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PREVALENCE OF MULTIDRUG RESISTANT SHIGA TOXIN PRODUCING *E. coli* IN THE MILK OF CATTLE, BUFFALOES, AND CAMEL

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Abstract: Milk is regarded as an important source of essential amino acids, vitamins, and trace elements. Milk from camel is consumed in many Arabian countries as a traditional Medicine. However, few reports investigated the microbiological quality of raw camel milk in a comparison with cattle and buffalo's milk. This study was conducted to investigate the bacteriological quality of raw milk collected from cattle, buffaloes, and camel. Besides, Isolation and identification of different *E. coli* serotypes were carried out. Moreover, molecular confirmation of the recovered *E. coli* isolates via amplification of *16S rRNA* and detection of shiga toxin coding genes (*stx1*, and *stx2*) were done using PCR. Additionally, the antibiogram of the recovered *E. coli* isolates was screened using the disk diffusion method. The obtained results indicated that milk samples collected from camels had the lowest microbial counts compared with that of cattle and buffaloes. *E. coli* was isolated from the collected samples of cattle, buffaloes, and camel at 50%, 20%, and 10%, respectively. Various *E. coli* serotypes were identified in the present study with the ability of three and four *E. coli* isolates recovered from cattle milk samples to harbor *stx1*, and *stx2*, respectively. All recovered *E. coli* isolates showed multidrug resistance profiling. Therefore, pasteurization of milk prior to use, and adoption of strict hygienic measures are highly recommended to avoid the risk of human exposure to shiga toxin producing *E. coli*.

Key words: shiga toxin; *E. coli*; milk; camel; drug resistance

Introduction

Milk is regarded as a major source for the nutrients needed to maintain the human health. Milk proteins such as casein and whey proteins have been proven to be crucial for a number of human metabolic, physiological, and other nutritional processes. Additional benefits of milk proteins include their ability to function as antioxidants, boost the immune system, and guard against gastrointestinal infections. Even while other animal and plant proteins include potential bioactive peptides, milk proteins are now the primary source of many physiologically active peptides (1, 2). Milk is a rich source of trace elements such as calcium and magnesium. Milk sources in Arab countries involve cattle, buffalo, and camel. The latter is particularly regarded as an ethnic food with many therapeutic functions and used in the traditional medicine (3). However, milk is implicated in the transmission of several foodborne pathogens such as *E. coli*, *Salmonella spp.*, *Listeria spp.*, and others (4, 5).

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Microbial contamination of milk is controlled by the hygienic practices adopted during the milking process, processing, and distribution. The poor microbiological quality of a food product is a direct indicator for the hygienic procedures followed during production of such a product (6).*Escherichia coli (E. coli)* is a typical resident of both human and animal digestive systems. Its detection in animal products therefore refers to fecal contamination. *E. coli* is moreover one of the foodborne pathogens associated with the emergence of various human sickness outbreaks (7). Shiga toxin producing *E. coli* (STEC) is the cause of numerous hospitalizations of people all over the world (8). Several non O157 *E. coli* pathotypes are classified as STEC like O26, O45, O103, O111, O121, and O145 (9). One major task of the food safety and zoonoses sector is the continuous monitoring and surveillance of the foodborne pathogens like *E. coli* in various food subjects.

Microbial drug resistance is a challenging problem that poses serious risks to the public's health. In livestock production, antimicrobials are frequently used to prevent and control bacterial infections. However, the continuous and the uncontrolled usage of antimicrobials may result in the emergence of organisms with multidrug resistance properties (10, 11).

Taken the previous notes, this study was conducted to investigate the prevalence of *E. coli* in the milk of cattle, buffalo, and camel retailed in Egypt. Furthermore, detection of shiga toxin coding genes (*stx1*, and *stx2*) in the recovered *E. coli* isolates was done using PCR. Moreover, the antibiogram of the serodiagnosed-*E. coli* pathotypes was screened using the disk diffusion method.

Material and methods

Collection of Samples

Sixty random milk samples were collected from cattle, buffaloes, and camel (20/each). The collected milk samples (100 mL/each sample) were purchased from local stores and dairy farms in Sharkia governorate, Egypt. Samples were apparently normal with no alterations in their sensory characteristics. Samples were transferred cooled without delay to the laboratory for bacterial isolation and identification.

Bacteriological examinations

Milk samples were processed for bacteriological analysis in accordance with the APHA-recommended technique (12). Briefly, 25 mL of each sample were mixed with 225 ml of sterile buffered peptone water 0.1% to create a dilution of 10^{-1}. Further tenfold decimal serial dilution were created by aseptically transferring one ml from the prepared dilution (10^{-1}) to another sterile tube containing 9 ml of sterile 0.1% buffered peptone water.

Total bacterial Count (TBC)

Using plate count agar, TBC was estimated according to the method of APHA (12). Cultured plates were incubated for 24 h at 35 ± 2 °C, all colonies including pinpoint size were recorded.

TBC/g = average No. of colonies × reciprocal of dilution

Counted colonies expressed as log 10 cfu/g.

Determination of most probable number (MPN) of Coliforms

The three-tube approach was employed to calculate the MPN of coliforms (12). Three test tubes containing MacConkey broth with inverted Durham's tubes were inoculated with one mL of each generated dilution. The test tubes were then incubated at 37°C for 24-48 hours. Positive tubes that produced gas and acid were recorded. The recommended tables were used to determine MPN of coliforms that were most likely to exist.

Determination of MPN of E. coli

In tubes containing 7 ml of *E. coli* (EC) broth (Himedia, Mumbai), loopfuls from positive tubes demonstrating acid and gas productions on MPN of coliforms assays were inoculated. The tubes were then incubated at 44.5°C for 24-48 hours (12). Tubes with acid and gas production were recorded as positive. According to the suggested tables, the MPN of *E. coli* was calculated.

Isolation of Escherichia coli

Using the APHA procedure, *E. coli* was isolated using Eosin Methylene Blue (EMB) agar (12). A loopful of EC broth from each positive tube was

spread onto EMB agar. The inoculated plates underwent a 24-hour incubation period at 37°C. *E. coli* colonies typically had a dark purple core and were metallic green in appearance. Purified suspected colonies were sub-cultured onto nutrient slope agar and incubated for further examinations. Staining and biochemical assays were used to identify the isolates. Using specific *E. coli* antisera sets, the recovered *E. coli* isolates were subjected to serological identification (Difco, Detroit, USA) (13).

Bacterial DNA preparation and detection of 16S rRNA- E. coli specific gene and shiga toxin producing genes in the identified isolates

Each isolate of *E. coli* from the glycerol stock underwent DNA extraction using the previously described procedure (8). A PCR analysis was used to confirm isolation of E. coli via amplification of E. coli specific *16S rRNA*, and identify genes encoding the shiga toxins (*stx1*, and *stx2*). Table 1 displayed the amplified product sizes and primer sequences. A Thermal Cycler was used to carry out the amplification (Eppendorf, Hamburg, Germany). PCR assays were performed utilizing Dhanashree and Mallya's technique (14). A denaturing step at 95°C for three minutes was followed by 35 cycles of 95°C for 20 seconds, 58°C for 40 seconds, and 72°C for 90 seconds as the amplification conditions. The last cycle lasted 5 min at 72°C. The reference strains *E. coli* O157:H7 Sakai and *E. coli* K12DH5α were used as positive and negative strains, respectively. On a 2% agarose gel electrophoresis (Applichem, Germany, GmbH) in a 1x TBE buffer stained with ethidium bromide, amplified DNA products were visualized.

Antibiotic resistance of the recovered E. coli

The disc diffusion method was used to assess the antibiogram of the recovered *E. coli* isolates. Nutrient agar and the used antimicrobial discs were acquired from Oxoid in Hampshire, United Kingdom. We used the Clinical and Laboratory Standards Institute's (CLSI) experimental guidelines (16). Additionally, using the method outlined by Singh et al. (17), the Multiple Antibiotic Resistance (MAR) index for each tested *E. coli* isolate was calculated as follows.:

MAR index = No. of resistance / Total No. of tested antibiotics

Isolates classified as intermediate were considered sensitive for MAR index

The tested antimicrobial discs (Oxoid Limited, Hampshire, UK) were ampicillin (10 µg) (AM), cephalothin (30 µg) (CET), chloramphenicol (30 µg) (C), ciprofloxacin (5 µg) (CIP), enrofloxacin (5 µg) (ENR), erythromycin (15 µg) (E), gentamicin (10 µg) (GEN), kanamycin (30 µg) (K), nalidixic acid (30 µg) (NA), neomycin (30 µg) (N), oxacillin (1 µg) (OX), oxytetracycline (30 µg) (OXY), penicillin (10 IU) (P), and trimethoprim/sulfamethoxazole (25 µg) (SXT).

Statistical analysis:

All values are expressed as means ± SD. Statistical significance was evaluated using the Tukey–Kramer HSD test. $P < 0.05$ was used to indicate statistical significance using JMP statistical package, SAS Institute Inc., Cary, NC.

Table 1: Oligonucleotides' sequences used in the present study

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
16S rRNA (F)	5′ CTTTCAGCGGGGAGGAAGG ′3	390	(15)
16S rRNA (R)	5′ TCAACCTCCAAGTCGACATCGT ′3		
stx1 (F)	5′ ACACTGGATGATCTCAGTGG ′3	614	(14)
stx1 (R)	5′ CTGAATCCCCCTCCATTATG ′3		
stx2 (F)	5′ CCATGACAACGGACAGCAGTT ′3	779	
stx2 (R)	5′ CCTGTCAACTGAGCAGCACTTTG ′3		

Results

All examined milk samples from all animal species had normal organoleptical characteristics at sensory evaluation (Data are not shown). Bacteriological examination of the examined milk samples at the present study revealed that the average values of TBC were 4.84 ± 0.28, 3.69 ± 0.15, and 2.69 ± 0.21-log cfu/g in the examined cattle, buffaloes, and camel milk samples, respectively (Fig. 1A). The calculated mean values of the Most probable number of coliforms (MPN) were 3.20 ± 0.15, 2.59 ± 0.24 and 1.71 ± 0.22-log MPN/g in the same examined samples, respectively (Fig. 1B).

MPN of *E. coli* was also evaluated using EC broth. The calculated values of MPN of *E. coli* in the examined samples were 2.92 ± 0.15, 2.13 ± 0.11, and 1.17 ± 0.08-log MPN/g, respectively (Fig. 2A). The prevalence rates (%) of *E. coli* in the examined samples were 50% in cow's milk samples, 20% in buffalo's milk samples, and 10% in the examined camel's milk samples, respectively (Fig. 2B). Serotyping of the recovered *E. coli* revealed six serotypes, namely *E. coli* O2:H6 at 31.25%, *E. coli* O26:H11 at 25%, *E. coli* O55:H7 at 18.75%, *E. coli* O78:H- at 12.5%, *E. coli* O86:H11 at 6.25%, and *E. coli* O127:H6 at 6.25% (Fig. 3). All recovered *E. coli* isolates had *16S rRNA* as detected by PCR. Besides, detection of shiga toxin coding genes (*stx1*, and *stx2*) among the recovered *E. coli* isolates demonstrated that *stx1* could be detected in three *E. coli* serotypes (*E. coli* O2:H6, *E. coli* O26:H11, and *E. coli* O55:H7) recovered from cow's milk. While *stx2* could be detected in four *E. coli* serotypes (*E. coli* O2:H6, *E. coli* O26:H11, *E. coli* O55:H7, and *E. coli* O78:H) recovered from cow's milk (Table 2).

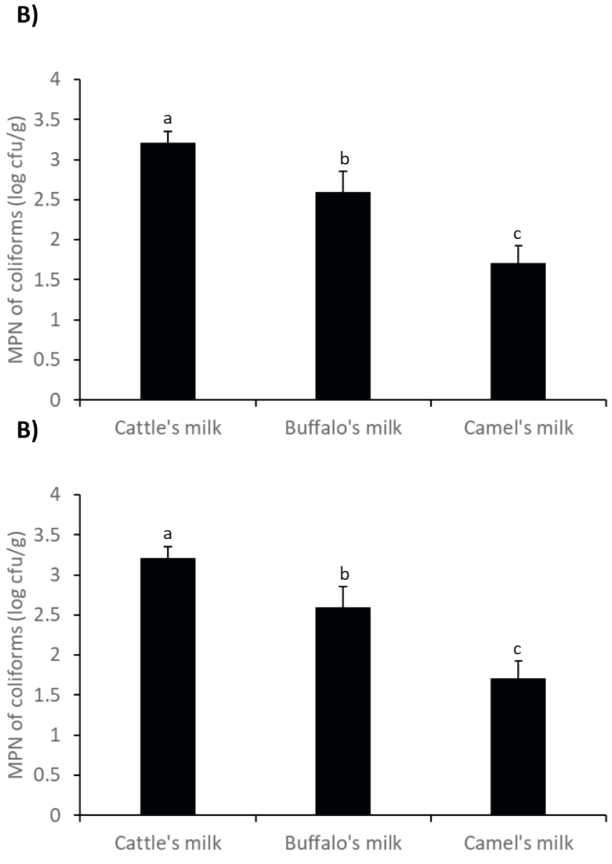

Figure 1: A) Total bacterial count B) MPN of coliforms. Values represent means ± SD in the examined milk samples of cattle, buffaloes, and camel. Columns with different letter are significantly different at $p < 0.05$

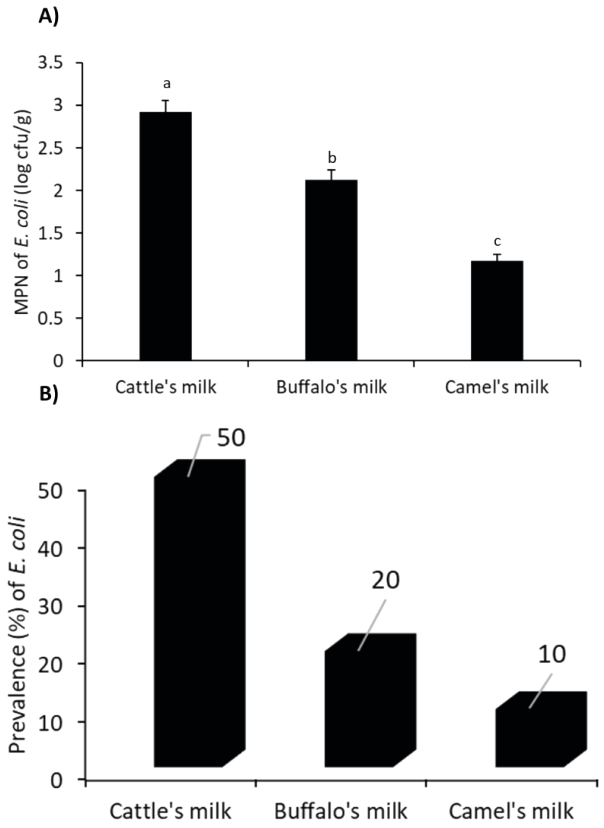

Figure 2: A) MPN of *E. coli*, B) prevalence (%) of *E. coli* isolation (n = 20/each). Values of MPN of E. coli represent means ± SD in the examined milk samples of cattle, buffaloes, and camel. Columns with different letter are significantly different at $p < 0.05$

Table 1: Antimicrobial resistance profiling and detection of *16S r*RNA, *stx1*, and *stx2* genes in the recovered *E. coli* isolates from milk samples collected from cattle, buffaloes, and camel

Isolate number	Serotype	Origin	*16S rRNA*	*stx1*	*stx2*	antimicrobial resistance profiling	MAR index
1	*E. coli O2: H6*	Cow's milk	+	+	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T, P, SXT	1
2	*E. coli O2: H6*	Cow's milk	+	-	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T	0.857
3	*E. coli O2: H6*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N	0.714
4	*E. coli O2: H6*	Buffalo's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA	0.642
5	*E. coli O2: H6*	Camel's milk	+	-	-	AM, CN, C, CP, En, E	0.428
6	*E. coli O26: H11*	Cow's milk	+	+	+	AM, CN, C, CP, En, E, G, K, P, K, NA	0.785
7	*E. coli O26: H11*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, P	0.571
8	*E. coli O26: H11*	Buffalo's milk	+	-	-	AM, CN, C, CP, En, E	0.428
9	*E. coli O26: H11*	Camel's milk	+	-	-	AM, G, K, NA, T	0.357
10	*E. coli O55: H7*	Cow's milk	+	+	+	AM, CN, C, CP, En, E, G, K, NA, N, OX, T	0.857
11	*E. coli O55: H7*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N	0.714
12	*E. coli O55: H7*	Buffalo's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA	0.643
13	*E. coli O78: H-*	Cow's milk	+	-	+	AM, CN, C, CP, En, E, G, K, NA, N, P	0.785
14	*E. coli O78: H-*	Buffalo's milk	+	-	-	AM, CN, C, CP, En, E, K, NA	0.571
15	*E. coli O86: H11*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX, P	0.857
16	*E. coli O127: H6*	Cow's milk	+	-	-	AM, CN, C, CP, En, E, G, K, NA, N, OX	0.786
						Average MAR index	0.688

Table 2: Antimicrobial resistance rates among the recovered E. coli serotypes

	O2		O26		O55		O78		O86		O127	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
AM	5	100	4	100	3	100	2	100	1	100	1	100
CN	5	100	3	75	3	100	2	100	1	100	1	100
C	5	100	3	75	3	100	2	100	1	100	1	100
CP	5	100	3	75	3	100	2	100	1	100	1	100
En	5	100	3	75	3	100	2	100	1	100	1	100
E	5	100	3	75	3	100	2	100	1	100	1	100
G	4	80	3	75	3	100	1	50	1	100	1	100
K	4	80	2	50	3	100	2	100	1	100	1	100
NA	4	80	2	50	3	100	2	100	1	100	1	100
N	3	60	2	50	2	66.66	1	50	1	100	1	100
OX	2	40	2	50	1	33.33	0	0	1	100	1	100
T	2	40	1	25	1	33.33	0	0	0	0	0	0
P	1	20	2	50	0	0	1	50	1	100	0	0
SXT	1	20	0	0	0	0	0	0	0	0	0	0

No. Number of isolates, %: Percentage of isolates, AM: Ampicillin; CN: Cephalothin; C: Chloramphenicol; CP: Ciprofloxacin; En: Enrofloxacin; E: Erythromycin; G: Gentamicin; K: Kanamycin; NA: nalidixic acid; N: Neomycin; OX: Oxacillin; T: Oxytetracycline; P: Penicillin; SXT: trimethoprim/sulfamethoxazole

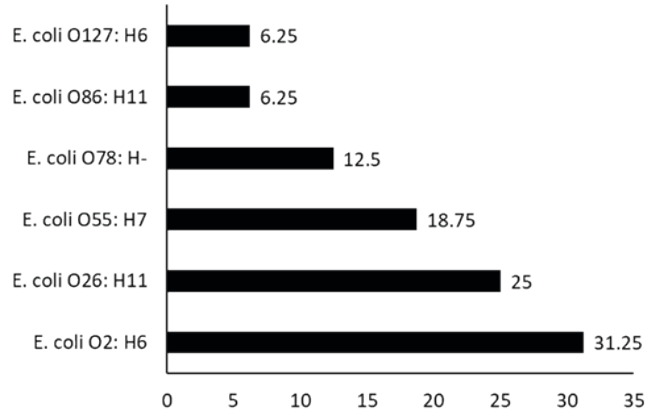

Figure 3: Prevalence (%) of different *E. coli* serotypes recovered in the present study

Tables 2 and 3 show the drug resistance profiling of the recovered *E. coli* pathotypes in the current investigation. The resistance profile of *E. coli O2: H6* was 100% to AM, CN, C, CP, En, and E; 80% to G, KA, and NA; 60% to N, and less than 50% to the other tested antimicrobials. 78.94% to polymyxin B; 73.68% nalidixic acid and less than 50% to other tested antimicrobials. *E. coli O26: H11* had 100% resistance to AM; 75% to CN, C, CP, En, E, and G; While 50% or less to the other tested antimicrobials. *E. coli O55: H7* had 100% resistance to AM, CN, C, CP, En, E, G, K, and NA, 66.66% to N; While less than 50% to the other tested antimicrobials. *E. coli O78: H-* had 100% resistance to AM, CN, C, CP, En, E, G, NA, and N; While 50% or less to the other tested antimicrobials. The recovered isolate of *E. coli O86: H11* had 100% resistance to all tested antimicrobials, but sensitive to T, and SXT. Similarly, the recovered isolate of *E. coli O127: H6* had 100% resistance to all tested antimicrobials, but sensitive to T, P, and SXT. The recovered isolates had an average MAR index of 0.688.

Discussion

Milk is regarded as an essential nutrient for all ages due to its high content of protein, fat and minerals. Camel milk in particular has been used for centuries as a food and as a traditional medication for many diseases such as diabetes, allergy, and asthma (18). However, milk can be easily contaminated during the production cycle starting from milking, processing, packaging, and distribution. Few reports investigated the bacteriological status of camel milk in comparison with the milk of other species. In the present study, the bacteriological status of the milk collected from three animal species, cattle, buffaloes, and camel, was evaluated. Cattle milk had significantly ($p < 0.05$) the highest bacterial counts in terms of TBC, MPN of coliforms, and MPN of *E. coli*, followed by samples collected from buffaloes. While camel had the lowest counts of such parameters. Similarly, *E. coli* was isolated from the milk samples of cattle, buffaloes, and camel at 50%, 20%, and 10%, respectively. In agreement with the recorded results of the present study, camel milk was found contaminated at higher levels in Samara-Logia Town of Afar National Regional State, Northeast Ethiopia, compared to the recorded values in the present study, as TBC, and total coliforms counts (log 1o cfu/mL) were 6.37, and 4.87, respectively. *E. coli* was also isolated at higher rate 24.6% in the same study (19). *E. coli* was also isolated from raw milk marketed at Taif region (Western Saudi Arabia) (20). Raw milk samples collected from Peninsular, Malaysia had higher TBC counts compared with the present study. Besides, *E. coli O157:H7* was isolated at 33.5%, while not detected in the current investigation (21). Raw cow's milk collected from Shahrekord, Iran was found contaminated with coliforms and *E. coli* at higher rates at 79%, and 69%, respectively. In Egypt, raw milk marketed in Dakahlia governorate was found highly contaminated as TBC and total coliforms counts were recorded at 5.46×10^7 and 8.42×10^6, respectively (23). The obtained results of the present study revealed identification of several *E. coli* pathotypes. Similarly, *E. coli O26:H11, E. coli O55:H7, E. coli O78:H-, E. coli O111:H4, and E. coli O127:H6* were recovered and identified from dairy products retailed in Egypt (15). Shiga toxin coding genes are also detected in three *E. coli* isolates for *stx1*, and 4 isolates for *stx2*. Likely, shiga toxin related genes were also detected in E. coli isolated from raw milk in Taif, Saudi Arabia (20). Consumption of raw milk was associated with the onset of several outbreaks and food poisoning cases worldwide. For instances, an outbreak of *E. coli O157: H7* associated with raw milk consumption was reported in the Pacific Northwest, United States during 2005 (24). Moreover, raw milk consumption was linked to foodborne infection cases with shiga toxin producing *E. coli* in several European countries (25).

Development of drug-resistant pathogens, which is viewed as a serious health threat, is mostly

caused by the uncontrolled use of antibiotics in animal production (10, 11). The recovered *E. coli* pathotypes in the current investigation were characterized by a high prevalence of multidrug resistance. Studies from Brazil, Egypt, and Romania all reported isolation of multidrug resistant *E. coli* in raw milk (26, 27, 28). Therefore, it is strongly advised that antimicrobials be used responsibly in the livestock production and in dairy farms.

In conclusion, the current investigation identified and isolated multidrug-resistant and shiga toxin-producing *E. coli* from milk sold in Zagazig, Egypt, from cows, buffaloes, and camels. Therefore, when handling milk, extra hygiene precautions should be used. Additionally, it is strongly advised against consuming any raw milk, even from camel milk. Furthermore, it is strongly recommended to do ongoing research on the occurrence of STEC in milk.

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Conflict of interest: None

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