APPLICATION OF PROTEIN ANTIGENS IN A MULTIPLEX LUMINEX XMAP ASSAY FOR SERODIAGNOSIS OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTIONS IN DAIRY CATTLE

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis, a chronic intestinal disease in ruminants, and has also been implicated in the etiology of Crohn's disease. Clinical disease is characterized by a progressive weight loss and chronic diarrhea, and most or all animals ultimately die from this disease. Commercial ELISA tests for serodiagnosis of MAP-infected cows have a poor sensitivity and are therefore not effective as a tool for disease control. In recent years, a whole range of new antigens was identified for the development of new, more sensitive diagnostics. We used three protein antigens in a Luminex microsphere-based multiplexed assay for the detection and quantification of serum antibodies against MAP.

Materials & methods

Three recombinant protein antigens Ag1, Ag2 and Ag7 with molecular weights 35, 15 and 9 kDa, respectively (Willemsen et al., 2006) were over expressed in *Escherichia coli*, purified and coupled to MagPlex microspheres. As a conjugate, biotinylated llama antibodies to bovine IgG were used. For setting up the test endpoint serum samples of cows, experimentally infected with MAP (Nos 1349, 1362, 1363) and endpoint serum samples of cows immunized with Ag2 (No. 8546) and Ag7 (No. 1728) were used. As negative control served a serum sample of a cow from a certified MAP-free herd was used. For validation of the test, serum samples of cattle, diagnosed as shedders of MAP bacteria by faecal culture (n=80) and serum samples of cows originating from certified MAP-free herds (n=80) were used. The sera were also tested with a commercial available ELISA test kit (Institut Pourquier, ELISA Paratuberculosis Antibody Screening).

Results

From the results it appeared that 1 ug/ml of Ag1 coupled to beads was sufficient to obtain high Mean Fluorescence Intensity (MFI) units on positive serum sample and low signals on a negative serum sample (Figure 1). For Ag5 and Ag7 high MFIs were obtained with beads coated with 5 and 25 ug/ml, the MFIs were lower at a concentration of 1 ug/ml on beads. Therefore, we decided to use beads coated with 5 ug/ml of the antigens.

The optimal dilution of serum samples was determined as well. Irrespective the concentration of the antigen on the beads, the highest MFIs were obtained at dilutions of serum samples ranging from 1:100 to 1:400 (Figure 1), so we decided to test serum samples at a dilution of 1:200. Interestingly, low MFIs were obtained at dilutions of 1:25 and 1:50 (Figure 1). Possibly, at these dilutions there is an excess of antibodies which leads to these artefactually low results, also described as a high dose hook effect (Selby, 1999).

Table 1: Sensitivity of the multiplex Luminex test and ELISA test calculated at specificities of 0.95 and 1.00

Antigen	Sensitivity at a specificity of	
	0.95	1.00
Antigen 1	0.23	0.15
Antigen 2	0.19	0.14
Antigen 7	0.13	0.09
Ag 1, Ag 2 & Ag 7*	0.33**	0.24
ELISA***	0.31	0.23

* Test result positive when one of the antigens is positive.
** Calculated specificity = 0.88
*** ELISA Paratuberculosis Antibody Screening (Institut Pourquier)

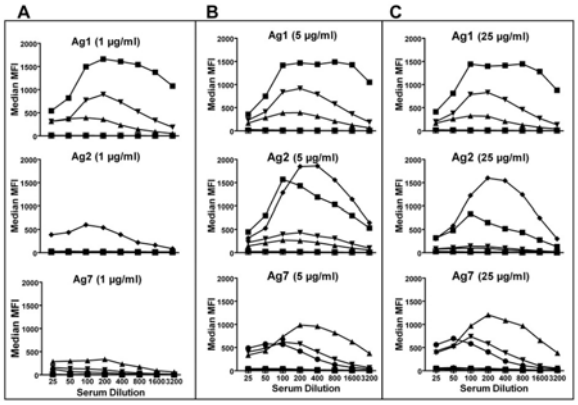

Figure 1: The optimal dilutions of antigen and test sera were determined by checkerboard titration in multiplex Luminex tests (A, B and C). Serum samples of cows 1349 (● ; anti-MAP antibodies), 1362 (▲;anti-MAP antibodies) 1363 (▼;anti-MAP antibodies), cow 1728 (◆; anti-Ag7 antibodies), cow 8546 (●; anti-Ag2 antibodies) and cow UU74864 (□; MAP-free) served as known negative and positive controls.

Discussion & conclusions

Commercial immunodiagnostic tests for the control of paratuberculosis infections in dairy cattle are nearly all based on the use of crude MAP antigens in ELISA formats. All these tests have reported a low diagnostic sensitivity and improvement is needed. Here we describe the development of a Luminex microsphere-based assay that measures simultaneously the levels of antibodies to three different purified specific MAP antigens. Comparsion of the results of the multiplex bead immunoassay with the commercial ELISA test on serum samples of naturally MAP infected and cows free for a MAP infection showed that sensitivity of both tests was nearly the same. These results indicate the potential of a Luminex multiplex assay. Its flexible and open format makes it possible to increase the sensitivity of the assay by incorporation of additional microbead sets with immunodiagnostic antigens to MAP.

References

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