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REVIEW ARTICLES

Peptides and current methods on bovine tuberculosis diagnosis

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Abstract: Bovine tuberculosis (bTB) is a respiratory disease caused by *Mycobacterium bovis* that mainly infects cattle and adversely affects animal health and the livestock economy. Additionally, bTB affects human health as a zoonotic disease. Therefore, implementing diagnostic tests and slaughter policies campaigns is a valuable strategy to control this disease. The tuberculin skin test (TST) and the interferon-gamma (IFN-γ) assay are applied as current *ante-mortem* bTB diagnostic approaches. In addition, the choice of antigens is critical for the bTB diagnostic technique. The sensitivity of currently TST tests range from 40 to 95%. Thus, available tests present limitations with methodology and/or antigen used. In this scenario, several antigens are derived from inactivated cells or proteins, although peptides have been increasingly studied and used due to their numerous advantages. From this perspective, this review provides an overall literature review of the current *ante-mortem* bTB diagnostic tests, their advantages and limitations, and the peptide antigens used to improve test performance.

Highlights
- The incidence of Bovine tuberculosis in different countries and regions were cataloged;
- The main aspects of the current ante-mortem diagnostic of bTB are discussed;
- The remain problems of bTB and the need to develop efficient diagnostic tests are highlighted;·
- The tests that employ peptide antigens were the focus of the discussion.

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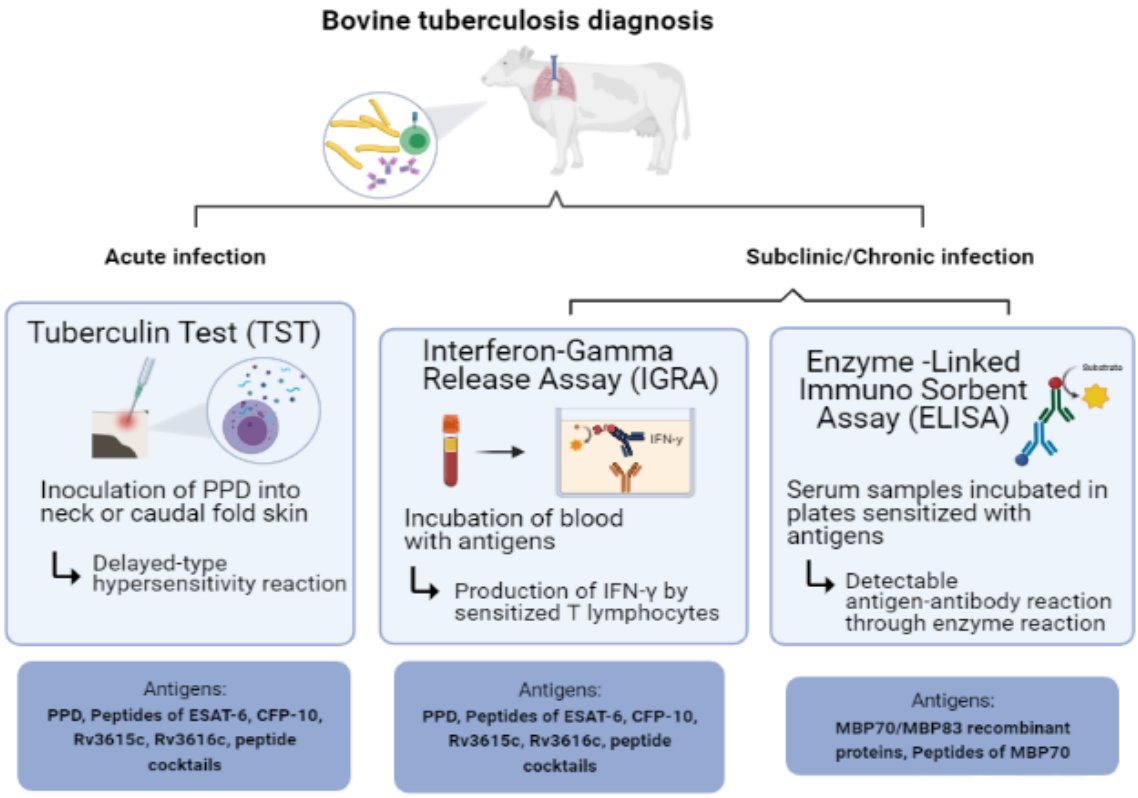
GRAPHICAL ABSTRACT



Introduction

The etiological agent of bovine tuberculosis (bTB) is *Mycobacterium bovis*, a bacterial member of the *M. tuberculosis* complex (MTC) (Trost et al., 2016) that mainly affects cattle, although other mammals are susceptible, e.g., buffaloes, goats, sheep, and humans (World Organization for Animal Health, 2019b). Those individuals affected by the disease suffer from granulomatous lesions in the respiratory and alimentary tracts and progressive weight loss (Kuria, 2019). Globally, bTB is estimated to result in an annual agricultural revenue loss of billions of dollars owing to the loss of milk, meat, and carcass (Pérez-Morote et al., 2020). Moreover, bTB restricts animal trading and negatively impacts the welfare of affected farming families. Additionally, tuberculosis (TB) is a major public health concern, especially in developing countries (Djafar et al., 2020).

In 2016, the World Health Organization (WHO) estimated 147,000 new cases of zoonotic TB in humans and 12,500 zoonotic TB-related deaths. Unfortunately, the estimates of the global burden of this disease are imprecise because of the lack of data from human and animal populations in most countries, especially in nations where bTB is endemic and laboratory infrastructure is limited (World Organization for Animal Health, 2019b). Bovine tuberculosis is a global problem in every continent. One hundred and eighty-eight countries reported bTB cases to the World Organization for Animal Health (OIE) between January 2017 and June 2018, 82 of which reported widespread occurrence. Among these 82 nations, 29 (35.4%) reported both livestock and wildlife, whereas 51 (62.2%) reported only livestock cases and 2 (2.4%) exclusively in wildlife (World Organization for Animal Health, 2019a) (Table 1). Some countries do not report the prevalence of bTB through their official agriculture and livestock organizations. However, the disease prevalence can be obtained from studies in specific cities or regions. For example, one study reported that the prevalence of bTB among 1529 animals in Sicily (Italy) was 3.3%. In Ethiopia, the herd prevalence of bTB was 51.2% in selected regions in the eastern part of the country, with at least one animal testing positive in each farm. One survey of 1856 farms from five districts in Bangladesh revealed that the prevalence of bTB was 45.6%. Study done in a province of the northern part of Morocco reported that the prevalence of bTB among 1194 animals was 57.7%.

Worldwide efforts have been made implemented to control bTB through eradication campaigns or programs. Australia has led the global example of disease eradication and has been declared bTB-free since 1997. The Australian National Brucellosis and Tuberculosis Eradication Campaign relies on systematic skin testing using the caudal fold test, the slaughter of reactive animals, and cattle regulation (Carneiro & Kaneene, 2018). Other eradication programs usually share these guidelines. In Latin America, Brazil has a National Program for the Control and Eradication of Brucellosis and Tuberculosis, which classifies territory states according to the prevalence of cases to further define applications of animal health defense procedures. Generally, this program involves the slaughter of positive animals, training of veterinarians in diagnostic procedures, and standardized tests. Approximately

Table 1. Incidence of bovine tuberculosis in different regions and countries. The cattle population and disease prevalence data were gathered from the Food and Agriculture Organization of the United Nations (FAO) database, official agriculture and livestock organizations, and studies carried out in specific cities or regions.

Region	Country	Bovine livestock population in 2019 (million numbers)	Prevalence of bTB (%)	Reference
Asia	Bangladesh	24.1	45.6	Islam et al. (2020)
	China	63.5	2.4	Gong et al. (2021)
	India	193.4	7.3	Srinivasan et al. (2018)
Africa	Ethiopia	63.2	51.2	Kemal et al. (2019)
	Morocco	3.3	57.7	Azami et al. (2018)
Europe	Spain	6.6	2.3	MAPA, España (2020)
	England	9.4	5.5	Animal and Plant Health Agency (2020)
	Ireland	6.5	4.2	Department of Agriculture Food and the Marine (2021)
	Italy	6.4	3.3	Abbate et al. (2020)
North America	United States of America	94.8	<0.001	U.S. Department of Agriculture (2017)
	Canada	11.5	1.6	World Organization for Animal Health (2019c)
South America	Brazil	214.6	1.3	MAPA, Brazil (2020)
	Uruguay	11.4	0.02	MGAP, Uruguay (2019)
	Argentina	54.4	0.46	Garcia et al. (2021)
Oceania	Australia	24.7	0.01	Sergeant et al. (2017)
	New Zealand	10.1	0.09	OSPRI New Zealand (2016)

10 million diagnostic tests for bTB were performed in Brazil between 2014 and 2018 MAPA, Brazil (2020). In Uruguay, control strategies aiming to improve bTB control use a combination of two different tests, regular testing every three months and mathematical modeling (Picasso-Risso et al., 2021). In effect, accurate and early diagnosis of bTB is a key factor in determining its prevalence and epidemiological status, enabling authorities to establish preventive measures and eradicate the disease.

Tests currently being used presents limitations of accuracy which can lead to false diagnosis and/or false idea of the epidemiological status. From this perspective, this review discusses the major *ante-mortem* diagnostic techniques for bTB, their current applications and limitations, with a focus on the test antigens, especially synthetic peptides, by providing an overview on merging approaches, test parameters, and platforms.

Bovine tuberculosis *ante-mortem* diagnosis

Cell-mediated response plays a central role during *M. bovis* infection, and tuberculin and IFN-γ assays are used to detect this response (Lyashchenko et al., 2020). On the other hand, humoral marker detection tests such as ELISA can be applied to identify animals in advanced phases of infection and tested negative in the tuberculin test. In this context, humoral marker detection tests can be used to complement the results of cellular response tests (Garbaccio et al., 2019). A compilation of diagnostic tests for bTB screening is listed (Table 2), and the specifications of each are given in the subsequent sections.

Tuberculin (TST)

The primary diagnostic test for cattle TB is the Tuberculin (TST). The Purified Protein Derivative (PPD) is used as the antigen for the test and comprises several antigens derived from *M. bovis*. The test is based on the delayed-type hypersensitivity reaction. In order to perform TST, PPD is inoculated into the skin of the neck or caudal fold and the skin thickness is measured before and after inoculation (World Organization for Animal Health, 2018). The result is interpreted as negative if the thickness is ≤ 2 mm and the animals show no clinical symptoms, e.g., edema, exudation, inflammation, or pain of the lymphatic ducts at the region of application. Animals with clinical symptoms or skin thickness > 4 mm are interpreted as positive. Tuberculin, which was first developed by Koch at the end of the nineteenth century, is an important tool for diagnosis and eradication programs in many countries. However, PPD comprises a mixture of proteins, lipids, and carbohydrates from an *M. bovis* (World Organization for Animal Health, 2018) AN5 culture. Therefore, PPD specificity markedly decreases once the animals are sensitized to environmental nonpathogenic mycobacteria, which may contain some antigens present in PPD (Roperto et al., 2017). Furthermore, the effectiveness of tuberculin varies between various manufacturers as its composition has not been completely characterized. TST

Table 2. Overview of the latest diagnostic tests and methods for bovine tuberculosis: sensitivity, specificity and antigen used. Horizontal lines divide the table in test category, as the same antigen can be presented in different tests. Commercial assays such as ELISA INDEXX and IGRA BOVIGAM can be found with symbols alongside their sensitivity and specificity.

Test	Evaluation type	Antigen	Sensitivity - Specificity (%)	Reference
Tuberculin (TST)	Skin thickness, cellular response	PPD	63.70 – *not shown*	Casal et al. (2017)
		PPD	87.6– 83.6	Singhla et al. (2019)
		Peptides of ESAT6, CFP10, Rv3615c (DST)	76 - *not shown*	Srinivasan et al. (2019)
		7 recombinant proteins, Rv3616c peptide cocktail (MDT)	71-100 – 100	Middleton et al. (2021)
IGRA	IFN-γ levels	PPD	85– 90.4†	Al-Mouqatea et al. (2018)
		Peptides of ESAT6, CFP10, Rv3615c (DST)	*not shown*	Srinivasan et al. (2019)
		Peptide cocktail of ESAT6, CFP10	76.2 – 96.5	Picasso-Risso et al. (2019)
		Peptide cocktails: ESAT-6/CFP-10 and Rv3615c	91 – 96	Coad et al. (2019)
		7 recombinant proteins, Rv3616c peptide cocktail (MDT)	100 – 97-100	Middleton et al. (2021)
ELISA	Antibody detection	MBP70, MPB83 recombinant proteins	63– 98‡	Waters et al. (2011)
		Recombinant proteins, MBP70 peptide	93.1 – 98.4%	Whelan et al. (2008)

†BOVIGAM commercial test; ‡IDEXX ELISA commercial test

performance varies depending on different factors, e.g., the dose applied, PPD preparation, the site of application, and data interpretation. It can be observed high variability in values of sensitivity in different studies, such as 63.70% (Casal et al., 2017), 75.3-95.2% with a median of 87.6% (Singhla et al., 2019) and a variation between 40.1% and 92.2% in the same study (Casal et al., 2012). A variability on specificity is sometimes presented as well, between 74.2% and 92.8% (Singhla et al., 2019). Hence, ongoing efforts are being conducted to identify new antigens not present in environmental mycobacteria by selecting proteins that belong to the MTC, especially to *M. bovis*. Moreover, TST should be associated with low costs to allow its widespread application.

An optimal diagnostic test should differentiate infected from vaccinated animals (DIVA). Srinivasan et al., (2019) developed a novel TST (DST) with antigens derived from specific *M. bovis* proteins. The test consists of 13 peptides representing these three antigens: ESAT-6, CFP10, and ESX-1 (Rv3615c) and can identify a higher percentage of naturally infected animals than the PPD test. DST could identify DIVA after vaccination (Srinivasan et al., 2020). Synthetic peptides that mimic the Rv3616c antigen have been included with other protein antigens to develop a new TST test for bovine tuberculosis, called MDT (Middleton et al., 2021). The test showed 100% of specificity and greater sensitivity than the DST previously mentioned, with relative sensitivities of 100% and 73% for the MDT and DST, respectively in experimentally infected cattle, and 71% and 29% respectively in naturally-infected cattle. Moreover, a comparison with different numbers of residues peptides was made (20 versus 40-mer), and the MDT of 40-mer peptides induced a strong skin test response (Middleton et al., 2021).

Interferon-Gamma Release Assay (IGRA)

The IGRA or IFN-γ test is an alternative diagnostic test for bTB. IGRA has been used in parallel with TST in many countries to prevent tuberculosis outbreaks (Guétin-Poirier et al., 2020; Jang et al., 2020). In order to perform IGRA, the blood from sampled animals is incubated with antigens to stimulate the production of IFN-γ by sensitized T lymphocytes (World Organization for Animal Health, 2018). Then, the IFN-γ levels are measured using a sandwich enzyme immunoassay. The BOVIGAM® (Prionics AG, Schlieren, Switzerland) IFN-γ assay is a commercial test that contains antigens from bovine tuberculin PPD. Field studies have reported that IFN-γ is more sensitive than TST, with similar or lower specificity (Keshavarz et al., 2016). Studies performed between 1991 and 2006 revealed that the sensitivity and specificity values of BOVIGAM® were 87.6 and 96.6%, respectively, based on 15 field studies (de la Rua-Domenech et al., 2006). On the other hand, recent studies have reported that the sensitivity and specificity of the BOVIGAM® test ranged between 78-85% and 90.4-91.4% (Al-Mouqatea et al., 2018; Picasso-Risso et al., 2019), respectively.

The BOVIGAM™ PC-EC stimulating antigen (Prionics AG, Schlieren-Zurich, Switzerland) is a commercial IGRA based on a peptide cocktail derived from ESAT-6 and CFP10. Compared to the PPD test, the commercial test showed slightly lower sensitivity and higher specificity (Thermo Fisher Scientific, 2019). Furthermore, in a study comparing the accuracy of IGRA tests, the one with peptide antigens derived from ESAT-6/CFP10 demonstrated higher sensitivity and specificity (Picasso-Risso et al., 2019). Srinivasan et al. (2019) tested DST (peptide-based TST using ESAT-6, CFP10, and Rv3615c proteins). The cocktail of synthetic peptides showed enhanced performance compared to a construction containing these

three antigens as a fusion protein in IGRA and skin tests. Similarly, the previously mentioned MDT was evaluated for its potential to stimulate IFN-γ production (Middleton et al., 2021). The MDT test, composed of recombinant proteins and a cocktail of synthetic peptides, induced higher levels of IFN-γ than the DST test in experimentally and naturally infected animals (100% sensitivity in both groups). In addition, non-infected control animals showed a strong response when tested with avian and bovine PPD and no response for MDT, showing great specificity.

Studies have focused on adding other biomarkers alongside IFN-γ to better detect *M. bovis*. Two peptide cocktails, ESAT-6/CFP-10 and Rv3615c, were tested in comparison with avian and bovine PPD to measure the IFN-γ and CXCL10 readouts (Coad et al., 2019). Although the CXCL10 measuring sensitivity was lower than IFN-γ (which was 100%) with any antigens, the new biomarker could complement sensitivity by identifying TST-positive animals that did not have an IFN-γ readout when using peptide cocktails as antigens.

Enzyme-linked Immuno Sorbent Assay (ELISA)

ELISA is widely used for diagnosing many human and animal diseases. The test is based on detecting antibodies against *M. bovis*, which prevail in advanced stages of the infection, thus providing an indirect way to diagnose bTB. Different antigens and methods have been investigated for ELISA-based bTB diagnosis since the 1980s, and antigens such as MBP70, ESAT-6, CFP10, and MBP83 have been examined to establish a reliable ELISA for bTB (Sun et al., 2021). However, developing antibody assays entails various limitations, including decreased sensitivity, especially in samples from early infection stages. Several studies have attempted to increase the performance of ELISA. The ELISA IDEXX *M. bovis* Ab Test (IDEXX, Maine, USA), a commercial kit for field testing, detects antibodies against MPB70 and MPB83 proteins and can be used as a supplementary test in bTB eradication programs (Paulo Alex Machado Carneiro et al., 2021). The sensitivity and specificity of the test have shown values of 63 and 98%, respectively. However, sensitivity can be increased with TST (Waters et al., 2011).

A synthetic peptide-based ELISA can be developed by peptide-based capture, wherein peptides are directly immobilized in wells of the microtiter plate by the adsorption procedure (Pandey et al., 2021), which was the case of a study that synthesized peptides derived from the known immunogenic MBP70 protein. However, peptides were tested alongside other recombinant proteins, and the ELISA test results showed sensitivity and specificity values of 93.1% and 98.4%, respectively (Whelan et al., 2008). Another study explored peptides from different regions of MBP70, recognized by anti-MBP70 chicken antibodies, but no further tests were conducted with cattle sera (Modise, 2012).

Peptide antigens

The use of synthetic peptides for diagnostic purposes has increased in the past few years since they are highly pure and can be chemically modified (Pandey et al., 2021). Many immunological and bioinformatic techniques exist to identify, select and design immunodominant peptides. They are used to screen the pathogen proteins and search within them for the most antigenic regions. In this way, it is possible to reduce the time needed to develop a diagnostic test and to find the antigens that will result in tests with the best performance. Some online tools available for linear B-cell epitope prediction are ABCPred, Bebipred 2.0, among others. Ellipro and Discotope are available for prediction of discontinuous or conformational B-cell epitopes (both available through The Immune Epitope Database - IEDB). Immunological approaches are based on the interaction between antibody and antigen, e.g., PEPSCAN (Lorenzo et al., 2021), phage display (Ramli et al., 2019) and combinatorial peptide libraries (Bozovičar & Bratkovič, 2019). Meanwhile, as mentioned, the bioinformatic tools make use of amino acids properties and epitopes databases to predict immunogenic regions (Bai et al., 2018; Ortega-Tirado et al., 2020). Hence, validating new peptide markers for diagnostic purposes has become more accessible, less expensive, and more effective.

Peptides can be easily synthesized using the standard solid-phase method, in which molecules are covalently bonded to solid support material, and amino acids are synthesized step-by-step using Fmoc protective groups. Highly pure amino acids with Fmoc are available for this purpose (>99%). The constant improvement in protection strategies during synthesis and increasing purity levels have made peptides more accessible in terms of industrial-scale use (Behrendt et al., 2016). Other approaches are being explored in peptide production, e.g., micro-flow technology, which has a shorter production time, fewer risks with dangerous compound reagents, and ready scale-up with high reproducibility (Fuse et al., 2018). Using synthetic peptides as antigens is cost-effective since their manufacture overcomes the limitations associated with using recombinant proteins, expressed in heterologous systems (*i.e.*, *E. coli*) such as formation of inclusion bodies (Bhatwa et al., 2021) with its laborious steps (purification and refolding. These synthetic molecules can be evaluated individually or as a cocktail of peptides with one or more antigens, simplifying experimental validation. Furthermore, whole proteins can be replaced with peptides to reduce nonspecific binding, thus evaluating the immune response for a single antigenic epitope (Vedova-Costa et al., 2021), improving test sensitivity, a major problem in bTB assays as cross-reactions with the environment and MTC bacteria are common.

In the last few years, several studies have highlighted the potential of synthetic peptides in diagnostic tests for various diseases, e.g., bovine anaplasmosis (Quiroz-Castañeda et al., 2019), bovine coxiellosis (Yadav et al., 2020), leishmaniosis (Link et al., 2017), capro-ovine listeriosis (Malla et al., 2021), toxoplasmosis (Alves et al., 2019), sensorineural hearing loss caused by cytomegalovirus (Zavaglio et al., 2021), and COVID-19 (Polvere et al., 2022). The accuracy values for most of these test's range between 60 and 100% for sensitivity and 73 and 100% for specificity.

Overview

This review discusses the methods currently used for diagnosing of bTB, their sensitivity/specificity, and

advantages/disadvantages. Bovine tuberculosis is a major worldwide problem. In summary, the research articles discussed here presented studies of different uses of the characterized proteins such as ESAT-6 and/or CFP10, like novel approaches with peptides of these antigens. In addition, some articles explored the use of Rv3615c in the peptide form, but rarely the MBP70 and/or MBP83 proteins. Commonly, it is used defined groups for evaluating the test accuracy, which are generally cattle natural infected by *M. bovis* and noninfected cattle control from tuberculosis-free farms. The number of animals used for the standardization of tests ranged from 25 to 279. For the TST, the antigen is inoculated in experimentally infected cattle to evaluate its efficacy. Samples taken are different for each test, as sera is used for ELISA and Peripheral Blood Mononuclear Cells (PBMCs) are used for IGRA. Sensitivity and specificity were calculated using commercially available statistics program such as Prism 4 and 7. Within these programs different statistical approaches were used: analysis of variance repeated measures, Tukey's multiple comparisons test, Student t test and Bayesian approach; all of those with the 95% confidence interval (CI).

It can be observed that although PPD delivers great specificity, it stills lack sensitivity in most tests. TST using PPD as an antigen provides variable results as it can be seen in different articles presenting values with wide differences. Also, it presents the problem of cattle being sensitized by environmental mycobacteria, which may be resolved with the use of more specific antigens. Therefore, the TST is rapid and easy-managing, but lacks novel antigens to increase its performance. In the last few years, ELISA has been the most studied method by many research groups since the current commercial tests lack a suitable performance. Nevertheless, the antigens used are recombinant proteins, and if peptides are included, they are usually the already known ESAT-6/CFP10 cocktails. Although not sufficiently explored, other peptide antigens could present satisfactory results and constitute a good alternative. In this scenario, immunoinformatics can help identify and select new peptide candidates, whereas peptide-manufacturing technologies are widespread, making them more accessible. In addition, it is now easier and less expensive to efficiently test novel antigens. Hence, it is necessary to research these antigens and develop simple, rapid, and sensitive diagnostic methods for bTB to mitigate the disease's adverse effects. This is especially true in non-developed regions, where fewer alternatives are available, and new findings could facilitate applying the guidelines for bTB control.

Conflict of interests

None.

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