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USE OF EXPERIMENTAL JOHNIN IN THE GAMMA-INTERFERON TEST IN CATTLE INFECTED BY *Mycobacterium avium* subsp. *paratuberculosis*: PRELIMINARY DATA

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Paratuberculosis, γ-IFN test, Johnin

INTRODUCTION

The diagnosis of Paratuberculosis (PTB), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is usually based on serology and fecal culture, able to detect advanced stages of infection only. The Gamma Interferon (γ-IFN) test, used for *ante-mortem* diagnosis of bovine Tuberculosis (bTB), due to *Mycobacterium bovis* (MB) infection, together with Skin Test (IDT), may be used also for PTB diagnosis as additional test. Moreover, the development of an immunological test able to distinguish between animals infected with MB or MAP and, at the same time, allowing an early diagnosis of PTB, could support the control of both these diseases. The γ-IFN test detects the amount of cytokine produced by T lymphocytes of infected animals, in response to a stimulation carried out with the traditional bovine and avian purified protein derivatives, respectively extracted from MB (PPDB) and from *M. avium* (PPDA). Similarly, the Johnin PPD (PPDJ) is produced from a culture of MAP. In order to achieve an early diagnosis of PTB, in this preliminary study, 3 new PPDJs were produced, then, they have been used in the γ-IFN test and finally compared to classic PPDs, to reduce false positive reactions for bTB.

MATERIALS & METHODS

1°- Johnin production

20 Italian MAP isolates, were genotyped by amplification of Mini and Microsatellite loci, to be used for production of PPDJs. Two strains were selected: the one with the most and the one with the least frequent genetic profile observed in Italy, they were identified as Strain A and Strain B respectively. The strain ATCC 19698 (Strain C) was used for the third batch of Johnin. The three MAP strains were cultured for 4 months at 37°C in Watson-Reid (WR) modified broth, and then the bottles were autoclaved at 100°C for 3 hours. Cells were removed and the proteins extracted by precipitation with trichloroacetic acid. The precipitate was then washed and dissolved in phosphate phenolate buffer and glycerine, with a final protein content of 1 mg/ml.

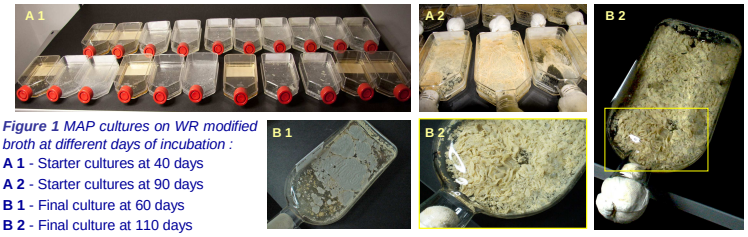


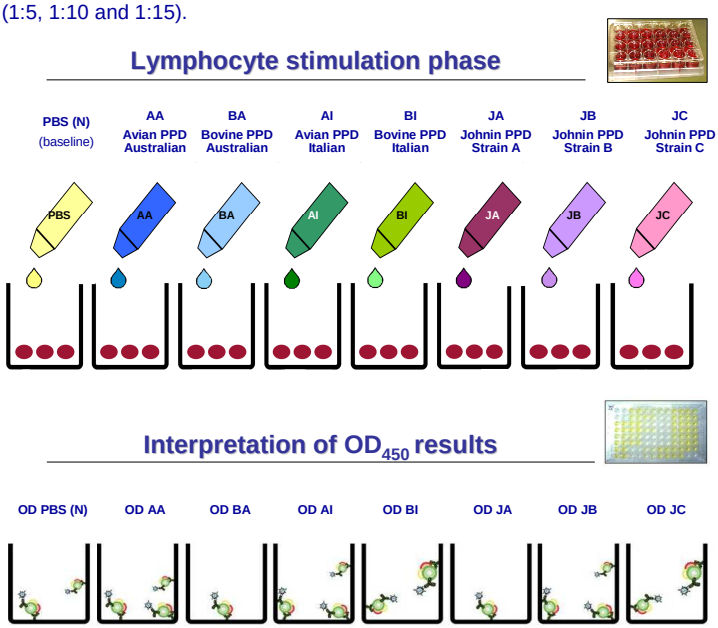
Figure 1 MAP cultures on WR modified broth at different days of incubation :
A 1 - Starter cultures at 40 days
A 2 - Starter cultures at 90 days
B 1 - Final culture at 60 days
B 2 - Final culture at 110 days

2° - Field trials

24 IDT tuberculosis negative bovines from 3 bTB officially free (OF) dairy herds were selected. In these herds, clinical cases of PTB had been reported. Each animal was tested in parallel by ELISA test for PTB (IDVet), PCR and culture for MAP from faeces. An animal was considered PTB positive if resulted positive in at least one test; on this basis, 16 out of 24 animals were classified as PTB infected while 8 were negative in all tests.

3° - In vitro stimulation and γ-IFN test

The heparinized blood samples of the 24 bovines were dispensed in aliquots of 1 ml and stimulated respectively with: "Phosphate Buffered Saline" (PBS 0.01 M, pH 7.2), used as baseline value; 30 μg of PPDB and 30 μg of PPDA (AgriQuality Australia Pty Ltd, Victoria, Australia); 10 μg of Italian PPDB and 10 μg of Italian PPDA; three experimental PPDJ (strains A, B, C) with three different dilutions (1:5, 1:10 and 1:15).



The evaluation of γ-IFN was performed with the Elisa kit BOVIGAM® on the plasma collected after 24 hours of incubation. The samples have been considered positive when the Optical Density value (OD450 nm) was ≥ 2 OD of the baseline value.

4 - Statistical interpretation of results

A 2-way analysis of variance, using the procedure Proc GLM of SAS® v9.2 software, was applied to evaluate the "dilution factor" of Johnin (1:5, 1:10; 1:15) and the "strain factor" (A, B, C). The relative specificity of PPDB and PPDJ in the diagnosis of bTB (bTB OF farms) was calculated using the IDT as Gold Standard (GS). The relative sensitivity of PPDJ in the diagnosis of PTB was calculated using as GS positivity in ELISA and/or PCR and/or Bacteriological test.

Production of IFN-γ in lymphocytes stimulated with PPDs and Johnina A, B, C

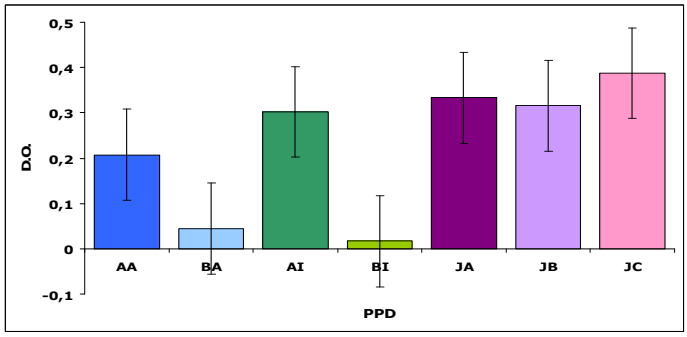


Figure 3 *Production of γ-IFN in lymphocytes of 16 animals PTB positive. Values are expressed as mean differences between the OD450 and the value doubled of OD450 found with only PBS (± standard deviation).*
***AA**: Australian Avian PPD; **BA**: Australian Bovine PPD; **AI**: Italian Avian PPD; **BI**: Italian Bovine PPD, **JA**, **JB**, **JC**: Johnin set up with MAP strains A, B, C*

RESULTS

The results of the lymphocyte stimulation with the different PPDs are shown in Figure 3. The analysis of variance showed statistically significant differences between the mean OD_{450} of PPDJ for both the "dilution factor" of Johnin (F test = 28.44, p<0.0001) and for the "strain factor" (F test = 9.82, p<0.0001). Regarding dilutions, the 1:5 seems to stimulate the highest production of γ-IFN for all three PPDJs and the difference was statistically significant. Considering the strain factor, strain B stimulates a lower production of γ-IFN, while there are no statistically significant differences between strain A and C. For bTB diagnosis, the comparative use of PPDA and PPDJ against PPDB, in the 24 bTB negative cattle, yielded a 100% (CI95: 85-100%) specificity. Finally for the diagnosis of PTB, all the three PPDJs, at 1:5 dilution, showed a 69% (CI95: 41-93%) sensitivity.

DISCUSSION & CONCLUSIONS

These preliminary results confirm the high specificity of PPDAs and Johnins, when used in γ-IFN test for the bTB diagnosis (no false positive results).

For PTB diagnosis, the low sensitivity is influenced by the tests used as GS, able to detect advanced stages of infection only. Instead, the use of PPDJ in γ-IFN test seems to be able to reveal earlier stages of infection; in fact, 3 out of 8 subjects previously classified as negatives to PTB, but positive to PPDJ γ-IFN-test, became true positive 6 months after the and of this work. In particular, two bovines tested positive to the serological test and the other one by culture.

Regarding the different ability to elicit a production of γ-IFN showed by the strain B in the study may be justified with its limited distribution. In fact strain B is poorly represented in Italy with a subsequent reduced immunological memory in animals. Therefore, in the framework of a future larger scale longitudinal study, during the choosing process of the correct Johnin to be used in γ-IFN test, the geographical diffusion of the strain to be used for PPDJ production should be taken in consideration.

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