4.3.7 Diagnostic tools to detect pathogens causing tuberculosis in cattle and prevent their transmission through dairy products to humans

Flábio R. de Araújo[1], Nalvo F. Almeida[2] and Martin J. Zumárraga[3]

[1] Embrapa Beef Cattle, Campo Grande, MS, Brazil
Presenter's e-mail: flabio.araujo@embrapa.br

[2] School of Computing, Federal University of Mato Grosso do Sul, Brazil

[3] Biotechnology Institute, National Institute of Agricultural Technology (INTA), Buenos Aires, Argentina

Mycobacterial species that cause tuberculosis in humans and animals belong to the *Mycobacterium tuberculosis* complex (MTBC), including: *M. tuberculosis*, *M. africanum* and *M. canettii*, which are mainly human pathogens; *M. bovis* and *M. caprae*, which are mainly ruminant pathogens; *M. microti*, a pathogen of small rodents; *M. pinnipedii*, from marine mammals; *M. mungi*, from mongooses; and *M. orygis*, from oryx.

Members of the MTBC have been associated with food-borne transmission to humans. The consumption of contaminated raw dairy products has been recognized as a major cause of transmission of *M. bovis* to humans, generally associated with the development of extra pulmonary tuberculosis. Another MTBC species that infects man is *M. caprae*. Although the transmission to humans by raw dairy products has not been proven formally for *M. caprae*, the relatedness of the pathogens and the epidemiological settings suggest that this is probably the case.

Current bovine tuberculosis (bTB) eradication programmes are based on a screening and slaughter policy, using mainly the intradermal tuberculin test, which detects the cell-mediated immunity (CMI) to the injection of purified protein derivative (PPD), a mixture of proteins prepared after a heat treatment and lysis of *M. bovis* AN5 (bovine PPD) and *M. avium* D4ER or TB56. The single intradermal tuberculin test (SITT) and the caudal fold tuberculin test (CFTT) both use bovine tuberculin, while the comparative intradermal tuberculin test (CITT) uses both bovine and avian PPD. The CITT is used to differentiate between animals infected with *M. bovis* and those responding to bovine tuberculin as a result of exposure to other mycobacteria.

Advantages of the intradermal tuberculin tests and reasons for their wide use are low costs and low logistical demands, and a well-documented use. Limitations include difficulties in administration and interpretation of results, need for a second-step visit, low degree of standardization and imperfect test accuracy. False-negative reactions are also a concern, since infected cattle may remain in herds. In Brazil, there is strong evidence of a resurgence of bTB in accredited-free herds due to infected cattle not being responsive on CITT. Also, when results of intradermal tests are inconclusive, it is necessary to wait at least 60 days before repeating the SITT or applying a CITT. This mandatory interval requires cattle to be kept in quarantine and it increases the risk of spreading the disease to herdmates and potentially to humans.

The interferon-gamma (IFN-γ) assay also detects the CMI on tuberculosis-infected cattle. In this test, sensitized lymphocytes from infected cattle are incubated *in vitro* for 16–24 hours with PPD and the released IFN-γ is detected with a sandwich enzyme-linked immunosorbent assay (ELISA) that uses two monoclonal antibodies to bovine gamma interferon. In animals that are difficult or dangerous to handle, the advantage of the IFN-γ test over the skin test is that the animals need to be captured only once. Another advantage is that since lymphocyte stimulation is done *in vitro*, it is not necessary to wait 60–90 days to repeat the test when the initial test is inconclusive.

Bovine tuberculosis is an infection that triggers predominantly a CMI during early and intermediate phases of the infection. Therefore, the main diagnostic techniques used worldwide in eradication programmes are based on the detection of the CMI: intradermal tests and interferon-gamma (IFN-γ) assay. As the disease progresses, there is a decrease in CMI and the development of serological responses. The importance of antibodies for the diagnosis of bovine tuberculosis has been debated because of the variable sensitivity (18–73 percent) reached with serological assays in preliminary studies, although high specificity has been observed (88–96 percent)

Recent studies have re-established interest in serological assays as diagnostic tests to detect false-negative animals in the intradermal tests and the IFN-γ assay. In animals with experimental infection, the serological response has been shown to increase after performing intradermal tests (anamnestic effect), leading to an improvement of the sensitivity of these techniques. The serological response varies depending on the different antigens. The Brazilian Agricultural Research Corporation (Embrapa) has developed an ELISA for detection of *M. bovis* antibodies based on a fusion recombinant antigen with the hydrophilic domains of 6 kilodilation early secreted antigen test (ESAT-6), MPB70 and MPB83 proteins. This ELISA has been used to detect infected animals missed by the CITT.

In Brazil, the control of bTB is regulated by the Brazilian National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis (PNCEBT). These regulations involve the slaughter of cattle with positive reactions to the intradermal tuberculin test (*ante-mortem* diagnosis) and the inspection of carcasses for gross lesions in abattoirs (*post-mortem* diagnosis). However, there is increasing pressure from beef markets for a definitive diagnosis of tuberculosis in cattle exhibiting lesions compatible with tuberculosis (LCT). Since 2012, the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) determined that farms with cases of bovine/bubaline tuberculosis cannot export beef to the Customs Union of Belarus, Kazakhstan and Russia. All lots of animals from a farm with suspicious animals are sequestered and the LCT are submitted to an official laboratory for aetiological diagnosis.

The culture is considered to be the "gold standard" and definitive test for the confirmation of bovine tuberculosis. However, the microbiological diagnosis of *M. bovis* is an extremely slow procedure which may take as long as 2–3 months. An additional 2–3 weeks are required for the biochemical identification of isolates. Therefore, the need for more rapid diagnostic systems is evident. Molecular diagnostic systems, particularly those based on real-time PCR technology, are faster.

A nested-PCR technique was developed by our research group that showed a clinical sensitivity value of 76 percent with tissue samples from animals that exhibited positive results in the CITT, as well as from those with LCT that rendered positive cultures. A clinical specificity value of 100 percent was

detected with tissue samples from animals with CITT-negative results, with no visible lesions and negative cultures. Nested-PCR allowed the identification of *M. bovis* in tissues with a performance that was similar or superior to the culture. Individual results from the nested-PCR were obtained in a short period of time (two days), in contrast with the culture which took up to 90 days.

It is a priority to improve and simplify diagnosis of bTB. The excretion of mycobacteria in milk is intermittent, and up to 30 percent of infected cows eliminate it by milk. Because milk samples are very easy to collect, a new strategy based on PCR in bulk tank samples has been developed at INTA in order to detect herds infected with *M. bovis*. The touchdown (TD) modification programme of PCR was used to amplify *M. bovis* insertion sequence IS6110, since sensitivity increases significantly when compared with conventional PCR. In individual milk samples, 55 percent of PPD-positive cows were shown to be positive and 95 percent of PPD-negative cows were negative to TD-IS6110 respectively. Besides, in infected herds, 47 percent of samples were positive whereas in herds with official free-of-tuberculosis-certification (TFC), 62 percent were negative and 38 percent were positive by TD-IS6110 PCR, respectively. TD-IS6110 PCR in bulk tanks could be used as a vigilance strategy for negative skin test in herds with official TFC since the negative predictive value was 95 percent. This method has been incorporated since 2012 in the Plan of Control and Eradication of Tuberculosis of Santa Fe province, Argentina, which produces 41 percent of the total milk production of the country.

To the extent that programmes for the eradication of bTB advance, more effective genotyping techniques are required in order to trace back the remaining outbreaks. A research group which involves Embrapa Gad de Corte, INTA, Universidade Federal de Mato Grosso do Sul, LANAGRO-MG, Instituto Biológico and Universidade de São Paulo has been working on the sequencing of genomes of South American strains of *M. bovis* and comparing these with genomes from the United States of America, in conjunction with the USDA-ARS. These studies will give us a better understanding of bTB and its relationship to specific phenotypes of all strains investigated; they will also generate important data for local epidemiological studies.