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M. bovis infection in pigs: Improvement of the γ-IFN assay efficiency in this species using a maintenance medium

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ABSTRACT

The interferon-gamma (IFN-γ) test measures cell mediated immune response (CMI) during the early stages of tuberculosis infection. Although Bovine Tuberculosis (BT) spread in feral pigs is widely documented in literature, the effectiveness of IFN-γ in this species has been only recently reported. One of the major obstacle of this assay is that whole blood samples should be stimulated with purified protein derivative (PPD) cocktail within 8 h from the blood sampling.

This study set up a defined broth culture in which lymphocytes, the cell population predominantly responsible for IFN-γ production, are maintained in a steady-state and their vitality is preserved. The IFN-γ production measured from the samples added with the maintenance medium and stored at 4 °C was similar to the enzyme-linked immunosorbent assay (ELISA) optical density values obtained from the same assay performed within 8 h from sampling.

1. Introduction

BT is a re-emerging zoonosis in different ecological scenarios in the world. Pigs (wild boar, domestic and feral pigs), sheep, goats, buffalo and a variety of wildlife and farmed species, are susceptible [8].

The epidemiological role of reservoir or spillover hosts done by some species, such as wild boars, possums and badgers is closely related to the ecosystem in which they live and to the related ecological factors (animals, environment and population). Pigs may be considered as reservoir or spillover host in relation to the habitat and the ecosystem considered [2–5]. In order to evaluate the disease prevalence is important to study possible risk factors (e.g. presence of wildlife reservoir). Thus, pigs as well as cattle, should be considered in the tuberculosis eradication programs to maximize the chances of eradication.

Intra vitam tests, which are officially used to detect TB in cattle, are skin test and γ-IFN assay, both based on the immune cells response [11].

Skin test interpretation is based on the observation of clinical signs in the site of injection (heat, pain, necrosis, exudation, edema) and on the measurement of the skin-fold thickness 72 h after injection of protein purified derived bovine (PPDB) intradermally. False-positive reactions may be caused by sensitization due to other mycobacteria or if there are local inflammatory processes. False negatives can also be caused by a state of immunological anergy.

In pigs the use of this test is difficult to perform, the requirement of their immobilization for injection execution and the difficulties of the reading and interpretation related to the wild nature of this species (mainly if feral pigs or wild boar) and often, also, the black colour of the bristles complicate the routine use of this test in this species [6].

Therefore serology tests are more convenient [4], the gamma-interferon assay uses ELISA as detection method for the gamma interferon production, after stimulation of the blood samples with PPD. The advantages of the IFN-γ assay are due to the capability of detecting early stages of the infections, make a single intervention on the animal and an objective evaluation on the infection status of the subject examined of TB infection [3]. Previous studies showed the feasible use of the IFN-γ assay as an intra vitam test for the surveillance and management of *M. bovis* infection in pigs [7]. The use of both tests (skin test and gamma interferon assay) in parallel allows the detection of infected animals before they become a source of infection for other animals and for the environment.

Regarding humoral immunity tests, they are less considered for diagnostic purpose because it rises towards the later stages of the

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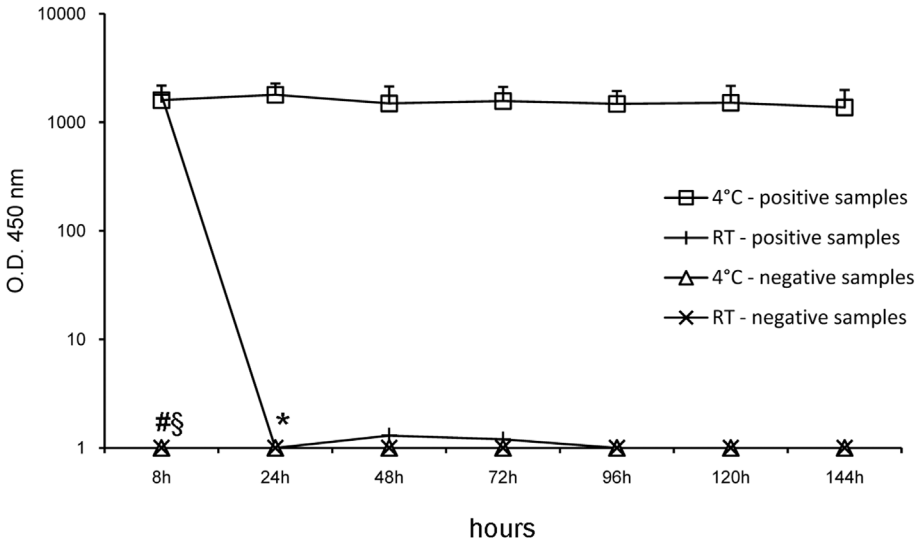

Fig. 1. IFN-γ production under different conditions. Data are expressed as the means ± SDs of 20 determinations, each conducted on a different animal. *, $p < 0.05$ 4 °C positive samples vs RT positive samples, #, $p < 0.05$ 4 °C RT positive samples vs RT negative samples, §, $p < 0.05$ 4 °C positive samples vs 4 °C negative samples. Differences were considered significant at a p value of < 0.05.

disease and it is indicative of the failure of the immune innate response. In this phase the host may be at its most infectious state [12].

The use of IFN-γ test appears, thus, to be fundamental for an effective surveillance of tuberculosis in pigs. However, it is not always applicable during the TB controls due to the time required by the standard protocol. In fact, it is recommended that blood samples used for the gamma interferon test have to be transported to the laboratory within 8 h from the sampling and not later than 24 h after blood collection (manual OIE) [3]. This is an obvious limit, in particular in sparsely populated and extended areas or in mountain territories where the possibilities to reach on time the laboratories are scarce [3]. The aim of this study was to develop a sampling method involving a less restrictive schedule, but still able to provide reliable results.

To overcome these logistic difficulties in order to implement an epidemiological survey of TB in pigs using gamma interferon test, we individuated the best conditions to storage the samples pending their transfer to the laboratory.

2. Materials and methods

2.1. Experimental design

We recruited two animal groups: one composed by infected pigs which came from an infected farm and which were positive to both tests, skin test and IFN-gamma assay [a] and another composed by pigs which came from a BT free farm, who were negatives to both tests. Whole blood samples were collected from each animal and used to perform both the standard test and a modified version where blood were added with a “maintenance” medium. This medium contains Roswell Park Memorial Institute (RPMI 1640) [b] and Fetal Calf Serum (FCS)[c] at 0,3% in order to preserve the vitality and the ability of lymphocytes to respond to antigenic stimulus for extended time points. Samples were stored at 4 °C in the maintenance medium (1:1) and were daily stimulated for the next 6 days after sampling. All samples were assayed in triplicate, and optical densities (ODs) were measured on an ELISA plate reader at 450 nm.

2.2. Animals and sample collection

Blood was collected into lithium heparin vacuum blood tubes[d] from 20 tuberculin skin test positive adult pigs of 18–24 months old. Fifty ml of blood were collected for each animal 20 days after the skin test. Twenty whole blood samples from bovine tuberculosis (BT) free pigs were used as controls.

2.3. Whole blood stimulation culture

IFN-γ standard test was performed according to test manufacturer's instructions for an extended period (8 hours-6 days after sampling).

For the modified IFN-γ protocol, blood was collected into lithium heparin blood tubes containing a maintenance medium (1:1 dilution). The maintenance medium used consisted of RPMI-1640 medium, containing 0,3% FCS L-glutamine (2 mM) and penicillin–streptomycin (100 μg/ml) [e].

Every day in the next six days after sampling an aliquot of each blood sample (1,5 ml), which was stored at 4 °C, was dispensed into a 24-well tissue culture plate and incubated at 37° C and 5% CO_2 in a humidified incubator; after that, the concentration of FCS in the medium was adjusted to 10% before the antigenic stimulation. Thus the gamma interferon test was performed as indicated in the manufacturer's instructions. All samples were assayed in duplicate and optical densities (ODs) were measured on an ELISA plate reader at 450 nm. The cells were always stimulated with100 μl (50000 UI/ml) of PPDB [f].

2.4. Statistical analysis

To determine the differences between samples treated with the modified protocol, one-way ANOVA was applied. Relative importance of difference of samples versus control from animals TB free was analyzed using Holm-Sidak method comparison test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Standard IFN-γ assay is not reliable if stimulated more than 8 h after sampling

Blood samples from 20 tuberculin skin test positive animals were stimulated with PPDB after 8 h following the standard procedures. The production of IFN-γ from blood samples (stored at room temperature and stimulated with PPDB following the standard protocol) was still evident when samples were stimulated 8 h after sampling, but it was completely abrogated when samples were stimulated 24 h after sampling (Fig. 1). Therefore the IFN-γ test has to be performed within 8 h from sampling to reach reliable results.

3.2. The conservation in a maintenance medium at 4 °C preserves cell viability

IFN-γ production was determined in whole blood samples diluted in