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

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SHORT COMMUNICATION

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*

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)
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 (κ > 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 dodeylsulfate-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 (κ). 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 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 paratuberculosis (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 (κ > 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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