Temporal variation of faecal shedding of *Escherichia coli* O157:H7 in a dairy herd producing raw milk for direct human consumption

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Abstract

The objective of this study was to analyse over time the evolution of *E. coli* O157:H7 faecal shedding in a dairy herd producing raw milk for direct human consumption. The study was performed between October 2012 and September 2013 in an average size Italian dairy farm where animals are housed inside the barn all over the year. The farm housed about 140 animals during the study – 70 cows and 70 calves and heifers. Twenty-six animals were randomly selected from both the cows and young animals group, and faecal sampling was performed rectally six times two months apart in each animal. Eleven animals were culled during the study and a total of 285 faecal samples were collected. At each faecal sampling, three trough water samples and two trough feed samples were also collected for a total of 36 water samples and 24 feed samples. Samples were analysed by real time polymerase chain reaction (RT-PCR) and culture. Overall, 16 (5.6%) faecal samples were positive for *E. coli* O157 by RT-PCR. Cultural examination found 9 (3.1%) samples positive for *E. coli* O157; all the isolates were positive for *stx1*, *stx 2* and *eae* genes. One (4.1%) feed sample was positive for *E. coli* O157 by RT-PCR; none of the water samples was positive for *E. coli* O157. The model highlighted a general significant reduction of the number of positive samples observed during the study from the first to the sixth sampling (P=0.000) and a positive relation between the presence of positive samples and average environmental temperature (P=0.003). The results of the study showed that in an Italian dairy farm housing animals all year, faecal shedding of *E. coli* O157 followed the same temporal trend reported for other types of farming. The enhanced faecal shedding during warmer months may have a significant impact on environmental contamination and the safety of raw milk and its by-products.

Introduction

The sale of raw milk for human consumption by self-service automatic vending machines has been allowed in Italy since 2004. After a case report of haemolytic uremic syndrome related to the consumption of raw milk (Scavia *et al*., 2009), the Italian Health Ministry published an ordinance (10 December 2008) establishing that vending machines should bear the notice *Milk must be boiled before consumption*. Despite this, some consumers continue to drink raw milk (Giacometti *et al*., 2012d). The presence of pathogenic bacteria in raw milk has been well documented both in Europe and in the United States, but the isolation rate reported has varied considerably (Oliver *et al*., 2005, 2009). The most frequent pathogenic bacteria involved in outbreaks due to raw milk consumption in Europe and worldwide are *Campylobacter jejuni* and human pathogenic *Escherichia coli* (Claeys *et al*., 2013). *E. coli* O157 was among the most frequently detected pathogenic bacteria in raw milk sold by self-service vending machines (Giacometti *et al*., 2012b, 2013) in Italy, and the only reported foodborne outbreaks due to raw milk consumption were due to *E. coli* O157:H7 and *Campylobacter jejuni* (Giacometti *et al*., 2012a). A seasonal trend of *E. coli* O157:H7 infection has been reported in humans (Renter and Sargeant, 2002; Money *et al*., 2010) and the increase in human infection was supposed to follow the increase in *E. coli* faecal shedding by cattle. In addition, infectious outbreaks were associated with private water supplies contaminated by bovine faeces, visits to dairy farms and cattle density in a given geographical area (Renter and Sergeant, 2002; Money *et al*., 2010). The seasonal variation of *E. coli* O157:H7 shedding in cattle faeces was previously reported but often in cross-sectional studies or in farming conditions different from those common in Italian dairy herds where cattle is housed all the year long. The present study is a longitudinal analysis of the evolution of *E. coli* O157:H7 faecal shedding in a dairy herd producing raw milk to evaluate the potential temporal variation of raw milk contamination. Moreover, faecal shedding between young and adult animals was compared.

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Materials and Methods

The study was performed between October 2012 and September 2013 on a typical average size Italian dairy farm. The farm housed about 140 animals during the study, 70 cows (older than 24 months) and 70 calves and heifers (2-24 month-old). Cows and young animals were housed in two different parts of the barn: cows were housed in cubicles and divided into three groups depending on their milk yield; younger animals were housed on straw in a yard with an external paddock and grouped in five pens depending on their age. Feeding of animal groups remained the same throughout the study. Twenty-six animals were randomly selected from both the cow group and young animals group and faecal sampling was performed rectally six times two months apart in each animal. Eleven animals were culled during the study and a total of 285 faecal samples were collected. At each sampling three trough water samples and two trough feed samples were collected for cows and young animals for a total of 36 water samples and 24 feed samples. Data on rainfall and environmental temperature were collected from the website http://weather.yahoo.com/italia/emilia-romagna/bologna-711080/

Escherichia coli O157 detection

Five grams of faeces, 25 g of feed and 25 mL of water were diluted tenfold (w/v) in buffered peptone water, and incubated at 37±1°C for 21±3 h. Bacterial DNA was extracted from 1 mL of enriched broth using the *Gen elute™*

bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) as described by the manufacturer. Reference strain *E. coli* O157:H7 ATCC35150 was included in each set of analysed samples as positive extraction and amplification control.

All primers and probes used in this study were described in ISO 13136:2012 and published previously (Nielsen and Andersen, 2003; ISO, 2012)

Real time PCR (RT-PCR) for the detection of *E. coli* O157 serogroup-associated *rfbE* gene was conducted in a 25 µL reaction volume using the following reaction mixture: 1 X TaqMan® Universal PCR Master mix (Applied Biosystems, Carlsbad, CA, USA), 900 nM each of the forward and reverse primers, 250 nM of the labelled probe and 4 µL DNA template. A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems) was included in each RT-PCR reaction. Real time-PCR thermal cycling was conducted using a StepOne Plus system (Applied Biosystems). The cycling parameters were: 95°C hold for 10 min for initial denaturation of the DNA and activation of the hot-start Taq polymerase, followed by 40 cycles of amplification of 95°C for 15 s, and 60°C for 60 s.

Positive samples were further characterised by multiplex RT-PCR targeting the virulence genes *eae*, *stx1* and *stx2*. In a 25 µL reaction volume, the following reaction mixture was used: 1 X Taqman®Universal PCR Master mix (Applied Biosystems), 450 nM each of the forward and reverse primers, 100 nM of each labelled probe and 4 µL DNA template. The PCR instrument and programme were the same as those used for the previous reaction.

In addition, isolation of *E. coli* O157 was also attempted from serogroup-specific RT-PCR positive samples. Enriched faecal samples were plated on tryptone bile x-glucuronide agar and incubated for 18-24 h at 37±1°C. Up to 50 colonies with *E. coli* morphology were picked up and point-inoculated on nutrient agar. Pools of ten colonies were tested by RT-PCR for *E. coli* O157 detection, then colonies from positive pools were tested singly by RT-PCR as described above in order to identify the *E. coli* O157 strain and to detect *eae*, *stx1* and *stx2* genes.

Statistical analysis

Data analysis was performed using Stata® 11.2 (StataCorp, College Station, TX, USA). Logistic regression random-effects models for longitudinal/panel data were built to evaluate the influence of animal age (young *vs* adult), sampling (from first to sixth), average monthly temperature (°C) and rainfall (mm) on *E. coli* O157 faecal shedding. Individual animal identifier was set as the panel variable; sampling month was set as the time variable. The relevant variables to be included in the final model were selected using manual backward elimination and log-likelihood ratio test. Significance was set at P<0.05. For descriptive statistics, robust standard errors allowing possible intra-panel correlations were used to calculate 95% confidence intervals.

Results

Overall 16 (5.6%) faecal samples were positive for *E. coli* O157 by RT-PCR examination. Cultural examination identified nine (3.1%) samples positive for *E. coli* O157; all the isolates were positive for *stx1*, *stx 2* and *eae* genes. Fourteen animals (25.9%) were positive by RT-PCR at least once during the study, and two animals resulted positive twice. Table 1 details the prevalence of RT-PCR-positive faecal samples in young and adult animals during the study period. Table 2 shows the prevalence of RT-PCR-positive faecal samples at the six sampling times. One (4.1%) feed sample was positive for *E. coli* O157 by RT-PCR, but no detection could be made by cultural examination; none of the water samples was positive for *E. coli* O157.

Logistic regression showed no significant differences between young and adult animals (P=0.671 in the full model) or a significant influence of average monthly rainfall (P=0.613 in the full model).

The final model highlighted a general significant reduction of the number of positive samples observed during the study from the first to the sixth sampling (P=0.000) and a positive relation between the presence of positive samples and average temperature (P=0.003), as reported in Table 3. In detail, the final logistic regression model output showed a significant increase in *E. coli* O157:H7 prevalence with temperature [odds ratio (OR)>1] and a significant decrease at following sampling times (OR<1). Likelihood ratio test demonstrated that the full model did not perform better than the final model. Wald chi-square test confirms the significance of the final model. The prevalence predicted over time (month) by the final model is reported in Figure 1: a significant increase was observed in the summer months.

Table 1. Overall percentage of positive faecal samples (prevalence) and robust 95% confidence interval in young and adult animals.

Age	Samples (n)	Prevalence (%)	95% CI
Young	154	6.5	2.3-10.7
Adult	131	4.6	1.3-7.8

CI, confidence interval.

Table 2. Overall percentage of positive faecal samples (prevalence) and robust 95% confidence interval at 6 different sampling times.

Sampling	Samples (n)	Prevalence (%)	95% CI
1st	52	15.4	5.3-25.5
2nd	51	2.0	0.0-5.9
3rd	48	2.1	0.0-6.3
4th	47	6.4	0.0-13.6
5th	46	4.3	0.0-10.4
6th	41	2.4	0.0-7.3

CI, confidence interval.

Table 3. Final logistic regression model output showing a significant increase in *Escherichia coli* prevalence with temperature.

E. coli O157	OR	SE	z	P	95% CI	
Temperature	1.193132	0.0708786	2.97	0.003	1.061995	1.340462
Sampling	0.4937161	0.0966556	-3.61	0.000	0.3363847	0.7246335

E. coli, Escherichia coli; OR, odds ratio; SE, standard error; CI, confidence interval. Random-effects logistic regression: number of observations=285; group variable=animal; number of groups=54; Log likelihood=-53.380248. Likelihood-ratio test: LR chi-square=0.56. Assumption: finalmodel nested in fullmodel P=0.7548; Wald chi-square=13.57 P=0.0011.

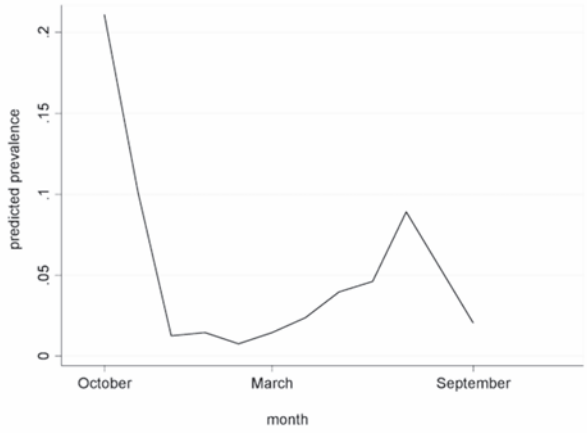

Figure 1. Prevalence predicted over time by the final logistic regression model. Prevalence is reported as proportion not percentage.

Discussion

The wordwide reported prevalence of *E. coli* O157:H7 in cattle varies widely ranging from 1 to 28%, most frequently <5% (Renter and Sargeant, 2002). This study found *E.coli* O157 prevalence rates similar to those reported by other studies on dairy herds (Stanford *et al*., 2005; Renter and Sargeant, 2002). Real time PCR detection of *E. coli* O157 with a limited number of the corresponding strain isolations precludes in-depth epidemiological study based on molecular characterisation of isolates, but it is a valid indicator of the carrier condition of cattle (Fernandez *et al*., 2009).

Our study showed no difference in the prevalence of positive faecal samples between young and older animals, at variance with other reports of a higher prevalence of *E. coli* O157 shedding in younger animals (Stanford *et al.*, 2005) than in cows (18.1-22.9% *vs* 10.7-11.1%). This difference is thought to be due to the type of sampling performed: the results of Stanford *et al*. (2005) was produced by a cross-sectional study performed sampling animals of different age in five dairy farms. On the contrary, our study was a longitudinal analysis in which the same proportion of animals of different ages (calves, heifers and cows) was randomly chosen and repeatedly sampled for one year; thus, the ageing of animals during the study may have influenced the results as previously reported for *Campylobacter jejuni* (Ellis-Iversen *et al*., 2009) and this may also explain the reduced prevalence detected during the study.

The increased prevalence of *E. coli* O157-positive faecal samples in warmer months (from July to October) is in agreement with previous reports (Renter and Sargeant, 2002; Fernandez *et al*., 2009; Money *et al*., 2010; Stanford *et al*., 2005) both in cattle and dairy herds. The reason for increased *E. coli* O157 faecal shedding during summer remains unsettled: a higher *E. coli* O157 survival ability outside the host, the increased house fly population, environmental factors (rainfall, increased temperature, day length), changes in feeding and hormone levels in the host have been proposed (Money *et al*., 2010). None of these causes can be excluded in our study except for feeding changes.

A recent report (Giacometti *et al.,* 2012c) demonstrated a relation between the detection of Verocytotoxin-producing *E. coli* in dairy herds and the general hygiene condition of the farm (feed trough, water trough and bedding cleanliness) and a higher *E. coli* O157 survival ability in faeces at higher temperature (22°C *vs* 4°C) (Bach *et al.,* 2005). The association between higher environmental temperatures and the prevalence of faecal shedding found in our study suggests that the higher survival of *E. coli* O157 in faeces outside the host may play a role in enhancing *E. coli* O157.

Conclusions

Our study shows that in an Italian dairy farm housing animals all the year long, faecal shedding of *E. coli* O157 followed the same temporal trend as that reported for other types of farming. The enhanced faecal shedding during warmer months may have a significant impact on environmental contamination and the safety of raw milk and its by-products.

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