Evaluation of residues of β-lactam, sulfonamide, tetracycline, quinolone, fluoroquinolone e pyrimidine in raw milk

Luciana Martins VALENÇA1*, José do Egito de PAIVA2, Severino Benone Paes BARBOSA1, Irapuan Oliveira PINHEIRO3, Ângela Maria Vieira BATISTA1, Marcelo José Ferreira Batista da SILVA1, Elizabeth Sampaio de MEDEIROS4

Abstract
Food quality and safety have been a concern of the world population, especially in relation to the chemical and microbiological hazards present in food. The presence of antimicrobial residues at levels above the maximum residue limit makes milk unsuitable for human consumption. In this context, the objective of this study was to analyze 184 raw milk samples from the dairy region of Pernambuco state, in order to evaluate the presence of 31 antimicrobial residues from six different groups (β-lactam, sulfonamide, tetracycline, quinolone, fluoroquinolone and pyrimidine). The samples were collected from producers who supply the dairies with and no inspection service and the analyses were performed by liquid chromatography coupled to mass spectrometry. In the qualitative analysis of multiresidues, no sample presented violation for the antimicrobials analyzed. In the quantitative analysis of β-lactams, only one sample of producers who supply milk to dairies with state inspection service presented a result above the maximum residue limit for cloxacillin (464 μg.L⁻¹). It is concluded that raw milk produced in the dairy region of the state of Pernambuco has a low rate of violation of the values established by the current legislation for antimicrobial residues.

Keywords: mastitis; maximum residue limit; inspection service; antimicrobial residue; LC-MS/MS.

Practical Application: Contribute to the elaboration of public policies, benefiting the producers and consumers.

1 Introduction
Dairy herds are often affected with mastitis, causing losses of financial resources and reduction of the quality of milk and dairy products (Bezman et al., 2015; Down et al., 2017; Machado & Bicalho, 2018). To prevent and combat this disease, antimicrobials are administered, but, during the grace period reported by the manufacturer, milk should be discarded, since antimicrobial residues are present and are not removed in their entirety by processing this raw material in industry (Calbert, 1951; Gajda et al., 2017; Poonia et al., 2017; Rossi et al., 2018; Tempini et al., 2018).

The consumption of milk with antimicrobial residue above the Maximum Residue Limit (MRL) represents a public health problem, since it can cause: allergic reactions, antimicrobial resistance, blood dyscrasias, gastrointestinal disorder, neurological disorder, cancer, among other effects (Titouche et al., 2013; Baynes et al., 2016; Delatour et al., 2018; Du et al., 2019). In addition, the presence of antimicrobials generates problems for the dairy, as they alter the results of analyses, inactivate or delay the activity of starter cultures in the production of cheese, yogurts, compromising the production of acids, resulting in failure of coagulation and maturation, causing modification of the sensory properties of dairy products (Calbert, 1951; Berruga et al., 2016; Gajda et al., 2017).

In Brazil, the control of residues of veterinary medicines in food is carried out through the National Program for the Control of Residues and Contaminants (PNCRC) of the Ministry of Agriculture, Livestock and Supply (MAPA). The PNCRC’s regulatory function is to control and monitor these residues in food. For this, the level of tolerance or MRL that refers to the presence of antimicrobials in foods of animal origin has been established (Brasil, 1999).

The objective in this study is to evaluate the presence of 31 residues of antimicrobials of six different groups (β-lactam, sulfonamide, tetracycline, quinolone, fluoroquinolone and pyrimidine), in raw milk, produced in the Agreste region, the dairy basin of the state of Pernambuco, and supplied to dairies with the Federal Inspection Service (FIS), State Inspection Service (SIS) and the No Inspection Service (NIS).

2 Materials and methods
The study covered eighteen municipalities representing the dairy region of Pernambuco, which accounts for 83.8% of milk production in the state (Instituto Brasileiro de Geografia e Estatística, 2018). The choice of producers was random and for each dairy we collected only one sample of the same producer, per season of the year, according to rainfall. The amount of raw
Antimicrobial residues in raw milk

milk samples collected, according to the inspection service and the time of collection is described in Table 1.

Before each collection, the raw milk was homogenized and, with the aid of a stainless steel ladle, approximately 200 mL were collected and placed in sterile vials. The ladles were sanitized with distilled water and alcohol at 70% v/v before and after each collection, routinely, to avoid cross-contamination. All vials were properly identified with univocal numbering and with necessary information to ensure traceability, such as: vial number, date and time of collection, sample temperature, dairy, type of inspection service, producer and municipality. All samples were placed in styrofoam boxes with ice cubes immediately after collection.

The samples were subsequently frozen in a freezer and sent to the Laboratory of Analysis of Pesticide Residues and Veterinary Medicines (RPM Laboratory), of the National Agricultural Laboratory of Rio Grande do Sul (LANAGRO-RS) to perform the qualitative analysis of multiresidues and quantitative analysis of residue β-lactams by liquid chromatography coupled to mass spectrometry (LC-MS/MS).

2.1 Qualitative analysis of multiresidues by LC-MS/MS

The qualitative analysis of multiresidues was performed according to the procedure described in the Screening Method for qualitative detection of twenty-five residues of antimicrobials from five different groups (sulfonamides, tetracyclines, quinolones, fluoroquinolones and pyrimidine), using LC-MS/MS, a method validated for milk samples and accredited by INMETRO (Laboratório Nacional Agropecuário, 2012).

2.2 Quantitative analysis of residue β-lactams by LC-MS/MS

All steps of the quantitative analysis to detect and quantify the residues of the six β-lactams (ceftiofur, penicillin G, penicillin V, oxacillin, cloxacillin and dicloxacillin) using LC-MS/MS, were carried out by methodology described by Jank et al. (2012).

The analytical parameters of the analytes analyzed in this method are described in Table 2.

3 Results

In the qualitative analysis of multiresidues, the samples did not present violation, indicating that they did not have the analytes studied in values above the MRL. However, six samples (6.5%), collected in the dry period, were positive for oxytetracycline, meaning that these samples presented concentration of this substance above the CCβ (CCβoxytetracycline = 25 μg.L⁻¹). The samples identified as positive by the screening method were analyzed in a specific confirmatory methodology for the tetracycline group. Among the six samples that were positive, three had concentrations above CCβ by confirmatory method, but lower than MRL (MRLβ-tetracycline group = 100 μg.L⁻¹), which were: two samples from SIS producers (49.7 μg.L⁻¹ and 31.4 μg.L⁻¹) and one sample from NIS producers (26.9 μg.L⁻¹); and three samples showed concentrations lower than the CCβ, which were: a FIS producer sample (11.9 μg.L⁻¹), a SIS producer sample (13.5 μg.L⁻¹) and an NIS producer sample (7.1 μg.L⁻¹).

In the quantitative analysis of residue β-lactams, the samples collected in rain period did not present concentrations above the MRL. However, three samples had penicillin G residues in a concentration lower than the MRL, which were: a producer sample from the FIS (0.2 μg.L⁻¹), a producer sample from the SIS (2.0 μg.L⁻¹) and a producer sample from the NIS (0.4 μg.L⁻¹). In the dry period, a producer sample from the SIS (1.1% of the samples collected in this season or 2.8% of the samples submitted to SIS collected in this season) presented cloxacillin residue in a concentration higher than the MRL (464 μg.L⁻¹). This result represents a concentration greater than 15 times the MRL for cloxacillin, according to Table 3.

4 Discussion

The results found may indicate the use of good agricultural practices in dairy production, because if the application of antimicrobial occurs during the period when the cow is not in production and the grace period indicated by the manufacturer of the antimicrobial for milk disposal is respected when application occurs in the period that the animal is lactating, the risk of passage of residues of antimicrobials into milk will be low (Andrew et al., 2009; Rossi et al., 2018). However, the results may also indicate low frequency of antimicrobial treatment and also low occurrence of clinical mastitis, which may be related to the low animal productivity (Kayitsinga et al., 2017).

A condition observed during the collections which may also have contributed to these results was the use of natural therapies by some producers, mainly small producers, in the treatment of mastitis, due to the low purchasing power of this group, this fact was also observed by Kayitsinga et al. (2017). On the other hand, the effect of dilution cannot be ruled out (Rassoul et al., 2014; Novaes et al., 2017), which occurs after the mixture of milk free of antimicrobial residue with contaminated milk, which may also reflect the reality found in these samples.

Table 2. Analytical parameters obtained for β-lactams in milk (μg.L⁻¹).

| β-lactams   | LOD | LOQ | CCα  | CCβ  |
|------------|-----|-----|------|------|
| Ceftriaxone| 10.0| 25.0| 120.4| 147.9|
| Penicillin G| 0.4  | 1.0 | 4.7 | 5.7 |
| Penicillin V| 0.4  | 1.0 | 4.7 | 6.1 |
| Oxacillin  | 3.0  | 7.5 | 36.5 | 53.7 |
| Cloxacillin| 3.0  | 7.5 | 35.6 | 52.8 |
| Dicloxacillin| 3.0 | 7.5 | 36.3 | 56.6 |

LOD - Limit of detection, LOQ - Limit of quantification, CCα - Limit of decision, CCβ - Detection capacity. Source: Jank et al. (2012).
Table 3. Maximum residue limit values for antimicrobial groups studied in raw milk.

| Group          | Analyte                                           | MRL (µg.L⁻¹) |
|----------------|---------------------------------------------------|--------------|
|                | Penicillin G, penicillin V                        | 4            |
| β-lactams      | Oxacillin, cloxacillin, dicloxacillin             | 30           |
|                | Cefotiofur                                        | 100          |
| Sulfonamide    | Sulfadimethoxine, sulfaquinoxaline, sulfadiazine, | 100          |
|                | sulfatiazole, sulfapyridine, sulfamethoxazole,    |              |
|                | sulfamethazine, sulfaclorpridazine, sulfadoxine, |              |
|                | sulfametoxazole, sulfametazine, sulfamerazine    |              |
| Tetracyclines  | Chlortetracycline, tetracycline, oxotetracycline  | 100          |
| Quinolones     | Oxolinic acid, nalidixic acid                     | 20           |
|                | Flumequine                                        | 50           |
|                | Norfloxacin                                       | 10           |
|                | Sarafloxacin                                      | 20           |
| Fluoroquinolones| Danofoxacin                                       | 30           |
|                | Ciprofloxacin, enrofloxacin                       | 50           |
|                | Difloxacin                                        | 100          |

Source: Jank et al. (2012); LANAGRO-RS/MET RPM 10 02 (Laboratório Nacional Agropecuário, 2012).

The low rate of violation of samples verified in this study is in accordance with the results reported by the PNCR in the years 2010 to 2017, since of the 3,370 milk samples analyzed only 12 (0.36%) were contaminated (Brasil, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018). Similar results were also reported by Bilandžić et al. (2011), Rassouli et al. (2014), Prado et al. (2015). However, higher amounts of antimicrobial residues were found by Aalipour et al. (2015), Layada et al. (2016), Orwa et al. (2017), Rama et al. (2017), Wang et al. (2017), Khanal et al. (2018).

5 Conclusion

This study concludes that raw milk produced in the dairy region of the state of Pernambuco has a low rate of violation of the MRL established by the PNCR, however, a more constant and effective control of inspection services is necessary in order to ensure greater safety for producers and consumers.

References

Aalipour, F., Mirlohi, M., Jalali, M., & Azadbakht, L. (2015). Dietary exposure to tetracycline residues through milk consumption in Iran. *Journal of Environmental Health Science & Engineering*, 13(1), 80. http://dx.doi.org/10.1186/s40201-015-0235-6. PMid:26600942.

Andrew, S. M., Moyes, K. M., Borm, A. A., Fox, L. K., Leslie, K. E., Hogan, J. S., Oliver, S. P., Schukken, Y. H., Owens, W. E., & Norman, C. (2009). Factors associated with the risk of antibiotic residues and intramammary pathogen presence in milk from heifers administered prepartum intramammary antibiotic therapy. *Veterinary Microbiology*, 134(1-2), 150-156. http://dx.doi.org/10.1016/j.vetmic.2008.09.022. PMid:18945559.

Baynes, R. E., Dedonder, K., Kissell, L., Mryk, D., Marmulak, T., Smith, G., Tell, L., Gehring, R., Davis, J., & Riviére, J. E. (2016). Health concerns and management of select veterinary drug residues. *Food and Chemical Toxicology*, 88, 112-122. http://dx.doi.org/10.1016/j.fct.2015.12.020. PMid:26751035.

Berruga, M. I., Molina, A., Althaus, R. L., & Molina, M. P. (2016). Control and prevention of antibiotic residues and contaminants in sheep and goat’s milk. *Small Ruminant Research*, 142, 38-43. http://dx.doi.org/10.1016/j.smallrumres.2016.02.023.

Bezman, D., Lemberskiy-Kuzin, L., Katz, G., Merin, U., & Leitner, G. (2015). Influence of intramammary infection of a single gland in dairy cows on the cow’s milk quality. *Journal of Dairy Research*, 82(3), 304-311. http://dx.doi.org/10.1017/S002202991500031X. PMid:26134490.

Bilandžić, N., Kolanović, B. S., Varenina, I., Scortichini, G., Annunziata, L., Brstilo, M., & Rudan, N. (2011). Veterinary drug residues determination in raw milk in Croatia. *Food Control*, 22(12), 1941-1948. http://dx.doi.org/10.1016/j.foodcont.2011.05.007.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento - MAPA. (1999). Altera o Plano Nacional de Controle de Resíduos em Produtos de Origem Animal - PNCR e os Programas de Controle de Resíduos em Carne - PCRC, Mel – PCRM, Leite – PCRL e Pescado – PCRP e dá outras providências (Instrução Normativa nº 42, de 20 de dezembro de 1999). *Diário Oficial [da] República Federativa do Brasil*.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento - MAPA. (2011). Publica os resultados do acompanhamento dos Programas de Controle de Resíduos e Contaminantes dos subprogramas de monitoramento e exploratório em Carnes (Bovina, Suína, Aves e Equina), Leite, Ovos, Mel e Pescado do exercício de 2010, na forma do Anexo a presente Instrução Normativa, em conformidade com a Instrução Normativa nº 08, de 29 de Abril de 2010 (Instrução Normativa de 2011). *Diário Oficial [da] República Federativa do Brasil*.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento - MAPA. (2012). Publica os resultados do acompanhamento dos Programas de Controle de Resíduos e Contaminantes dos subprogramas de monitoramento e exploratório em Carnes (Bovina, Suína, Aves e Equina), Leite, Ovos, Mel e Pescado do exercício de 2011, na forma dos Anexos à presente Instrução Normativa, em conformidade com a Instrução Normativa nº 24, de 09 de Agosto de 2011 (Instrução Normativa nº 7 de 04 de abril de 2012). *Diário Oficial [da] República Federativa do Brasil*.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento - MAPA. (2013). Publica os resultados do acompanhamento dos Programas de Controle de Resíduos e Contaminantes dos subprogramas de monitoramento e exploratório em Carnes (Bovina, Suína, Aves e Equina), Leite, Ovos, Mel e Pescado do exercício de 2012, na forma dos Anexos à presente Instrução Normativa, em conformidade com a Instrução Normativa nº 11, de 22 de Maio de 2012 (Instrução Normativa nº 7 de 27 de março de 2013). *Diário Oficial [da] República Federativa do Brasil*.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento - MAPA. (2014). Publica os resultados do acompanhamento dos Programas de Controle de Resíduos e Contaminantes dos subprogramas de monitoramento e exploratório em Carnes (Bovina, Suína, Aves e Equina), Leite, Ovos, Mel e Pescado do exercício de 2014. *Diário Oficial [da] República Federativa do Brasil*.
Antimicrobial residues in raw milk

Brasil. Ministério da Agricultura, Pecuária e Abastecimento - MAPA. (2016). Resultados gerais do subprograma de monitoramento e subprograma exploratório do plano nacional de controle de resíduos e contaminantes – PNCRC 2015. Brasília: MAPA.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento – MAPA. (2017). Resultados gerais do subprograma de monitoramento e subprograma exploratório do plano nacional de controle de resíduos e contaminantes – PNCRC 2016. Brasília: MAPA.

Brasil. Ministério da Agricultura, Pecuária e Abastecimento – MAPA. (2018). Resultados do plano nacional de controle de resíduos e contaminantes – PNCRC 2017. Brasília: MAPA.

Calbert, H. E. (1951). The problem of antibiotics in milk. Journal of Milk and Food Technology, 14(2), 61-64. http://dx.doi.org/10.4315/0022-2747-14.2.61.

Delateur, T., Racault, L., Bessaïre, T., & Desmarchelier, A. (2018). Screening of veterinary drug residues in food by LC-MS/MS. Background and challenges. Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 35(4), 633-646. http://dx.doi.org/10.1080/19440049.2018.1426890. PMid:29234075.

Down, P. M., Bradley, A. J., Breen, J. E., & Green, M. J. (2017). Factors affecting the cost-effectiveness of on-farm culture prior to the treatment of clinical mastitis in dairy cows. Preventive Veterinary Medicine, 145, 91-99. http://dx.doi.org/10.1016/j.prevetmed.2017.07.006. PMid:28903881.

Du, B., Wen, F., Zhang, Y., Zheng, N., Li, S., Li, F., & Wang, J. (2019). Presence of tetracyclines, quinolones, lincomycin and streptomycin in milk. Food Control, 100(2), 171-175. http://dx.doi.org/10.1016/j.foodcont.2019.01.005.

Gajda, A., Nowacka-Kozak, E., Gbilyk-Sikorska, M., & Posyniak, A. (2017). Tetracycline antibiotics transfer from contaminated milk to dairy products and the effect of the skimming step and pasteurisation process on residue concentrations. Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 35(1), 66-76. http://dx.doi.org/10.1080/19440049.2017.1397773. PMid:29076394.

Instituto Brasileiro de Geografia e Estatística – IBGE. (2018). Pesquisa da pecuária municipal. Rio de Janeiro: IBGE. Retrieved from https://sidra.ibge.gov.br/tabelas/74#resultado.

Jank, L., Hof, R. B., Tarouco, P. C., Barreto, F., & Pizzolato, T. M. (2012). β-lactam antibiotics residues analysis in bovine milk by LC-ESI-MS/MS: a simple and fast liquid-liquid extraction method. Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 29(4), 497-507. http://dx.doi.org/10.1080/19440049.2011.604044. PMid:21988179.

Kayitisinga, J., Schewe, R. L., Contraseras, G. A., & Erskine, R. J. (2017). Antimicrobial treatment of clinical mastitis in the eastern United States: The influence of dairy farmers’ mastitis management and treatment behavior and attitudes. Journal of Dairy Science, 100(2), 1388-1407. http://dx.doi.org/10.3168/jds.2016-11708. PMid:27939551.

Khanal, B. K. S., Sadiq, M. B., Singh, M., & Anal, A. K. (2018). Screening of antibiotic residues in fresh milk of Kathmandu Valley, Nepal. Journal of Environmental Science and Health, Part. B, Pesticides, Food Contaminants, and Agricultural Wastes, 53(1), 57-86. http://dx.doi.org/10.1080/03601234.2017.1375832. PMid:29083954.

Laboratório Nacional Agropecuário – LANAGRO/RS. (2012). Método de ensaio (pp. 1-15). RS: Laboratório Nacional Agropecuário — Rio Grande do Sul/Laboratório de Análise de Resíduos de Pesticidas e Medicamentos Veterinários, Brasília: Ministério da Agricultura Pecuária e Abastecimento.

Layada, S., Benouareth, D. E., Coucke, W., & Andjelkovic, M. (2016). Assessment of antibiotic residues in commercial and farm milk collected in the region of Guelma (Algeria). International Journal of Food Contamination, 3(19), 1-16. http://dx.doi.org/10.1186/s40550-016-0042-6.

Machado, V. S., & Bicalho, R. C. (2018). Preparum application of internal teat sealant or intramammary amoxicillin on dairy heifers: effect on udder health, survival, and performance. Journal of Dairy Science, 101(2), 1388-1402. http://dx.doi.org/10.3168/jds.2017-13415. PMid:29224874.

Novaes, S. F., Schreiner, L. L., Silva, I. P., & Franco, R. M. (2017). Residues of veterinary drugs in milk in Brazil. Ciência Rural, 47(8), 1-7. http://dx.doi.org/10.1590/0103-8478cr20170215.

Orwa, J. D., Matofari, J. W., Muliro, P. S., & Lamuka, P. (2017). Assessment of sulphonamides and tetracyclines antibiotic residue contaminants in rural and peri urban dairy value chains in Kenya. International Journal of Food Contamination, 4(1), 1-11. http://dx.doi.org/10.1186/s40550-017-0050-1.

Poonia, A., Jha, A., Sharma, R., Singh, H. B., Rai, A. K., & Sharma, N. (2017). Detection of adulteration in milk: A review. International Journal of Dairy Technology, 70(1), 23-42. http://dx.doi.org/10.1111/1471-0307.12274.

Prado, C. K., Ferreira, F. D., Bando, E., & Machinski, M. J. (2015). Oxytetracycline, tetracycline, chlorotetracycline and doxycycline in pasteurised cow’s milk commercialised in Brazil. Food Additives & Contaminants. Part B, Surveillance, 8(2), 81-84. http://dx.doi.org/10.1080/19393210.2014.968881. PMid:25247384.

Rama, A., Lucatello, L., Benetti, C., Galina, G., & Bajraktari, D. (2017). Assessment of antibacterial drug residues in milk for consumption in Kosovo. Journal of food and drug analysis, 25(3), 525-532. http://dx.doi.org/10.1016/j.jfda.2016.07.007. PMid:28911638.

Rassoul, A., Amanzi, Z., Bahonar, A., Shams, G.H., & Abdolmaleki, Z. A. (2014). A trace analysis of oxytetracycline and tetracycline residues in pasteurized milk supplied in Tehran: a one-year study (April 2011- March 2012). Iranian Journal of Veterinary Medicine, 8(2), 119-123. http://dx.doi.org/10.22059/ijvm.2014.51409.

Rossi, R., Saluti, G., Moretti, S., Diamanti, L., Giusepponi, D., & Galarini, R. (2018). Multiclass methods for the analysis of antibiotic residues in milk by liquid chromatography coupled to mass spectrometry: a review. Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 35(2), 241-257. http://dx.doi.org/10.1080/19440049.2017.1393107. PMid:29087807.

Temptini, P. N., Ay, S. S., Karle, B. M., & Pereira, R. V. (2018). Multidrug residues and antimicrobial resistance patterns in waste milk from dairy farms in Central California. Journal of Dairy Science, 101(9), 8110-8122. http://dx.doi.org/10.3168/jds.2017-43498. PMid:30126599.

Titouche, Y., Hakem, A., Houali, K., Yabir, B., Malki, O., Chergui, A., Chenouf, N., Yahiaoui, S., Labiad, M., Ghenim, H., Kechih-Bounar, S., Chirila, F., Nadas, G., & Fit, N. I. (2013). Detection of antibiotics residues and antimicrobial resistance patterns in waste milk from contaminated milk collected in the region of Guelma (Algeria). International Journal of Food Contamination, 3(19), 1-16. http://dx.doi.org/10.1186/s40550-016-0042-6.

Wang, H., Ren, L., Yu, X., Hu, J., Chen, Y., He, G., & Jiang, Q. (2017). Antibiotic residues in meat, milk and aquatic products in Shanghai and human exposure assessment. Food Control, 80, 217-225. http://dx.doi.org/10.1016/j.foodcont.2017.04.034.