EVALUATION OF ANIMAL DRUG RESIDUE DETECTION METHODS*

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SUMMARY

Animal drug residues in milk have been a major source of concern in the United States for several years. Many new methods to detect various residues at low levels have been introduced, revised or improved. At present the only "official" method approved by federal regulatory officials is for the detection of beta-lactam type antibiotics and is the Bacillus Stearothermophilus disc assay. The Food and Drug Administration (FDA) HPLC (High Pressure Liquid Chromatography) procedure is the "official" confirmatory method. A study was undertaken to evaluate 14 detection methods with 24 animal drug residues in milk to determine the sensitivity of each method and its ability to detect a residue at the FDA "recommended violative" level. No method tested is quantitative but give only a positive or negative indication of the presence of the drug. Milk samples were contaminated or "spiked" with known amounts of commonly available antimicrobial agents in 3 to 6 concentration levels. Fifteen replicates of each milk preparation were tested with applicable methods. The data revealed a total of 13 methods capable of detecting 23 drug residues at their respective levels of concern. Therefore, it would appear there exists adequate methodology for supplementation of the Bacillus stearothermophilus disc assay in regulatory milk testing.

INTRODUCTION

The entire U.S. dairy industry is sensitized to the presence of animal drug residues in milk. Many new methods have been introduced, and some others revised and improved, to detect various residues at low levels. These methods will be included in the 16th edition of Standard Methods for the Examination of Dairy Products, (1) to be published 1991/92. The present situation is quite simple; the "official" method for detection of beta-lactams is the Bacillus stearothermophilus disc assay (2) and "official" confirmatory methods are those HPLC procedures developed by FDA (3).

The U.S. dairy industry has, for some time, anticipated some progress or change in the Pasteurized Milk Ordinance to accommodate new methods as to their potential to supplement the Bacillus stearothermophilus disc assay as an "official" method. New tests designed to detect residue of animal drugs have been reported (4,5). In an attempt to offer scientific data to assist in this potential change, the objective of this study was to evaluate 14 detection methods with 24 animal drug residues in milk to determine the sensitivity of each method and its ability to detect a residue at the "recommended violative" level. This level does not necessarily correspond to safe or tolerance levels, or safe concentration.**

It is imperative that dairy processors, producers, and regulatory agencies have adequate information concerning the most appropriate detection method for a particular situation so they are able to make an educated assessment of the product.

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**"Safe" level-used by FDA as guides for prosecutorial discretion. They do not legalize residues found in milk that are below the safe level. They are not and cannot be transformed into established tolerances.

Established Tolerance Level - a concentration of a marker residue in the target tissue selected to monitor for total residues of the drug in the target animal.

Level of Concern - or "recommended violative" level, a level at which regulatory action will be taken but does not occur at a tolerance or safe level or concentration.

Safe Concentration - concentration of total residues considered safe in edible tissues.

MATERIALS AND METHODS

With the establishment by FDA's Center for Veterinary Medicine of "safe" and established tolerance levels, levels of concern (4.8 ppb penicillin), and safe concentrations for animal drug residues in milk, test kit manufacturers have target levels of detection for methodology development. This has led to numerous methods now being available. The reported study evaluated the 14 methods in Table 1, which were conducted according to manufacturer's training.

Table 1. Antibiotic residue detection tests which were evaluated and thier manufacturers.

Test	Manufacturers
Agri-Screen	Neogen Corp. Lansing, Mi
BR Test	Glengarry Biotech, Apple Hill, Ontario, Canada
Charm II	Charm Sciences, Inc., Malden, MA
Charm Cowside	Charm Sciences, Inc., Malden, MA
Charm Farm	Charm Sciences, Inc., Malden, MA
CITE	IDEXX Corp. Portland, ME
Delvotest P	Gist-Brocades, King of Prussia, PA
Disc Assay	(See process description in Standard Methods for the Examination of Dairy Products, 15th ed, (1)
EZ-Screen	Environmental Diagnostics, Inc., Burlington, NC
LacTek	Idetek, Inc., San Bruno, CA
Penzyme	SmithKline Beecham Animal Health, West Chester, PA
Penzyme III	SmithKline Beecham Animal Health, West Chester, PA
Signal	SmithKline Beecham Animal Health, West Chester, PA

These methods were evaluated for their ability to detect the 24 animal drug residues in Table 2 at their respective "level of concern."

Table 2. Twenty-four drug substances were added to milk samples for residue determinations.

Penicillin G	Sulfamerazine
Cephapirin	Sulfathiazole
Cloxacillin	Sulfadizaine
Ceftiofur (Naxcel)	Bovine Triple Sulfa - Sulfapyridine
Ampicillin	- Sulfamethazine
Amoxicillin	- Sulfathiazole
Tetracycline	Poultry Triple Sulfa - Sulfamerazine
Chlortetracycline	- Sulfamethazine
Oxytetracycline	- Sulfaquinoxaline
Erythromycin	Novobiocin
Tylosin Tartrate	Polymixin B
Sulfamethazine	Gentamycin Sulfate
Sulfadimethoxine	Neomycin Sulfate
Streptomycin Sulfate	Spectinomycin

Raw, unhomogenized milk was collected 2-3 times weekly from 4 first-lactation dairy cows which had not received any drug treatment in the prior year. Drug residues from frozen stock buffer solutions were diluted and coded daily in fresh milk accordingly for the levels tested. These levels usually numbered 5-6, dependent upon the specific residue, and included a zero control, the "level of concern," and usually the detection method's claim level or minimum level of detection (MLD). On day of testing, each residue level was analyzed by each appropriate method 5 times (5 subsamples). This entire protocol, 14 methods x 24 residues x 5 levels x 5 samples, was replicated 4 times. The first replicate utilized commercial veterinary preparations which were found to be insoluble and of non-exact concentrations. Therefore, pure drug compounds were used for replicates 2-4 and the first replicate's data was not included.

"Probe It" analysis of SAS (Statistical Analysis System, Cary, NC) was used to determine necessary positive results required for a 90-95% confidence limit.

RESULTS AND DISCUSSION

A total of 20,876 analyses were conducted. Table 3 is an example of the individual data for a single antibiotic (ampicillin). Table 4 summarizes the data for oxytetracycline. It should be noted that "positives" were determined versus a zero control and not compared to an internal standard (except with Agri-Screen), as spiked samples constituted multiple internal standards. For the methods Charm Farm, Delvotest, Delvotest SP, Penzyme, and Penzyme III, "caution" was recorded as "positive." The "Probe

lt" analysis indicated a needed result of 13 positives out of 15 analyses for 90% confidence limits, which we then interpreted as the minimum "tested" level of detection.

Table 3. Detection of ampicillin by evaluated methods, minimum levels of detection (MLD).*

ppb

Methods	Claim MLD	0	0	10	50	MLD[b]
BR Test		0[a]	15	15	15	≤10
Charm II	3	0	15	15	15	≤ 5
Charm Cowside		0	8	15	15	≤10
Charm Farm	5	0	15	15	15	≤ 5
CITE - visual	8	0	15	15	15	≤10
- instrument		0	0	15	15	≤10
Delvotest P	4	0	0	15	15	≤10
Delvotest SP	4	0	0	15	15	≤10
Disc Assay[c]	10	0	0	15	15	≤10
LacTek	0	12	15	15	≤10	
Penzyme	0	5	14	15	≤10	
Penzyme III	0	9	15	15	≤10	

*Tolerance level of 10 ppb
[a]# out of 15 tests postive
[b]≥ 13 out of 15 analyses positive
[c]# of 15 with Zones ≥ 16 mm

Table 4. Detection of oxytetracycline by evaluated methods.*

ppb

Methods	Claim MLD	0	30	80	250	1,000	MLD[b]
BR Test		0[a]	1	1	3	15	≤1,000
Charm II	3	0	15	15	15	15	≤30
Charm Farm+	150	0	5	15	15	15	≤80
CITE - visual	40	0	13	15	15	15	≤30
- instrument		0	15	15	15	15	≤30
Delvotest P+	500	0	0	0	15	15	≤200
Delvotest SP	500	0	0	0	15	15	≤1,000
Disc Assay[c]	1,000	0	0	0	0(3)[d]	14(1)	≤1,000

*"Safe" level of 30 ppb
[a]# out of 15 tests positive
[b]≥ 13 out of 15
[c]zones ≥ 16
[d]zones ≥ 14 mm but <16mm
+15 at 200 ppb

Table 5 summarizes the minimum tested levels of detection and the safe/tolerance levels for each of the 24 drugs tested. Amoxicillin detection was adequate at the tolerance level by all evaluated methods except for the "official" beta-lactam method - the disc assay. Ampicillin was detected by all tested methods at or below its established tolerance level. Ceftiofur was detected at its relatively high safe concentration of 1,000 ppb by all evaluated methods except LacTek. Cephapirin was adequately detected by all evaluated methods. Chlortetracycline was detected by Charm II and CITE at its "safe" level. Cloxacillin was detected at its tolerance level by only LacTek. Erythromycin was adequately detected at its "safe" level by only Charm II. Gentamicin was detected at its "safe" level by Charm II, CITE, LacTek, and Signal. Neomycin was detected at its tolerance level by Charm Farm, Delvotest P, and Signal. Novobiocin was only detected by Charm II at its tolerance level. Oxytetracycline was detected at its "safe" level by Charm II and CITE. Penicillin was detected at its level of concern by all methods except BR Test, which did detect the drug at its "safe" level.

Table 5. Minimum Tested levels of detection of each residue by evaluated methods given in parts per billion (ppb).

Number	Residue	Safe/ Tolerance	Agri-Screen	BR Test	Charm II	Charm Cowside	Charm Farm	CITE[1]	Delvotest P	Delvotest SP	Disc Assay	EZ-Screen	LacTek[2]	Penzyme	Penzyme III	Signal[3]
1	Penicillin	10/0/4.8[4]		10	2.5	5	2.5	5	2.5	5	5		5	2.5	5	
2	Cephapirin	/20		10	5	5	20	5	10	5	10		10	10	5	
3	Cloxacillin	/10		100	20	50	50	100	50	50	50		10	50	20	
4	Ceftiofur	1,000[5]		100	5	10	50	10	50	100	100		>100	50	50	
5	Ampicillin	/10		10	5	10	5	10	10	10	10		10	10	10	
6	Amoxicillin	/10		5	5	10	5	10	10	10	50		5	5	5	
7	Tetracycline	/80		1,000	5		50	30	420	>1,000	>1,000					
8	Chlortetra-cycline	30/0		>1,000	5		150	30	200	1,000	1,000					
9	Oxytetra-cycline	30/		1,000	30		80	30	200	1,000	1,000					
10	Erythromycin	50/0		1,000	50		1,000		400	400	1,000					
11	Tylosin	50/		50	150		50		100	100	1,000					
12	Sulfamethazine	10/	100	1,000	5	10	20	5	>1,000	1,000	>1,000	10	10			10
13	Sulfadi-methoxine	10/10	>1,000	100	5	10	10	10	>1,000	1,000	>1,000	5	100			
14	Sulfamerazine	10/	1,000	1,000	5	5	100	100	>1,000	1,000	>1,000	100	100			
15	Sulfathiazole	10/	>1,000	1,000	5	100	100	10	>1,000	1,000	>1,000	>1,000	>1,000			
16	Sulfadiazine	10/	>1,000	1,000	5	5	10	1,000	>1,000	250	>1,000	>1,000	>1,000			
17	Novobiocin	/100		>1,000	100		750		1,000	>1,000	>1,000					
18	Polymixin B	/		>1,000			>1,000		>1,000	>1,000	>1,000					
19	Gentamicin	30/		>500	30		150	30	150	250	500		30			30
20	Neomycin	/150		>500	>500		150	>500	150	500	>500		>500			10
21	Streptomycin	125/0		>1000	10		>1,000	>1,000	>1,000	>1,000	>1,000		>1,000			
22	Spectinomycin	/		>1,000	30		>1,000	>1,000	>1,000	>1,000	>1,000					

[1]Residues 1-6 tested with CITE Probe Beta-Lactam, Residues 7-9 tested with CITE Probe Tetracycline, Residues 12 tested with CITE Probe Sulfa Trio, Residues 19-22 tested with CITE Probe Gentamicin.
[2]Residues 1-6 tested with LacTek Beta-Lactam Milk, Residues 12-16 tested with LacTek Milk Sulfa methazine, Residues 19-21 tested with LacTek Gentamicin.
[3]Residue 12 tested with Signal sulfamethazine, Residue 19 tested with Signal Gentamicin, Residue 20 tested with Signal Neomycin.
[4]Level of concern, or "recommended violative" level.
[5]Safe concentration/no tolerance needed.

Polymixin B was not detected by any of the methods evaluated at any of the levels used (up to 1,000 ppb). Spectinomycin was only adequately detected by Charm II at any of the levels tested. Streptomycin, again, was only detected at its "safe" level by Charm II. With the sulfonamide group of drugs being of major concern, 7 residues were tested for adequate detection, including 2 multiple sulfa drugs to evaluate any possible "additive effect" in detection. Sulfadiazine was detected at its 'safe" level by Charm II, Charm Cowside, and Charm Farm. Sulfadimethoxine detection at its "safe"/tolerance level was accomplished by Charm II, Charm Cowside, Charm Farm, CITE, and EZ-Screen. Sulfamerazine was detected at its "safe" level by Charm II and Charm Cowside. Sulfamethazine, the sulfa drug which has caused the most concern, was detected at its "safe" level by Charm II, Charm Cowside, CITE, EZ-Screen, LacTek, and Signal. Sulfathiazole was detected by only Charm II and CITE at its "safe level." The additive detection of sulfa drugs was evaluated using a bovine triple-sulfa (sulfapyridine, sulfamethazine, and sulfathiazole in unknown proportions), and a poultry triple-sulfa (sulfamerazine, sulfamethazine, and sulfaquinoxaline in unknown proportions). Only Charm II and Charm Farm appeared to detect the sum of sulfa drugs, with other methods such as CITE, EZ-Screen, and LacTek demonstrating specific sensitivity to one or more of the included drugs. Tetracycline was detected at its "safe," level by Charm II, Charm Farm, and CITE. Tylosin was detected by BR Test and Charm Farm at its tolerance level.

CONCLUSIONS AND DISCUSSION

In this study, milk from a source for which there was very little liklihood of contamination with antimicrobiol products was utilized. Known amounts of antimicrobial products were then added to the milk (spiked). An alternative approach would have been to treat animals with the drugs, then collect the milk, analyze it for drug content and perform the detection tests. this second approach was ruled out because of the difficulty of determining exact residue levels in a milk sample containing an unknown amount of drug. Even high pressure liquid chromatography (HPLC), which is utilized by the Food and Drug Administraiton in the United States as the confirmatory test for residue violaitons, is basically a qualitative test. Attempts have been made, with varying success, to interpret HPLC results in a quantitative manner (3). in addition, milk samples with residues produced by drug administration to animals may often contain drug metabolites with varying activities that would impact upon test results. While this is an important phenomenon it is an issue beyond the scope of this study.

Milk utilized in this study was obtained from clincially normal cows. This milk is judged to be similar to milk that moves into marketing channels. Milk from cows with clinical conditions or recovering from clinical conditions could contain substances that would interfere with the residue detection tests examined. Applying the results of this trial to testing of milk from individual cows that have been treated for clinical conditions to assess marketability of milk should be done with caution for this reason.

Minimum "tested" levels of detection of each spiked residue by each evaluated method are given in Table 5. These levels should be interpreted as detection at less than or equal to, and not as an absolute minimum level of detection. The data reveals a total of 13 methods capable of detecting 21 drug residues (and 2 cumulative sulfa drugs) at their respective levels. It would appear there exists adequate methodology for supplementation of the Bacillus stearothermophilus disc assay in the regulatory examination of milk for antibicrobial residues.

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