JOURNAL OF FOOD QUALITY & HAZARDS CONTROL

SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 4 (2017) 9-13

An Investigation on Risk Factors Related to the Occurrence of *Escherichia coli* in Fluid Milk Marketed in Chittagong, Bangladesh

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HIGHLIGHTS

- *Escherichia coli* was found in 18.3% and 11.8% samples of markets and farm, respectively.
- Adding water hyacinth leaves to milk showed the strongest point estimate of effect odds ratio.
- Washing milk vat/container everyday with tube well water was recognized as a protective factor.
- Educational awareness should be highlighted to inform consumers and farmers about risk of *E. coli*.

Article type
Original article

Keywords
Risk Factors
Escherichia coli
Milk
Bangladesh

Article history
Received: 11 Sep 2016
Revised: 24 Oct 2016
Accepted: 18 Nov 2016

Acronyms and abbreviations
OR=Odds Ratio
CI=Confidence Interval
CFU=Colony Forming Unit

ABSTRACT

Background: *Escherichia coli* is one of the most important pathogens which could be transmitted by milk. The main aim of the present study was to assess risk factors related to the occurrence of *E. coli* in fluid milk marketed in Chittagong, Bangladesh.

Methods: Totally, 169 milk samples were collected from different entry points in Chittagong, Bangladesh; and also, 17 samples were directly collected from a dairy farm. The milk samples were microbiologically analyzed for detection of *E. coli*. A questionnaire was designed and then administered by two trained veterinarians who interviewed milk vendors at time of milk collecting. The data were analyzed by Stata 11 (Stata Corp, College Station, Texas, USA).

Results: *E. coli* was found in 18.3% and 11.8% milk samples obtained from markets and dairy farm, respectively. The results of univariable analysis showed that addition of water hyacinth leaves to milk had the strongest point estimate of effect Odds Ratio (OR 27.1) and high statistical significance (p=0.0007) despite wide 95% Confidence Intervals (CIs) of 2.8-1291.6. The final logistic regression model identified two variables as independent risk factors for the presence of *E. coli* in fluid milk including selling milk after 1-2 h of collection (OR 4.7, 95% CI 1.9-11.7), and also adding banana tree leaves into milk (OR 3.8, 95% CI 1.5-9.4). The final model identified washing milk vat/container everyday with tube well water (OR 0.1, 95% CI 0.05–0.3), as a protective factor.

Conclusion: The two main factors with great influence on risk of *E. coli* in fluid milk marketed in Chittagong, Bangladesh consisted of selling milk after 1-2 h of collection and also adding banana tree leaves into milk. Educational awareness should be highlighted to inform consumers and farmers in this regard.

Introduction

Raw milk may harbor a variety of food-borne pathogens. Gram-negative bacteria are the major microbial load in cold raw milk and mainly composed of psychrotrophic bacteria (Jay et al., 2005; Martins et al., 2006). The initial raw milk obtained from the mammary gland of healthy animal has usually low microbial load and

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To cite: Alam M.K., Akther S., Sarwar N. (2017). An investigation on risk factors related to the occurrence of *Escherichia coli* in fluid milk marketed in Chittagong, Bangladesh. *Journal of Food Quality and Hazards Control*. 4: 9-13.

the application of all hygienic measures during milking prevents milk from being contaminated (Hayes et al., 2001; Robinson, 2005).

Although there are several milk-based commercial companies have been operating in Bangladesh, the demand for safe fresh milk is rising. So, there are several groups of milk vendors collecting raw milk directly from the farmers and selling it at the markets that may be local or city-based, or directly use in households. The hygienic measures being taken from collecting fluid milk to sell are often unknown. If the proper hygienic approaches are not followed, the milk may be contaminated with zoonotic pathogens (Ray and Bhunia, 2007).

Escherichia coli is one of the most important pathogens which could be transmitted by milk and therefore attributed as one of the various milk-borne outbreaks. It is obvious that the processing conditions are of much importance regarding to safety of milk and dairy products (Avery et al., 2004; Betts, 2000; Jay et al., 2005). Thus, it is necessary to know the risk factors affecting hygienic status of milk to do future preventive actions for control of *E. coli*. With the above background, the main aim of the present study was to assess risk factors related to the occurrence of *E. coli* in fluid milk marketed in the Chittagong, Bangladesh.

Materials and methods

Sample collection points

Totally, 169 fluid milk samples were collected from different entry points in Chittagong city in Bangladesh including, Shikalbaha, Sholoshahar Railway Station, Jalalabad Market, Chittagong city Gate, Halishahar, Chittagong Port, and Chittagong Batali Road. Also, 17 samples were directly collected from a dairy farm located in Shikalbaha. All the samples were collected from September 2013 to March 2014.

Data collection and survey method

A questionnaire designed for this study was pretested according to Yien (2014) with some modifications. The questionnaire was then administered by two trained veterinarians who interviewed milk vendors at time of milk collecting. The main assessed variables during interviews were geographic location, amount of milk collected, stock information, flock health history, and overall farm management. Data collection sheet used during milk sample collection is shown in Table 1.

Identification of E. coli

For initial screening of *E. coli*, the collected samples were prepared by serial dilution. At first, 100 µl from each milk sample was transferred to 900 µl sterile peptone water (0.1%) and thoroughly mixed to give 1:10 dilution and then the serial dilutions were prepared. Each 100 µl diluted milk sample was inoculated onto MacConkey agar medium (Oxoid, Basingstoke, Hampshire, UK), incubated for 24 h at 37 $^{\circ}$C. The colony counts of large pink color colonies on medium were presented as Colony Forming Unit per ml (CFU/ml). At the same time, diluted milk samples were inoculated onto blood agar medium (Oxoid, Basingstoke, Hampshire, UK) enriched with 5% defibrinated bovine blood for total plate count by colony counter. Five large pink color colonies from MacConkey agar medium were homogenized and inoculated onto an eosin methylene blue (Oxoid, Basingstoke, Hampshire, UK) agar plate, incubated at 37 $^{\circ}$C for 24 h. Convectional biochemical tests were performed for identification of *E. coli* isolates using triple sugar iron agar, urea agar, methyl red broth, and simmon citrate agar.

Statistical analysis

The data were analyzed by Stata 11 (Stata Corp, College Station, Texas, USA). *P* value <0.05 in two-sided Fisher's exact test was considered as significant. Any variable with $p<0.20$ was considered for multivariable analysis. Logistic regression was applied for multivariable analysis. A backward stepwise variable selection strategy was used to construct a final model with a significance level of $p<0.05$.

Results

Out of 169 raw market milk samples, 31 (18.3%) were found to be infected with *E. coli*. Also, among 17 samples obtained from dairy farm, two (11.8%) were contaminated with *E. coli*. The highest and lowest *E. coli* counts were recorded as 1.20×10^{7} CFU/ml and 1.10×10^{4} CFU/ml, respectively.

The results of univariable analysis (Table 2) showed that adding water hyacinth leaves into milk had the strongest point estimate of effect Odds Ratio (OR 27.1) and high statistical significance (p=0.0007) despite wide 95% Confidence Intervals (CIs) of 2.8-1291.6. Protective factors (OR<1) for *E. coli* in fluid milk were washing milk vat/container everyday with tube well water (OR 0.1, 95% CI 0.04–0.3, $p\leq0.001$), selling milk after 0-1 h of collection (OR 0.2, 95% CI 0.09-0.5, p=0.0001), and having a milk vats/container made of enamel (OR 0.3, 95% CI 0.1–0.7, p=0.0014).

Nine variables were considered for inclusion in the logistic regression model to estimate independence of effects. The initial model of the multivariable analysis is shown in Table 3. However, the final logistic regression model identified two variables as independent risk factors for the presence of *E. coli* in fluid milk marketed in the Chittagong corporation shown in Table 4. They were selling milk after 1-2 h of collection (OR 4.7, 95% CI 1.9-11.7), and adding banana tree leaves into milk (OR 3.8, 95% CI 1.5-9.4). The final model also identified washing milk vat/container everyday with tube well water (OR 0.1, 95% CI 0.05–0.3), as a protective factor.

Table 1: Data collection sheet used during milk sample collection

Location:
Sample code:
Amount of milk (ml) collected:
1. (a) Is the milk your own farm/smallholding? Yes/No (b) If yes, local milking cows do you have? Yes/No (c) If yes, cross-bred milking cows do you have? Yes/No (d) If yes, both local and cross-bred milking cows do you have? Yes/No
2. (a) The milk is not yours, but you are a middleman in selling it? Yes/No (b) Not own milk, but collected from a single farm of another person? Yes/No (c) Not own milk, but collected from multiple farms? Yes/No
3. (a) What is the time (0-1 h) elapsed between milk collection and selling? Yes/No (b) What is the time (1-2 h) elapsed between milk collection and selling? Yes/No (c) What is the time (›2 h) elapsed between milk collection and selling? Yes/No
4. Is the milk vats/container made of mud? Yes/No
5. Is the milk vats/container made of plastic? Yes/No
6. Is the milk vats/container made of enamel? Yes/No
7. Is the milk vats/container made of stainless steel? Yes/No
8. Have you added any chemical into the milk? Yes/No; if yes then what is it?
9. Have you added date tree leaves into the milk? Yes/No
10. Have you added banana tree leaves into the milk? Yes/No
11. Have you added water hyacinth leaves into the milk? Yes/No
12. Do you use tube well water in washing your milk vats/container every day after selling of milk? Yes/No
13. Do you use water supplied from the city corporation to wash your milk vats/container every day after selling of milk? Yes/No
14. Do you use simple pond water/any surface water to wash your milk vats/container every day after selling of milk? Yes/No
15. Do you use any soap/bleach in washing the milk vats/container? Yes/No
16. Do you have any cutting/itching lesions/skin abrasions onto your fingers or into the finger commissures or nail beds? Yes/No

Table 2: Univariable analysis for risk of *E. coli* in fluid milk marketed in Chittagong, Bangladesh

Variable	No. of *E. coli* positive samples*	No. of *E. coli* negative samples**	OR (95% CI)	*P* value
Milk from own farm/smallholding	17	104	0.5 (0.2-1.2)	0.106
Having local milking cows	10	63	0.6 (0.2-1.5)	0.326
Having cross-bred milking cows	7	33	1.0 (0.3-2.6)	1.000
Not direct but middleman sourced milk	3	10	1.4 (0.2-6.0)	0.705
Single farm-sourced milk	6	30	0.9 (0.3-2.5)	1.000
Multiple farm-sourced milk	7	9	4.3 (1.2-14.2)	0.010
Selling milk after 0-1 h of collection	15	123	0.2 (0.09-0.5)	0.0001
Selling milk after 1-2 h of collection	18	30	4.9 (2.1-11.7)	0.0001
Milk vats/container made of mud	18	35	4.0 (1.7-9.5)	0.0005
Milk vats/container made of enamel	12	102	0.3 (0.1-0.7)	0.0014
Milk vats/container made of stainless steel	3	15	0.9 (0.2-3.6)	1.000
Adding date tree leaves into milk	2	21	0.4 (0.04-1.8)	0.380
Adding banana tree leaves into milk	22	53	3.8 (1.6-9.3)	0.0009
Adding water hyacinth leaves into milk	5	1	27.1 (2.8-1291.6)	0.0007
Washing milk vat/container everyday with tube well water	11	125	0.1 (0.04-0.3)	<0.001
Washing milk vat/container everyday with water supplied from city corporation	18	25	6.1 (2.5-14.9)	<0.001
Washing milk vat/container everyday with any pond/surface water	5	3	8.9 (1.6-59.7)	0.005
Use soap/bleach in washing milk vats/container	9	67	0.5 (0.2-1.2)	0.082
Presence of milker's hand-lesions	6	40	0.6 (0.2-1.7)	0.383

* Total No.=33
** Total No.=153
OR=Odds Ratio; CI=Confidence Interval

Table 3: Multivariable analysis of risk practices/factors associated with the presence of *E. coli* in fluid milk marketed in Chittagong, Bangladesh (Nine variables entered in the initial analysis)

Variable	OR	95% CI	*p* value
Washing milk vat/container everyday with water supplied from city corporation	0.6	0.1-3.7	0.619
Washing milk vat/container everyday with tube well water	0.1	0.01-0.5	0.007
Selling milk after 1-2 h of collection	3.4	1.2-9.6	0.021
Milk vats/container made of mud	2.3	0.4-12.6	0.329
Adding banana tree leaves into milk	2.7	1.0-7.2	0.053
Milk vats/container made of enamel	0.7	0.1-3.8	0.715
Use soap/bleach in washing milk vats/container	0.8	0.3-2.3	0.682
Multiple farm-sourced milk	1.7	0.3-8.7	0.536
Milk from own farm/smallholding	1.1	0.4-3.2	0.903

Logistic regression; initial model with 9 variables entered; $\chi2$ (9) for likelihood ratio test 53.32; pseudo R^2=0.308; No. of observation=186OR=Odds Ratio; CI=Confidence Interval

Table 4: Multivariable analysis of risk practices/factors associated with the presence of *E. coli* in fluid milk marketed in Chittagong, Bangladesh (final model)

Variable	OR	95% CI	*p* value
Washing milk vat/container everyday with tube well water	0.1	0.05-0.3	<0.001
Selling milk after 1-2 h of collection	4.7	1.9-11.7	0.001
Addition of banana tree leaves to milk	3.8	1.5-9.4	0.005

Logistic regression; 3 variables entered; $\chi2$ 2(3) for likelihood ratio test 47.15; p<0.001; pseudo R^2=0.271; significance of goodness-of-fit test (Hosmer-Lemeshow) 0.377; No. of observation=186
OR=Odds Ratio; CI=Confidence Interval

Discussion

Since milk has almost neutral pH with high water content and a variety of nutrients, it represents an ideal substrate for microbial growth (Jay et al., 2005). Milk samples marketed at Chittagong area had considerable contamination with *E. coli*. Our results are similar to fecal coliform counts of 4.2×10^7 CFU/ml in raw milk of Morocco expressed by Hadrya et al. (2012). Also, Aaku et al. (2004) and Arenas et al. (2004) observed that the total numbers of microorganisms in pooled raw milk were 5.5×10^6 CFU/ml and 10^6 to 10^7 CFU/ml, respectively. Zeinhom and Abdel-Latef (2014) stated that *E. coli* was detected in 26.7% and 16% of the milk sampled from markets and farms of Egypt, respectively.

The rates of microbial contaminations of cow's raw milk are influenced by some underlying factors. These are consisted of physiology of dairy cows, hygienic status of animal, and the environment in which they are housed and milked, methods of udder preparation before milking, cleaning and disinfection techniques of milking machines, personal hygiene of the people involved, etc. (Wiking et al., 2002). While acknowledging the possibility of milk contamination with any the above mentioned steps, the present study indicated that some practices may have high impact in contaminating the milk samples with *E. coli*. Several variables such as adding water hyacinth leaves into milk, washing milk vat/container everyday with any pond/surface water, washing milk vat/container everyday with water supplied from city corporation, selling milk after 1-2 h of collection, multiple farm-sourced milk, milk vats/container made of mud, and addition of banana tree leaves to milk seemingly had some contributions for the entrance of *E. coli* in the fluid milk being marketed in the Chittagong area which have not been published before in Bangladesh. However, there are some published reports about risk assessment of *E. coli* in milk samples produced in the other countries which their findings are mainly in agreement with the results of the present study. For example, Giacometti et al. (2016) indicated that the differential risk of *E. coli* O157 in Italian raw milk sold in vending machines, were milk mainly handled under standard conditions (4 $^{\circ}$C) and also the worst time-temperature field handling conditions. Awadallah et al. (2016) stated that mastitis in Egyptian dairy cows was an important risk factor associated with contamination of the produced milk with *E. coli*. Another work conducted by Giacometti et al. (2012) showed that boiling raw milk before consumption and strict control of temperatures by Italian farmers during raw drink distribution had significant impact on reduction of risk of *E. coli* O157 and *Campylobacter*. According to a survey regarding to the risk assessment of *E. coli* O157:H7 in unpasteurized milk marketed in some East African countries, the widespread practice of boiling milk before eating by consumers was identified as a major risk reducer (Grace et al., 2008).

It should be noted that the rate of bacterial multiplication is greater at higher temperatures and the temperature of milk just after milking is the optimum growth temperature for *E. coli* (Jay et al., 2005), so, there is a serious risk of *E. coli* contamination in milk samples. However, it was found that selling milk after 1-2 h of collection and also addition of banana tree leaves to milk were two independent risk practices in finding *E. coli* in fluid milk at Chittagong. It is difficult to explain how “selling milk after 1-2 h of collection” became an independent risk factor for finding *E. coli* in milk samples. It is assumed that this issue may be related to introduce the organism to milk sample during or just immediately after milking. One of the independent causal factors found in the present study was the addition of banana tree leaves to milk. Normally, banana tree leaves do not contain any pathogenic bacteria, but it may be possible to contaminate raw milk when it is contaminated with *E. coli* from fecal droppings of birds or carrying materials of fecal origins, either having washed in free-surface water or other direct or indirect means. Considering the findings of the present study, it seems necessary to recommend the milk sellers/producers in Chittagong to wash their milk vats/containers with tube well water every day in order to reduce the risk of *E. coli* contamination.

Conclusion

The two main factors with great influence on risk of *E. coli* in fluid milk marketed in Chittagong, Bangladesh consisted of selling milk after 1-2 h of collection and also addition of banana tree leaves to milk. Knowing that the raw milk consumption is an important source of *E. coli* infection, isolation of *E. coli* from milk samples revealed a zoonotic risk of raw milk consumption in this region. Educational awareness should be highlighted to inform consumers and farmers in this regard.

Conflicts of interest

The authors of this article declared that there is not any conflict of interest.

Acknowledgments

The authors are thankful for the financial and technical supports from Department of Food Processing and Engineering, Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh, during the present research.

This work was ethically approved by the local institutional review board. The authors performed this research as a part of thesis (Code number: 127) at Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh.

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