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Diagnostic Alternatives for *Mycobacterium paratuberculosis* in Domestic Ruminants: *"Well, Nobody's Perfect" (Some Like It Hot, 1959)*

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Abstract

The knowledge of the prevalence of an infection of such a microorganism as MAP (*Mycobacterium avium subsp. paratuberculosis*)- considering its very precise individualities, is the key issue when deciding which control measures to apply, in the precise individualities of a geographic region. The absence of scientific-based information about the prevalence of bovine paratuberculosis (PTB) could restrict the possibilities of systematic interventions for a problem already confirmed and known by the sanitary authorities, but without information on prevalence, epidemiology, and impact or economical losses, such confirmation is worthless. For the antemortem diagnosis of PTB in domestic ruminants, an important number of testing alternatives are available and recommended, including those used to detect MAP-cellular response, specific antibodies, MAP genes, and even only MAP growth. In this sense, the sensitivity and specificity of such tests vary significantly depending on the infection stage and inherent characteristics of the methods. In this reviewing appraisal, the advantages and disadvantages of currently available MAP diagnostic alternatives are revised, with the firm intention of proposing a response to a changing biological and methodological reality.

Keywords: Cattle; Diagnosis; Johne´s disease; Sensitivity; Specificity

Abbreviations: MAP: *Mycobacterium Avium Subsp. Paratuberculosis*; PTB: Paratuberculosis; IFN: Interferon; ELISA: Enzyme-Linked Immunosorbent Assay; PCR: Polymerase Chain Reaction

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The knowledge of the prevalence of an infection of such a microorganism as MAP (*Mycobacterium avium subsp. paratuberculosis*) - considering its very precise features, is the key issue when deciding which control measures to apply, in the also precise individualities of a geographic region. The absence of scientific-based information about the prevalence of bovine paratuberculosis (PTB) could restrict the possibilities of systematic interventions for a problem already confirmed and known by the sanitary authorities, but without information on prevalence, epidemiology, and impact or economical losses, such confirmation is worthless. Since the epidemiological information on this disease is still scarce, the economic impact on dairy health and production will remain uncalculated, and the potential zoonotic risk unattended, which is of main concern for all industrialized and non-industrialized countries.

An important number of testing alternatives are available and recommended for the antemortem diagnosis of PTB in domestic ruminants, including those aiming to detect anti-MAP immune cellular response and antibodies, MAP genes, or the bacteria itself on different matrices [1-4]. The test´s sensitivity and specificity vary depending on the infection phase and inherent characteristics of the tests [5].

On the first front of diagnostic alternatives are those based on the early immune response to MAP infection consists primarily of a cellular response characterized by the secretion of interferon (IFN)-γ [6-8], almost exclusively in those animals moving into stage II of the disease (this is, inapparent carrier adults), also presenting high MAP concentrations in their intestinal tissues [9]. Contrary to what one would think, such animals do not manifest

weight loss or diarrhea even with increased IFN-γ production by sensitized T cells pointing to specific mitogens and an increased anti-MAP antibodies antibody response [10-12]. Nevertheless, the results of this kind of in vitro test may not be representative of the general population and could be difficult to extrapolate and interpret [5].

The *in vivo* version of the previously mentioned test allows visualizing IFN-γ production from a measurable skin reaction, caused by the intradermal injection of johnin since the skin thickness is measured before and 72 hours after the inoculation of the purified antigen. Increases >2 mm indicate the occurrence of delayed-type hypersensitivity. However, sensitization to the *Mycobacterium avium* complex is widespread in livestock and other animals, and neither avian tuberculin nor johnin is highly specific. The specificity of this test is close to 80%, but its sensibility cut-off values had not been established so far [13-15].

Antibody-based tests being the most popular one, the enzyme-linked immunosorbent assay (ELISA), relies on the occurrence of an immune response against MAP infection [16]. ELISA is also the most extensively used to establish herd-level PTB status, in other words, for epidemiological studies. Several commercial ELISA kits for PTB diagnosis are currently available, and multiple studies have compared their accuracy and usefulness in different animal hosts and matrices [7,8,17-26]. ELISA has shown limitations, mainly those related to a low sensitivity, which is really related to the progress of the disease, rather than to limitations of the test itself, since the slow progression of MAP infection does not ensure an adequate detection capacity of animals in an early stage [6,16]. Nevertheless, the sensitivity of ELISA is the highest for animals in the clinical stage of the disease or those that excrete a considerable number of bacteria [27,28]. As an extra advantage, ELISA is highly specific with a low presentation of false-positive results [16,25,29], inexpensive, easy to perform, and quantitative results can be obtained in 1-2 hours in everyday practice [16,30].

From a more modern point of view, the detection of MAP genes by polymerase chain reaction (PCR) has shown advantages, including speed, specificity, lack of contamination, and no additional tests are needed to confirm MAP identity. On the other hand, moderate sensitivity, high cost, special equipment, and skilled personnel are required. Despite the latter, due to recent developments in PCR, it is being proposed for herd screening [1,31].

PCR performs as culture confirmatory test, but its direct application to clinical samples has been challenging, mainly due to the problems associated with complex matrices such as milk, feces, and blood, and, of course, the presence of inhibitors [32-34]. It is important to highlight those limits on detection, sensitivity, and specificity vary according to the targeted sequence and primer choice as well as the matrix tested and the PCR format (i.e. conventional gel-based PCR, reverse transcriptase PCR, nested PCR, real-time PCR, multiplex PCR). Molecular setups and methods for MAP enrichment or concentration are flexible, presenting pros and cons reliant on the matrices used and the way to be accomplished [35-36].

Last but not least, culture is still considered the gold standard for MAP detection and PTB diagnosis [5,37,38]. Its sensitivity is around 44% in MAP-infected cattle [5], due to the irregular shedding of bacteria and diverse features of the culture technique *per se* [39]. This result inevitably affects the sensitivity of the technique (culture) and the matrix (feces), leading to low detection performance, even of symptomatic animals. The specificity is almost 100% [5], even higher if the obtained isolates are confirmed as MAP by molecular methods such as PCR [5,40,41]. Disadvantages of culture include long detection time-mainly when a solid culture media is used, 12 to 16 weeks or longer, recognition of only animals actively eliminating MAP in feces, contamination risk with other mycobacteria or fungi, and the relatively high fees when compared to other tests [42]. The culture of MAP can be done either on liquid or solid media. Liquid culture methods have greater analytical and diagnostics sensitivity than solid medium and growth can be detected sooner, but a formal identification of MAP by genotypic methods is required (i.e. PCR), making the identification of MAP more difficult and expensive [39,43]. On the other hand, the identification of the organism is more difficult in liquid culture because the appearance of colonies and mycobactin dependence are not observable, and the growth of other organisms needs to be distinguished. Moreover, solid culture is more sensitive to recover C strains of MAP, since the ability of solid media to support their growth is well established [43].

Culture of pooled fecal samples as well as environmental sampling, are cost-effective approaches in the classification of herds as MAP-infected [17,44-51].

When a test combination is on the horizon -whatever the formula is, it must be considered that some infected cows have been producing antibodies several years prior to being able to find detectable quantities of MAP in feces. Or another way of saying it is that antibodies may not be detectable during the preliminary stages of infection, when MAP fecal shedding is minimal [16,52,53].

In any case, sampling of all adult cattle in every herd (mainly those over 2 years of age) -increasing the detection spectrum, and of the environment, the consideration of serialized testing, and the use of two or three tests have been suggested for herd screening and to increase the accurateness of MAP diagnosis, being the most reliable strategy when sanitary decisions must be made [1,31,54-57].

In the future, the improvement of laboratory diagnostic capacities is needed, increasing research foundations in the microbiologic, immunologic, epidemiologic, and economic aspects of the agent and the disease, even in consideration of the specific conditions of each biological or productive region, because "*Mama always said life was like a box of chocolates... you never know what you're gonna get*" (Forrest Gump).

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