Novel antigens used to detect cell-mediated immune responses over time in *Mycobacterium avium* subsp. *paratuberculosis* infected cattle

Heidi Mikkelsen¹,², Claus Aagaard³, Søren Saxmose Nielsen², Gregers Jungersen¹

¹National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark; ²Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark; ³State Serum Institute, Copenhagen, Denmark.

ABSTRACT

Early stage *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection of cattle can be detected by measuring specific cell mediated immune responses, using the interferon gamma (IFN-γ) test. Available IFN-γ tests are using purified protein derivatives of MAP (PPDj) which are crude products consisting of undefined antigens with possible cross reactions toward other environmental bacteria. The objective of the study was to optimize the IFN-γ test using different types of novel antigens for stimulation. Fourteen novel antigen candidates were selected for testing, including 4 peptides of the ESAT-6 family and 10 hypothetical proteins: 4 latency proteins, 3 secreted proteins, 2 proteins not present in *Mycobacterium avium* subsp. *avium* (MAA) and 1 from an immunological hot spot region. To determine variation of IFN-γ responses, three repeated tests was done with 4 and 5 week intervals on the same 30 heifers from a known MAP infected herd. Determination of cut-off for each antigen was based on samples from a non-infected herd, including 60 heifers. Based on PPDj stimulations, more than 50% of the heifers tested MAP positive at the first two samplings, whereas only 20% tested positive at third sampling. The result showed that PPDj detect a high percentage as MAP positive animals, as this crude antigen mixture is expected to induce non-specific IFN-γ production. However, the tested latency antigens, some secreted proteins and some peptides of the ESAT-6 family detected a comparable high percentage of animals as MAP positives. By combining novel antigens higher specificity might be obtained.

INTRODUCTION

Early MAP specific cell-mediated immune responses can be measured using the IFN-γ test (Wood et al., 1989). The IFN-γ test is a whole-blood proliferation assay, in which blood is cultured overnight with MAP antigens followed by collection of supernatant. The IFN-γ level in supernatants is then detected by an IFN-γ specific enzyme linked immunosorbent assay (ELISA). Available whole IFN-γ tests for MAP diagnosis are using PPDj, which is a crude undefined extract of MAP antigens. Lack of standardized PPDj is a major concern, as the preparation of PPDj and therefore the antigen composition varies between laboratories. In addition, PPDj are known to cross-react with environmental mycobacteria such as MAA leading to low specificity of the IFN-γ test. To induce the specificity of the IFN-γ test, well-defined and MAP specific antigens are needed. In this study 14 novel antigen candidates were selected for testing with the aim to increase specificity of the IFN-γ test. The novel antigen candidates included peptides of the ESAT-6 family (van Pinxteren et al., 2000), latency proteins (Leyten et al., 2006), secreted proteins (Cho and Collins, 2006), proteins not present in MAA and a protein from an immunological hot spot region.

MATERIALS AND METHODS

Blood samples were collected 3 times with 4 and 5 week intervals from the same 30 heifers 15-24 months of age in a Danish dairy herd known to be infected with MAP. On each sample date the whole blood samples incubated for 20-22 hours at 37°C in 5% CO₂ with the 14 novel antigens, PPDj, a negative (PBS) and a positive control (Staphylococcal enterotoxin B; SEB) in parallel cultures. The 14 novel antigens candidates included 4 peptides of the ESAT-6 family (MAP160, esxH, esxK and esxU) and 10 hypothetical proteins: 4 latency proteins (MAP2467c, MAP2768c, MAP3273c, and MAP3701c), 3 secreted proteins (MAP217, MAP1662 and MAP2888) 2 proteins not present in *Mycobacterium avium* subsp. *avium*
(MAA) (MAP87 and MAP3776) and 1 from an immunological hot spot region (MAP3783). Following overnight culture, the culture plates were centrifuged and the supernatants collected and stored at -20°C until further analysis. The antigen specific IFN-γ production in supernatants were determined by an in-house ELISA as described elsewhere (Mikkelsen et al., 2009). The level of IFN-γ (pg/ml) was calculated using linear regression on log-log transformed readings from the two-fold dilution series of a reference standard with known IFN-γ concentration.

Samples were excluded if the IFN-γ value in the SEB-stimulated sample was below 1500 pg/ml or the PBS-stimulated sample was higher than 250 pg/ml. Based on these exclusion criteria, one heifer was excluded at first and second sampling and two heifers were excluded at the third sampling. To determine the cut-off for each of the 14 novel antigens and PPDj, blood samples were collected from 60 heifers 15-24 months of age from a non-infected herd. Samples from the non-infected herd heifer were excluded, if the IFN-γ value in SEB-stimulated sample was below 1500 pg/ml or the PBS-stimulated sample was higher than 75 pg/ml. The remaining samples were used to calculate the cut-off for each antigen. The cut-off for each antigen was calculated as: mean IFN-γ response + (1.96 × standard deviation of IFN-γ response).

RESULTS
Figure 1 shows the percentage of positive calves detected by each antigen at the three sampling dates.

Figure 1. Percent positive calves detected by each antigen by the IFN-γ test. The novel antigens tested were divided into four groups: ESAT-6 family members, latency proteins, secreted proteins and other antigens. Cut-off for each antigen, to distinguish between test-positives and test-negatives, was calculated based on results from a negative herd. The same 30 heifers were tested at the three samplings. Data presented here are from 29 heifers at sampling 1 and 2, and 28 heifers at sampling 3, after exclusion of invalid samples.
PPDj detected 50% of the heifers as MAP positives at the first sampling, 60% at the second sampling and less than 20% on the last sampling date. In comparison the antigen 85B (Ag85B), which share high homology with other mycobacteria such as MAA, detected 50% as positive on the first sampling and 60% as positive on the second and third sampling. In general, the antigens detected the highest percentage of heifers as positives on the second and third sampling with exception of PPDj. The groups of latency proteins and secreted proteins detected 50% to 60% as positive on the second and third sampling. Similarly, the three ESAT-6 family peptides (MAP160, esxH, esxU) detected 50% to 60% as positive on the second and third sampling. The two protein antigens selected as not present in MAA, detected 30% to 50% of the heifers as positives, whereas the protein antigen selected from an immunological hot spot region detected 30% to 40% of the heifers as positive.

DISCUSSION AND CONCLUSION
The IFN-\(\gamma\) responses fluctuated between the three sampling dates, which indicate the importance of repeated test for evaluation of novel antigen performance in the IFN-\(\gamma\) test. Surprisingly, PPDj detected less than 20% as positive on the third sampling, but 50% to 60% at the first two sampling dates. For the majority of the antigens, the highest percentage of positive animals was detected at the second and third sampling dates. There is no evident explanation for the low percentage of animals detected by PPDj on the third sampling date and this result do not agree with the result for the majority of the antigens. On the other hand, the observed fluctuations may emphasise the need for an alternative to PPDj in the IFN-\(\gamma\) test. For optimization of the IFN-\(\gamma\) test well-characterised antigens should be included to induce specificity to MAP and reduce cross-reactions to environmental mycobacteria. To obtain high specificity of the IFN-\(\gamma\) test a combination of perhaps three novel antigens should be included. The optimal combination of novel antigens to be included in a MAP specific IFN-\(\gamma\) test remains to be selected.

ACKNOWLEDGEMENTS
Technicians Abdellatif El Ghazi and Sardar Ahmad have done most of the ELISAs. This study was co-funded by the European Commission within the Sitxh Framework Programme, as part of the project ParaTBTools (contract no. 023106 (FOOD)).

REFERENCES


