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# Four-Year Monitoring of Foodborne Pathogens in Raw Milk Sold by Vending Machines in Italy

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## ABSTRACT

Prevalence data were collected from official microbiological records monitoring four selected foodborne pathogens (*Salmonella, Listeria monocytogenes, Escherichia coli* O157:H7, and *Campylobacter jejuni*) in raw milk sold by self-service vending machines in seven Italian regions (60,907 samples from 1,239 vending machines) from 2008 to 2011. Data from samples analyzed by both culture-based and real-time PCR methods were collected in one region. One hundred raw milk consumers in four regions were interviewed while purchasing raw milk from vending machines. One hundred seventy-eight of 60,907 samples were positive for one of the four foodborne pathogens investigated: 18 samples were positive for *Salmonella,* 83 for *L. monocytogenes,* 24 for *E. coli* O157:H7, and 53 for *C. jejuni* in the seven regions investigated. No significant differences in prevalence were found among regions, but a significant increase in *C. jejuni* prevalence was observed over the years of the study. A comparison of the two analysis methods revealed that real-time PCR was 2.71 to 9.40 times more sensitive than the culture-based method. Data on consumer habits revealed that some behaviors may enhance the risk of infection linked to raw milk consumption: 37% of consumers did not boil milk before consumption, 93% never used an insulated bag to transport raw milk home, and raw milk was consumed by children younger than 5 years of age. These results emphasize that end-product controls alone are not sufficient to guarantee an adequate level of consumer protection. The beta distribution of positive samples in this study and the data on raw milk consumer habits will be useful for the development of a national quantitative risk assessment of *Salmonella, L. monocytogenes, E. coli* O157, and *C. jejuni* infection associated with raw milk consumption.

The sale of raw milk for human consumption by self-service vending machines has been allowed in Italy since 2004 after the enactment of European Commission Regulation 853/2004 *(15).* Regional regulations were subsequently developed and are periodically reviewed in cooperation with the Italian Ministry of Health and local authorities. The regulations differ considerably among regions in terms of both microbiological limits and analytical methods. Since December 2008, raw milk vending machines must display the notice ''milk must be consumed after boiling'' *(29),* but despite this warning about 40% of consumers do not boil milk or perform any effective antimicrobial heat treatment *(21).* Several pathogenic bacteria have been reported in milk *(39, 40),* and raw milk consumption carries the risk of ingesting pathogenic bacteria that pose a health hazard *(31).* Outbreaks of *Escherichia coli* O157:H7 and *Campylobacter jejuni (2, 3)* infection and a case of hemolytic uremic syndrome *(43)* have been reported in Italy, and raw milk and raw milk cheeses are recognized sources of *Salmonella (7, 8, 34)* and *Listeria monocytogenes* infections in humans worldwide *(12).*

The current best practice for evaluating health risks associated with food is through quantitative risk assessment. Several surveys on the prevalence of foodborne pathogens at the herd level, raw milk distribution, consumer milk consumption habits, and home storage of milk *(19–21)* led to the development of a farm-to-table quantitative risk assessment of verocytotoxin-producing *E. coli* O157:H7 (VTEC) and *C. jejuni* related to consumption of raw milk *(18).* The model was accurate according to data reported by the Italian Ministry of Health on pediatric hemolytic uremic syndrome and a useful tool for assessing the risk of

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campylobacteriosis and hemolytic uremic syndrome at the national level. However, the model was developed on the basis of a limited scale study that considered only one province in one Italian region and has yet to be applied elsewhere. Because of the length of the Italian peninsula and its geographical diversity, ranging from plains to mountains, the climate of Italy differs considerably among regions. Therefore, the prevalence of foodborne pathogens in raw milk may differ significantly among regions because of different environmental conditions and the consequently different herd management systems and consumer habits.

The main objectives of this study were to (i) collect data on the prevalence of four selected foodborne pathogens (*Salmonella, L. monocytogenes, E. coli* O157:H7, and *C. jejuni*) in raw milk sold in self-service vending machines in seven Italian regions over a 4-year period, (ii) evaluate whether regional or temporal differences could affect these prevalences, and (iii) investigate consumer habits in the different regions. The results obtained by two analytical methods used to test raw milk in one region were compared, and the reliability of the PCR-based method was assessed in comparison with the official culture-based methods.

## MATERIALS AND METHODS

**Data collection.** Data were collected from official microbiological records monitoring raw milk samples from self-service vending machines in seven regions of Italy by the Regional Veterinary Authorities from 2008 to 2011. The seven regions, Emilia Romagna, Lazio and Tuscany (pooled data), Lombardy, Marche, Piedmont, Sicily, and Veneto, account for most of the 1,411 vending machines registered in Italy. Data previously reported *(6)* also were included.

All samples were analyzed at the Experimental Institutes for Zooprophylaxis in the different regions; all the laboratories and test procedures are accredited according to International Organization for Standardization (ISO) method 17025:2005 *(26)* by the ACCREDIA Italian accreditation body *(6).* In accordance with microbiological criteria stipulated in the national legal requirements of Intesa Stato Regioni *(28),* we considered data on *Salmonella, L. monocytogenes, E. coli* O157:H7, and *C. jejuni.*

The number of vending machines in each region and the number of samples collected from vending machines may differ among regions, but in all regions at least one sample was tested from each vending machine each year for these four foodborne pathogens in accordance with the official ISO cultural method (ISO 16654:2003, ISO 6579:2002/C1:2004, ISO 11290-1:1996/AM1:2004, and ISO 10272-1:2006) *(23–25, 27).* A total of 1,239 vending machines (87.73% of the 1,411 registered vending machines) were sampled, and 60,907 samples were analyzed.

In Lombardy, data from samples analyzed by both culture-based (ISO procedures) and real-time PCR (RT-PCR) methods as described in a previous study *(19)* were collected. For *E. coli* O157:H7 during years 2008 through 2010 samples positive for *eae* and *vt1* and/or *vt2* were subjected to RT-PCR to detect the O157 serotype of *E. coli.* In 2011, the PCR method was improved to detect also VTEC serotypes other than O157 as described *(19)*: samples positive for *eae* and *vt1* and/or *vt2* were subjected to RT-PCR to detect *E. coli* serovars O26, O103, O111, O145, and O157.

**Statistical analysis.** To account for the uncertainty in pathogen prevalence estimation in milk samples sold in Italy, beta distributions were modeled starting from official data regarding pathogen prevalence in the seven Italian regions based on the following formula: $s + 1$; $N - s + 1$, where $s$ is the portion of positive milk samples recorded and $N$ is the total number of milk samples tested.

To represent this uncertainty, the 5th and 95th percentiles of the distribution were calculated. This distribution reasonably contains the actual prevalence of raw milk pathogens in the sampled areas.

Data analysis was carried out as a two-step process. First, a univariate analysis was conducted in which the overall difference (considering all 4 years of sampling) of the proportion of positive samples of each pathogen observed among sampled regions was tested with Pearson's $\chi^2$. Because of the large number of samples collected, the $P$ value for significance of this test was set at $<0.001$. A nonparametric test for trends across ordered groups *(10),* an extension of the Wilcoxon rank-sum test, was used to test the annual prevalence trend of each pathogen across the regions. The $P$ value for significance of this test was set at $<0.05$. Second, a multivariate analysis was conducted in which the proportion of samples positive for each pathogen was analyzed using mixed effects logistic regression. Region (Emilia Romagna, Lazio plus Tuscany, Lombardy, Marche, Piedmont, Sicily, and Veneto), years (2008, 2009, 2010, and 2011), and their interaction were considered independent variables, with significance set at $P < 0.05$. Statistical analyses were performed using Intercooled Stata 7.0 software (Stata Corporation, College Station, TX).

**Consumer habits.** In 2011, 100 consumers of raw milk in the four regions (25 consumers each in Piedmont, Lombardy, Veneto, and Emilia Romagna) accounting for the largest number of raw milk vending machines (1,167 of the 1,238 considered in the study) were interviewed while purchasing raw milk at vending machines. They were asked about their habits regarding the use of insulated bags to transport raw milk home, the average duration of transport, the custom of boiling milk before consumption, and the composition of the family in relation to potentially sensitive consumers (0 to 5 years old and 6 to 14 years old). Data on the habits of consumers of raw milk reported by the Emiliano Romagnolo Center of Veterinary Epidemiology (CEREV) as collected with an on-line form *(17)* also were used.

## RESULTS

Of the 60,907 samples tested, 178 were positive for one of the four foodborne pathogens investigated. Overall in the seven regions, 18 samples were positive for *Salmonella,* 83 for *L. monocytogenes*, 24 for *E. coli* O157:H7, and 53 for *C. jejuni,* with prevalences of 0 to 0.96% for *Salmonella,* 0.21 to 1.63% for *L. monocytogenes,* 0 to 1.50% for *E. coli* O157:H7, and 0 to 2.22% for *C. jejuni* in the different regions. Table 1 summarizes the results of the official monitoring over the 4-year period, including the number of raw milk samples tested, the number and percentage of positive samples, and the mean and 5th and 95th percentiles of the beta distribution modeled from the univariate analysis.

Univariate analysis showed that the overall prevalence for all pathogens considered, with the exception of *L. monocytogenes,* differed significantly ($P < 0.001$) between regions (Table 1). A positive trend ($P < 0.05$) in the prevalence of *L. monocytogenes* and *C. jejuni* was observed in regions sampled from 2008 and 2011, with prevalences of 0.3 to 0.8% and 0.2 to 0.6%, respectively (Table 2). The multivariate analysis revealed that the only variable that

TABLE 1. *Prevalence of* Salmonella, L. monocytogenes, E. coli *O157:H7, and* C. jejuni *isolated from raw milk samples in seven Italian regions, 2008 through 2011, and beta distributions from the univariate analysis*

| Pathogen, region | No. of negative samples | No of positive samples | Prevalence (%) | Beta distribution (%) | 5th percentile (%) | 95th percentile (%) |
|---|---|---|---|---|---|---|
| *Salmonella* | | | | | | |
| Emilia Romagna | 1,398 | 1 | 0.07 | 0.14 | 0.03 | 0.34 |
| Lazio and Tuscany | 626 | 6 | 0.96 | 1.11 | 0.53 | 1.88 |
| Lombardy | 10,788 | 7 | 0.06 | 0.07 | 0.04 | 0.12 |
| Marche | 196 | 0 | 0.00 | 0.51 | 0.03 | 1.51 |
| Piedmont | 826 | 2 | 0.24 | 0.36 | 0.10 | 0.76 |
| Sicily | 288 | 1 | 0.35 | 0.69 | 0.12 | 1.63 |
| Veneto | 1,280 | 1 | 0.08 | 0.16 | 0.03 | 0.37 |
| Pearson's $\chi^2$ (6) = 43.1139, $P$ = 0.000 | | | | | | |
| *L. monocytogenes* | | | | | | |
| Emilia Romagna | 1,398 | 3 | 0.21 | 0.29 | 0.10 | 0.55 |
| Lazio and Tuscany | 627 | 4 | 0.64 | 0.79 | 0.31 | 1.45 |
| Lombardy | 10,561 | 54 | 0.51 | 0.52 | 0.41 | 0.64 |
| Marche | 194 | 1 | 0.52 | 1.02 | 0.18 | 2.41 |
| Piedmont | 817 | 11 | 1.35 | 1.47 | 0.85 | 2.22 |
| Sicily | 307 | 5 | 1.63 | 2.26 | 1.07 | 3.80 |
| Veneto | 1,277 | 5 | 0.39 | 0.47 | 0.21 | 0.82 |
| Pearson's $\chi^2$ (6) = 19.6106, $P$ = 0.003 | | | | | | |
| *E. coli* O157:H7 | | | | | | |
| Emilia Romagna | 1,400 | 1 | 0.07 | 0.14 | 0.03 | 0.34 |
| Lazio and Tuscany | 602 | 9 | 1.50 | 1.66 | 0.90 | 2.59 |
| Lombardy | 10,849 | 9 | 0.08 | 0.09 | 0.05 | 0.14 |
| Marche | 177 | 0 | 0.00 | 0.56 | 0.03 | 1.67 |
| Piedmont | 827 | 1 | 0.12 | 0.24 | 0.04 | 0.57 |
| Sicily | 182 | 0 | 0.00 | 0.54 | 0.03 | 1.62 |
| Veneto | 1,058 | 4 | 0.38 | 0.47 | 0.19 | 0.86 |
| Pearson's $\chi^2$ (6) = 75.0332, $P$ = 0.000 | | | | | | |
| *C. jejuni* | | | | | | |
| Emilia Romagna | 1,374 | 8 | 0.58 | 0.65 | 0.34 | 1.05 |
| Lazio and Tuscany | 589 | 5 | 0.85 | 1.02 | 0.44 | 1.77 |
| Lombardy | 10,698 | 22 | 0.21 | 0.21 | 0.15 | 0.29 |
| Marche | 191 | 0 | 0.00 | 0.52 | 0.03 | 1.55 |
| Piedmont | 810 | 18 | 2.22 | 2.34 | 1.54 | 3.27 |
| Sicily | 293 | 0 | 0.00 | 0.34 | 0.02 | 1.01 |
| Veneto | 1,274 | 0 | 0.00 | 0.08 | 0.00 | 0.23 |
| Pearson's $\chi^2$ (6) = 98.6775, $P$ = 0.000 | | | | | | |

remained significant was the year for *C. jejuni* ($P$ = 0.002). The overall prevalence (considering all sampled regions) for *C. jejuni* increased from 0.22 and 0.23% in 2008 and 2009, respectively, to 0.65 and 0.54% in 2010 and 2011, respectively.

The comparison of the data from Lombardy from 4 years analyzed in parallel by the culture method and the RT-PCR method revealed that the RT-PCR method detected *Salmonella, L. monocytogenes, E. coli* O157, and *C. jejuni* in 0.12, 1.29, 0.51, and 1.35% of the samples, respectively,

TABLE 2. *Trend of the prevalence of* L. monocytogenes *and* C. jejuni *in raw milk samples, 2008 through 2011*

| | *L. monocytogenes*[a] | | | *C. jejuni*[b] | | |
|---|---|---|---|---|---|---|
| Year | No. of positive samples | No. of negative samples | Prevalence (%) | No. of positive samples | No. of negative samples | Prevalence (%) |
| 2008 | 13 | 4,501 | 0.29 | 10 | 4,560 | 0.22 |
| 2009 | 29 | 5,616 | 0.51 | 13 | 5,679 | 0.23 |
| 2010 | 22 | 2,673 | 0.82 | 17 | 2,597 | 0.65 |
| 2011 | 19 | 2,391 | 0.79 | 13 | 2,393 | 0.54 |

[a] $P < 0.01$, $r(z) = 3.36$.
[b] $P < 0.01$, $r(z) = 3.07$.

TABLE 3. *Number of raw milk samples analyzed from the Lombardy region and number and percentage of positive samples as determined by the PCR and culture methods*

| Evaluation | *Salmonella* | *L. monocytogenes* | *E. coli* O157:H7 | *C. jejuni* |
|---|---|---|---|---|
| Total no. of samples analyzed | 15,402 | 15,181 | 15,095 | 15,229 |
| No. (%) of samples positive by PCR | 19 (0.12) | 196 (1.29) | 77 (0.51) | 207 (1.35) |
| No. (%) of samples positive by culture | 7 (0.04) | 54 (0.35) | 9 (0.05) | 22 (0.14) |
| PCR/culture ratio | 2.71 | 3.62 | 8.55 | 9.40 |

whereas the culture method detected these pathogens in 0.04, 0.35, 0.05, and 0.14% of samples, respectively (Table 3). In 2011, with implementation of the RT-PCR method, 112 samples were VTEC positive *(vt2* or *eae* + v*t1* and/or *vt2*). Serovar O157 was the most common *E. coli* serovar detected (15 samples) followed by O26, O103, and O145 in seven, six, and five samples, respectively. Serovar O111 was never detected, and in 79 samples no serovar could be assigned; no positive results were reported by culture method in samples from 2011.

Of the 100 consumers interviewed, 93% did not use insulated bags to transport raw milk home, 3% used these bags only in summer, and 4% always used them. The duration of transport ranged from a few minutes to 18 min, 37% of consumers did not boil milk before drinking (26% drank raw milk and 11% heated the milk without reaching the boiling point) and 63% of consumers boiled the raw milk before consumption. Analysis of the 100 consumer interviews conducted in this study and the 45 collected in the CEREV study were used to identify sensitive populations among raw milk consumers; 5.57% of consumers were younger than 5 years old, and 6.69% of consumers were 6 to 14 years old.

## DISCUSSION

This study reports the prevalence of four foodborne pathogens in official samples collected during monitoring of raw milk sold by self-service vending machines over a 4-year period (2008 through 2011). The findings emphasize that end-product controls alone are not sufficient to guarantee an adequate level of consumer protection. The reported pathogen prevalence is in line with data reviewed by Oliver et al. *(39, 40)* and with the European data cited by Dontorou et al. *(14)* for VTEC O157:H7 (0 to 3%) but lower than that reported by Desmasures et al. *(13)* for *Salmonella* (5.8%) and *L. monocytogenes* (2.9%) and by Meyer-Broseta et al. *(35)* for *L. monocytogenes* (2.4%) in France. Previous Italian studies performed at the provincial *(19)* and regional *(6)* levels confirmed the low prevalence of raw milk contamination in comparison to international findings. This lower prevalence probably is due to the fact that farmers intending to sell raw milk must implement specific practices such as self-monitoring for meeting microbiological and chemical criteria for milk and management systems for meeting higher standards of good dairy farming practices than other types of dairy farms.

The comparison of the two sample evaluation methods revealed that RT-PCR is 2.71 to 9.40 times more sensitive than the culture method (Table 3). The higher sensitivity of PCR methods has been reported *(4, 9, 30, 44, 45),* including for raw milk samples in Italy *(1, 5, 19, 20)*. The fact that the RT-PCR method detects dead cells should be taken into account in comparing the sensitivity of the two methods, but this difference has no impact on the development of a risk assessment of consumption of raw milk sold in vending machines. According to the precautionary principle, the detection of a pathogen, potentially viable or not, in a risk pathway represents a key factor for modulating risk because raw milk does not receive any antimicrobial treatments and foodborne pathogens are able to survive throughout the shelf life *(21)*.

The higher sensitivity of the RT-PCR versus culture method must also be considered when conducting a quantitative risk assessment based on qualitative cultural analytical methods. The standard volume of sample analyzed in a qualitative culture method is usually 25 ml, and its sensitivity is usually assumed to be 1 cell in 25 ml or 25 g. Because PCR-based methods are about 10 times more sensitive, they are considered able to detect 0.1 cell in 25 ml (or 1 cell in 250 ml). Nero et al. *(38)* found that the isolation rate of *L. monocytogenes* and *Salmonella* Enteritidis in raw milk is greatly influenced by the level of any contaminating microflora and is seldom possible when the pathogen level is 2.0 log CFU/ml and the mesophilic aerobe levels are 4 to 5 log CFU/ml. This contamination scenario is common in raw milk sold by self-service vending machines in Italy *(19)*. Consequently, the sensitivity of microbiological methods is probably generally overestimated and can lead to an underestimation of the true prevalence of pathogen-positive samples and, consequently, of the estimated risk of infection or illness.

The detection of *eae, vt1,* and *vt2* genes by PCR methods revealed that non-O157 VTEC strains are more prevalent than O157 VTEC strains in raw milk sold in vending machines, in agreement with two previous surveys performed on dairy farms authorized to sell raw milk in the Emilia Romagna *(20)* and Marche *(41)* regions and with data reported internationally *(33, 37)*. Recently published surveys indicated that non-O157 VTEC strains also are more prevalent (72%) than O157 VTEC strains in fecal samples collected from patients in the United States *(36)* and that the incidence of non-O157 VTEC infections is increasing *(22)*. These two observations indicate that testing raw milk for only O157 VTEC strains according to Italian legislation is inadequate to ensure consumer safety. The true prevalence of non-O157 VTEC strains in milk currently remains undetermined in most of the regions investigated, and consequently official controls must be implemented for consumer protection and risk evaluation.

Data on consumer habits confirm that some of their behaviors may enhance the health risk associated with raw milk consumption: 37% of consumers do not boil raw milk before consumption, 93% never use insulated bags to transport raw milk home, and raw milk is consumed by children younger than 5 years of age. These data are in agreement with a study performed at the local level in which only 57% of consumers boiled milk before consumption and only 14% used insulated bags *(21)*. An interesting finding in the present study is the practice of consuming raw milk by adding it to tea. This behavior was not highlighted in previous studies *(11, 17, 21)* and could be typical of other cultures.

In spite of the different geographical features and climate, no significant difference in the occurrence of pathogens was observed among regions. The differences among regions in the prevalence of each pathogen as determined by univariate analysis were not confirmed in multivariate analysis, in which the prevalence of all four pathogens did not seem to be associated with the different regions. Bianchi et al. *(6)* found that the only factor affecting the probability of noncompliant raw milk samples was a previous instance of noncompliance, and these authors suggested the need for more attention in herd management and the implementation of biosafety procedures. Ricci et al. *(42)* also reported that farm management methods were strongly correlated with the risk of pathogen transmission to raw milk, more so than inadequate structures or the poor sanitary management of the herd.

The positive trend observed in the univariate analysis for *L. monocytogenes* and *C. jejuni* was confirmed in the multivariate analysis for only *C. jejuni,* whose prevalence was significantly associated with the year of sampling. The observed significant increase in samples positive for *C. jejuni* is in line with data from the European Food Safety Authority report *(16),* in which the European Union notification rate of confirmed cases of human campylobacteriosis showed a significant increasing trend from 2008 through 2011. The reasons for this trend are not completely understood. In five of the reported campylobacteriosis outbreaks, the implicated food vehicle was milk, and of these five outbreaks, three were attributed to raw or insufficiently heated milk, highlighting the risks related to consuming unpasteurized milk. The risk of campylobacteriosis and other diseases associated with the consumption of raw milk has been well documented *(16)*.

In spite of both specific and restrictive rules adopted and official monitoring, the number of raw milk samples out of compliance for the presence of foodborne pathogens appears to be static *(6)*. More rigorous testing could reduce the risks associated with unpasteurized milk consumption, but consumers can never be guaranteed that unpasteurized milk is pathogen free *(32)*. These facts emphasize the need for a consumer protection approach with specific educational programs demonstrating the importance of boiling milk before drinking. A more drastic measure to prevent raw milk–associated disease outbreaks is to refrain from consuming unpasteurized milk *(32)*.

The beta distribution of pathogen-positive samples in this study and data collected on raw milk consumer habits will be useful for the development of a reliable national quantitative risk assessment of *Salmonella, L. monocytogenes*, *E. coli* O157:H7, and *C. jejuni* infections related to the consumption of raw milk.

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