Survey of pyrethroid, macrocyclic lactone and antibacterial residues in bulk milk tank from Minas Gerais State, Brazil[1]

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ABSTRACT.- Picinin L.C.A., Toaldo I.M., Hoff R.B., Souza F.N., Leite M.O., Fonseca L.M., Diniz S.A., Silva M.X., Cerqueira M.M.O.P. & Bordignon-Luiz M.T. 2017. **Survey of pyrethroid, macrocyclic lactone and antibacterial residues in bulk milk tank in Minas Gerais State, Brazil**. *Pesquisa Veterinária Brasileira 37(2):97-104.* Departamento de Ciência e Tecnologia de Alimentos, Universidade Federal de Santa Catarina, Rodovia Admar Gonzaga 1346, Bairro Itacorubi, Florianópolis, SC 88034-001, Brazil. E-mail: lidia.picinin@gmail.com

A survey of veterinary drug residues in bulk milk tank from Minas Gerais State, Brazil, was carried out through a broad scope analysis. Here, 132 raw milk samples were collected at 45 dairy farms in Minas Gerais from August 2009 to February 2010, and analyzed for 42 analytes, comprising pyrethroids, macrocyclic lactones and antibacterials, using liquid chromatography coupled with mass spectrometry in tandem mode and gas chromatography with electron capture detection. Within all milk samples, at least one veterinary drug residue was identified in 40 milk samples (30.30%) by confirmatory tests, whereas 16 samples (12.12%) showed the presence of at least two residues. With regard to the Brazilian maximum residue levels, 11 milk samples (8.33%) were non-compliant according to Brazilian Legislation. The veterinary drugs detected in the non-compliant milk samples include penicillin V (one sample), abamectin (one sample) and cypermethrin (nine samples). Furthermore, the antibacterial screening methods failed to identify most of the positive samples that were detected by confirmatory tests, leading to a large discrepancy between the screening and confirmatory antimicrobial tests. Thus, the present study indicated that the veterinary drugs residues still represents a great concern for the milk production chain.

INDEX-TERMS: Veterinary drug, pesticide, anthelmintic, antibiotic, raw milk.

RESUMO.- [**Resíduos de piretróides, lactonas macrocíclicas e antimicrobianos em amostras de leite de tanque no estado de Minas Gerais.**] Avaliou-se a presença de 42 analitos, incluindo piretróides, lactonas macrocíclicas e antimicrobianos em 132 amostras de leite de tanque proveniente de 45 propriedades leiteiras localizadas no Estado de Minas Gerais. Para tal, utilizou-se a cromatografia líquida acoplada a espectrofotometria de massas tandem e cromatografia gasosa com detector com captura de elétrons. Dentre todas as amostras de leite, 40 (30,30%) amostras de leite de tanque apresentaram a presença de pelo menos um analito, enquanto 16 amostras (12,12%) de leite demonstraram a presença de pelo menos dois analitos. Considerando os limites estabelecidos pela legislação brasileira, 11 amostras de leite (8,33%) seriam consideradas como não conforme. Ademais, os testes de triagem para detecção de antimicrobianos no leite não conseguiram identificar a maioria das amostras positivas nos testes confirmatórios, levando a grande discrepância entre estes testes. Desta forma, os resultados do presente estudo indicam que os períodos de descarte do leite, especialmente para piretróides,

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não foram plenamente respeitados por todos os produtores de leite. Além disto, uma discrepância entre os resultados dos testes confirmatórios e os testes de triagem foi observada.

TERMOS DE INDEXAÇÃO: Medicamentos veterinários, pesticidas, antelmínticos, antibióticos, leite cru.

INTRODUCTION

Milk is one of the most consumed foods worldwide and its production has shown a continuous increase (Oliveira et al. 2010). Brazil is one of the largest producers with with around 35.200 ton of milk per year. Minas Gerais State, situated in the southeast of the country, is the major milk producer, and it is responsible for approximately 27% of the national milk production (IBGE 2014).

The use of veterinary drugs in food-producing animals has resulted in healthier, more productive animals, lower disease incidence and reduced morbidity and mortality in humans and animals, and production of abundant quantities of nutritious, high-quality, and low-cost food for human consumption. In spite of these benefits, there is considerable concern from public health, food safety, and regulatory perspectives about the use of veterinary drugs in food-producing animals. Over the last two decades, development of antimicrobial resistance resulting from the use of veterinary drugs that could also impact treatment of diseases affecting the human population that has become a significant global public health concern (Oliver et al. 2011).

As milk is an essential food, the maximum residue levels (MRLs) for veterinary drugs are markedly lower in milk than in other food matrices, suggesting a larger consumption and recommendation for young children and older people (Bilandžić et al. 2011). Furthermore, it is noteworthy that some veterinary drug residues are stable during storage, remain in the fermentation process and can become concentrated throughout the food chain (Whelan et al. 2010), strengthening the importance of an investigation of veterinary drug residues in raw milk that is the initial vehicle for the whole milk food chain (Nebot et al. 2012). Thus, in attempt to protect human health and ensure a high-quality product, several governmental authorities and international committees (Brasil 1999, European Commission 2010, FAO/WHO 2011) have set MRLs for various veterinary drug residues.

Antimicrobials, macrocyclic lactones and pyrethroids are the most widely used veterinary drugs in dairy cattle management for disease therapy and control of bacterial and parasitic infections (Bilandžić et al. 2011, Toaldo et al. 2012). Therefore, most of these studies that have assessed the presence of veterinary drug residues in bulk tank milk in Brazil have used rapid screening methods (Cerqueira et al. 2014, Trombete et al. 2014) or analyzed a single or limited class of active compounds (Ciscato et al. 2002, Hernandes et al. 2009, Pacheco-Silva et al. 2014). Furthermore, several authors have noted the problem of false-positive results in certain microbiological inhibitor tests. One possible cause for false-positive results in these screening tests is the presence of indigenous antimicrobial agents and free fatty acids (Moats et al. 1995, Molina et al. 2003, Le Breton et al. 2007). Although these screening methods (i.e. immunological or microbial inhibition assays) are widely used to detect the presence of veterinary drugs in foods, more accurate chromatographic methods coupled with highly specific and sensible detection systems, such as tandem mass spectrometry, are required to identify, confirm the presence of and quantify these compounds (Molina et al. 2003, Samanidou & Nisyriou 2008, Toaldo et al. 2012) because they provide full or complementary information enabling the unequivocal identification and quantification of the analyte at levels of interest (Samanidou & Nisyriou 2008).

In order to accomplish an effective and feasible approach regarding chemical residues in Brazilian milk, we present a broad scope analysis of veterinary drugs using combined analytical methods based on liquid chromatography coupled to tandem mass spectrometry and gas chromatography with electron capture detection. Thus, this work was carried out to investigate the presence of 42 analytes, comprising antibacterials, pyrethroids and macrocyclic lactones in bulk milk tank samples from Minas Gerais State, Brazil. Furthermore, the results of three antibacterial screening tests were compared with the confirmatory tests.

MATERIALS AND METHODS

Milk samples

Raw milk samples were collected from August 2009 to February 2010 from 45 dairy farms in the mesoregion Metropolitana de Belo Horizonte of Minas Gerais State, Brazil. A total of 132 samples were collected from bulk milk tanks after milk homogenization. Given the extensive sampling that was required and the costs of the analyses, dairy herds were randomly selected based on the list of farms from the most important dairy plant of the Minas Gerais State by considering their levels of daily milk production. Twenty-seven (60%) of the dairy farms had production levels of ≤500 L/day, five (11.11%) had production levels of between 501 and 1,000 L/day and 13 (28.89%) had levels of >1,000 L/day.

Veterinary drug residues

The milk samples were stored frozen at -18°C until the quantitative analysis of veterinary drug residues was carried out. In the present study, a milk sample was assumed to be positive if any analytical residue was detected, regardless of the drug concentration. Furthermore, the sample was considered to be non-compliant if a drug was present at a concentration above the Brazilian MRL (BMRL) value (Brasil 1999).

Chemicals and standard solutions

Pesticides. The analytical standards γ-cyhalothrin (γCYH), λ-cyhalothrin (λCYH), cyfluthrin (CYF), cypermethrin (CYP), deltamethrin (DEL), fenvalerate (FEV) and permethrin (PER), which all possessed levels of 95% certified purity, were obtained from Sigma-Aldrich (St. Louis, MO, USA). For the pyrethroids, standard stock solutions of 1 mg mL^{-1} were prepared individually in acetonitrile (ACN) and stored in a refrigerator at 8°C. Working standard solutions containing all of the pyrethroids were diluted with ACN to give calibration solutions ranging from 5.0ng mL^{-1} to 80.0ng mL^{-1}. All of the standard solutions were stored at -20°C.

Macrocyclic lactones and antibacterials. The analytical standards penicillin G (PNG), penicillin V (PNV), ceftiofur (CFT), cloxacillin (CLX), dicloxacillin (DCX), oxacillin (OXA), chlorte-

tracycline (CTC), doxycycline (DOX), tetracycline (TC), oxytetracycline (OTC), oxolinic acid (OXO), nalidixic acid (NALIDIX), flumequine (FLU), difloxacin (DIFLO), ciprofloxacin (CIPRO), enrofloxacin (ENRO), norfloxacin (NOR), sarafloxacin (SARA), trimethoprim (TMP), sulfadimethoxine (SDMX), sulfaquinoxaline (SQX), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridine (SPY), sulfamethoxazole (SMA), sulfamethazine (SMZ), sulfachloropyridazine (SCP), sulfisoxazole (SFX), sulfadoxine (SDX), sulfamerazine (SMR), ivermectin (IVR), eprinomectin (EPR), emamectin (EMA), doramectin (DOR), abamectin (ABA) and moxidectin (MOX) were purchased from Sigma Aldrich (St. Louis, MO, USA) as Vetranal™ analytical-grade standards (> 95% certified purity).

For the β-lactams, stock standard solutions were prepared by dissolving all of the compounds in polypropylene glycol 3000 in acetate buffer (pH = 4.5) at concentrations ranging from 0.5 to 3.75mg mL^{-1}. For the tetracyclines, sulfonamides and fluoroquinolones, a stock standard solution of each compound was prepared by dissolving 10mg of the analytical standard in 10mL of the appropriate solvent (acetonitrile for the tetracyclines and sulfonamides, methanol with a couple of drops (~2) of 1 M NaOH for the fluoroquinolones and methanol for the TMP). Aliquots of each stock solution were diluted to obtain final concentrations of 10μg mL^{-1} and 1μg mL^{-1}, which were then stored at -20°C.

For the macrocyclic lactones, individual stock solutions of 1.0mg mL^{-1} were prepared by dissolving 10mg of each standard in 10mL of ACN. The working solutions were prepared by combining aliquots of each stock solution in ACN to obtain final concentrations of 1μg mL^{-1} for ABA, IVR and MOX, 1.5μg mL^{-1} for DOR and 2.0μg mL^{-1} for EPR in ACN. EMA was used as an internal standard, and its working solution was prepared at 1.0μg mL^{-1} in ACN.

All of the reagents were of analytical grade unless otherwise indicated. Acetic acid, trichloroacetic acid, ACN (MeCN, MS-grade), ethanol and methanol (MeOH, MS-grade) were purchased from Merck (Darmstadt, Germany). Formic acid and triethylamine were supplied by J.T. Baker (Phillipsburg, NJ, USA). Deionized ultra-pure water (<18.2 MΩ cm resistivity) was obtained from the Milli-Q SP Water System (Millipore, Bedford, MA, US). Disodium ethylenediamine tetraacetate (Na_2EDTA) was obtained from Sigma. Ammonium acetate (analytical grade) was obtained from Mallinckrodt-Baker (Phillipsburg, NJ, USA).

Equipment and chromatographic conditions

Gas Chromatograph with Electron Capture Detector (GC--ECD) for pyrethroids. The analytical system that was used for this study was a Trace GC Ultra gas chromatograph (Thermo) equipped with a splitless injection system, AI 3000 autosampler and ^{63}Ni electron-capture detector fitted with either an OV-5 fused silica capillary column (15.0m × 0.25mm × 0.1μm film thickness) or an OV-35 fused silica capillary column (15.0m × 0.25mm × 0.25μm film thickness). The programmed temperature cycle for the OV-5 column was as follows: 100°C (1 min) to 250°C at a rate of 20°C min^{-1}, followed by an increase to 260°C (3 min) at 5°C min^{-1} and finally, to 330°C (5 min) at 20°C min^{-1}. The conditions for pyrethroid separation using the OV-35 column were similar to those used for the OV-5 column. The injector and detector were programmed at 240°C and 340°C respectively. Nitrogen at 1.0mL min^{-1} was used as the carrier gas. One microliter of each sample was injected in splitless mode. The identification of the peaks was based on comparisons of the retention times of compounds in the standard solutions.

Liquid Chromatography coupled with Mass Spectrometry in tandem mode (LC-MS/MS) multi-residue screening. Prior to the quantitative analysis, a LC-MS/MS multi-residue screening method was applied for the qualitative analysis of fluoroquinolones, tetracyclines, sulfonamides and trimethoprim in milk. The LC-MS/MS screening analysis was performed using a Waters Alliance 2795 system (Milford, USA) coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, USA) with an electrospray source. The multi-residue separation was performed in a Waters Symmetry C18 LC column (75 mm × 4.6 mm, 3.5 μm). A Phenomenex C18 column (4.0mm × 3.0mm) was used as the guard column. A gradient elution program with solvent A (aqueous solution of 0.1% formic acid) and solvent B (ACN with 0.1% formic acid) was used. The chromatographic conditions and mass spectrometry parameters were chosen according to Bittencourt et al. (2011). The detection was performed in multiple reaction monitoring mode, and the 2*m/z* transitions were monitored for each analyte. The *m/z* transitions that were associated with the retention times of the analytes provided confirmatory data, fulfilling the requirements for a confirmatory analysis. For positive results, quantitative methods were applied to determine the analyte concentrations.

LC-MS/MS (macrocyclic lactones and antibacterials). The LC system used in this study was an Agilent 1100 series LC (Santa Clara, CA, USA) with a quaternary pump, a vacuum degasser and an autosampler, which was coupled with an API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an electrospray ionization interface. The chromatographic separation of the β-lactams was performed using a Phenomenex Synergy® C18 analytical column (150 × 3.0mm, 4.0μm) that was preceded by a security guard C18 column (4.0 × 3.0mm, 5μm) (Phenomenex). A binary mobile phase was used with a flow of 500μL min^{-1} for a total run time of 12 min. Mobile phase component A was an aqueous solution of 0.1% formic acid, and component B was ACN with 0.1% formic acid. The detailed chromatographic and mass spectrometry conditions are provided in Jank et al. (2011).

For the tetracyclines, separation was performed on an Xterra C18 column (2.1 mm × 100 mm, 1.7 μm) preceded by a security guard C18 column (4.0 × 3.0 mm, 5 μm) (Phenomenex). The mobile phase was applied in gradient mode, using water with 0.05% formic acid (solvent A) and ACN with 0.05% formic acid (solvent B).

The separation of the fluoroquinolones was performed on a Waters Symmetry C18 column (75mm × 4.6mm, 3.5μm) with a Phenomenex C18 (4.0mm × 3.0mm) guard column via a gradient elution process, using an aqueous solution of 0.1% formic acid as solvent A and ACN with 0.1% formic acid as solvent B. For the sulfonamides and trimethoprim, the separation was performed on a Zorbax® XDB C18 column (150 × 4.6mm, 5μm) (Agilent). The mobile phase was composed of 10mM ammonium acetate that was acidified with 0.01% acetic acid (solvent A) and methanol (solvent B) using a gradient system. The detailed parameters are described in Hoff et al. (2009).

The separation of the macrocyclic lactones was performed on a Luna C18 column (150mm × 2.1mm, 5μm) that was preceded by a guard column (4mm × 3mm, 5μm) consisting of the same packing material (Phenomenex, Torrance, CA, USA). All of the analytes were eluted after 4 min using an isocratic mobile phase consisting of 50 mM ammonium acetate buffer (pH 5):ACN (5:95, v/v) at 0.2mL min^{-1}. The electrospray voltage and source temperature were set at 4500 V and 500°C, respectively.

Sample preparation procedure

Pyrethroids. The milk samples (5.0mL) were extracted with 10mL of ACN in 50mL polypropylene tubes, shaken for 20 min and then incubated in a freezer at -20°C for approximately 12 hours. After this period, the organic phase, which contained an organic solvent with the extracted compounds, remained as a liquid, whereas the aqueous phase and lipidic fraction of the milk froze. The supernatants were passed through previously cooled

glass wool containing anhydrous sodium sulfate (2.0g). The extracts were concentrated until dry in a water bath (38-40°C) under gentle nitrogen flow. The residues were dissolved in 1.0mL of ACN and analyzed by GC-ECD.

Qualitative determination of fluoroquinolones, tetracyclines, sulfonamides and trimethoprim. Aliquots (500µL) of the raw milk samples were extracted in microcentrifuge tubes (1.5mL) by the addition of 20µL of 20mM EDTA and 200µL of acidified ethanol (3% acetic acid). The samples were vortexed for approximately 15s and then centrifuged at 10,000*g.* Aliquots of the supernatants were diluted with water:ACN (1:1, v/v, both with 0.1% formic acid) in high-performance liquid chromatography (HPLC) vials and subjected to multi-residue analyses. Ten microliter volumes were injected into the LC-MS/MS system.

Antibacterials. β-lactams were determined as described in Jank et al. (2011). The extraction procedure consisted of the sequential addition of 1.0mL of ACN to 2.0mL of milk four times, with vortexing between each addition. The sample was then mixed in a head-over-head shaker for 15 min; 1.0g of sodium chloride was added, and the sample was mixed in the shaker for an additional 15 min. The samples were centrifuged for 5 min at 3,000*g*. Aliquots of the supernatant were transferred to HPLC vials, and a 10µL volume was injected into the chromatograph.

For the quantitative analysis of tetracyclines, 500µL of milk was placed in a microcentrifuge tube (2.0mL), and 5µL of 100mM EDTA was then added. After mixing for 15s, 200µL of acidified ethanol (3% acetic acid) was added. The samples were then mixed (15s) and centrifuged for 10 min at 12,000rpm. An aliquot of the supernatant (350µL) was diluted with water (650µL) in an HPLC vial and subjected to LC-MS/MS analysis.

The quantitative analysis of fluoroquinolones was performed using the same procedure as described above in "Qualitative analysis of fluoroquinolones, tetracyclines, sulfonamides and trimethoprim"; a matrix-matched calibration curve and a specific mass spectrometry method were used for fluoroquinolone quantification.

To determine the presence of sulfonamides and trimethoprim, 500µL of milk was vortexed for 15s to homogenize, and the analyte extraction was performed with 200µL of acidified ethanol (3% acetic acid). The extract was mixed for 15s and centrifuged for 10 min at 12,000rpm. An aliquot of the supernatant (350µL) was diluted with water (650µL) in an HPLC vial, and 10µL was injected into the chromatograph for LC-MS/MS analysis.

Macrocyclic lactones. The extraction of the samples was performed as described in Rübensam et al*.* (2011). For the procedure, 5.0mL of milk was extracted with four aliquots of 2.5mL of ACN using liquid-liquid extraction with low temperature purification. To this mixture, 2g of sodium chloride was added, and the sample was shaken until the salt dissolved. Then, the mixture was centrifuged. The top phase was transferred to a 15mL polypropylene centrifuge tube and incubated in a freezer for 12h at -20°C. The remaining liquid phase was then transferred to a new 50mL centrifuge tube and evaporated in a water bath (50-55°C) under a gentle nitrogen flow until completely dry. Finally, the dry extract was reconstituted in 1mL of ACN for further LC-MS/MS analysis.

Quality assurance and method performance. All of the samples were subjected to all of the previously described methods. First, the samples were analyzed by a LC-MS/MS screening method for antibacterial residues. For each positive result, a LC-MS/MS quantitative and confirmatory analysis was performed. All of the methods have been fully validated and are currently in routine use in the laboratory network of the Ministry of Agriculture, Livestock and Food Supply of Brazil (MAPA) for the NRCCP (Lins et al. 2012). The results were corrected for recovery. All of the applied methods met the internal criteria for residue analysis. The performance data of all analytical methods are summarized in Table 1.

Antibacterial screening tests. The milk samples were also analyzed using three commercially available antibacterial screening methods, including the DelvoTest® Accelerator SP 5-pack NT DA (Royal Gist-Brocades NV, Delft, Netherlands), Charm MRL β-lactam test and Charm Tetracycline test (Charm Science Inc., Malden, MA), all of which were performed according to the manufacturers' protocols. The DelvoTest® Accelerator SP 5-pack NT DA is based on the growth inhibition of *Geobacillus stearothermophilus* var. *calidolactis* and can detected a range of antimicrobials with different levels (Nagel et al. 2011). The Charm MRL β-lactam test can detect 14 beta-lactams (amoxicillin, ampicillin, cefacetrile, cefalexin, cefalonium, cefalozin, cefoperazone, cefquinome, ceftiofur, cefuroxime, cephapirin, cloxacillin, dicloxacillin, penicillin G). The Charm Tetracycline test can detect tetracycline, oxytetracycline and chlortetracycline.

Statistical analysis. The number of positive raw milk samples in the DelvoTest® Accelerator SP 5-pack NT DA, Charm MRL β-lactam test and Charm Tetracycline test and the number of milk samples with antibacterials, β-lactams and tetracyclines residues, respectively, detected by confirmatory tests were compared using the Pearson's chi-square test. Statistical analysis was performed using the InfoStat statistical software (Córdoba, Argentine).

RESULTS AND DISCUSSION

The veterinary drug residues that were found in the raw milk in the present study are summarized in Table 2. At least one veterinary drug residue was detected in 40 samples (30.30%) by confirmatory tests, whereas 16 samples (12.12%) showed the presence of at least two residues. With regard to the BMRLs, 11 milk samples (8.33%) from eight dairy farms (17.78%) were non-compliant. Three farms (6.67%) had CYP residues above the BMRLs at two of the three samplings. The BMRLs for PNV, ABA and CYP are 4, 10 and 20 µg kg^{-1}, respectively (Brasil 1999). According to Brazilian Legislation and considering the overall volume of 94,000 liters for daily production in the selected properties, 6.4% of total milk would be proven to be non-compliant and should be discarded, without considering that the milk contaminated with veterinary drug residues from a dairy farm was transported together with raw milk from other farmers without veterinary drugs residues.

This survey indicates that the withdrawal periods of some veterinary drugs, especially pirethroids (6.8% above the BMRLs), have not been fully respected by all dairy farmers. Furthermore, it is noteworthy that the presence of some veterinary drugs residues (i.e. avermectins) in milk, even below the BMRLs, is indicative of problems with good veterinary practices, since their use is not allowed in lactating dairy cows according to Brazilian legislation (Brasil 2009). From another point of view, some authors have found antimicrobial residues in milk beyond the recommended withdrawal period after intramammary administration (Martins et al. 2016). Besides this, another factor that can be associated with the presence of pyrethroid residues in raw milk is the use of veterinary drugs in products destined to animal feed, such as pyrethroid pesticides, or contaminated water (Iftikhar et al. 2014). On the other hand, the application of some veterinary drugs used on dairy farms can be a source of widespread and persistent environment contami-

nation that can remain in the soil or water for a period of over 20 years (Gassner et al. 1997, Zuccato et al. 2000, Iftikhar et al. 2014). Highlighting the data found on pesticide residues in raw milk, pyrethroid compounds are toxic and can accumulate in an organism causing severe neurological (Burns et al. 2013) and respiratory symptoms (Hudson et al. 2014), as well as, can adversely affect endocrine and immune systems (Du et al. 2010, Costa et al. 2013).

Table 1. Method performance data for the analysis of veterinary drugs and pesticides in raw milk samples

Technique Analyte	LOD	LOQ	CCα	CCβ	Recovery (%)	Linearity range (µg kg-1)	MRL (µg kg-1)
							Brazil[a]
LC-MS/MS							
PNG	0.4	1.0	4.7	5.7	54.2	0.4 - 40	4
PNV	0.4	1.0	4.7	6.1	63.7	0.4 - 40	4
CFT	10.0	25.0	120.4	147.9	41.9	10 - 200	100
CLX	3.0	7.5	35.6	52.8	79.3	3 - 100	30
OXA	3.0	7.5	36.5	53.7	73.8	3 - 100	30
DCX	3.0	7.5	36.3	56.6	81.3	3 - 100	30
CTC	10.0	25.0	108.0	122.0	49.8	10 - 300	100
DOXI	25.0	50.0	135.0	174.0	46.3	25 - 300	100
TC	10.0	25.0	112.0	148.0	60.3	10 - 300	100
OTC	10.0	25.0	114.0	151.0	54.7	10 - 300	100
OXO	10.0	20.0	117.6	135.2	76.0	10 - 200	-
NALIDIX	10.0	20.0	114.4	128.7	79.0	10 - 200	-
FLU	10.0	20.0	112.8	125.6	79.0	10 - 200	50
DIFLO	10.0	20.0	115.4	130.8	73.0	10 - 200	-
CIPRO	10.0	20.0	112.5	125.0	81.0	10 - 200	100
ENRO	10.0	20.0	111.8	123.6	81.0	10 - 200	100
NOR	10.0	20.0	113.6	127.3	80.0	10 - 200	-
SARA	10.0	20.0	114.4	128.9	76.0	10 - 200	-
TMP	10.0	20.0	109.7	118.0	89.0	10 - 200	-
SDMX	15.0	25.0	112.0	125.0	84.0	10 - 200	100
SQX	15.0	25.0	115.0	130.0	71.0	10 - 200	100
SDZ	15.0	25.0	113.0	126.0	73.0	10 - 200	100
STZ	15.0	25.0	115.0	128.0	65.0	10 - 200	100
SPY	15.0	25.0	112.0	121.0	69.0	10 - 200	100
SMA	15.0	25.0	119.0	134.0	74.0	10 - 200	100
SMZ	15.0	25.0	121.0	138.0	77.0	10 - 200	100
SCP	15.0	25.0	108.1	116.2	80.0	10 - 200	100
SFX	15.0	25.0	ND	ND	63.5	10 - 200	100
SDX	15.0	25.0	107.4	114.8	77.0	10 - 200	100
SMR	15.0	25.0	106.6	113.2	74.5	10 - 200	100
ABA	0.2	0.6	10.6	11.3	102.0	2.5 - 25	10
DOR	0.6	1.9	16.0	17.3	101.4	3.75 - 37.5	15
EPR	1.1	3.4	22.0	23.7	101.3	5 - 40	20
IVR	0.3	0.9	10.7	11.6	101.8	2.5 - 25	10
MOX	0.1	0.4	10.5	11.0	95.4	2.5 - 25	10
GC-ECD							
γCYH	2.0	10.0	32.4	38.4	86.8	2 – 100	25
λCYH	2.0	10.0	40.3	50.3	91.3	2 – 100	25
CYF	5.0	20.0	94.1	135.7	101.7	5 – 100	20
CYP	5.0	15.0	26.7	33.6	95.6	5 – 100	20
DEL	2.0	5.0	27.4	34.5	73.9	2 – 100	20
FEV	2.0	5.0	49.8	58.2	79.1	2 – 100	40
PER	2.0	5.0	26.6	33.1	64.8	2 – 100	50

[a] Set by Brazilian Maximum Residue Levels. LOD = limit of detection; LOQ = limit of quantification; CCα = decision limit; CCβ = detection capability; MRL = maximum residue levels; LC-MS/MS = liquid chromatography coupled with mass spectrometry in tandem mode; GC:ECD = gas chromatograph with electron capture detector; PNG = penicillin G; PNV = penicillin V; CFT = ceftiofur; CLX = cloxacillin; OXA = oxacillin; DCX = dicloxacillin; CTC = chlortetracycline; DOXI = doxycycline; TC = tetracycline; OTC = oxytetracycline; OXO = oxolinic acid; NALIDIX = alidixic acid; FLU = flumequine; DIFLO = difloxacin; CIPRO = ciprofloxacin; ENRO = enrofloxacin; NOR = norfloxacin; SARA =: sarafloxacin; SDMX = sulfadimethoxine; SQX = sulfaquinoxaline; SDZ = sulfadiazine; STZ = sulfathiazole; SPY = sulfapyridine; SMA = sulfamethoxazole; SMZ = sulfamethazine; SCP = sulfachloropyridazine; SFX = sulfisoxazole; SDX = sulfapyridine; SMA = sulfamethoxazole; SMR = sulfamerazine; TMP = trimethoprim; ABA = abamectin; DOR = doramectin; EPR = eprinomectin; IVR = ivermectin; MOX = moxidectin; γCYH = γ-cyhalothrin; λCYH = λ-cyhalothrin; CYF = cyfluthrin; CYP = cypermethrin; DEL = deltamethrin; FEV = fenvalerate; PER = permethrin.

Table 2. Residues of veterinary drugs and pesticides in 132 raw milk samples from dairy farms in Minas Gerais State, Brazil

Classes	Analytes	Detected analytes	Positive samples (%)	Detection levels (g kg-1)[a]	Mean + SE (g kg-1)	Non-compliant samples (%)[b]
β-lactam	PNG, PNV, CFT, CLX, NV OXA, DCX	1 (0.8) P	8.0-8.0	8.0 + 0.0	1 (0.8)	
		CFT	7 (5.3)	13.4-69.4	21.8 + 8.1	0 (0.0)
		OXA	2 (1.5)	4.2-4.4	4.3 + 0.1	0 (0.0)
		DCX	2 (1.5)	3.8-3.9	3.8 + 0.1	0 (0.0)
Tetracyclines	CTC, DOXI, TC, OTC					
OTC	3 (2.3)	16.3-25.0	20.0 + 2.6	0 (0.0)		
(Fluoro) quinolones	OXO, NALIDIX, FLU, DIFLO, CIPRO, ENRO, NOR, SARA					
CIPRO	3 (2.3)	16.6-39.4	31.6 + 7.6	0 (0.0)		
		ENRO	3 (2.3)	3.0-6.0	4.0 + 1.0	0 (0.0)
Sulfonamides	SDMX, SQX, SDZ, STZ, SPY, SMA, SMZ, SCP, SFX, SDX, SMR	ND	ND	ND	ND	0 (0.0)
Sulfonamide synergists	TMP	ND[b]	ND	ND	ND	0 (0.0)
Macrocyclic lactones	ABA, DOR, EPR, IVR, MOX	ABA	7 (5.3)	2.2-18.3	5.2 + 2.2	1 (0.8)
		DOR	3 (2.3)	0.9-2.5	1.7 + 0.5	0 (0.0)
		IVR	10 (7.6)	0.4-2.9	1.5 + 0.3	0 (0.0)
Pyrethroids	γCYH, λCYH, CYF, CYP, DEL, FEV, PER	CYP	12 (9.1)	1.6-251.0	87.1 + 26.6	9 (6.8)
		DEL	2 (1.5)	1.0-1.9	1.5 + 0.5	0 (0.0)

[a] Maximum and minimum concentration levels of the milk samples with veterinary drugs residues. [b] Set by Brazilian Maximum Residue Levels. ND = not detected; PNG = penicillin G; PNV = penicillin V; CFT = ceftiofur; CLX = cloxacillin; OXA = oxacillin; DCX = dicloxacillin; CTC = chlortetracycline; DOXI = doxycycline; TC = tetracycline; OTC = oxytetracycline; OXO = oxolinic acid; NALIDIX = nalidixic acid; FLU = flumequine; DIFLO = difloxacin; CIPRO = ciprofloxacin; ENRO = enrofloxacin; NOR = norfloxacin; SARA = sarafloxacin; SDMX = sulfadimethoxine; SQX = sulfaquinoxaline; SDZ = sulfadiazine; STZ = sulfathiazole; SPY = sulfapyridine; SMA = sulfamethoxazole; SMZ = sulfamethazine; SCP = sulfachloropyridazine; SFX = sulfisoxazole; SDX = sulfadoxine; SMR = sulfamerazine; TMP = trimethoprim; ABA = abamectin; DOR = doramectin; EPR = eprinomectin; IVR = ivermectin; MOX = moxidectin; γCYH = γ-cyhalothrin; λCYH = λ-cyhalothrin; CYF = cyfluthrin; CYP = cypermethrin; DEL = deltamethrin; FEV = fenvalerate; PER = permethrin.

Lobato et al. (2006) found a higher percentage of ivermectin (17.8%, n=168) residue in bovine milk samples from the Brazilian retail market, although none of them were above the BMRLs, as found here. Furthermore, the Brazilian Program of Analysis of Veterinary Drugs Residues in Food from Animal Origin (Brasil 2009) found a higher percentage of most of veterinary drugs residues in UHT milk and dry milk, as follows: 41.29% and 52.17% of positive samples for ivermectin residue; 2.80%, and 7.25% of positive samples for abamectin residue; 4.09% (0.22% above BMRLs) and 27.54% (5.80% above BMRLs) of positive samples for doramectin residue, 0.65% and 2.14% of positive samples for β-lactams residue, 1.72% and 15.83% of positive samples for tetracyclines residue, 3.87% and 4.41% of positive samples for sulfathiazole residue, 4.52% and 23.53% of positive samples for sulfamethazine residue, and 3.01% and 0.74% of positive samples for sulfadimethoxine residue, for UHT milk and dry milk, respectively.

Prado et al. (2015) found a low percentage (3%, n=100) of pasteurized cow's milk commercialized in the State of Paraná with oxytetracycline, tetracycline, chlortetracycline and doxycycline residues, similarly to the present study. The data from the Brazilian official program (National Residues and Contaminants Control Plan) in 2014 found only one raw milk sample (0.32%, n = 309) with antimicrobial residues above the BMRLs, likewise the findings of the present study. In contrast to the findings of the present study, they encountered no milk sample with antiparasitic (n=217) and pyrethroid (n=65; analyzing only three analytes: cypermethrin, permethrin and deltamethrin) residues above the BMRLs (Brasil 2015). It should be regarded that the pyrethroid residues surveys in milk samples were not analyzed by the Brazilian official program in the previous years.

With this in mind, it should be noted that Brazilian governmental monitoring programs - National Sanitary Surveillance Agency (ANVISA; Program of Analysis of Veterinary Drug Residues in Food from Animal Origin) and Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA; National Residues and Contaminants Control Plan) conducted investigations of veterinary drug residues in food from animal origins, especially regarding antimicrobial and anthelmintic residues (Pacheco-Silva et al. 2014). Thus, this study demonstrated the crucial importance of the dairy industry and government regulatory agencies in applying continuous monitoring programs to offer a safe product without any risk to consumers. For instance, our study demonstrated that almost all milk samples were compliant with BMRLs for antimicrobial (99.20%) and macrocyclic lactones (99.20%) residues. In contrast, pyrethroid residues were found to be above the BMRLs in 6.80% of milk samples.

The importance of veterinary residues in raw milk even in levels below the BMRLs should be highlighted due the fact that the raw milk is essential for the production of dairy products. As a result, some veterinary drugs residues may be found in greater concentration in milk products than milk from which these were manufactured due to concentration effect and/or affinity for lipid moiety and ther-

mal stability (Bajwa & Sandhu 2014, Macedo et al. 2015). For instance, in a survey of macrocyclic lactones residues in butter, Macedo et al. (2015) found a high incidence of the presence of avermectins in butter samples, in which ivermectin, doracmectin and abamectin were detected in 89.5% (0.3 to 119.4μg kg^{-1}), 76.3% (0.6 to 64.6μg kg^{-1}) and 55.2% (0.7 to 4.5μg kg^{-1}) of the butter samples in the metropolitan area of Rio de Janeiro - Brazil, respectively.

The number of positive bulk tank milk samples detected by the DelvoTest® SP 5-PACK NT DA - Accelerator, Charm MRL Beta-lactam test and Charm Tetracycline were four (3.03%), eight (6.06%) and six (4.55%) samples, respectively. From those positive milk samples for antimicrobial residues in the screening tests, only two (50.00%), one (12.50%) and two (33.30%) bulk tank milk samples were detected antimicrobials by liquid chromatography coupled with mass spectrometry in tandem mode spectrometry, respectively. In addition, the Charm Tetracycline screening test detected only one (33.3%, n=3) milk sample with tetracycline (OTC) residues. In addition, almost all samples (82.35%) that were positive for antimicrobial residues, as detected by confirmatory tests, were negative in screening tests. Furthermore, the one raw milk sample above the BMRLs for antimicrobials (PNV) was not detected by any screening tests. In addition, the number of positive raw milk samples in the Charm Tetracycline and DelvoTest® SP 5-PACK NT DA - Accelerator antibacterial screening tests were statistical different from the number of milk samples positive for tetracyclines (tetracycline, oxytetracycline and chlortetracycline; P=0.016) and all antibacterials (P=0.02) detected by confirmatory tests, respectively. Nonetheless, the number of positive milk samples in the Charm MRL Beta-lactam test was not statistical different from the number of raw milk samples positive for β-lactams antimicrobials (penicillin G, penicillin V, ceftiofur, cloxacillin, oxacillin and dicloxacillin) tested here by confirmatory tests (P=0.84).

These discrepancies could be due to the detection limits of the tests and the many analytes investigated by the confirmatory tests here (Molina et al. 2003). In agreement with our data, Moats et al. (1995) reported an inconsistency between Charm Tetracycline screening test results when compared with high-pressure liquid chromatography to detect oxytetracycline. Le Breton et al. (2007) also reported that some antimicrobials such as oxytetracycline, dihydrostreptomycin, trimethoprim and cefquinome were not detected or only detected with low sensitivity with the Delvotest SP NT antimicrobial screening kit.

The veterinary drug residues were detected in a significant percentage of bulk tank milk samples, particularly the pyrethroid residues, using confirmatory methods. Furthermore, the antibacterial screening methods failed to identify most of the positive samples that were detected by confirmatory tests, leading to a discrepancy between the screening and confirmatory antimicrobial tests. Thus, the present study indicated that the veterinary drugs residues still represents a great concern for the milk production chain. In face of, our findings indicated the need of a more strict monitoring of the veterinary drugs residues in milk produced in Minas Gerais State, especially regarding pyrethroid residues. To reach this objective, continuous monitoring programs should be applied in order to offer a safer product to consumers.

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Authors' contribution.- LCAP participated in acquisition, analysis and interpretation of data, and edited the manuscript. IMT and RBH participated in acquisition and analysis of data.
FNS participated in conceptual advice, drafted, edited and critically revised the manuscript content. SAD and MXS participated in the interpretation of data, study design and statistical analysis.
MOL, LMF, MMOPC and MTB-L served as principal investigators, who designed the experiments, supervised studies, critically revised the manuscript and provided the final approval of the version to be published.

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