REVIEW ARTICLE

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Proposal for the design of studies of intramammary antimicrobials to be conducted in Brazil for registration with the Ministry of Agriculture and Livestock

Proposta de delineamento de estudos de antimicrobianos intramamários a serem realizados no Brasil para registro no Ministério da Agricultura e Pecuária

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ABSTRACT

Before being made available to the Brazilian market, all veterinary medicines must be registered with the Ministry of Agriculture and Livestock after their efficacy, safety, and quality have been proven through clinical studies and scientific literature. Depending on the product class, the studies required by the regulatory authority must be carried out per Brazilian regulations and international reference guides. Some international organizations pursue the harmonization of these requirements, aiming at the mutual acceptance of studies conducted in different regions to facilitate international trade and reduce the use of animals in clinical research. Mastitis is one of the most prevalent and costly diseases in dairy cows, and it is associated with negative impacts on milk production and quality, cow welfare, and the profitability of the dairy industry. Normative Instruction No. 26/2009 determines a few rules for conducting studies for registering intramammary antimicrobials for cows with mastitis. However, the regulated sector and researchers report difficulties in following the recommendations of specific guidelines, which complement this regulation due to the peculiarities of Brazil's production systems. This review article aims to provide subsidies and orientations that scientifically support the conduction and critical analysis of clinical studies proving the efficacy of intramammary products for treating clinical and subclinical mastitis in cows. Considering the need for scientific rigor in the studies, the recommendations available in international guidelines, and the need to adapt the protocols to the current situation of veterinary clinical research in Brazil, this document is intended to contribute to the internal harmonization of experimental protocols that support both the regulated sector and the regulatory authority.

Keywords: Intramammary antimicrobial product. Bovine mastitis. Clinical study design. Veterinary drug. MAPA regulatory.

RESUMO

Todo medicamento veterinário, antes de ser disponibilizado ao mercado brasileiro, deve ser registrado no Ministério da Agricultura e Pecuária após comprovada sua eficácia, segurança e qualidade através de estudos clínicos e/ou literatura científica. A depender da classe de produto, os estudos que são exigidos pela autoridade regulatória devem ser realizados conforme normativas brasileiras e/ou guias internacionais de referência. A harmonização dessas exigências é perseguida por alguns organismos internacionais, visando à aceitação mútua de estudos conduzidos em diferentes regiões para facilitar o comércio internacional e permitir a redução do uso de animais em pesquisas clínicas. A mastite

é uma das doenças mais prevalentes e onerosas em vacas leiteiras e está associada a impactos negativos na produção e na qualidade do leite, no bem-estar das vacas e na rentabilidade do setor. A Instrução normativa nº 26/2009 determina algumas diretrizes para a condução dos estudos para o registro de antimicrobianos intramamários para vacas com mastite, mas o setor regulado e os pesquisadores relatam dificuldades em seguir as orientações de guias específicos, que complementam essa norma, devido às peculiaridades dos sistemas de produção brasileiros. Este artigo de revisão busca fornecer subsídios e orientações que amparem cientificamente a condução e a análise crítica dos estudos clínicos de comprovação da eficácia de produtos intramamários indicados para o tratamento de mastites clínicas e subclínicas. Considerando a necessidade de rigor científico dos estudos, as recomendações disponíveis nos guias internacionais e a necessidade de adequação dos protocolos à atual conjuntura da pesquisa clínica veterinária no Brasil, este documento visa contribuir para que sejam harmonizados internamente protocolos experimentais que subsidiem tanto o setor regulado quanto a autoridade reguladora.

Palavras-chave: Produto antimicrobiano intramamário. Mastite bovina. Delineamento de estudo clínico. Medicamento veterinário. Normativas MAPA.

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The Forum for Technical-Scientific Discussion on the Rational Use of Antimicrobials (ATMs), with a focus on bovine mastitis (BM), held on July 3 and 4, 2023, in the city of São Paulo/SP, Brazil, was planned to highlight the challenges in clinical research and discuss the causes of the disease, its clinical aspects, diagnosis, prevention, and treatment. From the event, recommendations were proposed for clinical study protocols to ensure the scientific quality of the data that support the registration of IMM ATMs with the Ministry of Agriculture and Livestock of Brazil (MAPA).

Mastitis is one of the most prevalent and costly diseases in dairy cows and the predominant cause of ATM use in adult dairy cows (Seegers et al., 2003; Ruegg, 2017). The disease harms the quantity and quality of milk produced, cow welfare, and the sector's profitability. These impacts lead to economic losses and various costs to the dairy industry that are influenced by several factors and are often underestimated by producers (Aghamohammadi et al., 2018; DeGraves & Fetrow, 1993). It also has public health implications because of global bacterial resistance concerns (Tang et al., 2017). Because BM is a disease with multifactorial causes, it is necessary to adopt control programs that include prevention measures, good agricultural practices, and safe and effective veterinary products.

Diagnosing BM by laboratory methods is essential for defining strategies for preventing, controlling, and treating cases (Adkins & Middleton, 2018). There are different laboratory diagnostic methods, such as standard plate microbiological culture, chromogenic microbiological culture, Matrix-Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF), and molecular techniques based on bacterial DNA amplification and sequencing (PCR), among others. These methods have advantages and disadvantages, but none are 100% accurate. However, four points are essential for the correct choice of method: collection procedures, the characteristics of the pathogenic agent, and its accuracy and cost-effectiveness (Ashraf & Imran, 2018). Thus, the method of diagnosing BM is highly relevant and must be associated with the correct interpretation of the results; in this manner, actions to combat BM can be defined and implemented effectively. In Brazil, Zambelli (2023) conducted a longitudinal study from 2012 to 2020 with 3,793 dairy farms that submitted milk samples for mastitis diagnosis to a reference laboratory (VidaVet, 2023). From a total of 679,706 microbiological results, 30% were diagnosed with contagious pathogens, and a slight reduction in the prevalence of these pathogens was observed during the study period.

The development of the disease depends on several factors, including the cow's inflammatory response, the dynamics of the agent in the mammary gland, and the mechanisms of microorganism escape, which influence the interpretation of laboratory test results (Fredebeul-Krein et al., 2022). Targeted treatments supported by laboratory diagnosis help restore animal welfare, milk quality for the final consumer, the productive and reproductive performance of the herd, and consequently, the farm's profitability.

Regarding the treatment and prognosis of BM, there are extreme cases, from those in which the cure is spontaneous (i.e., without the need for treatment) to cases in which treatment is not indicated due to the minimal chance of cure. Thus, by using the most appropriate antimicrobial agent or even choosing not to treat the animal, the ATMs are used rationally, which aligns with global concern about bacterial resistance to ATMs. However, for the proper use of the products available for treatment, it is essential that clinical studies follow the most appropriate protocols and that the information in the package insert reflects the evidence provided by well-conducted studies, i.e., proof of efficacy and safety in the treatment of clinical or subclinical BM in lactating or dry cows, target pathogens, and frequency and duration of treatment, among others. In approximately 70% of cases treated, it can be inferred that there was a cure through bacteriological evaluation. With the use of intramammary (IMM) ATMs, the cure rate is approximately 69%; in the association of IMM ATMs + systemic ATMs, the cure rate is approximately 68%, while without treatment, the cure rate reaches 60% (Ruegg, 2021). The treatment of dry cows, i.e., preventively treating cows with IMM ATMs when drying off, has been carried out by approximately 80% of large farms, approximately 70% of medium farms, and 50% of small farms (Martin, 2022). In general, the massive treatment of dry cows is carried out. In Brazil, few producers report that they analyze the history of the cows in this decision-making process or if they opt for selective treatment.

Brazil has the third largest dairy herd in the world, with approximately 17,065,000 heads, placing it the fifth largest milk producer worldwide (United States Department of Agriculture, 2023). MAPA is legally responsible for the inspection of products for veterinary use, as well as for the registration and licensing of these products, as determined by Article 3 of Decree-Law No. 467/1969 (Brasil, 1969) and Article 24 of the Regulation annexed to Decree No. 5053/2004 (Brasil, 2004). This regulation represented a milestone for Brazil to begin its alignment with developed countries with the requirements for veterinary products to be marketed in Brazilian territory.

Approximately 50 IMM ATMs indicated for BM treatment and prevention are currently registered with MAPA (Brasil, 2023). To be registered, all medicines must prove their efficacy and safety through clinical studies (for cows and humans, consumers of dairy products). These complex studies are, therefore, costly but essential to establish the best dosage, safety of use, and susceptibility of pathogens.

Specific requirements for the registration of veterinary ATMs were put forth after the publication of MAPA Normative Instruction No. 26 of July 9, 2009 (IN 26/2009) (Brasil, 2009). This regulation approved the "*Technical Regulation for the Manufacture, Quality Control, Commercialization and Veterinary Use of ATM Products*", which provides guidelines for conducting studies on ATMs' efficacy, safety, and withdrawal period. IN 26/2009, however, makes only a nominal reference to IMM products in Article 12, in which IMM-administered products must be sterile. In relation to studies to prove efficacy, in a generalized manner for all ATM products, this regulation advises that studies a) be performed with the recommended dosage; b) demonstrate efficacy against etiologic agents and in all animal species for which the product is indicated; c) may be performed *in vivo* with naturally or experimentally infected animals under controlled conditions; they may be performed *in vivo* with healthy animals, correlating the pharmacokinetic profile of the administered drug and the effective plasma concentration, with *in vitro* studies for the determination of the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) of each etiologic agent for which the product is determined; d) determine MIC and MBC values according to the protocols standardized by the *Clinical and Laboratory Standards Institute* (CLSI); e) preferably be carried out with a strain culture bank from Brazil; f) have a sample size that is statistically justified or by means of internationally recognized references; and g) be carried out in accordance with good veterinary clinical practice (GCP), according to nationally or internationally recognized references.

As anticipated in this normative act, IN 26/2009 regulates the execution of the regulation approved by Decree 5053/2004. It cannot transpose or innovate concerning the norm it complements. Therefore, in IN 26/2009, only basic principles are defined to prove the ATM withdrawal period's efficacy, safety, and definition. Any details regarding the design of the studies should be based on specific guides prepared by experts and international regulatory authorities, which are currently the technical references available and accepted by MAPA (Brasil, 2004). Therefore, the current review was based on guidelines from agencies in different countries (Australian Pesticides and Veterinary Medicines Authority, 2023; European Medicines Agency, 2016, 2017b; National Mastitis Council, 2001; South Africa, 2013; U.S. Food & Drug Administration, 2018).

In general, these guides have several similarities: a) the essentiality of carrying out field studies under practical conditions of use; b) multicenter, representative studies of the conditions under which the product will be marketed, taking into account differences in animal husbandry systems,

geographical location and climate, and studies carried out following GCP; c) inclusion in studies of cows with clinical or subclinical BM, depending on the indication of the product under test; d) treatment success measured through clinical and bacteriological parameters; e) bacteriological cure, measured by isolation of the causative agent; f) clinical cure assessed by the appearance of the milk and the clinical conditions of the udder (no inflammatory signs such as pain, heat, flushing, turgor, or loss of function); and g) all indicators (microbiological and clinical) evaluated before and after treatment.

The studies must be conducted in at least two geographic and climatological regions for the Food and Drug Administration (FDA) and the Department of Health of the Republic of South Africa (DoH). Concerning the FDA, the guidelines recommend evaluation in at least six different herds. On the other hand, the DoH guide suggests that at least three herds should be used. The EMA and DoH stress that the number of cows in a single herd should not exceed 20% of the total number of animals included in the study. In addition, the FDA guidelines warn that herds selected for clinical BM studies must have enough affected cows to allow for a block design. The study protocol should define the number of herds and blocks in each herd. This suggests that a prestudy should be carried out with a survey of the possible infections of the available herds so that this previous sampling facilitates the homogeneous distribution of pathogens among the treatment groups (U.S. Food & Drug Administration, 2018).

However, regarding FDA guidelines, the interested party must justify the choice of the positive control product concerning the indications and the target population of cows proposed for the product under test. In this sense, according to the American guidelines, treatment options for the control group include a) in clinical BM studies, i) use of a registered product or ii) milking three to four hours apart for 36 hours; b) in subclinical BM studies, i) no use of any product or ii) administration of placebo (formulation without the active ingredient).

For the Australian Pesticides and Veterinary Medicines Authority (APVMA), the cattle used in these evaluations must belong to at least three different herds, and studies conducted in Australia are recommended; however, if the evaluation is performed in other countries, the design applied, the bacterial strains, the methodology for defining the bacterial infection, and the sampling protocols must follow the criteria established by the APVMA. The APVMA also highlights the following general conditions for field studies: diagnosis in an accredited pathology laboratory; commitment and profile of the producer; individual and unambiguous identification of animals; proximity to the farm of a trained microbiology laboratory; exclusion of cows with chronic or recurrent BM; and care taken not to include false-positive cases in studies (Australian Pesticides and Veterinary Medicines Authority, 2023).

The European Medicines Agency (EMA) guideline advises that cows should be selected from herds whose animals have individual identification and health records, as well as a monthly tank somatic cell count history. In addition, the number of daily milkings should be the same in all herds participating in the study. Studies should be controlled and randomized, and masking should be applied to treatment groups (European Medicines Agency, 2016).

Considering the SCC, the EMA guideline indicates that one of the inclusion criteria in studies of subclinical BM is an SCC>200,000 cells/mL in one of the pretreatment samples (European Medicines Agency, 2017b). The FDA notes that the quarter's SCC should be evaluated within the cure (success) and non-cure (failure) groups. This assessment of the SCC trend will indicate whether further studies are needed to assess safety (inflammation) (U.S. Food & Drug Administration, 2018). According to DoH criteria, the SCC must be ≥ 300,000 cells/mL for BM to be determined (South Africa, 2013).

Each regulatory body referenced in this document states that sampling and bacteriological diagnosis should be conducted with a methodology standardized by the National Mastitis Council (2001) or another scientific reference. In addition, these governmental agencies suggest that the product's efficacy must be demonstrated statistically for each bacterium individually, and pathogens must be related to the intended use of the product, i.e., whether they are prevalent in lactating cows or dry cows.

This review's purpose is to provide recommendations based on international guidelines for studies on the efficacy of IMM ATMs conducted in Brazil, ensuring alignment with the directives outlined in IN 26/2009 (Brasil, 2009).

It is vital to define clinical and subclinical BM in the context of the studies to define the animal inclusion and exclusion criteria. Clinical BM presents clear signs of inflammation in one or more quarters (swelling, warmth, pain, flushing) and changes in milk appearance (clots or flakes, watery appearance, discoloration), with or without general clinical signs (fever, loss of appetite), but with a high somatic cell count (SCC>200,000 cell/mL) in quarter milk and with or without bacteriological isolation in milk (Smith et al., 2001). All ATM products for BM in lactating dairy cows should be adequate for clinical BM. In contrast to clinical BM, subclinical BM is not characterized by overt inflammatory symptoms. This condition is asymptomatic and is diagnosed through the analysis of udder secretions that appear clinically normal. Unlike clinical

manifestations, subclinical BM lacks acute and visible changes in the udder tissue. The secretions from the affected quarters have an increased somatic cell count (SCC>200,000 cells/mL for a composite sample) and positive bacteriological isolation (International Dairy Federation, 2013).

Initially, it is advised that field studies should be carried out in Brazil. Suppose studies have been conducted in other countries. In that case, it is understood that these investigations can be considered a weight of evidence on the product's efficacy under test, provided that the protocols applied have followed the same criteria established in this review. However, if studies conducted in other countries have not evaluated the efficacy of the product against pathogens that are important causes of infectious BM in Brazil, it is understood that complementary studies should be conducted to evaluate the efficacy against these bacteria. After all, it is understood that there is no technical justification for placing on the national market a product indicated for BM that has not proven its efficacy against the primary pathogens circulating in Brazilian territory.

However, it is essential to note that the same species of bacteria may have different resistance profiles depending on the environment and the selection pressure to which they are exposed, and excessive use or misuse of antimicrobials in dairy farms leads to the development of antimicrobial-resistant bacteria (Abdi et al., 2021). For studies carried out in other regions of the world, it is recommended that technical reasoning be presented based on internationally accepted scientific studies regarding the bacteria tested (genus and species) and their resistance profile compared to the same strains prevalent in Brazil.

Given the definitions of BM for the studies and the strict criteria for inclusion, exclusion, and withdrawal of animals, which will be detailed below, it is understood that there is no need to determine, *a priori*, the minimum number of herds that should be included in the studies. However, the interested parties must technically justify the choice of animals, herds, and farms based on some criteria, taking into account individual milk production at the time of treatment; race or genetic makeup; the number of lactations; date of parturition; individual CCS history; history of treatments for BM; general herd health records; production systems (type of housing, feeding, management for drying the cow, use of ATMs); the number of cows in the herd; milking method; teat disinfection procedures; geographic location of the farm and climate of the region; and technical justification for relevant changes in the study design (with the reference guides), such as, for example, the absence of a negative control group.

There are exclusion criteria for animals that are common to the studies: a) cows with a concomitant disease that compromises the study (e.g., metritis, retained placenta, respiratory disease); b) cows that have received ATM or anti-inflammatory treatment previously that may influence the study; c) cows that have received previous treatment that produces an immune-mediated response against BM and that may influence the study; d) cows with teat end lesions (e.g., hyperkeratosis); e) cows with clinical signs requiring systemic treatment; and f) cows with a history of average milk production of less than five liters per day or that at the time of selection are producing less than five liters per day. It is important to emphasize that for the inclusion of animals that were previously treated with any drug that may influence the study, the interested party must technically justify the period since the last treatment based on information about the time of permanence of the drug or its active residues in the breast tissue.

The individual cow is typically the most suitable experimental unit for clinical trials. While the mammary quarter may act as the experimental unit for IMM products under certain conditions, as per Thorburn (1990), this methodology is often not advisable. The rationale for this recommendation is that the physicochemical properties of the pharmaceutical agent, coupled with the inflammatory state of the mammary quarters, might facilitate the transference of the medication across different quarters (Gehring & Smith, 2006; Thorburn, 1990; Ziv & Sulman, 1975). Hence, when employing the mammary quarter as an experimental unit, it is crucial to substantiate the absence of experimental bias, as emphasized by Thorburn (1990).

Lactating cows: recommended study protocols

Cows should be systematically selected from all three phases of lactation: early, mid, and late, except for those in the last 30 days before lactation cessation. The group should include a balanced distribution of individuals with high (>30 L/day), medium (between 10 and 30 L/day), and low (between 5 and 10 L/day) milk production to ensure a comprehensive representation of lactational productivity variations. Any animal selection that is not included in these requirements must be technically justified based on scientific data from the literature.

Products indicated for clinical mastitis

Only those cows with clinical BM in a single mammary quarter should be selected for the study as long as they do not present symptoms that require systemic treatment. If any of the cows involved develop clinical BM in other quarter(s) during the study, they should be excluded.

The treatment unit and the statistical unit are the mammary quarter, and several quarters must be selected to allow the product's efficacy to be statistically demonstrated for each bacterium individually. The treatment groups are the treated and positive control groups, noting that only the quarter affected will be treated.

In the pretreatment evaluation, the milk from the affected quarter should be positively isolated for the target bacterium(s) in two pretreatment samplings. Exceptions should be made for contagious pathogens of the mammary gland (*Staphylococcus aureus* and *Streptococcus agalactiae*), given their characteristic of intermittent elimination through milk. Thus, only a positive pretreatment result qualifies the animal for inclusion in the study. For all other agents, both pretreatment specimens must be positive for the agent. An SCC of the fourth should be performed, and the cow should be clinically assessed, including udder and milk appearance.

In the posttreatment evaluation, bacteriology should be performed on two samples from all quarters between days 14 and 28 post-treatment, with at least seven days between examinations. A clinical examination should also be performed at the time of the first sampling, and if there is no clinical cure, the cow should be excluded from the subsequent sampling. An SCC is performed only in the second sampling, i.e., the SCC will not be performed in animals that did not present a clinical cure in the first posttreatment sampling.

Treatment success occurs when there is a clinical cure during the first posttreatment sampling and bacteriological cure in both posttreatment samplings. In posttreatment evaluations, cows that are infected with pathogens different from the original pathogen targeted for treatment with the product and show recovery from this targeted agent can be included as those with a bacteriological cure. These new infections should be included in the final report and, depending on their incidence, will require in-depth analysis.

For the purpose of the evaluation, the following are considered failures: when there is no clinical cure in the first posttreatment sampling; if the pathogen isolated at the time of inclusion is still present in at least one of the posttreatment samples; and if additional treatment for BM is needed during the trial period.

Products indicated for subclinical mastitis

Cows with subclinical BM in a single mammary quarter should be selected for the study, from which the same bacteria should be isolated in two pretreatment samples. In addition to isolation, there should be an SCC > 200,000 cells/mL in at least one pretreatment sampling.

Unlike the study of products for clinical BM discussed previously, this protocol recommends a design in which there is a treated group and a negative control group. That is, no product will be administered, or only a placebo will be used (which is the same formulation used in the treated group but without containing the active ingredient). Only the selected quarter of the cows in the treated group will receive the ATM. Cows in the control (negative) group will not be treated.

The treatment unit and the statistical unit are the mammary quarter. Several quarters with subclinical BM should be selected to allow the product's efficacy to be statistically demonstrated for each bacterium individually.

In the pretreatment period, the same pathogen should be isolated in two samplings with an SCC>200,000 cells/mL in at least one of these samplings. The two samplings, with an interval of one to three days between them, should be performed individually for each mammary quarter. If a pathogen is isolated in only one of these samples, a third (for confirmation) should be taken. The SCC should be determined in one of these samples.

In the posttreatment evaluation, bacteriology should be performed on two samples from all quarters between days 14 and 28 after treatment, with at least seven days between exams. The SCC is performed only in the second sampling.

Treatment succeeds when the parent pathogen is not detected in post-treatment samplings. As a secondary parameter, the decrease in SCC is evaluated, which must be substantial. The SCC is a secondary outcome for evaluating efficacy, but the study report should present the statistical analysis of these data. The relevant decrease in SCC supports the conclusion that the product under test is adequate. In posttreatment evaluations, cows that are infected with pathogens different from the original pathogen targeted for treatment with the product and show recovery from this targeted agent can be included as those with a bacteriological cure. These new infections should be included in the final report and, depending on their incidence, will require in-depth analysis.

Treatment failure occurs when the parent pathogen is present in one of the posttreatment samples or if any additional treatment for BM is needed during the trial period.

Dry cows: recommended study protocols for the evaluation of products indicated for the treatment of subclinical mastitis in drying and the prevention of new infections during the dry period

In addition to the exclusion criteria standard for all studies, some points should be highlighted in ATM studies indicated

during drying. Although some international guidelines for the study of ATM efficacy for dry cows establish that cows that in the previous dry period received treatment for clinical or subclinical BM should be excluded from the study, this is a very restrictive criterion since 40 to 50% of cows have subclinical BM, and approximately 8 to 10% have clinical BM; therefore, there is a high probability that these animals received treatment in the previous dry period. Furthermore, if this exclusion criterion were established, it would be necessary to restrict the studies only to those farms that control individual SCCs monthly, and the control of bulk SCCs would not be helpful.

In investigations intended to substantiate the effectiveness of treating subclinical BM in dry cows and those aimed at demonstrating the prevention of new infections, it is crucial to ensure that the bovine subjects are suitably prepared for the drying-off procedure and possess a projected dry period of no less than 35 days. These two studies may be conducted concurrently on the same animal but must be localized to distinct mammary quarters to maintain experimental integrity and clear differentiation of outcomes.

For the study of the treatment of subclinical BM during drying, cows with at least one quarter infected subclinically, that is, the presence of the same pathogen in two pretreatment samplings and an SCC>200,000 cells/mL in one of the samplings, should be selected.

For the study on the prevention of new infections during the dry period, the selected cows should have healthy mammary quarters, meaning free from any pathogens in two pretreatment samplings and a somatic cell count (SCC) of less than 200,000 cells/mL in at least one of the samples.

In these protocols, separating the animals into a treated group and a negative control group is also recommended. The unit of treatment is the cow, and the statistical unit is the individual mammary quarter. To evaluate a product indicated for the treatment of subclinical BM in drying, several quarters with subclinical BM should be selected to allow the efficacy of the product to be statistically demonstrated for each bacterium individually. In the case of products indicated for prevention, several quarters with negative isolation should be selected to allow the preventive efficacy of the product to be statistically demonstrated.

At the time of drying, all mammary quarters of the cows in the treated group will be treated, meaning infected and noninfected quarters of the same cow. Establishing appropriate measures regarding animal welfare is essential for cows in the control group.

In the pretreatment period, the SCC of the quarter should be performed for one of the milk samples, and the cow should be clinically assessed, including udder and milk appearance. In the protocol for treating subclinical BM, within one week prior to drying, bacteriology of two milk samples from all quarters, with an interval of one to three days between collections, should be performed. The quarter to be treated must have positive isolation for the target bacteria in both milk samples, and it is necessary to repeat sampling if one of the samples is negative. The protocol for preventing new infections during the dry period will have the same interval between samplings. If bacterial isolation occurs in only one of the two initial samples, a third sample should be obtained for confirmatory analysis.

When posttreatment assessment is initiated after calving, the cow should be clinically assessed, including udder and milk appearance, with two bacteriological evaluations of all quarters. The first sampling should be performed before the first regular milking (usually five days after calving, after colostrum has been eliminated), and the second should be performed between four and seven days after the first. The SCC is determined only in the second sampling.

Successful treatment of subclinical BM occurs when the original pathogen is not detected in either posttreatment sampling. In posttreatment evaluations, cows that are infected with pathogens different from the original pathogen targeted for treatment with the product and show recovery from this targeted agent can be included as those with a bacteriological cure. These new infections should be included in the final report and, depending on their incidence, will require in-depth analysis.

Treatment failure occurs when the original pathogen is detected in at least one of the posttreatment samplings or if, during the trial period, there is an additional need for treatment for BM.

Prevention is considered successful if no pathogen is isolated in any of the postpartum samples. However, if any pathogen is detected in at least one of the posttreatment samplings or if there is an additional need for treatment for BM during the trial period, these cases are considered failures of the product indicated for prevention.

Statistical considerations

Every study protocol should describe and justify the statistical tools and the criteria for interpretation. For each target pathogen whose efficacy is claimed, individual cases of cures and treatment failures shall be presented separately by herd and investigator. The description of the statistical analysis should include the definition of the population studied, the sample size calculation, and the confounding variables (confounding factors). The study must have sufficient

testing power to demonstrate significant efficacy for each target pathogen separately. Data should be expressed as the number of clinically cured animals and the number of bacteriologically cured animals. Individual SCC geometric means before and after treatment should be compared using appropriate statistical methods.

Statistical methods applied to discrete data should follow the recommendations established in the literature or national or international reference guides, for example, the Brazilian Guide to the Production, Maintenance or Use of Animals for Teaching or Scientific Research Activities of CONCEA (Brasil, 2015) or the EMA/CVMP/EWP/81976/2010 Committee for Medicinal Products for Veterinary Use (CVMP) Guideline on Statistical Principles for Veterinary Clinical Trials (European Medicines Agency, 2022).

Treatment of cows with chronic or recurrent infections

In all the guidelines referenced in this article, there is no mention of study protocols to prove the efficacy of products indicated for chronic and recurrent BM. The probable reason for this absence lies in what is recommended by the scientific literature, which is abundant in evidence that these cows, in general, should be eliminated from the herds, as they constitute permanent sources of infection for the others (in contagious BM). Therefore, the inclusion of these animals in the studies is not indicated, nor is there an indication in the package insert for treating cows under these conditions.

Use of a negative control group design

Due to the usual management of dairy farms in Brazil, it is expected that there will be difficulty in supplying cows to compose the negative control group. Mainly because this is difficult to accept by good-quality farms, impacting the difficulty of selecting cows that meet the inclusion criteria of studies where the product to be tested should be compared with the absence of any treatment. In Europe, it is agreed that conducting a negative-controlled clinical trial needed for BM infections with a high spontaneous cure rate in lactating cows is usually not acceptable under field conditions. Therefore, for such cases, a dose confirmation study should be performed under laboratory conditions with a negative control group (European Medicines Agency, 2016).

Pharmacokinetics/Pharmacodynamics (PK/PD) models for mastitis treatment

PK/PD models allow estimation of the probability of bacteriological eradication of therapeutic protocols using simulations and are considered complementary to clinical studies of efficacy in the field. To this end, the following are required: a) a pharmacokinetic model that allows simulating the concentrations of the drug in milk, carried out through an *in vivo* study, with different doses of the ATM, and subsequent construction of the PK model; and b) a PK/PD index, i.e., the magnitude of the amount of drug in the milk that determines the desired bacteriological reduction, normalized by the MIC. This is performed utilizing an *in vitro* study of the bacterial death curve and subsequent determination of the most appropriate PK/PD index (AUC/MIC, T>MIC, or Cmax/MIC).

The following benefits are highlighted among the outcomes of these complementary studies: a) providing an estimate of the doses that have a high probability of reaching the PK/PD ratio (desired bacterial reduction), according to the MIC of the bacteria (European Medicines Agency, 2016; Fernández-Varón et al., 2021); and b) estimation of the withdrawal period corresponding to each treatment protocol when the dose proposed in the package insert is changed, i.e., when there is off-label use of the ATM (Li et al., 2018).

In vitro susceptibility tests

According to critical regulatory authorities such as the EMA, FDA, and DoH, it is necessary to carry out, prior to treatment, an *in vitro* susceptibility test for all bacteria isolated in each herd of origin of the animals. The MIC and MIC breakpoint for isolated BM pathogens are determined according to CLSI manuals to assess the susceptibility of organisms to the drug. In other words, it is inferred that animals (or herds) infected with strains of bacteria classified according to the cutoff point as intermediate or resistant to the active ingredient could not be included in the study. However, for the protocols suggested in this review, it is understood that this would be complementary data and not a criterion for selecting herds or cows. If it were a selection criterion before treatment, it would create a tremendous additional difficulty in selecting animals or livestock. However, it is understood that, especially for cases considered treatment failure (lack of efficacy), evaluating *in vitro* susceptibility data is essential.

Final considerations and remarks

The rational use of ATM drugs involves determining an appropriate dosage of the drug to the sensitivity of the target bacterial population. Within this context, clinical studies for the registration of IMM drugs must be guided by recommendations representative of bacterial sensitivity and

the country's virulence factor profile. In addition, to enable compliance with the requirement of IN 26/2009 related to the use of CLSI protocols, the tests must be carried out with strains representative of those circulating in the country. Considering that Brazil has continental characteristics and that both virulence factors and the susceptibility profiles of bacteria to ATMs are dynamic, MAPA must act in a central strategic way, fostering and consolidating data from bacterial resistance monitoring centers to monitor and supply representative bacterial samples for testing. To this end, MAPA can take advantage of preexisting infrastructures in universities, laboratories accredited by MAPA, and private laboratories accredited by Inmetro and Embrapa, which have national coverage and collections of institutional microorganisms that follow their standards based on ISO 17025 and ISO 20387.

At the same time, to make this monitoring more refined, MAPA, together with entities representing the sector (e.g., SINDAN, ALANAC, ABIQUIFI, Alliance for the Responsible Use of ATMs, etc.), can regularly disclose consumption profiles of georeferenced ATM classes in Brazil. These data have already begun to be provided by the sector through the "Agromonitora" system, whose information is collected annually by the World Organization for Animal Health (WHOA). Information on the sale of ATM drugs for veterinary use is provided annually by the companies that hold the registrations of these products through the completion of a digital form made available by MAPA to compose the monitoring of the use of ATM drugs in animals, as provided for in the National Action Plan for the Prevention and Control of Resistance to ATMs within the scope of Agriculture (PAN-BR-AGRO).

Finally, it should be emphasized that despite evidence of drug safety and efficacy, field studies should always be complemented by post-registration information, i.e., pharmacovigilance data. These data reflect, in fact, the consequences of using the product in "real-world" conditions, where its efficacy and safety are tested in the most diverse conditions of breed, management, environment, etc.

Conflict of Interest

The authors declare that they do not have conflict of interest with respect to this manuscript.

Ethics Statement

It was not necessary to submit it to the ethics committee as it was a literature review.

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