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Evaluation of a *Bacillus stearothermophilus* tube test as a screening tool for anticoccidial residues in poultry

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A *Bacillus stearothermophilus* var. *calidolactis* C953 tube test was evaluated for its ability in detecting the residue of selected anticoccidial drugs in poultry, specically sulfamethazine, furazolidone, and amprolium. Various concentrations of each drug were injected into chicken liver and kidney tissues and these tissues were tested to determine the drug detection limits for each drug. The detection limit was defined as the drug concentration at which 95% of the test results were interpreted as positive. The limits of detection in liver tissue were 0.35 μg/ml for furazolidone, 0.70 μg/ml for sulfamethazine and 7.80 μg/ml for amprolium. In kidney tissues, they were 0.30 μg/ml for furazolidone, 0.54 μg/ml for sulfamethazine, and 7.6 μg/ml for amprolium. It was concluded that this tube test could be used to screen for the residue of these three drugs in poultry.

Key words: anticoccidial drug, *Bacillus stearothermophilus*, detection limit, poultry tissues

Introduction

The production of broiler meat has been on the rise in Kenya and represents an increased opportunity to generate income. Its rise in popularity is based on the availability of a ready market for such meat, low capital requirement, minimal space requirement, proximity to hatcheries, and the availability of a wide selection of animal feeds in urban areas [6]. Production is affected, however, by various diseases, of which coccidiosis is particularly important [3,4]. Coccidiostats are widely used to prevent this condition, to find animals that have the disease and need treatment, and to improve feed conversion efficiency and the rate of animal growth [1,8]. Harmful coccidial residues may appear in foods because of the extensive use of age of anticoccidial drugs in animal husbandry [1,4,7,8].

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Despite the common use of antimicrobials, routine monitoring of animals raised for food for anticoccidal residue is lacking. As in most low-income countries, this can be attributed to the high costs of analysis for coccidial residues and the lake of affordable screening methods [11,13].

The tube test, which was developed to detect drug residues levels in milk at the Codex alimentarius maximum residue limit (MRL), is a low-cost microbiological method with potential for use in low-income countries [9,13]. *Bacillus stearothermophilus* var *calidolactis* C953 is used as the test organism at a pH of 7.0 to 8.0 to detect a broad spectrum of antimicrobials in milk [13]. The applicability of this method to other food matrices has not been tried yet. This study was determine whether this technique could be used to detect the residue of 3 drugs that are frequently used to control coccidial infevtions: sulfamethazine, furazolidone, and amprolium.

Materials and Methods

Preparation of solutions

A stock solution (1 mg/ml) of sulfamethazine (Sigma, Netherlands) was made by dissolving sulfamethazine in ethanol and adding enough distilled water to create a 100 ml solution. Furazolidone (Cosmos, Kenya) was dissolved in distilled water to produce a stock solution of 1 mg/ml. Amprolium (Cosmos, Kenya) was dissolved in distilled water to produce a stock solution of 1 mg/ml.

The sulfamethazine stock solution was diluted further in either a pH 8.0 phosphate buffer (solution B) or distilled water (solution C) to produce 100, 10, 2.5, and 1.0 μg/ml solutions. The frazolidone and amprolium solutions were diluted further in distilled water to produce solutions of the same concentrations.

Working solutions of the 3 drugs were prepared in using distilled water: sulfamethazine and furazolidone, each at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 4.0 μg/ml and amprolium at concentrations of 1, 2, 4, 6, 8, 10, 12 and 14 μg/ml. These preparations were tested in the *B. sterothermophilus* var. *calidolactis* tube diffusion test using replicates of 10 to determine the limits of detection.

Control samples

Distilled water was used as the negative control and a 50 μg/ml solution of each drug was used as a positive control in our search for the limits of detection of each drug using the tube diffusion test. Liver or kidney tissue that was free of any antimicrobial drugs (ie, "blank tissues") was used as the negative control. Then 5 ml samples of blank kidney and liver tissues was injected with a 50 μg/ml solution of each drug that was prepared by adding 5 ml of the 100 μg/ml drug solution or 5 ml of the negative-control solution.

Tissue spiking

Solutions containing 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, and 4.0 μg/ml of sulfamethazine- and furazolidone-contaminated liver and kidney tissues were prepared using appropriate working solutions and blank tissue. Liver tissue was contaminated with sulfamethazine in a pH 8.0 phosphate buffer. Solutions of 1, 2, 4, 6, 8, 10, 12 and 14 μg/ml amprolium-contaminated liver and kidney tissues were prepared for use in the same manner.

Tube test

The tube diffusion was prepared as described by Nouws *et al*. [9]. In brief, it entailed the addition of 2 ml of bromocresol purple (2.5 μg/ml), 2 ml of a *B. stearothermophilius* var *calidolatcis* C953 spore suspension (10^7 spores/ml) and 0.3 ml of trimethoprim (50 μg/ml) to a 100 ml of agar count plate (Difco, USA) at 63°C. The pH of the medium was then adjusted to 8.00 ± 0.02 using 1 M NaOH solution at 63°C. Subsequently, the medium was distributed among the test tubes in 1 ml portions. The tubes were then placed in an upright position, and the agar was allowed to solidify at room temperature. The prepared tubes were used on the same day or kept at 4°C to 5°C for a maximum of 2 days. The detection limit of the tube for each drug was determined by constructing a dose response curve for each. The detection limit was defined as the concentration at which 95% of the test results was positive.

Assay procedure

Each drug was assayed by adding 0.33 ml of the drug solution to the test tubes so that there were 10 tubes for each concentration of the drug. The tubes were allowed to stand for 1 h to allow the drug solution to diffuse into the media. Any drug solution remaining after that time was removed by decanting. The tubes were then covered with an aluminum foil and incubated in a water bath at 63°C for 4.0 to 4.5 h. The results could be read immediately, because the negative control solutions turned from purple to yellow.

The contaminated tissue samples were centrifuged for 5 min to allow tissue debris to fall out of solution. The supernatant was added to the tubes such that each tube containing a replicate of each drug concentration received 0.33 ml. The tubes were allowed to stand for 1 h to allow the supernatant to diffuse into the media. The supernatant remaining after that time was removed by decanting. The tubes were then covered with aluminum foil and incubated in a water bath at 63°C for 4 to 5 h. The results could be read immediately the negative controls turned from purple to yellow.

Results

The results for sulfamethazine-contaminated tissue are shown in Table 1. The tube test indicated a detection limit of 0.5 mg/ml whenthe sulfamethazine solution was prepared in distilled water. A detection limit of 0.7 μg/ml was obtained in liver tissue injected with sulfamethazine. In sulfamethazine-contaminated kidney tissue, the limit of detection was 0.54 μg/ml. A 100% positive response was observed with drug concentrations exceeding 0.5 μg/ml.

The results for furazolidone-contaninated tissue are shown in Table 2. The tube method was able to detect furazolidone in solutions that had been prepared in distilled water at concentrations smaller than 1 μg/ml. A 100% positive response for furazolidone was obtained at a concentration of 0.3 μg/ml , and it was detected in all liver and kidney tissues samples injected with at least 0.35 μg/ml of this drug. Thus, t-test indicated a limit of detection of 0.35 μg/ml for furazolidone and could detect it a concentration as low as of 0.30 μg/ml in all kidney samples into which it had been injected.

The results for amprolium-contaminated tissue are shown in Table 3. A detection limit of 5.7 μg/ml was observed in amprolium solutions prepared with distilled water. A detection limit of 7.8 μg/ml was observed in contaminated liver tissues and 7.6 μg/ml in contaminated kidney tissues.

Table 1. Positive rate of detection for sulfamethazine contaminated liver and kidney tissues (unit: %)

Sulfamethazine concentration (μg/ml)	Solutions in distilled water	Contaminated liver tissue	Contaminated kidney tissue
0	0	0	0
0.1	20	0	40
0.2	50	20	70
0.4	90	50	70
0.6	100	90	100
0.8	100	100	100
1.0	100	100	100
2.0	100	100	100
4.0	100	100	100
50*	100	100	100

Note: The responses are determined from replicates of 10 at each drug concentration.
*: Control

Table 2. Positive rate of detection for furazolidone contaminated liver and kidney tissues (unit: %)

Furazolidone concentration (μg/ml)	Solutions in distilled water	Contaminated liver tissue	Contaminated kidney tissue
0	0	0	0
0.1	70	80	70
0.2	90	80	80
0.4	100	100	100
0.6	100	100	100
0.8	100	100	100
1	100	100	100
2	100	100	100
4	100	100	100
50*	100	100	100

Note: The responses are determined from replicates of 10 at each drug concentration.
*Control

Table 3. Positive rate of detection for amprolium contaminated liver and kidney tissues (unit: %)

Amprolium concentration (μg/ml)	Solutions in distilled water	Contaminated liver tissue	Contaminated kidney tissue
0	0	0	0
1	30	0	40
2	50	0	50
4	60	30	60
6	100	60	70
8	100	100	100
10	100	100	100
12	100	100	100
14	100	100	100
50*	100	100	100

Note: The responses are determined from replicates of 10 at each drug concentration.
*Control

Discussion

Drug residues in food animals being raised for human consumption may pose a public health hazard. Consumer protection can be ensured by screening such animals for residues [1,8]. The presence of antimicrobial residue in foods is of particular concern in low-income countries, because legislation regarding maximum tolerance levels for marketed products is often lacking and violation of the time set to terminate drug therapy occurs regularly [11,13].

The tube test is a microbial inhibitor test in which *B. stearothermophilus* spores are grown in agar with bromocresol purple as the pH indicator. The tubes differ with respect to pH value, supplements and antibiotics [9]. Normal microbial growth causes the pH indicator to change from purple to yellow in solution. Substances that inhibit normal microbial growth cause the color of the pH indicator to remain purple.

B. stearothermophilus has been shown to be sensitive to beta-lactam drugs in milk [10]. The applicability of this method in other foods has not been explored prior to this study. Using the tube diffusion test, we were able to detect sulfamethazine and furazolidone at concentrations smaller than 1 μg/ml. In a previous study using a *B. stearothermophilus* disk plate, sulfamethazine and furazolidone could only be detected at levels of 1μg/ml and above [8].

The type of organism used to find drug residue influences the detection limit. In the this study, sulfamethazine-contaminated kidney tissue appeared to be better suited for detection of the drug residue compared with liver tissue, because the tube diffusion test indicated lower limits of detection in this tissue. Similarly, furazolidone-contaminated kidney tissue had a lower limit of detection compared with contaminated liver tissues.

The higher limits of detection for the coccidiostasts could be attributed to the insensitivity of *B. stearothermophilus* to other compounds [13].The growth of *B. stearothermophilus* is mainly inhibited by beta-lactam drugs and to a lesser extent by other antibiotics [13]. In a other study, a higher sensitivity to salinomycin was reported in chicken tissues using the 4 plate method compared with the disk assay used with *B. stearothermophilus* [3].

When used to validate the STAR protocol in screening or antibiotics residues in milk, *B. stearothermophilus* was found to be sensitive to sulfonamides and beta-lactam drugs [5]. When used as the test organism in the inhibitor test, *B. stearothermophilus* was found to be unsuited for detecting tetracyclines up to the MRL in muscle tissue [12]. A rapid method of detecting sulfonamides in muscle tissue that uses *B. stearothermophilus* has been described [2]. The investigators were also able to use this method to detect sulfamethazine in tissues and solutions at levels of 75 to 150 ppb.

Our findings are thus in agreement with those of other studies in which *B. stearothermophilus* was used to find sulfamethazine and furazolidone at concentrations smallwer than 1 μg/ml. In our study, this organism demonstrated a lack of sensitivity to amprolium, however, which it could only detect at concentrations greater than 5 μg/ml, which exceeded the recommended Codex alimentarius MRL of 1 mg/kg in chicken.

The results of this study suggest that the *B. stearothermophilus* tube test has the potential to useful in detecting anticoccidial residue in poultry. Further studies are recommended to improve its sensitivity to a wider range of drugs at established Codex alimentarius MRLs.

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