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Short communication

# Thin-layer chromatographic detection of ivermectin in cattle dung

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**Abstract**

A qualitative method based on thin-layer chromatography (TLC) is described that reliably detects ivermectin as a fluorescent derivative in extracts of cattle dung. The limit of detection (LOD) was ≤40 ng/g of wet dung. These observations were statistically documented and shown visually using digital imagery. Residues were detected in fresh dung deposited by animals treated 10 days previously with a topical dose (500 μg/kg body weight) of ivermectin.

*Keywords:* Ivermectin

## 1. Introduction

Ivermectin (22,23-dihydroavermectin $B_1$) is a broad-spectrum parasiticide with efficacy against a variety of cattle nematodes and arthropods [1]. Regardless of the method of application (oral, subcutaneous, topical), ivermectin is excreted primarily in the dung of the treated animal [2] where it is associated with reductions in numbers of dung-breeding insects [3–5]. This phenomenon is of interest for conflicting reasons. Residues may be beneficial in reducing populations of pestiferous insects breeding in the dung of treated animals [6,7]. However, by reducing overall insect activity, residues also may slow the degradation of dung deposited by treated animals [8–10] and, consequently, reduce available grazing area and soil nitrogen [11–13] in pastures. In either event, methods for the detection of ivermectin residues in cattle dung are of immediate practical value.

Current methods of detecting ivermectin residues in dung use HPLC and fluorescence detection. These methods are extremely sensitive with reported limits of detection (LOD) ranging from 1 to 50 ng ivermectin/g dung wet weight [14–18]. Recently, Taylor et al. [19] reported a method for detecting ivermectin residues in cattle serum using TLC. Although this method was not quantitative, its LOD (1–2 ng/ml) was equal to, or better than, HPLC methods with UV detectors for detecting ivermectin residues in plasma and serum [20–22] and used common laboratory equipment in combination with straightforward extraction, derivatization and cleanup techniques. In the present study, we examined the utility of this TLC method for the detection of ivermectin residues in cattle dung.

## 2. Experimental

### 2.1. Reagents

1-Methylimidazole (>99%) and ammonia (2 *M* solution in methanol) were purchased from Aldrich

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(Milwaukee, WI, USA). Trifluoroacetic anhydride (>99%) was obtained from Pierce (Rockford, IL, USA). Acetonitrile, methyl *tert.*-butyl ether, hexane, acetone and methanol were OmniSolv glass-distilled grade (supplied by BDH, Edmonton, Canada). Distilled-in-glass decane was obtained from Caledon Laboratories (Edmonton, Canada). Water was distilled and further purified by a Barnstead NANOpure II system. Alumina activity grade III (i.e., 100 g alumina plus 7 g water) was prepared using Alumina W200 Neutral for column chromatography obtained from ICN Nutritional Biochemicals (Cleveland, OH, USA). Activated charcoal (Medicinal, Lot No. 20374) was obtained from the J.F. Hartz (Toronto, Canada).

Ivermectin (Lot No. L-640 471-000W109) was supplied as a white solid (≥80% 22,23-dihydroavermectin $B_{1a}$) by Merck (Rahway, NJ, USA). A stock solution of ivermectin (2 mg/ml) was prepared in acetonitrile and stored at −15°C. Working solutions of ivermectin were appropriately prepared by dilution with acetonitrile to give 5, 10, 20, 40 or 100 ng of ivermectin in 25 μl of solution. A commercial pour-on formulation of ivermectin (Ivomec, Lot No. 608822 S) was obtained from MSDAGVET, a division of Merck Agvet (Kirkland, Canada).

### 2.2. Dung samples

For use in detecting ivermectin in spiked samples of dung, fresh dung deposited by untreated heifers was collected and frozen at −40°C until use. Dung was then thawed and samples (1 g wet weight) were spiked with 5, 10, 20, 40 or 100 ng of ivermectin. Unspiked samples of the same dung were used as negative controls.

To test the TLC method using treated cattle, we collected fresh dung deposited by cattle (ca. 460 kg body weight per animal, $n=14$ animals) dosed with a single topical application of ivermectin at the recommended rate (500 μg/kg body weight). Two dung samples (1 g wet weight) were collected from each of four animals on Days 1–10 and Day 14 post-application. No effort was made to collect dung from the same four animals each time. Dung samples were frozen at −40°C after collection until tested for ivermectin residues. Ivermectin is stable in frozen dung for at least 7 weeks [18], and is stable in dung on pastures at summer temperatures for at least 45 days [15]. Animals were housed in covered pens and maintained on a constant diet of barley silage.

### 2.3. Extraction and derivatization of ivermectin from dung

Dung samples were extracted with methanol (5 ml) in test tubes (100×13 mm) equipped with PTFE-lined screw caps. The solution was sonicated (20 min), extracted using a reciprocal shaker (Ames aliquot mixer for 20 min), centrifuged (10 min at 320 *g*), and decanted into a clean tube where it was evaporated to dryness at 40°C under nitrogen (ca. 2 h). The residue was dissolved in acetone (5 ml) and loaded on to a column containing 2 g of alumina in a Pasteur pipet. The reservoir capacity of pipets was increased by attaching the cut end of a pipet to the end of an intact pipet using tygon tubing. The enlarged reservoir increased the head pressure on the column, which reduced processing time. The column was rinsed twice with acetone (2×5 ml) and ivermectin was then eluted with 8–9 ml of methanol. Activated charcoal (2.5 mg) was added to the methanol solution, which was then mixed using a reciprocal shaker (3 min), centrifuged (10 min at 320 *g*), filtered through methanol-washed glass wool and evaporated to dryness under nitrogen (ca. 2 h). The dung residue was dissolved in 200 μl of acetonitrile for derivatization.

Ivermectin was reacted with trifluoroacetic anhydride and 1-methylimidazole as described in detail by Taylor et al. [19], to produce a fluorescent derivative visually detectable under a long-wavelength UV lamp. In brief, 1-methylimidazole–acetonitrile (1:1, v/v) was mixed on ice with the acetonitrile solution containing the dissolved dung residue. Trifluoroacetic anhydride–acetonitrile (1:1, v/v) was subsequently added, and the solution mixed again. After warming to room temperature, a 2 *M* solution of ammonia in methanol was added to the solution, which was then concentrated by evaporation under nitrogen, reconstituted in water, and extracted with hexane.

Reference samples were prepared as outlined above by adding working solutions of ivermectin (5, 10, 20, 40 or 100 ng) to 200 μl of ice-cold acetonitrile.

### 2.4. Thin-layer chromatography

The TLC procedure was described by Taylor et al. [19]. In brief, precoated silica-gel 60 $F_{254}$ plastic sheets (i.e., plates) were developed at room temperature using a mixture of hexane–acetone–decane–methanol (59:30:10:1, v/v). Plates were viewed in a dark room under a 340–380 nm UV lamp (Blak-Ray B100A, Ultraviolet Products, San Gabriel, CA, USA) equipped with a 100-W General Electric mercury light (HR100PSP44-4).

To test the reliability of the TLC method, plates were viewed independently by fifteen participants. Each participant was shown one of 5 plates with each plate having 3 lanes. The centre lane was a reference solution containing the fluorescent derivative from 40 ng of ivermectin. One outside lane was spotted with an extract from dung spiked with 40 ng of ivermectin. The second outside lane was spotted with an extract from dung that did not contain ivermectin. This information was conveyed to each participant, but not the position of the outside lane (left vs. right) containing ivermectin. Each participant then was asked to identify the outside lane containing ivermectin. To reduce the possibility of bias, the position of this lane was randomly determined when plates were prepared, and participants were asked not to discuss their conclusions with others. Results were analyzed using a chi-square test with Yates correction for continuity [23].

## 3. Results and discussion

### 3.1. Sample pretreatment

Coextracted pigments confound the detection of avermectins in plant matrices. In response to this problem, Payne et al. [18] used solid-phase extraction columns and multiple hexane extractions to remove green pigments from dung extracts tested for ivermectin residues. A comparable method was used to remove pigments from vegetable extracts tested for abamectin residues [24]. Fox and Fink [25] used alumina to separate ivermectin from unidentified interferences that were co-extracted into methanol from feed samples. Our own efforts to remove green pigments from dung extracts using $C_{18}$ Sep-Paks as per Sommer et al. [15] were unsuccessful. However, activated charcoal has been previously used to remove chlorophylls from plant extracts [26] and worked well in the current study.

Interference that masked the ivermectin derivative on TLC plates was reduced by treating dung extracts with activated charcoal prior to spotting. Dung extracts were initially green in colour and contained compounds which appeared red under UV light. Adding activated charcoal visibly reduced levels of green pigment in dung extracts, which were likely chlorophylls removed from the dung during extraction with methanol. Methanol is recommended for extracting chlorophylls from plant tissues and chlorophylls appear red under UV light [27].

Fig. 1 documents the removal of plant pigments from dung extracts using activated charcoal. Lanes A and C both were spotted with extracts from dung (1 g wet weight) spiked with 100 ng ivermectin. However, only the extract used in Lane A was treated with activated charcoal prior to spotting. Whereas the ivermectin derivative was clearly seen in Lane A, it was largely masked in Lane C by dark bands associated with plant pigments. Lane B was spotted with a reference solution of 100 ng ivermectin (after derivatization).

Fig. 2 shows that other compounds extracted from dung are unlikely to be mistaken for the ivermectin derivative. Lanes A and C both were spotted with dung extracts treated with activated charcoal. However, dung extracted for Lane A was first spiked with 100 ng ivermectin. Whereas the ivermectin derivative was clearly seen in Lane A, there was no corresponding compound with a similar $R_F$ in Lane C. Lane B was spotted with a reference solution of 100 ng ivermectin (after derivatization). Although not apparent in the monochromic Fig. 2, the characteristic whitish-blue fluorescence of the ivermectin derivative under UV light further distinguishes it from other compounds, which typically appeared red.

We cannot explain the apparent selectivity of charcoal in adsorbing pigments from extracts of ivermectin-containing dung, but observed that recoveries of ivermectin seemed to decrease when the amount exceeded 2.5 mg. Hence, the amount of charcoal used must be optimized to remove plant pigments without excessively adsorbing the ivermectin. Because the level of plant pigments co-extracted

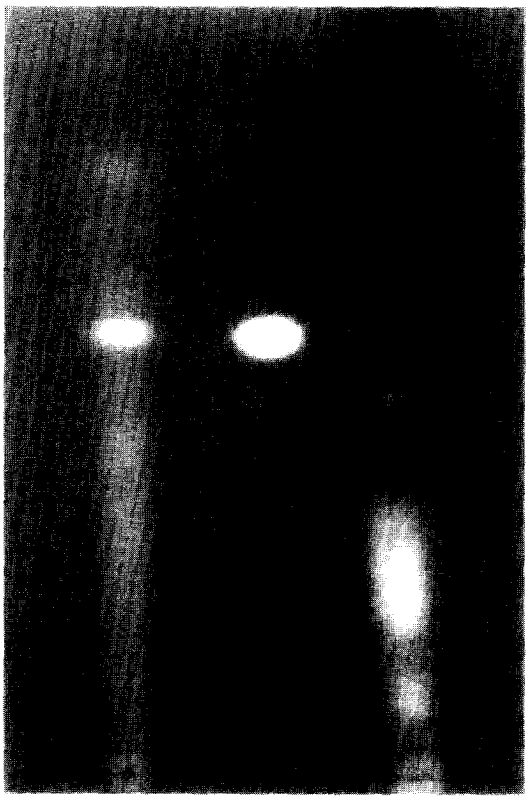


Fig. 1. TLC bands obtained for (A) a solution treated with activated charcoal after extraction from dung spiked with 100 ng ivermectin, (B) a reference solution of 100 ng ivermectin and (C) a solution not treated with activated charcoal after extraction from dung spiked with 100 ng ivermectin. All samples were derivatized.

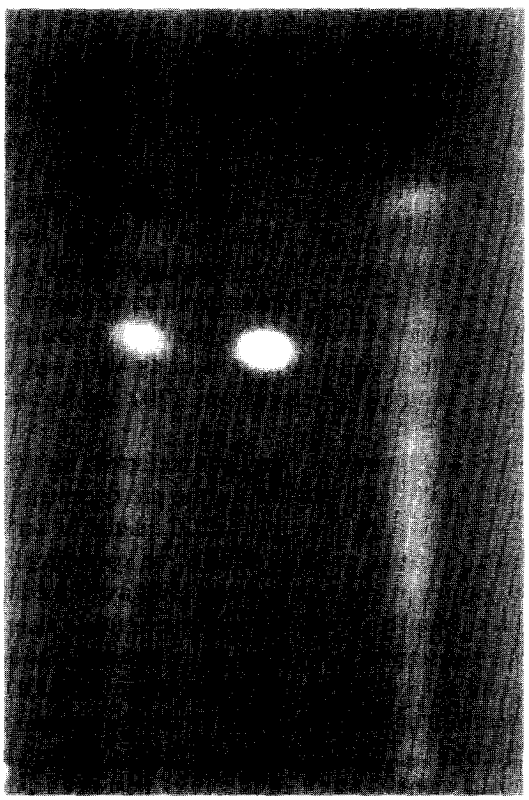


Fig. 2. TLC bands obtained for (A) a solution treated with activated charcoal after extraction from dung spiked with 100 ng ivermectin, (B) a reference solution of 100 ng ivermectin and (C) a solution treated with activated charcoal after extraction from unspiked dung. All samples were derivatized.

from dung will vary with the diet of the animal, the amount of charcoal to be added may require individual optimization.

### 3.2. LOD

The LOD of the TLC method described in the present study was 40 ng/g and as low as 10 ng/g in some samples (Table 1). Confirmation was obtained when each of fifteen participants correctly identified which of two lanes had been spotted with an extract from dung spiked with ivermectin (40 ng). The probability of this result occurring by random chance was <0.001 ($X^2=13.07$; $X^2_{0.001,\ v=1}=10.83$).

Table 1
TLC detection of ivermectin in dung (1 g wet weight) spiked with ivermectin

| Ivermectin added (ng) | Sample number | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 100 | +[a] | + | + | + | + |
| 40 | + | + | + | + | + |
| 20 | + | + | + | −[b] | − |
| 10 | + | + | + | − | − |
| 5 | − | − | − | − | − |

[a] Detectable.
[b] Undetectable.

### 3.3. Application

Ivermectin was detected in the dung of at least some animals for up to 10 days post-treatment with detection generally obtained for up to 7 days post-treatment (Table 2). Using HPLC, Sommer et al. [15] detected ivermectin residues in the dung of cattle treated 5 days previously with a topical application of ivermectin (500 μg/kg), but not in the dung deposited 13 days after application. Ivermectin residues have been detected in the dung deposited by cattle treated 12–14 days previously with subcutaneous injections of ivermectin (200 μg/kg body weight) [15,17,28].

The TLC method may be preferred for rapid screening of dung samples for the presence/absence of ivermectin residues, but HPLC methods will be required if these residues need to be quantified. We note, however, that the greater sensitivity of the latter method may be of more academic interest than of practical significance because ivermectin residues have biological activity at levels below the LOD of either TLC or HPLC methods. Neither technique can currently detect the presence of ivermectin residues in fresh dung more than 2 weeks post-application, but the horn fly, *Haematobia irritans*, is reportedly unable to develop in dung deposited by cattle treated up to 35 days previously with ivermectin [7]. Further examples of reduced insect emergence from dung deposited by animals treated several weeks previously with ivermectin are reported in Strong [4].

Table 2
TLC detection of ivermectin in the dung of treated cattle

| Day post-treatment | Animal | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Day 1 | +/+[a] | +/+ | −/−[b] | −/− |
| Day 2 | +/+ | +/+ | +/+ | −/− |
| Day 3 | +/+ | +/+ | +/+ | +/+ |
| Day 4 | +/+ | +/+ | +/+ | +/+ |
| Day 5 | +/+ | +/+ | +/+ | +/+ |
| Day 6 | +/+ | +/+ | +/+ | −/− |
| Day 7 | +/+ | +/+ | +/+ | −/− |
| Day 8 | +/+ | +/+ | −/− | −/− |
| Day 9 | +/−[c] | +/− | +/− | −/− |
| Day 10 | +/− | +/+ | +/+ | −/− |
| Day 14[d] | −/− | −/− | | |

[a] Detectable in each of two samples tested.
[b] Undetectable in each of two samples tested.
[c] Detectable in one of two samples tested.
[d] Only two animals tested.

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