Analysis of interferon-gamma producing cells during infections by Yersinia enterocolitica *O:9 and* Brucella abortus *in cattle*

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Brucella abortus,
Cattle,
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Yersinia enterocolitica O:9.

Summary
One of the major constraints in the diagnosis of animal brucellosis is the cross-reactivity that occurs between *Brucella* and *Yersinia* surface antigens. With the aim to find a method to distinguish *Brucella* from *Yersinia* infection, the expansion of interferon gamma producing (IFN-γ⁺) T cell subsets obtained from peripheral blood mononuclear cells (PBMC) isolated from cattle either infected by *Brucella abortus* or experimentally immunized with *Yersinia enterocolitica* O:9 were compared. The lymphocytes were analyzed by flow cytometry after PBMC were in vitro re-exposed to *Yersinia* or *Brucella* antigens. The results highlighted a statistically significant difference in the expansion of the CD4⁺ and CD8⁺ IFN-γ⁺T cells occurring when PBMC of animals immunized with *Yersinia* are *in vitro* exposed to *Y. enterocolitica* O:9 antigen but not to *Brucella* antigen. This method could thus be suggested in those cases where results obtained by serodiagnosis need to be further clarified.

Analisi dell'interferon gamma prodotto nelle cellule durante le infezioni da Yersinia enterocolitica *O:9 e* Brucella abortus *nei bovini*

Parole chiave
Brucella abortus,
Bovini,
Cellule IFN-γ⁺T,
Yersinia enterocolitica O:9.

Riassunto
Uno dei principali limiti nella diagnosi delle brucellosi animali è la cross-reattività che si verifica tra gli antigeni di superficie di *Brucella* e *Yersinia*. Con l'intento di cercare un metodo in grado di discriminare tra le infezioni causate dai due patogeni, nel presente lavoro è stata messa a confronto l'espansione di linfociti T in grado di produrre interferon gamma (IFN-γ⁺), ottenuti da cellule mononucleate di sangue periferico (PBMC) isolate da bovini con infezione da *Brucella abortus*, con quella indotta in animali sperimentalmente immunizzati con *Yersinia enterocolitica* O:9. I linfociti sono stati quindi analizzati mediante citofluorimetria dopo che le PBMC erano state riesposte *in vitro* ad antigeni di *Yersinia* o *Brucella*. I risultati ottenuti hanno evidenziato una differenza statisticamente significativa nell'espansione di linfociti T CD4⁺ e CD8⁺ / IFN-γ⁺ nei casi in cui le PBMC di animali immunizzati con *Yersinia* erano state esposte *in vitro* agli antigeni di *Y. enterocolitica* O:9, che invece non si verificava nel caso di esposizione ad antigeni di *Brucella*. Questo metodo, pertanto, potrebbe risultare di interesse per la conferma di casi sierologici con diagnosi dubbia e/o per l'esclusione di potenziali cross-reattività.

Introduction

Brucellosis is an infectious disease mainly affecting cattle, swine, goats, sheep and dogs caused by members of the genus *Brucella*. Brucellosis is the most common zoonotic infection worldwide (Pappas *et al.* 2006, Skendros *et al.* 2011), and the Mediterranean basin is an acknowledged endemic region of human brucellosis (Seimenis *et al.* 2006). The infection is transmitted to humans mostly by ruminants, through direct contact with infected materials or indirectly by ingestion of animal

products (milk and derivatives) or by inhalation (Olsen and Palmer 2014).

Infected cows can abort, once only after the exposure to the pathogen, usually at about 7 months of pregnancy; otherwise, newborn calves are very weak and usually die shortly after birth. *Brucella* colonization of the udder results in a severe drop in milk production, which may also present swelling and inflamed knees (hygromas) (Olsen and Palmer 2014), while bulls with brucellosis usually become sterile (Carvalho Neta *et al.* 2010). *Brucella* is able to survive in immune cells and persist in tissues of the reticuloendothelial system (De Jong *et al.* 2010), using several strategies of immune evasion. One of these includes the production of cyclic glucan molecules that impede the lipid raft-mediated vacuole maturation to interfere with the membrane transport systems (Skendros and Boura 2013). Moreover, LPS from *Brucella abortus* bears a non canonical lipid A active only at very high concentration, therefore even if the LPS is recognized by TLR4 receptors of phagocytes it does not induce early activation of macrophages and consequent production of pro-inflammatory cytokines (Barquero-Calvo *et al.* 2007). Brucellosis diagnosis in livestock is mainly based on serological methods; the Italian national eradication program in ruminants, based on test-and-slaughter policy, prescribes to perform the official diagnosis by two serological tests, Rose Bengal Test (RBT) and Complement Fixation Test (CFT) (O.M. 24-06-2015). Unfortunately, they both lack of specificity due to the use of the whole lipopolysaccharide fraction of *Brucella* cell envelope as antigen (Corbel 1985, Nielsen *et al.* 2004). In fact, the most relevant constraint of the serological diagnosis consists in the frequently observed cross-reactions after the animal exposure to other microorganisms which all share common features with *Brucella* polysaccharide 'O' chain, e.g. *Yersinia enterocolitica* O:9, *Salmonella enterica* serotype *typhimurium* and *Escherichia coli* O157 (Muñoz *et al.* 2005, Nielsen *et al.* 2004).

Despite the presence of a wide body of literature showing efforts to discover antigens that can be useful in discriminating between *Brucella* and other Gram negative bacteria infections (Denoel *et al.* 1997, Guzman *et al.* 2012, Ko *et al.* 2012), the lack of *Brucella* specific antigen still highlights the need to develop new diagnostic methods, in order to improve the efficacy of eradication strategies and to avoid un-necessary animal sacrifices.

The reaction of cattle immune system has been investigated in terms of Delayed Type Hypersensitivity, PBMC proliferative response and interferon gamma (IFN-γ) secretion by whole lymphocyte fraction in presence of *Brucella* antigens, detected by Enzyme Linked Immunosorbent Assay (ELISA) (Weynants *et al.* 1995). It has also been demonstrated that IFN-γ secreted by peripheral blood in response to a commercial *Brucella* antigen,[a] was not functional to distinguish between infection caused by *Brucella* or *Yersinia enterocolitica* O:9, representing the most important source of false positive reactions in the serological diagnosis of brucellosis in animals and humans (Kittelberger *et al.* 1997). The role of cell-mediated immunity in *Brucella* infection has been analyzed in murine models (Skyberg *et al.* 2011, Weynants *et al.* 1998). Protective immunity requires activated antigen-presenting cells (mainly macrophages and dendritic cells) as well as $CD4^+$, $CD8^+$ and $\gamma\delta^+$T lymphocytes activation. In particular, $CD4^+$IFN-γ^+ T cell subset was described as essential for the clearance of *Brucella* infection in murine model activating killing mechanisms in macrophages as well as other type of cells that are reservoir of replicating bacteria (Skendros and Boura 2013). *Brucella* may thus survive in host macrophages and dendritic cells evading adaptive immune mechanisms (Carvalho Neta *et al.* 2010, Kittelberger *et al.* 1997).

To our knowledge, the IFN-γ^- producing lymphoid subsets following initial *in vivo* and then *in vitro* re-exposure to *Brucella* and *Yersinia* antigens in cattle have not been identified yet. It was reported that $CD4^+$ IFN-γ^+ lymphocytes play a key role in containing *Brucella* infection in cattle (Skendros and Boura 2013). The novelty of our approach is represented by the effort to detect the IFN-γ^+T cell subsets during natural *Brucella* infection and experimental *Yersinia* immunization after *in vitro* re-exposure with specific antigens. This approach was designed to evaluate the potential diagnostic use of these parameters to discriminate between the two infections in cattle.

Materials and methods

Animal trial

A total number of 15 female cattle were enrolled for the study.

Blood samples were collected weekly by venipuncture in heparinized tubes and tubes with no anticoagulant from the groups of cattle. Sera were obtained after centrifugation and stored at - 20°C until used for diagnostic purposes.

In group 1, the animals were immunized with inactivated *Yersinia enterocolitica* O:9 (Charolaise breed, 6-8 months old). Group 2 (Holstein breed, 2-5 years old) consisted of cattle naturally infected with *B. abortus*. Group 3 (Charolaise breed, 1-3 years old) was the control group, including seronegative

animals obtained from farms officially free of Brucellosis.

Animal trial was accomplished following current European legislation (Directive 2010/63/UE and following Commission implementing Decisions) and the corresponding Italian law (D. Leg. 26, March 4th, 2014). The whole procedure was conducted according to the regulations of the Italian Health Ministry (Decreto Ministeriale No. 101/2006-A). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

As for Group 1, five cattle were immunized with 100 ml of heat inactivated (65 °C for 1.5 minutes) *Y. enterocolitica* O:9 (10^{12} Colony Forming Units – CFU – *per os*). A 10 ml dose containing 10^{12} CFU with aluminium hydroxide as adjuvant was also administered subcutaneously, in the neck region, weekly for two months. Antibodies against *Y. enterocolitica* O:9 were measured weekly for each animal by a specific CFT procedure, using a commercial antigen,[b] as recommended by the manufacturer. Sera showing at least 75% of fixation at 1:10 dilution were considered positive. Furthermore, the same serum samples from each animal were tested by RBT and CFT specific for brucellosis before the experimental procedure, resulting all negative.

As for Group 2, we collected samples from naturally infected cattle resulted positive to the official serological tests. Sera showing agglutination and/or at least 50% of fixation at 1:4 (20 IU/ml) dilution were diagnosed as positive, as prescribed by the National legislation (O.M. 24-06-2015). They were also tested for *Y. enterocolitica* O:9 isolation from faeces and resulted all negative.

PBMC cultivation

PBMC were isolated from heparinized blood samples of each experimental group as described elsewhere (La Manna *et al.* 2011). Then, 5 x 10^5 PBMC from each sample were cultured for 48 h at 37 °C-5% CO_2 with two different stimuli: YeO:9[b] (10 µg/ml) and *B. abortus*[a] (10 µg/ml), in a final volume of 0.2 ml/well of complete RPMI[c] in 96 well U-bottomed microplates[d]. The concentration of stimuli added to the wells was chosen following a dose-response curve assay for the best antigen-specific expansion of T cell subsets (data not shown).

After 48 h of culture, PBMC were collected and stained with Fluorescein Isothiocyanate (FITC) labelled monoclonal antibodies (mAb) -anti CD8 (clone CC63, mouse anti cow IgG2a),[e] -anti CD4 (clone 44.38, mouse anti cow IgG2a)[e] and -WC1 (clone 19.19, mouse anti sheep – cross reactive with cow- IgG1a)[e]. After 30 minutes of incubation at 4 °C, cells were washed three times, fixed and permeabilized using a specific commercial reagent[f] and then incubated with Phicoeritrina (PE) labelled anti IFN-γ mAb (clone CC302, mouse anti cow IgG1)[e] for 30 minutes at 4 °C, washed three times and resuspended in 500 µl of Phosphate Buffer Solution.

IFN-γ measurement and data analysis

Stained PBMC were acquired by flow cytometry analysis using a cytometer[g] and a commercial software.[g] Each acquisition was performed collecting 10,000 events of lymphocytes region gated using Forward scatter (FSC) and Side Scatter (SSC) parameters.

Acquired cells were analyzed in order to detect the subset of IFN-γ producing cells; the percentage of IFN-γ+ cells detected in cultures with RPMI only (negative control) was subtracted from the same measurement observed in cells cultured with antigens. Statistical analysis of data was performed by Mann Whitney test, for any significant difference among the average percentages of a three times-repeated sample for each experimental group.

In order to evaluate a parameter able to discriminate between animals immunized with *Y. enterocolitica* and naturally infected with *B. abortus*, the ratio between the percentages of IFN-γ+ cells in response to *Brucella* and *Yersinia* antigens was also analyzed. The confidence value was fixed at 0.05 unless otherwise specified.

Results

IFN-γ+ cells expansion in response to *Yersinia*

Results showed that in animals immunized with *Y. enterocolitica* an expansion of IFN-γ+ lymphocytes was detected following the re-exposure to Ye O:9 (2.36% in immunized versus 0.36% in uninfected controls) but not to *Brucella* antigen (0.21%) ($p < 0.05$) (Figure 1A). There was a statistically significant difference in the expansion of CD4+ IFN-γ+T cells when PBMC from animals immunized with *Yersinia* were exposed to Ye O:9, but not to *Brucella* antigen (1.26% vs 0.14%) (Figure 1B). The percentage of CD8+ IFN-γ+T cells was 0.93% in *Yersinia*-immunized animals versus 0.08% in seronegative controls. Taking into account that γδT-lymphocytes are represented in a consistent amount in cattle in an age-dependent manner (Guzman *et al.* 2012) and are relevant in murine as well as in bovine immune response to *Brucella* (Skyberg *et al.* 2011), the role of γδ+ IFN-γ+ T cells was also analyzed (Figure 1D). Results show that these cells display a minor contribution to IFN-γ production in tested animals. The comparison of γδ+ IFN-γ+ T cells *in vitro* expansion was statistically different when PBMC of

Yersinia treated animals were exposed to *Yersinia* and *Brucella* antigen ($p < 0.05$).

IFN-γ+ cells expansion in response to *Brucella*

The results obtained in cattle naturally infected by *Brucella* (Figure 2) showed a statistically significant increase of IFN-γ+ lymphocytes when the cells were stimulated both by *Brucella* and *Yersinia* antigens in comparison with the control group ($p < 0.05$) (Figure 2A). Data showed that in the same animals IFN-γ+ lymphocytes are similarly represented when *in vitro* exposed to *Yersinia* (5.99%) or *Brucella* (5.70%). The analysis of T-cell subsets showed that CD4+ T cells produce a similar percentage of IFN-γ+ cells in response to both antigens, thus confirming cross-reactivity in cell-mediated immunity (Figure 2B). *Brucella abortus*-infected samples showed a higher percentage of IFN-γ+ T lymphocytes compared to controls, with statistically significant differences. IFN-γ+ PBMC and CD4+ IFN-γ+ in *Brucella abortus*-infected samples were also higher than in *Yersinia*-infected animals, maybe due to natural infections with other cross-reactive Gram-negative bacteria (Kittelberger *et al.* 1997).

Statistical analysis

In details, CD4+ IFN-γ+ T-cell subset expanded similarly in response to *Yersinia* (2.96% for cattle positive for *Brucella abortus* and *in vitro* stimulated with *Yersinia*

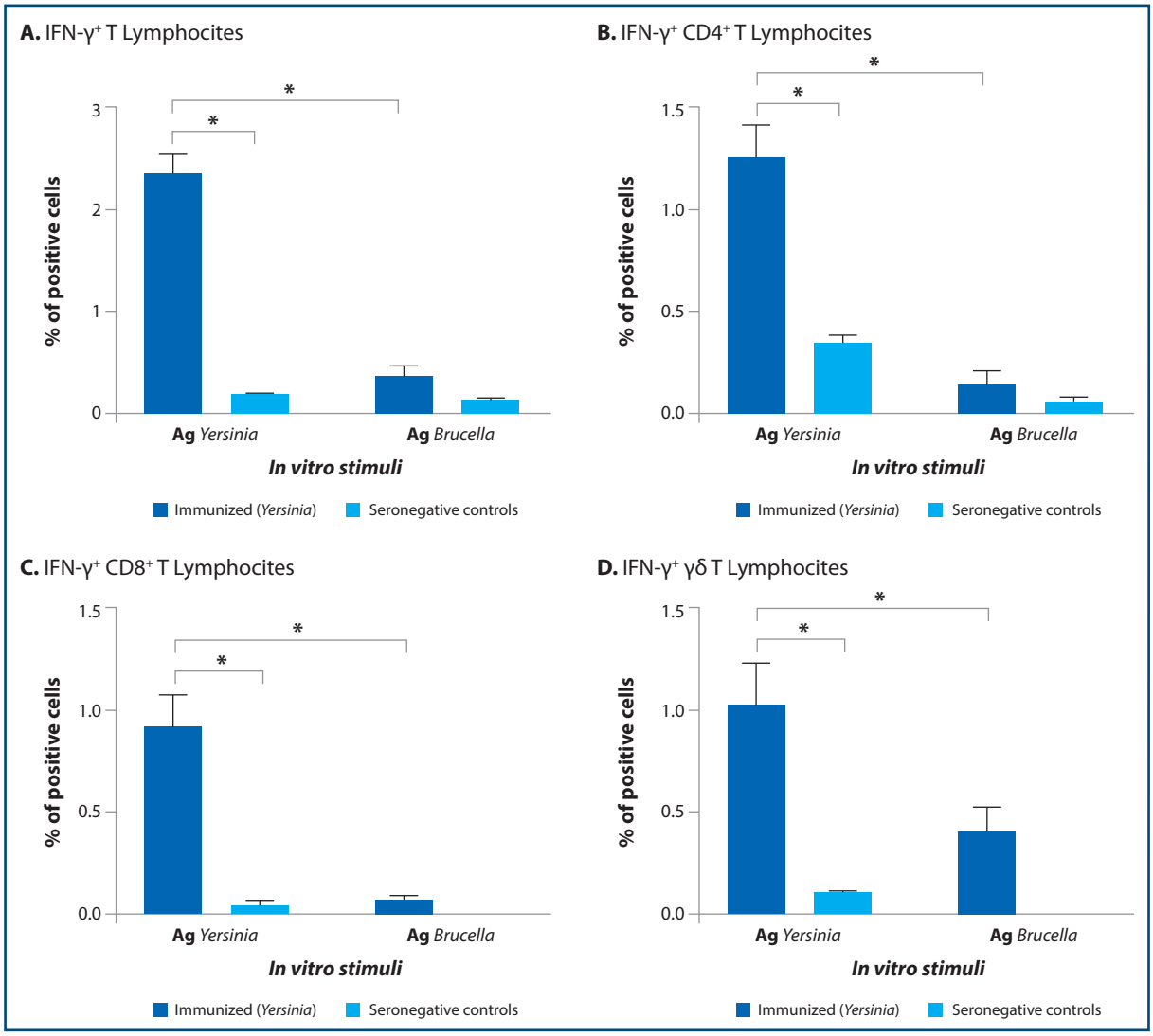

Figure 1. *Difference of IFN-γ production between cells stimulated with the two antigens in* Y. enterocolitica *immunized animals.* **A.** In *Yersinia*-immunized animals the antigen-specific stimulation resulted in a statistically significant difference of IFN-γ+ T-lymphocytes with respect to the seronegative controls in all the cellular subpopulation analyzed. In these animals, also a higher percentage of IFN-γ+ T-lymphocytes was detected, when compared with the same cells stimulated with *Brucella* antigen. **B.** Detection of CD4+ IFN- γ+ T-lymphocytes. **C.** Detection of CD8+ IFN-γ+ T-lymphocytes confirmed a similar contribute of these two subsets to total IFN-γ+ T-lymphocytes in the two experimental groups, with a slightly higher CD4+ T cells detection. **D.** Detection of γδ+ IFN-γ+ T cells amounted to 0.1% in response to YeO:9 and to 0.04% in seronegative animals. *$p < 0.05$.

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antigen) and *Brucella* (2.77% *in vitro* stimulated with *Brucella* antigen). We did not observe any statistically significant difference when CD8⁺ cells were analyzed in response to the two antigens (Figure 2C). Data regarding the analysis of γδT cells revealed that the difference of IFN-γ production was not statistically significant between animals positive for *Brucella* and *in vitro* stimulated with both antigens, while a statistically significant difference was detected when PBMC from infected and seronegative cattle were exposed to *Yersinia* (Figure 2D).

The Mann-Whitney test has been performed to study the difference in the ratio of IFN-γ⁺ lymphocytes stimulated with *Yersinia* over those stimulated with *Brucella* antigen, between the animals immunized with *Yersinia* versus those infected with *Brucella*. Our results showed that this difference is statistically significant ($p < 0.05$). Following the same approach, we also compared the ratios: between CD4⁺ IFN-γ⁺ T cells and CD8⁺ IFN-γ⁺ T cells within the same groups. When the percentages of CD4⁺IFN-γ⁺ T cells are analyzed ($p < 0.05$), the difference was statistically significant, whereas it was not for CD8⁺ IFN-γ⁺ T cells.

Conclusions

Previous data related to antigen-specific IFN-γ production measured by ELISA in *Y. enterocolitica*-experimentally infected cattle re-exposed to *Brucellergene*[a] are available (Kittelberger *et al.* 1997), but in the present work, for the first time, the production of antigen-specific CD4⁺- CD8⁺- and γδ- IFN-γ⁺ T lymphocytes were assessed by flow cytometry in animals naturally infected with *Brucella*

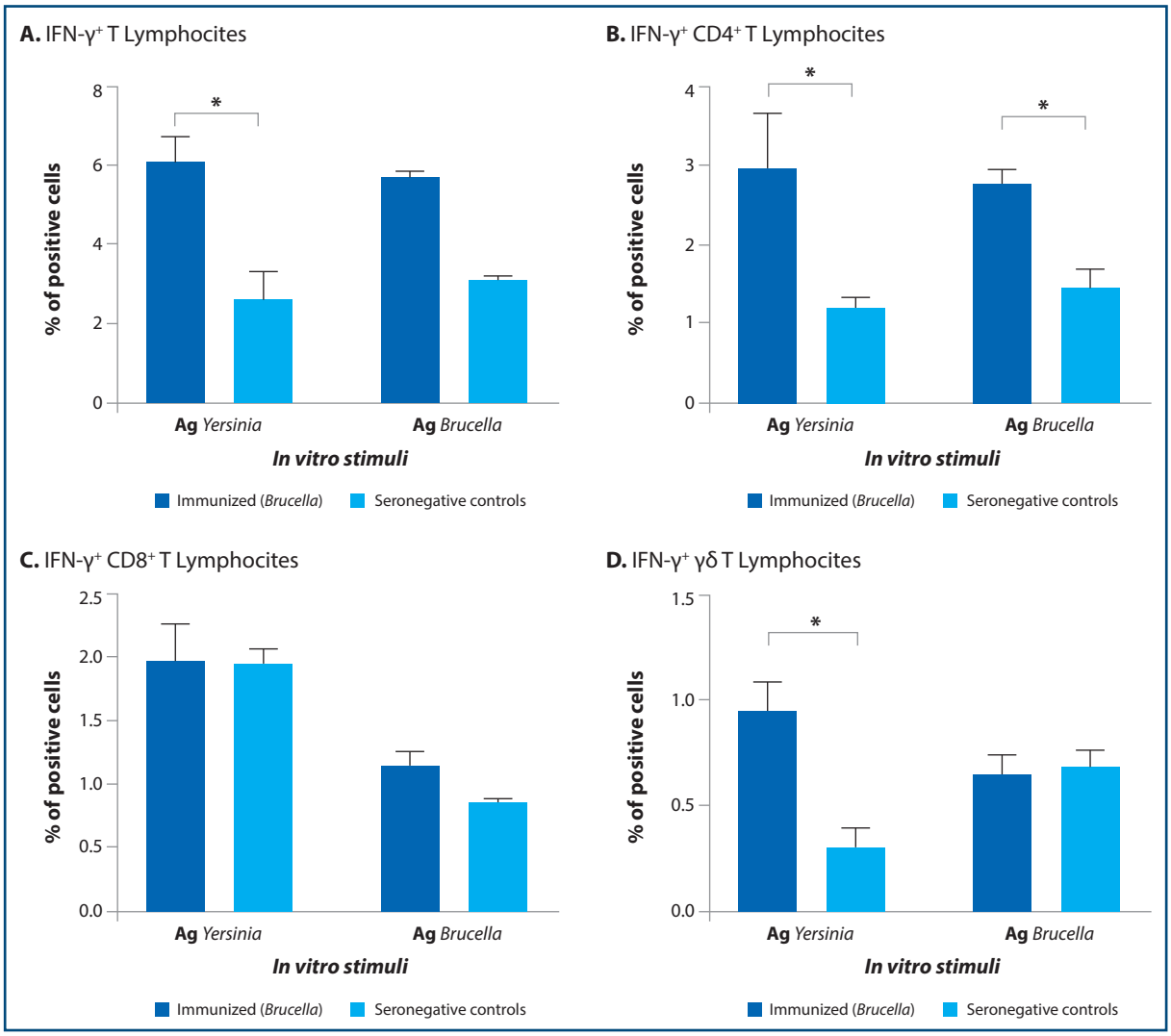

Figure 2. *Difference of IFN-γ production between cells stimulated with the two antigens in* Brucella *infected animals.* **A.** In cattle naturally infected by *Brucella* IFN-γ⁺ T-cells detection is similar when cells are *in vitro* exposed to *Yersinia* or *Brucella* antigens. **B.** CD4⁺ T cells produce IFN-γ when exposed to both antigens. Statistical significances were assessed and percentages of IFN-γ⁺ and CD4⁺ IFN-γ⁺ T cells were compared to seronegative controls. **C.** No statistically significant expansion of CD8⁺ IFN-γ⁺ T cellswas observed in response to both the antigens. **D.** No statistically significant expansion of γδ⁺ IFN-γ⁺ T cellswas observed in response to both the antigens. *$p < 0.05$.

and experimentally immunized with *Yersinia* after *in vitro* re-exposure to the specific antigens. Our data show that in animals immunized with *Yersinia* there is an antigen-specific expansion of $CD4^+$ and $CD8^+$ T lymphocytes without any appreciable cross-reactivity, at least with *Brucella*. In cattle naturally infected with cross-reactivity between the *in vitro Yersinia* and *Brucella* stimulated cells has been observed and $CD4^+$ T lymphocytes were the main producer of IFN-γ. The increase of $CD4^+$ IFN-γ^+ T cell subset was expected, since its crucial role has been already demonstrated in a murine model for the control of *Brucella* infection (Baldwin and Parent 2002, Vitry *et al.* 2012).

The γδ T cells do not play a key role in response to both antigens either *in vivo* or *in vitro*, being a minor IFN-γ^+ producing T cell subset when compared to $CD4^+$ and $CD8^+$ lymphocytes, as also reported by previous results (Vitry *et al.* 2012).

The work carried out during this study allowed the detection, for the first time in cattle, of lymphocyte populations, i.e. $CD4^+$ and $CD8^+$IFN-γ^+T cells, that expand differently when PBMC of animals immunized with *Yersinia* are *in vitro* exposed to *Yersinia* or to *Brucella*. Even if this scenario was not described viceversa, the analysis of the expansion of IFN-γ^+ cells in response to *Brucella* and *Y. enterocolitica* could be useful to sort out between *Yersinia* and *Brucella* infection in cattle in case of doubtful results with the official serological methods. Anyway, further studies will be necessary to assess the efficacy of this method also with animals naturally infected with *Yersinia*.

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Sources and manufacturers

a. Brucellergene, OCB® Zoetis, USA.
b. *Yersinia enterocolitica* O:9 YOP® - InstitutVirion/ Serion GmbH, Würzburg ,Germany.
c. Gibco Media of Fisher Scientific UK Ltd, Loughborough, UK.
d. Nunc A/S, Roskilde, Denmark.
e. AbDSerotec, Kidlington, UK.
f. FIX & PERM® Cell Fixation and Cell Permeabilization Kit – Thermofisher Scientific, Waltham, Massachusetts, USA.
g. FACScan and Cell Quest Pro, Becton Dickinson, New Jersey, USA.

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