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Determination of the depletion of furazolidone residues in chicken tissues using a *Bacillus stearothermophilus* test

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ABSTRACT

This study assessed the applicability of a *Bacillus stearothermophilus* test for detection of the depletion of furazolidone anticoccidial drug residues in chicken tissues. Thirty-three Ross breed chicken were dosed orally with furazolidone (2 mg/kg body weight) daily for 5 days. After the last treatment the birds were sacrificed in groups of three at intervals of 1, 3, 6, 10, 24, 48, 144, 240, 360 and 480 h. Liver, kidney and breast muscle samples were collected and immediately analysed at each interval for furazolidone residues. Serum was separated from the blood collected from jugular veins into bottles containing glass beads and analysed. Supernatant from the homogenates was analysed for the presence or absence of furazolidone residues using the *B. stearothermophilus* test. Semi quantification of furazolidone residues was done by comparing the obtained zones of inhibition on a *Bacillus subtilis* test plates and the zones from calibrated standard curves. Qualitatively the *B. stearothermophilus* test was positive for furazolidone residues up to 480 h post treatment in serum samples, 360 h in liver samples, 480 h in kidney samples and 360 h in muscle samples. Semi quantitatively from the *B. subtilis* test plates, serum samples were observed to have the highest furazolidone concentration of 3.2 μg/ml. The lowest furazolidone concentration was recorded in kidney tissues at 0.21 μg/ml. Kidney and serum tissues appear to be good tissues for testing of furazolidone tissues. The Kidney samples would appear best for post-mortem screening of furazolidone residues while serum could be used antemortem screening of depletion in chicken.

1. Introduction

Anticoccidial drugs are widely used for therapeutic and prophylactic purposes in intensive poultry rearing (Chapman, 2000; Cabadaj, Nagy, Popelka, Mate, & Bugarsky, 2002). Furazolidone, (*N*-(5-nitro-2-furfurylidodene)-3-2-oxazolidone) is an example of an anticoccidial drug that has been used for years for treatment of bacterial and protozoal infections (Nazifi & Asasi, 2001; O'Keefle et al., 2004). While the administration of furazolidone is prohibited in food producing animals in the United States and European countries (Cooper et al., 2005; Vahl, 2005), the drug is commonly used in poultry in the Middle East, Far East and Africa as a coccidiostat.

Furazolidone is rapidly and completely degraded in liver, kidney and muscle tissues of calves (Zuidema et al., 2005). Biotransformation of furazolidone results in formation of protein bound metabolite, 3-amino-2-oxazolidinone (AOZ) which has been shown to have a long residence time in tissues (McCracken, Van Rhijn, & Kennedy, 2005; Cooper et al., 2005). Due to this, a long withdrawal period prior to slaughter of treated animals is usually observed. The presence of anticoccidial residues in poultry meat may have serious toxicological consequences (Zuidema et al., 2005). It is therefore important to avoid these residues in chicken tissues (Bergwertt, 2005).

There are several screening methods which are used to detect the residues in foods (Lee, Lee, & Ryo, 2001). These methods are often expensive and out of reach for low income countries. Hence, affordable screening tests need to be used. The *Bacillus stearothermophilus* tube test is a low cost microbiological test whose potential for screening antibiotic residues in milk has been demonstrated (Shitandi & Kihumbu, 2005). The use of this test had been limited to milk and trials in other food matrixes not been investigated. In a previous study the *Bacillus stearothermophilus var. calidolactis C953* tube test was evaluated for its ability in detecting the residue of selected anticoccidial drugs spiked *in vitro* into poultry, especially sulfamethazine, furazolidone, and amprolium (Shitandi et al., 2006). 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 findings suggested that the *B. stearothermophilus* test could be used to screen for the residue of these three drugs in poultry. The applicability of the *B. stearothermophilus* test *in vivo* with anticoccidial drugs was however uncertain.

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The present study describes *in* vivo studies aimed at assessing applicability of the *B. stearothermophilus* test for detecting the depletion of furazolidone residues in chicken tissues.

2. Materials and methods

2.1. Propagation of B. stearothermophilus var calidolactis C953 spores

A loop of the original *B. stearothermophilus var calidolactis C953* spore suspension (supplied from Rikilt dlo laboratories, the Netherlands) was inoculated into 10 ml of nutrient broth (®Difco, Detroit, MI, USA), and incubated at 55 °C for 24 h. The resulting bacteria suspension was spread on nutrient agar (®Difco, Detroit, MI, USA) supplemented with 31.3 mg/L manganese sulphate (®Merck, Darmstadt, Germany) for improvement of sporulation. The Petri dishes were incubated in plastic bags to prevent drying at 63 °C for 3 days. The spores were harvested using scalpel scraping and physiological saline solution (0.85% NaCl), and the spores washed with physiological saline solution and centrifuged at 2700 rpm for 10 min three times. Finally the suspension was heat treated at 80 °C for 10 min to kill the bacterial cells and kept at 5 °C. The final concentration of the spores was counted on plate count agar (®Difco, Detroit, MI, USA) at 55 °C for 24 h.

2.2. Preparation of the tube test

Plate count agar was melted and kept at 63 °C in a water bath. To 100 ml of the medium, 2 ml bromocresol purple solution (2.5 mg/ml), 0.3 ml of trimethoprim (50 μg/ml) and 2 ml *B. calidolactis* spore suspension (10^7 spores/ml) were pipetted. The final concentration of spores was 2.10^5 spores/ml agar. The final concentration of trimethoprim/ml was 0.15 g/ml medium. The pH of the medium was adjusted to pH 8 ± 0.02 by NaOH solution (1 M at 63 °C). Subsequently the medium was distributed in 1 ml portions in test tubes. The test tubes were placed in an upright position to allow the media to solidify. The prepared test tubes were used on the same day or kept at 5 °C for a maximum of 2 days.

2.3. Preparation of standards

One-hundred milligrams of furazolidone (®Cosmos, Kenya Limited) was weighed and dissolved into a 100 ml flask and dissolved in 100 ml distilled water to produce a solution of 1000 μg/ml (stock A). Stock A was further diluted with distilled water producing solutions of 100 μg/ml (stock B). Liver and kidneys tissues were obtained from birds which were untreated with furazolidone. The liver and kidney samples were homogenized with distilled water at a ratio of 1:2 (tissue:distilled water). The homogenates were centrifuged for 5 min at 3000×g to eliminate tissue debris, known to inhibit diffusion of the drug into the medium. Supernatant from the extracts was used to dilute stock B to produce working solutions standards of, 10, 4, 2, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 μg/ml.

2.4. Preparation of standard curves

Trypticase soy agar medium (®Becton Dickson, USA) was spiked with *Bacillus subtilis* spores at a concentration of 10^5 spores/ml at 50 °C. Eight millilitres of trypticase soy agar medium (®Becton Dickson, USA) was poured into 100 × 15 mm Petri dishes and allowed to solidify. The prepared plates were left on the bench at room temperature for three hours after which wells were bored (7 mm internal diameter). Exactly 0.02 ml of a standard solution prepared was pipetted into each well.

Three wells were bored in each plate and one plate used for each standard. Plates were pre-incubated at room temperature for about 3 h, followed by incubation at 29 °C for 16 h. Zones of inhibition around the wells were measured to the nearest millimeter with a ruler and the mean zone of inhibition calculated for the three replicates of each concentration. The mean zones of inhibition were plotted against tissue homogenate drug concentration to establish the relationship between the tissue standard concentration and zones of inhibition.

A linear relationship between the standard concentrations and zones of inhibition, corrected for the diameter of the punched hole (7 mm) was obtained by addition of a linear trend line to the plotted curve of standard concentrations versus inhibition zones. This line was used to estimate the levels of furazolidone residues in chicken tissues. The correlation coefficient (r) was calculated using a regression analysis computer program based on the least square regression analysis.

2.5. Determination of depletion rates

Thirty-three broiler chickens (Ross Breed) which were all three weeks old, were purchased from a local producer and allowed 3 weeks to acclimatise. The birds had access to water and antibiotic free-broiler feed (the feed was obtained from Ranalo Feeds Nakuru, Kenya). The birds were gavaged and a commercial oral suspension of furazolidone given once daily for 5 days at a dosage of 2 mg/kg of body weight.

After the last treatment the birds were killed in groups of three at intervals of 1, 3, 6, 10, 24, 48, 144, 240, 360 and 480 h. Liver, kidney and breast muscle samples were collected and immediately analysed at each interval for furazolidone residues. Blood was collected from jugular veins into bottles containing glass beads which acted to remove clotting factors from blood. Serum was then separated from the blood and analysed.

The liver, kidney and muscle samples were homogenized with distilled water at a ratio of 1:2 (tissue:distilled water). The homogenates were centrifuged for 5 min at 3000×g to eliminate tissue debris, which appeared to inhibit diffusion of the drug into the medium. Supernatants (0.1 ml) from the homogenates was analysed for residues using the *B. stearothermophilus* while 0.02 ml was used for the *B. subtilis* test plate. Semi quantification of furazolidone residues was done by comparing the obtained zones of inhibition on a *B. subtilis* test plates and the zones from the standard curves.

2.6. Controls

Three birds served as negative controls with furazolidone was not administered to them. The birds were killed at the end of the treatment for the experimental birds and their liver, kidney and

Table 1
Furazolidone residues (mean ± SD) depletion rates in chicken tissues following a 5 day oral administration at a dose of 2 mg/kg

Hour[a]	Furazolidone (ppm)			
	Serum	Liver	Kidney	Muscle
1	3.22 ± 0.38	0.84 ± 0.19	0.67 ± 0.26	0.54 ± 0.09
3	3.14 ± 0.64	0.34 ± 0.07	0.41± 0.06	0.34 ± 0.07
6	2.34 ± 0.57	0.88 ± 0.14	1.31 ± 0.09	0.66 ± 0.19
10	2.72 ± 0.28	0.64 ± 0.00	0.46 ± 0.15	0.55 ± 0.15
24	2.4 0 ± 0.39	1.18 ± 0.39	0.41 ± 0.13	0.50 ± 0.22
48	2.28 ± 0.71	0.92 ± 0.19	0.46 ± 0.15	0.45 ± 0.06
144	2.16 ± 0.14	0.67 ± 0.63	0.41 ± 0.06	0.25 ± 0.21
240	1.91 ± 0.39	0.67 ± 0.16	0.92 ± 0.29	0.00 ± 0.00
360	1.81 ± 0.19	0.45 ± 0.13	0.46 ± 0.15	0.00 ± 0.00
480	0.88 ± 0.22	0.00 ± 0.00	0.21 ± 0.37	0.00 ± 0.00

[a] Hours after withdrawal of treatment.