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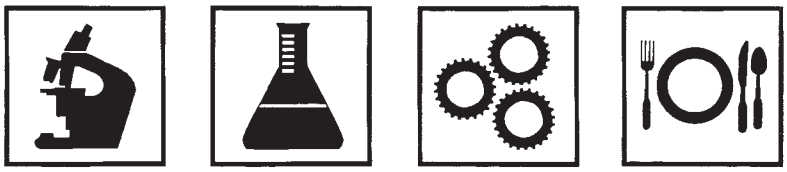

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ABSTRACT

β-Lactam antibiotics are the most commonly used drugs on dairy farms. β-Lactam residues in milk are kept out of the human milk supply with good agricultural practices and mandatory truck screening performed by the dairy industry under Appendix N of the Pasteurized Milk Ordinance. Flunixin, a nonsteroidal and anti-inflammatory drug, appears in dairy cattle tissue residues with a frequency similar to the occurrence of penicillin G. This creates concern that flunixin residues could be in milk and would go undetected under current milk screening programs. A single test that combines mandatory β-lactam screening with voluntary flunixin screening is an economical approach for monitoring and controlling for potential flunixin or 5-hydroxyflunixin, the primary flunixin metabolite marker in milk. The objective of this study was to validate a β-lactam and flunixin rapid lateral flow test (LFT) and compare the results obtained with a liquid chromatography-triple quadrupole tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of flunixin and 5-hydroxyflunixin in raw milk with a limit of detection of <1 ppb, equivalent to 1 ng/ml. Using the LFT, three combined manufactured lots of test strips detected penicillin G at 2.0 ppb, ampicillin at 6.8 ppb, amoxicillin at 5.9 ppb, cephapirin at 13.4 ppb, ceftiofur (total metabolites) at 63 ppb, and 5-hydroxyflunixin at 1.9 ppb at least 90% of the time with 95% confidence. The LFT also detected incurred flunixin milk samples that were analyzed with the LC-MS/MS and diluted to tolerance in raw milk. The detection levels for the LFT are lower than the U.S. safe levels or tolerances and qualify the test to be used in compliance with U.S. milk screening programs.

β-Lactam drugs are the most commonly used antibiotics in dairy cattle health management in the United States *(8)*. Proper drug management includes disposal of milk for prescribed withhold times to prevent drug residues from passing into the human food chain. It is a public health requirement that all milk tankers prior to unloading into dairies be tested for β-lactam drugs using methods recommended to the National Conference of Interstate Milk Shipments and evaluated by the U.S. Food and Drug Administration (FDA) as dictated in the Pasteurized Milk Ordinance Appendix N program *(19)*. Tests are evaluated for specificity, sensitivity, and ability to detect incurred levels at drug tolerances or safe levels expressed as parts per billion, which is equivalent to nanograms per milliliter. Numerous β-lactam drug-screening tests and other FDA-evaluated and National Conference of Interstate Milk Shipments–approved antibiotic screening tests for tetracyclines, sulfa drugs, and chloramphenicol are listed in FDA memorandum M-A-85 *(20)*.

Flunixin (2-{[2-methyl-3-(trifluoromethyl) phenyl]amino}-3-pyridinecarboxylic acid), is a nonsteroidal anti-inflammatory drug administered intravenously to cattle and is sometimes used in combination with antibiotic treatment. Its presence has been detected in the tissues of dairy cattle with a frequency comparable to that of penicillin G according to the national tissue monitoring program conducted by the U.S. Department of Agriculture Food Safety Inspection Service *(16)*. Since the drug is detected in dairy cattle, there is concern that the milk from flunixin-treated cows may be contaminated with the drug and that the drug would not be detected under the current evaluated screening methods recommended for milk tankers. Thus, there is a need for rapid, sensitive, and reliable procedures for the detection of drug residues in milk.

A method for determination of flunixin in milk by liquid chromatography (LC) and confirmation by gas chromatography–mass spectrometry (MS) has been reported *(11)*. Analysis of samples from cattle treated by intravenous administration of a normal dose of 2.2 mg/kg of body weight of [^{14}C]flunixin for 3 consecutive days indicated that 5-hydroxyflunixin was the major residue in bovine milk. Therefore, 5-hydroxyflunixin has been established as the marker residue to be used for monitoring of flunixin residues in bovine milk *(5)*. More recently, an LC–tandem MS (LC-MS/MS) method was developed for the determination and confirmation of 5-hydroxyflunixin at 1- and 2-ppb levels, respectively, in unpasteurized bovine milk *(1)*.

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Screening tests are substantially less time consuming and less expensive than chromatographic techniques, such as LC-MS/MS. Recently, a new 8-min immunological and receptor-based lateral flow test (LFT) has been developed for the detection of flunixin and five of the β-lactam drugs approved for dairy cattle in the United States. The new lateral flow assay is similar in principle to the SL6 β-lactam test evaluated and approved in 2003, which used an antibody for cloxacillin to achieve detection of a sixth β-lactam drug that was not detected by the β-lactam receptor binder *(3)*. In the flunixin lateral flow method, a flunixin antibody replaces the cloxacillin antibody, so that while detection of cloxacillin at U.S. tolerance is lost, the detection of flunixin and flunixin metabolites is gained. Another commercially available enzyme-linked immunosorbent assay method claims to detect flunixin in tissue and milk *(10)*. This method takes more than 30 min and is unable to measure both the β-lactam drugs and flunixin at the same time.

A third-party laboratory evaluation and validation has been designed by the FDA's Center for Veterinary Medicine (FDA-CVM) for evaluating screening methods for flunixin in bulk commingled milk for sensitivity and selectivity, and it is similar to the β-lactam screening test protocol used to validate bulk tanks and tanker trucks as specified in Appendix N of the Pasteurized Milk Ordinance *(20–22)*. The objective of this study was to validate the new β-lactam and flunixin combination test following the FDA-CVM protocol developed in association with the Institute for Food Safety and Health (IFSH). Separate method validations were done for flunixin and the β-lactam drugs so that the screening method could be used to comply with mandated β-lactam drug screening criteria specified in Appendix N of the Pasteurized Milk Ordinance.

MATERIALS AND METHODS

Materials. Methanol (LC-MS grade), acetonitrile (LC-MS grade), water (LC-MS grade), formic acid (99 to 100%), ethyl acetate (high-performance LC [HPLC] grade), and acetone (HPLC grade) were purchased from Fisher Scientific (Pittsburg, PA). Amoxicillin, trihydrate ampicillin, sodium cephapirin, and potassium penicillin G standards and 31 drugs used in interference tests were from United States Pharmacopeia (Rockville, MD). The analytical standards, flunixin and deuterated flunixin(d_3), were purchased from Sigma-Aldrich (St. Louis, MO), and 5-hydroxyflunixin was obtained from Schering-Plough Research Institute (Union, NJ).

Gram-positive (G+), coagulase-negative (*Streptococcus* spp. and *Staphylococcus aureus*), gram-negative (G−) (*Pseudomonas aeroginosa, Enterobacter, Escherichia coli,* and *Klebsiella* spp.), and G+/G− bacteria were isolated from mastitic cow's milk by the Dairy Quality Control Institute (Mounds View, MN). Freshly grown cultures of isolates in nutrient broth (equal mixes) were prepared and diluted into milk at targeted concentrations of 150,000 and 300,000 CFU/ml.

The Charm FLUBL6 β-lactam and flunixin test (LFT) contains either 100 test strips, one positive control of a 2-ppb flunixin standard, and one positive control of a 5-ppb penicillin G standard or 500 test strips, five positive controls of a 2-ppb flunixin standard, and five positive controls of a 5-ppb penicillin G standard (Charm Sciences, Lawrence, MA). A Rapid One Step Assay (ROSA) 56°C incubator, a ROSA Pearl Reader, and a fixed-volume 300-μl pipet with 200- to 1,000-μl disposable pipet tips were supplied by Charm Sciences.

Specificity and selectivity study for β-lactam drugs and flunixin in raw milk. Fresh raw commingled cow's milk from local dairy producer tanks or silos tested to be β-lactam free using the SL β-lactam test *(13)* and Charm II β-lactam quantitative assay *(18)* was used for the spiking experiments. The age of the milk from cow milking was 5 days or less, and the milk was maintained at 4°C.

β-Lactam drugs were dissolved in buffers as described in the *United States Pharmacopeia* and prepared on the day of testing *(15)*. 5-Hydroxyflunixin was dissolved in 50% methanol-water to yield 100-mg/liter stock solutions. Standard stock solutions were then diluted in milk for a final concentration of 1 mg/liter to be used for the spiking tests. These stock solutions were made fresh daily.

Each drug's specificity and sensitivity was evaluated on a separate day. Thirty test samples were prepared at concentrations that define the screening test dose response as determined by an FDA statistician based on the manufacturer's submitted data, as follows: 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 ppb (ng/ml) for amoxicillin; 4.0, 5.0, 6.0, 7.0, 8.0, and 10.0 ppb for ampicillin; 8.0, 12.0, 14.0, 16.0, 18.0, and 20.0 ppb for cephapirin; 1.0, 2.0, 2.5, 2.75, 3.0, and 5.0 ng/ml for penicillin G; and 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 ppb for 5-hydroxyflunixin. Sixty samples of the raw milk were prepared for a negative control (0 ng/ml) on the day of the test. Samples were scrambled and blind coded and tested according to the manufacturer's instructions (Fig. 1), and tests conducted consistent with good laboratory practices *(17)*. For these experiments, there was a team of sample preparers and coders in the test laboratory that then passed the samples to testers without their knowledge of the sample, preparation, or order sequence.

Incurred residue studies for β-lactam drugs and flunixin in raw milk. Incurred residue samples were obtained by treating lactating cows with an intramammary infusion under the supervision of a veterinarian. Samples containing penicillin G, amoxicillin, cephapirin, ampicillin, and ceftiofur were tested and commingled in 2007 and stored at −80°C *(12)*. These concentrations were verified by HPLC analysis prior to use *(2, 3, 7, 9, 14, 24)*. In the spring of 2010, CVM collected incurred residue milk samples by treating three cows with flunixin after it was verified that the cows were healthy and had not been treated for the preceding 30 days. These incurred samples were provided to the IFSH for testing *(1)*. Daily a.m. and p.m. milkings were collected until the prescribed drug clearance withhold time (4 days) and frozen. Samples were tested by the HPLC-MS/MS reference method *(4)* for 5-hydroxyflunixin to identify samples probably containing the drug above the safe level or tolerance (SL/T) but no more than four times greater than the SL/T. The frozen commingled samples were then extracted for the LC-MS/MS test to identify and quantify the drug content in the samples *(2, 4)*. Specific samples were chosen for dilution with negative commingled cow's milk (milk collected and prequalified as negative prior to injection) to produce test samples.

In one incurred study, ceftiofur samples were prepared at 20, 40, 55, 60, 65, 70, 80, and 100 ppb of total ceftiofur and ceftiofur metabolite (desfuroylceftiofur acetamide). Thirty replicates of each concentration and 60 negative samples were blind coded (300 total samples), frozen at −80°C, and then sent to the IFSH to be evaluated. In another study, amoxicillin, ampicillin, cephapirin, 5-hydroxyflunixin, and penicillin G incurred samples containing the SL/T detection concentrations, the 1/10, 1/4, and 1/2 SL/T detection concentrations, and the 90% sensitivity with 95% confidence (90%/95%) levels were prepared. Ten replicates of

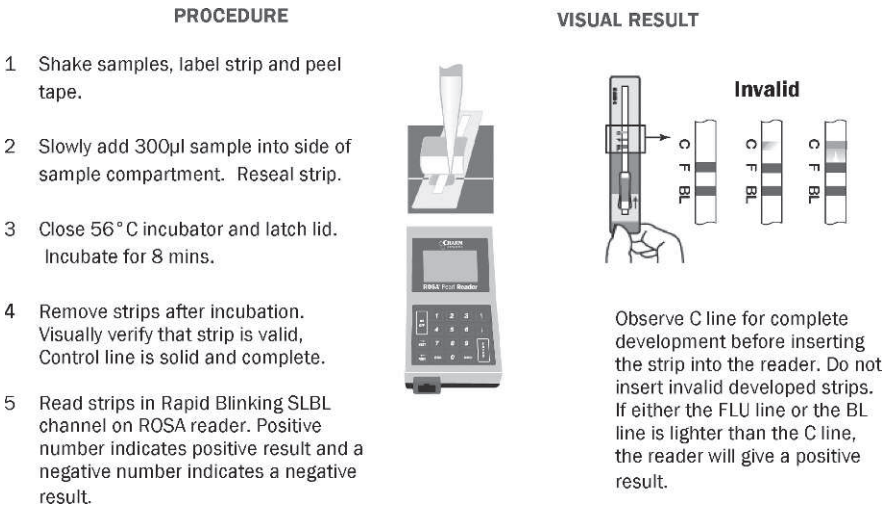

FIGURE 1. *β-Lactam and flunixin test procedure.*

each sample and 60 negative samples were blind coded (310 total samples), frozen at −80°C, and sent to the IFSH at the Illinois Institute of Technology for analysis.

Interference studies for β-lactam drugs and flunixin in raw milk. In the somatic cell interference study, the Dairy Quality Control Institute laboratory identified farm raw milk with a high somatic cell count (1.1 million somatic cells per ml) and qualified it as negative for β-lactam with the Charm II β-lactam quantitative assay and negative for flunixin with the test assay. The drugs were later verified by LC-MS/MS to be less than the 1-ppb limit of detection. Milk was spiked with 4.0 ppb of penicillin G, 20.0 ppb of cephapirin, and 2.0 ppb of 5-hydroxyflunixin. Negative samples were divided into 60 replicates, and the drug-fortified samples were each divided into 30 replicates. The 150 blind-coded samples were tested by technicians not involved in sample coding or preparation.

In the bacterial interference study, the Dairy Quality Control Institute laboratory prepared blind-coded samples (negative, 4.0 ppb of penicillin G, 20.0 ppb of cephapirin, and 2.0 ppb of 5-hydroxyflunixin) by fortifying milk with freshly prepared bacterial G+, G−, and G+/G− cultures at 150,000 and 300,000 CFU/ml. Each of the samples was then divided into 5 replicates, except the negative G+/G−, which was split into 20 replicates. The total number of samples was 165. Samples were prepared, blind coded, and tested at the independent laboratory by technicians who were not involved with sample preparation or coding.

Sample preparation method for quantitation of flunixin and 5-hydroxyflunixin in raw milk using LC-MS/MS. Individual stock solutions of flunixin, 5-hydroxyflunixin, and d_3-flunixin were prepared in methanol at 500 μg/ml. All standard solutions were stored at −20°C. Intermediate standard solutions of 50 μg/ml containing flunixin and 5-hydroxyflunixin were prepared by diluting the stock solutions with methanol. Working standards at concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 25 ng/ml in 50% methanol-water were used to construct a standard calibration curve. Each working standard contained 5 ng/ml of the internal standard, d_3-flunixin.

Untreated milk (control) from CVM was sent to IFSH to be used for validation and recovery studies, as well as the milk with incurred samples. Standard mixtures containing 1 μg/ml flunixin and 5-hydroxyflunixin in 20% methanol-water were used for spiking the control milk samples at 2.5, 10, and 50 ng/ml. Each spiked or unspiked milk sample also contained 50 ng/ml d_3-flunixin.

CVM-supplied incurred flunixin milk samples were tested 8, 24, 32, 48, and 56 h after injections. All samples were extracted for flunixin and 5-hydroxyflunixin.

Two milliliters of a homogenized sample was transferred into a 50-ml centrifuge tube, 1.5 ml of 0.1 N HCl was added, and then the sample was vortexed (Vortex Genie 2, Fisher Scientific, Pittsburg, PA) for 3 min at high speed. The sample was then diluted to 30 ml with 50% acetone–ethyl acetate and vortexed for 1 min, sonicated (Branson Ultrasonic Cleaner 1510, Branson Ultrasonics Corp., Danbury, CT) for 30 s, and then centrifuged (Eppendorf 5810R, Fisher Scientific) at a relative centrifugal force of 2,830 for 5 min at 20°C. The supernatant (1.5 ml) was transferred to a clean 15-ml test tube. The solvent was evaporated to dryness under a gentle stream of nitrogen at 50°C (Pierce Reacti-Therm III-heating/stirring module, Pierce, Rockford, IL). The residue was dissolved in 1 ml of 50% methanol-water, vortexed for 1 min, centrifuged at 7,500 rpm (relative centrifugal force of 2,830) for 5 min at 20°C, and filtered through a 0.2-μm polytetrafluoroethylene filter prior to analysis.

Analysis was performed on an Agilent 1200 Series HPLC equipped with an Agilent 6460 triple quadrupole MS (LC-MS/MS). Separations were carried out with an Agilent Zorbax Extend-C_{18} column (100 by 2.1 mm) at 40°C. A gradient elution was used, consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient conditions were as follows: initial, 90% A and 10% B; 5 min, 10% A and 90% B; 6 min, 100% B; 7 min, 100% B with a flow of 0.4 ml/min. The MS was operated in positive electrospray ionization mode with jet stream technology. The gas temperature was 300°C, and the flow rate was 10 liters/min. The nebulizer was set at 35 lb/in^2, and the sheath gas temperature and flow rate were 350°C and 11 liters/min, respectively. The capillary and nozzle voltages were 3,500 and 1,000 V, respectively. Three different fragment ion transitions were monitored for 5-hydroxyflunixin (*m/z* 313→295, *m/z* 313→280, and *m/z* 313→109), flunixin (*m/z* 297→279, *m/z* 297→264, and *m/z* 297→109), and the internal standard, d_3-flunixin (*m/z* 300→282, *m/z* 300→262, and *m/z* 300→112). The fragment voltage was 122 for all three of the compounds, and the collision energies were 20, 32, and 50, respectively, for the flunixin and d_3-flunixin product ions and 20, 36, and 50, respectively, for the 5-hydroxyflunixin product ions.

RESULTS AND DISCUSSION

Sensitivity, selectivity, and lot-to-lot repeatability study. Each experimental set of blind-coded samples was

TABLE 1. *Summary of independent laboratory evaluation of dose-response of spiked drug into commingled raw milk using β-lactam and flunixin test*[a]

	% positive (no. positive/no. tested)				
Concn (ppb [ng/ml])	Penicillin G	Ampicillin	Amoxicillin	Cephapirin	5-Hydroxyflunixin
0	0 (0/60)	0 (0/60)	0 (0/60)	0 (0/60)	0 (0/60)
0.2					13 (4/30)
0.4					3 (1/30)
0.8					50 (15/30)
1.0	50 (15/30)				
1.2					57 (17/30)
1.6					83 (25/30)
2.0	90 (27/30)				100 (30/30)
2.5	100 (30/30)				
2.75	100 (30/30)				
3.0			37 (11/30)		
4.0		87 (26/30)	57 (17/30)		
5.0	100 (30/30)	73 (22/30)	83 (25/30)		
6.0		83 (25/30)	97 (29/30)		
7.0		87 (26/30)			
8.0		100 (30/30)	100 (30/30)	97 (29/30)	
10.0		100 (30/30)	100 (30/30)		
12.0				80 (24/30)	
14.0				83 (25/30)	
16.0				100 (30/30)	
18.0				100 (30/30)	
20.0				100 (30/30)	
90%/95% level (ng/ml [ppb])	2.0	6.7	5.9	13.4	1.9
SL/T (ng/ml [ppb])	5.0	10.0	10.0	20.0	2.0

[a] Each column represents a blinded study of 60 negative raw milk samples and 180 spiked samples. The 90% sensitivity with 95% confidence (90%/95%) level is a statistical analysis of the drug response.

divided into 10 equivalent laboratory breakpoints (subsets), with an equal number of specific lot numbers (001, 002, or 003) performed in each subset. The IFSH laboratory-determined drug sensitivity data using fortified samples are expressed as the percentage of positives and number of positives per number of replicates in Table 1. Data analyses were performed to determine the minimum 90%/95% levels *(18)* and to establish statistical parameters for the numerical readout of the qualitative result *(5)*. The 90%/95% levels determined from the fortified-drug data were less than or equal to the SL/T values for all the claimed drugs. In addition, the 90%/95% determinations were not more sensitive than 2 ppb for penicillin G or lower than 50% of SL/T for ampicillin, amoxicillin, cephapirin, and 5-hydroxyflunixin. The 90%/95% levels met the sensitivity specifications of the evaluation protocols *(21, 22)*. The sensitivity determinations were similar to the manufacturer's data with the exception of penicillin G, which was 2 ppb at the IFSH laboratory and closer to 3 ppb as tested by the manufacturer. The selectivity of the LFT was measured with 60 negative samples in each of the five dose-response experiments. In all five experiments, there were 0 positives from 60 samples. All five experiments met FDA-CVM 90%/95% specifications.

TABLE 2. *Total ceftiofur (parent and ceftiofur-related metabolites) incurred study results*[a]

Concn (ppb [ng/ml])	% positive (no. positive/no. tested)
Negative	1.7 (1/60)
20	0 (0/60)
40	37 (11/30)
55	87 (26/30)
60	97 (29/30)
65	100 (30/30)
70	100 (30/30)
80	97 (29/30)
100	100 (30/30)

[a] Samples were prepared as commingled dilutions from incurred samples that had the ceftiofur metabolites detected in milk by using HPLC. Negative milk ($n = 60$) and prepared incurred dilutions ($n = 180$) were scrambled and blind coded before testing.

Ceftiofur sensitivity was determined based on incurred residues because the variety of ceftiofur-related metabolites on which the tolerance is based cannot be duplicated in fortified samples (Table 2). The 90%/95% level determined for the three combined lots was 63 ppb, which met SL/T sensitivity claim specifications. There was 1 positive sample among the 60 zero-drug samples meeting the FDA 90%/95% specifications. It is speculated that this false-positive sample was a testing sequence error with an 80-ppb sample. These samples were tested one right after the other in the testing sequence; the negative sample was the only one of 60 replicates that tested positive, and the 80-ppb sample was the only one of the replicates that tested negative.

TABLE 3. *Drug 90%/95% levels of three lots determined by the independent laboratory's data*[a]

Drug	Lot 001 (ppb)	Lot 002 (ppb)	Lot 003 (ppb)
Penicillin G	1.9	2.5	2.3
Ampicillin	5.6	9.1	7.0
Amoxicillin	6.7	6.7	5.7
Cephapirin	12.5	17.0	14.3
Ceftiofur total metabolites	78	64	55
5-Hydroxyflunixin	2.2	2.0	1.9

[a] The spiked drug sensitivity (90% sensitivity with 95% confidence [90%/95%]) analysis used 30 replicate analyses of each drug with 10 replicates of three different lots (001, 002, and 003). Sensitivities calculated by probit analysis are reported as parts per billion and are equivalent to nanograms per milliliter.

The test strips used in the independent laboratory evaluations were an even mixture of three manufactured lots. Therefore, dose-response data for each lot were determined for the three lots. Table 3 compares the replicates ($n = 10$) of the three lots and their calculated 90%/95% levels. In one case, lot 001 5-hydroxyflunixin, the sensitivity, 2.2 ppb, was greater than the 2.0 SL/T, reflecting the larger uncertainty of the 95% confidence when fewer than 30 replicates are used in the calculation. In most cases, the levels determined for the three lots are consistent to within 20% of each other, with the exceptions of lot 002 ampicillin, 9.1 ppb, and lot 002 cephapirin, 17.0 ppb, which had 100% observed positives at 8 and 16 ppb, respectively, but due to curve irregularities did not have 95% confidence levels until the concentrations were just lower than the SL/T. Overall, the data indicate that the lot performances are repeatable.

The results of the fortified β-lactam, flunixin, and ceftiofur incurred sensitivity experiments were that the drugs were detected at or below SL/T. The principle of the β-lactam and flunixin test detection uses bacterial receptors and antibody in a lateral flow design similar to other ROSA tests manufactured by Charm Sciences. The receptors have affinity to all β-lactam drugs and are down-regulated by a proprietary technique to achieve detection near regulatory levels without being hypersensitive *(13)*. The antibody is specific to flunixin and 5-hydroxyflunixin, the major metabolite and marker for flunixin in milk.

Ruggedness. The manufacturer's evaluation data submitted to the FDA included seven ruggedness perturbations following an approved multivariate experiment *(23)*. The ruggedness perturbations selected were similar to those of other ROSA tests, including ambient temperature, milk temperature, pipet volume, incubator temperature, time to pipet multiple samples before starting timer, incubation time, and time to read results after test is completed *(3, 12, 13)*.

The ruggedness parameters of the LFT were determined by multivariate analysis using 12 replicates and examining their results, *t* test analysis, and significance tests. Perturbations were considered minor if the *P* value was <0.05 and more significant if the *P* value was <0.01. Perturbations that did not have an effect were incubation temperature ($\pm 1^{\circ}$C), milk temperature (0 to 7°C), a time of 90 s to pipet multiple samples, a time of 5 min to read results after test completion, incubation for 9 min instead of 8 min, and ambient temperatures of 10 to 35°C. A perturbation that did have an effect was a milk pipetting volume of 270 μl, which is 10% less than the target 300 μl. This perturbation in combination with another perturbation of low ambient temperature produced two false-negative flunixin results of 2.0 ppb. Negative 3.5-ppb penicillin G and 16.0-ppb cephapirin results were correct, but the readings were significantly shifted from those for the control with the 10% low milk volume. Additional ($n = 6$) tests looking specifically at 5% pipet variance (300 $\pm$15 μl), without the other perturbations in combination, showed no significant effect.

Incurred β-lactam and flunixin study. Incurred flunixin samples were prepared from collected milkings that were analyzed by LC-MS/MS. The concentration of flunixin in the untreated control milk was <1 ng/ml, reached 3.5 ng/ml 8 h after treatment, and was reduced to <1 ng/ml at 24, 32, 48, and 56 h after treatment. The concentration of 5-hydroxyflunixin in the untreated milk was <1 ng/ml, reached 25 ng/ml at 8 h after treatment, and was reduced to <1 ng/ml 24, 32, 48, and 56 h after treatment (Fig. 2).

The positive responses of amoxicillin, cephapirin, and 5-hydroxyflunixin incurred samples were very similar to those of the spiked samples (Table 4). At 1.9 ppb of hydroxyflunixin, the 90%/95% level of the assay, the results were 90% positive. At 2.0 ppb, there were 100% positive results. The ampicillin samples at 3.0 ppb were 100% positive, demonstrating that incurred ampicillin samples had about twice the sensitivity determined with fortified samples. This increase in sensitivity with the ampicillin incurred samples may be explained by a metabolite that is not quantified by the HPLC but that is cumulatively detected along with the parent compound by the screening method. Metabolites in these samples were observed previously by HPLC *(24)*. Similar results have been observed previously with other incurred studies *(3, 12, 13)*. The penicillin G results were 100% positive at 5.0 ppb, the SL/T, but only 70% positive at 2.5 ppb. This is slightly less sensitive than expected from the spiked-sample results determined in the IFSH laboratory and more consistent with the sensitivity determined in the manufacturer's laboratory. The sensitivity to penicillin G is between 2.0 and 3.0 ppb using the incurred and all of the spiked-sample data. The β-lactam and flunixin test detected antibiotics at levels in incurred samples with sensitivities similar to or greater than those in fortified samples, demonstrating that the test reliably detects drugs under the prescribed conditions of administration to lactating cows.

Interference and frozen milk studies. Intrafamily animal drug cross-reaction was evaluated using a series of cocktails containing animal drugs at 100 ppb (sulfadiazine, sulfanilamide, sulfathiazole, sulfamethazine, sulfapyridine, sulfadimethoxine, tetracycline, oxytetracycline, chlortetracycline, doxycycline, gentamicin, neomycin, streptomycin, ivermectin, erythomycin, pirlimycin, tilmicosin, novobiocin,

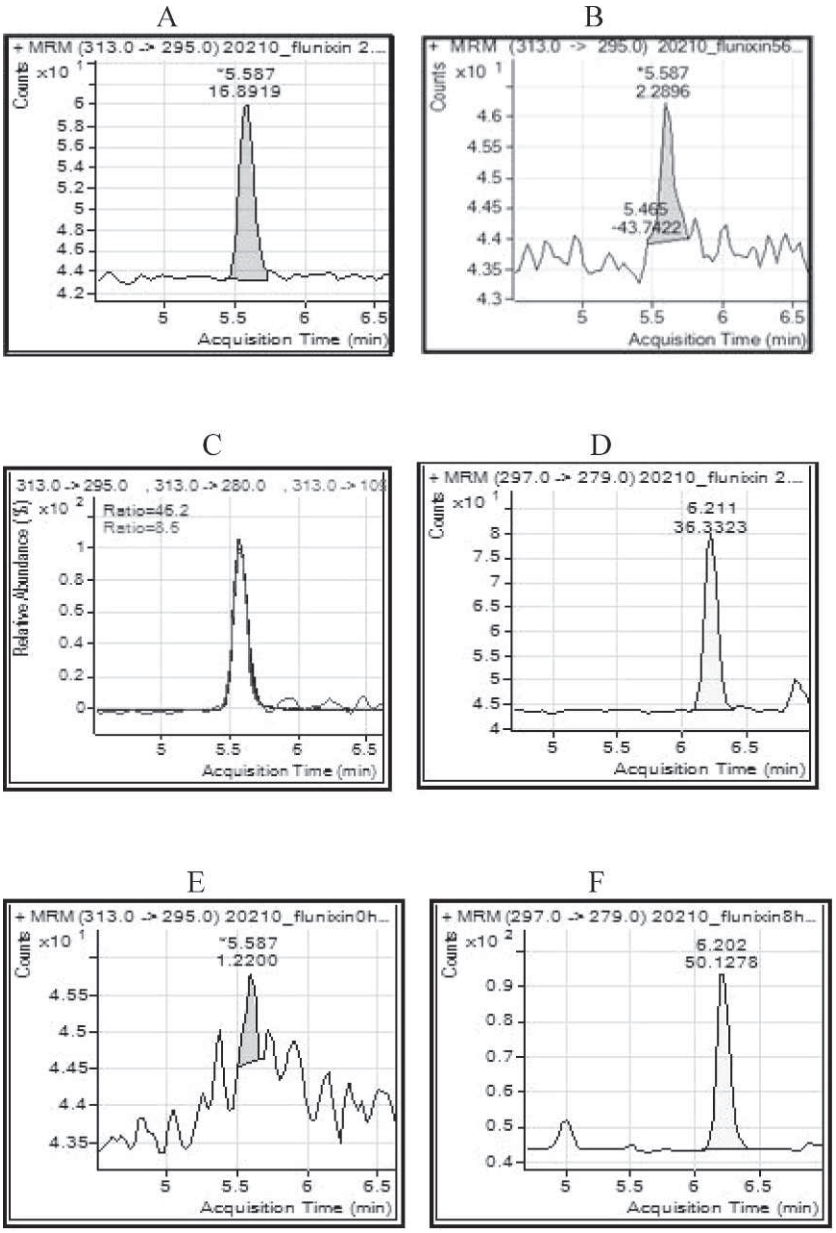

FIGURE 2. *HPLC–MS-MS chromatograms of 5-hydroxyflunixin (multiple reaction monitoring transitions of 313→295 and 313→280) and flunixin (multiple reaction monitoring transition of 297→279). Results are shown as follows: (A) 2.5 ppb of 5-hydroxyflunixin fortified in milk; (B) untreated (control) milk, <1 ppb of 5-hydroxyflunixin; (C) incurred milk at 8 h posttreatment, 26 ppb of 5-hydroxyflunixin; (D) 2.5 ppb of flunixin fortified in milk; (E) untreated (control) milk, <1 ppb of flunixin; and (F) incurred milk at 8 h posttreatment, 3.5 ppb of flunixin.*

enrofloxacin, florfenicol, furosemide, trichlormethiazide, thiabendazole, chlorothiazide, oxytocin, phenylbutazone, dexamethasone, para-aminobenzoic acid, dipyrone, and the nitrofuran metabolites). These drugs were added to negative raw milk and raw milk spiked with 3.5 ppb of penicillin G, 16 ppb of cephapirin, and 2 ppb of 5-hydroxyflunixin and tested. Interfamily cross-reaction was determined using six replicates, and the concentration was found that gives at least five positive of six responses with a reading intensity equivalent to that of 3.5 ppb of penicillin G.

TABLE 4. *Concentration response of incurred drug residues*[a]

Penicillin G		Ampicillin		Amoxicillin		Cephapirin		5-Hydroxyflunixin	
Concn (ppb)	% positive	Concn (ppb)	% positive	Concn (ppb)	% positive	Concn (ppb)	% positive	Concn (ppb)	% positive
5	100	10	100	10	90	20	100	2.0	100
2.5	70	6	100	8.0	100	14	100	1.9	90
1.25	10	3	100	4.0	30	7.0	10	0.95	40
0.63	0	1.5	30	2.0	0	3.5	0	0.48	10
0.25	0	0.6	10	0.8	0	1.4	0	0.19	0

[a] Negative milk replicates ($n = 60$) were blind coded with 150 incurred residue samples diluted to the indicated drug parts per billion (ppb) levels based on HPLC of β-lactam drugs and LC-MS/MS of the flunixin marker in the incurred milk samples ($n = 10$). There were 59 of 60 negative results of the negative samples, or a 1.7% positive rate.

TABLE 5. *Results for the four different interference studies examining challenges with bacteria, somatic cells, chemicals, and frozen milk*[a]

Interference challenge	Spiking material	Negative milk	Penicillin G, 4.0 ppb[b]	Cephapirin, 20.0 ppb[c]	5-Hydroxyflunixin, 2.0 ppb
Bacteria	G+/G− mix, 110,000–150,000 CFU/ml	0/20	5/5	5/5	5/5
	G+/G− mix, 250,000–340,000 CFU/ml	0/20	5/5	5/5	5/5
	G+, 120,000–160,000 CFU/ml	0/5	5/5	5/5	5/5
	G+, 280,000–320,000 CFU/ml	0/5	5/5	5/5	5/5
	G−, 110,000–150,000 CFU/ml	0/5	5/5	5/5	5/5
	G−, 220,000–250,000 CFU/ml	0/5	5/5	5/5	5/5
	Control milk, 64,000–78,000 CFU/ml	0/5	5/5	5/5	5/5
Bacterial summary		0/65	35/35	35/35	35/35
Somatic cells	1,100,000 somatic cells/ml	0/60	30/30	30/30	30/30
Interfering chemicals	6 Sulfa drugs	0/3	3/3	3/3	3/3
	4 Tetracyclines	0/3	3/3	3/3	3/3
	3 Aminoglycosides	0/3	3/3	3/3	3/3
	4 Macrolides and ivermectin	0/3	3/3	3/3	3/3
	Dewormers and pesticides	0/3	3/3	3/3	3/3
	Steroids	0/3	3/3	3/3	3/3
	Enrofloxacin, para-aminobenzoic acid, nitrofuran metabolites, and florfenicol	0/3	3/3	3/3	3/3
Chemical interference summary		0/21	21/21	21/21	21/21
Frozen milk[d]	Thawed weekly and tested at 1, 2, 3, 4, and 8 wk	(0/25) 0/35	(25/25) 35/35	(25/25) 35/35	(25/25) 31/35

[a] Results are expressed as number of positive readings/number tested. Each experiment was prepared as scrambled blind-coded samples containing negative and positive spiked milk samples. G+, gram-positive bacteria; G−, gram-negative bacteria.
[b] Penicillin G was spiked at 4.0 ppb in the freeze-thaw, somatic, and microbial interference studies and 16.0 ppb in the interfering chemicals study.
[c] Cephapirin was spiked at 20.0 ppb in the freeze-thaw, somatic, and microbial interference studies and 16.0 ppb in the interfering chemicals study.
[d] Results in parentheses are through week 4 only.

There were no effects on the LFT from somatic cells, bacteria, or 31 structurally different animal drugs. Somatic cells at 1.1 million/ml did not interfere with positive or negative results. Bacterial cells at 110,000 to 310,000 CFU/ml did not interfere with positive or negative results. Two false-negative 2.0-ppb flunixin results that occurred in two different chemical interference cocktail experiments were not repeated when tested again ($n = 6$).

Milk and milk fortified with 4.0 ppb of penicillin G, 20 ppb of cephapirin, and 2 ppb of 5-hydroxyflunixin were prepared in qualified negative raw milk, frozen in blind aliquots, and repeatedly thawed each week for 8 weeks and tested.

Frozen milk thawed repeatedly at 1, 2, 3, and 4 weeks had no effect on results; however, at 8 weeks the 5-hydroxyflunixin sample showed only 6 positives of 10 replicates. Table 5 summarizes the results from the four studies. All the results support stability of frozen milk up to 4 weeks of age with no interference from unrelated microbes, veterinary drugs, or physiologic constituents that could appear in raw milk.

Table 6 summarizes interfamily cross-reactivity experiments to cloxacillin and other β-lactam drugs that are not used in U.S. dairy management practices. The listed cross-reactivity levels are based on at least five positives of six replicates and with an average reader value equivalent to the average reading of 3.5 ppb of penicillin G.

Real-time shelf life study data for kits that had undergone simulated 72 h, 37°C shipping stress and 12-month refrigerated storage showed less than a 10% change in detection levels compared with manufacturer- and IFSH-determined dose-responses.

TABLE 6. *Cross-reactivity of other β-lactam drugs not used in U.S. dairy management practices at the estimated 90%/95% level*[a]

Drug	Concn (ppb)
Cefacetrile	30
Cefalexin	50
Cefalonium	5
Cefadroxil	30
Cefazolin	30
Cefoperazone	9
Cefquinome	75
Cefuroxime	20
Cloxacillin	75
Dicloxacillin	60
Nafcillin	200
Oxacillin	100
Ticarcillin	100

[a] Drug concentration parts per billion (ppb) reported are based on six replicates that provide five or six positive results with reader values equivalent to those for 4 ppb of penicillin G.

Significance. The β-lactam and flunixin test is a lateral flow receptor assay designed to detect the flunixin metabolite 5-hydroxyflunixin and five β-lactam drugs at their SL/T in raw milk. The β-lactam and flunixin test meets the conditions specified under the FDA-CVM protocol established for milk screening tests. The test takes 8 min to detect penicillin G at 2.0 ppb, ampicillin at 6.7 ppb, amoxicillin at 5.9 ppb, cephapirin at 13.4 ppb, ceftiofur total metabolites at 63 ppb, and 5-hydroxyflunixin at 1.9 ppb with 95% confidence in raw commingled cow's milk. These drugs, which represent flunixin and five of the six approved β-lactam drugs for use in lactating cow's in the United States, can be detected by this test method from incurred milk at levels equivalent to or more sensitive than in drug-fortified milk samples. The detection levels of fortified samples are less than the drug's SL/Ts but not so overly sensitive as to disqualify the test from acceptance as an Appendix N screening method under the Pasteurized Milk Ordinance. The β-lactam and flunixin test method met the other approval criteria for ruggedness, lack of causes of interference, stability while frozen, selectivity, incurred samples, lot repeatability, and quality assurance. While flunixin is not a drug required in milk truck screening, the inclusion of this drug in a screening test that also detects five β-lactam drugs gives the dairy industry the flexibility to screen for drugs commonly used on the farm while complying with the screening required under Appendix N of the Pasteurized Milk Ordinance.

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