Universidade Federal de Minas Gerais

Systematic review of the immune response to *Brucella abortus* S19 and RB51 vaccines and analysis of cross-reactivity of monoclonal antibodies in buffaloes (*Bubalus bubalis*)

Kelly Mara Gomes Godoy

Belo Horizonte

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade Federal de Minas Gerais como requisito parcial para obtenção do Título de Mestre em Ciência Animal.

Área de Concentração: Medicina Veterinária Preventiva

Orientação: Andrey Pereira Lage

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“Depois de algum tempo você aprende... que o tempo não é algo que possa voltar para trás, portanto, plante seu jardim e decore sua alma ao invés de esperar que alguém lhe traga flores, e você aprende que realmente pode suportar... que realmente é forte e que pode ir muito mais longe depois de pensar que não se pode mais. Descobre que realmente a vida tem valor e que você tem valor diante da vida! Nossas dúvidas são traidoras e nos fazem perder o bem que poderíamos conquistar, se não fosse o medo de tentar. ”

Veronica Shoffstall

RESUMO

Esta dissertação aborda dois estudos interrelacionados, focando na resposta imunológica de búfalos (*Bubalus bubalis*) frente a vacinação contra a brucelose e na ampliação de anticorpos monoclonais (mAbs) disponíveis comercialmente para uso em citometria de fluxo com células de bubalinos. A primeira parte é uma revisão sistemática que analisa a literatura existente sobre cepas vacinais de *Brucella abortus*, especificamente B19 e RB51, usadas na imunização de búfalos. A revisão incluiu 25 estudos, totalizando 65 ensaios. A vacina B19 induziu respostas imunológicas humorais e celulares, com a persistência de anticorpos proporcional à idade dos animais à vacinação. A vacina RB51 demonstrou segurança e induziu tanto resposta imunológica humoral quanto celular, além de permitir a diferenciação de animais infectados de vacinados (DIVA), apresentando resposta imunológica sem comprometer a gestação ou lactação dos animais. No entanto, a eficácia das vacinas não pôde ser avaliada por uma metanálise em funçãoda heterogeneidade dos métodos nos estudos analisados. A segunda parte da dissertação aborda a limitação de pesquisas sobre a ativação e regulação do sistema imunológico dos búfalos, causada pela escassez de anticorpos monoclonais comerciais específicos para esses animais. Para superar essa limitação, foi avaliada a reatividade cruzada de 16 mAbs comerciais anti-marcadores celulares (CD4, CD8, CD14, CD21, CD25, CD28, CD45RB, CD45RO, WC1 e CD80) e anti-citocinas e outras moléculas efetoras do sistema imunológico (TNF-α, IFN-γ, IL-4, IL-17, granzima B e perforina). Todos os mAbs foram analisados por meio de citometria de fluxo. Os resultados mostraram que a maioria dos anticorpos monoclonais avaliados (15/16) teve boa reatividade cruzada, exceto o anticorpo anti-CD4. Esta pesquisa oferece uma abordagem promissora para expandir os dados sobre a imunologia de búfalos. Em conjunto, esses estudos contribuem para o entendimento da imunização de búfalos contra a brucelose e destacam a necessidade de mais pesquisas sobre a imunologia desses animais, especialmente em relação ao desenvolvimento de ferramentas específicas para estudos imunológicos mais avançados.

Palavras-chave: vacinação, resposta imune, citometria de fluxo, imunofenotipagem, brucelose.

ABSTRACT

This dissertation addresses two interrelated studies, focusing on the immune response of buffaloes (*Bubalus bubalis*) to vaccination against brucellosis and on the expansion of commercially available monoclonal antibodies (mAbs) for use in flow cytometry of buffalo cells. The first part is a systematic review that analyzes the existing literature on vaccine strains of *Brucella abortus*, specifically S19 and RB51, used in the immunization of buffaloes. The review included 25 studies, totaling 65 trials. The S19 vaccine induced humoral and cellular immune responses, with antibody persistence proportional to the age of the animals to vaccination. The RB51 vaccine showed safety and induced both humoral and cellular immune responses, in addition to allowing the separation of infected and vaccinated animals (DIVA), providing an immune response in the vaccination of buffaloes without compromising the gestation or lactation of the animals. However, the efficacy of the vaccines could not be evaluated by a meta-analysis due to the heterogeneity of the methods in the analyzed studies. The second part of the dissertation addresses the limitations of research on the activation and regulation of the buffalo immune system, caused by the scarcityof commercial monoclonal antibodies specific for these animals. To overcome this limitation, the cross-reactivity of 16 commercial mAbs against cellular markers (CD4, CD8, CD14, CD21, CD25, CD28, CD45RB, CD45RO, WC1 and CD80) and anti-cytokines and other immune system markers (TNF-α, IFN-γ, IL-4, IL-17, granzyme B and perforin) was evaluated. All mAbs were analyzed by flow cytometry. The results demonstrated that most of the monoclonal antibodies evaluated (15/16) had good cross-reactivity, except the anti-CD4 antibody. This research offers a promising approach to expand the data on buffalo immunology. Collectively, these studies contribute to the understanding of buffalo immunization against brucellosis and highlight the need for further research on the immunology of these animals, especially in relation to the development of more advanced immunological studies.

Keywords: Vaccination, immune response, flow cytometry, immunophenotyping.

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LIST OF ABBREVIATIONS

2-ME	2 Mercaptoethanol
AAT	Antígeno acidificado tamponado
AL	Anel em leite
BD	Becton, Dickinson & Company
BFA	Brefeldin A
BLAST	Basic Local Alignment Search Tool
BPAT	Buffered Plate Agglutination Test
BVD	Bovine viral diarrhea
CD	Cluster of differentiation
CEUA	Ethics Committee for Animal Use
CF	Complement Fixation
CF-RB51	Complement Fixation Test with RB51 antigen
CFU	colony forming unit
CNN	National Collective Catalog of Periodicals
DIVA	Differentiation between Infected and Vaccinated Animals
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assays
FAO	Food and Agriculture Organization
FITC	Fluorescein isothiocyanate
FPA	Fluorescence Polarization Immunoassay
FSC-A	Forward scatter area
FSC-H	Forward scatter height
GTA	Animal Transit Guide
IBGE	Instituto Brasileiro de Geografia e Estatística
IFN-γ	Interferon gamma
IL	Interleukin
IQR	Interquartile range
LPS	lipopolysaccharide

mAbs	monoclonal antibodyes
MFF	Mac’s Fac’s Fix
MHC	Major histocompatibility complex
NCBI	National Center for Biotechnology Information
PBS	Phosphate buffered saline
PDO	Protected Designation of Origin
PE	Phycoerythrin
PH	Potential of hydrogen
PICOT	Population, intervention, comparison, outcome, and time
PMA	Phorbol 12-myristate 13-acetate
PNCEBT	Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose
PRISMA	Preferred Reported Items for Systematic Reviews and Meta-Analyse
RBT	Rose Bengal Test
SAT	Serum Tube Agglutination Test
SPAT	Standard Plate Agglutination Test
SSC-A	Side scatter area
TNF-α	Tumor necrosis factor alpha
UFLA	Universidade Federal de Lavras
USA	United States of America
WOAH	World Organization for Animal Health

SUMMARY

1. INTRODUCTION

The domestic buffalo (*Bubalus bubalis*) is a production animal known for its rusticity and suitability for meat and milk production, in addition to be used for traction (Gama Pantoja *et al.*, 2022). Due to its importance in livestock farming, studies have been developed to understand how the immune system of these animals behaves in the face of vaccine responses and infections by pathogens, such as *Brucella abortus,* which causes brucellosis (Davis *et al.*, 2001; Grandoni *et al.*, 2023). The efficacy of vaccination against brucellosis in cattle is well established in the literature. However, in buffaloes, the literature on the subject is still scarce and not very well established. To better understand how the dynamics of vaccination against brucellosis in buffaloes is being studied, it is necessary to carry out a systematic review of the existing literature to understand about the state-of-art on the efficacy of vaccines in buffaloes.

In brucellosis, the main immune response mechanism involved in protection is cellular and not humoral (Nicoletti, 1990; Olsen *et al.*, 2005; Dorneles *et al.*, 2015a). In the literature, many studies on the humoral response of brucellosis vaccines in buffaloes are available, however, there is only few studies about the cellular immunity generated by vaccination. A valuable tool for studying the cellular immune response is flow cytometry. Flow cytometry is a tool that provides multiparametric analysis of cells or particles present in a solution. One of the main applications of flow cytometry is immunophenotyping, in which cellular antigens are searched in order to characterize and quantify cell populations and the production of intracellular cytokines in a sample. This is possible through the use of monoclonal antibodies (mAb), labeled with fluorescent molecules (fluorochromes), which will bind to specific sites of interest in the cells (McKinnon, 2018; Vaz *et al.*, 2018). However, there are few commercially available mAbs against buffalo markers, which hinders more in-depth research on the regulatory mechanisms of the buffalo immune response. In the literature, there are several articles that demonstrate the presence of cross-reactivity of mAbs between different species. Considering that mAb production is a laborious, time-consuming and expensive process, a better way to identify buffalo immunological markers is to search for through cross-reactivity with commercially available mAbs anti-bovine and human cellular markers.

2. LITERATURE REVIEW

2.1 The importance of buffalo in livestock farming

The domestic buffalo (*Bubalus bubalis*) is a production animal known for its hardiness and suitability for meat and milk production, in addition to being used for traction (Gama Pantoja *et al.*, 2022). These animals adapt well to low-quality pastures or to places that are difficult to access, such as swampy areas (Borghese, 2005).

The world's buffalo population is approximately 204 million heads, with 98% of these animals belonging to the Asian continent. In South America, 0.8% of the buffalo population is concentrated, with Brazil having the largest number of animals (FAO, 2023b). According to the Instituto Brasileiro de Geografia e Estatística (IBGE), in 2022, Brazil had approximately 1,598,268 heads of buffalo, with Pará being the largest producing state, with approximately 644,672 heads. In Minas Gerais, the number of heads was 84,713 (IBGE, 2022).

Buffalo meat production in 2021 was 4.3 million tons, with 93.5% of production concentrated in Asia, especially India. Only 0.6% of buffalo meat is produced in the Americas, with 26 million tons produced in South America (FAO, 2023a). Buffalo meat production in Brazil is underestimated, since it does not yet have a national identification standard. It is estimated that 90% of all buffalo meat produced in Brazil is marketed as beef (Corrêa *et al.*, 2004).

Buffalo is of great importance in dairy production, since buffalo milk production represents 15% of global milk production, being the second species that contributes the most to milk production in the world. In developing countries, about one third of milk production is made up of buffalo milk (FAO, 2023a). In Brazil, in 2018, a production of 87,472 tons of buffalo milk was estimated (Minervino *et al.*, 2020). According to the 2017 Agricultural Census (IBGE), Minas Gerais is the largest producer of buffalo milk in the country (IBGE, 2022).

2.2 Brucellosis

2.2.1 Definition

Brucellosis is a disease caused by bacteria of the genus *Brucella* and affects a large number of animal species. In addition to being a chronic disease, it is also one of the most important zoonotic diseases worldwide (Corbel, 2006; Neta *et al.*, 2010).

2.2.2 Etiology and susceptible hosts

The genus Brucella is composed of species classified as "classical," associated with brucellosis in terrestrial mammals, and emerging or environmentally associated species, called "non-classical." The distinction between these groups is based on phylogenomic analyses, ecological characteristics, and pathogenic potential. Classical species include B. melitensis, B. suis, B. abortus, and B. canis—recognized as zoonotic—and, more rarely, B. ceti and B. pinnipedialis. Species such as B. ovis, B. neotomae, B. papionis, and B. microti are less associated with human infection. B. ovis is considered non-pathogenic to humans, and the zoonotic impact of B. neotomae is still uncertain (Hartigan, 1997; Moreno, 2014; Suárez-Esquivel *et al*., 2020; Moreno *et al*. 2022; She *et al*., 2023).

Bacteria of the genus Brucella demonstrate a preference for certain hosts, although this affinity is not absolute. For example, B. melitensis preferentially infects goats, B. suis pigs, B. abortus cattle, B. canis dogs, B. ovis sheep, and B. neotomae rodents of the genus Neotoma (Hartigan, 1997; Moreno, 2014; Suárez-Esquivel *et al*., 2020; Moreno *et al*. 2022; She *et al*., 2023).

2.2.3 Brucella abortus

Brucella abortus , in addition to having cattle as its preferred host, is also described in other animals such as bison (*Bison bison*), elk (*Cervus elaphus*) (Scurlock *et al.*, 2010), camels (*Camelus dromedarius*) (Alamian *et al.*, 2019) and buffaloes (*Bubalus bubalis*) (Fosgate *et al.*, 2011). In Brazil, this agent is of great importance in cattle and buffaloes, being the target of a control and eradication program in the country (Poester *et al.*, 2002; Ferreira Neto *et al*., 2016).

2.2.3.1 Pathogenesis

Infection by *B. abortus* occurs through damaged skin, conjunctiva, respiratory mucosa and the intestinal tract, the latter being the most common route of infection in cattle and buffaloes (Ko *et al.*, 2003; Neta *et al.*, 2010; Fosgate *et al.*, 2011). As it is a facultative intracellular microorganism, it is most often found inside cells, except when bacteremia and dissemination throughout the host's body occur (Moreno *et al.*, 2002). After infection of the animal, the bacteria are phagocytosed by macrophages, and within the phagosome it inhibits phagosome-lysosome fusion, allowing its survival and multiplication within the macrophages and dissemination to other host cells. The dissemination of *Brucella spp* to the lymphatic organs, sexual organs, mammary gland, liver and spleen occurs through contaminated macrophages (Ko *et al.*, 2003).

Its pathology can be divided into three phases: incubation, in which the host cells are invaded without clinical signs; acute phase, in which the microorganism multiplies and spreads to other tissues; and the chronic phase in which *Brucella* adapts to the intramacrophage environment and extends its intracellular persistence for an indefinite period (Figueiredo *et al.*, 2015).

2.2.3.2 Clinical signs

The clinical signs associated with brucellosis in cattle and buffaloes are directly linked to reproductive problems such as abortions in the final third of gestation, especially in the first gestation after infection, birth of weak calves and infertility in infected females. Clinical signs are visible mainly in females, but cases of orchitis can be observed in males to a lesser extent (Poester *et al.*, 2006; Neta *et al.*, 2010; Fosgate *et al.*, 2011; Megid, 2016).

2.2.3.3 Zoonosis

Because it causes a major productive impact on animals, and it is a highly infectious to humans, brucellosis is a disease listed in the Terrestrial Animal Health Code of the World Organization for Animal Health (WOAH) (WOAH, 2018). The spread of the disease to humans occurs mainly through the ingestion of milk and dairy products produced with raw milk and can cause serious debilitating disease (Corbel, 2006). Control of human brucellosis is achieved through control of the disease in animals (WOAH, 2022).

2.2.3.4 Diagnosis and control

The diagnosis of brucellosis can be performed by direct and indirect diagnostic methods. Direct methods include the polymerase chain reaction (PCR) test and the isolation of *B. abortus* in the laboratory, which is considered the gold standard. However, for isolation, it is necessary to use biosafety level 3 laboratories, in addition to presenting a high risk of contamination for those who will handle the collected materials, both in the field and in the laboratory. Indirect methods rely on serological tests that are more viable and economical, and are the most widely used tests for the diagnosis of brucellosis (Moreno *et al.*, 2002; Corbel, 2006). Some of the most commonly used indirect tests are: Rose Bengal Test (RBT), Tube Agglutination Test (SAT), 2-Mercaptoethanol (2-ME), Complement Fixation (FC), Fluorescence Polarization (FPA), and

Indirect Enzyme-Linked Immunoassay (iELISA). (McKenna *et al.*, 2006; Andrade *et al.*, 2024). According to a systematic review, the tests that presented the best diagnostic sensitivity and diagnostic specificity were the bacterial suspension iELISA and the FPA, respectively (Andrade *et al.*, 2024).

Serological tests for brucellosis are generally performed and interpreted in series, with a screening test followed by a confirmatory test (McKenna *et al.*, 2006; Andrade *et al.*, 2024). In Brazil, in 2001, the Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose (PNCEBT) was established, which regulates the standards for diagnosis and control of brucellosis and tuberculosis (BRASIL, 2017). The diagnostic tests recommended by PNCEBT are the Rose Bengal Test (AAT) test and the Milk Ring Test (AL) as screening tests that can be performed by accredited veterinarians. Confirmatory tests must be performed by official or accredited laboratories, namely the 2-Mercaptoethanol (2-ME) Test, Complement Fixation (FC) Test and the Fluorescent Polarization (FPA) Test (BRASIL, 2017).

To control and eradicate the disease, the program recommends the slaughter of positive animals and requires the vaccination of female cattle and buffaloes between 3 and 8 months of age with S19 vaccine. Female heifers over 8 months of age, that were not vaccinated with S19, must be vaccinated with RB51. In order to issue the Animal Transit Guide (GTA), all cattle and buffaloes to be transported must be properly immunized against brucellosis (BRASIL, 2017).

2.2.3.5 Vaccination of cattle and buffaloes against brucellosis

One of the most effective ways to control brucellosis in herds is through animal vaccination (Olsen *et al.*, 2005; Dorneles *et al.*, 2017). In Brazil, after the implementation of the PNCEBT, vaccination was the action that evolved the most in the program. The most widely used vaccine strains in the world against brucellosis are S19 and RB51.

2.2.3.5.1 S19 Vaccine

The S19 vaccine is widely used to immunize cattle against brucellosis (WOAH, 2022). It is a live attenuated vaccine derived from a virulent isolate of *Brucella abortus* biovar 1 strain 19 that was attenuated after being forgotten on the bench at room temperature for about 1 year (Olsen *et al.*, 2005). Furthermore, it is a vaccine with low pathogenicity, moderate antigenicity, and high immunogenicity (Olsen *et al.*, 2005; Dorneles *et al.*, 2015a).

The S19 vaccine induces an antibody response that makes it impossible to differentiate it from a natural infection by routine serological tests (Olsen *et al.*, 2005). This occurs because S19 strain and the smooth strains of *Brucella* spp. present in their lipopolysaccharide (LPS) a perosamine homopolymer called the O-chain, which is an immunodominant antigen that stimulates a greater immune response against it. In addition, the antigens used in serological tests are, for the most part, specific for antibodies produced against the O-chain (Schurig *et al.*, 1991).

To avoid vaccine interference in indirect diagnostic tests, vaccination is recommended in prepuberal animals (Olsen *et al.,* 2005). The WOAH recommendation is to vaccinate female cattle and buffaloes from 3 to 6 months of age, while the PNCEBT recommends the vaccination of female cattle and buffaloes from 3 to 8 months of age (BRASIL, 2017; WOAH, 2022). The younger the cattle are vaccinated, the faster the decline in antibody titers induced by vaccination, with animals vaccinated at 3 months of age taking approximately 3 months for their vaccine titers to drop, and those vaccinated at 8 months of age taking 12 months for their vaccine titers to decrease (Manthei, 1968; Cheville *et al.*, 1996; Oliveira *et al.*, 2022). For this reason, the PNCEBT recommends serological testing of these animals after 24 months of age (BRASIL, 2017). Regarding the recommended dose, the WOAH recommends vaccination of animals with a single dose of 5-8x10^{10} colony forming units (CFU). For adult animals, a reduced dose of 3×10^{8} CFU/dose to 5×10^{9} CFU/dose can be used, with the risk of presenting antibody persistence (WOAH, 2022). Oliveira *et al.* (2022) showed, through a systematic review and meta-analysis, that for cattle, the 10^{9} CFU/dose of the S19 vaccine is the most indicated for the prevention of abortions. However, there are no specific WOAH recommendations for vaccinating buffaloes with the S19 strain, and there are a limited number of articles on buffalo vaccination in the literature, which use very different methodologies.

In buffaloes, Nardi Júnior *et al.* (2012) demonstrated that vaccination of heifers aged 3 to 8 months with the same dose recommended by WOAH for cattle, 5-8x10^{10} CFU/dose , does not promote persistence of vaccine antibodies in animals over 24 months of age. According to Normative Instruction No. 15/2004 of the Brazilian Ministry of Agriculture SDA nº 15/2004 and nº 10/2017, female cattle and buffaloes should be vaccinated between 3 and 8 months of age with a single dose of B19 vaccine containing 6×10^{10} CFU/dose (BRASIL, 2004; 2017). In buffaloes, there are studies that evaluate the immunological response to the application of reduced doses of the S19 vaccine. In one study, Shome *et al.* (2020), tested four doses of S19 in buffaloes aged 4 to 5 months, $4x10^{10}$ CFU/dose, $4x10^{9}$ CFU/dose, $1.5\text{-}2.0x10^{9}$ CFU/dose, $3.0\text{-}4.0x10^{8}$ CFU/dose, subcutaneously, with the first dose being recommended for vaccination of cattle and buffaloes in India, and the others as reduced doses. He evaluated the cellular response of the vaccinated animals, showing that only the first reduced dose presented an immunological response similar to the recommended dose ($4x10^{9}$ CFU/dose). The author also highlights that to know the efficacy of the vaccine in buffaloes, a more robust study is needed, with challenge of vaccinated animals. In another study, Caporale *et al.* (2010) vaccinated buffaloes aged 5 to 6 months with a reduced dose of S19, $6\text{-}12x10^{9}$ CFU/dose, challenged these animals after 69 days and tested them through serological tests, and isolation of *B. abortus* after necropsy. The animals vaccinated with S19 maintained a persistence of antibodies detectable in the serological tests, and did not present isolation of the challenge strain in the lymph nodes, showing that the S19 vaccine, in addition to stimulating a humoral response in buffaloes, was also effective in protecting the animals at the dose tested.

The studies found in the literature on the vaccination of buffaloes with S19 mostly address the humoral response of the animals after vaccination. Among them, we found differences between the vaccine doses, number of animals, age range of the buffaloes, duration of the studies, route of administration of the vaccine, among others. To better understand the data found in the studies, it is necessary to conduct a systematic review to group and summarize the available data on the vaccination of buffaloes with the S19 vaccine.

2.2.3.5.2 RB51 Vaccine

Another widely used vaccine against brucellosis in cattle is *B. abortus* RB51. RB51 is a rough strain that was obtained from successive passages of the *B. abortus* 2308 strain in media with subinhibitory doses of rifampicin and penicillin (Schurig *et al.*, 2002). The RB51 strain minimally expresses the O chain of lipopolysaccharide (LPS), which induces a low antibody response against this epitope. This allows the RB51 to be used as a DIVA (Differentiation infected from vaccinated animals) vaccine, since it does not interfere with routine diagnostic tests (Schurig *et al.*, 1991; Olsen *et al.*, 2005).

In PNCEBT, it is recommended only for cattle as an alternative to vaccination with S19 for animals that were not vaccinated up to 8 months of age or as a facultative vaccine for adult animals (BRASIL, 2017). The methodology for applying this vaccine is slightly different in each country that uses it. The WOAH recommendation for the RB51 vaccine is a dose of 1–3.4

× 10^{10} CFU/dose, for 4 to 12 month old cattle, and for adults a reduced dose of 1–3 × 10^{9} CFU/dose (WOAH, 2022).

For buffaloes, the recommended dose of the vaccine has not yet been established. Fosgate *et al.* (2003) evaluated the efficacy of vaccination of buffaloes aged 5 to 20 months vaccinated with a single dose of 1-3.4 x 10^{10} CFU/dose of the RB51. The animals were challenged with a field strain and the animals were monitored through serological tests and microbiological diagnosis. The study showed that the RB51 vaccine, at the same dose indicated for young animals, is not effective in preventing *B. abortus*. In another study, Diptee *et al.* (2006) evaluated the biological safety and antibody response of buffaloes aged 6 to 10 months vaccinated with RB51. Different doses were tested: a single dose of 2.02x10^{10} CFU/dose; two doses of 2.02x10^{10} CFU/dose with a 4-week interval; single dose of 4.04x10^{10} CFU/dose; two doses of 4.04x10^{10} CFU/dose with a 4-week interval. The study showed that the RB51 vaccine is safe for buffaloes at all doses tested. In the same study, the elimination of RB51 from the lymph nodes occurred in 2 weeks, and the higher doses implied higher and more persistent antibody titers. This does not mean that the vaccine is more effective, since antibodies do not play a protective role in brucellosis. Caporale *et al.* (2010) vaccinated female buffaloes with a triple dose of RB51 indicated for cattle, 3-10.2 x 10^{10} CFU/dose, and revaccinated the animals 30 days apart. The animals were challenged 33 days after revaccination, and the results demonstrated that the triple dose appears to be able to provide protection against brucellosis.

In cattle, the literature on doses and efficacy of the RB51 vaccine against brucellosis presents more consolidated responses. For buffaloes, however, the information is still very limited and mostly seeks to show the humoral response of vaccinated animals. In existing studies, there are differences between vaccine doses, number of applications, age of animals, route of administration, among others. A systematic analysis of the available studies may allow the acquisition of consolidated responses on the efficacy of the RB51 strain in buffaloes.

2.3 Flow Cytometry

Flow cytometry is a tool that provides multiparametric analysis of cells or particles present in a solution. Cytometers are capable of analyzing the size, internal complexity and fluorescence intensity of the analyzed molecules (McKinnon, 2018). One of the applications of flow cytometry is immunophenotyping, which consists of searching cellular antigens in order to characterize and quantify the cell populations in a sample. This is possible through the use of monoclonal antibodies (mAbs), labeled with fluorescent molecules (fluorochromes), which will bind to specific sites of interest in the cells (Vaz *et al.*, 2018).

Using a methodology that promotes the lysis of whole blood, it becomes possible to evaluate the main leukocyte components of the blood, such as monocytes, lymphocytes and granulocytes. For the cells to be evaluated by the flow cytometer, they must be dispersed in a fluid suspension (buffer) (Vaz *et al.*, 2018). They will be captured by the cytometer and transported in a continuous flow until they pass through a beam of light, where the cell will be read through the optical system Cytometers are capable of analyzing the size, internal complexity and fluorescence intensity of the analyzed molecules (McKinnon, 2018).

The cytometer reading occurs by the scattering of light generated from the incidence of light. The light beams that fall directly (Forward Scatter – FSC) will help measure the size of the cell, and those that fall at 90° (Side Scatter – SSC) will help measure the complexity of the cell (McKinnon, 2018). In addition, the fluorochromes present in the marked cells, when passing through the light beam, will be excited, emitting fluorescence at specific wavelengths that will be captured by the optical system. All the optical information read will be converted into numerical data through an electronic system (Costa, 2020). Through graphs, cellular events can be represented, quantified and interpreted through a multiparametric analysis (Vaz *et al.*, 2018).

One of the most important components in immunophenotyping is the monoclonal antibody (Vaz *et al.*, 2018). It is produced through hybridomas, which are the fusion of myeloma cells and B cells from an animal immunized with a known antigen of interest. Each hybridoma clone will produce an antibody specific to an epitope of the antigen of interest (KÖHler *et al.*, 1975). However, mAb production is a laborious, time-consuming and expensive process (Carbonetti *et al.*, 2017)

There are few commercially available mAbs against buffalo immune markers, which hinders more in-depth research on the regulatory mechanisms of the buffalo immune response (Davis *et al.*, 2001). In the literature, there are several articles that demonstrate the presence of cross-reactivity between immunological markers of different species (Davis *et al.*, 1987; Pedersen *et al.*, 2002; Dorneles *et al.*, 2015b; Moreira *et al.*, 2015). Some mAbs against bovine immunological markers can recognize conserved epitopes in orthologous molecules of other animals, such as goats and sheep, but there is little data on these cross-reactions with buffalo immunological markers (Davis *et al.*, 2001; Dorneles *et al.*, 2015b).

Davis *et al.* (2001), carried out a study testing more than 200 mAbs against bovine cell markers, where 138 of the mAbs showed cross-reactivity with buffalo cell markers. Other studies such as (De Matteis *et al.*, 2016; Grandoni *et al.*, 2020; Petrini *et al.*, 2021; De Matteis *et al.*, 2023) use anti-bovine and anti-human monoclonal antibodies as a tool to evaluate cross-reactivity in buffalo cells, and to study the immune response of these animals to pathogens and vaccines. The reason for testing the cross-reactivity of existing mAbs is because it is a more economical method than the production of new monoclonal antibodies, in addition to their production being laborious (Pedersen *et al.*, 2002; Moreira *et al.*, 2015).

To date, the number of articles in the literature that seek to characterize the immune response of buffaloes is low. Obtaining mAbs that react to buffalo immune markers will aid future research on the immune response of these animals to an infection or vaccine challenge. Furthermore, the search for commercially available mAbs makes it possible for other research groups to conduct immunological characterization studies in buffaloes. Due to the need to increase the number of monoclonal antibodies available for use in studies of the immune response of buffaloes, it is necessary to search for commercially available markers and clones that present cross-reactivity with buffalo cells and cytokines.

3. GENERAL OJECTIVES

To analyze different immunological aspects of buffaloes (*Bubalus bubalis*) through a systematic review of brucellosis vaccination and an evaluation of monoclonal antibody cross-reactivity.

4. SPECIFIC OBJECTIVES

To carry out a systematic review on the immune response of the vaccine against brucellosis in buffaloes (*Bubalus bubalis*).

To assess the cross-reactivity of human and cattle mAbs against cattle and humans that present cross-reactivity with immunological markers of buffaloes.

5. REFERENCES

ALAMIAN, S.; DADAR, M. *Brucella abortus* contamination of camel milk in two Iranian regions. *Preventive Veterinary Medicine***,** v. 169, p. 104708, 2019.

ANDRADE, R. S.; OLIVEIRA, M. M. D.; BUENO FILHO, J. S. D. S. *et al*. Accuracy of serological tests for bovine brucellosis: A systematic review and meta-analysis. *Preventive Veterinary Medicine***,** v. 222, p. 106079, 2024.

BORGHESE, A. *Buffalo production and research*. Roma (Italy): REU Technical Series, 2005.

BRASIL. Regulamento Técnico para produção e controle de qualidade da vacina contra Brucelose e antígenos para diagnóstico da brucelose e Tuberculose Animal. Instrução Normativa Nº 15, de 19 de fevereiro de 2004. SECRETARIA DE DEFESA AGROPECUÁRIA, M. D. A., PECUÁRIA E ABASTECIMENTO. Brasilia: *Diário Oficial da União*. 57**:** 25-26 p. 2004.

BRASIL. Regulamento Técnico do Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal – PNCEBT. IInstrução Normativa N° 10 de 3 de março de 2017. SECRETARIA DE DEFESA AGROPECUÁRIA, M. D. A., PECUÁRIA E ABASTECIMENTO. Brasilia: *Diário Oficial da União* 116**:** 4-8 p. 2017.

CAPORALE, V.; BONFINI, B.; DI GIANNATALE, E. *et al*. Efficacy of *Brucella abortus* vaccine strain RB51 compared to the reference vaccine *Brucella abortus* strain 19 in water buffalo. *Vet Ital***,** v. 46, n. 1, p. 13-9, 5-11, 2010.

CARBONETTI, S.; OLIVER, B. G.; VIGDOROVICH, V. *et al*. A method for the isolation and characterization of functional murine monoclonal antibodies by single B cell cloning. *J Immunol Methods***,** v. 448, p. 66-73, 2017.

CHEVILLE, N. F.; OLSEN, S. C.; JENSEN, A. E. *et al*. Effects of age at vaccination on efficacy of *Brucella abortus* strain RB51 to protect cattle against brucellosis. *Am J Vet Res***,** v. 57, n. 8, p. 1153-6, 1996.

CORBEL, M. J. Brucellosis in humans and animals. *WHO Library*, p. 102, 2006.

CORRÊA, A.; TRAMOSO, E. Búfalos. *Revista Produz***,** v. 1, n. 6, p. 36-43, 2004.

COSTA, R. N. *Introdução à Citometria de Fluxo: Um manual básico para iniciantes*. Curitiba: Independently published, 2020.

DAVIS, W. C.; KHALID, A. M.; HAMILTON, M. J. *et al*. The use of crossreactive monoclonal antibodies to characterize the immune system of the water buffalo (*Bubalus bubalis*). *J Vet Sci***,** v. 2, n. 2, p. 103-9, 2001.

DAVIS, W. C.; MARUSIC, S.; LEWIN, H. A. *et al*. The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. *Vet Immunol Immunopathol***,** v. 15, n. 4, p. 337-76, 1987.

DE FIGUEIREDO, P.; FICHT, T. A.; RICE-FICHT, A. *et al*. Pathogenesis and immunobiology of brucellosis: review of Brucella-host interactions. *Am J Pathol***,** v. 185, n. 6, p. 1505-17, 2015.

DE MATTEIS, G.; GRANDONI, F.; SCATÀ, M. C. *et al*. Evaluation of leptin receptor expression on buffalo leukocytes. *Vet Immunol Immunopathol***,** v. 177, p. 16-23, 2016.

DE MATTEIS, G.; SCATÀ, M. C.; ZAMPIERI, M. *et al*. Flow cytometric detection of IFN-γ production and Caspase-3 activation in CD4(+) T lymphocytes to discriminate between healthy and Mycobacterium bovis naturally infected water buffaloes. *Tuberculosis* v. 139, p. 102327, 2023.

DIPTEE, M. D.; ADESIYUN, A. A.; ASGARALI, Z. *et al*. Serologic responses, biosafety and clearance of four dosages of *Brucella abortus* strain RB51 in 6-10 months old water buffalo (*Bubalus bubalis*). *Vet Immunol Immunopathol***,** v. 109, n. 1-2, p. 43-55, 2006.

DORNELES, E. M.; SRIRANGANATHAN, N.; LAGE, A. P. Recent advances in *Brucella abortus* vaccines. *Vet Res***,** v. 46, n. 1, p. 76, 2015a.

DORNELES, E. M.; ARAÚJO, M. S.; TEIXEIRA-CARVALHO, A. *et al*. Cross-reactivity of anti-human cytokine monoclonal antibodies used as a tool to identify novel immunological biomarkers in domestic ruminants. *Genet Mol Res***,** v. 14, n. 1, p. 940-51, 2015b.

DORNELES, E. M. S.; OLIVERIA, L. F.; LAGE, A. P. *Brucella abortus* vaccines: use in control programs and immune response. *J Bacteriol Mycol***,** v. 4, p. 1044, 2017.

FAO. Dairy animals. 2023a. Avaialble at: https://www.fao.org/dairy-productionproducts/production/dairy-animals/en/. Accessed on: 27/09/2023.

FAO. Crops and livestock products. 2023b. Avaialble at: https://www.fao.org/faostat/en/#data/QCL/visualize. Accessed on: 20/09/2023.

FERREIRA NETO, J. S.; SILVEIRA, G. B. da; ROSA, B. M., *et al*. Analysis of 15 years of the National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis, Brazil. *Semina ciênc. agrar* , v. 37, n. 5, p. 3385-3402, 2016.

FOSGATE, G. T.; DIPTEE, M. D.; RAMNANAN, A. *et al*. Brucellosis in domestic water buffalo (*Bubalus bubalis*) of Trinidad and Tobago with comparative epidemiology to cattle. *Trop Anim Health Prod***,** v. 43, n. 8, p. 1479-86, 2011.

FOSGATE, G. T.; ADESIYUN, A. A.; HIRD, D. W. *et al*. Evaluation of brucellosis RB51 vaccine for domestic water buffalo (*Bubalus bubalis*) in Trinidad. *Prev Vet Med***,** v. 58, n. 3-4, p. 211-25, 2003.

GAMA PANTOJA, L. S.; AMANTE, E. R.; MANOEL DA CRUZ RODRIGUES, A. *et al*. World scenario for the valorization of byproducts of buffalo milk production chain. *Journal of Cleaner Production***,** v. 364, p. 132605, 2022.

GRANDONI, F.; MARTUCCIELLO, A.; PETRINI, S. *et al*. Assessment of Multicolor Flow Cytometry Panels to Study Leukocyte Subs*et al*terations in Water Buffalo (*Bubalus bubalis*) During BVDV Acute Infection. *Front Vet Sci***,** v. 7, p. 574434, 2020.

GRANDONI, F. A.-O.; SIGNORELLI, F.; MARTUCCIELLO, A. *et al*. In-depth immunophenotyping reveals significant alteration of lymphocytes in buffalo with brucellosis. *Cytometry. Part A : the journal of the International Society for Analytical Cytology***,** v. 103(6), p. 528–536, 2023.

HARTIGAN, P. J. Human brucellosis epidemiology and clinical manifestations. *Irish Veterinary Journal***,** v. 50, n. 3, p. 179-180, 1997.

IBGE. Rebanho de Bubalinos (Búfalos) – Tamanho de rebanho (cabeças). . 2022. Avaialble at: https://www.ibge.gov.br/explica/producao-agropecuaria/bubalinos/br Accessed on: 25/10/2023.

KO, J.; SPLITTER, G. A. Molecular host-pathogen interaction in brucellosis: Current understanding and future approaches to vaccine development for mice and humans. *Clinical Microbiology Reviews***,** v. 16, n. 1, p. 65-78, 2003.

KÖHLER, G.; MILSTEIN, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature***,** v. 256, n. 5517, p. 495-497, 1975.

MANTHEI, C. A. Application of Research to Bovine Brucellosis Control and Eradication Programs. *Journal of Dairy Science***,** v. 51, n. 7, p. 1115-1120, 1968.

MEGID, J.; MATHIAS, L. A. Brucelose. In: ROCA (Ed.). Doenças Infecciosas em Animais de Produção e de Companhia. Rio de Janeiro: Roca, v.1, 2016. chap. 3, p.1294.

MCKENNA, S. L. B.; DOHOO, I. R. Using and Interpreting Diagnostic Tests. *Veterinary Clinics of North America: Food Animal Practice***,** v. 22, n. 1, p. 195-205, 2006.

MCKINNON, K. M. Flow Cytometry: An Overview. *Curr Protoc Immunol***,** v. 120, n 1, p. 1 - 5, 2018.

MINERVINO, A. H. H.; ZAVA, M.; VECCHIO, D. *et al*. *Bubalus bubalis*: A Short Story. *Front Vet Sci***,** v. 7, p. 570413, 2020.

MOREIRA, M. L.; DORNELES, E. M.; SOARES, R. P. *et al*. Cross-reactivity of commercially available anti-human monoclonal antibodies with canine cytokines: establishment of a reliable panel to detect the functional profile of peripheral blood lymphocytes by intracytoplasmic staining. *Acta Vet Scand***,** v. 57, n. 1, p. 51, 2015.

MORENO, E.; SILVA, J. M.; LETESSON, J. J.; GORVEL, J. P.; MORIYÓN, I. Pathogenicity and Its Implications in Taxonomy: The *Brucella* and *Ochrobactrum* Case. *Pathogens*, v. 11, n. 3, p. 377, 2022.

MORENO, E. Retrospective and prospective perspectives on zoonotic brucellosis. *Front Microbiol***,** v. 5, p. 213, 2014.

MORENO, E.; CLOECKAERT, A.; MORIYÓN, I. Brucella evolution and taxonomy. *Veterinary Microbiology***,** v. 90, n. 1, p. 209-227, 2002.

NARDI JÚNIOR, G.; RIBEIRO, M. G.; JORGE, A. M. *et al*. Serological profile of buffalo (*Bubalus bubalis*) female calves vaccinated with standard *Brucella abortus* strain 19 vaccine using rose bengal, 2-mercaptoethanol and complement fixation tests. *Biologicals***,** v. 40, n. 2, p. 158-161, 2012.

NETA, A. V. C.; MOL, J. P. S.; XAVIER, M. N. *et al*. Pathogenesis of bovine brucellosis. *The Veterinary Journal***,** v. 184, n. 2, p. 146-155, 2010.

NICOLETTI, P. Vaccination against Brucella. *Advances in biotechnological processes***,** v. 13, p. 147–168, 1990.

OLIVEIRA, M. M.; PEREIRA, C. R.; DE OLIVEIRA, I. R. C. *et al*. Efficacy of *Brucella abortus* S19 and RB51 vaccine strains: A systematic review and meta-analysis. *Transboundary and emerging diseases***,** v. 69 (4), p. e32-e51, 2022.

OLSEN, S. C.; STOFFREGEN, W. S. Essential role of vaccines in brucellosis control and eradication programs for livestock. *Expert Review of Vaccines***,** v. 4, n. 6, p. 915-928, 2005.

PEDERSEN, L. G.; CASTELRUIZ, Y.; JACOBSEN, S. *et al*. Identification of monoclonal antibodies that cross-react with cytokines from different animal species. *Vet Immunol Immunopathol***,** v. 88, n. 3-4, p. 111-22, 2002.

PETRINI, S.; MARTUCCIELLO, A.; GRANDONI, F. *et al*. Evaluation of Safety and Efficacy of an Inactivated Marker Vaccine against Bovine alphaherpesvirus 1 (BoHV-1) in Water Buffalo (*Bubalus bubalis*). *Vaccines***,** v. 9 (4), p. 355, 2021.

POESTER, F. P.; GONÇALVES, V. T. S. P.; LAGE, A. P. Brucellosis in Brazil. *Veterinary Microbiology***,** v. 90, n. 1, p. 55-62, 2002.

POESTER, F. P.; GONÇALVES, V. S. P.; PAIXÃO, T. A. *et al*. Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. *Vaccine***,** v. 24, n. 25, p. 5327-5334, 2006.

SCHURIG, G. G.; SRIRANGANATHAN, N.; CORBEL, M. J. Brucellosis vaccines: past, present and future. *Vet Microbiol***,** v. 90, n. 1-4, p. 479-96, 2002.

SCHURIG, G. G.; ROOP, R. M.; BAGCHI, T. *et al*. Biological properties of RB51; a stable rough strain of *Brucella abortus*. *Veterinary Microbiology***,** v. 28, n. 2, p. 171-188, 1991.

SCURLOCK, B. M.; EDWARDS, W. H. Status of brucellosis in free-ranging elk and bison in Wyoming. *J Wildl Dis***,** v. 46, n. 2, p. 442-9, 2010.

SHE, R.; ANGLEWICZ, C.; JERKE, K.; RELICH, R.; GLAZIER, M.; FILKINS, L.; SCHUETZ, A. *Brucella* and *Ochrobactrum*: Taxonomic updates for laboratories. Washington, DC: *American Society for Microbiolog*y, 2023. Disponível em: https://asm.org/guideline/brucella-and-ochrobactrum-taxonomic-updates-for-la. Acesso em: 07 jul. 2025.

SHOME, R.; KILARI, S.; SAHARE, A. *et al*. Evaluation of the immune responses against reduced doses of *Brucella abortus* S19 (calfhood) vaccine in water buffaloes (*Bubalus bubalis*), India. *Vaccine***,** v. 38, n. 45, p. 7070-7078, 2020.

SOLER-LLORÉNS, P. F.; QUANCE, C. R.; LAWHON, S. D. *et al*. A Brucella spp. Isolate from a Pac-Man Frog (Ceratophrys ornata) Reveals Characteristics Departing from Classical Brucellae. *Front Cell Infect Microbiol***,** v. 6, p. 116, 2016.

SUÁREZ-ESQUIVEL, M.; CHAVES-OLARTE, E.; MORENO, E. Brucella Genomics: Macro and Micro Evolution. *International journal of molecular sciences***,** v. 21 (20), p. 7749, 2020.

VAZ, A. J.; MARTINS, J. D. O.; TAKEI, K. *et al*. *Imunoensaios: Fundamentos e aplicações*. Rio de Janeiro: Guanabara Koogan, 2018.

WOAH. Listed disease - Brucellosis. 2018. Avaialble at: http://www.oie.int/en/animal-health-inthe-world/oie-listed-diseases-2018/. Accessed on: 20/09/2023.

WOAH. Brucellosis (infection with *Brucella abortus*, B . Melitensis and B . Suis). OIE Terrestrial Manual, 2022. Avaialble at: https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf. Accessed on: 20/09/2023.

CHAPTER 1- Immune response of *Brucella abortus* S19 and RB51 vaccine strains in buffaloes (*Bubalus bubalis*): A systematic review

Abstract

This systematic review aimed to analyze the available studies in the literature that used vaccine strains of *Brucella abortus* to immunize buffaloes (*Bubalus bubalis*) and evaluate the immune response induced by the brucellosis vaccine. The search for articles was conducted on seven platforms: CABI, Cochrane, PubMed, Scielo, Science Direct, Scopus, and Web of Science. One study was characterized by full-text analysis, while clinical trials consisted of single comparisons between a vaccine protocol and a control group or other experimental groups. Initially, 1,846 articles were found. Eighteen articles were selected by screening, and one article was included at the suggestion of an expert. Based on the inclusion and exclusion criteria, 25 studies were included in the systematic review, totaling 65 clinical trials. The most commonly used vaccine strain was S19, present in 27 [41.5% (27/65)] trials, mainly at a dose of 10^{10} CFU [59.3% (16/27)] and administered subcutaneously. The persistence of antibodies induced after vaccination with S19 was evaluated in 20 [74% (20/27)] trials, while only one study evaluated the cellular response. Only two studies conducted an experimental challenge to evaluate vaccine efficacy, using the B. abortus 544 strain at a dose of 10^7 CFU, administered conjunctivally. The RB51 vaccine was used in 33 [50.8% (33/65)] trials, with the most common dose also being 10^{10} CFU, administered subcutaneously. All studies with RB51 evaluated the ability to differentiate between infected and vaccinated animals (DIVA), while the cellular response was analyzed in only four studies. The safety of RB51 was evaluated in two [20% (10)] studies with non-pregnant females and in one [10% (1/10)] study with pregnant females. In the RB51 trials that performed vaccine challenge, the Trinidad 1 strain was the most commonly used, present in two [20% (10)] studies and administered via the conjunctival, subcutaneous, and intravenous routes. Due to the heterogeneity of the methodologies used across the studies, a meta-analysis was not possible. In conclusion, the results suggest that both S19 and RB51 induce humoral and cellular responses in buffaloes. The persistence of S19-induced antibodies appears to be directly related to age at vaccination. However, based on the studies analyzed, it was not possible to determine the protective efficacy of the S19 and RB51 strains in buffaloes.

Keywords: Water buffalo, vaccination, immune response, brucellosis, *B. abortus*

1. Introduction

Brucellosis is a disease caused by bacteria of the genus *Brucella* and affects a large number of animal species (Corbel, 2006). In addition to being a chronic disease, it is also one of the most important zoonotic diseases worldwide (Corbel, 2006; Pereira *et al.*, 2020). *Brucella abortus* has cattle as its preferred host, but it also infects and is important for other animals such as bison (*Bison bison*), elk (*Cervus elaphus*) (Scurlock *et al.*, 2010), camels (*Camelus dromedarius*) (Alamian *et al.*, 2019) and buffalo (*Bubalus bubalis*) (Fosgate *et al.*, 2011; De Massis *et al.*, 2019; Jaismon *et al.*, 2023). Vaccination of calves is the main strategy of a brucellosis control program, as it promotes a significant reduction in the prevalence of the disease (Olsen *et al.*, 2005; Dorneles *et al.*, 2015a; Dorneles *et al.*, 2017). In this scenario, S19 and RB51 are the most widely used vaccines worldwide for controlling brucellosis in cattle (Dorneles *et al.*, 2015a). S19 is a live attenuated vaccine derived from a virulent isolate of *B. abortus* biovar 1 that was attenuated after being forgotten on the bench at room temperature for about 1 year (Buck, 1930; Olsen *et al.*, 2005; Dorneles *et al.*, 2015a). The S19 vaccine contains a perosamine homopolymer called O-chain in its lipopolysaccharide (LPS), an immunodominant antigen, to which most of the antibodies triggered by vaccination and infection are driven, making it impossible to differentiate both using conventional serological tests (Olsen *et al.*, 2005; Andrade *et al.*, 2024). On the other hand, the RB51 is a rough *B. abortus* biovar 1 strain that was obtained from successive passages of the *B. abortus* 2308 strain in media with subinhibitory doses of rifampicinand penicillin (Schurig *et al.*, 1991; Schurig *et al.*, 2002). The RB51 strain minimally expresses the LPS O chain and thereby, it is a DIVA vaccine (Differentiating Infected from Vaccinated Animals) that does not interfere with disease diagnostic tests (Schurig *et al.*, 1991; Olsen *et al.*, 2005; Dorneles *et al.*, 2015a; Oliveira *et al.*, 2022; Andrade *et al.*, 2024).

The World Organization for Animal Health (WOAH) recommends vaccination with S19 of female cattle 3 to 8 months of age with a single dose of $5–8 \times 10^{10}$ colony forming units (CFU) and, for adult animals, a reduced dose of 3×10^{8} CFU to 5×10^{9} CFU (WOAH, 2022). For RB51, a dose of $1–3.4 \times 10^{10}$ CFU is recommended for 4 to 12 month old cattle, and for adults a reduced dose of $1–3 \times 10^{9}$ CFU (WOAH, 2022). However, there are no specific recommendations by WOAH for vaccinating buffaloes with S19 or RB51 strains. Furthermore, there are a limited number of articles in the literature on the brucellosis vaccination of buffaloes and that use very different methodologies (Bonaduce *et al.*, 1985; Chaudhuri *et al.*, 2021; Afzal *et al.*, 2000; Chand *et al.*, 2013; Iovane *et al.*, 2007; Roxo *et al.*, 1994; Jacobo *et al.*, 2005; Rivera *et al.*, 2016; Fosgate *et al.*, 2003; Galiero *et al.*, 2009; Pereira *et al.*, 2015; Caporale *et al.*, 2010; Diptee *et al.*, 2007; Ramnanan *et al.*, 2012; Diptee *et al.*, 2005; Diptee *et al.*, 2006; Ramnanan *et al.*, 2018; El-Razik *et al.*, 2011; Domingues *et al.*, 1992; De Massis *et al.*, 2023; Shome *et al.*, 2020; Bora *et al.*, 2008; Jamal *et al.*, 2003; Nardi Júnior *et al.*, 2012; Poester *et al.*, 1999). Therefore, data on the literature is very heterogeneous on the vaccination of buffaloes against brucellosis using the two worldwide used vaccines, S19 and RB51.

The prevalence of brucellosis in buffaloes worldwide is estimated at 9.7%, but this number may be an underestimate due to the limited data available on the prevalence of this disease in buffalo herds (Shi *et al.*, 2021). Therefore, brucellosis is still a disease that can cause production losses and pose a public health risk. Thus, the aims of this systematic review were to organize and discuss the literature on vaccination of water buffaloes against brucellosis.

2. Material and Methods

The literature review was conducted using PRISMA (Preferred Reported Items for Systematic Reviews and Meta-Analyses) methodology (Page *et al.*, 2021) (Table S1).

2.1 Search strategy and study selection

The search was conducted on August 22, 2023, and keywords were used to identify studies in seven search platforms: CABI, Cochrane, PubMed, Scielo, Science Direct, Scopus, and Web of Science. The PICOT (population, intervention, comparison, outcome, and time) used for the search was: buffalo (population), vaccination, S19, strain 19, RB51, *Brucella* (intervention), abort*, serology*, efficacy, protection, immunity, sera, serum (comparison), effectiveness, efficacy, protection, immunity (outcome), with no restrictions on the time that study was published. The search terms used for each platform are detailed in Table S2.

After the searches, the references retrieved in each platform were selected and exported to the EndNote program (version X7-Bld 7072, Thomson Reuters, 2013). An automatic search for duplicates was then performed and these were manually analyzed and excluded by a team member (KMGG). All records that lacked information that made it impossible to identify the reference were excluded from the searches. The remaining references were imported into the web-based software Rayyan (Rayyan Systems Inc., USA, 2002), in which the title and abstract analyses were performed.

The selection by title and abstract was performed by two collaborators: KMGG and MSG. All titles that addressed the term “brucellosis” or “buffalo” were selected for the abstract analysis. The abstracts that addressed the terms “vaccination” or “brucellosis”, or “water buffalo” were selected for the full-text analysis. Disagreements in the selection were solved by consensus between the two collaborators. The full text selection was performed by 4 collaborators: KMGG, MSG, APL and EMSD. A complementary search was also performed in the reference lists of all selected articles, to identify studies not recovered during the initial search.

2.2 Inclusion and exclusion criteria

The inclusion criteria used were: (i) articles from all countries and (ii) from all years; (iii) written in English, Italian, Spanish, French or Portuguese; (iv) focused on buffalo (*Bubalus bubalis*), (v) vaccination, and (vi) brucellosis; and (vii) original research studies. Articles that did not address the evaluation of vaccine response, either humoral or cellular, were excluded from the review. All studies that aimed only to validate diagnostic tests or that evaluated only the presence of antibodies or vaccine strains in buffalo milk were also excluded. The complete inclusion and exclusion criteria are presented in Table S3.

2.3 Type of Studies Included

Cross-sectional and cohort studies of original research were included in the selection. Case-control, case series and case reports were excluded, as well as conference proceedings, book, book chapters and reviews.

2.4 Data extraction

In this review, a study was characterized by the full text, while trials comprised a single comparison of a vaccination protocol with a control group or with other experimental groups. The data from the studies were extracted by one of the collaborators (KMGG). The extracted data include: first author, year of publication, country where the study was conducted, number of animals used, number of animals per group, age at vaccination, vaccine strain, vaccine dose, route of administration, use of revaccination, revaccination dose, interval until revaccination, challenge strain, challenge dose, challenge route, type of immune response analyzed (humoral or cellular), immunological tests used, and duration of study.

3. Results

3.1. Selected studies

The literature review retrieved studies from 1926 to 2022. The search strategy using seven databases identified 1,839 records, of which 263 duplicates were excluded and 189 were excluded due to insufficient reference information to identify the full text. Of the 1,387 remaining records, 814 were excluded after analyzing the titles, and then another 509 were excluded after reading the abstract, both based on the inclusion and exclusion criteria (Table S3), and 3 studies did not have the full text found. Among the 61 full text assessed for eligibility, 22 studies were selected. Subsequently, through the search of the reference list of these full texts, 18 articles were selected, as well as one article was also included due to an expert suggestion (APL) (De Massis *et al.*, 2023). After screening the studies selected from the reference lists using the same criteria previously established, three were included, totaling 25 studies to compose the systematic review. A summary of the data from the article selection is presented in Figure 1.

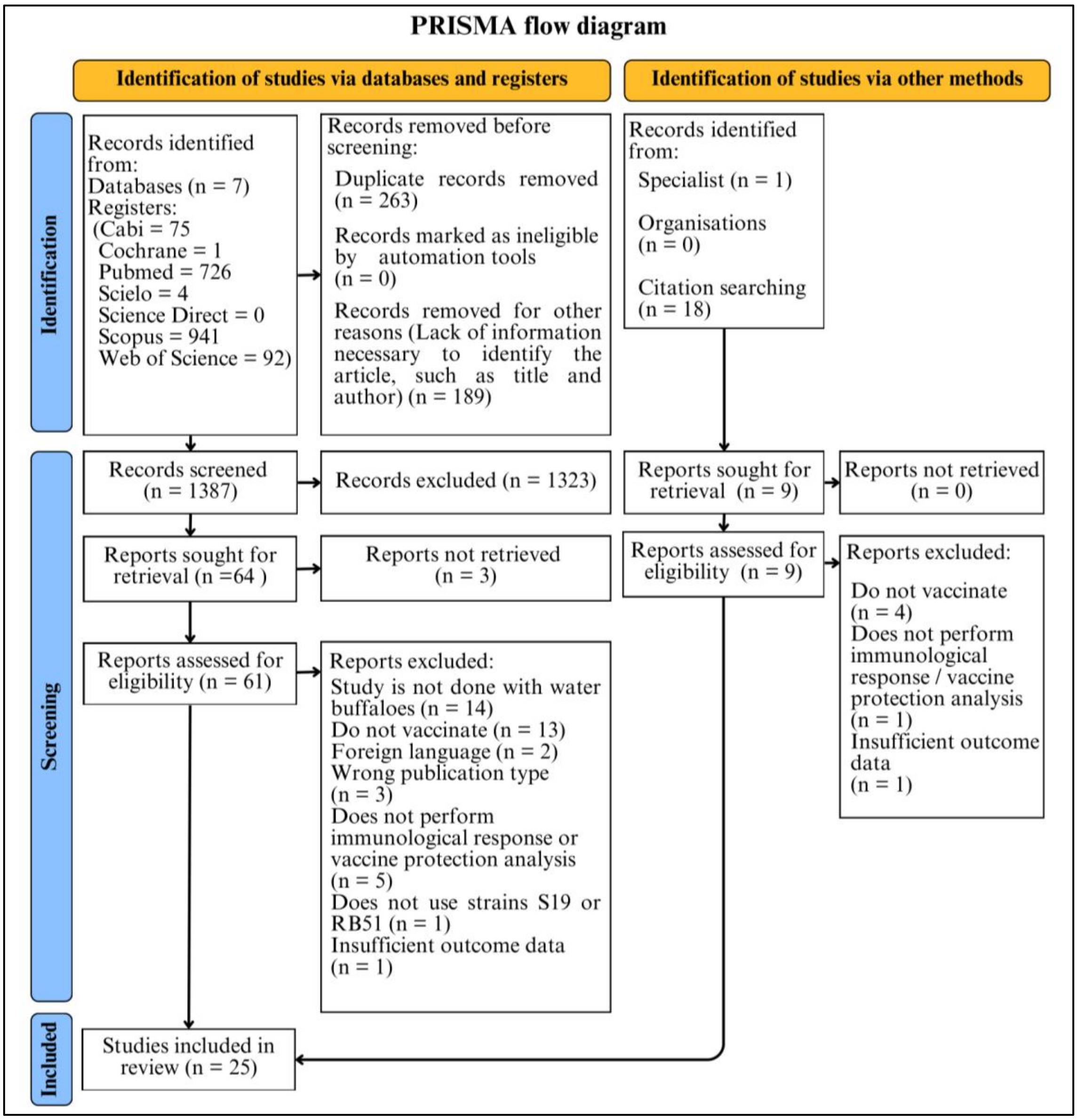

Figure 1. PRISMA flowchart used to select studies for this systematic review.

Among the 25 selected articles, 8 were cohort studies, 3 were prevalence studies, and 14 were studies evaluating the immune response. It is possible for a study to contain more than one trial, therefore, of the 25 selected studies, 10 [40% (10/25)] presented only one trial per study, while 15 [60% (15/25)] showed more than one trial per study, summing 65 analyzed trials. Regarding the year of publication of the studies, 4 of the 25 studies [16% (4/25)] were published before the year 2000, while the other articles [84% (21/25)] were published from 2000 to 2023 (Figure 2).

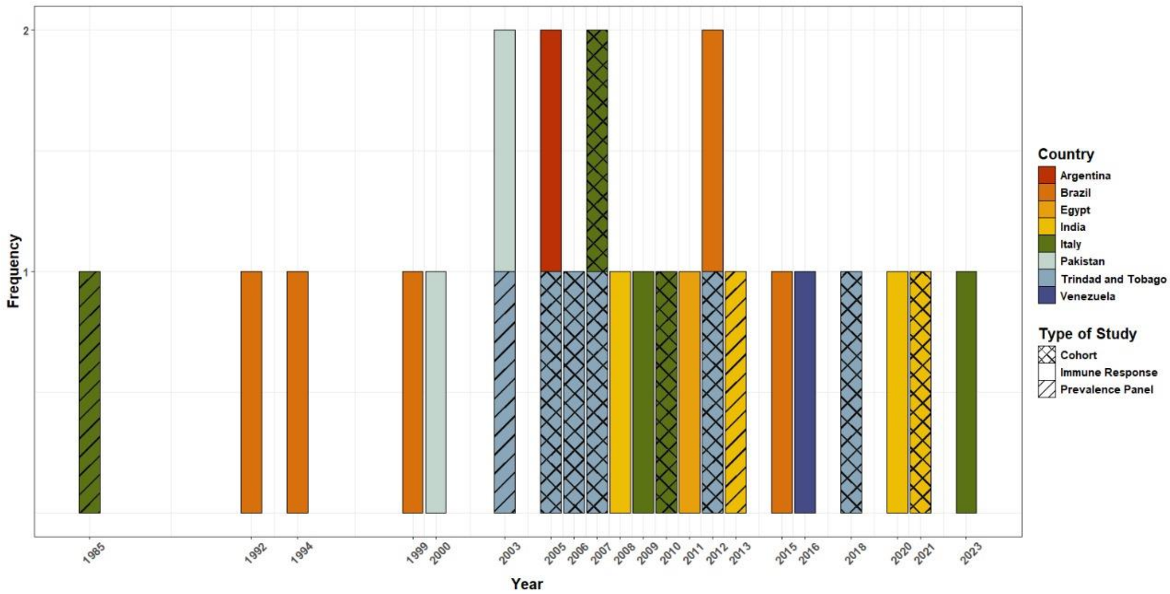

Figure 2. Frequency of the type of study, country and year of publication of the studies included in the systematic review.

3.2 Animals used and immunological response analyzed

Of the analyzed studies, all were carried out on water buffaloes, although 18 [72% (18/25)] did not report the breed used. Among the reported breeds the most frequent were Murrah [8% (2/25)] (Chand *et al.*, 2013; Chaudhuri *et al.*, 2021), Mediterranean [8% (2/25)] (Iovane *et al.*, 2007; Galiero *et al.*, 2009), Nili-Ravi [4% (1/25)] (Afzal *et al.*, 2000) and Egyptian [4% (1/25)] (El-Razik *et al.*, 2011). One study [4% (1/25)] (Domingues *et al.*, 1992) used Jafarabadi and Murrah breeds.

The number of animals used in the studies ranged from 5 (Jamal *et al.*, 2003) to 737 (Bonaduce *et al.*, 1985), with a mean of 60.64 (± 143.24) and median of 30 [Interquartile range (IQR) = 16]. The number of animals vaccinated per study ranged from 5 to 737, with a mean of 56.68 (± 144.09) and median of 21 (IQR = 15). The number of animals in the control group had a mean of 6.6 (± 3.37) and median of 144.1 (IQR = 2). Regarding the sex of the animals used, 18 [72% (18/25)] studies used only females, whereas 6 [24% (6/25)] studies used both males and females. Of the studies that used both males and females, in 4 [66.6% (4/6)] males were vaccinated (Fosgate *et al.*, 2003; Diptee *et al.*, 2005; Diptee *et al.*, 2006; Diptee *et al.*, 2007), and in 2 [33.3% (2/6)] they were used only in the control group for serology comparison (Pereira *et al.*, 2015; De Massis *et al.*, 2023). Only one [4% (1/25)] study did not specify whether it used only females (Afzal *et al.*, 2000).

Regarding the analyses of the immunological response of vaccinated animals, 20 [80% (20/25)] studies performed only analysis of the humoral response and 2 [8% (2/25)] performed only analysis of the cellular response (Diptee *et al.*, 2007). Another three [12% (3/25)] studies analyzed both the humoral and the cellular response (Table S4).

3.3 Vaccine strain, dose and route of administration

Eleven studies [44% (11/25)] tested only S19 strain, 10 [40% (10/25)] used only RB51 strain, and 2 [8% (2/25)] assessed both strains. Moreover, one [4% (1/25)] study used S19 strain together with 45/20 (Bonaduce *et al.*, 1985) and one [4% (1/25)] study used S19 strain with *S19Δper* mutant strain (Chaudhuri *et al.*, 2021).

Considering the 65 trials, 27 [41.5% (27/65)] used the S19 strain, 33 [50.8% (33/65)] used the RB51 strain, 4 [6.2% (4/65)] used the *S19Δper* strain and one used the 45/20 strain [1.5% (1/65)]. The tested dose of S19 vaccine ranged from 3-4 x 10^8 CFU to 1-2 x 10^{10} CFU, being 10^{10} CFU [59.3% (16/27)] the most tested dose among the trials, followed by 10^9 CFU [11.1% (3/27)] and 10^8 CFU [7.4% (2/27)]. Furthermore, 4 [22.2% (6/27)] trials did not report the dosage in CFU (Afzal *et al.*, 2000; Pereira *et al.*, 2015). Revaccination of animals with S19 strain was evaluated in only one trial, using the same dose used in first vaccination (5 x 10^9), with an interval of 4 months (Chand *et al.*, 2013).

For the trials that used RB51, the tested dose ranged from 1 x 10^{10} CFU to 3.4 x 10^{11} CFU, with 10^{10} CFU [94% (31/33)] being the most commonly used one, followed by 10^{11} CFU [3% (1/33)]. Only one [3% (1/33)] trial did not clearly state the dose used (Iovane *et al.*, 2007). Booster vaccination was performed in 21 [63.7% (21/33)] trials, while 11 [33.3% (11/33)] used a single dose of the vaccine. In one [3% (1/33)] trial, it was not clear whether the animals received a booster dose (Iovane *et al.*, 2007). Of the trials that performed revaccination, 16 [76.2% (16/21)] used the same dose equal at primer vaccination and booster, while 5 [52,38% (5/21)] trials used different doses, with the primer vaccination of double dose of 1-3.4 x 10^{10} CFU / dose, and booster dose of 1-3.4 x 10^{10} CFU / dose (Rivera *et al.*, 2016). Regarding the revaccination interval for trials using RB51, the most used one was 4 weeks [38.1% (11/21)] followed by, 18 weeks [9.5% (2/21)], 20 weeks [4.8% (1/21)] and 6 months (24 weeks) [33.3% (7/21)]. The *S19Δpe*r mutant strain was used in only one study, and tested doses ranged from 1 x 10^9 CFU to 4 x 10^{10} CFU (Chaudhuri *et al.*, 2021). The 45/20 vaccine was also tested in only one study and the vaccine dose evaluated was 3-4 x10^{11} (Bonaduce *et al.*, 1985) (Table S4).

The most commonly used route of administration in the trials with S19 and RB51 was subcutaneous [71.7% (43/60)], followed by the conjunctival [1.7% (1/60)] (Chand *et al.*, 2013). Sixteen [26.6% (16/60)] trials did not indicate the route of vaccine administration (Roxo *et al.*, 1994; Afzal *et al.*, 2000; Jacobo *et al.*, 2005; Rivera *et al.*, 2016). In the four trials that evaluated the *S19Δper* mutant vaccine, the route used was subcutaneous, while in the trial using the 42/20 vaccine the route of administration was intramuscular.

3.4 Vaccination age and vaccination of pregnant animals

Most of the animals used in the trials aged between 3 and 12 months [78.5% (51/65)], however, 10 [15.4% (10/65)] trials used animals older than 12 months. In 2 trials, the animals were between 5 and more than 20 months old (Fosgate *et al.*, 2003; Chand *et al.*, 2013), and in two trials the age of the animals used was not reported (Iovane *et al.*, 2007; Galiero *et al.*, 2009). Only one study performed vaccination with the RB51 strain in pregnant buffaloes, at 3 to 6 months of gestation (Galiero *et al.*, 2009) (Table S4).

3.5 Effectiveness study

Two studies [8% (2/25)] carried out an observational field study using the S19 strain. The study by Bonaduce *et al*. (1985) pointed out the occurrence of abortion, placenta retention, and persistent antibody titers in buffaloes vaccinated with S19. Chand *et al*. (2013) carried out a study in a herd with a 31.62% prevalence of brucellosis, and pointed out that after vaccinating buffalo calves with a dose of 4 x 10^{10} CFU/dose and adult buffaloes with reduced doses of 5 x 10^{9} CFU/dose and a booster after 4 months with the same dose, the number of abortions in the herd was reduced.

Only one study [4% (1/25)] evaluated the efficacy of the RB51 vaccine, through an observational study under field conditions, in which vaccinated animals (1-3.4x10^{10} CFU/dose) were placed with animals infected by B. abortus, on a property with a prevalence of 56% of brucellosis cases (Fosgate *et al*., 2003).

3.6 Challenge: strains, dose, and route of exposure

Only 5 [20% (5/25)] studies tested vaccine efficacy through controlled clinical trials. Among these, 12 [18.4% (12/65)] trials performed challenge, 2 [16.7% (2/12)] in animals vaccinated with S19 strain (Caporale *et al.*, 2010; Chaudhuri *et al.*, 2021), 8 [66.6% (8/12)] in animals vaccinated with RB51 strain (Diptee *et al.*, 2007; Iovane *et al.*, 2007; Caporale *et al.*, 2010; Ramnanan *et al.*, 2018) and 2 [16.7% (2/12)] in animals vaccinated the *S19Δper* strain (Chaudhuri *et al.*, 2021). The strain used to challenge animals vaccinated with S19 was *B. abortus* 544, used in both [100% (2/2)] trials (Caporale *et al.*, 2010; Chaudhuri *et al.*, 2021). To challenge animals vaccinated with RB51, the strains used were the Trinidad 1 [50% (6/8)]

(Diptee *et al.*, 2007; Ramnanan *et al.*, 2018), strain 24E [33.3 (4/8)] (Diptee *et al.*, 2007), *B. abortus* 544 [8.3% (1/8)] (Caporale *et al.*, 2010) and CZV S19 Fatro vaccine strain [8.3% (1/8)] (Iovane *et al.*, 2007). It is worth to mention that in one of these studies, four trials were performed using the Trinidad 1 strain followed by 24E strain as challenge (Diptee *et al.*, 2007). For animals vaccinated with *S19Δper* vaccine, the vaccine strain used for challenge in both [100% (2/2)] trials was *B. abortus* 544 (Chaudhuri *et al.*, 2021).

The challenge dose ranged from 1 x 10^7 CFU to 1 x 10^{10} CFU, with 1 x 10^7 CFU being the most used challenge dose. The most used challenge route was conjunctival [50% (6/12)]. In four [33,3% (4/12)] trials from the same study, the same animals were also challenged via conjunctival and subcutaneous routes, by more than one strain (Trinidad 1 and E24) (Diptee *et al.*, 2007). Moreover, in other two [16,7% (2/12)] trials from the same study, the used challenge route was intravenous (Ramnanan *et al.*, 2018). The vaccination and challenge data are detailed in Table S4.

3.7 Post-vaccination serology

Of the trials conducted with S19 vaccine, all performed at least one serological test [100% (27/27)] after vaccination. Among the tests most used were Rose Bengal Test (RBT) [66.6% (18/27)], followed by 2-Mercaptoethanol test (2-ME) [51.8% (14/27)], Serum Tube Agglutination Test (SAT) [66.6% (18/27)], Enzyme-linked Immunosorbent Assays (ELISA) [25.9% (7/27)], Standard Plate Agglutination Test (SPAT), Buffered Plate Agglutination Test (BPAT), [11.1% (3/27)], Complement Fixation Test (CF) [7.4% (2/27)].

All trials using RB51 also performed at least one serological test [100% (33/33)] after vaccination. Among the conventional tests used to evaluate whether there was seroconversion of vaccinated animal were BPAT [51.5% (17/33)], RBT [30.3% (10/33)], CFT [24.2% (8/33)], SPAT [21.2% (7/33)], Fluorescence Polarization Immunoassay (FPA) [21.2% (7/33)] and SAT [6% (2/33)]. Other tests specific for detection of anti-RB51 antibodies were used to evaluate the vaccine response, such as ELISA [54.5% (18/33)], CF-RB51 [45.4% (15/33)] and RB51 Dot-blot Assay [21.2% (7/33)]. Three studies evaluated the shedding of the RB51 strain, which involved multiple biopsies of superficial cervical lymph nodes from buffaloes after vaccination and the results are in Appendix S5 (Diptee *et al.*, 2005; Diptee *et al.*, 2006; Ramnanan *et al.*, 2018).

Antibody clearance of the vaccine for the S19 strain showed wide variation between trials, with a minimum of 69 days and a maximum of over 56 months. For vaccination with RB51 strain, clearance ranged from 49 days to over 49 weeks. Data of the trials used, vaccine clearance and strain used are shown in Table S5.

3.8 Post-vaccination cellular response

The cellular response was evaluated in 20 [30.7% (20/65)] assays. In animals vaccinated with the S19 strain, the cellular immune response was analyzed in 4 [20% (4/20)] assays using ELISA and flow cytometry (Shome *et al*., 2020). The results of Shome *et al*. (2020) indicate that animals vaccinated with RB51 showed an increase in CD4 and CD8 cells, with this increase being considered dependent on the dose used. The TH1 response, assessed by IL-12, TNF-α, and IFN-γ, showed lower responses in animals vaccinated with reduced doses.

For the RB51 strain, the cellular immune response was analyzed in 16 [80% (16/20)] assays (Diptee *et al*., 2005; Iovane *et al*., 2007; Galiero *et al*., 2009; Rivera *et al*., 2016). The methodologies used to evaluate the cellular response in the assays for both strains were ELISA [55% (11/20)] (Diptee *et al*., 2005; Rivera *et al*., 2016) and skin test [25% (5/20)] (Iovane *et al*., 2007; Galiero *et al*., 2009). For Iovane *et al*. (2007), cult tests were performed in animals vaccinated with doses of 1-3.4 x 10^{10} CFU, 1-3.4 x 10^{10} CFU with booster, three times the dose of 1-3.4 x 10^{10} CFU with booster and ten times the dose of 1-3.4 x 10^{10} CFU, and for Galiero *et al*. (2009), the dose of three times 1-3.4 x 10^{10} CFU. At all doses tested, the culture test was positive, demonstrating a cellular response, even in animals vaccinated with the standard dose. The other two studies used standardized bovine ELISA tests to quantify IFN-γ (Diptee *et al*., 2005; Rivera *et al*., 2016). The study by Diptee *et al*. (2005) shows that IFN-γ production in animals vaccinated with RB51 was low. Furthermore, the histology of the lymph nodes of vaccinated animals was evaluated and showed that animals vaccinated with a dose of 2.02 x 10^{10} CFU, either as a single dose or booster, presented multiple germinal centers in the cortical region of the lymph node without visualization of expansion of the intercortical zone. Rivera *et al*. (2016) show that the peak of IFN-γ production in vaccinated animals occurs around the fourth week after primary vaccination.

3.9 Vaccine safety

The safety of the RB51 vaccine was tested in two studies with non-pregnant cows [20% (2/10)] (Iovane *et al.*, 2007; De Massis *et al.*, 2023). In the analysis by Iovane *et al*. (2007), they tested a vaccine dose ten times higher than the 3.4 x 10^{10} CFU dose, and the animals showed no clinical reaction. In the study by De Massis *et al*. (2023), buffaloes were vaccinated between 5 and 9 months of age with a triple dose (three times the 1-3.4 x 10^{10} CFU dose) and revaccinated after 20 weeks. They were then impregnated at 24 months of age, and the effect of vaccination on pregnancy, parturition, and lactation of these animals was monitored. The results showed that the vaccine did not cause any changes in pregnancy, parturition, or lactation. Furthermore, the presence of the vaccine strain in milk, colostrum, cotyledons, vaginal swabs, lymph nodes, spleen, and other organs of the vaccinated animals was evaluated, and no RB51 was isolated. There was also no isolation of the strain in control animals.

Only one [10% (1/10)] study on the safety of the RB51 vaccine was performed in pregnant cows (Galiero *et al.*, 2009). None of the studies that tested S19 performed the vaccine safety evaluation. The study carried out by Galiero *et al*. (2009), in which pregnant buffaloes were vaccinated with a triple dose of the vaccine (three times the dose of 1–3.4 x 1010 CFU/dose) at 3 to 6 months of gestation, and the result showed that four animals (4/10) aborted, with the vaccine strain being isolated in only one fetus. Furthermore, none of the milk samples collected from vaccinated animals in the study by Galiero *et al*. (2009) presented the vaccine strain.

3.10 Post-challenge assessed outcomes

Post-challenge evaluation was performed by means of serological tests and isolation of the challenge strain in lymph nodes, liver, spleen, uterus, vaginal swab, and fetal contents. Of the two studies that used S19 and performed experimental challenge, one assay analyzed the presence of the challenge strain (*B. abortus* 544) in the lymph nodes, which was not identified in any vaccinated animal after 31 days of challenge (Caporale *et al.* 2010). In the study by Chaudhuri *et al.* (2021), an assay showed rapid clearance of the challenge strain after 30 days of inoculation in animals vaccinated with S19.

For the RB51 strain, Caporale *et al.* (2010) also challenged animals vaccinated with RB51 strain, and the challenge strain was found in only one vaccinated animal. Diptee *et al.*, 2007 challenged animals with Trinidad 1 and E24 strains, and, as result, seroconversion was observed in BPAT and c-ELISA after challenge in all vaccinated pregnant animals, regardless of the dose used. Ramnanan *et al.* 2018 carried out challenge with the Trinidad 1 strain in pregnant buffalo cows, and it was observed that all animals that were vaccinated twice with a dose of 1-3.4 x 10^{10} CFU presented seroconversion in BPAT, and in addition, the challenge strain was isolated from 8 calves of these buffalo cows. Of the animals vaccinated with a dose of 1-3.4 x 10^{10} CFU and booster after 18 weeks, it was observed that seven animals (7/9) presented seroconversion in BPAT, and the challenge strain was found in 5 calves of these buffalo cows. The study by Iovane *et al.* (2007) challenged animals vaccinated with RB51 vaccine using the CZV S19 Fatro strain, but it was not isolated in any challenged animals. Data from studies that performed challenges are shown in Table S4.

4. Discussion

This systematic review aimed to analyze the vaccine response of buffaloes vaccinated against *Brucella abortus*. Since only two studies evaluated the 45/20 and S19Δper vaccines, and both vaccines are not commercially used, the discussion of this review will focus only on S19 and RB51 strains, which were at the aim of the systematic review. Furthermore, S19 and RB51 strains are the most widely worldwide used vaccines for the control of brucellosis (Olsen *et al.*, 2005; Dorneles *et al.*, 2015a; Oliveira *et al.*, 2022). However, there are no official recommended doses of these vaccines for buffaloes (WOAH, 2022). Therefore, there are several studies seeking to understand whether vaccination with S19 and RB51 strains can induce a protective immune response in buffaloes. Thus, this review presents the main immunological responses observed, to date, in buffaloes vaccinated with S19 and RB51 strains.

The main strategy in a brucellosis control program is vaccination of calves (Olsen *et al.*, 2005; Dorneles *et al.*, 2017). In cattle, studies on protection, dose and efficacy of vaccines against brucellosis are well established (Oliveira *et al.*, 2022; Serpa Gonçalves *et al.*, 2025), while in buffaloes the topic has not yet been sufficiently explored and is still dispersed in the literature. Among the selected articles, a high heterogeneity in the design of the studies, as well as, in some studies, the absence of important information, such as dose, age of the animals, route of vaccine administration and the difficult to extract data from tables and graphs, precluded a better grouping and analysis of the data. Indeed, due to these huge variations and lack of consistent information, it was not possible to carry out a meta-analysis for protection of *B. abortus* vaccines in buffaloes based on the data available in the literature.

Moreover, approximately 80% of the selected studies used only humoral immunity to evaluate the vaccine response in buffaloes. This finding is probably because the evaluation of the humoral response is faster and easier to perform and analyze by routine diagnostic tests. Nonetheless, in brucellosis, the cellular response is the main immune response mechanism involved, while antibodies play a secondary role in protection (Olsen *et al.*, 2005; Dorneles *et al.*, 2014; Dorneles *et al.*, 2015c). Therefore, based on the studies that only evaluated the humoral response, it is not possible to say that buffaloes developed a protective immune response to vaccination, and thereby that the vaccines are effective. The efficacy of a vaccine can only be evaluated in studies that performed a controlled experimental study (controlled clinical trials) with animal challenge (Knight-Jones *et al.*, 2014). However, even in studies that performed an experimental challenge, the number of animals per group in 11 [44%(11/25)] analyzed studies was less than or equal to 6 animals, leading to a low analytical power (Afzal *et al.*, 2000; Jamal *et al.*, 2003; Diptee *et al.*, 2005; Diptee *et al.*, 2006; Diptee *et al.*, 2007; Iovane *et al.*, 2007; Bora *et al.*, 2008; Caporale *et al.*, 2010; Rivera *et al.*, 2016; Shome *et al.*, 2020; Chaudhuri *et al.*, 2021). This low number of animals used in clinical trials is certainly because large animal challenges experiments are very expensive and difficult to perform. Studies using the assessment of cellular immune response in buffaloes, which could be used to find correlates of protection and reduce the need for challenge experiments, are also restricted due to the low availability of commercial reagents, such as monoclonal antibodies or cytokine detection kits (ELISA, Luminex, etc) validated for buffaloes (Grandoni *et al.*, 2017; Capítulo 2 de Godoy, 2025).

Although protection cannot be inferred from the analysis of the data available in the literature on brucellosis vaccination of buffaloes, the persistence of vaccine antibodies demonstrated that S19 is efficient in stimulating humoral response. The wide variation in antibody clearance in studies with S19 was related to differences in experimental design, such as age, vaccine dose and booster. Two studies with S19 analyzed antibody clearance according to the age of vaccination and showed that clearance of animals vaccinated at 6 months ranged from 69 days to 6 months, while in animals aged 8 to 9 months ranged from 190 days to 12 months (Table S5). Thus, they showed that the younger the animals are vaccinated with S19 strain, the lower is the persistence of vaccinal antibodies, similar to what was observed in cattle (Nicoletti, 1990). Only one study, which vaccinated buffalo calves between 4 and 10 months of age with the dose of 6×10^{10} CFU, demonstrated vaccinal antibody persistence over 56 months in more than 100 animals (Bonaduce *et al.*, 1985). This study showed that a large number of animals presented vaccine persistence until adulthood. In cattle, the persistence of vaccine antibodies in adult females vaccinated with S19 between 3 and 8 months of age is about two animals for every 100,000 vaccinated calves (Meyer *et al.*, 1969; Jones *et al.*, 1976; Dorneles *et al.*, 2015a). What could justify the result of the (Bonaduce *et al.*, 1985) study would be an unequivocal interpretation of the serological analyses, since the study carried out the vaccination of buffaloes calves with negative serological tests, but which were on a property with a prevalence of brucellosis of 30%. In other words, the study may have vaccinated calves in the early stages of *B. abortus* infection, which did not present seroconversion in the test, with the persistence of antibodies being related to the infection and not to the vaccination. No studies have evaluated clearance of the S19 vaccine strain in buffaloes.

In adult cattle the S19 vaccine can be used at a reduced dose of 3–10 × 10^9 CFU / dose in an attempt to reduce the period of persistence of vaccine antibodies (Dorneles *et al.*, 2015a). Similarly, the use of reduced vaccination in adult cattle was used as a strategy to reduce outbreaks in infected herds, but its use was discontinued because it caused interference with routine testing and abortions in pregnant animals (Corner *et al.*, 1981; Nicoletti, 1981; Dorneles *et al.*, 2015a). The use of reduced doses of the strain S19 may be economically beneficial for countries with large buffalo populations, however, in none of the reduced-dose studies the animals were challenged, and, although the vaccine showed lower vaccine persistence, the protection was not evaluating and is unknown. Furthermore, due to its DIVA properties, RB51 would probably be more suitable for vaccination of those animals (Dorneles *et al*., 2015b; Oliveira *et al*., 2022).

Only one study evaluated the cellular response in calves vaccinated with S19 vaccine (Shome *et al.*, 2020). However, in their methodology (Shome *et al.*, 2020), ELISA tests standardized for cattle were used to identify IL-12 and TNF-α, without first analyzing their functionality for buffalo cytokines. In addition, flow cytometry was performed with monoclonal antibodies without informing which clone and whether this clone is specific or presents cross-reactivity with buffalo cells. Also, it did not inform whether peripheral blood culture with *Brucella abortus* antigen was performed to count only CD4 and CD8 cells stimulated by vaccination. In other words, although the study indicates that the S19 vaccine stimulated a cellular response in buffaloes, due to the lack of information provided in the methodology, we cannot characterize the cellular response in buffaloes induced by the S19 vaccine.

However, Chaudhuri *et al.* (2021) showed in their study that challenged animals presented few histopathological changes, and the bacterial count of the lymph nodes, liver, and spleen showed a rapid reduction after vaccination, compared to the control group. Caporale *et al.* (2010) indicated that after the challenge, no vaccinated animal presented bacterial isolation in the spleen, retropharyngeal, and supramammary lymph nodes. From these data, we can affirm that the S19 vaccine promotes a cellular immune response. Regarding vaccine efficacy, Caporale *et al.* (2010) state that the power of the study was low, requiring a larger number of animals to identify differences between the vaccinated group and the control group. Therefore, the results of this study cannot be used to evaluate the efficacy of the S19 vaccine in buffaloes. *B. abortus* strain 544, used as challenge strain in studies the S19 vaccine, is one of the most widely used in clinical trials in cattle (Oliveira *et al.*, 2022). Furthermore, in a murine model, strain 544 did not present a significant difference from the *B. abortus* 2308 strain for use in challenge (Miranda *et al*., 2015), and the dose of 10^7 CFU used in challenges is the closest to a natural infection (Manthei, 1959). From those data, we can affirm that the S19 vaccine promotes a cellular immune response, but we cannot confirm its efficacy in buffaloes.

Regarding vaccine safety, a study reported the occurrence of abortion in buffaloes vaccinated during calfhood (Bonaduce *et al.*, 1985). However, the study considered all cases of abortion and retained placenta in buffaloes as a result of vaccination with S19 when they were calves, without carrying out any type of test to diagnose the cause of abortion in the animals, given that the property had a prevalence of 30% of animals positive for brucellosis. Young female cattle vaccinated with S19 may, in some cases, present persistence of the strain in the reproductive tract, causing abortion (Corner *et al.*, 1981; Dorneles *et al.*, 2015b). Although it is possible that abortions caused by the S19 strain occur in buffaloes, to prove this a study would be necessary in which the presence of the strain in fetal material or in the aborted fetus was evaluated, and this was not carried out in the study by Bonaduce *et al*., 1985)

All trials with RB51, 33 [100% (33/33)], tested its DIVA characteristic in buffaloes, and there was no reactivity of the vaccinated animals in routine tests against *B. abortus*, confirming that RB51 is a DIVA vaccine for buffaloes. This is important because it shows that the RB51 vaccine is ideal for vaccinating adult animals that were not vaccinated as young animals with S19. In four [40% (4/10)] studies, male buffaloes were vaccinated. However, the use of males in the study does not allow us to assess the protection of the vaccine against abortions, which is the main clinical sign of brucellosis. Furthermore, vaccination of males is not recommended, as they are not epidemiologically important in the transmission of the disease and there is risk of testicular infection and infertility, as has already been reported for cattle vaccinated with S19. (Lambert *et al.*, 1963; Dorneles *et al.*, 2015a).

The most used standard dose of RB51 was 10^{10} CFU, and in 23 [69,7% (23/33)] trials animals between 4 and 10 months of age were used. Another 10 trials used adult animals but maintained the dose of 10^{10} UFC. In cattle, the standard dose of 1-3.4 x 10^{10} UFC is generally recommended for calves and a dose of 1 x 10^9 UFC is recommended for adult animals (WOAH, 2022). In buffaloes, an effectiveness study conducted by Fosgate *et al.* (2003) they pointed out that the RB51 vaccine at the recommended dose for cattle (1-3.4 x 10^{10} UFC) is not effective. However, analyzing the methodology of this study, it is possible to identify some points that raise doubts on the reliability of the results. Initially, the authors state several times that the animals were thin and in poor physical condition, and that some animals died. The nutritional status of the animals may have caused a reduction in the activity of the cellular and humoral response of these animals, leading to a vaccine failure due to malnutrition, or even a difficulty in presenting seroconversion (McFarlane *et al.*, 1973; Schaible *et al.*, 2007; Hoang *et al.*, 2015). In addition, the study shows a statistical power, which is the ability to find a difference when it exists, of only 22%, which is a very low value to estimate the reliability of the study (Dohoo *et al.*, 2012). This occurred because the final number of animals compared was lower than planned, since during the course of the study, there were deaths of animals and low seroconversion. It is worth noting that the presence of pregnant females is the main risk factor for the spread of brucellosis in a herd (Olsen *et al*., 2010). In other words, in the study by Fosgate *et al*. (2003), there is no evidence that the infected animals were pregnant. Furthermore, it was reported that the entire herd was vaccinated with RB51, which could explain the low seroconversion of the animals.

Based on the work of Fosgate *et al*. (2003), other studies sought to test higher and booster doses of RB51 for buffaloes. Based on the results of Fosgate *et al*. (2003), in one study, Diptee *et al*. (2005) assumed that the RB51 vaccine does not promote a cellular immune response based on the low production of IFN-gamma after stimulation. However, they performed an ELISA test using bovine anti-IFN-gamma antibodies without first evaluating their functionality in labeling buffalo cytokines. Therefore, the study does not present sufficient data to assume that the RB51 vaccine does not induce a good cellular response at the different doses tested. In another study, Diptee *et al*. (2006) showed that higher doses with a booster presented greater antibody clearance. In other words, the RB51 vaccine is capable of stimulating the immune system of buffaloes. Iovane *10.*, 2007, highlighted that a triple dose (three times the dose 1-3.4 x 1010 CFU) with a booster would be the most appropriate for immunizing buffaloes against brucellosis. However, this study corroborates this statement based on the animals' humoral response, a fact that is not relevant to the protection of animals against brucellosis.

The booster dose intervals commonly used in the RB51 trials were 4 to 20 weeks. One study showed that revaccination with an 18-week interval resulted in greater persistence of vaccine antibodies (Ramnanan *et al*., 2012). Administering a booster dose with a short interval (4 weeks) may affect the B and T cell differentiation pathway, resulting in a weaker immune response (Pettini *et al*., 2021).The evaluation of the clearance of the vaccine strain RB51 was assessed by three studies (Diptee *et al.*, 2005; Diptee *et al*., 2006; Ramnanan *et al*., 2018). The analyses performed in both studies appear to be the same and present the same results. They show that animals vaccinated with the dose of 2.02 x 10^{10} CFU without a booster presented clearance of the RB51 strain in the cervical lymph node within 4 to 6 weeks. In only one animal vaccinated with a double dose (twice the dose of 2.02 x 10^{10} CFU) was the vaccine strain detected two weeks after vaccination. Furthermore, the vaccine strain was not found in any animal that received the booster. These results differ from those presented for cattle, with clearance of the RB51 strain observed from 6 to 14 weeks using the dose of 1.6 x 10^{10} (Olsen *et al*., 1999; Oliveira *et al*., 2022). Furthermore, the vaccine strain was not isolated in the lymph node of animals vaccinated with higher doses in the studies by Diptee *et al*. (2005) and Diptee *et al*., (2006). This demonstrates that the RB51 vaccine in buffaloes is efficiently eliminated from the animal's organism, without inducing persistent infection in the animals. Another important analysis conducted by Diptee *et al*. (2006) is whether the strain was excreted via the nasal, ocular, preputial, or vaginal routes. They found that no vaccine strain was isolated from any animals in the control or vaccinated groups, even those vaccinated with a double dose (2.02 x 10^{10} CFU) and a booster. In other words, the vaccine is not capable of causing horizontal infection, even with higher doses.

Regarding the safety of the RB51 vaccine, WOAH recommends administering one to three times the recommended dose to test its safety, evaluating for 21 days whether there are local and systemic effects (WOAH, 2022). In the analysis by Iovane *et al*. (2007), they tested a vaccine dose ten times higher than the 3.4 × 10^{10} CFU dose, and the animals showed no clinical reaction. Therefore, we can state that the RB51 vaccine does not cause any local or systemic damage in buffaloes, even at high doses. The absence of isolation of the bacteria in the milk and colostrum of buffaloes vaccinated as calves in the study by De Massis *et al*. (2023) is consistent with the study by Longo *et al*. (2009), which also conducted with buffaloes using the 1.02 × 10^{11} CFU vaccine dose and booster vaccination. Furthermore, the lack of isolation of the strain in other samples in the study by De Massis *et al*. (2023) can be explained by the rapid elimination of the vaccine strain demonstrated by Diptee *et al*. (2005) and Diptee *et al*. (2006). Therefore, the RB51 vaccine when administered to young buffaloes, even at higher doses and with booster vaccination, is safe and does not interfere with pregnancy. The only study that tested vaccination of buffaloes vaccinated during pregnancy with RB51 was conducted by Galiero *et al*. (2009). The study shows that the triple-dose RB51 vaccine (three times the dose of 1–3.4 x 10^{10} CFU/dose) can induce abortion in pregnant animals. This result differs from a study with cattle, conducted by Palmer *et al*. (1997), in which they vaccinated cows at 6 months of gestation with a dose of 10^{9} CFU/dose of the RB51 vaccine and observed no abortions. The induction of abortion in the study by Galiero *et al*. (2009) may be associated with the high dose of the vaccine, since the study used adult animals, and the dose used was higher than the dose recommended by WOAH for adult cattle (1–3 × 10^{9} CFU). Furthermore, none of the animals in the study showed excretion of the vaccine strain in the milk. This finding agrees with the study by Miranda *et al*. (2016) carried out in cattle, in which they vaccinated cows in the postpartum period with the RB51 vaccine at a dose of 1.3 × 10^{10} CFU/dose, and excretion of the strain in the animals' milk was also not observed. However, also in cattle, Samartino *et al*. (2000) demonstrated that pregnant cows vaccinated with RB51 at a dose of 1.5 × 10^{9} CFU during gestation presented elimination of the vaccine strain in the milk, although for a short period. This demonstrates that, in buffaloes, vaccination of animals with doses higher than those recommended for cattle does not induce excretion of the strain in the milk.

In evaluating the cellular response at all doses tested, the culturing test showed a positive result, demonstrating a cellular response even in animals vaccinated with the standard dose. The other two studies used standardized ELISA tests for cattle to quantify IFN-γ, without first evaluating the function and standardizing the test for buffaloes. Therefore, the study by Diptee *et al*. (2005) shows that IFN-gamma production in animals vaccinated with RB51 was low, which may indicate a poor cellular immune response, especially in animals vaccinated with a single dose of 2.02 x 10^{10} CFU. Furthermore, it also evaluated the histology of the lymph nodes of vaccinated animals and showed that animals vaccinated with a dose of 2.02 x 10^{10} CFU, whether as a single dose or booster, presented multiple germinal centers in the cortical region of the lymph node without visualization of expansion of the intercortical zone, indicating a good B cell response but a poor T cell response. This differs from the results found in cattle, where a strong Th1-mediated immune response with high IFN-gamma production was observed (Dorneles *et al*., 2014; Dorneles *et al*., 2015c). Therefore, it is believed that the RB51 vaccine induces a cellular response, but additional immunological studies are needed to confirm and characterize the cellular immune response in vaccinated buffaloes.

Four studies conducted a challenge to evaluate the efficacy of the RB51 vaccine administered at different doses (Diptee *et al*., 2007; Iovane *et al*., 2007; Caporale *et al*., 2010). In the study by Diptee *et al*. (2007), two field strains, Trinidad 1 and 24E, isolated from naturally infected animals, were used for the challenge, without prior testing to evaluate the virulence of the strains in buffaloes. The study initially performed two challenge attempts, each with a single strain, at a dose of 10^7 CFU/dose administered conjunctivally over a short period of time (5 weeks), but the challenge was unsuccessful. To perform the challenge test in a study, it is necessary to use a known pathogenic sample (Manthei, 1968; Miranda *et al*., 2015; Oliveira *et al*., 2022). Therefore, the challenge may not have been successful due to the low pathogenicity of the strain. A final challenge attempt was performed with both strains at doses of 1.2 x 10^8 CFU via the conjunctiva and 1.1 x 10^{10} CFU via the subcutaneous route, simultaneously, and seroconversion and isolation of the challenge strain in regional lymph nodes were observed. However, when using a challenge strain, it is necessary to know in advance whether the sample is inducing abortion in the animals and the best concentration to use, as very low concentrations may not infect the animals, and very high concentrations can induce abortion, even in vaccinated animals (Manthei, 1968; Miranda *et al*., 2015). Therefore, this study was unable to evaluate the efficacy and protection of the vaccine by the challenge performed. In another study, Iovane *et al*. (2007) used the CZV S-19 Fatro strain (vaccine strain) to challenge an animal, but did not observe seroconversion. However, the S19 strain is an attenuated strain and is not recommended as a challenge strain (Manthei, 1968; Dorneles *et al.*, 2015a; Oliveira *et al.*, 2022). Similarly, the S19 strain is apparently not pathogenic for buffaloes, since it is also used to immunize these animals, and there are no reports that it has caused abortion in vaccinated animals. In the study by Ramnanan *et al.* (2018), the vaccine challenge was also performed using the Trinidad 1 strain, previously tested in two pregnant females infected intravenously. In the study, they used a dose of 10^8 CFU of the strain intravenously to challenge pregnant buffaloes that had been vaccinated and revaccinated with the standard dose of RB51 (Table S4), showing significant colonization of the challenge strain in the fetal tissues of the vaccinated animals. However, the study performed an intravenous challenge on the animals, which does not correspond to a natural route of infection for brucellosis and also used a dose 10 times higher than that indicated for the challenge of cattle. Again, vaccine failure may be linked to the concentration of exposure to the challenge strain received by the animal, and abortion may be associated with the high concentration of the challenge strain and not with vaccine ineffectiveness (Manthei, 1968; Oliveira *et al.*, 2022). The Caporale *et al.* (2010) study challenged animals vaccinated with a triple dose (3-10 x 10^{10} CFU/dose) of RB51 with strain 544 at a dose of 10^7 CFU/dose via the intraconjunctival route, and the strain was isolated in only one animal. However, the author himself states that the power of the study was low, requiring a larger number of animals to identify differences between the vaccinated group and the control group. Therefore, the results of this study cannot be used to assess the efficacy of the RB51 vaccine in buffaloes.

5. Conclusion

In conclusion, studies with S19 vaccine have mostly analyzed the persistence of vaccine antibodies and have shown that the vaccine induces humoral responses, and that antibody persistence is directly proportional to the age at which the vaccine is administered. S19 vaccine induces a cellular response in buffaloes, but more targeted studies are still needed to understand the pattern of this cellular response. As for the RB51 strain, it was possible to prove its DIVA characteristic in buffaloes. In addition, it stimulates humoral and cellular responses in vaccinated animals, but more targeted studies are needed to characterize the cellular response. Regarding the safety of the vaccine, the RB51 strain has been shown to be safe for vaccinating buffaloes, even at high doses, and for vaccinating calves, without interfering with the animals' gestation, lactation, or birth. However, in pregnant animals, it can cause abortion. Therefore, it is possible to state that the two vaccine strains induce immune response in buffaloes, but further research is still needed to understand whether these responses are effective in protecting against brucellosis.

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7. References

AFZAL, M.; ASHRAF MIRZA M FAU - JAHANGIR, M.; JAHANGIR, M. Immune response of buffaloes to vaccination with *Brucella abortus* strain 19. *Revue scientifique et technique (International Office of Epizootics)***,** v. 9(3), p. 867–870, 2000.

ALAMIAN, S.; DADAR, M. *Brucella abortus* contamination of camel milk in two Iranian regions. *Preventive Veterinary Medicine***,** v. 169, p. 104708, 2019.

ANDRADE, R. S.; OLIVEIRA, M. M. D.; BUENO FILHO, J. S. D. S. *et al.* Accuracy of serological tests for bovine brucellosis: A systematic review and meta-analysis. *Preventive Veterinary Medicine***,** v. 222, p. 106079, 2024.

BONADUCE, A.; QUESADA, A.; MARTONE, F. *et al.* Immunization of buffaloes on a farm in southern Italy with attenuated (strain 19) and inactivated (strain 45/20) brucellosis vaccines. *Acta Medica Veterinaria***,** v. Vol. 31, No. 1/2, p. 3-25 ref. 103, 1985.

BORA, S.; MAHATO, G.; BARMAN, N. N. *et al.* Immune response of buffalo calves to vaccination with *Brucella abortus* strain-19 vaccine. *Buffalo Bulletin (September 2008)***,** v. 27, n. 3, p. 19, 2008.

BUCK, J. M. Studies on vaccination during calfhood to prevent bovine infectious abortion. J Agri Res, v. 41 p. 667–689, 1930.

CAPORALE, V.; BONFINI, B.; DI GIANNATALE, E. *et al.* Efficacy of *Brucella abortus* vaccine strain RB51 compared to the reference vaccine *Brucella abortus* strain 19 in water buffalo. *Vet Ital***,** v. 46, n. 1, p. 13-9, 5-11, 2010.

CHAND, P.; CHHABRA, R.; JALE, I. S. *et al.* Control of brucellosis on an infected Murrah buffalo farm with reduced dose of *Brucella abortus* S19 vaccine administered by conjunctival route in adult animals. *The Indian Journal of Animal Sciences***,** v. 83, n. 4, 2013.

CHAUDHURI, P.; SAMINATHAN, M. A.-O.; ALI, S. A.-O. X. *et al.* Immunization with *Brucella abortus* S19Δper Conferred Protection in Water Buffaloes against Virulent Challenge with B. abortus Strain S544. *Vaccines***,** v. 9(12), p. 1423, 2021.

CORBEL, M. J. Brucellosis in humans and animals. *WHO Library*, p. 102, 2006.

CORNER, L.; ALTON, G. G. Persistence of *Brucella abortus* strain 19 infection in adult cattle vaccinated with reduced doses. *Research in veterinary science***,** v. 31(3), p. 342–344, 1981.

DE MASSIS, F.; ZILLI, K.; DI DONATO, G. *et al.* Distribution of Brucella field strains isolated from livestock, wildlife populations, and humans in Italy from 2007 to 2015. *PLOS ONE***,** v. 14, n. 3, p. e0213689, 2019.

DE MASSIS, F.; SACCHINI, F.; D'ALTERIO, N. *et al. Brucella abortus* Strain RB51 Administered to Prepubescent Water Buffaloes, from Vaccination to Lactation: Kinetics of Antibody Response and Vaccine Safety. *Microorganisms***,** v. 11 (8), p. 2078, 2023.

DIPTEE, M. D.; ADESIYUN, A. A.; ASGARALI, Z. *et al.* Evaluation of cell-mediated immune responses and bacterial clearance in 6–10 months old water buffalo (*Bubalus bubalis*) experimentally vaccinated with four dosages of commercial *Brucella abortus* strain RB51 vaccine. *Veterinary Immunology and Immunopathology***,** v. 106, n. 3, p. 209-220, 2005.

DIPTEE, M. D.; ADESIYUN, A. A.; ASGARALI, Z. *et al.* Serologic responses, biosafety and clearance of four dosages of *Brucella abortus* strain RB51 in 6-10 months old water buffalo (*Bubalus bubalis*). *Vet Immunol Immunopathol***,** v. 109, n. 1-2, p. 43-55, 2006.

DIPTEE, M. D.; ASGARALI Z FAU - CAMPBELL, M.; CAMPBELL M FAU - FOSGATE, G. *et al.* Post-exposure serological and bacteriological responses of water buffalo (*Bubalus bubalis*) to *Brucella abortus* biovar 1 following vaccination with *Brucella abortus* strain RB51. *Revue scientifique et technique (International Office of Epizootics)***,** v. 26(3) p. 669–678, 2007.

DOHOO, I. R.; MARTIN, S. W.; STRYHN, H. *Methods in Epidemiologic Research*. 2nd. Charlottetown Prince Edward Island (Canada): VER Inc., 2012.

DOMINGUES, P. F.; LANGONI, H.; PADOVANI, C. R. *et al.* Pesquisa de aglutininas anti-*Brucella* sp em soro de bezerras bubalinas (*Bubalus bubalis*) vacinadas com dose padrão ou reduzida da amostra B-19. *Arquivos Brasileiros de Medicina Veterinária e Zootecnia***,** v. V. 44, n 6, p. p. 491 - 500, 1992.

DORNELES, E. M.; SRIRANGANATHAN, N.; LAGE, A. P. Recent advances in *Brucella abortus* vaccines. *Vet Res***,** v. 46, n. 1, p. 76, 2015a.

DORNELES, E. M.; TEIXEIRA-CARVALHO, A.; ARAÚJO, M. S. *et al.* T lymphocytes subsets and cytokine pattern induced by vaccination against bovine brucellosis employing S19 calfhood vaccination and adult RB51 revaccination. *Vaccine***,** v. 32, n. 46, p. 6034-8, 2014.

DORNELES, E. M.; LIMA, G. K.; TEIXEIRA-CARVALHO, A. *et al.* Immune Response of Calves Vaccinated with *Brucella abortus* S19 or RB51 and Revaccinated with RB51. *PLoS One***,** v. 10, n. 9, p. e0136696, 2015b.

DORNELES, E. M. S.; OLIVERIA, L. F.; LAGE, A. P. *Brucella abortus* vaccines: use in control programs and immune response. *J Bacteriol Mycol***,** v. 4, p. 1044, 2017.

EL-RAZIK, K. A. A.; EL-HAFEZ, S. M. A.; SHELL, W. S. *et al.* The immunological response of RB51 vaccinated buffalo calves using brucella periplasmic proteins as ELISA antigen. *African Journal of Biotechnology***,** v. 10, n. 51, p. 10562-10566, 2011.

FOSGATE, G. T.; DIPTEE, M. D.; RAMNANAN, A. *et al.* Brucellosis in domestic water buffalo (*Bubalus bubalis*) of Trinidad and Tobago with comparative epidemiology to cattle. *Trop Anim Health Prod***,** v. 43, n. 8, p. 1479-86, 2011.

FOSGATE, G. T.; ADESIYUN, A. A.; HIRD, D. W. *et al.* Evaluation of brucellosis RB51 vaccine for domestic water buffalo (*Bubalus bubalis*) in Trinidad. *Prev Vet Med***,** v. 58, n. 3-4, p. 211-25, 2003.

GALIERO, G.; MARTUCCIELLO, A.; ASTARITA, S. *et al.* Innocuità del vaccino *Brucella abortus* RB51 nella bufala mediterranea. *Large Anim Rev***,** v. 15, n. 1, p. 19-22, 2009.

GODOY, K. M. G.; SERPA GONÇALVES, M.; DORNELES, E. M. S. *et al. Evaluation of Cross-Reactivity of Monoclonal Antibodies Against Cytokines and Cellular Markers of Bovine and Human Origin to Identify Immunological Markers in Buffaloes (Bubalus bubalis)*. In production. Belo Horizonte, Minas Gerais: Universidade Federal de Minas Gerais, 2025. 21.

GRANDONI, F.; ELNAGGAR, M. M.; ABDELLRAZEQ, G. S. *et al.* Characterization of leukocyte subsets in buffalo (*Bubalus bubalis*) with cross-reactive monoclonal antibodies specific for bovine MHC class I and class II molecules and leukocyte differentiation molecules. *Dev Comp Immunol***,** v. 74, p. 101-109, 2017.

HOANG, T.; AGGER ELSE, M.; CASSIDY JOSEPH, P. *et al.* Protein Energy Malnutrition during Vaccination Has Limited Influence on Vaccine Efficacy but Abolishes Immunity if

Administered during Mycobacterium tuberculosis Infection. *Infection and Immunity***,** v. 83, n. 5, p. 2118-2126, 2015.

IOVANE, G.; MARTUCCIELLO, A.; ASTARITA, S. *et al.* Vaccino *Brucella abortus* RB51: primi resultati sull'innocuità ed attività immunogena nella bufala mediterranea. *Progr vet***,** v. 62 (1), p. 19-21, 2007.

JACOBO, R. A.; STORANI, C. A.; CIPOLINI, M. F. *et al.* Vaccinated antibodies in inoculated veals with C 19. *Revista de Medicina Veterinaria (Buenos Aires)***,** v. Vol. 86 , No. 5, p. 195-198 ref. 15, 2005.

JAISMON, P. A.; SUSHMITHA, A. P.; VERMA, M. R. *et al.* Prevalence of bovine brucellosis in India: a meta-analysis. *Veterinary Quarterly***,** v. 43, n. 1, p. 1-9, 2023.

JAMAL, S. M.; AFZAL, M.; AHMED, S. The immune response of guinea pigs and buffalo calves to the locally prepared *Brucella abortus* strain 19 vaccine. *OIE Revue Scientifique et Technique***,** v. 22, n. 3, p. 893-897, 2003.

JONES, L. M.; BERMAN, D. T. The role of living vaccines in prophylaxis. *Developments in biological standardization***,** v. 31, p. 328-334, 1976.

KNIGHT-JONES, T. J. D.; EDMOND, K.; GUBBINS, S. *et al.* Veterinary and human vaccine evaluation methods. *Proceedings of the Royal Society B: Biological Sciences***,** v. 281, n. 1784, p. 20132839, 2014.

LAMBERT, G.; MANTHEI, C.; DEYOE, B. L. Studies on *Brucella abortus* infection in bulls. *American journal of veterinary research***,** v. 24, p. 1152–1157, 1963.

LONGO, M.; MALLARDO, K.; MONTAGNARO, S. *et al.* Shedding of *Brucella abortus* rough mutant strain RB51 in milk of water buffalo (*Bubalus bubalis*). *Preventive Veterinary Medicine***,** v. 90, n. 1, p. 113-118, 2009.

MANTHEI, C. A. Summary of controlled research with Strain 19. *Proceedings. United States Live Stock Sanitary Association***,** v. , p. 91-97 pp., 1959.

MANTHEI, C. A. Application of Research to Bovine Brucellosis Control and Eradication Programs. *Journal of Dairy Science***,** v. 51, n. 7, p. 1115-1120, 1968.

MCFARLANE, H.; HAMID, J. Cell-mediated immune response in malnutrition. *Exp Immunol.* **,** v. 13(1), p. 153-64, 1973.

MEYER, M. E.; NELSON, C. J. Persistence of *Brucella abortus*, strain 19 infection in immunized cattle. *Proceedings, annual meeting of the United States Animal Health Association***,** v. 73, n. 0082-8750 (Print), p. 159–165, 1969.

MIRANDA, K. L.; DORNELES, E. M. S.; PAULETTI, R. B. *et al. Brucella abortus* S19 and RB51 vaccine immunogenicity test: Evaluation of three mice (BALB/c, Swiss and CD-1®) and two challenge strains (544 and 2308). *Vaccine***,** v. 33, n. 4, p. 507-511, 2015.

MIRANDA K. L.; POESTER F. P.; DORNELES E. M. *et al*. *Brucella abortus* RB51 in milk of vaccinated adult cattle. *Acta Trop,* v. 160, p. 58-61, 2016.

NARDI JÚNIOR, G.; RIBEIRO, M. G.; JORGE, A. M. *et al.* Serological profile of buffalo (*Bubalus bubalis*) female calves vaccinated with standard *Brucella abortus* strain 19 vaccine using rose bengal, 2-mercaptoethanol and complement fixation tests. *Biologicals***,** v. 40, n. 2, p. 158-161, 2012.

NICOLETTI, P. Prevalence and persistence of *Brucella abortus* strain 19 infections and prevalence of other biotypes in vaccinated adult dairy cattle. *Journal of the American Veterinary Medical Association***,** v. 178(2), p. 143–145, 1981.

NICOLETTI, P. Vaccination against Brucella. *Advances in biotechnological processes***,** v. 13, p. 147–168, 1990.

OLIVEIRA, M. M.; PEREIRA, C. R.; DE OLIVEIRA, I. R. C. *et al.* Efficacy of *Brucella abortus* S19 and RB51 vaccine strains: A systematic review and meta-analysis. *Transboundary and emerging diseases***,** v. 69 (4), p. e32-e51, 2022.

OLSEN, S.; TATUM, F. Bovine Brucellosis. *Veterinary Clinics of North America: Food Animal Practice***,** v. 26, n. 1, p. 15-27, 2010.

OLSEN, S. C.; STOFFREGEN, W. S. Essential role of vaccines in brucellosis control and eradication programs for livestock. *Expert Review of Vaccines***,** v. 4, n. 6, p. 915-928, 2005.

OLSEN, S. C.; BRICKER, B.; PALMER, M. V. *et al.* Responses of cattle to two dosages of*Brucella abortus*strain RB51: serology, clearance and efficacy. *Research in Veterinary Science***,** v. 66, n. 2, p. 101-105, 1999.

PAGE, M. J.; MCKENZIE, J. E.; BOSSUYT, P. M. *et al.* The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *Bmj***,** v. 372, p. n71, 2021.

PALMER, M. V.; OLSEN SC FAU - CHEVILLE, N. F.; CHEVILLE, N. F. Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. *American journal of veterinary research***,** v. 58(5), p. 472–477, 1997.

PEREIRA, A. P. M.; DO ROSÁRIO CASSEB, A.; NEVES, L. M. *et al*. Humoral immune response of buffalo heifers (*Bubalus bubalis*) vaccinated with S19 strain of *Brucella abortus* Resposta imune humoral de bubalinos (*Bubalus bubalis*) vacinados pela cepa S19 de *Brucella abortus*. *Archives of Veterinary Science***,** v. 20, n. 1, p. 56-61, 2015.

PEREIRA, C. R.; COTRIM DE ALMEIDA, J. V. F.; CARDOSO DE OLIVEIRA, I. R. *et al.* Occupational exposure to Brucella spp.: A systematic review and meta-analysis. *PLOS Neglected Tropical Diseases***,** v. 14, n. 5, p. e0008164, 2020.

PETTINI, E. A.-O.; PASTORE, G.; FIORINO, F. *et al.* Short or Long Interval between Priming and Boosting: Does It Impact on the Vaccine Immunogenicity? . *Vaccines***,** v. 9 (3), p. 289, 2021.

POESTER, F. P.; RECKZIEGEL, P. E. Persistência de reações sorológicas em búfalas (*Bubalus bubalis*) vacinadas com brucella abortas amostra 19. *Pesquisa Agropecuária Gaúcha***,** v. 4, n. 1, p. 39-41, 1998.

RAMNANAN, A.; DIPTEE, M.; ASGARALI, Z. *et al.* Serological and bacteriological responses of water buffalo (*Bubalus bubalis*) vaccinated with two doses of *Brucella abortus* strain RB51 vaccine. *Tropical Animal Health and Production***,** v. 44, n. 7, p. 1451-1458, 2012.

RAMNANAN, A.; ADESIYUN, A. A.; CAMPBELL, M. *et al.* Experimental study on *Brucella abortus* strain RB51 vaccinated water buffalo (*Bubalus bubalis*) challenged with virulent B. abortus strain during pregnancy. *Journal of Buffalo Science***,** v. 7, n. 2, p. 17-29, 2018.

RIVERA, S.; FORASTIERO, A.; LÓPEZ, C. *et al.* IFN-γ as a marker of immune response in water buffalo vaccinated with RB51. *REDVET***,** v. 17 (6), p. 061606, 2016.

ROXO, E.; BARUSELLI, P. S.; REIS, C. A. B. *et al.* Antibody level variation in buffaloes vaccinated against brucellosis with strain 19. *Proceedings, 4th World Buffalo Congress, São Paulo, Brazil, 27-30 June 1994.***,** v. Volume 3., p. 440-442, 1994.

SAMARTINO, L. E.; FORT, M.; GREGORET, R. *et al.* Use of *Brucella abortus* vaccine strain RB51 in pregnant cows after calfhood vaccination with strain 19 in Argentina. *Preventive Veterinary Medicine***,** v. 45, n. 3, p. 193-199, 2000.

SCHAIBLE, U. E.; KAUFMANN, S. H. E. Malnutrition and Infection: Complex Mechanisms and Global Impacts. *PLOS Medicine***,** v. 4, n. 5, p. e115, 2007.

SCHURIG, G. G.; SRIRANGANATHAN, N.; CORBEL, M. J. Brucellosis vaccines: past, present and future. *Vet Microbiol***,** v. 90, n. 1-4, p. 479-96, 2002.

SCHURIG, G. G.; ROOP, R. M.; BAGCHI, T. *et al.* Biological properties of RB51; a stable rough strain of *Brucella abortus*. *Veterinary Microbiology***,** v. 28, n. 2, p. 171-188, 1991.

SCURLOCK, B. M.; EDWARDS, W. H. Status of brucellosis in free-ranging elk and bison in Wyoming. *J Wildl Dis***,** v. 46, n. 2, p. 442-9, 2010.

SERPA GONÇALVES, M.; DE OLIVEIRA, M. M.; ANDRADE, R. S. *et al.* Systematic review on the effectiveness of *Brucella abortus* S19 and RB51 vaccine strains in field studies. *Vaccine***,** v. 45, p. 126649, 2025.

SHI, J. F.; GONG, Q. L; ZHAO, B. *et al.* Seroprevalence of Brucellosis in Buffalo Worldwide and Associated Risk Factors: A Systematic Review and Meta-Analysis. Front *Vet Sci.* v. 8, p. 649252, 2021.

SHOME, R.; KILARI, S.; SAHARE, A. *et al.* Evaluation of the immune responses against reduced doses of *Brucella abortus* S19 (calfhood) vaccine in water buffaloes (*Bubalus bubalis*), India. *Vaccine***,** v. 38, n. 45, p. 7070-7078, 2020.

WOAH. Brucellosis (infection with *Brucella abortus*, B . Melitensis and B . Suis). OIE Terrestrial Manual, 2022. Avaialble at:

https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf. Accessed on: 20/09/2023.

CHAPTER 2 - Evaluation of Cross-Reactivity of Monoclonal Antibodies Against Cytokines and Cellular Markers of Bovine and Human Origin to Identify Immunological Markers in Buffaloes (*Bubalus bubalis*)

Abstract

Research related to the investigation of the activation and regulation of the immune system of domestic buffaloes is still very limited. The main factor linked to this limitation is the scanty availability of commercial monoclonal antibodies (mAb) against buffalo immune markers that allow the use of multiparametric techniques, such as flow cytometry, to study the modulation of the immune system of these animals. The evaluation of the cross-reactivity of commercially available mAbs against other species for cytokines and components of the immune system of buffaloes, is the fastest and least expensive way to expand the tools to assess immune response in those animals. Therefore, sixteen commercial mAbs anti-cell markers (CD4, CD8, CD14, CD21, CD25, CD28, CD45RB, CD45RO, WC1, and CD80) and anti-cytokines or other effector molecules of the immune system (TNF-α, IFN-γ, IL-4, IL-17, granzyme B and perforin) that recognize molecules from bovine and man were evaluated in whole blood samples from buffaloes and cattle. Two experiments were performed: Experiment 1, with six buffaloes, evaluated the specificity of mAb binding, and experiment 2, with twelve buffaloes and twelve cattle, evaluated the cross-reactivity of the mAbs. Samples were processed and analyzed by flow cytometry. Of the mAbs analyzed, 93.7% showed specific binding and cross-reacted with cells, cytokines and molecules of buffalo immune system. Only the CD4 clone (6.3%) used did not show cross-reactivity in buffaloes. Those results amplify the spectrum analyses of immune response of buffaloes.

Keywords: Vaccination, immune response, flow cytometry, immunophenotyping

1. Introduction

The domestic buffalo (*Bubalus bubalis*) is known for its robustness and ability to produce meat and milk, as well as for its use in draft work (Borghese, 2005; Gama Pantoja *et al.*, 2022). Its population in the world is approximately 204 million head, with 98% of these animals belonging to the Asian continent, 1% in South America, 0.7% in Africa, and 0.2% in Europe (FAO, 2023a). Buffaloes are responsible for 15% of global milk production, making them the second largest contributor to global production. The five largest producers of buffalo milk are India, Pakistan, China, Egypt and Nepal (FAO, 2023a). Italy is the sixth largest producer of buffalo milk in the world and the largest producer in Europe, in addition to producing buffalo mozzarella, protected by the European Union's Protected Designation of Origin (PDO) regime (Minervino *et al.*, 2020). In developing countries, about one-third of milk production comes from buffalo milk (FAO, 2023b). About 93.5% of buffalo meat production is concentrated in Asia, especially in India. In India and Pakistan, buffalo meat production in 2021 was 4.3 million tons (FAO, 2023b).

Despite their hardiness, buffaloes are susceptible to many diseases that affect cattle, albeit they generally present less evident clinical signs (Borghese, 2005). To understand the particularities of the manifestation of each disease in buffaloes, it is necessary to have means to analyze the functioning of the immune system of these animals, with flow cytometry being a fundamental tool for research (Davis *et al.*, 2001; Grandoni *et al.*, 2017). Flow cytometry provides multiparametric analyses of cells or particles in suspension (McKinnon, 2018), and one of its applications is immunophenotyping, which examines cellular antigens to characterize and quantify specific cell populations and intracellular cytokines. This is made possible by the use of monoclonal antibodies (mAbs) labeled with fluorescent molecules that bind to specific target sites on cells (Owens *et al.*, 2000). However, few mAbs are commercially available for buffalo, limiting more in-depth studies on the regulatory mechanisms of their immune response (Grandoni *et al.*, 2017).

Since the discovery of mAb production using inbred mice, a vast number of markers have been developed for research and diagnosis in humans and animals. Currently, there has been an increasing number of commercial mAbs developed for domestic species, such as swine, ruminants (cattle, goats, and sheep), poultry (chickens), fish, and companion animals (horses, cats, and dogs) (Entrican *et al.*, 2020). However, the production of new mAbs is laborious, time-consuming, and expensive (Pedersen *et al.*, 2002). In this context, due to the need to expand immunological studies in other animal species of economic, epidemiological, and environmental importance, studies have mainly sought cross-reactivity between existing mAbs from more studied species such as humans and cattle, and species that do not have or have a restricted variety of specific mAbs, such as buffaloes (Davis *et al.*, 1987; Mosaad *et al.*, 2006; Grandoni *et al.*, 2017).

Due to the wide variety and commercial availability of mAbs against humans, many researchers test their reactivity against cells and cytokines from other species, such as cattle, goats, sheep, dogs, pigs, buffaloes, bison, llamas and camelids (Mosaad *et al.*, 2006; Griebel *et al.*, 2007;

Dorneles *et al.*, 2015a; Moreira *et al.*, 2015). Some studies have tested the cross-reactivity of bovine markers in buffaloes, due to the evolutionary proximity between the species, and the focus of these studies was the identification of MHC I, MHC II and CD (clusters of differentiation) molecules (Davis *et al.*, 2001; Mosaad *et al.*, 2006; Grandoni *et al.*, 2017). From this, they confirmed that mAbs against cattle can recognize conserved epitopes in orthologous molecules of other species, such as buffaloes, resulting in cross-reactivity ((Davis *et al.*, 2001; Mosaad *et al.*, 2006; Grandoni *et al.*, 2017; Entrican *et al.*, 2020). However, there are only few studies available that seek to identify cross-reactivity for cytokines and other components of the buffalo immune system. Furthermore, some mAb clones may bind to different epitopes of the same marker, which may cause differences in cross-reactivity. Therefore, mAb clones against the same marker should be tested to expand the availability of reagents for immunological studies in buffaloes.

The study of the adaptive immune response is very important to understand mechanisms associated with the function of B and T lymphocytes, as well as the induction of immunological memory to produce rapid and specific responses against pathogens. Furthermore, it is possible to understand the effectiveness of the induction of cellular and humoral responses after vaccination and thereby seek for correlates of protection (Dorneles *et al.*, 2015c; Pollard *et al.*, 2021; Chi *et al.*, 2024). In addition, the study of components of the adaptive immune response of buffaloes allows the development of studies to understand the host/pathogen interaction, in addition to understanding the mechanisms of the protective immune response of these animals, favoring the evaluation of potential vaccines.

Thus, the objective of this study was to evaluate the cross-reactivity of commercial mAbs against bovine and human cell markers and cytokines in buffaloes.

2. Materials and Methods

2.1 Monoclonal antibodies

Sixteen murine-derived monoclonal antibodies were used in this study, comprising ten against bovine cell markers (CD4, CD8, CD14, CD21, CD25, CD28, CD45RB, CD45RO, CD80, WC1) and three against bovine cytokines (TNF-α, IFN-γ, IL-4), and three against human immune system components and cytokine (IL-17, Granzyme B, and Perforin). Details on mAbs specificity, clone identification, fluorochrome marker, manufacturer, and working dilution are listed in Table 1.

2.2 Similarity among buffalo and bovine or human immune molecules recognized by the selected mAbs

The similarities between buffalo molecules and bovine or human specific immune molecules recognized by the selected mAbs were assessed by comparing the amino acid sequences via BLAST (Camacho *et al*., 2009). An amino acid sequence was identified for each of the 16 molecules analyzed in the study, according to the target species, using a reference genome per species (GCF_002263795.3; GCF_019923935.1; GCF_000001405.40) selected from the National Center for Biotechnology Information (NCBI). All available isoforms of each molecule were also selected (Apendix S6). Then, a comparative analysis was performed with amino acid sequences of domestic buffaloes in BLAST, according to alignment and identity. For amino acids that presented more than one isoform, an average between the identity values was calculated.

2.3 Animals and material collection

Eighteen healthy adult crossbred buffaloes (*Bubalus bubalis*) from a herd in Oliveira, Minas Gerais, Brazil were used. Additionally, 12 healthy adult crossbred cows (*Bos taurus*) from a herd in Lavras, Minas Gerais, Brazil, were also included in the study. Sera from Balb/C mice from the collection of serum of the Laboratório Integrado de Sanidade Animal e Saúde Coletiva – LISASC (Integrate Laboratory of Animal and Public Health) from Universidade Federal de Lavras (UFLA) were also used. The use of the bovine and buffalo animals (CEUA/UFLA 038/23) and the mouse sera (CEUA/UFLA 027/2017) in this study was approved by the Ethics Committee for Animal Use (CEUA) of UFLA.

Table 1. Summary of anti-bovine and anti-human monoclonal antibodies (mAbs) against cell markers and citokines used in the cross-reaction test.

Target	**Clone**	**Isotype**	**Target species**	**Fluorochrome**	**Manufacturer**	**Final dilution**	**Similarity to buffalo (%)**	**Cross reactivity**
CD4 [E]	CC8	IgG2a	Bovine	FITC	Bio-Rad Laboratories	1:400	85.67	-
CD8 [E]	CC63	IgG2a	Bovine	FITC	Bio-Rad Laboratories	1:400	95.45 [A]/ 94.33 [B]	+
CD14 [E]	CC-G33	IgG1	Bovine	FITC	Serotec	1:50	97.31	+
CD21 [E]	CC21	IgG1	Bovine	FITC	Serotec	1:400	92.75	+
CD25 [E]	IL-A111	IgG1	Bovine	PE	Serotec	1:100	95.72	+
CD28 [E]	CC219	IgG1	Bovine	PE	Serotec	1:100	99.49	+
CD45RB [E]	CC76	IgG1	Bovine	FITC	Bio-Rad Laboratories	1:100	98.60	+
CD45RO [E]	IL-A116	IgG3	Bovine	PE	Bio-Rad Laboratories	1:20	98.33	+
CD80 [E]	IL-A159	IgG1	Bovine	PE	Bio-Rad Laboratories	1:100	96.31	+
Granzime B [I]	GB11	IgG1	Human	PE	R & D Systems	1:50	71.49	+
IFN- γ [I]	CC302	IgG1	Bovine	PE	Bio-Rad Laboratories	1:100	99.15	+
IL4 [I]	CC303	IgG2a	Bovine	PE	Serotec	1:100	99.26	+
IL17 [I]	eBio64DEC17	IgG1	Human	PE	Invitrogen	1:100	74.45	+
Perforin [I]	δG9	IgG2b	Human	PE	BD Biosciences	1:50	74.63	+
TNF-α [I]	CC327	IgG2b	Bovine	ALEXA 488	Serotec	1:50	95.58	+
WC1 [E]	CC15	IgG2a	Bovine	FITC	Serotec	1:100	93.35	+

[E] - Extracellular marker, [I] - Intracellular marker, [A] - Alpha Chain, [B] - Beta Chain

2.4 Experimental design

Experimental animals were divided into two experimental groups. Experiment 1, using of six buffaloes, evaluated the specificity of binding of mAbs against bovine or human to buffalo cellular markers or cytokines. In this step,a pool of mouse sera were used as a blocking agent (5% mouse serum), preventing nonspecific binding by the fragment crystallizable (Fc) portion of the mAbs (Hulspas *et al.*, 2009). Experiment 2, used 12 buffaloes and 12 cows to evaluate the cross-reactivity of mAbs against bovine and human cell markers or cytokines to components of buffalo immune system.

Ten milliliters of whole blood was collected from each animal (buffaloes and cattle) of both experiments. The blood was collected into vacuum heparinized tubes (Vacuplast, Brazil) and kept at room temperature until processing, which was completed within 24 hours from collection. The intracellular and extracellular labeling protocols were the same for all animals in experiments 1 and 2.

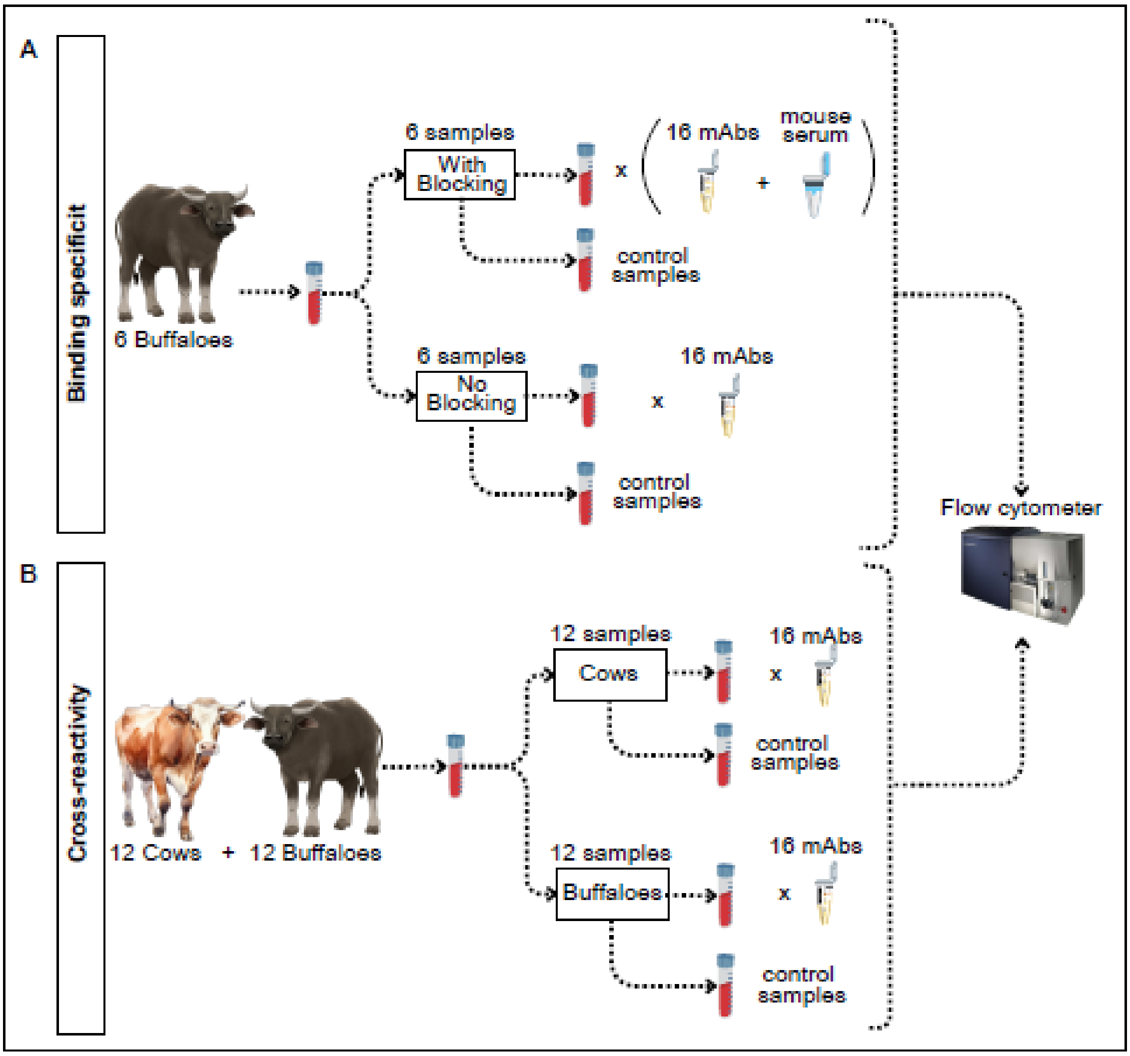

Figure 1. Experimental design of cross-reactivity analysis. Analysis of the samples used to evaluate the binding specificity of mAbs in buffalo cells (A) and to evaluate cross-reactivity of commercially available monoclonal antibodies against bovine and human cellular markers and cytokines against buffalo molecules, by flow cytometry (B).

2.5 *In vitro* culture to stimulate nonspecific cytokine production

The protocols used for *in vitro* culture, intracellular, and extracellular staining were adapted from Dorneles *et al.* (2015b). The dilutions of the mAbs used were based on previous marking tests performed in bovine cells. For each animal, all mAbs were tested at the dilutions listed in Table 1.

Initially, samples for intracellular staining underwent short-term *in vitro* stimulation. Briefly, each centrifuge tube received 1 mL of collected blood, 910 µL of RPMI (Sigma Aldrich, USA), 2 µL of ionomycin (1 mg/mL) (Sigma Aldrich, USA), 50 µL of phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, USA), and 40 µL of brefeldin A (BFA) (Sigma Aldrich, USA). PMA and ionomycin stimulate cytokine production, while brefeldin A prevents their release into the extracellular environment (Pedersen *et al.*, 2002). Samples were incubated for 4 hours at 37°C in a 5% CO_2 atmosphere.

2.6 Intracellular cytokine immunophenotyping

After incubation, 220 µL of EDTA (20 mM) (Synth, Brazil) was added to each tube to stop cell activation, followed by a 10-minute incubation at room temperature. The tubes were centrifuged (521 × g for 7 min at 18°C), and 100 µL of the cell suspension were transferred to polystyrene tubes. Then, 3 mL of lysis solution (Billig 1x) (2.85 g sodium citrate, Synth, Brazil; 800 µL heparin—5000 IU/mL, Blau Farmacêutica, Brazil; 54 mL formaldehyde PA, Synth, Brazil; 30 mL diethylene glycol, Synth, Brazil) was added during vortex homogenization, followed by 10 minutes of incubation at room temperature. The tubes were centrifuged (521 × g for 7 min at 18°C), and the supernatant was discarded.

Next, 3 mL of PBS-P permeabilization buffer (0.01 M PBS, pH 7.2, supplemented with 0.5% bovine serum albumin and 0.5% saponin, all from Sigma Aldrich, USA) were used to wash the samples, followed by a 10-minute incubation at room temperature. After centrifugation (521 × g for 10 min at 18°C), the supernatant was discarded, and antibodies were added according to the dilutions provided in Table 1. A control tube (without mAb) and one tube per marker were prepared for each blood sample. For experiment 1, one tube per marker containing 15 µL of total serum blocking buffer (PBS-P supplemented with 5% mouse serum) was also prepared. Samples were incubated for 30 min at room temperature, protected from light, and then centrifuged (521 × g for 7 min at 18°C), with the supernatant discarded.

Afterward, 1 mL of PBS-P was added, followed by vortex homogenization, centrifugation (521 × g for 10 min at 18°C), and supernatant removal. Finally, 150 µL of Mac's Fac's Fix (MFF) fixation solution (10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, 6.63 g/L sodium chloride, PH 7.2, all reagents from Sigma Aldrich, USA) was added to preserve cell integrity, and the samples were stored refrigerated at 4°C until analysis.

2.7 Extracellular staining

Polystyrene tubes containing 90 µL of blood and 10 µL of mAbs dilutions according to in Table 1, were prepared. For each blood sample, a control tube (without monoclonal antibodies) and one tube per antibody were prepared. For experiment 1 samples, tubes containing 15 µL of total serum blocking buffer (PBS-P supplemented with 5% mouse serum) and antibodies were also included. Samples were incubated for 30 minutes at room temperature, protected from light.

Then, 3 mL of lysis solution (Billig 1x) was added to each tube during vortex homogenization, followed by 10 minutes of incubation at room temperature, protected from light. The tubes were centrifuged (344 × g for 7 min at 18°C), and the supernatant was discarded. Then, 1 mL of PBS-W (0.01 M PBS, pH 7.2, supplemented with 0.5% bovine serum albumin) was added, followed by vortex homogenization and centrifugation (344 × g for 10 min at 18°C). Finally, 150 µL of Mac's Fac's Fix (MFF) (10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, 6.63 g/L sodium chloride, PH 7.2, all reagents from Sigma Aldrich, USA) were added, and the samples were stored refrigerated at 4°C until analysis.

2.8 Sample acquisition and gating strategy

Samples were analyzed using a BD FacsLyric™ flow cytometer (Becton, Dickinson & Company, USA), analyzing 50,000 events for samples from experiment 1 animals, and a minimum of 14,000 events for samples from experiment 2 animals. Data were processed with FlowJo software, version 10.9.0/2023 (Becton, Dickinson & Company, USA). The analysis involved three gating steps to determine the count and frequency of stained cells. The first step selected the population of interest, lymphocytes and monocytes, based on cell morphology (SSC-A x FSC-A). The second step excluded cells detected as doublets by the cytometer (FSC-H x FSC-A). The third step selected stained cells. Control tubes were analyzed using dot plots with two fluorochromes, such as FITC vs. PE or PE vs. Alexa 488, to confirm the absence of stained cells. All those steps are illustrated in Figure 2.

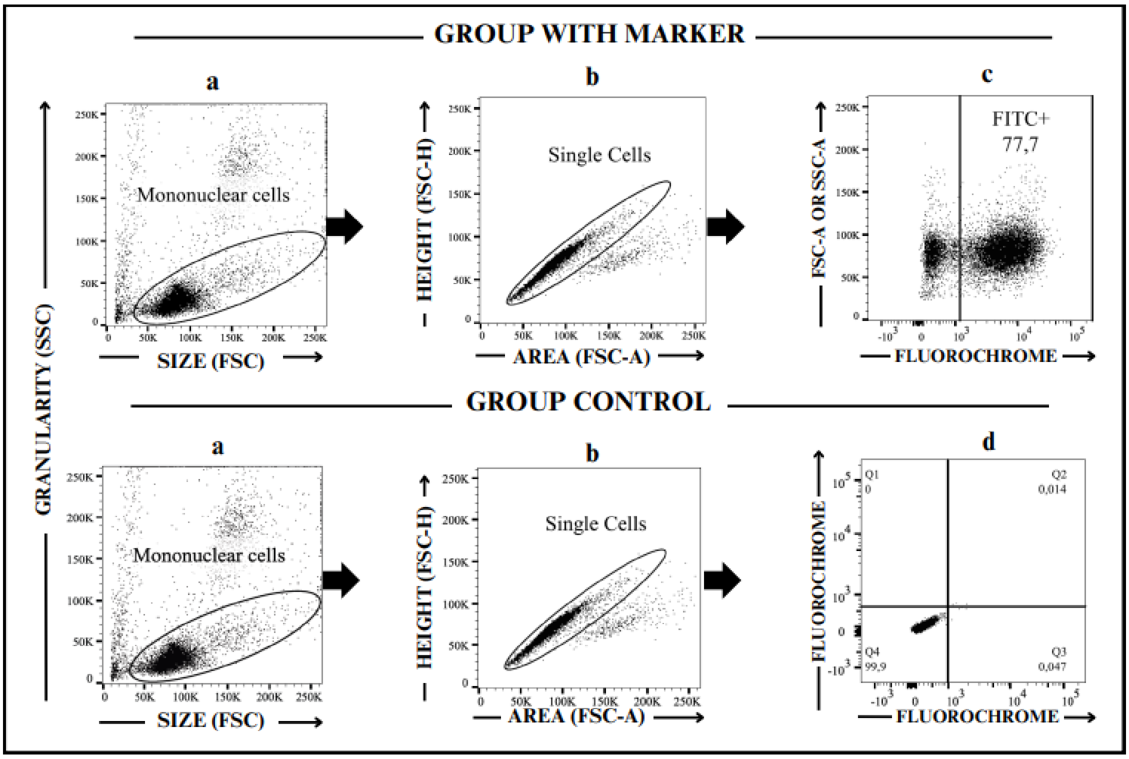

Figure 2. Gating strategies. Gates used to select the monocyte population from the labeled samples (a, b, and c), with graph "a" based on the complexity (SSC-A) and size (FSC-A) of the cells, followed by graph "b" with an analysis of forward scatter based on height (FSC-H) and total area (FSC-A), to exclude any cell clusters that may cause interruption in the final reading. Finally, graph "c" uses forward scatter (FSC-H) or side scatter (SSC-H) and the fluorochrome that labels sample to identify the labeled cells. For the control samples (a, b, and d), the first two steps mentioned above (a and b) were used, and the third step (d) uses two of the fluorochromes that were used to label the study samples to highlight the absence of labeling in the selected cells.

2.9 Statistical Analysis of the Data

Statistical analyses were performed using R Core Team (2024) software version 4.4.2. For the results of experiments 1 and 2, extracted from the FlowJo™ software, mean, median and standard deviation were calculated using the frequency of labeled cells. All samples were evaluated for distribution by the Shapiro–Wilk test and Q-Q plot (Conover, 1999). For data that presented normal distribution, the Student's t-test was performed; for samples that did not present Gaussian distribution, the nonparametric Wilcoxon signed rank test was applied to compare the cell labeling of animals with total serum block (mouse serum) and animals without block and the nonparametric Mann-Whitney U test was used to compare buffalo and cattle samples. Differences were considered significant with a p-value < 0.05 (Conover, 1999; Dohoo *et al.*, 2012).

3. Results

The similarity analysis performed between the amino acid sequences of surface proteins and cytokines showed homology values above 85% between bovine and buffalo. Regarding the similarity between cytokines and molecules of the immune system of humans and buffaloes, the values ranged from 71 to 74%. The results of the similarity analysis are presented in Table 1 and Supplementary Table 1.

Regarding the assessment of binding specificity, Figures 3 and 4 show that cell and cytokine labeling in both blocked and non-blocked animals was very similar. Furthermore, all markers, except IL-17 and CD28, did not show statistical differences in labeling between blocked and non-blocked samples. The markers IL-17 and CD28 showed statistical differences in labeling, being greater in blocked samples (Figures 3 and 4).

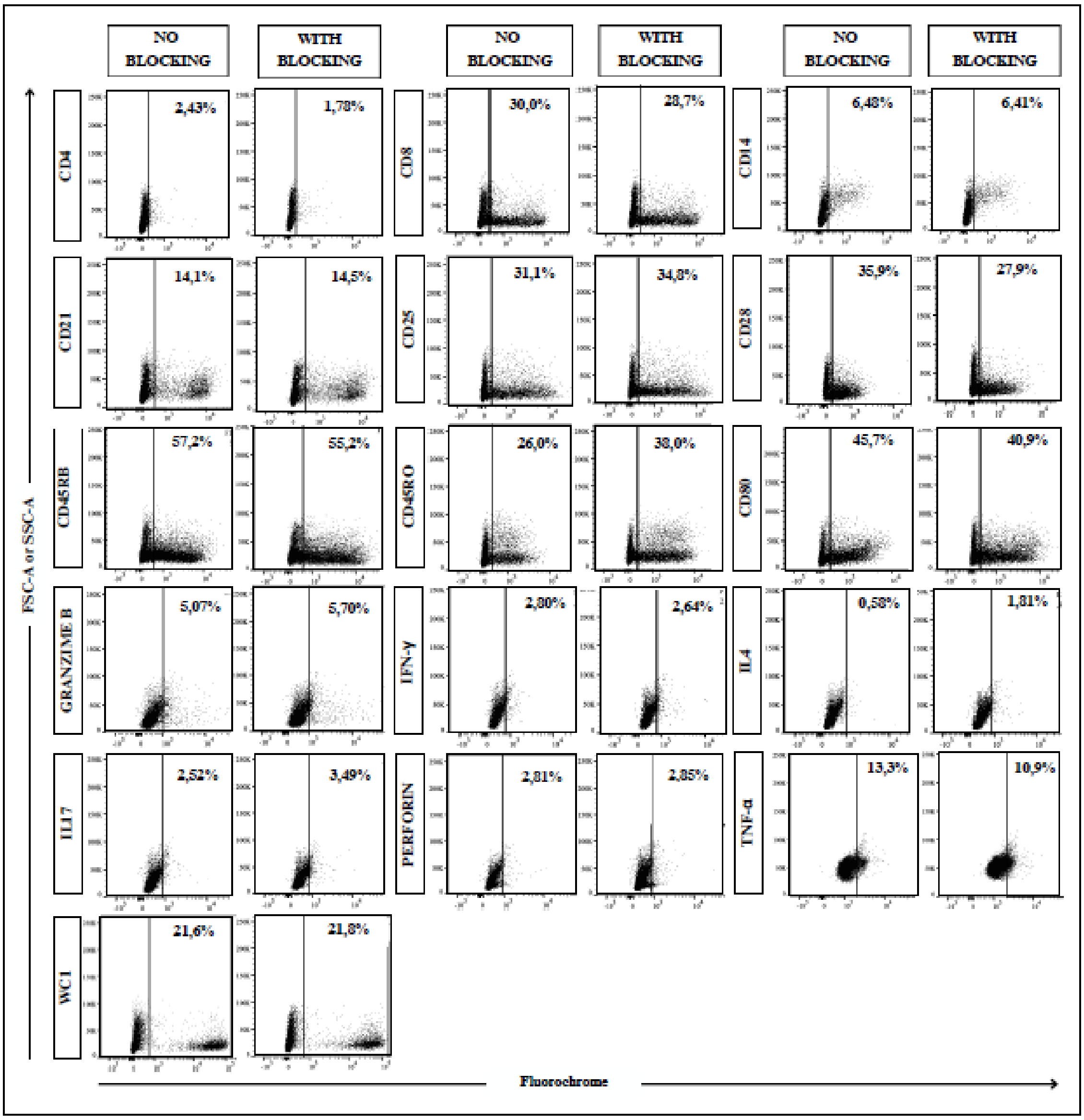

Figure 3. Binding specificity of anti-bovine monoclonal antibodies in buffaloes. Flow cytometry plots illustrating the labeling of anti-bovine mAbs (CD4, CD8, CD14, CD21, CD25, CD28, CD45RB, CD45RO, CD80, and WC1) and anti-cytokine and immune molecules from bovine (IL4, IFN-γ, and TNF-α) and man (Granizyme B, IL17, and Perforin) in representative samples from one animal, to compare the effects of blocking with 5% mouse serum on labeling. After extracellular labeling, samples were read on a flow cytometer evaluating 50,000 events. Mononuclear cells were sorted by side scatter (SSC) versus forward scatter (FSC), followed by an area-to-height analysis to select single cells. The final plots presented in this figure are composites of forward scatter (FSC-H) or side scatter (SSC-H) dots versus fluorochrome. Results are expressed as the percentage of marker-positive events.

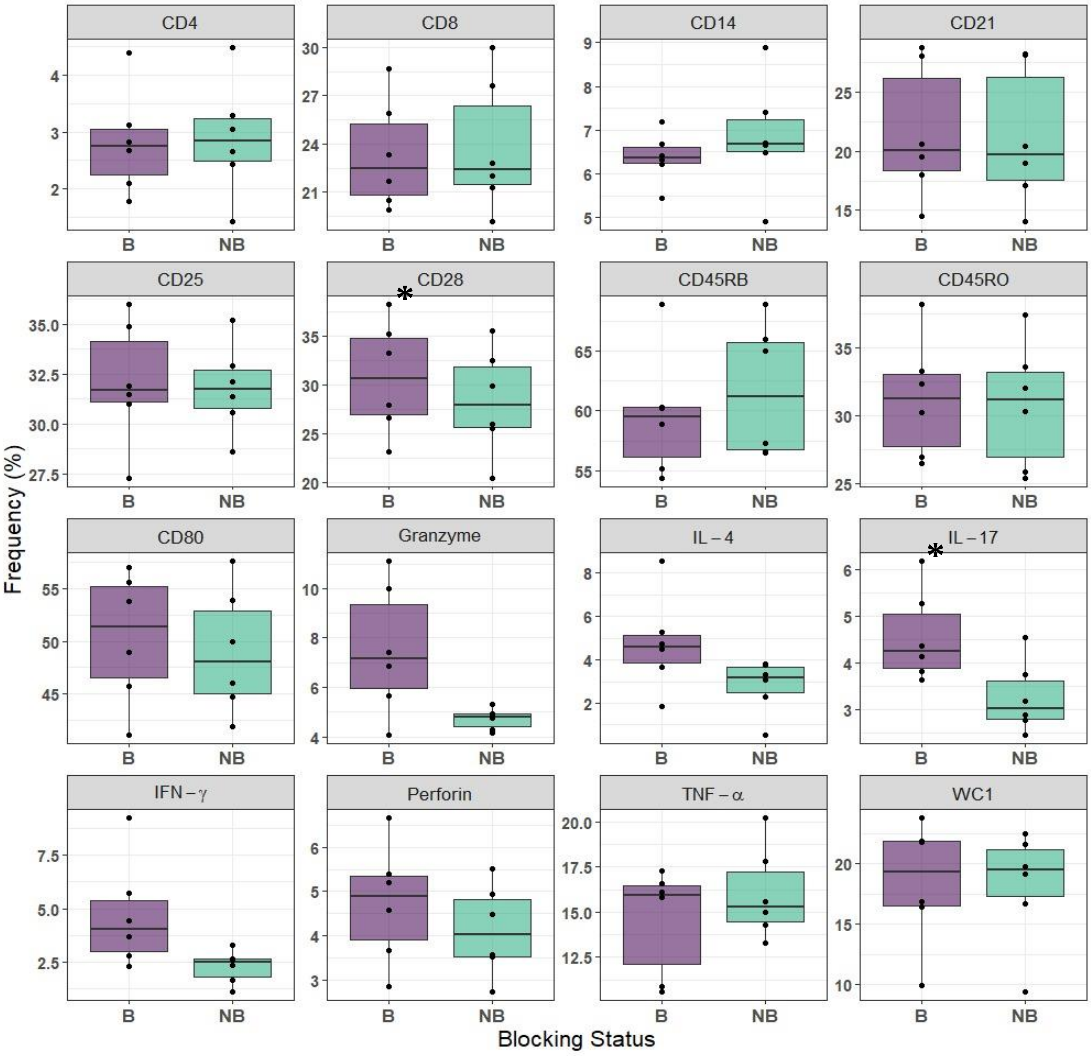

Figure 4. Binding specificity of mAbs against bovine and human immune molecules to buffalo cell antigens. The labeling frequency values of each monoclonal antibody from all animals were evaluated for distribution by the Shapiro-Wilk test and Q-Q diagram. Then, for samples that presented normal distribution, the t-test was performed, and for those that did not present Gaussian distribution, the nonparametric Wilcoxon test was applied to compare the cell labeling of animals with total serum blocking (mouse serum) and animals without blocking. * p-value < 0.05

B: Blocking; **NB**: Non-blocking.

In the evaluation of cross-reactivity, the results presented in Figure 5 show that the percentage of staining of cells, in both cytokines and components of the immune system, in buffaloes was similar to that in cattle. In some cases, such as CD14, CD25, CD28, CD80, WC1, Granzyme, IL-4, IFN-γ, perforin and TNF-α, the buffalo samples showed a higher frequency of staining compared to the cattle samples (Figure 5). Only the CD4 marker clearly showed much lower staining, if any, in buffaloes compared to cattle. The graph in Figure 8 shows that most mAbs against bovine and human presented statistically equal staining to buffaloes, except for the markers CD4, CD14, CD25, CD28, CD45RB, WC1 and Granzyme B. However, among the markers that presented statistical difference, only the CD4 and CD45RB markers presented greater staining in cattle, while the markers CD14, CD25, CD28, WC1 and Granzyme B presented greater staining in buffaloes.

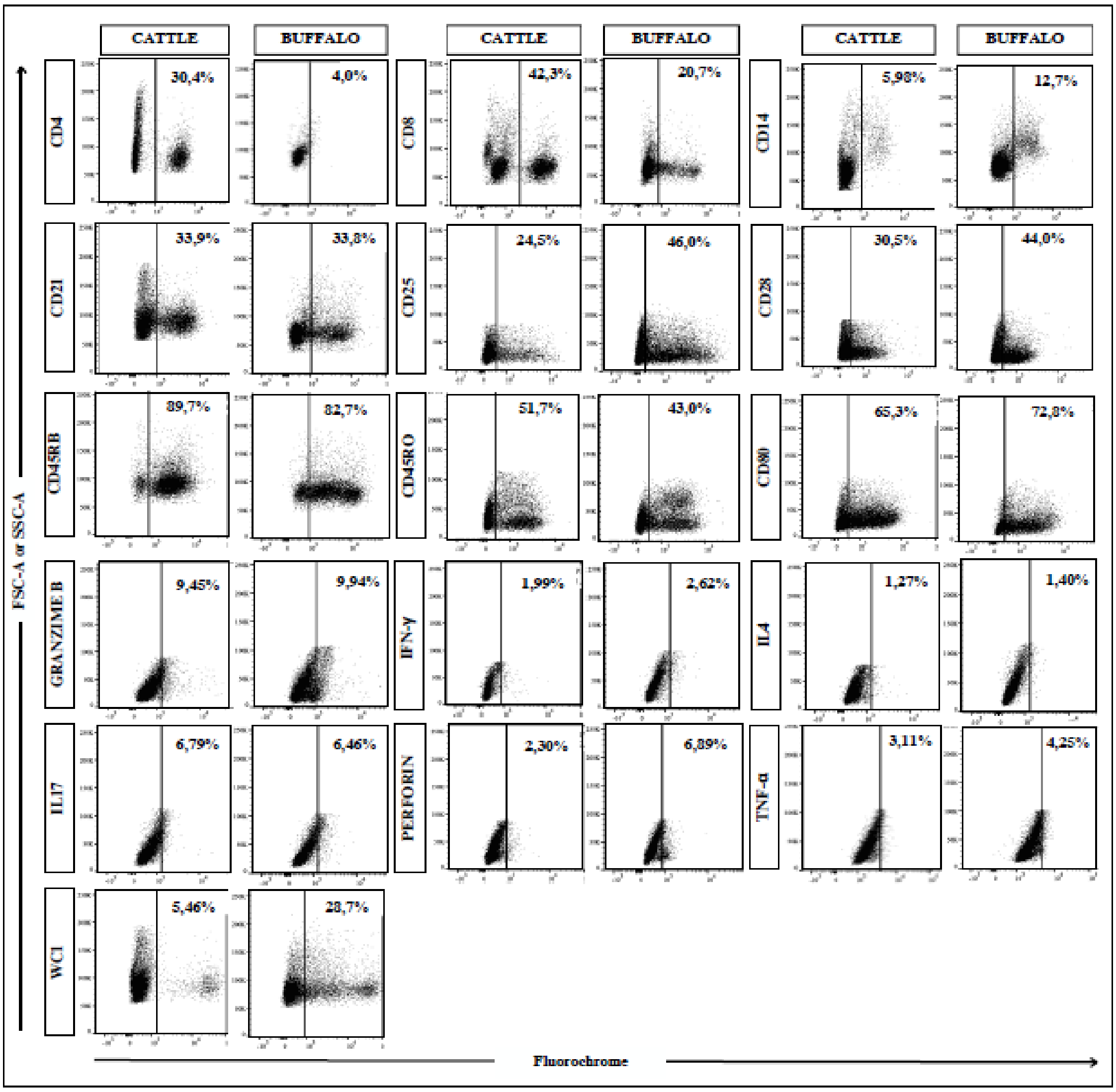

Figure 5. Cross-reactivity of anti-bovine monoclonal antibodies in buffaloes. Flow cytometry plots illustrating staining of anti-bovine mAbs (CD4, CD8, CD14, CD21, CD25, CD28, CD45RB, CD45RO, CD80, and WC1) and anti-cytokine mAbs bovine (IL4, IFN-γ, and TNF-α) and human (Granzyme B, IL17, and Perforin) immune molecules with representative samples from one bovine and one buffalo per marker. After extracellular staining, samples were read on a flow cytometer evaluating 14,000 events. Mononuclear cells were sorted by granularity versus size, followed by an area-to-height analysis to select single cells. The final plots presented in this figure are composites of forward scatter (FSC-H) or side scatter (SSC-H) dots versus fluorochrome. Results are expressed as the percentage of marker-positive events.

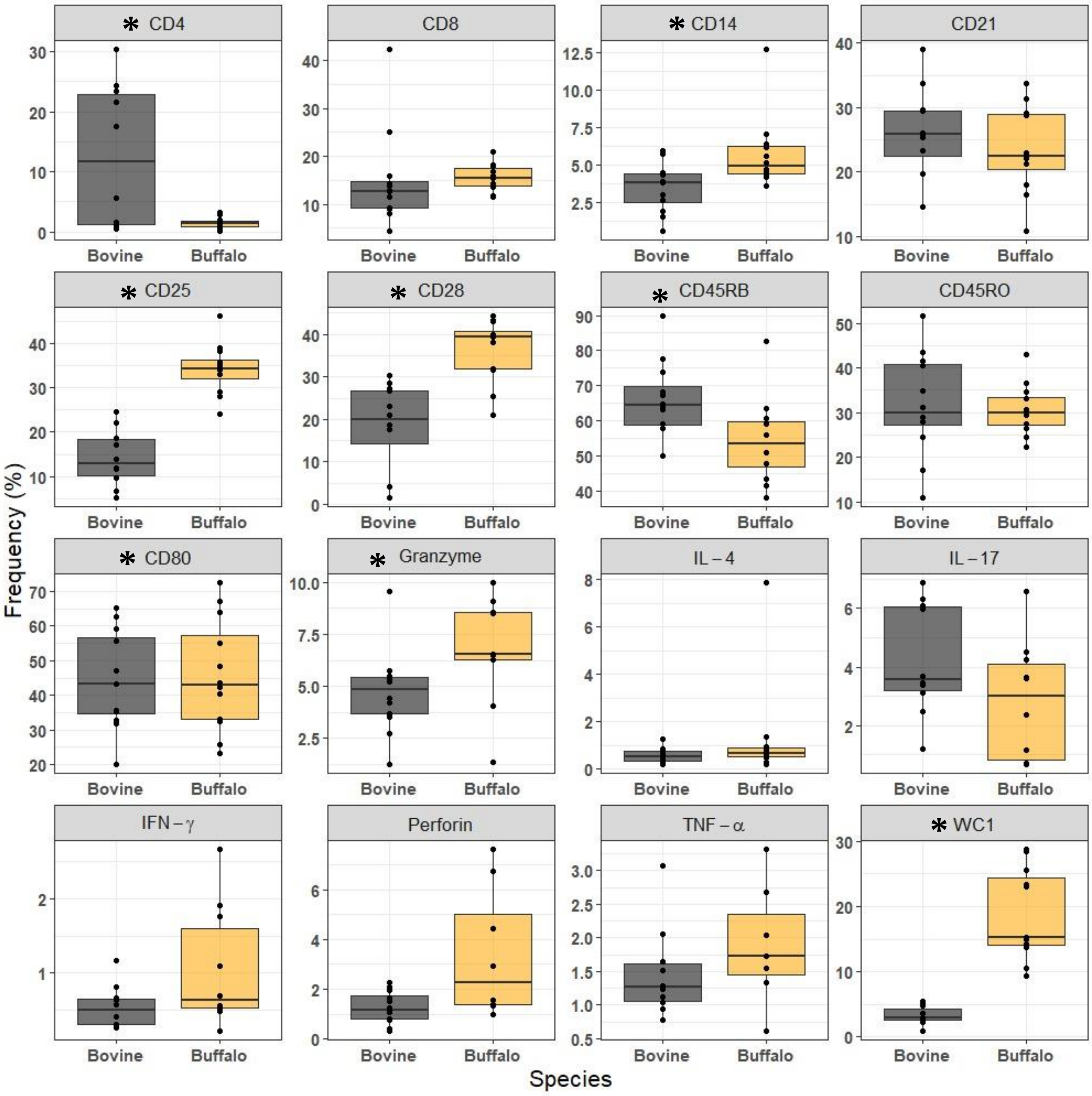

Figure 6. Cross-reactivity of monoclonal antibodies against human and bovine immune molecules to buffalo antigens. The labeling frequency values of each mAb from all animals were evaluated for distribution by the Shapiro-Wilk test and Q-Q plot. Then, for samples that presented normal distribution, the t-test was performed, and for those that did not present Gaussian distribution, the nonparametric Mann-Whitney U test was used to compare samples from buffaloes and cattle. * *p*-value < 0.05

4. Discussion

This study evaluated the cross-reactivity of 16 commercially available monoclonal antibodies, cytokine, and immune system molecules against bovine and human immune cells with buffalo immune system molecules. The results obtained showed that all mAbs tested but the one against CD4 showed cross-reactivity with buffalo antigens.

The similarities among the amino acid sequences of bovine or human and buffalo immune system molecules were greater than 70%, with most molecules showing similarities over 90%. It has been reported that cytokines with amino acid sequence homologies over 60% are more likely to present cross-reactivity (Scheerlinck, 1999), as it was observed in the present study. Although there are no reported homology parameters described to analyze the results of bovine or human and buffalo cell surface proteins, cross-reactivity was observed with the mAbs to molecules with amino acid similarity results greater than 88% (Tables 1 and Supplementary Table 1). Although the similarity of CD4 amino acid sequences between cattle and buffaloes was 88.35%, the mAb clone CC8 tested in the present study did not show cross-reactivity with CD4 on buffalo lymphocytes. A possible explanation could be the alteration change of two lysines in the CD4 amino acid sequence of buffaloes, leading to the loss on buffalo cells of the epitope identified by this clone (Grandoni *et al.*, 2020).

The difference in the number of events read in each experiment is probably related to problems in the execution of the technique. Some samples (7.6%) of the cross-reactivity experiment presented a low number of cells (less than 50,000), making it necessary to reduce the minimum number of events so as not to interfere with the statistical analysis of the work. The specificity and reliability of the observed cross-reactivity results were evaluated by the whole serum blocking assay, which showed that the staining pattern for mAbs tests was similar between animals with and without blocking by mouse serum, as it is depicted in Figure 3, and that mAbs did not bind nonspecifically by Fc portion of antibodies. Among the 16 mAbs tested, only mAbs against IL-17 and CD28 presented statistically significant differences on buffalo cell staining with and without serum blocking (Figure 4). In both cases, the blocked samples showed higher levels of staining, however no justifications were identified that could explain this result.

Studies have been carried out to better understand the immune response of buffaloes to infectious disease, through flow cytometry, such as brucellosis (Grandoni *et al.*, 2023), tuberculosis (De Matteis *et al.*, 2023) and bovine viral diarrhea (BVD) (Grandoni *et al.*, 2020). The use of multiparametric methods, such as immunophenotyping, is essential to characterize and quantify immune cell subsets on animals in those studies (Robinson *et al.*, 2023). Due to the lack of specific mAbs against buffaloes, attempts have been made to evaluate the cross-reactivity of mAbs against other species, such as humans and cattle. The markers used in this study allow the evaluation of T cell subsets ($CD4^+$ and $CD8^+$), γδ T cells ($WC1^+$), B cells ($CD21^+$), activated T cells ($CD25^+$), naive T cells ($CD45RB^+$), memory T cells ($CD45RO^+$), monocyte populations ($CD14^+$), costimulatory molecules ($CD28^+$ and $CD80^+$), cytokines (IFN-γ, IL4, IL17, TNF-α) and cytotoxicity markers (granzyme B and perforin) (Brusko *et al.*, 2009; Telfer *et al.*, 2015; Grandoni *et al.*, 2017; Krummey *et al.*, 2020; Chi *et al.*, 2024). This set of mAbs will allow the evaluation of the adaptive immune response of buffaloes, allowing the expansion of studies on the mechanisms of protection and immunological memory against pathogens through natural infection or through vaccines.

The analysis of the cross-reactivity graphs showed high similarity of labeling between buffalo and bovine cells in most samples, except for the CD4 mAb clone CC8, which clearly presents low labeling in buffaloes (Figures 5 and 6, Table 1). Clones of anti-CD4 (CC8), anti-CD8 (CC63), anti-CD21 (CC21), anti-IFN-γ (CC302), CD45RO (IL-A116), CD80 (IL-A159), and anti-IL-4 (CC303) mAbs have been previously used in other studies (Yang *et al*., 2012; De Matteis *et al*., 2016; Grandoni *et al*., 2017; De Massis *et al*., 2023). However, Yang *et al.* (2012) did not previously evaluate the used clones (anti-CD8 CC63, anti-IFN-γ CC302 and anti-IL-4 CC303) for cross-reactivity, but the results showed that cell and cytokine labeling were sufficient to evaluate the immune response of buffaloes. Regarding anti-CD4 (CC8) mAb, Yang *et al*. (2012) stated that there is cross-reactivity with buffalo cells, and they emphasized that the percentage of CD4 cells in cattle has always been significantly higher than in buffaloes. Grandoni *et al*. (2020) used the same anti-CD4 clone, CC8, to analyze its efficiency in water buffaloes, for labeling peripheral blood buffalo cells in a study that evaluated the response against bovine viral diarrhea virus (BVDV), but the results of this mAb were removed from the study under the allegation that it did not show good labeling. In our study, we decided to test the anti-CD4 mAb CC8 clone again for cross-reactivity with buffalo cells and understand the difference between the two previous studies and our results were similar to those of Grandoni *et al*. (2020) and we concluded that the anti-CD4 clone CC8 do not cross-react with buffalo cells. The use of clones already described in the literature also aimed to analyze the binding specificity of these mAbs in addition to analyzing the statistical data of cross-reactivity, since no study identified to date has performed a comparative statistical analysis between labeling in the target species and in buffaloes. There are already mAb clones to identify the other cellular markers (CD14, CD25, CD28, CD45RB and WC1) mentioned in this study; however, not all of them are commercially available (Davis *et al*., 2001; Mosaad *et al*., 2006; Grandoni *et al*., 2017). Therefore, this present work evaluates the cross-reactivity of commercially available mAb clones that have not yet been tested in buffaloes and, therefore, represent alternatives to those that already exist. Furthermore, there are few studies analyzing the cross-reactivity of anti-cytokine mAbs and immune system molecules in buffaloes (De Matteis *et al*., 2023). Therefore, our study brings mAbs that can help in the study of cytokines important for the cellular immune response of buffaloes. Furthermore, through mAbs validated for use in buffalo cells, it will be possible to understand how the adaptive immune response of these animals works against pathogens or vaccine responses.

It was also possible to observe that some mAbs marked more cells, cytokines or components of the buffalo immune system than bovine or human antigens that they were raised against. Anti-CD14, anti-CD25, anti-CD28, anti-granzyme B, anti-TNF-α and anti-WC1 mAbs clones tested exhibited a higher staining in buffaloes compared to cattle, and only CD45RO presented a statistically lower staining than cattle but still demonstrated cross-reactivity (Figure 6). Considering the difference in marking between buffaloes and cattle, it is necessary to first understand that the animals selected for the study, both buffaloes and cattle, were adult lactating females, ruling out age-related influences on the populations of immune cells studied (Davis *et al.*, 2001; Grandoni *et al.*, 2017). Another important point is that cattle were kept in a confined free stall system and buffaloes raised in a semi-intensive system, with access to pasture and a river, being more prone to challenges from pathogens and parasites, which could explain a higher marking rate in buffaloes than in cattle. Due to the low spontaneous production of intracellular cytokines, a nonspecific prior stimulation with ionomycin and PMA was used to identify the cytokines in this study (Schauer *et al.*, 1996). However, this stimulation was carried out in the same proportion for cattle and buffaloes, and the variations found may be associated with production by species. Furthermore, the animals were apparently healthy and well-fed, but our group did not have access to the disease and vaccination history of the two herds, and if the buffaloes had been vaccinated close to the time of blood collection or had suffered from any disease, that could have stimulated an increase in the animals' immune response. Also, another possibility that would justify higher marking rates in buffaloes would be a greater expression of the epitopes of interest in buffalo cells. The dilution used to perform flow cytometry in this study was based on a previous titration for cell and cytokine labeling in cattle, and it is necessary to adapt the dilution when these mAbs are used in buffaloes.

In conclusion, the present study expanded the number of commercially available mAbs against cytokines, cells markers and immune system components that can be used for studying and characterization of buffalo immune responses in different situations as vaccine response, clinical therapy, and parasitic and infectious diseases.

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6. References

BORGHESE, A. *Buffalo production and research*. Roma (Italy): REU Technical Series, 2005.

BRUSKO, T. M.; WASSERFALL, C. H.; HULME, M. A. *et al*. Influence of Membrane CD25 Stability on T Lymphocyte Activity: Implications for Immunoregulation. *PLOS ONE***,** v. 4, n. 11, p. e7980, 2009.

CAMACHO, C.; COULOURIS, G.; AVAGYAN, V. *et al*. BLAST+: architecture and applications . *BMC Bioinformatics* v. 10, n. 421, 2009.

CHI, H.; PEPPER, M.; THOMAS, P. G. Principles and therapeutic applications of adaptive immunity. *Cell***,** v. 187, n. 9, p. 2052-2078, 2024.

CONOVER, W. J. *Practical Nonparametric Statistical*. 3rd Edition. John Wiley & Sons Inc: 1999.

DAVIS, W. C.; KHALID, A. M.; HAMILTON, M. J. *et al*. The use of crossreactive monoclonal antibodies to characterize the immune system of the water buffalo (*Bubalus bubalis*). *J Vet Sci***,** v. 2, n. 2, p. 103-9, 2001.

DAVIS, W. C.; MARUSIC, S.; LEWIN, H. A. *et al*. The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. *Vet Immunol Immunopathol***,** v. 15, n. 4, p. 337-76, 1987.

DE MASSIS, F.; SACCHINI, F.; D'ALTERIO, N. *et al*. *Brucella abortus* Strain RB51 Administered to Prepubescent Water Buffaloes, from Vaccination to Lactation: Kinetics of Antibody Response and Vaccine Safety. *Microorganisms***,** v. 11 (8), p. 2078, 2023.

DE MATTEIS, G.; GRANDONI, F.; SCATÀ, M. C. *et al*. Evaluation of leptin receptor expression on buffalo leukocytes. *Vet Immunol Immunopathol***,** v. 177, p. 16-23, 2016.

DE MATTEIS, G.; SCATÀ, M. C.; ZAMPIERI, M. *et al*. Flow cytometric detection of IFN-γ production and Caspase-3 activation in CD4(+) T lymphocytes to discriminate between healthy and Mycobacterium bovis naturally infected water buffaloes. *Tuberculosis* v. 139, p. 102327, 2023.

DOHOO, I. R.; MARTIN, S. W.; STRYHN, H. *Methods in Epidemiologic Research*. 2nd. Charlottetown Prince Edward Island (Canada): VER Inc., 2012.

DORNELES, E. M.; ARAÚJO, M. S.; TEIXEIRA-CARVALHO, A. *et al*. Cross-reactivity of anti-human cytokine monoclonal antibodies used as a tool to identify novel immunological biomarkers in domestic ruminants. *Genet Mol Res***,** v. 14, n. 1, p. 940-51, 2015a.

DORNELES, E. M.; LIMA, G. K.; TEIXEIRA-CARVALHO, A. *et al*. Immune Response of Calves Vaccinated with *Brucella abortus* S19 or RB51 and Revaccinated with RB51. *PLoS One***,** v. 10, n. 9, p. e0136696, 2015b.

DORNELES, E. M. S.; TEIXEIRA-CARVALHO, A.; ARAÚJO, M. S. S. *et al*. Immune response triggered by *Brucella abortus* following infection or vaccination. *Vaccine***,** v. 33, n. 31, p. 3659-3666, 2015c.

ENTRICAN, G.; LUNNEY, J. K.; WATTEGEDERA, S. R. *et al*. The Veterinary Immunological Toolbox: Past, Present, and Future. *Front. Immunol.***,** v. 11, p. 1651, 2020.

FAO. Crops and livestock products. 2023a. Avaialble at: https://www.fao.org/faostat/en/#data/QCL/visualize. Accessed on: 20/09/2023.

FAO. Dairy animals. 2023b. Avaialble at: https://www.fao.org/dairy-productionproducts/production/dairy-animals/en/. Accessed on: 27/09/2023.

GAMA PANTOJA, L. S.; AMANTE, E. R.; MANOEL DA CRUZ RODRIGUES, A. *et al*. World scenario for the valorization of byproducts of buffalo milk production chain. *Journal of Cleaner Production***,** v. 364, p. 132605, 2022.

GRANDONI, F.; ELNAGGAR, M. M.; ABDELLRAZEQ, G. S. *et al*. Characterization of leukocyte subsets in buffalo (*Bubalus bubalis*) with cross-reactive monoclonal antibodies specific for bovine MHC class I and class II molecules and leukocyte differentiation molecules. *Dev Comp Immunol***,** v. 74, p. 101-109, 2017.

GRANDONI, F.; MARTUCCIELLO, A.; PETRINI, S. *et al*. Assessment of Multicolor Flow Cytometry Panels to Study Leukocyte Subs*et al*terations in Water Buffalo (*Bubalus bubalis*) During BVDV Acute Infection. *Front Vet Sci***,** v. 7, p. 574434, 2020.

GRANDONI, F. A.-O.; SIGNORELLI, F.; MARTUCCIELLO, A. *et al*. In-depth immunophenotyping reveals significant alteration of lymphocytes in buffalo with brucellosis. *Cytometry. Part A : the journal of the International Society for Analytical Cytology***,** v. 103(6), p. 528–536, 2023.

GRIEBEL, P. J.; ENTRICAN G FAU - ROCCHI, M.; ROCCHI M FAU - BESKORWAYNE, T. *et al*. Cross-reactivity of mAbs to human CD antigens with sheep leukocytes. n. 0165-2427 2007.

HULSPAS, R.; O'GORMAN, M. R.; WOOD, B. L. *et al*. Considerations for the control of background fluorescence in clinical flow cytometry. *Cytometry B Clin Cytom***,** v. 76, n. 6, p. 355-64, 2009.

KRUMMEY, S. M.; MORRIS, A. B.; JACOBS, J. R. *et al*. CD45RB Status of CD8+ T Cell Memory Defines T Cell Receptor Affinity and Persistence. *Cell Reports***,** v. 30, n. 5, p. 1282-1291.e5, 2020.

MCKINNON, K. M. Flow Cytometry: An Overview. *Curr Protoc Immunol***,** v. 120, n 1, p. 1 - 5, 2018.

MINERVINO, A. H. H.; ZAVA, M.; VECCHIO, D. *et al*. *Bubalus bubalis*: A Short Story. *Front Vet Sci***,** v. 7, p. 570413, 2020.

MOREIRA, M. L.; DORNELES, E. M.; SOARES, R. P. *et al*. Cross-reactivity of commercially available anti-human monoclonal antibodies with canine cytokines: establishment of a reliable panel to detect the functional profile of peripheral blood lymphocytes by intracytoplasmic staining. *Acta Vet Scand***,** v. 57, n. 1, p. 51, 2015.

MOSAAD, A. A.; ELBAGORY, A. R.; KHALID, A. *et al*. Identification of Monoclonal Antibody Reactions for Use in the Study of the Immune Response to Infectious Agents in Camel and Water Buffalo. *Journal of Camel Practice and Research***,** v. Vol. 13, n. 2, p. pp. 91-101, 2006.

OWENS, M. A.; VALL, H. G.; HURLEY, A. A. *et al*. Validation and quality control of immunophenotyping in clinical flow cytometry. *Journal of Immunological Methods***,** v. 243, n. 1, p. 33-50, 2000.

PEDERSEN, L. G.; CASTELRUIZ, Y.; JACOBSEN, S. *et al*. Identification of monoclonal antibodies that cross-react with cytokines from different animal species. *Vet Immunol Immunopathol***,** v. 88, n. 3-4, p. 111-22, 2002.

POLLARD, A. J.; BIJKER, E. M. A guide to vaccinology: from basic principles to new developments. *Nature Reviews Immunology***,** v. 21, n. 2, p. 83-100, 2021.

ROBINSON, J. P.; OSTAFE, R.; IYENGAR, S. N. *et al*. Flow Cytometry: The Next Revolution. *Cells***,** v. 12, n 14, p. 1875, 2023.

SCHAUER, U.; JUNG, T.; KRUG, N. *et al*. Measurement of intracellular cytokines. *Immunology Today***,** v. 17, n. 7, p. 305-306, 1996.

SCHEERLINCK, J.-P. Y. Functional and structural comparison of cytokines in different species. *Veterinary immunology and immunopathology***,** v. 72, n. 1-2, p. 39-44, 1999.

TELFER, J. C.; BALDWIN, C. L. Bovine gamma delta T cells and the function of gamma delta T cell specific WC1 co-receptors. *Cellular Immunology***,** v. 296, n. 1, p. 76-86, 2015.

YANG, J.; FU, Z.; FENG, X. *et al*. Comparison of worm development and host immune responses in natural hosts of schistosoma japonicum, yellow cattle and water buffalo. *BMC Veterinary Research***,** v. 8, n. 1, p. 25, 2012.

6. CONCLUSIONS

This dissertation addressed two crucial aspects related to the immunization of buffaloes (*Bubalus bubalis*) and the tools that allow understanding their immune system. First, the systematic review on the use of vaccine strains of *Brucella abortus* demonstrated that the S19 and RB51 vaccines induce immune responses in buffaloes. Although the studies were not able to confirm the efficacy of the vaccines in controlling brucellosis, the vaccines were associated with humoral and cellular responses. The RB51 vaccine was particularly highlighted for its safety, in addition to presenting the DIVA characteristic, essential for the differentiation between vaccinated and infected animals. Therefore, there is a need for further studies that seek to evaluate the efficacy of these vaccines in buffaloes, for the understanding of the best vaccine strategy to protect these animals. Second, the research carried out using commercial Mabs against bovine and human immune markers demonstrated cross-reactivity with cells and molecules of buffalo immune system, and opened new perspectives for the study of the modulation of the immune system of these species. The use of techniques such as flow cytometry, combined with the assessment of cross-reactivity, can accelerate the expansion the acquisition of data on the immune response of buffaloes, enabling advances in improving vaccination and disease control strategies.

7. APPENDIX

Appendix S1. Prisma checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	27
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	27 to 28
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	28 to 29
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	30
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	31
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	29
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	29
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	29 to 31
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	29 to 31
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	31
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	29 to 31

Section and Topic	Item #	Checklist item	Location where item is reported
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	29 to 31
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Not done
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	31
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	31
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	31
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Not done
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Not done
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Not done
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Not done
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Not done
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	31
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	31
Study characteristics	17	Cite each included study and present its characteristics.	Supplementary Table 4 and 5
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Not done
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Not done

Section and Topic	Item #	Checklist item	Location where item is reported
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Not done
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Not done
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Not done
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Not done
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Not done
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Not done
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	38 to 39
	23b	Discuss any limitations of the evidence included in the review.	38 to 46
	23c	Discuss any limitations of the review processes used.	37
	23d	Discuss implications of the results for practice, policy, and future research.	46 and 47
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Not registered
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Not prepared
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Not applied
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	18
Competing interests	26	Declare any competing interests of review authors.	Not done
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Not avaliable

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, *et al.* The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71.

Appendix S2. Search terms used in the search for references.

Cabi	((((buffalo*)) AND ((vaccin* OR S19 OR strain 19 OR RB51))) AND (brucel*)) AND ((abort* OR serolog* OR efficacy OR protection* OR immun* OR sera OR serum OR antibod* OR effectiveness OR prevent* OR control*))
Cochrane	((buffalo*) AND (vaccin* OR S19 OR strain 19 OR RB51) AND (brucel*) AND (abort* OR serolog* OR efficacy OR protection* OR immun* OR sera OR serum OR antibod* OR effectiveness OR prevent* OR control*))
Pubmed	(buffalo*) AND (vaccin* OR S19 OR strain 19 OR RB51) AND (brucel*) AND (abort* OR serolog* OR efficacy OR protection* OR immun* OR sera OR serum OR antibod* OR effectiveness OR prevent* OR control*))
Scielo	(buffalo*) AND (vaccin* OR S19 OR strain 19 OR RB51) AND (brucel*) AND (abort* OR serolog* OR efficacy OR protection* OR immun* OR sera OR serum OR antibod* OR effectiveness OR prevent* OR control*))
Scopus	ALL ((buffalo*) AND (vaccin* OR s19 OR strain 19 OR rb51) AND (brucel*) AND (abort* OR serolog* OR efficacy OR protection * OR immun* OR sera OR serum OR antibod* OR effectiveness OR prevent* OR control*))
Web of Science	ALL=(((buffalo*) AND (vaccin* OR S19 OR strain 19 OR RB51) AND (brucel*) AND (abort* OR serolog* OR efficacy OR protection* OR immun* OR sera OR serum OR antibod* OR effectiveness OR prevent* OR control*)))
Science direct	((buffalo*) AND (vaccin* OR S19 OR strain 19 OR RB51) AND (brucel*) AND (abort* OR serolog* OR efficacy OR protection* OR immun* OR sera OR serum OR antibod* OR effectiveness OR prevent* OR control*))

Appendix S3. Inclusion and exclusion criteria for selection of studies in this scoping review.

Inclusion criteria	*Exclusion criteria*
Papers of all countries	No vaccination performed
All years	Vaccination for diseases other than brucellosis
Written in English, Italian, Spanish, French or Portuguese	Species other than buffalo (*Bubalus bubalis*)
Articles that discuss buffaloes and brucellosis vaccination	Studies written in languages other than English, Italian, Spanish, French or Portuguese
Original research studies	Reviews
	Full text not available
	Proceedings
	Dissertations and theses
	Books

Appendix S4. Vaccination and challenge data from trials selected for systematic review

First author, Year	N V	N C	Vaccination				Challenge					
			Age	Strain	Dose	Route	Strain	Dose	Route	Time challenge	Positives vaccinated	Positives control
Afzal, 2000	2	-	6m	S19	UN	UN	-	-	-			
Afzal, 2000	4	-	8-9m	S19	UN	UN	-	-	-			
Afzal, 2000	4	-	11-12m	S19	UN	UN	-	-	-			
Afzal, 2000	6	-	18 - 33 m	S19	UN	UN	-	-	-			
Afzal, 2000	4	-	19 - 33 m	S19	UN	UN	-	-	-			
Bonaduce, 1985	612	-	4-10 m	S19	$6x10^{10}$	SC	-	-	-			
Bonaduce, 1985*	125	-	> 24 m	45/20	$3\text{-}4x10^{11}$	IM	-	-	-			
Bora, 2008	6	6	4-8 m	S19	$4\text{-}8x10^{10}$	SC	-	-	-			
Carporale, 2010	5	3	5-6 m	S19	$6\text{-}12x10^{10}$	SC	*B. abortus* S544	10^7	IC	69 d	0/5	3/3
Carporale, 2010*	5	3	5-6 m	RB51	(3x) $1\text{-}3x10^{10}$	SC	*B. abortus* S544	10^7	IC	69 d	1/5	3/3
Chand, 2013	33	-	4-8 m	S19	$4x10^{10}$	SC	-	-	-			
Chand, 2013*	106	-	> 8 m	S19	$5x10^9$	IC	-	-	-			
Chaudhuri, 2021	3	3	6-8 m	S19	$4x10^{10}$	SC	-	-	-			
Chaudhuri, 2021	3	3	6-8 m	S19Δper	$4x10^{10}$	SC	-	-	-			
Chaudhuri, 2021	3	3	6-8 m	S19Δper	$1x10^9$	SC	-	-	-			
Chaudhuri, 2021	4	3	6-8 m	S19	$4x10^{10}$	SC	*B. abortus* S544	$2x10^7$	IC	300 d	UN	UN
Chaudhuri, 2021	4	3	6-8 m	S19Δper	$4x10^{10}$	SC	*B. abortus* S544	$2x10^7$	IC	300 d	UN	UN
Chaudhuri, 2021	4	3	6-8 m	S19Δper	$2x10^9$	SC	*B. abortus* S544	$2x10^7$	IC	300 d	UN	UN
De Massis, 2023*	30	6	5-9 m	RB51	(3X) $1\text{-}3,4x10^{10}$	SC	-	-	-			
Diptee, 2005	6	6	6-10 m	RB51	$2,02x10^{10}$	SC	-	-	-			
Diptee, 2005*	6	6	6-10 m	RB51	$2,02x10^{10}$	SC	-	-	-			
Diptee, 2005	6	6	6-10 m	RB51	(2x) $2,02x10^{10}$	SC	-	-	-			
Diptee, 2005*	6	6	6-10 m	RB51	(2x) $2,02x10^{10}$	SC	-	-	-			

First author, Year	N V	N C	Vaccination				Challenge					
			Age	Strain	Dose	Route	Strain	Dose	Route	Time challenge	Positives vaccinated	Positives control
Diptee, 2006	6	6	6-10 m	RB51	$2{,}02x10^{10}$	SC	-	-	-	-	-	-
Diptee, 2006*	6	6	6-10 m	RB51	$2{,}02x10^{10}$	SC	-	-	-	-	-	-
Diptee, 2006	6	6	6-10 m	RB51	(2x) $2{,}02x10^{10}$	SC	-	-	-	-	-	-
Diptee, 2006*	6	6	6-10 m	RB51	(2x) $2{,}02x10^{10}$	SC	-	-	-	-	-	-
Diptee, 2007	6	6	6-10 m	RB51	$2{,}02x10^{10}$	SC	strain 24 E, strain Trinidad 1	$1{,}1x10^{7}$, $2{,}2x10^{7}$, $1{,}2x10^{8}$, $1{,}1x10^{10}$	IC, SC	57 w	6/6	6/6
Diptee, 2007*	6	6	6-10 m	RB51	$2{,}02x10^{10}$	SC	strain 24 E, strain Trinidad 1	$1{,}1x10^{7}$, $2{,}2x10^{7}$, $1{,}2x10^{8}$, $1{,}1x10^{10}$	IC, SC	57 w	6/6	6/6
Diptee, 2007	6	6	6-10 m	RB51	(2x) $2{,}02x10^{10}$	SC	strain 24 E, strain Trinidad 1	$1{,}1x10^{7}$, $2{,}2x10^{7}$, $1{,}2x10^{8}$, $1{,}1x10^{10}$	IC, SC	57 w	6/6	6/6
Diptee, 2007*	6	6	6-10 m	RB51	(2x) $2{,}02x10^{10}$	SC	strain 24 E, strain Trinidad 1	$1{,}1x10^{7}$, $2{,}2x10^{7}$, $1{,}2x10^{8}$, $1{,}1x10^{10}$	IC, SC	57 w	6/6	6/6
Domingues, 1992	20	-	3-8 m	S19	$7{,}5x10^{8}$	SC	-	-	-	-	-	-
Domingues, 1992	13	-	3-8 m	S19	$6x10^{10}$	SC	-	-	-	-	-	-
El-Razik, 2011	10	10	6m	S19	$1\text{-}2X10^{10}$	SC	-	-	-	-	-	-
El-Razik, 2011	10	10	6m	RB51	$1\text{-}3{,}4x10^{10}$	SC	-	-	-	-	-	-
Fosgate, 2003	16	16	5-20 m	RB51	$1\text{-}3{,}4x10^{10}$	SC	Natural infection	-	-	3 m	6/14	2/13
Galiero, 2009[P]	10	-	UN	RB51	(3x) $1\text{-}3{,}4x10^{10}$	SC	-	-	-	-	-	-
Iovane, 2007	12	3	4-9 m	RB51	$1\text{-}3{,}4x10^{10}$	SC	-	-	-	-	-	-
Iovane, 2007*	12	3	4-9 m	RB51	$1\text{-}3{,}4x10^{10}$	SC	-	-	-	-	-	-
Iovane, 2007*	13	3	4-9 m	RB51	(3x) $1\text{-}3{,}4x10^{10}$	SC	-	-	-	-	-	-
Iovane, 2007	2	3	4-9 m	RB51	(10x) $1\text{-}3{,}4x10^{10}$	SC	-	-	-	-	-	-
Iovane, 2007	7	3	UN	RB51	UN	SC	CZV S-19 Fatro	$1x10^{10}$	IC	12m	UN	-
Jacobo, 2005	13	-	6 m	S19	$1{,}5\text{-}3x10^{10}$	UN	-	-	-	-	-	-
Jacobo, 2005	14	-	7 m	S19	$1{,}5\text{-}3x10^{10}$	UN	-	-	-	-	-	-
Jacobo, 2005	23	-	8 m	S19	$1{,}5\text{-}3x10^{10}$	UN	-	-	-	-	-	-

First author, Year	N V	N C	Vaccination				Challenge					
			Age	Strain	Dose	Route	Strain	Dose	Route	Time challenge	Positives vaccinated	Positives control
Jamal, 2003	5	-	8-10,5 m	S19	$7x10^{10}$	SC	-	-	-	-	-	-
Nardi Júnior, 2012	21	-	3-8 m	S19	$5-8x10^{10}$	SC	-	-	-	-	-	-
Pereira, 2015	36	6	3-8 m	S19	UN	SC	-	-	-	-	-	-
Poester, 1999	19	-	4-10 m	S19	$6x10^{10}$	SC	-	-	-	-	-	-
Ramnanan, 2012*	12	8	6-10 m	RB51	$1-3,4x10^{10}$	SC	-	-	-	-	-	-
Ramnanan, 2012*	12	8	6-10 m	RB51	$1-3,4x10^{10}$	SC	-	-	-	-	-	-
Ramnanan, 2018*	9	5	6-10 m	RB51	$1-3,4x10^{10}$	SC	strain Trinidad 1	$1,1x10^{10}$	IV	6 months of pregnancy	9/9	5/5
Ramnanan, 2018*	7	5	6-10 m	RB51	$1-3,4x10^{10}$	SC	strain Trinidad 1	$1,1x10^{10}$	IV	6 months of pregnancy	5/7	5/5
Rivera, 2016*	5	10	1 y	RB51	$(2x)1-3,4x10^{10}$	UN	-	-	-	-	-	-
Rivera, 2016*	10	10	1 y	RB51	$(2x)1-3,4x10^{10}$	UN	-	-	-	-	-	-
Rivera, 2016*	10	10	2 y	RB51	$(2x)1-3,4x10^{10}$	UN	-	-	-	-	-	-
Rivera, 2016*	10	10	2-3 y	RB51	$1-3,4x10^{10}$	UN	-	-	-	-	-	-
Rivera, 2016*	10	10	3 y	RB51	$(2x)1-3,4x10^{10}$	UN	-	-	-	-	-	-
Rivera, 2016*	10	10	5-7 y	RB51	$1-3,4x10^{10}$	UN	-	-	-	-	-	-
Rivera, 2016*	10	10	>9 y	RB51	$(2x)1-3,4x10^{10}$	UN	-	-	-	-	-	-
Roxo, 1994	18	-	5-6 m	S19	$7-9\ x10^{10}$	UN	-	-	-	-	-	-
Shome, 2020	5	5	4-5 m	S19	$4x10^{10}$	SC	-	-	-	-	-	-
Shome, 2020	5	5	4-5 m	S19	$4x10^{9}$	SC	-	-	-	-	-	-
Shome, 2020	5	5	4-5 m	S19	$1,5-2x10^{9}$	SC	-	-	-	-	-	-
Shome, 2020	5	5	4-5 m	S19	$3-4x10^{8}$	SC	-	-	-	-	-	-

NV: number of vaccinated animals.
N C: number of control animals.
D: days
m: months.
y: years.
P: vaccination of pregnant animals.
SC: subcultaneous.
IM: intramuscular.
IC: intraconjuntival.
IV: intravenous
ON: oronasal.
UN: uninformed.
***:** These trials performed a booster vaccination.
The vaccine and challenge doses are in CFU/dose .

Appendix S5. Results of post-vaccination serological tests for brucellosis and elimination of antibodies and vaccine strain.

First author, Year	Age	Strain	Dose	Serological tests	Frequency of analysis	Seroconversion	Clerance Ab	Clerance strain
Afzal, 2000	6m	S19	UN	2ME,SAT,RBT	15 d	15 d	69 d	-
Afzal, 2000	8-9m	S19	UN	2ME,SAT,RBT	15 d	15 d	190 d	-
Afzal, 2000	11-12m	S19	UN	2ME,SAT,RBT	15 d	15 d	> 211 d	-
Afzal, 2000	18 - 33 m	S19	UN	2ME,SAT,RBT	15 d	15 d	> 211 d	-
Afzal, 2000	19 - 33 m	S19	UN	2ME,SAT,RBT	15 d	15 d	> 211 d	-
Bonaduce, 1985	4-8 m	S19	$6x10^{10}$	SAT	15 m	15 m	> 56 m	-
Bonaduce, 1985*	4-10 m	45/20	$3-4x10^{11}$	SAT	6/15/30/51 m	6 m	> 51 m	-
Bora, 2008	> 24 m	S19	$4-8x10^{10}$	2ME, SAT	15/30 d	15 m	56 m	-
Carporale, 2010	5-6 m	S19	$6-12x10^{10}$	RBT, CFT, CFT RB51	7/3- 4 d	11 d	> 33 d	-
Carporale, 2010*	5-6 m	RB51	(3x) $1-3x10^{10}$	RBT, CFT, CFT RB51	7/3-4 d	35 d	49 d	-
Chand, 2013	4-8 m	S19	$4x10^{10}$	RBT	30 d	1 m	> 3 m	-
Chand, 2013*	> 8 m	S19	$5x10^{9}$	RBT	30 d	1 m	2 m	-
Chaudhuri, 2021	6-8 m	S19	$4x10^{10}$	RBT, iELISA	7/14/28/42 d	7 d	300 d	-
Chaudhuri, 2021	6-8 m	S19Δper	$4x10^{10}$	RBT, iELISA	7/14/28/42 d	7 d	112 d	-
Chaudhuri, 2021	6-8 m	S19Δper	$1x10^{9}$	RBT, iELISA	7/14/28/42 d	7 d	70 d	-
Chaudhuri, 2021	6-8 m	S19	$4x10^{10}$	RBT, iELISA	7/14/28/42/70/112/300 d	7 d	> 42 d	-
Chaudhuri, 2021	6-8 m	S19Δper	$4x10^{10}$	RBT, iELISA	7/14/28/42/70/112/300 d	7 d	> 42 d	-
Chaudhuri, 2021	6-8 m	S19Δper	$2x10^{9}$	RBT, iELISA	7/14/28/42/70/112/300 d	7 d	> 42 d	-
De Massis, 2023*	5-9 m	RB51	(3x) $1-3,4x10^{10}$	RBT, CFT, CFT RB51	7/15/30 d	1 w	>109 w	-
Diptee, 2005	6-10 m	RB51	$2,02x10^{10}$	BPAT, cELISA	2 w	UN	UN	2 w
Diptee, 2005*	6-10 m	RB51	$2,02x10^{10}$	BPAT, cELISA	2 w	UN	UN	6 w
Diptee, 2005	6-10 m	RB51	(2x) $2,02x10^{10}$	BPAT, cELISA	2 w	UN	UN	4 w
Diptee, 2005*	6-10 m	RB51	(2x) $2,02x10^{10}$	BPAT, cELISA	2 w	UN	UN	6 w
Diptee, 2006	6-10 m	RB51	$2,02x10^{10}$	BPAT,CFT RB51, Dot blot RB51, cELISA	2 w	2 w	18 w	12 w

First author, Year	Age	Strain	Dose	Serological tests	Frequency of analysis	Seroconversion	Clerance Ab	Clerance strain
Diptee, 2006*	6-10 m	RB51	$2,02x10^{10}$	BPAT,CFT RB51, Dot blot RB51, cELISA	2 w	2 w	27 w	6 w
Diptee, 2006	6-10 m	RB51	(2x) $2,02x10^{10}$	BPAT,CFT RB51, Dot blot RB51, cELISA	2 w	2 w	> 27 w	6 w
Diptee, 2006*	6-10 m	RB51	(2x) $2,02x10^{10}$	BPAT,CFT RB51, Dot blot RB51, cELISA	2 w	2 w	> 27 w	6 w
Diptee, 2007	6-10 m	RB51	$2,02x10^{10}$	BPAT, cELISA	1 m	UN	UN	-
Diptee, 2007*	6-10 m	RB51	$2,02x10^{10}$	BPAT, cELISA	1 m	UN	UN	-
Diptee, 2007	6-10 m	RB51	(2x) $2,02x10^{10}$	BPAT, cELISA	1 m	UN	UN	-
Diptee, 2007*	6-10 m	RB51	(2x) $2,02x10^{10}$	BPAT, cELISA	1 m	UN	UN	-
Domingues, 1992	3-8 m	S19	$7,5x10^{8}$	SPAT	30 d	30 d	> 240 d	-
Domingues, 1992	3-8 m	S19	$6x10^{10}$	SPAT	30 d	30 d	> 240d	-
El-Razik, 2011	6m	S19	$1-2x10^{10}$	SAT, LPS-ELISA, PPA-ELISA	1 w	1 w	13 w	-
El-Razik, 2011	6m	RB51	$1-3,4x10^{10}$	SAT, LPS-ELISA, PPA-ELISA	1 w	1 w	11 w	-
Fosgate, 2003	5-20 m	RB51	$1-3,4x10^{10}$	SAT, SPAT, BPAT, RBT, CFT RB51, Dot blot RB51, cELISA	1/3/6 m	UN	> 1 m	-
Galiero, 2009	UN	RB51	(3x) $1-3,4x10^{10}$	SPAT, CFT, CFT RB51	30 d	30 d	> 90 d	-
Iovane, 2007	4-9 m	RB51	$1-3,4x10^{10}$	SPAT, CFT, CFT RB51, iELISA	15 d/30 m	30 d	< 150 d	-
Iovane, 2007*	4-9 m	RB51	$1-3,4x10^{10}$	SPAT, CFT, CFT RB51, iELISA	15 d/30 m	30 d	< 150 d	-
Iovane, 2007*	4-9 m	RB51	(3x) $1-3,4x10^{10}$	SPAT, CFT, CFT RB51, iELISA	15 d/30 m	30 d	> 350 d	-
Iovane, 2007	4-9 m	RB51	(10x) $1-3,4x10^{10}$	SPAT, CFT, CFT RB51, iELISA	15 d/30 m	30 d	< 150 d	-
Iovane, 2007	UN	RB51	UN	SPAT, CFT, CFT RB51	UN	30 d	UN	-
Jacobo, 2005	6 m	S19	$1,5-3x10^{10}$	2ME, SAT, BPAT	30 d	15 d	6 m	-
Jacobo, 2005	7 m	S19	$1,5-3x10^{10}$	2ME, SAT, BPAT	30 d	15 d	> 12 m	-
Jacobo, 2005	8 m	S19	$1,5-3x10^{10}$	2ME, SAT, BPAT	30 d	15 d	> 12 m	-
Jamal, 2003	8-10,5 m	S19	$7x10^{10}$	2M, SAT	7 d	7 d	> 91 d	-
Nardi Júnior, 2012	3-8 m	S19	$5-8x10^{10}$	2ME, RBT, CFT	15/30 d	15 d	270 d	-
Pereira, 2015	3-8 m	S19	UN	2ME, RBT	30 d*	30d	390 d	-
Poester, 1999	4-10 m	S19	$6x10^{10}$	2ME, SAT, RBT	30 d	30 d	300 d	-

First author, Year	Age	Strain	Dose	Serological tests	Frequency of analysis	Seroconversion	Clearance Ab	Clearance strain
Ramnanan, 2012*	7-10 m	RB51	1-3,4x10^{10}	BPAT, CFT RB51, Dot blot RB51	1, 2, 3, 4, 8, 12, 16, 18, 20, 24, 28, 32, 40, 48 w	1 w	36 w	18 w
Ramnanan, 2012*	7-10 m	RB51	1-3,4x10^{10}	BPAT, CFT RB51, Dot blot RB51	1, 2, 3, 4, 8, 12, 16, 18, 20, 24, 28, 32, 40, 48 w	1 w	48 w	> 18 w
Ramnanan, 2018*	7-10 m	RB51	1-3,4x10^{10}	BPAT	2, 4 d/ 1,2,4,6,8,10 w	UN	UN	-
Ramnanan, 2018*	7-10 m	RB51	1-3,4x10^{10}	BPAT	2, 4 d/ 1,2,4,6,8,10 w	UN	UN	-
Rivera, 2016*	1 y	RB51	(2x)1-3,4x10^{10}	RBT, FPA	once	UN	UN	-
Rivera, 2016*	1 y	RB51	(2x)1-3,4x10^{10}	RBT, FPA	once	UN	UN	-
Rivera, 2016*	2 y	RB51	(2x)1-3,4x10^{10}	RBT, FPA	once	UN	UN	-
Rivera, 2016*	2-3 y	RB51	1-3,4x10^{10}	RBT, FPA	once	UN	UN	-
Rivera, 2016*	3 y	RB51	(2x)1-3,4x10^{10}	RBT, FPA	once	UN	UN	-
Rivera, 2016*	5-7 y	RB51	1-3,4x10^{10}	RBT, FPA	once	UN	UN	-
Rivera, 2016*	>9 y	RB51	(2x)1-3,4x10^{10}	RBT, FPA	once	UN	UN	-
Roxo, 1994	5-6 m	S19	7-9 x10^{10}	2ME, SAT, SPAT, RBT	30 d	30 d	5 m	-
Shome, 2020	4-5 m	S19	4x10^{10}	SAT, RBT, iELISA	14/28/45/60/120 d	14 d	> 120 d	-
Shome, 2020	4-5 m	S19	4x10^{9}	SAT, RBT, iELISA	14/28/45/60/120 d	14 d	120 d	-
Shome, 2020	4-5 m	S19	1,5-2x10^{9}	SAT, RBT, iELISA	14/28/45/60/120 d	14 d	120 d	-
Shome, 2020	4-5 m	S19	3-4x10^{8}	SAT, RBT, iELISA	14/28/45/60/120 d	14 d	120 d	-

Detect Ab: Start of antibody detection.
Ab: antibody.
m: months.
d: days.
w: weeks
y: year
P: pregnant animals.
UN: uninformed.
RBT: Rose Bengal Test.
BPAT: Buffered Plate Agglutination Test

CF: Complement Fixation Test.
SAT: Serum Tube Agglutination Test
STAT: Standard Tube Agglutination Test.
SPAT: Standard Plate Agglutination Test.
ELISA: Enzyme-Linked Immunosorbent Assay.
2-ME: 2-Mercaptoethanol.
FPA: Fluorescence Polarization Immunoassay
***:** These trials performed a booster vaccination.
The vaccine and challenge doses are in CFU/ DOSE (colony forming unit)

Appendix S6. Analysis of similarity between amino acid sequences of molecules and immunological structures of buffaloes and cattle/humans

Structure/ Cytokine	Max Score	Total Score	Query Cover	E value	Percent Identity	Accession Length	Accession
CD4	702	702	97%	0.0	91,12	455	Query_3724140
CD4	694	694	97%	0.0	89,49	462	Query_3724139
CD4	566	566	97%	0.0	76,41	409	Query_3724141
CD8A	473	473	100%	1E-176	95,45	242	Query_8348155
CD8B	320	320	92%	3E-117	94,33	210	Query_11153
CD14	725	725	100%	0.0	97,32	373	Query_218481
CD14	723	723	100%	0.0	97,31	375	Query_218480
CD21	2969	2969	100%	0.0	97,35	1577	Query_558105
CD21	2680	4353	100%	0.0	97,15	1455	Query_558108
CD21	2678	4711	98%	0.0	97,07	1371	Query_558107
CD21	2216	2216	100%	0.0	76,35	1518	Query_558106
CD21	2156	4184	100%	0.0	95,86	1200	Query_558109
CD25	555	555	100%	0.0	96	275	Query_5040546
CD25	484	484	88%	4E-180	95,44	241	Query_5040547
CD28	456	456	100%	1E-169	99,54	265	Query_2491405
CD28	423	423	93%	2E-157	99,51	215	Query_2491407
CD28	364	364	81%	2E-133	99,44	247	Query_2491406
CD45RB	4561	4561	100%	0.0	98,69	2211	Query_2969388
CD45RB	4457	4457	98%	0.0	98,43	2172	Query_2969392
CD45RB	4456	4456	98%	0.0	98,61	2192	Query_2969391
CD45RB	4066	4066	89%	0.0	98,63	1995	Query_2969389
CD45RB	4064	4064	89%	0.0	98,68	1986	Query_2969390
CD45RB	3700	5165	87%	0.0	98,56	1801	Query_2969393
CD45RO	2511	2511	100%	0.0	99,1	1216	Query_3171948
CD45RO	2511	2511	100%	0.0	99,1	1216	Query_3171947
CD45RO	2434	2434	100%	0.0	96,79	1188	Query_3171949
CD80	585	585	100%	0.0	95,61	296	Query_3255360
CD80	541	541	92%	0.0	96,34	311	Query_3255364
CD80	541	541	92%	0.0	96,34	311	Query_3255362
CD80	541	541	92%	0.0	96,34	311	Query_3255361
CD80	541	541	92%	0.0	96,34	311	Query_3255359
CD80	541	541	92%	0.0	96,34	311	Query_3255358
CD80	474	474	80%	2E-175	96,61	237	Query_3255363
CD80	473	473	80%	1E-174	96,61	274	Query_3255365
GRANZYME B	344	344	97%	1E-125	72,37	246	Query_2162858
GRANZYME B	310	310	90%	1E-112	70,62	212	Query_2162859
IFN-γ	727	727	96%	0.0	99,15	355	Query_3873397
IL4	271	271	100%	1E-100	99,26	135	Query_8088215
IL17	216	216	88%	3E-78	74.45	153	Query_4194659

Structure/ Cytokine	**Max Score**	**Total Score**	**Query Cover**	**E value**	**Percent Identity**	**Accession Length**	**Accession**
PERFORIN	855	855	97%	0.0	74.63	554	Query_6861705
TNF-α	1256	1256	100%	0.0	95,58	696	Query_6068465
TNF-α	1256	1256	100%	0.0	95,58	696	Query_6068464
TNF-α	1256	1256	100%	0.0	95,58	696	Query_6068463
TNF-α	1256	1256	100%	0.0	95,58	696	Query_6068462
WC1	2692	2692	100%	0.0	94,14	1433	Query_6807913
WC1	2619	2619	100%	0.0	92,11	1403	Query_6864603
WC1	2606	2606	99%	0.0	93,01	1429	Query_1908665
WC1	2384	3753	100%	0.0	94,17	1300	Query_1967211