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Interferences on microbial inhibitor tests related to ivermectin treatment in lactating dairy goats

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Short title: Ivermectin and microbial inhibitor tests

This research communication reports interferences related to the administration of ivermectin in lactating dairy goats on the response of microbial tests for screening antibiotics in milk. Twenty-eight Murciano-Granadina goats, naturally infested with *Sarcoptes scabiei* var. *caprae*, were treated with a subcutaneous injection of ivermectin (200 µg/Kg b.w.). To prevent re-infestation, a second dose was applied seven days later. Individual milk samples were collected, daily, up to fifteen days post-treatment. Milk samples were analysed by microbial inhibitor tests (BRT MRL, Delvotest SP-NT MCS and Eclipse 100) and ivermectin residues were quantified by HPLC. A large number of positive results were obtained for all the microbial tests, especially on the first day after treatment (BRT MRL= 46.4%; Delvotest SP-NT MCS= 14.3%; and Eclipse 100= 17.8%). However, the highest concentration of drug residues in milk (24.3 ng/ml) was detected on the tenth day after treatment, when positive outcomes were relatively lower (BRT MRL= 17.8%; Delvotest SP-NT MCS= 10.7%; and Eclipse 100= 7.4%). Results herein suggest that factors related to the ivermectin treatment other than drug residues in milk, or alterations produced by the parasitic disease itself affecting the immune response of animals, could be the cause of false-positive results in microbial tests. It can be concluded that the application of ivermectin in dairy goats infested

40 with sarcoptes mange during lactation produces persistent drug residues in milk, and could
41 also cause false-positive results in microbial tests for screening antibiotics.

42 **Key words:** goat milk, ivermectin, microbial inhibitor tests

43 Monitoring the presence of antibiotic residues above the maximum residue limits (MRLs) in
44 foodstuffs of animal origin is mandatory in the European Union (Regulation (EC) No
45 853/2004). Microbial inhibitor tests are widely used for screening antibiotics in raw milk as
46 they are relatively inexpensive, user-friendly and have a high sample throughput. However,
47 they are non-specific and several factors related to milk composition (natural inhibitors or
48 somatic cell counts, among others), have been associated with the occurrence of false
49 positive results (Andrew, 2001; Althaus *et al*., 2003). Also, the presence of contaminants in
50 milk related to regular farming practices (detergents, disinfectants or antiparasitic
51 substances) may interfere with the microbial test responses (Romero *et al*., 2014; 2015).
52 However, available information about this aspect is rather limited.

53 Antiparastic treatments are often applied to prevent and control various parasitic diseases.
54 Sarcoptic mange, caused by the ectoparasite *Sarcoptes scabiei* var. *caprae,* is a common
55 parasitic disease in caprine livestock causing considerable economic losses as milk
56 production is decreased (Wallton & Currie, 2007). Additionally, it can have repercussions on
57 public health because of its potential transmission; therefore the antiparasitic treatment of
58 goats infested with sarcoptic mange becomes necessary.

59 Ivermectin (IVM) is one of the most effective drugs against scabies and other parasitic
60 diseases (McKellar & Benchaoui, 1996). However, its use is not authorised in animals from
61 which milk is produced for human consumption (Commission Regulation (EU) No 37/2010)
62 due to the high persistence of IVM residues in milk (Alvinerie *et al*., 1993; Fernanda *et al*.,
63 2004). Accordingly, the MRL for this substance in milk has not been established and,

64 therefore, the health impact of the illegal use of this drug in lactating dairy goats in unknown.
65 The European Medicine Agency (EMA, 2014) indicates that the Codex Alimentarius
66 establishes an MRL for IVM residues in milk at a concentration of 10 μg/kg, although more
67 studies about its potential effects are necessary to set the MRL in the European legislation.

68 The aim of this study was to evaluate the response of microbial inhibitor tests in milk from
69 dairy goats treated with IVM during lactation.

70 **Material and methods**

71 ***Animals, antiparasitic treatment and milk sampling***

72 Twenty-eight Murciano-Granadina goats in the fifth month of lactation (body weight 55-65
73 kg) from the Institute of Animal Science and Technology of Universitat Politècnica de
74 València (UPV, Valencia, Spain) were used. Animal procedures and management were
75 approved by the Ethics Committee of UPV.

76 Animals naturally infested with sarcoptic mange were treated with Ivomec® (Merial
77 Laboratorios S.A., Madrid, Spain; IVM 1.0%) by subcutaneous injection following the
78 manufacturer's recommendations for ovine species. The dose administered was 0.2 ml/10 kg
79 b.w. (200 μg of IVM per kg b.w.). To prevent re-infestation, a second dose was applied
80 seven days after the first treatment.

81 The animals were milked in a milking parlour (08:00 a.m.) and the sampling was carried out
82 for 15 days after the first administration. Milk samples were analysed by microbial screening
83 tests and aliquots were frozen (-80 °C) for ivermectin quantification by HPLC.

84 ***Microbial inhibitor tests***

85 Goat's milk samples were analysed in triplicate by microbial inhibitor tests: BRT MRL
86 (Analytik in MilchProduktions-und Vertriebs-GmbH, Munich, Germany), Delvotest SP-NT

MCS (DSM Food Specialties, Delft, Netherlands) and Eclipse 100 (ZEULAB S.L., Zaragoza, Spain). The tests were used according to each manufacturer's instructions. A negative control (antimicrobial-free goat milk) and a positive control (antimicrobial-free goat milk spiked with 4 μg/kg of benzylpenicillin) were included on each plate. Visual interpretation of the test results was carried out by three technicians assessing the colour change in the medium; classifying milk samples as "positive" when the colour remained purple/blue and "negative" when the colour changed to yellow.

Ivermectin quantification

Extraction and derivatisation procedures to quantify IVM were carried out following the technique described by Imperiale *et al*. (2004). IVM was analysed using a Waters HPLC system (Milford, MA, USA), which included two pumps (Mod. 515, Waters), an autosampler (Mod. 717 plus, Waters), and a fluorescence detector (Mod. 474, Waters) set at an excitation and emission wavelength of 365 and 475 nm, respectively. The separation in isocratic form was achieved using a Nova-Pack C18 column (Waters, 4 μm, 3.9x150 mm) coupled with a guard column Nova-Pack C18 (3.9x2 mm) at 35 ºC. The mobile phase consisted of a methanol/water mix (97:3, v/v) using a flow rate of 1.8 ml/min. IVM was identified by comparison with retention time of a reference standard and IVM concentration was calculated using an internal standard (abamectin, ABM). The standards of IVM (I8898) and ABM (31732) were provided by Sigma-Aldrich (Sigma-Aldrich Química, S.A., Madrid, Spain). The detection limit (LOD) and the quantification limit (LOQ) calculated for IVM were 0.13 ng/ml and 0.43 ng/ml, respectively. Average ivermectin recovery was 92.7% and the coefficient of variation (CV) values were ranging 4.8-8.9%.

Statistical analysis

The non-parametric Kruskal-Wallis test (K-W) was applied to analyse statistical differences (P <0.05) of IVM concentrations with respect to days post-treatment. Subsequently, the Bonferroni test was performed to establish the differences along the days post-treatment. Statgraphics Centurion XVI software (16.1.03) (StatPoint Technologies, Inc., Warrenton, VA, USA) was used for statistical analyses.

Results and discussion

As shown in Table 1, a large number of positive outcomes were obtained when antibiotic-free milk samples from goats treated with IVM were analyzed. Although the occurrence of positive results was observed throughout most of the experimental period (up to 14 days), the highest percentage was recorded, in all cases, on the first day post-treatment (BRT MRL= 46.4%; Delvotest SP-NT MCS= 14.3%; and Eclipse 100= 17.8%), the BRT MRL being the most affected test throughout the experimental period. These results could be related to the different composition of the culture medium of the tests (BRT MRL: Mueller-Hinton agar and black brilliant as redox indicator; Delvotest SP-NT MCS and Eclipse 100: Plate count agar and bromocresol purple as pH indicator).

Microbial screening tests present a lower false-positive rate (0.6-4.3%) in milk from individual goats (Beltrán *et al*., 2015) and, therefore, results obtained herein suggest that the administration of IVM may be related to these interferences.

A significant increase ($p<0.05$) of the concentration of IVM residues in goat's milk along the three days following the application of the drug (Figure 1) was observed. However, the concentration of IVM ranged from 8.9 to 24.3 ng/ml, values rather lower than the inhibitory concentration producing 5% of positive results in microbial tests (IC_5) calculated by Romero *et al.* (2015) in an *in vitro* study (BRT MRL= 41 mg/l; Delvotest SP-NT MCS= 83 mg/l and Eclipse 100= 109 mg/l). In addition, the highest occurrence of positive results was not related

134 to an elevated concentration of IVM residues in milk, suggesting that factors associated to
135 drug administration other than residues in milk could be the cause of the interferences found.
136 Thus, for example, IVM has been evaluated as immunomodulatory showing a favourable
137 potential use as positive modulator (Stankiewwicz *et al*., 1995; Sajid *et al*., 2007). Moreover,
138 several studies indicate that sarcoptes mange does not only produce visible dermatological
139 symptoms in mite-infested goats, but is also associated with physiological changes such as
140 highest concentration of biochemical indicators of oxidative stress in the blood (Ujival Dey,
141 2010), high total protein levels, γ-globulin and IgG in the serum plasma (Lastras *et al*., 2000),
142 and increased pro-inflammatory cytokine levels (Mullins *et al.* 2009) as well as high levels of
143 acute phase proteins in the blood (Rahman *et al*., 2010). The potential changes in the
144 immunological system of the animals as a result of the IVM activity or the immune response
145 of goats to combat the parasitic disease, could result in the presence of inhibitory substances
146 in milk capable of interfering with the response of microbial inhibitor tests for screening
147 antibiotics.

148 It can be concluded that the illegal application of IVM in lactating dairy goats infested with
149 sarcoptes mange produces persistent drug residues in milk and could also cause false-positive
150 results in microbial inhibitor tests for screening antibiotics.

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Table 1. Positive results in microbial inhibitor tests in milk from goats (n= 28) treated with ivermectin

Days post-treatment	Positive results (%)		
	BRT MRL	Delvotest SP- NT MCS	Eclipse 100
1	46.4	14.3	17.8
2	32.1	7.4	7.4
3	25.0	3.6	10.7
4	21.4	7.4	3.6
5	28.6	10.7	10.7
6	7.4	10.7	7.4
7	3.6	10.7	3.6
8	35.7	10.7	3.6
9	32.1	7.4	7.4
10	17.8	10.7	7.4
11	10.7	7.4	3.6
12	14.3	0.0	3.6
13	14.3	0.0	0.0
14	10.7	0.0	0.0
15	0.0	0.0	0.0
Average	20.0	6.6	5.7

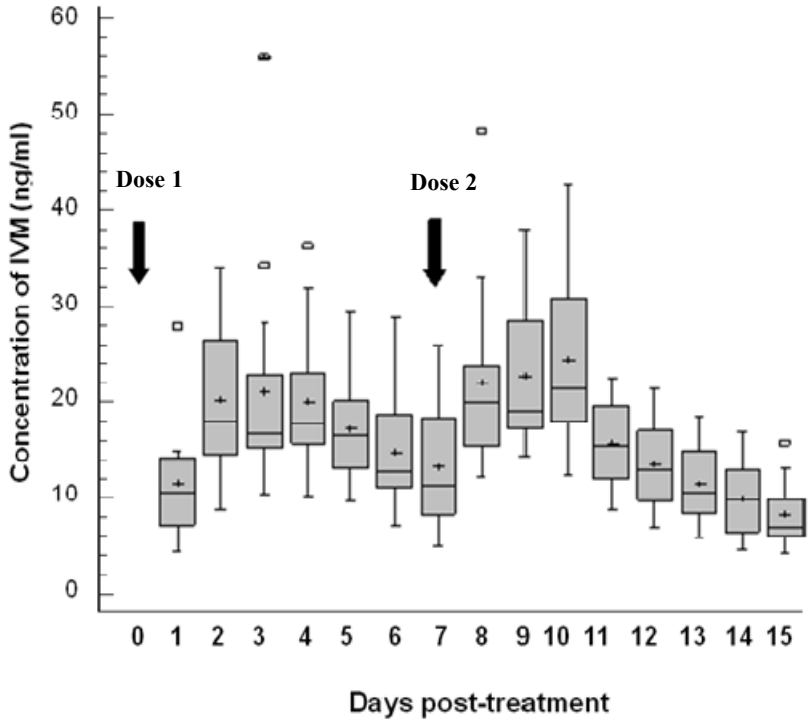

Figure 1. Box and whisker plots illustrating the concentration of ivermectin (IVM) in goat milk on the post-treatment days. The plots show the 25th and 75th percentiles (box), median (—), mean (+), 10th and 90th percentiles (whisker bars) and outliers (□).