The Veterinary Journal 217 (2016) 83–88

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Occurrence, genotyping, shiga toxin genes and associated risk factors of *E. coli* isolated from dairy farms, handlers and milk consumers

M.A. Awadallah, H.A. Ahmed *, A.M. Merwad, M.A. Selim

Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511 Zagazig, Egypt

ARTICLE INFO

Article history:
Accepted 29 September 2016

Keywords:
E. coli
STEC
ERIC-PCR
Risk factors
Dairy farms

ABSTRACT

The objectives of the current study were to determine the occurrence and genotypes of *E. coli* in dairy farms, workers and milk consumers and to evaluate risk factors associated with contamination of milk in dairy farms. Molecular characterization of shiga toxin associated genes and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) finger printing of *E. coli* from different sources were also studied. Paired milk samples and rectal swabs from 125 dairy cows, rectal swabs from 82 calves and hand swabs from 45 dairy workers from five dairy farms were collected. In addition, 100 stool samples from 70 diarrheic and 30 healthy humans were collected and examined for the presence of *E. coli.*

E. coli was isolated from milk (22.4%), dairy cattle feces (33.6%), calf feces (35.4%), dairy worker hand swabs (11.1%) and stools of milk consumers (2%, from diarrheic patients only). Only *stx1* was identified in seven of 12 *E. coli* O125 isolated from different sources. High genetic diversity was determined (Simpson's index of diversity, $D = 1$) and *E. coli* O125 isolates were classified into 12 distinct profiles, E1–E12. The dendrogram analysis showed that two main clusters were generated. Mastitis in dairy cows was considered a risk factor associated with contamination of the produced milk with *E. coli.* The isolation of *E. coli* from rectal swabs of dairy cows and calves poses a zoonotic risk through consumption of unpasteurized contaminated dairy milk. Educational awareness should be developed to address risks related to consumption of raw milk.

Introduction

Contamination of milk in dairy farms can be associated with risk factors such as mastitis, handling, type of management and hygienic conditions within the farm (Snow et al., 2012). It is essential to assess the role of raw milk and other possible sources of contamination within the farm in transmitting some zoonotic bacteria to milk consumers.

Shiga toxin-producing *E. coli* (STEC) causes human diseases, ranging from uncomplicated diarrhea to haemorrhagic colitis (HC) and life-threatening complications such as haemolytic uremic syndrome (HUS; Pradel et al., 2008). Molecular typing based on PCR techniques including enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) has been developed for typing of *E. coli* and other Gram-negative bacteria (Versalovic et al., 1991). The reproducibility and discriminatory power of the ERIC-PCR technique in typing of *E. coli* favor its application in studying the genetic relationship of the isolates from different sources (Oltramari et al., 2014).

The present study aimed to investigate the occurrence and molecular detection of shiga toxin associated genes and ERIC-PCR finger printing of *E. coli* isolates from dairy cattle, calves, milk handlers and milk consumers. In addition, risk factors associated with *E. coli* contamination of milk in dairy farms including type of milking, type of management and hygienic conditions of the farms were evaluated.

Materials and methods

Sampling

This study was conducted at Sharkia Governorate, Egypt, selected due to convenience in sampling during the period from May to September 2014. The study included sampling from five dairy farms selected in the study area after agreement of the farm owners to participate. The farms were small scale dairy farms containing 50 animals housed in groups in an open housing system. On each farm, all lactating cows and all calves were sampled. The calves were 1–2 months of age and were milk fed. Milk samples and rectal swabs were collected (125, each) from dairy cows and rectal swabs were collected from 82 calves. In addition, 45 hand swabs from milk handlers were collected from the five dairy farms. Stool swabs were also collected from diarrheic ($n = 70$) and apparently healthy ($n = 30$) milk consumers from a private laboratory in the same vicinity as the examined farms. Informed verbal/written consent for participation in the study was obtained from all participants and the study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt (Protocol ANWE-206; Approval date 1 April 2014).

Sample preparation

For collection of milk samples, teats were sterilized and at the middle of lactation milk was collected in sterile screw capped bottles. Twenty-five milliliters of each

* Corresponding author.
E-mail address: heba_ahmed@zu.edu.eg (H. Ahmed).

http://dx.doi.org/10.1016/j.tvjl.2016.09.014
1090-0233/

milk sample was homogenized with 225 mL of buffered peptone water (BPW; Oxoid, CM509). Animal feces were collected directly from the rectum into sterile plastic bags and then a swab was taken from each sample and immersed in BPW tubes. Human stool samples were collected in sterile containers and a swab was then taken into BPW. Finally, the entire surface of the dairy workers' hands was swabbed using sterile swabs. The swabs were then immersed in test tubes containing 9 mL BPW under aseptic conditions. All the samples in BPW were pre-enriched at 37 °C for 24 h.

Isolation and identification of E. coli

Isolation of *E. coli* was carried out according to Gómez-Duarte et al. (2010) and Feng et al. (2011) on EMB agar (Oxoid, CM69, Adelaide, Australia) by incubation at 37 °C for 18–24 h. One suspected colony from each plate was subjected to Gram staining and biochemical identification according to Cruickshank et al. (1975). Thirty-two *E. coli* strains isolated from different sources were serogrouped (Kok et al., 1996) by using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN CO., Japan) at the Animal Health Research Institute, Dokki, Giza, Egypt.

Molecular identification of shiga toxin associated genes using real-time PCR

Twelve isolates of serogroup *E. coli* O125 were identified from all examined sample sources. The DNA was extracted using QIAGEN kits (QIAamp DNA mini kit) according to the manufacturer's instructions. Real time PCR was performed using primers and TaqMan probe sets (AlphaDNA, Canada) targeting *stx*1 (STX1-TM-F, CATCGCGAGTTGCCAGAAT, STX1-TM-R, GCGTAATCCCACGGACTCTTC, STX1-TM-P, STX1-TM-P) and *stx*2 (STX2-TM-F, CCGGAATGCAAATCAGTC, STX2-TM-R, CAGTGACAAAACGCAGAACT, STX2-TM-P, FAM-ACTGAACTCCATTAACGCCAGATATGA-TAMRA) genes as reported by Chui et al. (2010).

ERIC-PCR fingerprinting

Two primer sets with the sequences ERIC-DG111-F 5′-ATG TAA GCT CCT GGG GAT TCA C-3′ and ERIC-DG112-R 5′-AAG TAA GTG ACT GGG GTG AGC G-3′ were used to amplify repetitive sequences contained in the chromosomal DNA of *E. coli* isolates using a single amplification profile (Versalovic et al., 1991). ERIC-PCR genotyping generates strain specific fingerprinting using primers directed to specific ERIC sequences to analyze the genetic diversity of the bacteria under investigation. *E. coli* ATCC 25922 was used as a standard strain in the reaction.

ERIC-PCR fingerprinting data were transformed into a binary code depending on the presence or absence of each band. Dendrograms were generated by the unweighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering routine. Cluster analysis and dendrogram construction were performed with SPSS, version 22 (IBM 2013). The discriminatory power of ERIC-PCR was measured by the Simpson's index of diversity (*D*) that indicates the average probability that a typing system will assign a different type to two unrelated strains randomly sampled from a population. A *D* value of more than 0.9 indicates good differentiation (Hunter, 1990).

Risk factors associated with E. coli *contamination of milk in dairy farms*

Open and closed ended questionnaire was developed to collect information about risk factors associated with milk contamination with *E. coli* in the farms under investigation. The questionnaire included information about the locality, milking, milk condition, hygiene practices (disinfection of milking, feed and water equipment and rodent control), feeding type and presence of *E. coli* in rectal swabs from cows. The questionnaire was completed during each visit to dairy farms by the farm owner.

Statistical analysis

The occurrence of different species within family *Enterobacteriaceae* was calculated as the proportion of the samples in which infection or contamination was detected by bacteriological examination. The 95% confidence interval (95% CI) was computed based on the exact binomial distribution. The occurrence and 95% CI were computed using WinPepi Package, Version 11.39. Differences were considered to be statistically significant at $P < 0.05$. The difference in the isolation of *E. coli* between different samples was analyzed using chi-square test (degree of freedom was noted as a subscript to the χ^2-statistics) that was computed in R Package Version 3.1.1 (the R Foundation for statistical computing platform[1]). Differences were considered to be statistically significant at $P < 0.05$. Bivariate logistic regression model was fitted to determine factors associated with *E. coli* contamination of milk samples at the investigated dairy farms using the computer program SPSS, version 22 (IBM 2013). Crude Odds Ratios (COR) and their 95% confidence interval (95% CI) were noted. *P* values <0.05 were considered statistically significant in the analysis.

[1] See: https://cran.r-project.org/bin/windows/base/old/3.1.1/ (accessed September 28 2016).

Results

E. coli was isolated from raw milk samples (22.4%; 28/125), dairy cattle feces (33.6%; 42/125), calf feces (35.4%; 29/82) and hand swabs from dairy workers (11.1%; 5/45) collected from the five dairy farms at Sharkia Governorate, Egypt. *E. coli* was also isolated from 2% (2/100) of stools from milk consumers attending a private laboratory at the same vicinity of the examined farms. *E. coli* was only detected in diarrheic people (2.9%; Table 1). The isolation rate of *E. coli* from diarrheic cows (53.8%; 7/13) was not different from that of non-diarrheic cows (31.3%; 35/112; Table 1; $\chi_1^2 = 0.652$; $P = 0.4$).

Serogrouping of *E. coli* isolates revealed that O125 was the only serogroup identified from all the sources under investigation, comprising 12 isolates. Other serogroups of O55, O18, O1, O27, O128, O168, O119, O151 and O158 were also identified. Only *stx1* gene was identified in seven of 12 *E. coli* O125 isolated from different sources. The genetic similarity of 12 *E. coli* O125 isolates from different sources was assessed using ERIC-PCR. The discriminatory index of ERIC-PCR in typing the isolates was calculated to be $D = 1$, indicating high discrimination of the technique. *E. coli* O125 isolates were classified into 12 distinct profiles namely E1–E12. The dendrogram analysis showed that two main clusters and three individual isolates were generated at linkage distance 12.5 (Fig. 1). Cluster I included isolates from rectal swabs from cows ($n = 2$), milk ($n = 2$) and hand swabs from dairy workers ($n = 2$), while Cluster II included one isolate from milk and two from rectal swabs from cows (Table 2; Fig. 1).

Analysis of risk factors associated with *E. coli* contamination of milk in dairy farms showed that mastitis in dairy cows is a significant risk factor (Table 3). Milk collected from cows with mastitis was four times more likely to be contaminated with *E. coli* than milk collected from normal cows (odds ratio [OR], 4; 95% CI, 1.07–14.99; $P < 0.05$). There were no statistically significant correlations with other risk factors (type of milking, disinfection of milking equipment, rodent control and feeding type) shown (Table 3; $P > 0.05$).

Table 1
Occurrence of *E. coli* in specimens from dairy cows, calves, milk handlers and milk consumers.

Samples	Sample condition	Number examined	No. of *E. coli* (%, 95% CI)
Rectal swabs – cows	Diarrheic	13	7 (53.8, 25.1–80.8)
	Non-diarrheic	112	35 (31.3, 22.8–40.7)
	Total	125	42 (33.6, 25.4–42.6)
Rectal swabs – calves	Diarrheic	50	17 (34, 21.2–48.8)
	Non-diarrheic	32	12 (37.5, 21.1–56.3)
	Total	82	29 (35.4, 25.1–46.7)
Milk	Normal milk	115	23 (20, 13.1–28.5)
	Mastitic milk	10	5 (50, 18.7–81.3)
	Total	125	28 (22.4, 15.4–30.7)
Hand swabs – dairy workers	Apparently healthy	45	5 (11.1, 3.7–24.1)
Milk consumers	Diarrheic	70	2 (2.9, 0.3–9.9)
	Non diarrheic patients	30	0
	Total	100	2 (2, 0.2–7)
Total		377	104 (27.6, 23.1–32.4)