SHORT COMMUNICATIONS
COMUNICAÇÕES CIENTÍFICAS

SEMINA
Ciências Agrárias

DOI: 10.5433/1679-0359.2022v43n5p2385

Most common pathogens from cows with subclinical mastitis in northwestern Paraná, Southern Brazil and their antimicrobial susceptibility

Patógenos mais comuns isolados de vacas com mastite subclínica no noroeste do Paraná e suas resistências à antimicrobianos

Josiane Ito Eleodoro[1]; Selwyn Arlington Headley[2]; Ana Paula Kuller Zanoni[3];
Lucienne Garcia Pretto-Giordano[4]; Rafael Fagnani[5]*

Highlights

Streptococcus mutans was the most frequent pathogen.
Staphylococcus spp. showed 42% overall resistance to Gentamicin + Amoxicillin.
The antibiotic treatment decisions should be based on prior resistance tests.

Abstract

Considering the high prevalence of subclinical mastitis and its impacts on milk production, thematic studies are needed and can provide strategic data for its control. This study aimed at investigating the most frequent microorganisms associated with subclinical mastitis in dairy cows in the northwest State of Paraná, Brazil and its sensitivity to antibiotics. A total of 478 foremilk samples were tested. Streptococci were the most frequently genus observed (63%), followed by staphylococci (31%). *Streptococcus mutans, S. sanguis,* and *S. uberis* were the most frequently identified species. For *Streptococcus* spp., the antimicrobial resistance was higher for Ceftiofur, whereas for *Staphylococcus* spp. it was also possible to verify a high occurrence of resistance for Gentamicin + Amoxicillin. Our results strengthen the knowledge of the microbiological agent and antibiotic-resistance patterns of pathogens at regional level, targeting future strategies to reduce the environmental spread of pathogenic strains resistant to antibiotics.

Key words: Antibiotic resistance. *Staphylococcus. Streptococcus.*

---

[1] Graduate Student, Master of Science Program in Animal Health and Production, Universidade Pitágoras UNOPAR, Arapongas, PR, Brazil. E-mail: josianeito95@hotmail.com

[2] Prof. Dr., Master of Science Program in Animal Health and Production, UNOPAR, Arapongas, PR, Brazil. E-mail: selwyn.headley@kroton.com.br

[3] Graduate Student, Master of Science Program in Animal Health and Production, UNOPAR, Arapongas, PR, Brazil. E-mail: anapaulakzanoni@gmail.com

[4] Prof. Dr., Agrarian Science Center, Preventive Medicine Department, Universidade Estadual de Londrina, UEL, Londrina, PR, Brazil. E-mail:lgiordano@uel.br

[5] Prof. Dr., Master of Science Program in Animal Health and Production, UNOPAR, Arapongas, PR, Brazil. E-mail: rafael.fagnani@unopar.br

* Author for correspondence

Received: Mar. 28, 2022 - Approved: May 31, 2022

Resumo

Considerando a alta prevalência de mastite subclínica e seus impactos na produção de leite, estudos temáticos são cada vez mais importantes e podem fornecer dados estratégicos para o controle dessa infecção. Este estudo teve como objetivo investigar os microrganismos associados à mastite subclínica em vacas leiteiras no noroeste do estado do Paraná e sua sensibilidade a antibióticos. Foram testadas 478 amostras de leite. Os estreptococos foram os micro-organismos mais frequentemente observados (63%), seguidos pelos estafilococos (31%). *Streptococcus mutans, S. sanguis* e *S. uberis* foram as espécies identificadas em maior frequência. Para *Streptococcus* spp., a resistência antimicrobiana foi maior para Ceftiofur, enquanto para *Staphylococcus* spp. foi possível verificar alta ocorrência de resistência para Gentamicina + Amoxicilina. Esses resultados ampliam os conhecimentos sobre os agentes microbiológico envolvidos em mastites subclínicas e seus padrões de resistência a antibióticos em nível regional, possibilitando estratégias para reduzir a propagação ambiental de cepas patogênicas resistentes a antibióticos.

Palavras-chave: Resistência microbiana. *Staphylococcus. Streptococcus.*

Mastitis in dairy cows is one of the main diseases that involve antibiotic therapy worldwide. Despite the many benefits of these drugs, there is concern regarding antibiotic residues in food intended for human consumption and the potential development and transmission of antimicrobial resistance (J. P. Oliver et al., 2020).

Although antibiotic resistance associated with the pathogens of subclinical bovine mastitis has been reported in Brazil and other countries, similar work has never been conducted in northwest Paraná, Brazil. This study is in line with global co-operative efforts, such as the Tripartite Alliance between the WHO, FAO, and OIE, to address antimicrobial resistance (Rousham et al., 2018). Regarding this issue, studies in this field could help to clarify the spread of drug-resistant strains of animal origin through environmental routes. It is well known that propagation of antimicrobial resistance is beyond geographical borders and can traverse among animals and humans across countries (Sharma et al., 2018).

This study aimed at investigating the most frequent microorganisms associated with subclinical mastitis in dairy cows in the northwest State of Paraná, Brazil and its sensitivity to antibiotics. The study was done on milk collected from 452 cows from 44 dairy farms located within 13 cities from the northwestern region of the State of Paraná, Southern Brazil (Figure 1).

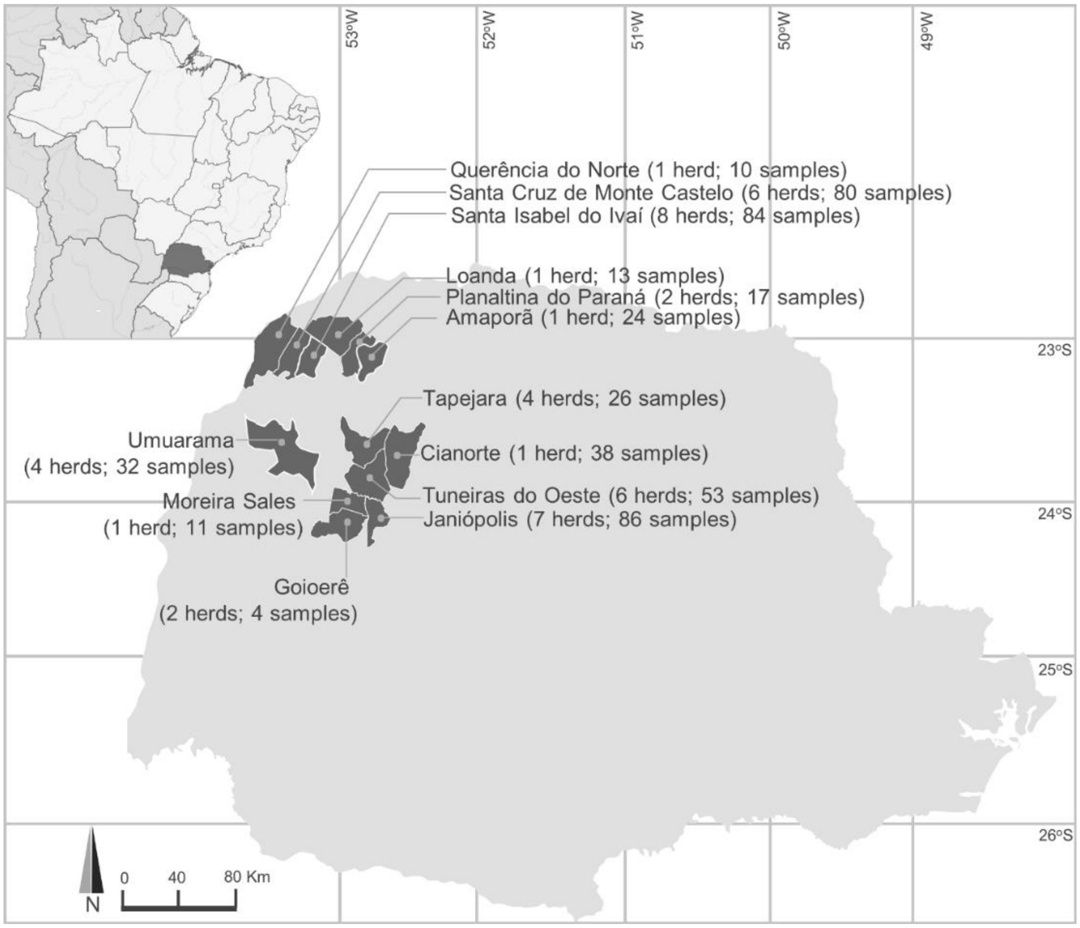

Figure 1. Geographic distribution of the 44 dairy herds and milk samples collected from the northwestern region of the State of Paraná (Brazil) between 2018 November and 2019 April.

A cross-sectional study was performed on commercial dairy herds that supplied milk to a dairy plant in Umuarama, Paraná. From November 2018 to April 2019 all commercial dairy herds (44) were recruited for the study. Out of these, foremilk samples exhibiting two consecutive positive scores on the California Mastitis Test (CMT) were considered for sampling. The CMT was routinely performed weekly at all farms. Most of these proprieties were small dairy farms, that used family labor and mechanical milking, having an average of 16 ± 5.9 cows per farm and mean daily milk production of 14 ± 7.4 L/cow. Most herds consisted of cows of the Holstein-Friesian breed.

A total of 478 foremilk samples were aseptically collected following the Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality (S. P. Oliver, 2004). Before milking, teat ends were cleaned with 70% ethanol and the first three squirts were discarded. A total of 10 mL of milk from each quarter were collected in sterile tubes. All samples were cooled with ice packs and immediately transported to the laboratory for microbiological analyses.

The microbiological cultures of milk samples were performed according to Winn et al. (2012), with approximately 10µL seeded on blood agar plates with 5% defibrinated ovine blood, followed by incubation at 37 °C for 48 h in aerobic conditions.

After incubation, a milk sample was considered positive if: (1) at least one colony of any pathogen was observed on the agar plate; (2) two or more colonies for coagulase negative staphylococci; (3) at least five colonies for *Bacillus* spp. Samples leading to the isolation of more than three species were considered as contaminated and discarded. Samples that produced three or less pathogens were considered as mixed infections.

The identification of colonies was performed by evaluation of morphology, hemolysis, and Gram staining. Further differentiation between genera were performed according to S. P. Oliver (2004) and Winn et al. (2012). For Gram positive cocci, catalase positivity was determined to distinguish between streptococci and staphylococci.

Staphylococci were further characterized by mannitol salt agar selective medium and tube coagulase test (ovine plasma). Coagulase positive and mannitol positive strains were characterized as *Staphylococcus aureus* (Kateete et al., 2010).

The streptococci characterization was confirmed by a combination of sorbitol and mannitol fermentation and the hydrolysis of arginine and aesculin. *Streptococcus mutans* were characterized by positive sorbitol and mannitol, negative arginine and positive aesculin. *Streptococcus uberis* or *S. parauberis* were characterized by positive sorbitol and mannitol, positive arginine and aesculin. *S. sobrinus* were characterized by negative sorbitol and positive mannitol. *Streptococcus sanguis* were characterized by negative sorbitol and mannitol, negative aesculin and positive arginine. *Streptococcus mitis* were characterized by negative sorbitol and mannitol, negative arginine and aesculin. Streptococci with β-hemolysis and negative aesculin were identified as *Streptococcus agalactiae* (Winn et al., 2012).

Corynebacterium spp. were identified based on macro and microscopical morphology by Gram staining, and culture characteristics, such as Gram-positive rods, pleomorphic, slightly curved with tapered or clubbed ends, with singly cells or in pairs, often in a "V" formation, similar to "Chinese letters". *Corynebacterium* spp. were further identified by nitrate reduction, urease activity and the presence of hemolysis on blood agar (Funke & Bernard, 2015).

Bacillus spp. were identified by their distinct phenotypic and morphological characteristics, such as Gram-positive rod-shaped and straight cells, often arranged in pairs or chains, with rounded or squared ends, and oval, round or cylindrical endospores (Bergey, 1994).

Small β-hemolytic colonies, Gram-positive rods with branching bacilli with negative reaction in the catalase were identified as *Trueperella* spp. (Tomazi et al., 2018).

The disk-diffusion method on Mueller-Hinton agar were performed following the Clinical and Laboratory Standards Institute (CLSI, 2018), classifying the isolated pathogens as susceptible or resistant. The antibiotics discs used were: (1) association of Amoxicillin and Clavulanic acid (AMC) (2:1), (2) association of Gentamicin and Amoxicillin (GEN) (1:1), and (3) Ceftiofur (CTF), all with antibiotic concentration of 30 µg per disc.

Differences in the distribution of pathogens were tested using the Chi square analysis when the absolute frequency were greater than 5 and using Fisher's test otherwise (both at 5% significance level). The proportion of isolates exhibiting resistant phenotypes was calculated and presented as prevalence of antimicrobial susceptibility, dividing the number of resistant isolates by the total isolates tested.

Regarding the proportion of herds affected, almost all (95%) showed at least one isolate. *Staphylococcus aureus* (27%), followed by *Streptococcus sanguis* (25%), and *Bacillus* spp. (25%) were the most frequently disseminated species across the 44 dairy herds studied (Table 1). A total of 478 foremilk samples from quarters with subclinical mastitis were analyzed, with positive microbiological culture being isolated from 62% (295/478) of these samples (Table 1).

Considering the sum of simple and mixed infections, Streptococci organisms were the most frequently isolated and contributed towards 63% (187/295) of all pathogens identified in cows with subclinical mastitis from Northern Paraná, followed by Staphylococci (31%) and other members of other genera (6.5%) (X^2= 146.29; p<0.01).

Although *Streptococcus mutans* (44%), *Streptococcus sanguis* (42%), and *Streptococcus uberis* (47%) were the most frequently identified species, the proportion between these three species were equally distributed (X^2= 0.63; p=0.73). The equal distribution between these members of *Streptococcus* identified in this study is not frequently described, since in most studies *Streptococcus uberis* is the most frequently identified species in cows with mastitis (Valmorbida et al., 2017). The clinical importance of this finding is that some virulence factors involved in streptococcal pathogenicity are species-specific, what can lead to the production of antibiotic-resistance proteins, an important information to improve the treatment and minimize clinical complications due to resistant strains (Abril et al., 2020).

Regarding the considerable proportion of *Streptococcus sanguis* (13%) found in this study, it is important to point out that this specie is not frequently identified in cattle. Most commonly, *S. sanguis* are associated with oral health, being involved in the development of dental caries (Gomez et al., 2021). This finding suggests contamination during the milking process through a route that deserve to be further investigate, which should be related with human contamination or via the oral-to-udder transmission among calves.

Among Staphylococci, the most frequently was *S. aureus,* with 10.5% of occurrence among the identified pathogens. Differently from most studies and literature reviews that consider *Staphylococcus* spp. as the most frequent pathogen isolated from subclinical mastitis in dairy cows, followed by *Streptococcus* spp., we found a higher proportion of streptococci, which reinforces that the etiology of subclinical mastitis can differ regionally, and consequently shows the need for periodic monitoring for its control.

Table 1
Frequency of pathogens isolated from cows with subclinical mastitis

Pathogens isolated	Absolute frequency*	Relative frequency (%)	Herds affected (%)
Simple infections			
Streptococcus mutans	44	14.9	10 (23%)
Streptococcus sanguis	42	14.2	11 (25%)
Streptococcus uberis	37	12.6	10 (23%)
Streptococcus mitis	9	3.1	3 (7%)
Streptococcus sobrinus	7	2.4	4 (9%)
Streptococcus agalactiae	1	0.3	1 (2%)
Streptococcus spp.	24	8.1	21 (48%)
Subtotal	164	55.6^{a}	31 (70%)
Staphylococcus aureus	31	10.5	12 (27%)
Coagulase negative staphylococci	16	5.4	11 (23%)
Coagulase positive staphylococci	22	7.5	18 (41%)
Subtotal	69	23.4^{b}	27 (61%)
Bacillus spp.	22	7.5	11 (25%)
Trueperella spp.	9	3.1	5 (11%)
Corynebacterium spp.	7	2.4	4 (9%)
Yeast	1	0.3	1 (2%)
Subtotal	39	13.2^{c}	14 (32%)
Mixed infections			
Streptococcus. spp.+ *Staphylococcus.* spp.+ *Corynebacterium*	1	0.3	1 (2%)
Streptococcus. spp.+ *Staphylococcus.* spp.	20	6.8	10 (23%)
Trueperella spp. + *Streptococcus.* spp.	2	0.6	2 (5%)
Subtotal	23	7.8	12 (27%)
Total	295	100	42 (95%)

Footnote: Number of samples containing at least one isolate; a,b,c: proportions followed by different letters in the same column are significantly different by the Chi-square test ($p<0.05$).

Thus, we suggest that the causative agents of bovine mastitis depend on small-scale regional conditions, but further studies should be conducted in this sense Overall, 22% isolates showed resistance to the antibiotics used in this study (i.e., association of amoxicillin and clavulanic acid, association of gentamicin and Amoxicillin, and Ceftiofur

(Table 2). According to chi-square test, the antimicrobial resistance was proportional (X^2=1.273; p=0.5292) between the antibiotics and/or association of antibiotics used.

However, when categorized by genus, the antimicrobial resistance was not proportional between the three categories of antibiotics. For *Streptococcus* spp., the antimicrobial resistance was higher for Ceftiofur (X^2=7.091; p=0.0289), whereas for *Staphylococcus* spp. the antimicrobial resistance was lower for Ceftiofur (X^2=6.889; p=0.0319). It was also possible to verify a high occurrence of Gentamicin + Amoxicillin resistant *Staphylococcus* spp. (Table 2).

Table 2
Occurrence of antimicrobial resistance of pathogens isolated from subclinical bovine mastitis in northwest Paraná, Brazil between 2018 November and 2019 April

Antibiotic evaluated (number of analyzed samples)	Antimicrobial resistance (%)			
	All samples	Streptococci organisms	Staphylococci organisms	Other organisms
Amoxicillin + clavulanic acid (n=198)	24.4a	24.8a	25.8b	20.1a
Ceftiofur (n=212)	23.5a	27.1b	10.5a	20a
Gentamicin + Amoxicillin (n=204)	19a	14a	41.7b	22.2a
Total (n=614)	22.3	22	25.7	20.7

^{a,b}: proportions followed by different letters in the same column are significantly different by the Chi-square test (p<0.05).

The high proportion of ceftiofur resistant *Streptococcus* spp. isolates suggested that extended-spectrum beta-lactamase producing strains may be circulating in the investigated dairy farms within Northwestern Paraná.

A possible practical application of these results is that ceftiofur continues to maintain its antibacterial activity against Staphylococci genus, but not against Streptococci genus. Consequently, to increase the cure rates, is advisable to avoid Ceftiofur therapy when the etiologic agent belongs to Streptococci genus. Otherwise, it is highly recommended to use Ceftiofur when the etiologic agent belongs to Staphylococci genus. It should be highlighted that for subclinical mastitis the most appropriate treatment is the administration of antimicrobials at the beginning of the dry-period (Scherpenzeel et al., 2018).

The high occurrence of Gentamicin + Amoxicillin resistant *Staphylococcus* spp. (Table 2) contradicts the results from Costa et al., (2018), who found that Gentamicin and Amoxicillin was the most efficacious antimicrobial agents against *Staphylococcus* spp. Taken together, these results highlights that the antibiotic-resistance patterns of pathogens may differ at regional level once the investigation of Costa et al. (2018) was done in the Northeast of Brazil.

Finally, some limitations need to be considered. First, it is important to point out that there is a close association between seasonality, bacterial pathogens, and occurrence of antimicrobial resistance (Boireau et al., 2018). Thus, the current resistance profile may differ from the findings above since this research was conducted between 2018 November and 2019 April.

Nevertheless, the identification of the microbiological agent is essential to the most appropriate choice of the drug to be used in therapy. When possible, etiology should be determined before treatment, and the spectrum of antimicrobial activity should be appropriate for the etiological agent (Ruegg, 2020).

We found a particular profile of pathogens with high rates of antimicrobial resistance circulating in cows with subclinical mastitis from northern Parana. Thus, it is important that the antibiotic treatment decisions should be based on prior resistance tests to avoid the environmental spread of pathogenic strains resistant to antibiotics in dairy cattle populations.

Acknowledgments and Funding Information

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript. The authors would like to thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior Coordination for the Improvement of Higher Education Personnel) and FUNADESP (Fundação Nacional de Desenvolvimento do Ensino Superior Particular-National for fellowship.

References

Abril, A. G., Carrera, M., Böhme, K., Barros-Velázquez, J., Rama, J. L. R., Calo-Mata, P., & Villa, T. G. (2020). Proteomic characterization of antibiotic resistance, and production of antimicrobial and virulence factors in streptococcus species associated with bovine mastitis. Could enzybiotics represent novel therapeutic agents against these pathogens? *Antibiotics, 9*(6), 1-24. doi: 10.3390/antibiotics9060302

Bergey, D. H. (1994). *Bergey's manual of determinative bacteriology.* Lippincott Williams & Wilkins.

Boireau, C., Cazeau, G., Jarrige, N., Calavas, D., Madec, J. Y., Leblond, A., & Gay, É. (2018). Antimicrobial resistance in bacteria isolated from mastitis in dairy cattle in France, 2006-2016. *Journal of Dairy Science, 101*(10), 9451-9462. doi: 10.3168/jds.2018-14835

Clinical and Laboratory Standards Institute. (2018). *Performance standards for antimicrobial susceptibility testing* (13th ed.) Wayne, PA: Clinical and Laboratory Standards Institute.

Costa, F. N., Belo, N. O., Costa, E. A., Andrade, G. I., Pereira, L. S., Carvalho, I. A., & Santos, R. L. (2018). Frequency of enterotoxins, toxic shock syndrome toxin-1, and biofilm formation genes in *Staphylococcus aureus* isolates from cows with mastitis in the Northeast of Brazil. *Tropical Animal Health and Production, 50*(5), 1089-1097. doi: 10.1007/s11250-018-1534-6

Funke, G., & Bernard, K. A. (2015). Coryneform Gram-positive rods. In J. H. Jorgensen, K. C. Carroll, G. Funke, M. A. Pfaller, M.

L. Landry, S. S. Richter, & D. W. Warnock (Eds.), *Manual of Clinical Microbiology* (11th ed, Cap. 28, pp. 474-503). doi: 10.1128/9781555817381.ch28

Gomez, D. L. S., Lozano, A. S., Alanis, H. F. M., Capetillo-Hernandez, G. R., Capetillo, E. G. T., Cepeda, S. E. N., & Solis-Soto, J. M. (2021). Streptococcus: an orthodontic point of view. *International Journal of Applied Dental Sciences, 7*(3), 237-241. doi: 10.22271/oral.2021.v7.i3d.1306

Kateete, D. P., Kimani, C. N., Katabazi, F. A., Okeng, A., Okee, M. S., Nanteza, A., & Najjuka, F. C. (2010). Identification of *Staphylococcus aureus:* DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. *Annals of Clinical Microbiology and Antimicrobials, 9*(1), 1-7. doi: 10.1186/1476-0711-9-23

Oliver, J. P., Gooch, C. A., Lansing, S., Schueler, J., Hurst, J. J., Sassoubre, L., & Aga, D. S. (2020). Invited review: fate of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in US dairy manure management systems. *Journal of Dairy Science, 103*(2), 1051-1071. doi: 10.3168/jds.2019-16778

Oliver, S. P. (2004). *Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality.* National Mastitis Council.

Rousham, E. K., Unicomb, L., & Islam, M. A. (2018). Human, animal and environmental contributors to antibiotic resistance in low-resource settings: Integrating behavioural, epidemiological and one health approaches. *Proceedings of the Royal Society B: Biological Sciences, 285*(1876), 1-9. doi: 10.1098/rspb.2018.0332

Ruegg, P. L. (2020). Understanding the economic impact of mastitis therapy - the role of duration and drug selection. *American Association of Bovine Practitioners Proceedings, 53*(1), 84-91. doi: 10.21423/aabppro20207976

Scherpenzeel, C. G. M., Hogeveen, H., Maas, L., & Lam, T. J. G. M. (2018). Economic optimization of selective dry cow treatment. *Journal of Dairy Science, 101*(2), 1530-1539. doi: 10.3168/jds.2017-13076

Sharma, C., Rokana, N., Chandra, M., Singh, B. P., Gulhane, R. D., Gill, J. P. S., & Panwar, H. (2018). Antimicrobial resistance: its surveillance, impact, and alternative management strategies in dairy animals. *Frontiers in Veterinary Science, 4*(2), 1-12. doi: 10.3389/fvets.2017.00237

Tomazi, T., Ferreira, G. C., Orsi, A. M., Gonçalves, J. L., Ospina, P. A., Nydam, D. V., & Santos, M. V. dos. (2018). Association of herd-level risk factors and incidence rate of clinical mastitis in 20 Brazilian dairy herds. *Preventive Veterinary Medicine, 161*(1), 9-18. doi: 10.1016/j.prevetmed.2018.10.007

Valmorbida, M. K., Santos Carneiro, D. C. dos, Prior, K. C., Griebeler, E., Troncarelli, M. Z., & Dezen, D. (2017). Etiology and in vitro antimicrobial susceptibility profile of strains isolated from bovine mastitis in dairy herds from the midwest region of Santa Catarina state, Brazil. *Acta Veterinaria Brasilica, 11*(4), 219-225. doi: 10.21708/avb.2017.11.4.7266

Winn, J. W., Allen, S., Janda, W., Koneman, E., Procop, G., Schreckenberger, P., & Woods, G. (2012). *Diagnóstico microbiológico: texto e atlas colorido.* Guanabara Koogan.