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Journal

Journal of Dairy Science, 97(3)

ISSN

0022-0302

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Publication Date

2014-03-01

DOI

10.3168/jds.2013-7421

Peer reviewed

HHS Public Access

Author manuscript

J Dairy Sci. Author manuscript; available in PMC 2015 March 04.

Published in final edited form as:

J Dairy Sci. 2014 March ; 97(3): 1513–1519. doi:10.3168/jds.2013-7421.

Multiresidue screening of milk withheld for sale at dairy farms in central New York State

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Abstract

Many of the drugs commonly used in lactating dairy cows result in residues in the milk, prohibiting its sale for human consumption. Milk withheld for sale because of drug treatment or from cows with high somatic cell counts is commonly called “waste milk.” One-third of dairy farms in the United States use waste milk to feed preweaned dairy calves. Limited information is currently available on the effect of this practice on the selection and dissemination of antibiotic-resistant bacteria. Pooled waste milk samples were collected from 34 dairy farms in central New York State with the objective of detecting the presence and quantity of drug residues in these samples. Samples were collected and refrigerated using ice packs and then stored at 4°C upon arrival at the Cornell laboratory (Ithaca, NY). Screening for β-lactam, tetracycline, and sulfonamide residues in the milk was performed using commercial enzyme-linked receptor-binding assay (SNAP) tests (Idexx Laboratories Inc., Westbrook, ME). Samples with a positive SNAP test were selected for screening using a multiresidue liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The SNAP tests revealed that 75, 14.3, and 7.1% of waste milk samples (n = 34) contained β-lactam, tetracycline, and sulfamethazine residues, respectively. Of the samples sent for LC-MS/MS (n = 28), half had detectable quantities of drug residues. The most prevalent drugs detected by LC-MS/MS were ceftiofur (39.2%; mean ± SE concentration = 0.151 ± 0.042 μg/mL), penicillin G (14.2%; mean ± SE concentration = 0.008 ± 0.001 μg/mL), and ampicillin (7.1%; mean ± SE concentration = 0.472 ± 0.43 μg/mL). In addition, one sample had detectable concentrations of oxytetracycline and one sample had detectable concentrations of sulfadimethoxine. These results provide insight on drug residues present in waste milk from select farm in upstate New York, and additionally indicate the need for additional studies targeting on-farm treatments that could degrade drug residues present in waste milk and reduce the potential effects on the biosphere from the disposal and use of waste milk as a feed source.

Keywords

waste milk; antibiotic resistance; drug residues; liquid chromatography-tandem mass spectrometry

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Introduction

According to the last USDA Animal and Plant Health Inspection Service (APHIS) report on antimicrobial drug use on US dairy operations, mastitis is the leading disease responsible for the use of antibiotics in cows, followed in descending order by lameness, respiratory disease, and reproductive disorders (USDA, 2008b).

Regardless of the benefits of using antimicrobial agents in food-producing animals, considerable concerns from public health, food safety, and regulatory perspectives arise from the potential for development of antimicrobial resistance (Oliver et al., 2011). The selection of resistant bacteria has generally been assumed to occur at concentrations between the MIC of the susceptible wild-type population and that of the resistant bacteria. Concentrations below the MIC of the susceptible population were considered to not inhibit growth of the susceptible bacteria and therefore were considered unable to cause selection pressure (Drlica and Zhao, 2007; Gullberg et al., 2011). However, studies using highly sensitive competition experiments have shown that selection of resistant bacteria can occur at extremely low antibiotic concentrations, selecting for resistant bacteria with compensatory mutations that counterbalance the decreased fitness cost caused by resistance (Davies et al., 2006; Andersson and Hughes, 2010; Kohanski et al., 2010; Gullberg et al., 2011). Furthermore, exposure of bacteria to antibiotics at sub-MIC levels has been shown to stimulate mutagenesis and recombination, leading to bacterial adaptation to various stresses, including antibiotic pressure (López and Blazquez, 2009; Thi et al., 2011).

Diseases have a great impact on the dairy industry, resulting in treatment expenses and production losses such as reduction in milk production and withholding of nonsaleable milk containing drug residues (Cha et al., 2010; Cha et al., 2011). To overcome production losses related to treating cows with antibiotics, 33% of dairy farms in the United States feed preweaned calves “waste milk,” the nonsaleable milk from cows that have milk withheld because of therapeutics or from cows with high SCC (USDA, 2008a). Feeding pasteurized waste milk instead of milk replacer to preweaned calves has been observed to result in an estimated saving of $0.69 per calf per day (Godden et al., 2005).

To the best of our knowledge, no currently available studies have identified the presence and the concentration of antimicrobial drugs in waste milk from dairy farms in the United States. The objective of this study was to identify the presence and measure the concentration of 27 drugs in the pooled waste milk of dairy farms located in central New York State.

Materials and Methods

Study Population

With the objective of identifying antimicrobial agents that could be present at any given time in the waste milk of a dairy farm participating in the study, 1 raw nonsaleable milk (waste milk) sample was collected from each farm. Milk samples from 34 dairy farms were collected by 3 veterinarians from herds in the following counties in central New York State: Cortland, Tompkins, Cayuga, Allegany, Livingston, Wyoming, Ontario, Wayne, and Steuben. Participating veterinarians identified study farms based on having typical

management practices for nonorganic dairy farms in upstate New York, routinely pooling nonsaleable milk, and willingness of the owner to participate.

Descriptive data from sampled farms was collected and revealed that the number of lactating cows ranged from 28 to 5,000 (median = 780), the number of preweaned calves ranged from 8 to 400 (median = 57.5), and the number of cows milked into the pooled waste milk bulk tank on the day of sampling ranged from 3 to 150 (median = 19.5). Twenty-one of these farms had more than 500 lactating cows and 13 had fewer than 500 lactating cows.

Milk Sample Collection and Processing

Collected waste milk samples were kept in a leak-proof container with ice packs and transported or mailed overnight to our laboratory at Cornell University where they were refrigerated at 4°C upon arrival. Milk samples that tested positive for antibiotic residues using the commercial enzyme-linked receptor-binding assay tests were stored at −18°C until sent by overnight mail in cold packaging to a commercial laboratory for drug residue quantification using liquid chromatography-tandem mass spectrometry (**LC-MS/MS**).

Commercial Enzyme-Linked Receptor-Binding Assays Screening Test

Three commercial enzyme-linked receptor-binding assay (**SNAP**) tests with limit of detection (**LOD**) for antibiotic residues at or below the US Food and Drug Administration (**FDA**) tolerance levels were used for the initial screening of milk samples for antimicrobial residues. The New SNAP Beta-lactam Test Kit (Idexx Laboratories Inc., Westbrook, ME) was used to screen for the following β-lactam drugs: penicillin (90/95% sensitivity at 0.003 μg/mL), amoxicillin (90/95% sensitivity at 0.0073 μg/mL), cephapirin (90/95% sensitivity at 0.0117 μg/mL), and ceftiofur residues (90/95% sensitivity at 0.012 μg/mL) in raw milk. In addition, the SNAP Sulfamethazine Test Kit (Idexx Laboratories Inc.), which detects sulfamethazine (LOD = 0.01 μg/ mL) residues in milk, and the SNAP Tetracycline Test Kit (Idexx Laboratories Inc.), which detects tetracycline (LOD = 0.05 μg/mL), oxytetracycline (LOD = 0.05 μg/mL), and chlortetracycline (LOD = 0.1 μg/ mL) residues in milk, were used as initial screening tests. The SNAP tests were used following the manufacturer's instructions.

Quantification of Drug Residues Using LC-MS/MS

Milk samples that tested positive for the presence of antibiotic residues for at least 1 of the 3 screening tests were sent to Eurofins CAL (Metairie, LA) for quantification by LC-MS/MS using a protocol that can detect and quantify 27 drugs. The protocol for LC-MS/MS at Eurofins CAL followed a laboratory information bulletin by the FDA (US FDA, 2011), which was modified and internally validated by Eurofins CAL to include testing for ceftiofur. Briefly, the reagents and materials used for LC-MS/MS were deionized water (18.2 MΩ·cm), high-purity chromatographic- and spectrophotometric-grade acetonitrile and methanol, and formic acid at 96% purity.

The limits of quantification (**LOQ**) for the 27 residues screened using LC-MS/MS were as follows: ampicillin (0.01 μg/mL), penicillin G (0.005 μg/mL), cloxacillin (0.01 μg/mL), cephapirin (0.01 μg/mL), ceftiofur (0.01 μg/mL), sulfamethazine (0.002 μg/mL),

sulfadiazine (0.002 μg/mL), sulfadimethoxine (0.002 μg/mL), sulfathiazole (0.002 μg/mL), sulfaquinoxaline (0.002 μg/mL), sulfapyridine (0.002 μg/mL), sulfachloropyridazine (0.002 μg/mL), sulfamerazine (0.002 μg/ mL), oxytetracycline (0.01 μg/mL), tetracycline (0.01 μg/ mL), chlortetracycline (0.01 μg/mL), doxycycline (0.01 μg/mL), tylosin (0.01 μg/mL), tilmicosin (0.01 μg/mL), erythromycin (0.05 μg/mL), sarafloxacin (0.05 μg/mL), enrofloxacin (0.05 μg/mL), ciprofloxacin (0.05 μg/mL), 5-hydroxyflunixin (0.002 μg/mL), bacitracin (0.25 μg/mL), thiabendazole (0.01 μg/mL), and virginiamycin (0.005 μg/mL). The concentrations of the stock solutions used in the LC-MS/MS corresponded to the active drug compound, and the amounts weighed were adjusted to take into account purity and any counter-ions.

Statistical Analysis

Descriptive analysis for the SNAP tests and LC-MS/ MS were done using Microsoft Office Excel 2010 (Microsoft Corp., Redmond, WA) and PROC FREQ and PROC UNIVARIATE procedures in SAS (SAS Institute Inc., Cary, NC).

Results

SNAP Tests

Antibiotic screening of waste milk using the SNAP tests revealed that 28 of the 34 milk samples tested positive for β-lactam drug residues. Additionally, 7.1, 14.3, and 3.6% (n = 28) of the samples tested positive for sulfamethazine, tetracycline, and simultaneously for all 3 antimicrobial classes, respectively (Table 1).

Quantification of Drug Residues Using LC-MS/MS

Half of the milk samples submitted for LC-MS/MS had detectable concentrations of one or more of the 27 drug residues screened by this analytical test. Ceftiofur was the most frequently detected β-lactam, present in 39.2% (n = 28) of the waste milk samples (mean ± SE = 0.151 ± 0.042 μg/mL), followed by penicillin G at 14.2% (0.008 ± 0.001 μg/mL), ampicillin at 7.1% (0.472 ± 0.43 μg/mL), cephapirin at 3.5%, and cloxacillin at 3.5% (Table 2). One sample had detectable concentrations of oxytetracycline, 1sample had detectable concentrations of sulfadimethoxine, and 1 sample had detectable concentrations of 5-hydroxiflunixin. The interpretation of LC-MS/MS results in our study is limited to samples testing positive on a screening test (SNAP tests), because only milk samples that tested positive for the presence of antibiotic residues for at least 1 of the 3 SNAP were screened using LC-MS/MS. Furthermore, these results do not represent the average concentration for all waste milk samples collected in the study.

Discussion

A study by Selim and Cullor (1997) screened 189 waste milk samples using SNAP tests and observed that 46, 30, and 63% of samples tested positive for β-lactam, tetracycline, and β-lactam or tetracycline drugs, respectively. In the present study, the prevalence of β-lactam residues was greater than that of tetracycline, possibly reflecting the shift in antibiotic classes used on dairy farms from 1997 to 2013, such as the increase in the use of

cephalosporins (Sawant et al., 2005; Raymond et al., 2006; Saini et al., 2012). To ensure that the residues in the milk were not the result of treatment of an individual animal with antimicrobials, samples were collected only from farms where the waste milk bulk tank contained milk pooled from 3 or more cows. In addition, to exclude the effect of antibacterial treatments on the concentration of antimicrobials in the waste milk, samples were collected before animals received any antibacterial treatments.

With the exception of penicillin, the LOQ for every drug in the LC-MS/MS panel was below the limit of detection (**LOD**) of SNAP tests; however, only half of the milk samples submitted for LC-MS/MS had one or more drug residues at detectable concentrations. The LC-MS/MS method offers analytical specificity superior to that of immunoassays or conventional HPLC for low-molecular-weight analytes and has higher through put than GC-MS (Grebe and Singh, 2011). A study by Gibbons-Burgener et al. (2001) evaluated the reliability of the commercial SNAP test for detection of β-lactam residues in the milk of cows diagnosed with mild clinical mastitis and observed that of 28 milk samples positive by a β-lactam SNAP test, only 11 had a β-lactam drug detected by HPLC. Factors that may cause false positives in SNAP tests include (1) high counts (colony-forming units) in the milk, which may affect the binding of components in the assay system and result in increased probability of a false-positive outcome; and (2) high butter milk fat, which hinders the movement of milk through the assay and causes a lack of chemical reaction, resulting in an increased probability of false positives (Van Eenennaam et al., 1993). Furthermore, increased probability of false-positive outcomes when using commercial drug residue screening tests in milk has been linked to increased milk protein content and SCC (Van Eenennaam et al., 1993; Andrew, 2000). In addition, a positive result when using the β-lactam SNAP test and a negative result for the presence of a β-lactam drug above the LOQ when using LC-MS/MS could have occurred because the 90/95% sensitivity for penicillin residues in the β-lactam SNAP test (90/95% LOD = 0.003 μg/mL) is below the LOQ for penicillin residues in the LC-MS/MS (LOQ = 0.005 μg/mL). In our study, milk samples were labeled as testing positive or negative for 1 of the 3 antimicrobial classes tested by using one SNAP test for each of these classes. The use of replicates for the same antimicrobial class is one alternative to decrease the number false-positive tests when using SNAP tests. We also recognize the cross-sectional nature of the sampling as a study limitation. We did not collect data to determine the variation of residue presence and concentration on the same farm over time. Therefore, the results should be considered a snapshot of a set of typical New York State dairy farms.

Discordance between results of SNAP tests and quantification using liquid chromatography has also been attributed to decomposition of drug residues during storage and transportation. A study by Riediker et al. (2004) showed that penicillin G, amoxicillin, and ampicillin spiked in milk at a concentration of 0.01 μg/ mL and suffered degradation, in most cases of more than 50% of initial concentration, when stored for 6 d at 4°C. Raw milk spiked with ceftiofur at a concentration of 0.1 μg/mL and stored for 14 d at 4°C retained 90 to 100% of the initial concentration (Karageorgou and Samanidou, 2010). Sulfonamide drugs spiked in milk and stored at −18°C, 4°C, and room temperature have been reported to retain 90 to 100% of the initial concentration after 4 weeks, 4 d and 6 h, respectively (Tolika et al.,

2011). Raw milk samples with tetracycline residues have been shown to have losses ranging from 4 to 13% after storage for 72 h at 4°C and from 3 to 18% after storage for 48 h at 25°C (Podhorniak et al., 1999).

The identification of penicillin G as the second most common drug detected in waste milk samples was not surprising because penicillin is reportedly the most common drug used on dairy farms (Zwald et al., 2004; Saini et al., 2012). Ceftiofur was the most common drug detected in waste milk and is the only third-generation cephalosporin licensed to treat food-producing animals in the United States. Whether ceftiofur is derived from sodium salts, hydrochloride salts, or free acid, it is rapidly metabolized to produce the central active metabolite desfuroylceftiofur (Hornish et al., 2003). Because parenteral administration of ceftiofur has a short half-life of 5 to 10 min in plasma following intramuscular administration in cattle, desfuroylceftiofur is the main metabolite residue of concern in milk after parenteral administration (Jaglan et al., 1990). Nevertheless, parenteral administration of commercially available ceftiofur at the approved label dosage does not result in ceftiofur or desfuroylceftiofur milk residues at concentrations above the tolerance levels established by the FDA (US FDA-NADA, 1995, 1998, 2012). Therefore, the most plausible source for the ceftiofur residues observed in the waste milk samples is not parenteral administration of the drug (unless it was administered above the label dosage), but a mastitis treatment for lactating cows using intramammary infusion of ceftiofur. The currently available commercial intramammary treatment for lactating cows using ceftiofur (Spectramast LC, Zoetis, New York, NY) was approved for the treatment of clinical mastitis in lactating dairy cattle in 2005 and results in drug residues in milk above the FDA tolerance concentrations, requiring a 72-h milk withdrawal period after the last treatment (US FDA-NADA, 2005).

Of the 3 sulfamethazine SNAP tests positive for residues in the waste milk samples, only one had quantifiable sulfonamide drugs identified by LC-MS/MS, namely sulfadimethoxine (Table 2). As described in the FDA Code of Federal Regulations, except for the approved use of sulfadimethoxine, sulfabromomethazine, and sulfaethoxypyridazine, sulfonamide drugs are prohibited from extra-label use in lactating dairy cattle (US FDA, 2012). Of the 3 antibiotics approved for use in cows, only sulfadimethoxine is marketed for use in lactating dairy cattle, with restrictions limiting its extra-label use as a sustained-release bolus or as an intramammary infusion of an injectable form, as determined by the Animal Medical Drug Use Clarification Act (AMDUCA; US FDA, 2006). Resistance to sulfadimethoxine on the dairy farm has been shown to vary drastically by bacteria species and site of isolation, with reports of sulfadimethoxine resistance in 72% (n = 256) of S*almonella* isolates from fecal samples of dairy cows (Cummings et al., 2013), and reports of sulfadimethoxine susceptibility in all *Staphylococcus aureus* isolates (n = 116) in milk from cows with clinical mastitis (Oliveira et al., 2012).

Of the 3 milk samples testing positive with the tetracycline SNAP test, only one had quantifiable tetracycline drugs identified by LC-MS/MS, namely oxytetracycline (Table 2). The low cost and multiple routes of administration of tetracycline make it one of the most widely used drugs on dairy farms (Zwald et al., 2004). A survey on antimicrobial resistance of *Salmonella enterica* isolates from milk bulk tanks and milk filters indicated that tetracycline resistance was the most common resistance phenotype, observed in 15.3% of

isolates (n = 176; Van Kessel et al., 2013). In addition, a study of the prevalence and antimicrobial resistance of *Campylobacter* in US dairy cattle revealed that, regardless of species, resistance to tetracycline was the highest among the antibiotics tested and was present in 49.4% of isolates (n = 532; Englen et al., 2007).

The nonsteroidal antiinflammatory drug flunixin meglumine was also identified in one of the waste milk samples by LC-MS/MS screening through its residue marker 5-hydroxyflunixin. Flunixin meglumine is approved for intravenous administration in cattle, although intramuscular and subcutaneous administrations are common routes of extra-label use in dairy cattle (US FDA-NADA, 2004). The extra-label use of drugs can modify the route, duration, and concentration of excretion, as shown in a study by Kissell et al. (2012). They observed that the administration of flunixin meglumine intramuscularly and subcutaneously in cows results in concentrations of 5-hydroxyflunixin above the tolerance limit after the 36-h withdrawal time established by the FDA for intravenous drug administration.

Currently, limited information is available to evaluate the effects of waste milk on the selection of resistant bacteria on the dairy farm. However, in vitro studies have shown potential for the dissemination and selection of antibiotic-resistant pathogens when exposed to low concentrations of antibiotics. In a study by Gullberg et al. (2011), the effect of low antibiotic concentrations was tested in single cultures where a susceptible wild-type and a resistant mutant carrying a tetracycline-resistant gene (Tn*10d*Tet) were grown separately in the presence of different concentrations of tetracycline. Concentrations far below the MIC for the susceptible bacteria reduced the exponential growth rate of the susceptible strain without any apparent effect on the resistant strain.

Several prospective methods are currently available to reduce the concentration of antibiotic residues in waste milk and include heat treatment, storage, and electrochemical methods. As previously discussed, temperature and storage time can result in degradation of antibiotics. Degradation of β-lactams has also been shown in the presence of various metal ions, where the ions catalyze the inactivation of the hydrolytic opening of β-lactams (Navarro et al., 2003; Michnik et al., 2004; Alekseev et al., 2006). Electrochemical oxidation of raw milk with an initial concentration of oxytetracycline of 100 mg/mL has resulted in an 83% reduction of this drug after a 6-h treatment (Kitazono et al., 2012). Heat treatment (120°C for 20 min) of milk containing β-lactam drugs has shown to degrade 47% of amoxicillin, 84% of ampicillin, 53% of cloxacillin, and 61% of penicillin G (Roca et al., 2011). In addition, biodegradation of ceftiofur has been shown to increase with the increase of temperature, with optimal biodegradation temperatures between 35°C and 45°C (Li et al., 2011). Pasteurization of waste milk used to feed preweaned calves is recommended to reduce bacterial contamination and limit the spread of disease, and furthermore may result in the reduction of the concentration of certain antibiotics (Elizondo-Salazar et al., 2010).

Studies evaluating the effect on the biosphere from the disposal and use of waste milk as a feed source for calves are timely and essential for the development of new intervention measures to counteract the development and spread of antimicrobial resistance.

Conclusions

The use of a SNAP test to detect antibiotic residues in pooled waste milk revealed that the most prevalent antibiotic class observed was β-lactam (74%), followed by tetracycline (14.3%), and sulfamethazine (7.1%). Analysis of these samples using LC-MS/MS revealed that the most prevalent detectable drug was ceftiofur (39.2%; mean concentration = 0.151 μg/mL) followed by penicillin G (14.2%; mean concentration = 0.008 μg/mL) and ampicillin (7.1%; mean concentration = 0.472 μg/mL). Further studies are necessary to evaluate effects on the development and dissemination of antibiotic resistance on dairy farms from using waste milk as feed source for calves.

Acknowledgments

We thank Pepi Leids and Melanie Hemenway from the New York State Cattle Health Assurance Program (NYSCHAP, Albany, NY) for assisting in the collection of samples for this project. Research reported in this publication was supported by the National Institute of Food and Agriculture (NIFA), USDA, under HATCH award number NYC-478400. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Food and Agriculture (NIFA) or the USDA. Research reported in this publication was partially supported by National Center for Research Resources, a component of the National Institutes of Health (NIH), under Award Number T32ODO011000. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

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Table 1

Distribution of drug residues in nonsaleable raw milk samples from dairy farms detected by 3 commercial enzyme-linked receptor-binding assay (SNAP) tests and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Description	Distribution (%)
Result of commercial SNAP screening test[1] (n = 34)	
Positive	82.3
Negative	17.7
Distribution by drug class (by SNAP tests[2]; n = 28)	
Only β-lactams	75.0
β-Lactams and tetracycline	14.3
β-Lactams and sulfamethazine	7.1
β-Lactams, sulfamethazine, and tetracycline	3.6
Distribution by drug class (by LC-MS/MS[3]; n = 28)	
Negative	50
Only β-lactams	43
β-Lactams and sulfonamide	3.5
β-Lactams and tetracycline	3.5

[1]Positive = milk samples testing positive for at least 1 of the 2 commercial SNAP tests used; negative = milk samples testing negative for all 3 SNAP tests used.

[2]Milk samples testing positive for the New SNAP Beta-lactam Test Kit, SNAP Tetracycline Test Kit, SNAP Sulfamethazine Test Kit (Idexx Laboratories Inc., Westbrook, ME).

[3]Negative = milk samples with no drug residues observed by LC-MS/MS; other categories show distribution of samples with drug residues belonging to the described drug classes at concentrations above the limit of quantification for LC-MS/MS.

Table 2

Drug residues in nonsaleable raw milk detected and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Drug residue	Distribution[1] (%) (n = 28)	Mean[2] (μg/mL)	SE[3]	LOQ[4] (μg/mL)	Tolerance[5] (μg/mL)
β-Lactams[6]	50				
Ampicillin	7.1	0.472 (n = 2)	0.438	<0.01	0.01
Cephapirin	3.5	0.033 (n = 1)	—	<0.01	0.02
Ceftiofur	39.2	0.151 (n = 11)	0.042	<0.01	0.1
Cloxacillin	3.5	0.055 (n = 1)	—	<0.01	0.01
Penicillin G	14.2	0.008 (n = 4)	0.001	<0.005	0
Sulfonamide					
Sulfadimethoxine	3.5	1.00 (n = 1)	—	<0.002	0.01
Tetracycline					
Oxytetracycline	3.5	0.01 (n = 1)	—	<0.01	0.3
Nonsteroidal antiinflammatory					
5-Hydroxyflunixin	3.5	0.003 (n = 1)	—	<0.002	0.002

[1] Percent of milk samples with drug residues at concentrations above the limit of quantification (LOQ) for LC-MS/MS for each individual drug or drug class described.

[2] Mean concentration of drug residues in nonsaleable raw milk samples collected from dairy farms and quantified by LC-MS/MS. Number of samples with drug residues within the limit of detection in parentheses.

[3] Standard error of drug residues quantified by LC-MS/MS.

[4] LOQ of LC-MS/MS for each drug tested.

[5] Food and Drug Administration (FDA) tolerance for drug residues in raw milk.

[6] Samples with detectable concentrations of one or more β-lactam drug residues measured by LC-MS/MS.