10^{8}$	**14**	**28.00**
Total	**50**	**100.00**

Table (3): Incidence of Enterobacteriaceae species in the examined samples (total number of isolates= 262).

Type of isolates	no.	%
Klebsiella pneumoniae* subsp.*ozaenae	**74**	**28.24**
Serratia fonticola	**60**	**22.90**
Enterobacter cloacae	**32**	**12.21**
Citrobacter freundii	**30**	**11.45**
Klebsiella pneumoniae* subsp.*pneumoniae	**27**	**10.31**
Enterobacter asburiae	**17**	**6.49**
Escherichia coli	**9**	**3.44**
Enterobacter intermedius	**8**	**3.05**
Proteus penneri	**2**	**0.76**
Hafnia alvei	**2**	**0.76**
Proteus mirabilis	**1**	**0.38**
Total	**262**	**100.00**

Table (4): Incidence of *E.coli* serovars in the examined isolates (no. = 9).

E.coli serovars	no. of isolates	%
O 146	**1**	**11.11**
O 27	**1**	**11.11**
Non-typed	**7**	**77.78**
Total	**9**	**100.00**

DISCUSSION

The Enterobacteriaceae is a family of Gram-negative, non-spore-forming bacteria and is one of the most important groups of bacteria known to man **(Baylis *et al.*,2011).** Results presented in (Table 1) reveal that, the 100% of the examined milk samples were positive for Enterobacteriaceae with a minimum of 9.0×10 CFU/ml, a maximum of 2.8×10^8 CFU/ ml and a mean value of 2.56×10^7 CFU/ml. Enterobacteriaceae count is taken as an index for the probable presence of enteric pathogens, which may constitute public health hazards **(Eman and El- Kaseh, 2008)**.These results were lower than that obtained by **Ibtisam and Mahboba (2007)** and higher than that obtained by **Eman and El- Kaseh (2008)**. Fourteen % of the examined samples of raw milk were positive for presence of *E.coli* either by selective EMB or by violet red bile glucose agar medium. The minimum count of *E.coli* was 1.30×10^3 MPN/ml, maximum count was 3.22×10^6 MPN/ml and the mean value was 5.65×10^5 $\pm0.11\times10^5$

MPN/ml. Higher count of *E.coli* was obtained by **Salman and Iman (2011), Pourhassan and Taravat Najafabadi (2011)**, while lower count was recorded by **Chye *et al.* (2004), Mubarack *et al.* (2011) and Uraz *et al.* (2014).** VITEK 2 system is a fully automated system for bacterial identification and antibiotic susceptibility testing. It is considered as a rapid method for identification of up to 20 samples within 8 hours **(Pincus, 2005)**. The highest frequency distribution of Enterobacteriaceae count in the examined samples lies within the range of 10^4- < 10^6 CFU/ml in percentage of 54 (Table 2). *Salmonella* failed to be isolated from all the examined samples neither by using violet red bile glucose agar nor by selective (XLD medium). The absence of *Salmonella* in the examined raw milk samples was agreed with obtained by **Ekici *et al.* (2004)**, while **Kivanc *et al.* (1992)** could detect *Salmonella* in two milk samples. Results presented in (Table 3) reveal that, the identification of Enterobacteriaceae isolated from the examined samples were *Escherichia coli, Klebsiella pneumoniae* subsp. *pneumoniae, Citrobacter freundii, Enterobacter cloacae* and *Serratia fonticola* in a percentage of 3.44, 10.31, 11.45, 12.21 and 22.90 from total number of isolates respectively. The highest percentage (28.24) of Enterobacteriaceae isolates was *Klebsiella pneumoniae* subsp. *ozaenae*, which was lower than that obtained by **Uraz *et al.* (2014).** Lower results were obtained by **Salman and Iman (2011) and Pourhassan and Taravat Najafabadi (2011).** *Klebsiella pneumoniae* is a world wide spread that can be responsible for arthritis, meningitis, appendicitis, cystitis, pneumonia and septicemia in newborns **(Bernabe *et al.*, 1998).** *Enterobacter* spp. results were higher than obtained by **Uraz *et al.* (2014)** and lower than **Salman and Iman (2011) and Pourhassan and Taravat Najafabadi (2011).** *Enterobacter* spp. was incriminated in urinary infection and septicemia **(Bernabe *et al.*, 1998).** *Citrobacter* spp. results were higher than obtained by **Salman and Iman (2011) and Uraz *et al.* (2014).** It can cause a wide range of infections; in urinary system, respiratory tract, bone, heart and bloodstream of humans **(Pavani, 2012)**. The natural habitat of *Serratia* spp. is a soil, plants and water, while *Serratia* mastitis in dairy cattle is associated with poor environmental, water quality and milking hygiene **(Hogan *et al.*, 1999)**. The obtained results of *Serratia* spp. were higher than obtained by **Salman and Iman (2011)** and lower than that obtained by **Uraz *et al.* (2014).** *Proteus* spp. results were lower than that obtained by **Pourhassan and Taravat Najafabadi (2011).** *Hafnia alvei* result was lower than obtained by **Uraz *et al.* (2014).** The presence of *E. coli, Enterobacter* spp., *Klebsiella* spp. and *Citrobacter* spp. as major

Coliforms is associated with low quality raw milk **(Jayarao and Wang, 1999 and FAO, 2008).** Gastroenteritis cases are associated with *Klebsiella pneumonia*, *E.cloacae*,*P.mirabilis*, *H.alvei* and *Citrobacter* spp. **(Kirov,1997).** *H.alvei, E.cloacae* and *P. mirabilis* are all opportunistic pathogens and may cause diarrhoea. *C. freundii* may be a normal inhabitant of the intestine, but some of them represent a potential hazard **(Schauer*et al.*,1995).**The isolated Enterobacteriaceae may not necessarily indicate a fecal contamination of milk, but it is an accurate indicator of bad sanitary practices during milking and handling processes. The presence of *E. coli* represents a risk that other enteric pathogens may be found **(Hayes *et al.*, 2001).** *Escherichia coli* are representing a portion of the intestinal tract normal microflora of warm-blooded animals and human. Also it is used as good indicator to detect and measure fecal contamination in the evaluation of food safety **(Borgatta *et al.,* 2012)**. The serological typing of the isolated *E.coli* were identified as O 146 and O 27 in a percentage of 11.11 for each, while non-typed isolates could be detected from samples in a percentage of 77.78 (Table 4). All EHEC strains produce Shiga toxins (Stx). So, EHEC strains are also called Shiga toxin-producing *E. coli* (STEC) **(Levine, 1987)**. STEC are recognized as important pathogens and have been associated with diarrhea and Hemolytic Uremic Syndrome **(Paton and Paton, 1999).** *E. coli* O146 strains belonging to the STEC group have been implicated in human disease **(Beutin *et al.*, 2004)** and have also been reported in healthy animals, such as sheep and cattle **(Beutin *et al.*, 1993 and Vettorato *et al.,* 2003)**. The Enterotoxigenic *E.coli* secretes toxins, which lead to the production of a watery diarrhea and severe dehydration in children **(Cohen and Gianella, 1995 and Fratamico *et al.,* 2002)**.

CONCLUSION

It is clear from the results obtained that, the rate of contamination of milk was influenced by poor hygienic conditions during milking and handling, as well as post-milking environmental contaminants. Lacking of proper cooling and storage with ambient summer temperature in Egypt are also factors that magnitudes the problem of the bacterial contamination. The objectionable heavy contamination of milk with different types of Enterobacteriaceae may result into serious changes in the products rendering it of inferior quality. Moreover, being an important vehicle for the transmission of milk-borne pathogens to consumers. Routine assessment of milk quality produced by small-scale livestock keepers has to be mandatory in

order to safeguard the public from milk-borne zoonotic infections. Strictly hygienic measures should be applied during milking and milk handling practices, achievable by educating small-scale livestock keepers on good animal husbandry practices. The behavior of consuming raw milk and its products made from raw milk should be discouraged. It is concluded that a gradual move to total Enterobacteriaceae (measure of food quality and spoilage) to improve assessment of food safety and quality rather than limiting examination for Coliforms and fecal Coliforms.

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