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Applicability of Screening Tests for Oxytetracycline in the Milk of Three Breeds of Goats

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ABSTRACT

Antibiotics are widely used in animal husbandry, and the presence of antibiotic residues in milk is a health hazard. The objectives of this study were to determine residual amounts of oxytetracycline in the milk of three breeds of goats using high-pressure liquid chromatography (HPLC) analysis and screening tests. It was also essential to explore the safe withdrawal period of oxytetracycline in lactating goats and examine the applicability of Charm ROSA and SNAP screening tests. The qualitative results of these tests were compared with the quantitative results of the HPLC method. Fifteen milking does, five each from the Alpine, LaMancha, and Nubian breeds, were selected from the herd at Prairie View A&M University. Milk samples containing antibiotic residues were deproteinized by HCl and acetonitrile, and then oxytetracycline was extracted from the supernatant. The residues of oxytetracycline in goat's milk up to 110 h after injection were qualitatively detected using the Charm ROSA test. Similarly, the SNAP test detected the antibiotic residues in milk up to 110 h after treatment. The HPLC results indicated that oxytetracycline residues in milk from Alpine goats were below the tolerance level (300 ng/ml) 82 h after drug treatment (72 h for LaManchas, 58 h for Nubians); however, the results of the screening tests would indicate longer withdrawal periods for milk from the breeds of goats studied, which would result in economic losses to goat's milk producers. The results of this study also indicated that oxytetracycline was not stable in raw goat's milk at refrigeration temperature or during pasteurization and that the concentrations decreased significantly. Commercial goat's milk is usually exposed to several hours of refrigeration and then to pasteurization. The results of this study indicated that, if oxytetracycline was present in raw goat's milk, the concentration would decrease significantly before it was marketed.

Key words: Goat's milk; Oxytetracycline; Screening tests; Withdrawal period

Milk has been an important food for humans since the domestication of dairy animals. It is a common component of the animal-derived food products that comprise many diets. With the expansion of the goat dairy industry and use of goat's milk in various sectors of food processing, methods to ensure the safety and quality of goat's milk and milk products have become necessary. Therefore, it is important to ensure that marketed goat's milk is safe for human consumption.

Antibiotics are widely used in animal husbandry for the treatment of diseases, health maintenance, and in some countries, at subtherapeutic levels as feed additives to suppress undesirable bacteria and to enhance the growth of food-producing animals *(11, 17, 21)*. The improper use of antibiotics for the control of diseases such as mastitis is the major source of drug residues found in milk *(21, 24)*. There are concerns that antibiotic residues in foods could significantly shift the resistance patterns in the microbial population in the human intestinal tract *(10, 11),* and there are also concerns about antibiotic resistance in foodborne pathogens such as *Salmonella* and *Campylobacter (17, 24)*.

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The presence of antibiotic residues in milk is known to interfere with the manufacture of several fermented dairy products by inhibiting starter activity, which can lead to monetary losses *(9, 10, 12)*. Milk supplies containing antibiotics above certain concentrations are illegal. Tolerances for residues of new animal drugs in food from animal products were determined for different antibiotics so that no unintended harmful effects would be likely to occur from these drugs *(9, 26)*.

Tetracyclines are a group of broad-spectrum antibiotics that are active against gram-positive and gram-negative bacteria, such as chlamydia, mycoplasmas, rickettsia, and protozoan parasites. The tetracycline group is currently used to treat goats with diseases such as mastitis, pinkeye, and urinary and enteric infections *(17)*. Although they are generally regarded as relatively nontoxic, they may produce a large number of adverse effects, some potentially life-threatening, in some individuals, including superinfection, diarrhea, ingestion, direct toxicity, irritation, dizziness, antianabolic effects, photosensitivity, and allergic symptoms.

Current methods for the detection of antibiotic residues in milk include tests such as microbiological inhibition tests, immunoassay tests, and chemical or physical methods such

as spectrophotometric and chromatographic methods *(3, 18, 23)*. The available microbiological tests are nonspecific for some antibiotics such as tetracyclines and penicillins, and immunoassays are usually quite expensive. A quantitatively accurate chemical method for detecting antibiotics in milk is high-performance liquid chromatography (HPLC). Considerable progress has been reported in the development of HPLC methods for determination of tetracyclines and other antibiotics in both milk and meat tissues *(2, 6–8)*. HPLC techniques are usually time-consuming; they include several sample preparation steps, require trained personnel, and are not applicable for daily use at milk-producing facilities. Thus, there is a need for a simple, fast, and sensitive screening procedure such as the Charm ROSA or SNAP test to qualitatively and economically determine the presence of tetracycline residues in goat's milk at dairy facilities.

The U.S. Food and Drug Administration (FDA) established guidelines for the use of oxytetracycline (Liquamycin LA-200) injections in lactating dairy cows in 1998. Prior to that, Liquamycin LA-200 was only approved for beef animals *(25)*. With this approval, Liquamycin LA-200 is labeled for administration to beef and dairy animals for treatment of pneumonia, shipping fever complexes associated with *Pasteurella* spp., *Haemophilus* spp., pinkeye, foot rot, bacterial enteritis, leptospirosis, wound infections, and acute metritis. The FDA and the dairy industry set a new tolerance level of 300 ng/ml for tetracyclines in milk when the approval notice for Liquamycin LA-200 was published in the Federal Register *(5)*. According to the Codex Alimentarius Commission (and member organization European Union) and regulatory organizations in some other countries, the maximum residue limit level for oxytetracycline in milk is 100 ng/ml (Veterinary Drug MRL Database, www.mrldatabase.com).

The Charm ROSA and SNAP tetracycline tests are quite sensitive and are commonly used to screen bovine milk for the presence of antibiotic residues. The Charm ROSA screening test was developed to qualitatively detect oxytetracycline residues in bovine milk at or above the tolerance level of 300 ng/ml. Most of the antimicrobial residue screening tests that are commercially available are either drug-specific or species-specific, and their applicability should be verified before testing goat's milk *(1, 4, 22)*. The applicability of the Charm ROSA screening test was examined on spiked goat's milk with an immunoassay *(3)*, according to the European Union maximum residue levels (100 ng/ml), and was not quantitatively verified. However, the applicability and accuracy of these screening tests according to the U.S. tolerance limit has not been examined on lactating goats.

The withdrawal period is the waiting time that must elapse before treated animals or their products can be processed for human consumption *(17)*. The withdrawal period of oxytetracycline for lactating cows is 96 h after the last treatment *(5)*. However, the withdrawal period for this antibiotic has not been determined for milking goats. Goat's milk producers currently use the withdrawal time recommended for lactating cows. Currently, there is considerable variation in the management practices associated with antibiotic use on dairy farms *(21)*. Goats are considered minor species, and there is no FDA-accepted screening test for tetracyclines in goat's milk *(25)*. It is important to qualitatively determine the residual amounts of these antibiotics in goat's milk using different screening techniques and to verify the results of these screening tests using a quantitative method to explore the safe withdrawal period of treated lactating goats. Information on the safe withdrawal periods for antibiotic residues in goat's milk is limited, and the depletion rate of oxytetracycline in the Alpine, Nubian, and LaMancha breeds has not been previously determined. Therefore, it was necessary to study the effectiveness of commercially available screening tests for determining oxytetracycline residues in goat's milk and also to determine a suitable test for routine daily use at farms and/or at processing facilities. Additionally, verification of the results of these screening tests with an accurate quantitative method (HPLC) to determine the exact levels of antibiotic residues in goat's milk is essential.

Therefore, the objectives of this study were (i) to qualitatively determine the presence of oxytetracycline residues in the milk of Alpine, Nubian, and LaMancha goats using the Charm ROSA and the SNAP tetracycline tests; (ii) to compare the results of these qualitative tests with a quantitative technique (HPLC); (iii) to explore the safe withdrawal period of oxytetracycline residues in milk of Alpine, Nubian, and LaMancha breeds treated intramuscularly with this antibiotic; and (iv) to determine the concentrations of residual oxytetracycline in fresh, pasteurized, and aged (72 h) raw goat's milk.

MATERIALS AND METHODS

Animal selection and treatment. Fifteen milking does, five Nubian, five Alpine, and five LaMancha, were randomly selected from the herd at the International Goat Research Center at Prairie View A&M University (TX). The selected does were average milk producers (2.3 to 2.8 kg/day), with body weights ranging from 55 to 75 kg, and they were in midlactation cycle. Each goat was intramuscularly injected with oxytetracycline (Liquamycin LA-200, Pfizer Inc., New York, NY) in the hind leg, according to animal body weight. A dose of 17.6 mg of oxytetracycline per kg of body weight was applied for all injections (1 ml/11.4 kg of body weight), according to the manufacturer's recommendation. One milliliter of Liquamycin LA-200 contained 200 mg of amphoteric oxytetracycline base in the aqueous solution (manufacturer's certified concentration). Each doe was given two antibiotic injections, one in the evening before milking and the second 48 h later, according to the recommended therapeutic practice.

Sample collection. The treated goats were milked by hand, and a sample from each goat was placed on ice in a container and was brought to the laboratory for analysis. Milk samples (approximately 100 ml) were collected twice daily, in the mornings and evenings after the second antibiotic injection, for up to 138 h. Each milk sample was divided into three portions. One portion of milk sample was pasteurized (63°C for 30 min) and frozen until analysis. The second portion of raw milk was aged in the refrigerator (4°C) for 72 h before analysis, and the third portion of the sample was analyzed the day it was collected. Using the HPLC system, all fresh milk samples from treated goats were analyzed for oxytetracycline residues until depletion.

Antibiotic residue testing. In addition to HPLC analysis, all milk samples from treated animals were also analyzed for oxytetracycline residues until depletion using the Charm ROSA tetracycline test (Charm Science Inc., Lawrence, MA), and the SNAP tetracycline test (IDEXX Laboratories, Inc., Westbrook, ME). The tests were performed according to the manufacturers' instructions. Briefly, a 1:2 dilution of samples and control milk was recommended by the manufacturer for the Charm ROSA tetracycline test because the sensitivity of this screening test is from 70 to 100 ng/ml, whereas the established tolerance level for tetracycline residues in milk is 300 ng/ml in the United States. The diluted samples and control milk were mixed thoroughly and were incubated at 55 ± 1°C. This was required for activation of the binding process for this test. This was a rapid binding assay test that used binders specific to tetracycline drugs and beta-lactam antibiotics. Meanwhile, refrigerated test strips supplied with the test kit were labeled and incubated in the ROSA incubator. Aliquots (approximately 300 ± 15 μl) of diluted milk samples were added into the well of the sample pad compartment. The samples were sealed with tape over the sample compartment and were incubated in the ROSA incubator for 10 min. After the required incubation period, test strips were removed for visual inspection and interpretation. Presumptive-positive samples were diluted as mentioned and were retested with the control samples.

Milk samples were mixed and diluted 1:10 with control milk for antibiotic residue screening by the SNAP tetracycline test, according to the manufacturer's recommendation. The reagent pellet that contained the enzyme for this test was present at each sample tube. The SNAP device was placed horizontally in the heating block, which was tempered at 45°C for at least 5 min. Approximately 450 ± 50 μl of the milk sample was added and mixed gently to dissolve the reagent pellet. The sample tube was incubated in the heating block for 3 min. The milk sample was poured into the sample well of the SNAP device, and the tube was discarded. The SNAP test was capable of determining tetracycline residues in milk after 8 to 10 min. After the incubation period, visual interpretation of the results was obtained by comparison of the samples and controls.

Preparation of mobile phase for HPLC analysis. Exactly 2.44 g of sodium-decanesulfonate was placed into a 250-ml flask and was completely dissolved into 100 ml of distilled and deionized water. The dissolved compound was sonicated (model 3510, Branson Ultrasonics Corporation, Danbury, CT) for 10 min and then was brought to exactly 1 liter with acetonitrile. The mixture was degassed for 5 min using an ultrasonic cleaner (Branson Ultrasonics Corporation). A 0.02 M H_3PO_4 solution was prepared by mixing reagent grade H_3PO_4 (1.35 ml) acid diluted with distilled and deionized water to 1 liter using a volumetric flask. The mobile phase was prepared by mixing 65% of 0.02 M H_3PO_4 and 35% of 0.01 M Na decanesulfonate-acetonitrile. The solution was gently mixed and filtered, and then it was degassed for 10 min.

Sample extraction. Five milliliters of milk was deproteinized by mixing with 1 ml of 1 M HCl and then 15 ml of acetonitrile in a 125-ml flask. Acetonitrile was added to the mixture in three portions, and the flask was swirled after each addition to mix the contents, according to the method of Moats and Harik-Khan *(13),* with the following modifications. After 5 min, the supernatant was filtered (filter paper #541, Whatman International Ltd., Maidstone, UK) and approximately 12 ml of filtrate was collected. The filtrate was transferred into a 50-ml recovery flask (Lab Glass, Vineland, NJ) and was placed into the Rotavapor concentrator (model RE-200, Buchi, Flawil, Switzerland) for evaporation under reduced pressure. The sample flask of the Rotavapor was placed into a distilled water bath at 36°C, and then vacuum was applied to the Rotavapor concentrator by an aspirator pump (model 7049-00, Cole-Parmer Instrument, Vernon Hills, IL). The contents were evaporated to approximately 1 ml or slightly less (but not to dryness) and were transferred (using disposable Pasteur pipettes) into a graduated evaporator receiver tube (Ace Glass Inc., Vineland, NJ). The final volume of the extract was adjusted exactly to 1 ml with rinses of small amounts of distilled water. The extract was drawn into a 3-ml disposable syringe (no. 305196, BD, Franklin Lakes, NJ) using 18-gauge, 1.5-in. (3.8-cm) injection needles (no. 305196, BD) and filtered through a 25-mm Acrodisc 0.45-μm HT Tuffryn membrane (Pall Corporation, Ann Arbor, MI). The filtered samples were placed into microcentrifuge tubes (1.5-ml polypropylene) until they were loaded into HPLC vials for analysis.

Standard preparation and spiking of milk. Antibiotic-free milk (control) was collected from the selected does prior to drug injection and was used for preparation of standards with oxytetracyline at known concentrations of 100, 200, 300, 400, and 500 ng/ml, according to the method of Moats and Harik-Khan *(13)*. The above standards were prepared from oxytetracycline with certified concentration (200 mg/ml) by dilution in mobile phase using volumetric flasks. A stock solution was made by dissolving 300 μl of oxytetracycline into 100 ml of mobile phase, and it was then diluted to the final concentration of 60 μg/ml. Five-milliliter samples of control milk were spiked using Hamilton pipettes to get the required concentrations of 100, 200, 300, 400, and 500 ng/ml. The spiked milk samples were extracted and analyzed by the following HPLC method.

HPLC analysis. The Waters HPLC system (Waters, Milford, MA), with 515 pump, 2489 UV detector, 717 autosampler, and Empower 2 software, was used for analyses. A ProteCol-GP C18 125 (150 by 4.6 mm i.d., 5 μm, 120 Å; SGE Analytical Science, Austin, TX) analytical column was used. The mobile phase was equilibrated for 30 min before sample injection, and the flow rate was 1 ml/min with the UV detection set at 380 nm. Oxytetracycline standards were analyzed at the beginning and at the end of each day to get accurate quantification. Portions (50 μl) of each standard and sample were injected into the system. Sample analyses were performed after duplicate standards with known concentrations were analyzed. A linear regression equation was established for each day of analysis, with the software using peak areas and concentrations of standards. The concentrations of oxytetracycline residues in samples were calculated using the linear regression equation for each day of analysis. The correlation coefficient (r) of the curve for each day of analysis ranged from 0.88 to 0.99 and could slightly differ due to HPLC response sensitivity and variability in sample extraction.

Statistical analysis. The results of the screening tests were analyzed by the PROC LOGISTIC procedure of SAS *(20)* to compare the time effect for each test. The observed responses were considered binary and were classified as "positive" for both positive and doubtful close to positive ($+d$) results or as "negative" for doubtful (d) and negative results. The logistic regression model used was

$$L_{CS} = \text{logit}[P_{CS}] = b + aT_{CS} + E_{CS}$$

where logit is a linear logistic model, that is, $\ln[P_{CS}/(1 - P_{CS})]$, P_{CS} is the probability of "negative" versus "positive" results (C,

TABLE 1. *Results of screening tests on the milk of 15 lactating does at different times after treatment with oxytetracycline*[a]

	Charm test											SNAP test										
Milking does	10 h	24 h	34 h	48 h	58 h	72 h	82 h	96 h	110 h	124 h	138 h	10 h	24 h	34 h	48 h	58 h	72 h	82 h	96 h	110 h	124 h	138 h
Alpine	+	+	+	+	+	+	+	+	*d*	−	−	+	+	+	+	+	+	+	+	+	−	−
Alpine	+	+	+	+	+	+	+	+	+*d*	+*d*	−	+	+	+	+	+	+	+	+	+	−	−
Alpine	+	+	+	+	+	+	+	+	+*d*	*d*	−	+	+	+	+	+	+	+	+	+	+	−
Alpine	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	−	−
Alpine	+	+	+	+	+	+	+	*d*	*d*	−	−	+	+	+	+	+	+	+	+*d*	*d*	−	−
Nubian	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	−	−
Nubian	+	+	+	+	+	+	+	+	+*d*	+*d*	−	+	+	+	+	+	+	+	+	+	−	−
Nubian	+	+	+	+	+	+	+	+	+	*d*	−	+	+	+	+	+	+	+	+	+	+*d*	−
Nubian	+	+	+	+	+	+	+	+	+*d*	−	−	+	+	+	+	+	+	+	+	+*d*	−	−
Nubian	+	+	+	+	+	+	+	+	+*d*	−	−	+	+	+	+	+	+	+	+	+	+	−
LaMancha	+	+	+	+	+	+	+	+	+	+*d*	−	+	+	+	+	+	+	+	+	+	−	−
LaMancha	+	+	+	+	+	+	+	+	*d*	−	−	+	+	+	+	+	+	+	+	+*d*	−	−
LaMancha	+	+	+	+	+	+	+	+	+*d*	*d*	−	+	+	+	+	+	+	+	+	+	−	−
LaMancha	+	+	+	+	+	+	+	+	*d*	−	−	+	+	+	+	+	+	+	+	+	−	−
LaMancha	+	+	+	+	+	+	+	+	+*d*	−	−	+	+	+	+	+	+	+	+	+	+*d*	−

[a] +, Sample is positive with antibiotic residues; *d*, result is doubtful; −, sample is negative without antibiotic residues; +*d*, doubtful, but close to positive.

Charm; S, SNAP), b is the intercept, a is the slope, T_{CS} is test kits (C or S), and E_{CS} is the random error term.

The following model was used to analyze data by the PROC MIXED procedure of SAS *(20)* for repeated measures. The least-square means option was used to determine differences among the concentrations of residual oxytetracycline in the fresh milk of three breeds of goats.

$$Y_{ijk} = \mu + B_i + E_{ik} + H_j + (B \times H)_{ij} + E_{ijk}$$

where Y_{ijk} is the mean concentration of antibiotic residues (i is Alpine, Nubian, or LaMancha; j is number of hours after injection [10, 24, 34, 48, 58, 72, 82, and 96]; k is the number of animals [five per breed]), μ is the population mean, B_i is the breed effect with three categories, H_j is hours after injection of antibiotic, $(B \times H)_{ij}$ is the interaction effect of breed and hours after antibiotic injections, and E_{ijk} is the random error term.

The data for concentrations of residual oxytetracycline in fresh, pasteurized, and aged (72 h) raw milk were analyzed by two-factorial design using the PROC GLM procedure of SAS *(20)*. The least-square means option was used to detect differences among means of the residual oxytetracycline in milk (fresh, pasteurized, or raw milk aged for 72 h) from the three goat breeds:

$$Y_{itk} = \mu + B_i + M_t + (B \times M)_{it} + E_{itk}$$

where Y_{itk} is the mean concentration of antibiotic residues, μ is the population mean, B_i is the breed effect, M_t is the milk type (t = fresh, pasteurized, or aged [72 h] raw milk), $(B \times M)_{it}$ is the interaction effect of breed and milk type, and E_{itk} is the random error term.

RESULTS

The results of the Charm ROSA and SNAP tests for the 15 milking does that were treated with oxytetracycline are shown in Table 1. Milk samples from the treated does were statistically positive according to the Charm ROSA test, from 10 to 110 h after treatment. The results of the Charm ROSA screening test differed significantly ($P < 0.05$) between 110 and 124 h after treatment, indicating that the numbers of milk samples with residual oxytetracycline at the tolerance level at 124 h after treatment were not significant. When the doubtful samples of 110 h after injection were tested again, 7 of 11 cases tested presumptive positive (close to positive), and the rest were doubtful when compared with the control. The Charm test efficiently detected oxytetracycline residues in milk for up to 96 h after drug administration. The readings of sample strips and color development were clear and easy to interpret up to 96 h after injection. However, at 110 h after the drug treatment, most samples gave doubtful results, mainly owing to the difficulty in color development and absence of the indicator line. According to this test, statistically, the levels of drug residues at 110 h after injection were positive, indicating levels of oxytetracycline in milk equal to or more than the tolerance level (300 ng/ml). Thus, according to the Charm ROSA test, animal withdrawal time after treatment with oxytetracycline would be 110 h. Most of the tested milk samples were negative at 124 h after injection, and a few samples were lower than the tolerance level, some giving doubtful results.

Similarly, the results of the SNAP test indicated that drug residues were clearly present in milk samples up to 110 h (ninth milking) after treatment and then gave two positive and two doubtful results at 124 h (10th milking) after injection. The results of the SNAP test differed significantly ($P < 0.05$) between 110 and 124 h after injection. At 110 h after treatment, only one sample showed a doubtful result, indicating the possibility of residual levels below the tolerance limit, and the rest were all positive or close to positive. At 110 h after treatment, the SNAP screening test gave fewer doubtful results compared with the Charm ROSA test. The results obtained by the SNAP test indicated that the withholding time of goat's milk would be 110 h (ninth milking). At 124 h after drug treatment, according to the SNAP test, only two samples were positive and two others were doubtful. This test showed that oxytetracycline

TABLE 2. *Concentrations of residual oxytetracycline in the milk of three breeds of goats as determined by HPLC at different milking intervals after treatment*[a]

	Alpine		Nubian		LaMancha	
Time (h)	Mean	SEM	Mean	SEM	Mean	SEM
10	1,567 A	129	1,406 A	204	1,732 A	122
24	1,537 A	176	1,337 A	303	1,379 AB	181
34	1,315 A	208	1,080 AB	303	1,019 BC	208
48	599 B	98	431 BC	154	379 DE	97
58	305 C	99	181 CD	156	558 CD	99
72	333 C	64	245 CD	101	281 E	64
82	273 C	44	204 CD	71	271 E	44
96	93 D	6	ND		ND	

[a] Concentrations are in nanograms per milliliter. Means within each column followed by different letters are significantly different ($P < 0.05$). ND, not detectable.

residues were present in milk samples at or above the tolerance level at 110 h (ninth milking) after animal injection. To verify the applicability and reliability of these screening tests, an accurate HPLC procedure that met the requirements of regulatory agencies for quantitative sensitivity was used.

HPLC analysis. The residual concentrations of oxytetracycline in goat's milk up to 96 h after treatment were determined using the modified HPLC technique *(13)*. The mean results for each breed indicated that the withdrawal period of oxytetracycline in treated Alpine does was 82 h (seventh milking) and, in Nubian goats, was 58 h (fifth milking) after drug treatment (Table 2). The LaMancha does at 72 h after injection (sixth milking) had mean residual antibiotic lower than the tolerance level. The mean concentration of oxytetracycline for the Alpine breed was 273 ± 44 ng/ml 82 h after drug administration (below the tolerance level), whereas the mean concentration of oxytetracycline in the milk of the Nubian breed was 181 ± 156 ng/ml 58 h after injection. The mean concentration of oxytetracycline for the LaMancha breed was 281 ± 64 ng/ml 72 h after injection. There was a difference among the withdrawal periods for Alpine, LaMancha, and Nubian breeds of goats. The results obtained by HPLC in this study indicated that oxytetracycline residues were present in the milk of the Alpine goats longer than in the milk from the other two breeds of goats. However, based on data from individual animals at 96 h after injection, all goats had residual concentrations of approximately 100 ng/ml or lower, which was below the detection limit of the technique. Hence, 96 h after injection of drug is a safe withdrawal period for all the goats.

The residual concentrations of oxytetracycline were not different ($P < 0.05$) among the three breeds of goats at each milking interval. The residual concentration of oxytetracycline in milk was at the highest level 10 h after treatment and then decreased over time. The mean concentrations of oxytetracycline in the milk of Nubian goats differed ($P < 0.05$) between 10 and 48 h after drug administration. The mean concentrations of oxytetracycline in the milk of Alpine goats differed ($P < 0.05$) between 10 and 48 h after drug injection, whereas for the LaMancha does, the concentrations of antibiotic differed ($P < 0.05$) among 10, 34, and 48 h after treatment. The rate of decrease of oxytetracycline in milk was higher from the initial milking times up to 48 h, and then the rate of decrease slowed (Table 2).

However, the mean concentration of drug residue in all of the treated does of the three breeds of goats was 249 ± 31 ng/ml at 82 h after injection of animals, which was lower than the tolerance level of this antibiotic in milk (Table 3). The overall results indicated that the withdrawal period for oxytetracycline in all treated goats was 82 h (seventh milking) after injection. The mean concentrations of the residues were higher at the beginning of the study and then started decreasing significantly 10, 34, and 48 h after drug administration (Table 3).

The concentrations of residual oxytetracycline in fresh, pasteurized, and aged (72 h) raw milk were assessed for each breed of goats from the milk obtained 10 h after treatment (Table 4). The concentrations of residual antibiotic in fresh, pasteurized, and aged (72 h) raw goat's milk differed significantly ($P < 0.05$) for the Alpine and LaMancha breeds, and the concentration of oxytetracycline in the fresh milk was significantly different than in the pasteurized or aged raw milk for the Nubians. The residual concentrations of oxytetracycline were not significantly different among the

TABLE 3. *Concentrations of residual oxytetracycline in the milk of all treated goats of three breeds at different milking intervals after treatment*[a]

Time (h)	Mean	SEM
10	1,569 A	91
24	1,418 AB	131
34	1,138 B	148
48	469 C	69
58	348 CD	69
72	286 D	45
82	249 D	31
96	ND	ND

[a] Concentrations are in nanograms per milliliter. Means followed by different letters are significantly different ($P < 0.05$). ND, not detectable.

TABLE 4. *Concentrations of residual oxytetracycline in milk from the three goat breeds, obtained 10 h after drug injection*[a]

	Alpine		LaMancha		Nubian	
Milk type	Mean	SEM	Mean	SEM	Mean	SEM
Fresh	1,529 A	141	1,838 A	154	1,782 A	224
Pasteurized	860 B	141	806 B	141	587 C	239
Raw, aged for 72 h	341 C	141	368 C	141	549 C	239

[a] Concentrations are in nanograms per milliliter. Means followed by different letters are significantly different in both columns and rows ($P < 0.05$).

three breeds of goats for fresh or aged (72 h) raw milks. The pasteurized milk from Nubians differed significantly from that of the Alpines or LaManchas. The concentrations of oxytetracycline in the fresh, pasteurized, and aged raw milk, from the 10 h milking, in all three treated goat breeds (Table 5) were significantly different ($P < 0.05$).

DISCUSSION

The modified HPLC technique was sensitive enough to determine concentrations of oxytetracycline below the requirement of regulatory agencies. The HPLC method could be used to determine the different withdrawal periods of the three breeds of goats. The results of this study indicated that the mean withdrawal periods for oxytetracycline residues in goat's milk were 82, 72, and 58 h for the Alpine, LaMancha, and Nubian breeds of goats, respectively. However, individual animals have a 96-h withdrawal period for oxytetracycline based on HPLC. This difference in the depletion rate of oxytetracycline from the milk of these animals could be because of genetic variations of the three breeds or to differences in the rates of metabolism of these breeds. Molina et al. *(14)* reported that lactating sheep with lower milk production showed a more prolonged withdrawal period after antibiotic therapy. Payne et al. *(16)* intramuscularly injected eight milking goats of mixed breeds with the same formulation of oxytetracycline that was used in this study and determined the concentrations of drug residues in milk over time. Their results were similar to this study, using a different HPLC technique.

The results of the Charm test and the SNAP test showed that the withholding time of lactating goat's milk not suitable for human consumption was 110 h after animal injection, with the exception of two samples tested by the SNAP test. The positive antibiotic residues found in these samples at 124 h after treatment may be due to the state of animal activity during the study or to lower than average milk-producing abilities of the two animals. The screening tests indicated that the drug residues in goat's milk tested at 96 or 110 h after treatment were either at or above the tolerance level. However, the actual levels of antibiotic residues in milk were lower than the tolerance limit, as verified by the results of the HPLC method. The withdrawal period that was determined by the HPLC procedure was shorter than the withdrawal period that was determined by the screening tests. The results of the HPLC method indicated that the withdrawal period for oxytetracycline residues in goat's milk for all treated does of the three breeds was 82 h (seventh milking). Similarly, Rule et al. *(19)* intramuscularly injected five lactating Murciano-Granadina goats with a long-acting formulation of oxytetracycline; based on a screening test (Delvotest SP, DSM Food Specialties, Delft, The Netherlands), they concluded that milk of these animals should not be used for human consumption for 3 days (72 h). The HPLC results indicated that these screening tests identified milk that met the regulatory requirements (less than 300 ng/ml) as having antibiotic residues at concentrations not permitted. These two qualitative screening tests were more sensitive, and they detected oxytetracycline residues in goat's milk even below the tolerance level up to 110 h after drug injection. The higher level of sensitivity of these screening tests gave erroneous results that necessitated longer withdrawal periods for treated animals. This would lead to economic losses to goat's milk producers.

TABLE 5. *Concentrations of residual oxytetracycline in milk from all treated Alpine, LaMancha, and Nubian goats 10 h after injection*[a]

Milk type	Mean	SEM
Fresh	1,716 A	102
Pasteurized	751 B	104
Raw, aged for 72 h	419 C	104

[a] Concentrations are in nanograms per milliliter. Means followed by different letters are significantly different ($P < 0.05$).

This demonstrated that Charm ROSA and SNAP qualitative screening tests had higher sensitivities for detection of oxytetracycline in goat's milk and could detect this drug even when goat's milk had residues below the tolerance level. Beltran et al. *(3)* also noticed that the Charm ROSA immunoreceptor screening test was able to detect levels in spiked ewe's and goat's milk that were lower than the European Union maximum residue limits of 100 ng/ml. The results of the current study clearly indicate that these screening tests, which were originally designed for lactating cows, were more sensitive for lactating goats and, when applied to goat's milk, caused economic losses. This points to the need to develop less sensitive and more economically applicable screening tests for fast, routine determination of withdrawal periods for milking goats on farms and in the goat dairy industry. Further studies are needed to check the applicability of SNAP and Charm tests using milk from different breeds of goats.

Additionally, the results of this study indicated that oxytetracycline was not stable in raw goat's milk at refrigerated temperatures and that it decreased significantly compared to fresh or pasteurized milk. It is conceivable that

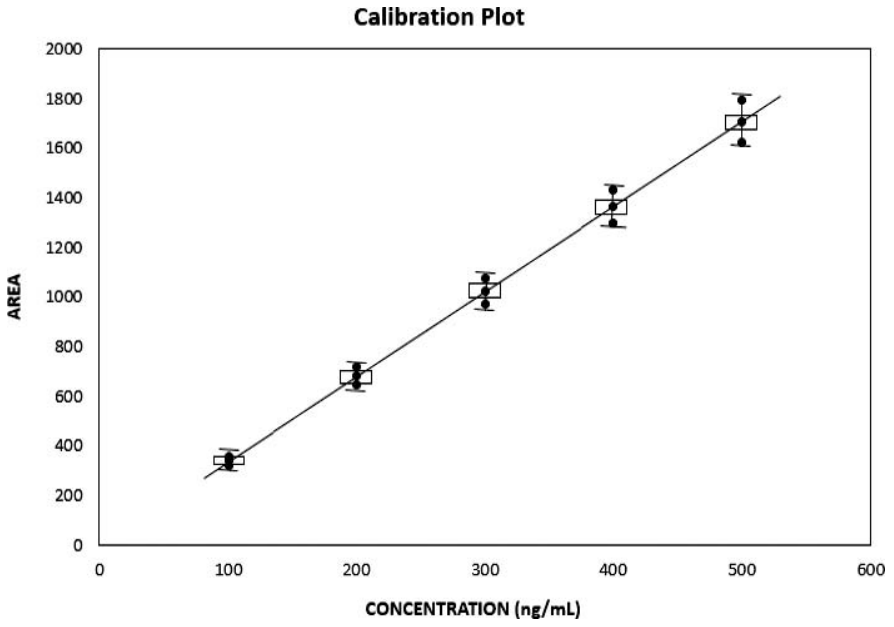

FIGURE 1. *Assay of oxytetracycline residues with six sets of spiked milk standards had the following equation:* $Y = a + bX$, *where* $a = -1.26$ *and* $b = 3.42$. *The correlation coefficient* $r = 88.8$.

the heat of pasteurization could have reduced the level of natural inhibitory enzymes in milk or the level of this antibiotic in pasteurized goat's milk. Yamaki et al. *(27)* and Molina et al. *(15)* also noticed that the level of "positive" and "doubtful" responses to antibiotic residue screenings of Delvotest SP (DSM Food Specialties) and BRT AiM (AiM-Analytik in Milch Produktions-und Vertriebs GmbH, Munchen, Germany) were lower in ewe's milk heated at 82°C for 10 min compared with unheated milk. However, 72-h aging of raw goat's milk in the refrigerator was not expected to have significantly lowered the concentrations of residual oxytetracycline compared with fresh or pasteurized milks. Based on this observation, it is speculated that either natural enzymes or microbial populations in raw milk may have reduced the level of this antibiotic compared with other milks. Podhorniak et al. *(18)* also noticed that tetracycline was not stable in raw cow's milk under storage conditions in the refrigerator. This finding has relevance for the level of oxytetracycline in marketed goat's milk. Commercial goat's milk is exposed to several hours of refrigerated storage in bulk tanks at milk-producing farms and then at the silo of milk cooperatives before it is processed. This usual delay in processing of commercial goat's milk would significantly reduce the amount of oxytetracycline, if present, in raw milk. If pasteurized milk is produced, the heat of pasteurization would further reduce oxytetracycline that may have been present in raw goat's milk. However, during the preliminary phase of this investigation it was observed that freezing of milk samples at –20°C did not affect the concentration of oxytetracycline.

Comparing the chromatograms of control milk with those of spiked standards indicated that oxytetracycline had a sharp peak at a concentration of 300 ng/ml. The antibiotic peak was clear with a high degree of resolution, and the analytical system used was sensitive in detecting oxytetracycline residues below the tolerance level (up to 100 ng/ml). The repeatability of this technique was measured with six sets of spiked milk standards (Fig. 1). The range of correlation coefficient (r) values for the calculation of oxytetracycline in milk samples was from 0.88 to 0.99. At 110 h after drug administration (ninth milking), the residues in milk could no longer be detected by the HPLC method. The HPLC method was quantitative and could be used to check the performance of commercial screening tests to determine whether or not violative residues were actually present in goat's milk.

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