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Commercially Available Antibodies to Human Tumour Necrosis Factor-α Tested for Cross-Reactivity with Ovine and Bovine Tumour Necrosis Factor-α using Flow Cytometric Assays

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[1]Department of Anatomy and Physiology, Swedish University of Agricultural Sciences, [2]Department of Ruminant and Porcine Diseases, National Veterinary Institute, and [3]Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Dernfalk J, Persson Waller K, Johannisson A: Commercially available antibodies to human tumour necrosis factor-α tested for cross-reactivity with ovine and bovine tumour necrosis factor-α using flow cytometric assays. Acta vet. scand. 2004, 45, 99-107. – A thorough understanding of the immune system, including the role of different cytokines, during inflammatory diseases in ruminants could lead to the development of new diagnostic methods and treatments. Tumour necrosis factor-α (TNF-α) is an important cytokine in the onset of the inflammatory responses. Unfortunately, the number of studies on cytokines, like TNF-α, in ruminants is limited due to a lack of species-specific reagents. As cytokines have remained rather conserved during evolution, cross-reactivity between animal species may occur. Therefore, the aim of the present study was to investigate 5 commercially available antibodies against human TNF-α for their ability to cross-react with ovine and/or bovine TNF-α, using a bead-based flow cytometric method. Two of the antibody clones (Mab 11 and 6401.1111) showed cross reactivity with ovine recombinant TNF-α in concentrations above 2.5ng/ml. However, none of the antibodies detected TNF-α in bovine milk, or serum containing known concentrations of bovine TNF-α, as earlier determined with ELISA. The results could be due to inability of the antibodies to cross-react between species, but quenching of the signal by matrix proteins might also have lowered the response.

cross-reactivity; particle based flow cytometry; pro-inflammatory cytokines; ruminants; bovine- ovine- human TNF-α.

Introduction

The inflammatory response during bacterial infections in ruminants is mediated by pro-inflammatory cytokines, such as tumour necrosis factor-a (TNF-a) (*Sordillo & Peel* 1992). TNF-α has an important role as a mediator of the inflammatory response as it induces expression of adhesion molecules on endothelial cells, activates neutrophils and macrophages, and induces production of nitric oxide and complement factors (*Tracey* 2002, *Rainard et al.* 2000). Quantitation of cytokines in biological fluids could be a useful tool in the diagnosis of inflammatory conditions since cytokine patterns give detailed information about the course of an inflammation. Infectious diseases, like mastitis, represent great health problems among dairy cows and sheep. A thorough understanding of how the immune system acts during such diseases could lead to the development of new diagnostic methods and/or treat-

ments. Therefore, the development of new techniques for measuring ruminant cytokines is warranted.

Several methods have been developed for cytokine detection, such as enzyme-linked immunosorbent assay (ELISA), enzyme-linked immuno spot assay (ELISPOT), bioassays, polymerase chain reaction (PCR) and intracellular staining (*Bienvenu et al.* 2000). Recently, multiplex cytokine assays have been developed, which measure several cytokines simultaneously in the same sample. In a study by *Carson & Vignali* (1999), 15 human cytokines were measured simultaneously in the same sample with a multiplex particle based flow cytometric method, the LabMAP™-assay (Luminex Corporation, Austin, TX, USA). Simultaneous quantification of several substances in a sample makes it possible to calculate the cytokine ratio. An altered cytokine ratio in an individual is considered a marker of disease state such as asthma and atopy (*Maggi* 1998).

Studies on cytokines in ruminants are limited by a lack of species-specific reagents. However, as cytokines have remained conserved during evolution, cross-reactivity between animal species may occur. In support for this contention, *Pedersen et al*. (2002) found that an antibody against ovine TNF-α cross-reacted with bovine and human TNF-α. To our knowledge, no studies on cross-reactivity between antibodies to human TNF-α and ovine and bovine cytokines have been published. Therefore, the aim of this study was to investigate the ability of a number of commercially available antibodies to human TNF-α to cross-react with ovine and/or bovine TNF-α using particle based flow cytometry. The assays were performed using two different procedures. Procedure one is called the LabMAP™-assay, where both coupling of antibodies to microspheres and detection assays were performed. The other is called the Fluorokine® MAP-assay (R&D Systems, Minneapolis, MN, USA), where microspheres already coupled with antibodies are used.

Materials and methods

Antibodies, recombinant proteins and samples

The antibodies to human TNF-α used were all commercially available (Table 1). Two clones of recombinant human TNF-α (rhuTNF-α) were used. One was purchased from Serotec Ltd (Oxford, England) and the other was included in the Fluorokine® MAP kit (R&D Systems). Recombinant ovine TNF-α (rovTNF-α) used was a kind gift from Dr. I. G. Colditz (CSIRO, Armidale, Australia).

Recombinant bovine TNF-a is not commercially available. Therefore, bovine serum and milk samples with known concentrations of TNF-α were used as positive controls in this study. Three bovine serum samples containing 14000, 15400 and 15600 pg TNF-α/ml, respectively, were used as positive controls. The samples were a kind gift from Dr. C. Røntved, Danish Institute of Agricultural Sciences, Tjele, Denmark, who earlier had measured the concentration of bovine TNF-α in the serum sam-

Table 1. List of anti-human TNF-a antibodies used in this study.

Capture antibodies	Company	Reporter antibodies
Mab1	BD Pharmingen (San Diego, CA, USA)	Mab11
Mab11	BD Pharmingen	6401.1111
6401.1111	BD Biosciences (San Diego, CA, USA)	Mab1
B-C7	Serotec (Oxford, England)	Mab11 or 6401.1111
Polyclonal	R&D Systems (Minneapolis, MN, USA)	Mab 11 or 6401.1111

ples using ELISA. The serum samples originated from blood collected from 3 Holstein-Friesian cows that were experimentally infected with different doses of *Escherichia coli* endotoxin lipolysaccharide (LPS) *in vivo*. The doses used were: 0.25, 1 or 2 μg LPS/kg bodyweight. Serum samples were maintained at -70 °C for 6 months before use.

Four bovine milk samples containing 2300, 3250, 13000 and 52000 pg TNF-α/ml, respectively, were used as positive controls. Concentrations of TNF-α in the samples were earlier determined with ELISA by Dr Bruckmaier, Agrar TU, Munich, Germany. The milk samples were collected from 4 cows where acute mastitis was experimentally induced with the introduction of *Staphylococcus aureus* into the udder (*Grönlund et al.* 2003). Milk samples were maintained at -70 °C for one year before use.

Two bovine serum samples and 2 bovine milk samples containing less than 100 pg/ml of TNF-α, originating from the same studies as the positive controls, were used as negative controls in this study. In experiments using rhuTNF-α and rovTNF-α, the buffer used was phosphate buffered saline (PBS) which also was used as a negative control.

Reagents

Microspheres were purchased from Luminex Corporation (Austin, TX, USA), human phycoerythrin (PE) -conjugated streptavidin from Dako A/S (Copenhagen, Denmark), N-hydroxysulfosuccinimide (Sulpho-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) from Pierce Biotechnology Inc. (Rockford, IL, USA), phosphate buffered saline (PBS) without $Ca^{2+}$and Mg^{2+} from the National Veterinary Institute (SVA) (Uppsala, Sweden), Tween 20 from Merck-Schuchardt (Hohenbrunn, Germany) and Bovine Serum Albumin (BSA) and morpholinoethanesulfonic acid (MES) from Sigma Chemicals (St. Louis, MS, USA). Finally, Fluorokine® MultiAnalyte Profiling Kit (Fluorokine® MAP) was obtained from R&D Systems (Minneapolis, MN, USA). The Luminex 100™ and Luminex XYP™ platform were purchased from Luminex Corporation.

Method optimization

Preliminary studies for the LabMAP™-assay were performed to determine the optimal detection system, which involved the assessement of different concentrations of capture and reporter antibodies, incubation times and temperatures. Two detection systems were tested, one direct method where the reporter antibody was PE-conjugated, and one indirect method where a biotinylated reporter antibody was detected by PE-conjugated streptavidin. In assays on rovTNF-α, best results were achieved by indirect detection, whereas the best detection system for rhuTNF-α was the direct detection system. Seven different concentrations of the different capture antibodies (5mg/ml, 0.5mg/ml, 50μg/ml, 5μg/ml, 100ng/ml, 50ng/ml and 10ng/ml) and reporter antibodies (3.2μg/ml, 1.6μg/ml, 800ng/ml, 400ng/ml, 320ng/ml, 200ng/ml, and 100ng/ml) were tested. Varying incubation times and temperatures were used for the incubation steps with antibodies (20 minutes at room temperature (RT) and 37 °C, 45 min at RT, 1 h at RT, 1.5 h at RT, 2 h at RT and 37 °C and overnight at RT). The best procedures for rhuTNF-α and rovTNF-α, with respect to signal intensity and background, are described below.

Coupling of monoclonal antibodies to microspheres

Coupling of antibodies to Luminex microspheres was performed in 1.7 ml eppendorf tubes (AB Göteborgs Termometerfabrik, Västra Frölunda, Sweden) according to the manu-

facturer's instructions (Luminex 100 System Training Material). Briefly, about 600000 microspheres were dissolved and washed twice with 100µl activation buffer (0.1 M sodium phosphate buffer, pH 6.2) before resuspension in 80µl activation buffer. Ten µl each of freshly prepared 50 mg/ml sulfo-NHS and 50 mg/ml EDC solutions were added to the suspension. The microsphere solution was incubated for 20 min in the dark at RT. The activated microspheres were washed twice with 100µl coupling buffer (0.1M MES diluted in dH_2O) before 500µl of TNF-α-antibody (0.5mg/ml) was added and the mixture was rotated for 2 h in the dark at RT (Fischer Hematology Mixer, Fisher Scientific International Inc., Hampton, NH, USA). After incubation the microspheres were washed twice with washing buffer (PBS with 0.05% Tween) and resuspended in PBS with 0.02% Tween (PBST) and 1mg/ml bovine serum albumin. The microspheres with attached antibodies were kept at 2-8°C, protected from light for at the longest for 14 days before use.

Cytokine assay using LabMAP™

RhuTNF-α (Serotec Ltd) or rovTNF-α was diluted in PBST to create appropriate standard curves. Bovine serum and milk samples were diluted 1:4 in PBST. Ninety ml cytokine solution, bovine serum or milk sample, was mixed with 3µl antibody-coupled microspheres, containing approximately 10 000 beads, and incubated for 20 min in the dark at RT. The mixture was subsequently washed twice with PBST. Forty ml biotinylated (5pg/ml) reporter antibody was added to rhuTNF-α samples whereas a PE-conjugated (0.3pg/ml) reporter antibody was added to the rovTNF-α samples. To milk and serum samples, either biotinylated reporter antibodies or PE-conjugated antibodies were added. All samples were incubated for 30 min in the dark at room temperature. After the incubation, rovTNF-α, milk and serum samples with PE-conjugated antibodies were washed twice with PBST and resuspended in 300ml PBS. Thereafter, they were transferred to 96-well microtiter plates (Göteborgs Termometerfabrik) and analysed on the Luminex 100™ analyser (Luminex Corp.). The rhuTNF-α, milk and serum samples with biotinylated reporter antibodies were washed twice with 100µl PBST. Then 40ml streptavidin-PE, diluted 1:100 in PBST, was added and the samples were incubated for 20 min protected from light at RT. Samples were then washed twice with PBST, resuspended in 300µl PBS and analysed on the Luminex 100™ analyser. All experiments were performed in duplicates or triplicates and repeated at least 2 times.

Cytokine assay using Fluorokine® MAP

The cytokine assay was performed according to manufacturer's instructions (R&D, Minneapolis, MN, USA). Briefly, RhuTNF-α (Serotec Ltd and R&D Systems) or rovTNF-α was diluted in PBST to appropriate standard curves. Bovine serum and milk samples were diluted 1:4 in PBST. A recovery experiment was conducted to verify if the assay could measure cytokines added to the milk or serum matrix. Duplicates of each sample were prepared and to one of the duplicates 50 µl of 2ng/ml rhuTNF-α was added. Fifty ml microsphere mixture was added to 50µl standard, or sample, and was incubated in the dark at RT on rotation (Fischer Hematology Mixer, Fisher Scientific International Inc.) for 2 h. The microspheres were washed 3 times with 100µl washing buffer after which 100µl of conjugate cocktail was added and the solution was incubated for another 2 h. After incubation, the microspheres were washed 3 times with washing buffer, resuspended in 100µl washing buffer and analysed in the Luminex 100™ analyser. All experiments were performed in duplicates or triplicates and repeated at least 2 times.

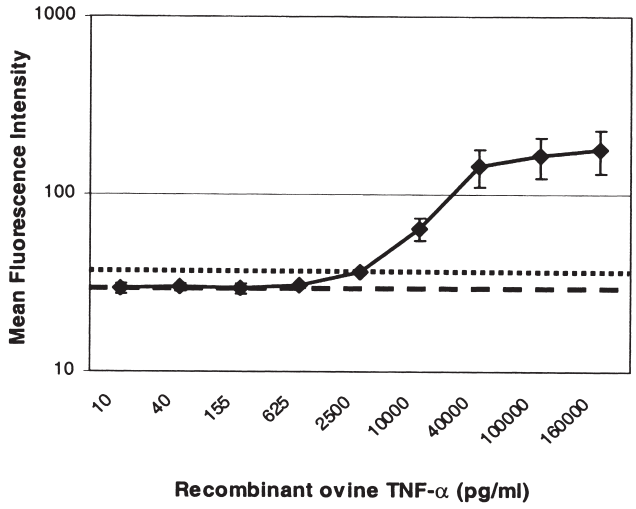

Figure 1. Standard curve of recombinant ovine TNF-α (-◆-) analysed with the LabMAP™-assay, using the antibodies to human TNF-a named Mab11 and 6401.1111. The curve is presented as mean fluorescence intensity versus concentration of standard. Results represent the mean (SD) of 4 standards performed in duplicate in 2 different experiments. The limit of detection for the assay is shown as a dotted line and was defined as the concentration corresponding to the signal 3 SD above the mean fluorescence of 6 samples without TNF-α. The mean fluorescence of the samples without TNF-α is shown as a dashed line.

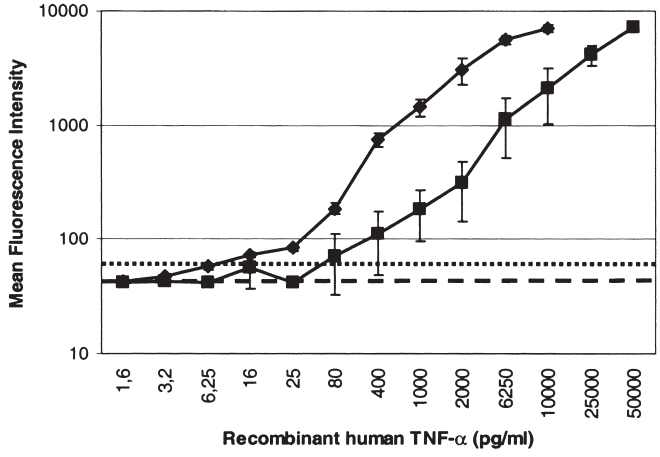

Figure 2. Standard curves of 2 different recombinant human TNF-α analysed with the Fluorokine® MAP-kit, TNF-α from Serotec Ltd (-■-) and TNF-α from R&D Systems (-◆-). Curves are presented as mean fluorescence intensity versus concentration of standards. Results represent the mean (SD) of 4 standards performed in duplicate in 2 different experiments. The limit of detection for the assay is shown as a dotted line and was defined as the concentration corresponding to the signal 3 SD above the mean fluorescence of 4 samples without TNF-α. The mean fluorescence of the samples without TNF-α is shown as a dashed line.

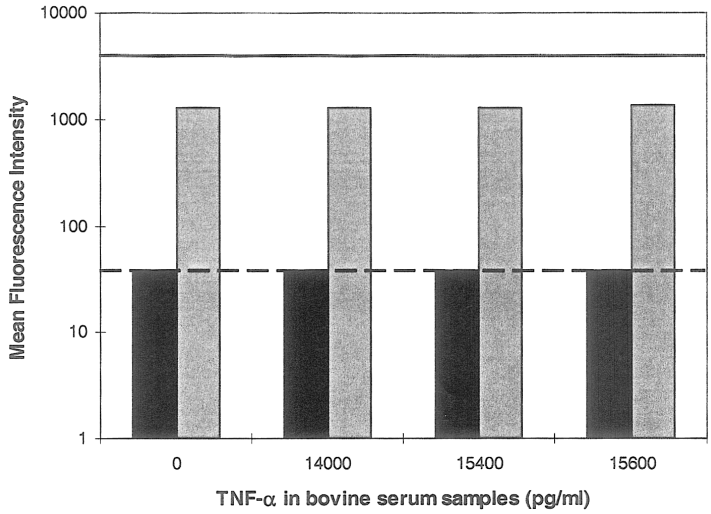

Figure 3. Mean fluorescence intensity from 3 bovine serum samples containing 14000, 15400 and 15600 pg TNF-α/ml, respectively, and 2 pooled serum samples not containing any TNF-α (0 pg/ml), as previously determined with ELISA. Concentrations of bovine TNF-α in the samples were determined with Fluorokine® MAP-kit, before (black bar) and after (grey bar) addition of 50 µl 2ng/ml recombinant human TNF-α. The dashed line marks the fluorescence of microspheres incubated with buffer only, whereas the solid line marks the fluorescence of microspheres incubated with buffer containing 50µl 2ng/ml recombinant human TNF-α.

Results

Cytokine assay using LabMAP™

Results from the cytokine assay by Luminex showed that all antibodies investigated detected rhuTNF-α (data not shown). In addition, the antibody combination Mab11 and 6401.1111 showed cross-reactivity with rovTNF-α when the cytokine concentration was above 2.5ng/ml (Fig. 1). However, none of the 7 different antibody combinations tested detected TNF-α in the bovine serum or milk samples (data not shown).

Cytokine assay using Fluorokine® MAP

The limit of detection of the Fluorokine® MAP-kit for rhuTNF-a was approximately 4pg/ml (Fig. 2). The kit detected both rhuTNF-α from R&D Systems, and rhuTNF-α from Serotec Ltd. The kit did not detect rovTNF-α (data not shown) nor bovine TNF-α in the serum or milk samples (Figs. 3 and 4). The recovery test showed that the kit detected rhuTNF-α when 50µl of 2ng/ml rhuTNF-α was added to the milk or serum samples (Figs. 3 and 4). The magnitude of the signals was however weaker in milk and serum compared with values achieved when measuring the same amount of rhuTNF-α in buffer. The mean percent recoveries in serum and milk, based on results from duplicate samples from two separate experiments, were 36.4 ± 1.6% and 59.8 ± 2.0%, respectively. When calculating the results, the mean fluorescence intensity from the experiments with rhuTNF-a in buffer was set to 100%. Thereafter, the mean fluorescence intensity from the experiments with rhuTNF-α serum and milk were compared to the experiments in buffer and the results are shown as percent of

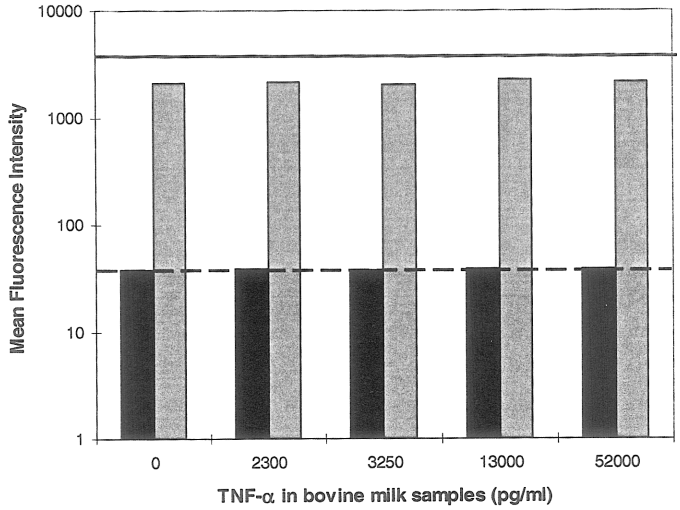

Figure 4. Mean fluorescence intensity from bovine milk samples containing 2300, 3250, 13000 and 52000 pg TNF-α/ml respectively and 2 pooled milk samples not containing any TNF-α (0 pg/ml), as previously determined with ELISA. Concentrations of bovine TNF-α were determined with Fluorokine® MAP-kit, before (black bar) and after (grey bar) addition of 50 µl 2ng/ml recombinant human TNF-α. The dashed line marks the fluorescence of microspheres incubated with buffer only, whereas the solid line marks the fluorescence of microspheres incubated with buffer containing 50 µl 2ng/ml recombinant human TNF-α.

rhuTNF-α in serum/milk ± standard deviation.

Discussion

All antibodies to human TNF-α investigated with the LabMAP™-assay in this study could detect rhuTNF-α. Those results indicate that the technique worked satisfactory. The antibodies against human TNF-α named Mab11 and 6401.1111 in combination also detected rovTNF-α in concentrations above 2.5ng/ml. However, this detection level is not satisfactory given that cytokines are very powerful signal molecules that give effects at low concentrations (*Kelso* 1998).

None of the antibodies against human TNF-α tested with the LabMAP™-assay could detect TNF-α in bovine serum or milk samples with known TNF-α concentrations. These results could be explained by the inability of antibodies to cross-react between species, or that the fluorescent signal was somehow quenched. Results of *Pedersen et al.* (2002) suggest that the TNF-α protein has remained conserved during evolution since other studies have shown that at least 60% amino acid homology is required between proteins for cross-reactivity to occur (*Scheerlinck* 1999). An 84% homogeneity is found between human and bovine TNF-α while ovine and bovine TNF-α shows more than 90% homogeneity when performing a genetic sequence comparison (http://www.ncbi.nlm.nih.gov/BLAST/). This indicates that human, bovine and ovine TNF-a have a high degree of similarity. However, our results do not completely support that theory, as none of the human antibodies tested in this study detected TNF-α in bovine samples and only one pair of antibodies detected rovTNF-α using the

LabMAP™-assay.

Both rhuTNF-α used in this study could be detected with the Fluorokine® MAP kit, and concentrations of ≥4 pg/ml of rhuTNF-α could be quantified. This detection limit could be compared to the quite high concentration of 2.5ng/ml that was required to achieve a signal when measuring rovTNF-α with the Lab MAP™-assay. However, the Fluorokine® MAP kit could not detect any rovTNF-α or bovine TNF-a in the serum and milk samples.

The recovery experiment where 50µl 2ng/ml rhuTNF-α was added to bovine milk and serum showed that the Fluorokine® MAP kit detected rhuTNF-α to a lower degree when added to bovine milk, or serum samples compared with quantifications in buffer, indicating a moderate quenching effect of the milk and serum. Both milk and serum are complex matrices that contain proteins and cells that may quench the signal from a fluorescent reporter antibody and thereby disturb the cytokine analysis (*Mire-Sluis et. al.* 1995, *Selby* 1999). Such an effect will not preclude cytokine detection when a high protein concentration is measured, but hampers the analysis when the concentration of protein is low. This experiment needs to be repeated adding varying concentrations of cytokine to normal bovine serum and milk since recovery experiments can show diverging results depending on the amount of cytokine added (*Cook et. al.* 2001).

In conclusion, our results indicate poor cross-reactivity between ovine/bovine TNF-α and the antibodies against human TNF-α used in this study making these antibodies unsuitable for further use in ovine/bovine immunoassays. However, the LabMAP™- and Fluorokine® MAP-assays may be valuable tools in studies on the roles of ovine and bovine cytokines during different inflammatory reactions.

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Sammanfattning

Kommersiellt tillgängliga antikroppar mot human Tumour Necrosis Factor-α testade för korsreaktivitet med ovin och bovin Tumour Necrosis Factor-α med hjälp av flödescytometriska analyser.

En grundlig förståelse för immunsystemet inklusive betydelsen av olika cytokiner vid inflammationssjukdomar hos idisslare kan leda till utveckling av nya diagnostiska metoder och behandlingar. En viktig cytokin i den tidiga inflammationsreaktionen är tumour necrosis factor-α (TNF-α). Tyvärr begränsas ofta studier rörande cytokiner som TNF-α hos idisslare av brist på artspecifika reagenser. Eftersom cytokiner har visats vara konserverade genom evolutionen förekommer korsreaktivitet mellan arter. Målet med den här studien var därför att med hjälp av partikelbaserad flödescytometri undersöka om 5 kommersiellt tillgängliga antikroppar mot humant TNF-α kunde korsreagera med ovint och/eller bovint TNF-a. Två av antikroppsklonerna (Mab 11 och 6401.1111) korsreagerade med ovint rekombinant TNF-α vid koncentrationer över 2,5 ng/ml. Ingen av de undersökta antikropparna detekterade dock TNF-α i bovina serum- och mjölkprov med kända koncentrationer av TNF-α tidigare bestämda med ELISA-analys. Resultaten kan tyda på en oförmåga hos antikropparna att korsreagera mellan arter men matrixproteiner kan också ha minskat signalens magnitud.

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