

## AN ALTERNATIVE FOR THE PREADSORPTION STEP IN THE PARATUBERCULOSIS SERODIAGNOSIS: *MYCOBACTERIUM FORTUITUM*

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### SHORT COMMUNICATION

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#### ABSTRACT

ELISAs for paratuberculosis employ a preadsorption step with *Mycobacterium phlei* to diminish unspecific reactions. As *M. fortuitum* is one of the most frequent environmental mycobacteria, the purpose of this pilot study was to evaluate its use as an alternative for the preadsorption in ELISAs for paratuberculosis. Results suggest that *M. fortuitum* can be an alternative instead of or associated to *M. phlei* with comparable results ( $\kappa > 0.8$ ) to conventional ELISAs using *M. phlei* as a preadsorption antigen.

**Key-words:** Mycobacteria, ELISA, *M. fortuitum*, *M. phlei*

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Paratuberculosis is a ruminant infection characterized by chronic intermittent diarrhea with bacillary excretion in feces. It progresses through several stages and, in the majority of cases, takes several years to manifest with clinical signs (11). The diagnosis is difficult, due to the low sensitivity of the tests developed so far. Available immunological and molecular assays may not identify all infected animals, and they may give a substantial number of false-positive results (5). Due to the fastidious growth of the agent, *Mycobacterium avium paratuberculosis* (Map), serological tests, mainly ELISAs, are widely used for diagnosis of the infection (4).

Since cross reactions with environmental mycobacteria were commonly reported in the first ELISAs (3), an absorption step of bovine sera with a suspension of killed environmental mycobacterium *Mycobacterium phlei* have been efficient for reducing such false positive reactions and therefore improving the test's specificity without reducing the sensitivity (3).

Some studies suggest that several atypical mycobacteria widely recovered from pastures could be ingested by cattle and possibly cause cross reactions in antibody tests for

paratuberculosis (10). Those atypical mycobacteria have been demonstrated to induce humoral immune response in cattle that contribute to false-positive serologic reactions even in commercially available preadsorbed serum ELISAs (13). In Brazil, the most frequently isolated environmental mycobacterium is *M. fortuitum*, which is ubiquitous in soil in the South and the Southeast regions of the country, where dairy cattle breeding is more common (7). *M. fortuitum* has also been reported to be the most frequent mycobacterial species in soil of other countries, as Argentina (12) or India (6).

Lyophilized *M. phlei* is normally imported and is not easy to obtain in Brazil. Due to the difference in prevalence of environmental mycobacteria and their role in the specificity of diagnosis tests, a pilot study that uses a local strain of mycobacteria instead of, or combined with *M. phlei* was designed, to check if this alternative increases the specificity of ELISAs tests.

A panel of 10 negative and four positive sera selected from our collection was used. One positive and one negative control serum, kindly offered by Dr. Michael Collins (Wisconsin, USA)

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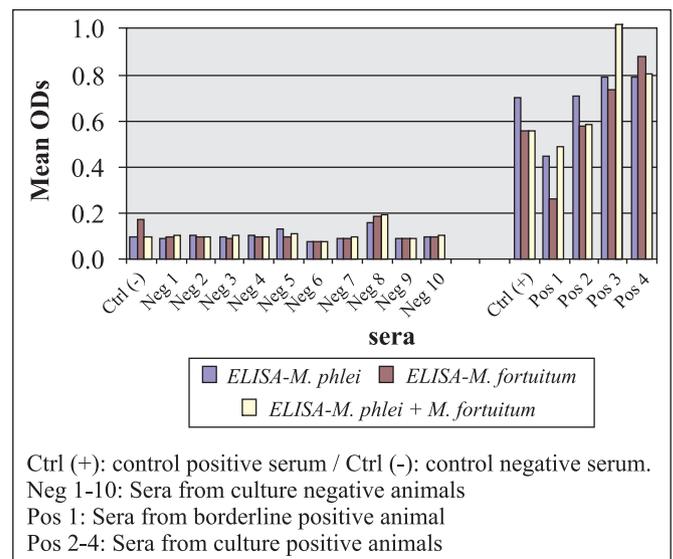
were also included. All animals that provided the positive and the negative sera had their status confirmed by bacteriological culture. All sera were tested by an ELISA test developed in our laboratory that uses protoplasmic paratuberculosis antigen (PPA) (8). This assay presented 100% sensitivity and 83.5% specificity, being comparable ( $\kappa > 0.5$ ) to commercial tests (9). Absorption of bovine sera was performed in three distinct ways: using *M. phlei* only, *M. fortuitum* only or a combination of *M. phlei* and *M. fortuitum*. With the exception of the preadsorption step, the exact same protocol, and a cut-off value of 0.35 was considered in all assays.

*M. phlei*-ELISA was performed as previously described (8). A lyophilized commercial *M. phlei* was reconstituted in saline solution in order to obtain a 5 mg/mL final concentration, according to manufacturer's instructions (Allied monitors-USA). Ten microliters of the suspension were mixed with an equal volume of suspect sera and incubated for 60 minutes at 37°C with constant agitation. After that, the suspension was diluted in 1 mL of TBST (Tris (Sigma) 10 mM, 0.9% NaCl, 0.2% Tween 20), and incubated overnight at 8°C. Sera and *M. phlei* solution were used in a final dilution of 1:100 each. *M. fortuitum*-ELISA was performed using a standard *M. fortuitum* strain (ATCC strain 6841) and cultivated as described in routine protocols (1). *M. fortuitum* was diluted as above in order to correspond to the commercial *M. phlei* solution (5 mg/mL) and used in the exact same conditions as described above.

In the *M. phlei* and *M. fortuitum*-ELISA, sera were mixed with a solution containing the same quantities of *M. phlei* and *M. fortuitum* that together presented a final concentration of 5 mg/mL. The protein concentration founded in 5 mg/mL of *M. fortuitum* was analyzed by the bicinchoninic acid (BCA) analysis (Pierce BCA Protein Assays Kit, USA), which demonstrated that *M. fortuitum* solution presented a protein concentration equivalent to *M. phlei* commercial solution. In order to compare the protein pattern of *M. phlei* and *M. fortuitum* protein extracts, 5 mg/mL of each were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel.

In order to analyze statistically the experiment, Chi-square test ( $\chi^2$ ) was used to compare the different protocols. Concordance between protocols was calculated using Kappa test ( $k$ ). Negative and positive control sera (one each), four sera of culture positive animals and 10 sera of culture negative animals were tested in three different conditions: as usual with *M. phlei*, with *M. fortuitum* and with a combination of *M. phlei* and *M. fortuitum* in the same concentration and volume, in the preadsorption step.

When the *M. fortuitum* preadsorption was used, four sera (two positive and two negative) plus the positive control serum presented lower ODs (Fig. 1) when compared to the standard assay (*M. phlei*-ELISA), possibly indicating a higher efficiency in eliminating unspecific antibodies, which could lead to cross-reactions with environmental mycobacteria and consequent



**Figure 1.** ELISA results of culture positive and negative bovine sera for paratuberculosis using *M. phlei* and *M. fortuitum* alone or associated at the preadsorption step.

false-positive results. In spite of the overall reduction on ODs values observed at the *M. fortuitum*-ELISA, three positive sera remained presenting much higher values (mean = 0.650) than negative sera (mean = 0.150), as expected (Fig. 1). Only one positive serum became negative with an OD value of 0.261 (cut-off = 0.35). This serum, when tested by *M. phlei*-ELISA, presented an OD value of 0.440, which is lower than the mean ODs of the positive sera used in this study. This same serum presented an OD of 0.489 when preadsorbed with the *M. phlei* and *M. fortuitum* suspension. This serum was obtained from an old cow that, in spite of being fecal-culture-positive and, consequently, presenting a PTB-positive status, might be immunologically sub-responding. Therefore, we classified the serum as being borderline. Statistical analysis revealed that, in spite of one serum having its final result altered, the assays using different preadsorptions demonstrated to be comparable ( $P < 0.01$ ) and no difference on efficacy could be detected between them ( $\kappa > 0.8$ ).

On the other hand, two of the positive sera and four of the negative sera preadsorbed with the *M. fortuitum* and *M. phlei* solution presented higher ODs than with the standard assay. Nevertheless, with this preadsorption step, no serum changed its final status and correlation between both tests was also high for those samples ( $\kappa > 0.8$ ). In spite of these differences, variation on ODs values observed among the three preadsorption assays was not significant ( $P < 0.01$ ).

The inclusion of a preadsorption step employing *M. phlei* was mandatory for increasing specificity of paratuberculosis ELISAs without interfering in the sensitivity (3). Nevertheless,

since atypical mycobacteria interfere with the results even in the preadsorbed commercially available serum ELISAs (13), other ways of reducing such interference must be achieved.

Protein patterns of both *M. phlei* and *M. fortuitum* were compared after separation by SDS-PAGE. This analysis demonstrated a very similar pattern of proteic bands. This finding suggests that some antigens may be shared between both microorganisms, leading to cross reactions. This is not unexpected, since it has been widely demonstrated that several mycobacteria species share proteins and other antigens (2). Therefore, it reinforces in a biochemical point of view the possibility of using *M. fortuitum* preparations as an alternative for the preadsorption step in paratuberculosis ELISAs.

*M. fortuitum* is a fast-growing mycobacterium with few requirements for its culture. It is also very frequent as a soil inhabitant in many countries and can be easily obtained and maintained by mycobacteria laboratories worldwide. Although only few sera were used in this study, these preliminary results suggest that *M. fortuitum*, alone or combined with *M. phlei*, may be considered as an alternative for the preadsorption step of ELISAs for paratuberculosis, with comparable results from those obtained with the standard assay that uses *M. phlei* at the preadsorption step. All the tested assays were capable to reduce cross-reactions with environmental mycobacteria and no significant difference was observed in the sensitivity or specificity of the assays in this study.

## RESUMO

### ***Mycobacterium fortuitum*: uma alternativa para a etapa de pré-adsorção no sorodiagnóstico da paratuberculose**

Ensaios de sorodiagnóstico de paratuberculose (ELISA) utilizam *Mycobacterium phlei* na etapa de pré-adsorção para diminuir reações inespecíficas. Uma vez que *M. fortuitum* é uma das micobactérias atípicas mais isoladas no Brasil, o objetivo central deste estudo foi averiguar a possibilidade de sua utilização como antígeno da etapa de pré-adsorção destes testes. Os resultados sugerem que *M. fortuitum* apresentou resultados comparáveis ( $\kappa > 0.8$ ) aos alcançados com *M. phlei* e que, portanto poderia ser uma alternativa ao invés ou associado a *M. phlei* na etapa de pré-adsorção de ELISAs para paratuberculose.

**Palavras-chaves:** Mycobacteria, ELISA, *M. fortuitum*, *M. phlei*

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