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Analysis of antigen-specific immune response in *Brucella* infection

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1. INTRODUCTION

Brucellosis is an ancient and one of the world's most widespread zoonotic diseases affecting both, public health and animal production (Ariza *et al*., 2007) which is caused by a Gram-negative, facultative intracellular bacteria of the genus *Brucella*. Sicily falls within the Italian regions with the highest percentage of positive herds (Pappas *et al.,* 2006).

The bacteria are transmitted from animals to humans by ingestion through infected food products, direct contact with an infected animal, or inhalation of aerosols. The disease is an old one that has been known by various names, including Mediterranean fever, Malta fever, gastric remittent fever, and undulant fever. Humans are accidental hosts, but brucellosis continues to be a major public health concern worldwide and is the most common zoonotic infection (Pappas *et al.,* 2006)

The target organs and tissues of *Brucella spp*. are placenta, mammary glands, and epididymis in animal reservoir host (Adams, 2002; Xavier et al., 2009; Neta et al., 2010).

Since 1920, in addition to *B. melitensis,B. abortus* and *B. suis*, at least 7 new species have been identified as belonging to the *Brucella* genus with several additional new species under consideration for inclusion (Oslen and Palmer, 2014). Now, this genus consists of at least ten nomospecies having characteristic host preferences and zoonotic potential. Of these, the following 4 have moderate-to-significant human pathogenicity, considered as bioweapons, and are listed as category B priority pathogens by the US Center for Disease Control (CDC):

- *Brucella melitensis (from sheep; highest pathogenicity)*
- *Brucella suis* (from pigs; high pathogenicity)
- *Brucella abortus* (from cattle; moderate pathogenicity)
- *Brucella canis* (from dogs; moderate pathogenicity)

Because the organism is highly infectious, it can be readily aerosolized and outbreaks might be difficult to detect due to non-specific symptoms associated with infection (Doganay and Doganay, 2013). The era of post-genomic technology offers new and exciting opportunities to understand the complete biology of bacteria belonging to *Brucella* genus. Recently, the genome sequences of *B. melitensis, B. suis* and *B. abortus* became available. These genomes are very similar in sequence, organization, and structure to other ubiquitous Gram-Negative bacteria and few fragments are unique among the genomes. The sequencing and annotation of these fragments paved the way for a highly comprehensive and rapid analysis of *Brucella* proteome; also comparative genomics provide insights into aspects of *Brucella* virulence that were only suspected before.

1.1 MORPHOLOGY

Brucella are coccobacilli or short rods 0.6 to 1.5 µm long by 0.5 to 0.7 µm in width. They are arranged singly and less frequently in pairs or small groups. The morphology of *Brucella* is fairly constant except in old cultures, where pleomorphic forms may be evident. *Brucella* are non-motile. They do not form spores, flagella, or pili. True capsules are not produced. *Brucella* are Gram-negative and usually do not show bipolar staining. They are not truly acid-fast but resist decolouration by weak acids, thus stain red by the Stamp's modification of Ziehl-Neelsen method, which is sometimes used for the microscopic diagnosis of brucellosis from smears of solid or liquid specimens.

1.2 CULTURE AND GROWTH CHARACTERISTICS

Brucella members are aerobic, but some strains require an atmosphere containing 5-10% carbon dioxide (CO2) added for growth, especially on primary isolation. The optimum pH for growth varies from 6.6 to 7.4, and culture media should be adequately buffered near pH 6.8 for optimum growth. The optimum growth temperature is 36-38°C, but most strains can grow between 20°C and 40°C. *Brucella* require biotin, thiamin and nicotinamide. The growth is improved by serum or blood, but haemin (V-factor) and nicotinamide-adenine dinucleotide (X-factor) are not required. The growth of most *Brucella* strains is inhibited on media containing bile salts, tellurite or selenite. Growth is usually poor in liquid media unless culture is vigorously agitated. Growth in static liquid media favours dissociation of smooth-phase cultures to non-smooth forms. Continuous and vigorous aeration will prevent this, provided a neutral pH is maintained. In semisolid media, CO2-independent *Brucella* strains produce a uniform turbidity from the surface down to a depth of a few millimetres, while cultures of CO2-requiring strains produce a disk of growth a few millimetres below the surface of the medium. On suitable solid media *Brucella* colonies are visible after 2 days incubation. After 4 days incubation, *Brucella* colonies are round, 1-2 mm in diameter, with smooth (S) margins, translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker. Smooth *Brucella* cultures, especially *B. melitensis* cultures, have a tendency to undergo variation during growth, especially with subcultures, and dissociate to rough (R) forms, and sometimes mucoïd (M) forms. Colonies are then much less transparent with a more granular, dull surface (R) or a sticky glutinous texture (M), and range in colour from matt white to brown in reflected or transmitted light. Intermediate (I) forms between S, R and M forms may occur in cultures undergoing dissociation to the non-smooth state. Changes in the colonial morphology are generally associated with changes in virulence, serological properties and phage sensitivity.

1.3 ANTIGENIC CHARACTERISTICS

All smooth *Brucella* strains show complete cross-reaction with each other in agglutination tests with unabsorbed polyclonal antisera, a cross-reaction which does not extend to non-smooth variants. Cross-reactions between non-smooth strains can be demonstrated by agglutination tests with unabsorbed anti-R

sera. Lipopolysaccharide (LPS) comprise the major surface antigens of the corresponding colonial phase involved in agglutination. The S-LPS molecules carry the A and M antigens, which have different quantitative distribution among the smooth *Brucella* strains. This is of value in differentiating biovars of the major species using absorbed monospecific A and M antisera. Serological cross-reactions have been reported between smooth *Brucella*and various other Gram negative bacteria*, e.g. Escherichia coli* O:116 and O:157, *Salmonella* group N(O:30) of Kaufmann-White, *Pseudomonas multophilia*, *Vibrio cholerae* and especially *Yersinia enterocolitica O:9.* These organisms can induce significant levels of antibodies which cross-react with S-LPS Brucella antigens in diagnostic tests.

1.4 TAXONOMY OF *BRUCELLA* SPECIES AND BIOVARS

Considering their high degree of DNA homology (> 90 % for all species), Brucellae have been proposed as a monospecific genus in which all types should be regarded as biovars of *B. melitensis* (Verger *et al.,* 1985). Since this proposal has not yet met with complete agreement, the old classification of the genus (and relevant nomenclature) into six species, i.e. *B. melitensis, B. abortus, B. suis, B. neotomaeae, B. ovis* and *B. canis* (Corbel and Brinley Morgan, 1984), is the classification used world-wide. The first 4 species are normally observed in the smooth form, whereas *B. ovis* and *B. canis* have only been encountered in the rough form. Three biovars are recognised for *B. melitensis* (1-3), seven for *B. abortus* (1-6 and 9), and five for *B. suis* (1-5).

Species identification is routinely based on lysis by phages and on somesimple biochemical tests (oxidase, urease). For *B. melitensis, B. abortus* and *B. suis*, the identification at the biovar level is currently performed byfour main tests, i.e. carbon dioxide (CO_2) requirement, production of hydrogen sulphide (H_2S), dye (thionin and basic fuchsin) sensitivity, and agglutination with monospecific A and M anti-sera (see Table 1). Moreover, a recently developed co-agglutination test, using latex beads coated with a pair of monoclonal antibodies directed against the rough lipopolysaccharide(R-LPS) and the 25 kDa outer membrane protein (Omp 25), respectively(Bowden *et al.,* 1997), makes it possible to accurately differentiate *B. ovis* from *B. canis* and the occasional rough isolates of the smooth *Brucella* species *B. melitensis* biovar 3 appears to be the most frequently biovar isolated in Mediterranean countries. The precise recognition of biovar 3,especially its differentiation from biovar 2 appears sometimes equivocal. Due to the use of insufficiently discriminating monospecific sera, a number of strains identified initially as biovar 2 were later confirmed as biovar 3 by expert laboratories.

Intermediate strains are occasionally found due to the instability reported for some of the phenotypic characteristics used for the current classification of *Brucella*. This situation sometimes impedes the identification of the species and their biovars. Therefore, the identification of stable DNA-specific markers is considered a high priority for taxonomic, diagnostic and epidemiological purposes.

Species	Biovar or colonial morphology	CO_2 requirement	H_2S production	Growth on dyes (1)		Agglutination in			
				Thionin	Basic Fuchsin	polyclonal sera anti-		latex - mAb anti-	
						A	M	R-LPS	omp25
B. melitensis	1	-	-	+	+	-	+	-	-
	2	-	-	+	+	+	-	-	-
	3	-	-	+	+	+	+	-	-
	Rough	-	-	+	+	-	-	+	+
B. ovis	Rough	+	-	+	-	-	-	+	-
B. abortus	1	+(2)	+	-	+	+	-	-	-
	2	+(2)	+	-	-	+	-	-	-
	3	+(2)	+	+	+	+	-	-	-
	4	+(2)	+	-	+	-	+	-	-
	5	-	-	+	+	-	+	-	-
	6	-	-	+	+	+	-	-	-
	9	+ or -	+	+	+	-	+	-	-
	Rough	+ or -(3)	+ or -(3)	+ or -(3)	+ or -(3)	-	-	+	+

(1) dye concentration, 20µg/ml in Blood Agar Base medium with 5% of serum (1:50,000)
(2) usually positive on primary isolation
(3) + or -, according to the original smooth type

Table 1. Biovar differentiation of *Brucella* species involved in sheep and goat brucellosis (SANCO.C.2/AH/R23/2001)

Several methods, mainly PCR-RFLP and Southern blot analysis of variousgenes or loci, have been employed to find DNA polymorphism which wouldenable the molecular identification and typing of the *Brucella* species andtheir biovars (Allardet-Servent *et al.,*1988; Ficht *et al.,*1990, 1996; Halling and Zehr, 1990; Halling *et al.,*1993; Fekete *et al.,*1992b; Ouahrani-Bettache *et al.,* 1996; Vizcaino *et al.,* 1997). Among thesemethods, detection of polymorphism by PCR-RFLP is considered to have an advantage over Southern blotting, since it is easier to perform and is lesstime-consuming when applied to large numbers of samples.

2 THE DISEASE

Pathogenically, *B. melitensis* infection in sheep and goats is similar to *B. abortus* infection in cattle. Nevertheless, differences are significant, and each species of *Brucella* causes a different disease (OIE Manual, 1996). *Brucella* are facultative intracellular parasites of the reticulo endothelial system. The virulence of *Brucella* varies considerably according to species, strain and the size of infecting inoculum. Host susceptibility is also variable and is associated with the reproductive status. Thus, in the field, all intermediate stages between typical acute infection and complete resistance may be observed. In addition, vaccinal immunity may modify the parasite-host relationship.

Transmission within the hosts may occur via ingestion of *Brucella* contaminated feed or water or licking an infected placenta, calf or fetus, or the genitalia of an infected animal soon after it has aborted or gave birth (Alexander *et al.,* 1981; Godfroid *et al.,* 2004). As the bacterial concentrations in fetal fluids or placenta

after abortion can be as high as 10^9 to 10^{10} colony-forming units (CFUs)/g and minimum infectious doses are estimated in the 10^3 to 10^4 CFU range, abortion events can laterally transmit brucellosis to many cattle that have contact with birthing materials (Olsen and Tatum, 2010).Moreover, transmission within the natural hosts can occur through milk or via semen or genital secretions during mating. Zoonotic transmission occurs most frequently via unpasteurized milk products in urban settings, while occupational exposure of farmers, veterinarians, or laboratory workers can result from direct contact with infected animals or tissues or fluids associated with abortion (Olsen and Palmer, 2014). Only rare cases of vertical and horizontal (Wyatt, 2010) transmission between humans have been reported (Ruben *et al.*, 1991; Mantur *et al*., 1996; Çelebi *et al.,* 2007; Meltzer *et al.,* 2010) and humans are generally considered to be incidental, or dead-end hosts for *Brucella* species (Meltzer *et al.,* 2010). The spillover of brucellae from wildlife to domestic ruminants is also possible (Mick *et al.,* 2014).

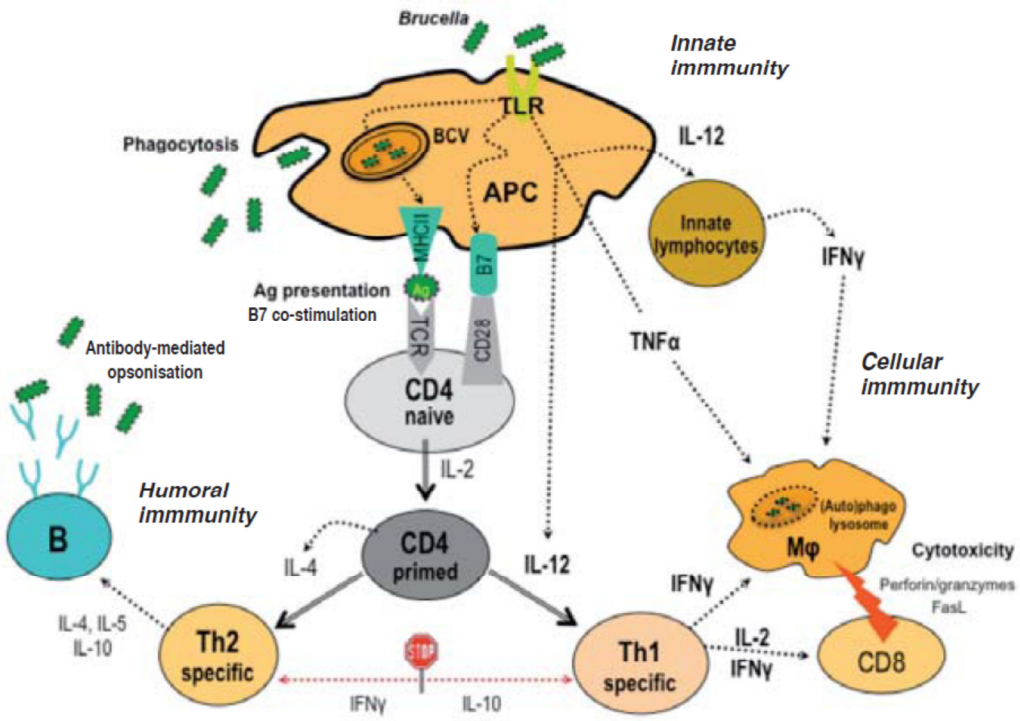
2.1 PHASES OF INFECTION

The infection in females follows a course very similar to *B. abortus* infection in cattle. The major route of infection appears to be through the mucous membranes of the oropharynx and upper respiratory tract or the conjunctiva. Other potential routes of infection are through the mucous membranes of the male or female genital tract. After gaining entrance to the body, the organisms encounter the cellular defences of the host, but generally succeed in arriving via the lymph channels at the nearest lymph node. The fate of invading bacteria is mainly determined by the cellular defences of the host, chiefly macrophages and T lymphocytes, though specific antibody undoubtedly plays a part. The outcome depends on the ruminant species infected, age, immune status of the host, pregnancy status, and the virulence and number of the invading *Brucella*. When the bacteria prevail over the body defences, a bacteraemia is generally established. This bacteraemia is detectable after 10 to 20 days and persists from 30 days to more than 2 months. If the animal is pregnant, bacteraemia often leads to the invasion of the uterus. At the same time, infection becomes established in various lymph nodes and organs, often in the udder and sometimes in the spleen. During this first stage of infection, the major clinical sign is abortion but other signs due to a localisation of *Brucella*may be observed (ie, orchitis, epididymitis, hygroma, arthritis, metritis, subclinical mastitis, etc.). However, numerous animals develop self-limiting infections or they become asymptomatic latent carriers and potential excretors. Abortion generally does not occur if the female becomes infected at the end of pregnancy. The second stage is characterised by either elimination of *Brucella* or, more frequently, by a persistent infection of mammary glands and supramammary and genital lymph nodes (Fensterbank, 1987) with constant or intermittent shedding of the organisms in the milk and genital secretions. Animals generally abort once, during the mid third of gestation, but reinvasion of the uterus occurs in subsequent pregnancies with shedding in 18 fluids and membranes. The pregnancy can also continue to full-term. The proportion of newly infected females that abort varies with the circumstances. The percentage of infected females lambing/kidding in a flock may reach 40%. Females that are born into an infected environment and subsequently infected, generally abort less than others. This explains the high level of abortions in newly

infected flocks and their relatively low frequency in flocks where infection is enzootic. The udder is a very important predilection site for *B. melitensis*. Infection in lactating non-pregnant goats is likely to lead to colonisation of the udder with excretion of *B. melitensis*in the milk (Renoux *et al.,* 1953). In goats, about two thirds of acute infections acquired naturally during pregnancy lead to infection of the udder and excretion of the bacteria in the milk during the subsequent lactation (Alton, 1985). In some goats excretion may cease during this lactation, but in many it persists and often continues during the next (Alton, 1962). Greatly reduced milk yield follows abortion, and infection of the udder following a normal birth also leads to a considerable reduction in yield. In spite of this, clinical signs of mastitis are seldom detectable in naturally infected goats (Alton, 1990). Sheep that abort often excrete the bacteria in the milk, but generally for not more than two months (Alton, 1990). However, exceptionally, excretion may continue for 140 days (Itabashi *et al.,*1938) and even 180 days (Biggi, 1956).

2.2. IMMUNE RESPONSE

Infection with *Brucella* usually results in the induction of both humoral and cell-mediated immune responses, but the magnitude and duration of these responses is affected by various factors including the virulence of the infecting strain, the size of infecting inoculum, pregnancy, sexual and immune status of the host (Joint FAO/WHO Expert Committee on Brucellosis, 1986) (Fig.1).



Ag: antigen
APC: antigen-presenting cell
B7: CD80/CD86 co-stimulatory molecules
BCV: *Brucella*-containing vacuole
CTL: cytotoxic lymphocyte
IFNγ: interferon gamma
IL: interleukin
MHC II: major histocompatibility complex type II
TCR: T-cell receptor
TLR: Toll-like receptor
TNFα: tumour necrosis factor alpha

Fig.1 **Simplified representation of immune response against *Brucella* (**Skendros, Boura, 2013*)* Phagocytosis and/or pattern recognition receptor (e.g. TLR) signalling lead to the activation of APC and the priming of naïve CD4+ T lymphocytes towards a Th1 phenotype (innate immunity). The Th1 cytokines (TNFα, IFNγ) enhance the anti-*Brucella* mechanisms of macrophages (Mφ) and induce the $CD8^{+}$CTL-mediated cytotoxicity against *Brucella*-infected Mφ (specifi c cellular immunity). Innate lymphocytes are early producers of IFN γ , linking innate to specific immunity. The Th2 response activates B lymphocytes (B) for antibody production, facilitating the phagocytosis of *Brucella* through opsonisation (specific humoral immunity). The Th2 cytokines (e.g. IL-10) inhibit the action of Th1 cytokines (e.g. IFNγ) and vice versa

2.3 HUMORAL IMMUNITY

Following infection by natural exposure, a serological response can be expected within 2 to 4 weeks, but the response is variable and may be absent altogether. Invasion of the pregnant uterus can be expected to produce a large and persistent rise of antibodies, but this may be delayed until after abortion or parturition at the normal time. Invasion of the lactating udder causes a lesser serological response, and localisation confined to a small number of lymph nodes may fail to stimulate any response at all, or only a minimal one. The pattern of the serological response in terms of immunoglobulin production has not been extensively studied in sheep and goats, but available information suggests close similarity to that in cattle, ie, production of IgM followed within a week or two by a predominance of IgG, with both isotypes falling to a low level in the more chronic stage of infection but with IgG predominating. The serological response is transient and sometimes missing in young sexually immature animals. As mentioned thereafter, the *B. melitensis* Rev.1 vaccine strain when applied under standard conditions (ie full dose via the subcutaneous route in young replacement animals) may induce a long lasting serological response to the agglutination test, that seriously interferes with serological screening for infected animals (Alton and Elberg, 1967; Elberg, 1981, 1996; Alton, 1990; MacMillan, 1990). As no differences have been found between the diagnostic antigens from field strains of *B. melitensis* and those from the Rev.1 vaccine, serological tests capable of distinguishing antibodies arising from infection and vaccination, respectively, have not been developed.

2.4 CELL MEDIATED IMMUNITY

Once the bacteria are phagocytosed by macrophages, DCs (Billard *et al.,*2007), and other antigen presenting cells (APCs),approximately 40–50% of the bacteria resist digestion within these cells. After initial encounter with *Brucella* antigens, APCs produce interleukin-1(IL-1),interleukin-6 (IL-6),tumor necrosis factor alpha (TNF-α), and gamma-interferon(IFN-γ) initiating innate immuneresponses (including natural killer cells) that may limit the initial spread of organisms. Infected APC in which organisms

residing with inunactivated phagolysosomes are likely to present some subset of peptidic *Brucella* antigens to CD4+ and CD8+ cells, and thus inducing a TH1 response associated with IFN-γ release. The functional consequences of antigen-specific IFN-γ release is unclear but does not lead to elimination of organisms during active, symptomatic infection and likely results in clinical symptomatology (i.e., fever, sweating, weightloss). Clonal T cell expansion is initiated with production of interleukin-2 (IL-2) and interleukin-12 (IL-12), which initiates a CD8+ cytotoxic response on *Brucella*-infected cells. Infected macrophages produce IL-12 and IFN-γ which regulate antigen presentation and may contribute to the limitation of intracellular bacterial replication through unknown mechanisms (Akbulut *et al.,*2005). The protective immunity requires activated antigen-presenting cells (mainly macrophages and dendritic cells) as well as $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-lymphocytes activation. Vitry*et al* demonstrated that $CD4^+$ IFN-γ^+ T cell subsets play a key role in containing *Brucella* infection (at least through in vitro infection) (Vitry*et al*, 2012) while Skendros*et al* demonstrated that $\gamma\delta^+$ T cell are of great importance in containing the infection, since they produce IFN-γ^+ and IL8 at different time post infection (Skendros*et al*, 2011).As mentioned above, after gaining entrance into the body, the organisms encounter the cellular defences of the host and the fate of invading bacteria is mainly determined by the cellular defences of the host, in particular macrophages and T lymphocytes. The most widely used correlates of CMI are lymphocyte stimulation, macrophage inhibition, delayed-type hypersensitivity and γ–interferon induction. Like the humoral response and depending on the method used to measure the cell mediated immunity the response can be expected as rapidly as a few weeks, but the response is variable and may not be detected.

2.5 EVADING IMMUNE SYSTEM

Many mechanisms contribute to allowing *Brucellae* to survive within the intracellular environment, and evade not only the innate immunity, but CD4+ - and CD8 + - mediated host cell killing (Covert *et al.,*2009). As *Brucella* cannot multiply outside their mammalian hosts, the most important aspect of *Brucella* ecology is their ability to establish an intracellular replicative niche and remain protected from the host immune responses (Bargen *et al.,* 2012). Brucellae lack classic virulence factors like toxins, fimbriae and capsules which raises the possibility that they might have unique and subtle mechanisms to penetrate host cells, elude host defenses, alter intracellular trafficking to avoid degradation and killing in lysosomes and modulate the intracellular environment to allow long-term intracellular survival and replication (Delrue *et al.,* 2004). The *Brucella* LPS O-polysaccharide appears to be a key molecule for cellular entry, to prevent complement-mediated bacterial lysis and to prevent apoptosis (i.e. programmed cell death) of the macrophages within which they reside allowing them to extend their longevity (de Bagüés *et al*., 2004; Lapaque *et al.,* 2005). *Brucella* has developed mechanisms to avoid innate immunity by minimizing stimulation of pattern recognition receptors (PRRs) of the host. The *Brucella* cell envelope has high hydrophobicity and its LPS has a non-canonical structure that elicits a reduced and delayed inflammatory response compared with other Gram-negative bacteria (Rittig et al., 2001) and has lower stimulatory activity on TLR4 receptors (Rittig *et al.,* 2003). The O side chain on the LPS can form complexes with the

major histocompatibility complex class II molecules that interfere with the ability of macrophages to present exogenous proteins. Brucella ornithine-containing lipids and lipoproteins in the outer membrane are poor activators of innate immunity. The rough (vaccine) strains (i.e., strains with lipopolysaccharide lacking the O-side chain) are less virulent because of their inability to overcome the host defense system (Rittig *et al.,* 2003). However, under in vitro conditions, up to 90% of virulent *Brucella* and 99% of nonvirulent *Brucella* may be killed following intracellular entry (Porte *et al.,* 1999).

2.6 SURVIVAL INSIDE HOST CELL

After entering into the host cell, smooth *Brucella* quickly traffic through the early endosomal compartment and depart the phagosome to form the modified phagosome (termed brucellosome). *Brucella* initially localize within acidified phagosomes (Rittig *et al.,* 2001), where they are exposed to free oxygen radicals generated by the respiratory burst of phagocytes. Brucellae have multiple mechanisms to detoxify free radicals. *Brucella* expresses 2 superoxide dismutases (SodA and SodC), which detoxify superoxide anions generated by the respiratory burst of phagocytes. Brucellae require acidification of the phagosomal compartment to a pH<4.5 before they display wild-type intracellular replication in initial stages of intracellular infection. Localization in an acidified environment induces expression of the VirB operon (virB 1–10), which controls expression of genes associated with a type IV secretion system. The VirB operon interacts with the endoplasmic reticulum to neutralize the pH of the phagosome (Anderson et al., 2008). The *Brucella*-induced modifications of the phagosome prevent fusion with the lysosome. Virulent *Brucella* strains express a cyclic glucan synthase that produces and secretes low molecular weight cyclic glucans. These molecules disrupt the lipid raft microdomain structures within intracellular membranes surrounding the bacteria. This modification of lipid raft distribution in phagosomal membranes inhibits phagosome maturation, prevents fusion with lysosomes (ArellanoReynoso *et al.,* 2005).

2.7 SURVIVAL OUTSIDE HOST CELL

Brucella may remain viable within the environment for a period of time. In general, the viability of *Brucella spp*. outside the mammalian host is enhanced by cool temperatures and moisture and decreased by high temperatures, dryness and direct exposure to sunlight. For example, *B. abortus* survives a couple of hours under direct sunlight but up to 185 days in the cold and shade. *Brucella abortus* also survives in aborted fetuses, manure and water for periods of up to 150 to 240 days (Saegerman *et al.,* 2010).

2.8 ZOONOTIC ASPECTS OF *BRUCELLA* INFECTIONS

Human brucellosis is widely distributed all over the world, with regions of high endemicity such Mediterranean, Middle East, Latin America and parts of Asia (Corbel, 1997, López-Merino, 1989). The true incidence of human brucellosis is unknown. Reported incidence in endemic-disease areas varies widely, from 200 per 100,000 population (López-Merino, 1989). Humans are accidentally infected and almost always dead-end hosts of *Brucella* infections. The disease is primarily an occupational risk in

exposed professions, i.e. veterinarians, farmers, laboratory technicians, abattoir workers, and others who work with animals and their products. People living near infected premises may also contract infection. The primary source is the animal and infection is contracted either by direct or indirect contact through the skin or mucous membranes or ingestion of contaminated products, especially fresh dairy products. The maximum danger is therefore during the lambing or kidding period. Dairy products are the 8 main source of infection for people who do not have direct contact with animals. The prevalence of human brucellosis acquired from dairy products is seasonal, reaching a peak soon after kidding and lambing. Abattoir workers handling infected animals are also at risk, especially from the contents of uteri and udders. The handling of raw wool has been identified as a potential source of infection of workers involved. Finally, *B. melitensis* is easily acquired by laboratory infection. Humans are susceptible to *B. abortus, B. melitensis, B. suis.B. melitensis and B. suis*often give rise to a severe and long lasting form of the disease. After an incubation period of 8 to 20 days, illness occurs in different forms. Asymptomatic infection is frequent and mainly due to *B. abortus*. It is characterised by antibody formation in persons with no history of symptoms consistent with brucellosis. The acute form of the disease is also common and symptoms include lassitude, headache and muscular or joint pain, and drenching sweats, especially at night. The manifestations of brucellosis are sometimes more pronounced or limited to a specific system or organ. This is then termed a *complication* when it occurs in the course of acute infection, or *localised brucellosis* when occurring in the absence of other signs of systemic illness. The most common localisations are spondylitis, peripheral arthritis, especially of the hip, knee and shoulder, or epididymo-orchitis. Nervous, genitourinary, hepatosplenic and cardiovascular complications may also be observed. Brucellosis is termed *chronic* when it includes one or more of the signs described above and persists or recurs over a period of six months or more. Finally*Brucella* dermatitis has traditionally been ascribed to "allergy" to *Brucella*. Familiarity with the manifestations of brucellosis and knowledge of the optimal laboratory studies are essential for the recognition of this reemerging zoonosis.*B melitensis, B abortus,* and *B suis* have been completely sequenced, and these sequencing data will help improve our understanding of the pathogenesis and the manifestations of this complex disease.

3. DIAGNOSTIC TESTS

The disease presents a great variety of clinical manifestations, making it difficult to diagnose clinically. Therefore, the diagnosis must be confirmed directly by isolation of *Brucella*, mostly from blood culture, or indirectly by the detection of immune response against its antigens. Clinically, identification to the genus level is sufficient to warrant initiation of therapy. The particular *Brucella* species involved does not affect the choice of therapeutic agents; however, speciation is necessary for epidemiologic surveillance and requires more detailed biochemical, metabolic, and immunologic testing.

The diagnosis of brucellosis based exclusively upon *Brucella* isolation presents several drawbacks (Orduña *et al.,*2000). The slow growth of *Brucella* in primary cultures means that diagnosis may take more than 7 days (Ariza, 1996, Rodríguez-Torres HWDO., 1987, Yagupsky, 1999). Besides, blood culture sensitivity is often low, ranging from 50-90 % depending on disease stage, *Brucella* species, culture medium, number of circulating bacteria and the culture technique employed (Gotuzzo *et al.,* 1986, Yagupsky, 1999). Hence, serological tests play a major role in diagnosis when the agent cannot be detected by blood culture. Yet, the interpretation of these tests is often difficult, particularly in patients with chronic brucellosis, in re-infections and relapses, and in endemic areas where a high portion of the population carries antibodies against brucellosis (Orduña *et al.,* 2000). Various serological tests have been used for the diagnosis of human brucellosis. The most common tests used are serum agglutination test (SAT), Coombs anti-*Brucella* test, Rose Bengal test and complement fixation test (Orduña *et al.,* 2000). During 9 the last decade, radioimmunoassays (Hewitt and Payne, 1984, Parrat *et al.,*1977) and in particular enzyme-immunoassays (Ariza *et al.,*1992, Gazapo *et al.,*1989, Saz *et al.,*1987) have also been used. Other tests have proved useful in some patients, such as the indirect immunofluorescence test, Brucellin counter-electrophoresis and passive haemagglutination test, but their value in clinical practice is still under assessment. Allergic tests reveal a delayed-type hypersensitivity; using conventional antigen preparations. Brucellin-INRA, a S-LPS free product was reported as reliable and innocuous, but further work is necessary.

3.1 DIRECT DIAGNOSIS: AGENT DETECTION

3.1.1 ISOLATION OF BACTERIA

The only unequivocal method for the diagnosis of brucellosis in smallruminants is based on the isolation of *Brucella* bacteria. (Alton*et al.,*1988).The presumptive bacteriological diagnosis of *B. melitensis*can be made by means of the microscopic examination of smears from vaginal swabs,placentas or aborted foetuses stained with the Stamp modification of theZiehl-Neelsen method. However, morphologically related micro-organisms such as *B. ovis*, *Clamydia psittaci* or *Coxiella burnetii*can mislead thediagnosis. Accordingly, the isolation of *B. melitensis* on appropriate culture media is recommended for an accurate diagnosis. Vaginal swabs and milk samples are the best samples to isolate *B. melitensis* from sheep and goats.The spleen and lymph nodes (iliac, mammary and prefemoral) are the most reliable samples for isolation purposes in necropsied animals (Marín *et al.,*1996a). *B. melitensis* does not require serum or CO_2 for growth and can be isolated on ordinary solid media under aerobic conditions at 37°C. However, the useof nonselective media cannot be recommended because of the over growing contaminants usually present in field samples, and selective media are needed for isolation purposes. The Farrell's selective medium, developed for the isolation of *B. abortus* from milk (Farrell, 1974), is also recommended for the isolation of *B. melitensis* (Alton *et al.,*1988). However, nalidixic acid and bacitracin, at the concentration used in this medium, may have inhibitory effects on some *B. melitensis* strains (Marín *et al.,*1996b). Thus, its sensitivity for the isolation of *B. melitensis* from naturally infected sheep is sometimes lower than that obtained with the less selective Thayer-Martin's modified medium (Marín *et al.,*1996a). The sensitivity of

bacteriological diagnosis is significantly increased by the simultaneous use of both the Farrell's and the modified Thayer-Martin's media (Marín *et al.,*1996b).Additional work should be carried out to develop a new selective medium that is more efficient and suitable for isolating all *Brucella* species.

3.1.2 OTHER METHODS FOR AGENT DETECTION

While culturing is a specific method, its sensitivity depends on the viabilityand numbers of *Brucella*within the sample, the nature of sample (foetal organs, foetal membranes, lymph nodes, etc.) and the number of specimens tested from the same animal (Hornitzky and Searson, 1986). The time required for culturing field specimens can be long and tissues or fluids that are only contaminated with a low number of *Brucella*may not be detected. Thus, in the case of tissues or fluids contaminated with non viable or a lownumber of *Brucella*, PCR could be a potentially useful method for the diagnosis of brucellosis. Several authors reported a good sensitivity of PCR for detecting of *Brucella* DNA on pure cultures (Fekete *et al.,* 1990a, 1990b). Others showed that PCR could be a potentially useful tool when used alone (PCR, AP-PCR, rep-PCR, ERIC-PCR) or in combination with labelled probes to differentiate some *Brucella* species and biovars (Fekete *et al.,* 1992b, Bricker and Halling, 1994, Tcherneva *et al.,* 1996). The possibility of PCR techniques to detect the DNA of dead bacteria, or in paucibacillary samples and even in samples highly contaminated with other micro-organisms, could potentially increase the rate of detecting animals infected with *Brucella.* However few studies have been performed with clinical or field samples (Fekete *et al.,*1992a; Rijpens *et al.,* 1996)and up to now, no technique has been demonstrated to be sensitive enough to replace classical bacteriology on all kinds of biological samples.

3.2 INDIRECT DIAGNOSIS: ANTIGEN OF *BRUCELLA* AND ANTIGEN DECTECTION

There is no scientific agreement on what should be the nature andcharacteristics of a universal antigen for diagnosing brucellosis due to smooth *Brucella* (*B. abortus, B. melitensis* and *B. suis*). One of the most controversial points concerning the serological diagnosis of *B. melitensis*infection in small ruminants is related to which *Brucella* species and biovarsare used in the production of the diagnostic antigens. The rose bengal test (RB) and the complement fixation test (CF) are the most widely used tests for the serological diagnosis of sheep brucellosis (Farina, 1985; MacMillan,1990). They are currently the official tests used in member states of theEuropean Union (Council Directive 91/68/EEC). The antigen suspensions(whole cells) used in both tests are made with *B. abortus* biovar 1 (an Adominant strain) (Alton *et al.,*1988) which means that, theoretically, infections due to M-dominant strains *B. melitensis* biovar 1; *B. abortus* biovars 4, 5 and 9; *B. suis* biovar 5) could be misdiagnosed (Alton *et al.,*1988; MacMillan, 1990). However, recent results showed that the sensitivity of the classical RB antigen prepared with *B. abortus* biovar 1 (A-dominant)was adequate for diagnosing ovine populations infected with the M dominant*B. Melitensis* biovar 1 (Blasco *et al.,*1994b).

The outer membrane of the bacteria contains the main antigens involved in the humoral response against *Brucella* (Díaz *et al.,*1968a). As in other gram negative bacteria, the outer membrane of smooth *Brucella* is composed of phospholipids, proteins and LPS (smooth lipopolysaccharide, S-LPS). The S-LPS is the immunodominant antigen. Most serological tests, particularly those using whole-cell suspensions as antigen (such as RB, CF), as well as ELISA , have been developed to detect antibodies to this antigen (Díaz *et al.,*1968a). The S-LPS of smooth *Brucella* is composed of an inner glycolipidic moiety (the core oligosaccharide plus the lipid A) and an outer polysaccharide chain (O-chain). This O-chain is the relevant antigenic moiety and is chemically composed of a perosamin homopolymer showing α-1,2 and α-1,3 linkages (Cherwonogrodzky *et al.,*1990). The O-chain polysaccharide of *B. abortus* biovar 1 (A dominant) possesses a fine structure with only a low-frequency (*ca.*2%) of α-1,3 linked 4,6 dideoxy-4-formamido-D-mannopyranoside residues. In contrast, the O-chain polysaccharide of *B. melitensis*biovar1 (M-dominant) contains repeated pentasaccharide units with one α-1,3 and four α-1,2 linkages. As a result, the A and M antigenic characteristics depend on the O- polysaccharides in which the frequency of α-1,3 linked residues varies. Studies with monoclonal
antibodies (Douglas and Palmer, 1988) have shown that the A epitope is related to portions of at least five sugars with α-1,2 linkages and that the M epitope includes sugars with α-1,3 linkages (thus its relevance in the O-chain of *B. abortus*biovar1 should not be important). Therefore, all biovars assigned as A-dominant should express few or no α-1,3 linked residues, while M-dominant strains possess a unique M epitope as well as a di-, tri- or tetrasaccharide with α-1,2 linkages, and can thus be considered to be contained within the A epitope structure(Bundle *et al.,*1989; Meikle *et al.,*1989; Cherwonogrodzky *et al.,*1990). The presence of common oligosaccharides of four or less sugars is consistent with the existence of a common (C) epitope. Indeed, this C epitope has been detected with the appropriate monoclonal antibodies (Douglas and Palmer, 1988) and can account for the high sensitivity of the antigens made from A-dominant strains (*ie B. abortus*biovar 1) at detecting M-dominant *B. melitensis*biovar 1 infections and *vice-versa* (MacMillan, 1990; Díaz-Aparicio *et al.,*1993). In fact, crude LPS extracts from either *B. melitensis* 16M (biovar 1, M-dominant) or *B. abortus*2308 (biovar 1, A-dominant) are equally sensitive in an indirect ELISA (i-ELISA) for diagnosing brucellosis in sheep infected by *B. melitensis* biovar 1. However, the native hapten and the S-LPS hydrolytic polysaccharides containing the O-chain and core sugars from *B. abortus* biovar 1 failed to react in precipitation tests with a large proportion of *B. melitensis* infected sheep, goats and cattle under conditions in which the same antigens obtained from *B. melitensis* biovar 1 detected most of those animals (Díaz-Aparicio *et al.,*1993). Therefore, further research is needed to clarify the practical

importance and interest of using species-specific diagnostic antigens for the different serological tests.

There is limited information on the value of outer membrane and innercytoplasmic proteins for the diagnosis of *Brucella* infection in sheep.

The immunoelectrophoretical patterns of cytoplasmic proteins show little differences between *Brucella s*pecies when assayed with polyclonal sera (Díaz *et al.,*1967, 1968b). These inner antigens are considered specific for the genus, being useful to differentiate infections due to *Brucella* from those due to bacteria whose LPS cross-reacts with the *Brucella* S-LPS, as is the case with *Yersinia enterocolitica*O:9. However, a cross-reactivity among cytosolic proteins of *B. melitensis* and those obtained from *Ochrobatrum anthropi*, an opportunistic human pathogen, has been reported recently (Velasco *et al*.1997). The *Brucella* cytoplasmic antigens have been used successfully for the allergic diagnosis of brucellosis in sheep and goats (Blasco *et al.,*1994b). Moreover, these cytoplasmic antigens have been reported to be sensitive and specific enough for the diagnosis of brucellosis in sheep and goats when used in precipitation tests (Díaz-Aparicio *et al.,* 1994). In contrast, when these cytoplasmic antigens are used in the i-ELISA, the sensitivity obtained is not adequate due to the high background IgG reactivities with sera from *Brucella* free animals (Díaz-Aparicio *et al.,* 1994). An important drawback of diagnostic test susing uncharacterised cytosolic proteins is the lack of specificity when testing Rev.1 vaccinated sheep and goats. But a partially purified cytosoluble protein of 28 kDa (CP28) from the cytosoluble protein extract (CPE) of *B. melitensis* has been reported as being able to differentiate Rev.1 vaccinated from *B. melitensis* infected ewes when used in i-ELISA (Debbarh *et al.,*1995). However, this test is less sensitive than both the RB and CF tests for diagnosing *B. melitensis* infected ewes. The corresponding *B. melitensis*16M *bp26*gene was expressed in *Escherichia coli* and monoclonal antibodies were produced (Cloeckaert *et al.,* 1996a). Sequence analysis of the cloned gene revealed that it was nearly identical to the recently published *B. abortus bp26*gene, coding for a periplasmic protein (Rossetti *et al.,* 1996). A competitive ELISA (c-ELISA)using CPE as antigen and some of these monoclonal antibodies showed improved sensitivity for diagnosing infected sheep, and no antibody response was detected in Rev.1 vaccinated sheep (Debbarh *et al.,* 1996).

Several authors have attempted to identify the main polypeptide specificities of the antibody response to outer-membrane protein (OMP) extracts of *B. melitensis* by using either immunoblotting or c-ELISAs with specific monoclonal antibodies (Tibor *et al.,* 1996). While OMPs of 10, 17, 19, 25-27and 31-34 kDa were found as potential antigens for the diagnosis of

brucellosis in sheep by immunoblotting or ELISA, the antibody response to them was very low and heterogeneous in *B. melitensis* infected sheep(Zygmunt *et al.,* 1994a).

3.3. SEROLOGICAL TESTS

Considering that infections by *B. abortus* and *B. melitensis* cause two different diseases in any of the susceptible host species, it is striking that no specific serological tests for *B. melitensis* infection of sheep have been developed. Instead, it is widely assumed that the serological tests used for *B. abortus* infection in cattle are also adequate for the diagnosis of *B. melitensis* infection in small ruminants. Accordingly, the RB and CF test are the most widely used tests for the serological diagnosis of brucellosis in sheep and goats (Farina, 1985; Alton, 1990; MacMillan, 1990). There is a considerable body of knowledge available on the diagnostic value of tests such as iELISA, cELISA, FPA, Coombs test or immunocapture test for ruminants in general. However, such knowledge does not enable to make a clear choice regarding the selection of a test for use in the serodiagnosis of ovine and caprine brucellosis (OIE Manual, 2000). Occurrence of false-positive serological reactions in surveillance and diagnostic testing for animal brucellosis is an important problem. Most commonly they are caused by infections with *Yersinia enterocolitica* serotype O:9 (YeO: 9), as this bacterium possesses an almost identical O-antigen LPS chain to that of *Brucella abortus*. *Brucella* LPS contain *Brucella spp*. specific M- and C-epitopes, as well as common A- or C/Y-epitopes that are shared with *Ye O: 9*, and a number monoclonal antibodies (mAbs) specifically recognizing these epitopes have been produced. However, although the exact structures of the various epitopes remain unclear, it appears that LPS epitopes span 2–5 overlapping polysaccharide units and that antibodies generated against the common A- or C/Y-epitopes are able to block the specific binding to adjacent M- and C-epitopes in competitive ELISAs. This means that antibodies generated by an infection with *YeO:9* will also inhibit binding of *Brucella*-specific mAbs in diagnostic competitive ELISAs. Attempts to overcome the LPS cross reactions with the use of *Brucella* protein antigens is hampered by the strong humoral immune dominance of smooth LPS in *Brucella* infections. This is an area of continued research and new candidates for antigen preparations continue to be evaluated. Attempts to produce phage-displayed peptide mimotopes of the Brucella-specific LPS epitopes have not been successful when used in vaccines or indirect ELISAs. Diagnostic use of cellular mediated immune responses to discriminate these two infections is a potential alternative to serological assays. This approach has been successful in cattle (Blasco *et al.,*1994b).

4.THESIS OBJECTIVES

4.1 PRELIMINARY DATA

To overcome the limitations of serological diagnosis, including false positive reactions caused by other pathogens, specific antigens for diagnosis of brucellosis other than those LPS-based are required. These antigens shouldn't show any cross-reaction with whole of *Yersinia enterocoliticaO:9*, *Vibrio cholerae,*

Salmonella typhimurium, and Escherichia coli O157. Several study were published on the use of alternative immunogenic proteins of *Brucella* genus to minimize cross reactions in brucellosis diagnosis, but so far none of these have produced any good outcome. Therefore, attempts to find a protein antigen for differentiating *Brucella*-infected from cross-reactive animals remain the principal aim of many studies.

Several authors have indeed demonstrated that the *Brucella* polysaccharide 'O' chain shares common features with various other bacteria (Nielsen et al., 2004; Muñoz et al, 2005). The relative lack of specificity and sensitivity of this test has often been presented as a major drawback for the diagnosis of brucellosis in cattle (Koet al., 2012). These factors bring up the need to develop new diagnostic methods able to distinguish between *Brucella* and other bacteria, in order to avoid worthless animal sacrifices. An independent confirmatory assay built on a non-LPS antigen and not expressed by an effective vaccine would be extremely useful.

Many potentially diagnostically useful protein antigens have been reported, too many to list here, but some examples are Lumazine synthase , copper/zinc superoxide dismutase (Cu/Zn SOD) L7/L12 , and malate dehydrogenase . Perhaps the most frequently cited is BP26 – also known as outer membrane protein (OMP)28 (Pappas *et al.,*2006). The Broad Institute has recently sequenced 25 *Brucella* genomes, wich along with the pre-existing genomes provide a sequence from every biovar, as well as other isolates of veterinary interest (http://www.broadinstitute.org/annotation/genoma/brucella_group/MultiHome.html). In addition, three new genomes were recently sequenced by The Centre of Disease Control and annotated by PATRIC (Snyder et al., 2007). Looking at the genomic differences between *Brucella* and the other pathogens there are small differences that are of potential interest to obtain an hypothetical immunogenic and high specific protein. These discriminating sequences could be synthesized in vitro and then used to test their ability to stimulate the immune system to produce specific cytokines that could be used for differential diagnosis of brucellosis. Vitry *et al* demonstrated that $CD4^+$ IFN-γ^+ T cell subsets play a key role in containing *Brucella* infection (at least through in vitro infection) (Vitry *et al* , 2012) while Skendros *et al* demonstrated that $\gamma\delta^+$ T cell are of great importance in containing the infection, since they produce IFN-γ^+ and IL8 at different time post infection (Skendros *et al*, 2011).

After a thorough review of the literature, objectives for the investigation of immune response and immunogenic proteins, in order to find a *Brucella* specific non-LPS protein(s) able to activate characteristic *Brucella* $CD4^+$ and $CD8^+$IFN-γ^+lymphocytes were drawn up.

Objective I: Bioinformatic studies. In order to set up a specific diagnosis to discriminate against *Brucella* infection from other diseases we carried out bioinformatic study of several identified non-LPS *Brucella* proteins. These included both genetic and immunological characterization of non-LPS immunogenic candidate antigens of *Brucella* in order to characterize their sequences and immune features, and to select the best non cross-reactive non-LPS epitopes, to employ during experimental infections, in order to provide important information for developing new diagnostic tools.

Objective II: **Experimental infections in cattle**

The research aimed to study the IFN-γ^+ T cell subsets during natural *Brucella* infection and experimental *Yersinia* immunization, in order to evaluate the potential diagnostic use of cell-mediated immune response to distinguish *Brucella* from *Yersinia* infections in cattle. We studied the release of cytokines from PBMCs (Peripheral Blood Mononuclear Cells) collected from infected animals, after stimulation with *Brucella* antigens. The cytokines were detected in cell culture supernatants by using both an enzyme immunoassay method and cytofluorimetric analysis. The novelty of the 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. The results allowed to deepen knowledge on interaction between *Brucella* specific antigens and host immune cells and the identification of a specific cytokine pathway for differential diagnosis of brucellosis.

5. MATERIALS AND METHODS

5.1 BACTERIAL STRAIN SEQUENCE ANALYSIS

Genotypic identification of the bacterial strains was carried out by 16S rRNA gene sequencing (Weisburg *et al*., 1991). *Brucella* and *Yersinia* cultures were grown on TSA plates. Extraction of genomic DNA was accomplished using the QIAamp DNA Mini Kit (Qiagen,Valencia, CA). The strains were genetically identified by 16S rRNA gene sequencing using the primers rD1 (5′ AAGGAGGTGATCCAGCC-3′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and the AmpliTaq Gold® 360 DNA Polymerase (Life Technologies, Carlsbad, CA, USA). DNA sequences were determinated by using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequences were compared with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) (Altschul *et al.,* 1997) databases. The last database compares a given sequence to those of type strains only. The isolates were considered to represent the species in question if 97% or higher similarity was detected (Stackebrandt and Goebel, 1994). The bacterial strains identified were employed to perform experimental infection in cattle and the future mouse models.

5.2 PROTEIN BIOINFORMATICS STUDIES

The bioinformatic study was carried out on several identified non-LPS *Brucella* proteins characterizing their sequences. The proteins selected were characterized for their amino acid sequences from PubMed (http://www.ncbi.nlm.nih.gov/pubmed) shown below:

PROTEIN	ACCESSION NUMBER	AMINO ACID SEQUENCE
ISOCITRATE LYASE	>gi|492965089|ref|WP_006071281.1|	MTDFYSLIPSAPKGRFDGIERAHTAEDVKRLRGSVEIKYSLAEM GANRLWKLIHEEDFVNALGALSGNQAMQMVRAGLKAIYLSGW QVAADANTASAMYPDQSLYPANAGPELAKRINRTLQRADQIET AEGKGLSVDTWFAPIVADAEAGFGGPLNAFEIMKAYIEAGAAG VHFEDQLASEKKCGHLGGKVLIPTAAHIRNLNAARLAADVMGT PTLIVARTDAEAAKLLTSDIDERDQPFVDYEAGRTAEGFYQVKN GIEPCIARAIAYAPYCDLIWMETSKPDLAQARRFAEAVHKAHPG KLLAYNCSPSFNWKKNLDDATIAKFQCELGAMGYKFQFITLAG FHQLNYGMFELARGYKDRQMAAYSELQQAEFAAEADGYTAT KHQREVGTGYFDAVSLAITGSQSSTTAMKESTETAQFKPAAE
ENOYL-COA HYDRATASE	>gi|489056931|ref|WP_002967057.1|	MNDFETLHIAVDHRGVARLTLNRPEQHNALSGRMIDELTTAAL HLADNEAVRIVILTGAGTSFCAGGDLGWMREQVNATRAQRIEE ARKLALMLKALRDLPKPLIGRINGQAYGGGVGLISVCDAAISIS GARFGLTETKLGLIPATISPYVVARIGEANALRTFTSARLFDAEE GRRIGLLHDVVEAERLDAAVEAEIKPYFSTAPAAVAASKRLVH ALGAPIDEAVIDMTLTRLADTWETPEAAEGIAAFFAKRAPAWK GGD
SUGAR BINDING PERIPLASMIC PROTEIN	>gi|38258624|sp|Q8YCE2.2|SP39_	MHKLLKLAAMGTAACALLAGMAPVANAQEKQNVEVLHWWT SGGEASALEVLKKDLESKGISWTDMPVAGGGGTEAMTVLRAR VTAGNAPTAVQMLGFDIRDWAEQGALGNLDTVASKEGWEKVI PAPLQEFAKYDGHWIAAPVNIHSTNWMWINKAALDKAGGKEP TNWDELIALLDNFKAQGITPIAHGGQPWQDATIFDAVVLSFGPD FYKKAFIDLDPEALGSDTMKQAFDRMSKLRTYVDDNFSGRDW NLASAMVIEGKAGVQFMGDWAKGEFLKAGKKPGEDFVCMRY PGTQGAVTFNSDMFAMFKVSEDKVPAQLEMASAIESPAFQSAF NVVKGSAPARTDVPDTAFDACGKKAIADVKEANSKGTLLGSM AHGYANPAAVKNAIYDVVTRQFNGQLSSEDAVKELVVAVEAA K
COML,COMPETENCE LIPOPROTEIN	>gi|81852175|sp|Q8YI58|Q8YI58_	MRSAKSTCCFKRERRYQRAGDRMTSFKFTGVTKTALLSGTIAV LIPLAGCASKNDDIDLTKYVETIDPADKLYNEGLANLDAGRLDE AAKKFAAIDRQHPYTEWARKALVMAAFTNYRKGNYEEAISMA KRYNTLYPTSPESAYAYYIIGLSYFRQIPDVTRDQAASRRAIAAM QEVIDRFPNSEYTDDAKTKIRVARDQLAGKEMQIGRYYLERKE YLAAIKRFRGVVEEYSNTRQVEEALARLVEAYYALGLTSEAQM AASVLGKNFPDSQWYKDSYKLLQSGGLQPRENGNSWLAKAGA LITGGSS
PERIPLASMIC DIPEPTIDE TRANSPORT PROTEIN	>gi|81852305|sp|Q8YIL0|Q8YIL0	MDDKYDPEAAKKALEAAGVKDLSMKIWAMPVSRPYMPNARR TAELMQADLAKVGVKADIVSMEWGKYLKKSSEKDRDGAVIM GWTGDNGDPDNFLGTLLGCAGLGNNNRAQWCYKPFEDLIQKA KTSTSQEERTKLYEEAQAVFKEQAPWDTIAHSTVFVPMSAKVT GFKQSPLGDYRFEEVDISE
MOLECULAR CHAPERONED NAK	>gi|493164348|ref|WP_006170012.1|	MAKVIGIDLGTTNSCVAVMDGKNAKVIENAEGARTTPSIIAFTD GDERLAGQPAKRQAVTNPEGTLFAVKRLIGRRYDDPMVTKDK DLVPYKIVKGDNGDAWVEVHGKKYSPSQISAMILQKMKETAES YLGETVTQAVITVPAYFNDAQRQATKDAGKIAGLEVLRIINEPT AAALAYGLDKSEGKTIAVYDLGGGTFDVSVLEIGDGVFEVKST NGDTFLGGEDFDIRLVEYLVAEFKKESDIDLKNDKLALQRLKEA AEKAKIELSSSQQTEINLPFITADQTGPKHLAIKLSRAKFESLVDD LVQRTVEPCKAALKDAGLKAGEIDEVVLVGGMTRMPKIQEVV

		KAFFGKEPHKGVNPDEVVAMGAAIQGGVLQGDVKDVLLLDVT PLSLGIETLGGVFTRLIERNTTIPTKKSQTFSTAEDNQSAVTIRVF QGEREMAADNKLLGQFDLVGIPPAPRGVPQIEVTFDIDANGIVN VSAKDKGTGKEHQIRIQASGGLSDADIEKMVKDAEANAEADKK RRESVEAKNQAESLVHSTEKSLAEYGDKVSADDKKAIEDAIAA LKTSLEGEDAEDIKAKTQALAEVSMKLGQAMYEAAAAEGAGA EGGEQASSSKDDVVDADYEEIDDNKKSS
CATALASE	>gi|493053174|ref|WP_006112539.1|	MTDRPIMTTSAGAPIPDNQNSLTAGERGPILMQDYQLIEKLSHQ NRERIPERAVHAKGWGAYGTLTITGDISRYTKAKVLQPGAQTP MLARFSTVAGELGAADAERDVRGFALKFYTQEGNWDLVGNNT PVFFVRDPLKFPDFIHTQKRHPRTHLRSATAMWDFWSLSPESLH QVTILMSDRGLPTDVRHINGYGSHTYSFWNDAGERYWVKFHF KTMQGHKHWTNAEAEQVIGRTRESTQEDLFSAIENGEFPKWKV QVQIMPELDADKTPYNPFDLTKVWPHADYPPIDIGVMELNRNP ENYFTEVENAAFSPSNIVPGIGFSPDKMLQARIFSYADAHRHRLG THYESIPVNQPKCPVHHYHRDGQMNVYGGIKTGNPDAYYEPNS FNGPVEQPSAKEPPLCISGNADRYNHRIGNDDYSQPRALFNLFD AAQKQRLFSNIAAAMKGVPGFIVERQLGHFKLIHPEYEAGVRK ALKDAHGYDANTIA
RIBOSE TRANSPORT SYSTEM SUBSTRATE-BINDING PROTEIN	>gi|496220735|ref|WP_008934772.1|	MFKKGMRVLFAAAAALPLIASTAWAEGLMTIIVNDPSNPYWFT EGEVAKKTAEGLGYKAVVGGHKGDTNTESNLIDTAITNKSVAII LDPANADGSVGAVKRAIAANIPVFLINAEINQEGLAKAQLVSNN AQGAALGATQWVESVGDKGKYVELFGAPSDNNAATRSNGYET VLSQYPDLVRVGKDVANWDRT
GLUTAMINE AMIDO TRANSFERASE	>gi|490822964|ref|WP_004685054.1|	MPSTRRIFMPIRTIVWGENIHEQINETVRSIYPEGMHNTIAGALN EDGAIEATTATLQEPEHGLLTERLAQTDVLVWWGHKDHGGVS DDVVERVARRVFEGMGLIVLHSGHFSKIFKRLMGTPCALKWRE AGERVWVVNRGHPIAQGLEETFVLENEEMYGEQFSVPEPLETV FISWFAGGEVFRSGMTWRRGAGNVFYFRPGHETYPTYHDANV RTVLRNAVKWAYNPQPAWTGIHTAPNVPVEKALEPIVERGPKL HKAGEAGYR
SN-GLYCEROL-3-PHOSPHATE-BINDING PERIPLASMIC PROTEIN UGPB	>gi|493105393|ref|WP_006138486.1|	MFTRLITTSALTGAIALTIGSQAFAQTELAWWHGMTGANNEMV NELSKEFNESQSEYKIVPVYKGNYPETLNAGIAAFRSKQPPAILQ VFDAGSGVMMAAEGAIVPAAEVLEKGGYKFDKSQYLPGIVAY YSKPDGTMLSFPYNSSSPILYYNKDAFKKAGLDENKPPKTWPEV FEAAKKIKASGASPCGFTST

The blastp alignment tool (protein-protein BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) was used to detect the matching scores between *Brucella* protein and the corresponding *Yersinia* one.

The proteins selected were characterized for their immune features, in order to find B- and/or T-cell epitopes to be used as potential stimuli. We used specific tools able to predict IC50 values for peptides binding to specific MHC molecules (http://tools.immuneepitope.org/main/html/tcell_tools.html),antibody epitope (http://tools.immuneepitope.org/tools/bcell/iedb_input), to detect the presence of transmembrane regions (TM) (http://www.cbs.dtu.dk/services/TMHMM/) and the presence of signal peptide (SP) (http://www.cbs.dtu.dk/services/SignalP/),

5.3 EXPERIMENTAL INFECTIONS IN CATTLE

Blood samples were collected from three groups of cattle (N=5 for each group) experimentally immunized with *Yersinia enterocolitica* O:9 (Ye O:9) (Charolaise breed, 6-8 months old), naturally infected with *B. abortus* (confirmed by positivity to the official serological tests) (Holstein breed, 2-5 years old), and from officially Brucellosis free farms (as serological negative controls). PBMC were isolated from heparinized blood samples (La Manna *et al.,* 2011) and *in vitro* stimulated with *Yersinia enterocolitica* O:9 (*Yersinia enterocolitica* O:9 YOP® - InstitutVirion/Serion GmbH, Germany - 80 µg/ml) and *B. abortus*whole antigens (Brucellergene, OCB®Synbiotics, Kansas City, MO US - 80 µg/ml) alternatively. 5 x 10^5 PBMC/well were cultured for 48 h andthen collected. For cell acquisition and flow cytometry analysis a FACScan cytometer (Becton Dickinson, U.S.A.) was used, by staining the cells with anti-surface-FITC-labeled and anti-IFN-γ PE-labeled mAbs. Surface immunofluorescence was detected by incubation of cells with anti-CD8 (FITC-labeled) anti-CD4 (FITC–labeled) and anti-WC1 (FITC-labeled) at 4°C. After three washes, the cells were fixed and permeabilized by Leucoperm™ reagent set (AbDSerotec, U.K.) and then incubated with anti-IFN-γmAb PE-labeled for 30 minutes at 4°C. After three washes, the lymphocytes were analyzed using a FACScan cytometer (Becton Dickinson, U.S.A.) using CELL-QUEST PRO software (Becton Dickinson, U.S.A.). Each analysis was performed collecting 10.000 events of live lymphocytes. The cells were gated according to their physical appearance using the forward scatter (FSC) and side scatter (SSC) parameters, to exclude debris and apoptotic cells.

6.RESULTS AND CONCLUSIONS

6.1. NUCLEOTIDE SEQUENCE BLAST RESULTS

Brucella and *Yersinia* strain sequences compared using the BLAST search gave the following nucleotide sequence and alignment results:

NULEOTIDE SEQUENCE	**BLAST RESULT**
TGGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCA GCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT GGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATC CCCGCGCTTAACGTGGGNACNGCATTTGAAACTGGCAAGCTAGAGT CTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA GAGATCTGNAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAA GACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA TACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTG CCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCT GGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTNNCGGGGG CCNGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA GAACCTTACCTACTCTTGACATCCACGGAATTTAGCAGAGATGCTT TAGTGNCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCA GCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC CTTATCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAAGGAGAC TGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATC ATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATAC AAAGTGAAGCGAACTCGCGAGA	>gi|176358|gb|M59292.1|YEPRR16SA *Yersinia enterocolitica* 16S ribosomal RNA (99%)

GTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCG AAGGCGGCTCACTGGACCATTACTGACGCTGAGGTGCGAAAGCGT GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC GATGAATGTTAGCCGTCGGGGTGTTTACACTTCGGTGGCGCAGCTA ACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAAC TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT TTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATCCCG GTCGCGGTTAGTGGAGACACTATCCTTCAGTTAGGCTGGACCGGAG ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG TTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATT CAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAA GGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTAC ACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGCACGCGAGT GTGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCA ACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCAT GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA CCATGGGAGTTGGTTTTACCCGAAGGCGCTGTGCTAACCGCAAGGA GGCAGGCGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAAC AAGGTAGCCGTAGGGGAACCTGCGGCTG	>gi|694174373|gb|KM117186.1| *Brucella melitensis*strain 46/VPH 16S ribosomal RNA gene (100%)

6.2 PROTEIN BIOINFORMATICS RESULTS

6.2.1 BLASTP ALIGNMENT RESULTS

The BLASTP alignment tool (protein-protein BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) detected the following matching scoresbetween*Brucella* protein and the corresponding *Yersinia* one:

Protein	**%Identities**
isocitratelyase	61
enoyl-CoA hydratase	63
sugar-binding periplasmic protein	--
Periplasmic Dipeptide TransportProtein	72
Coml, Competence Lipoprotein	--
moleculerchaperoneDnaK	64
Catalase	64
ribose transport system substrate-binding protein	76
sn-glycerol-3-phosphate-binding periplasmic protein ugpB	67
glutamine amidotransferase	73

No significant similarity was found between two *Brucella* and *Yersinia* proteins, *Sugar-binding periplasmic protein* and*Competence Lipoprotein*, since blastp cannot find any reliable alignment, proving they arespecific for *Brucella* protein set.

6.2.2 MHCI BINDING PREDICTIONRESULTS

Protein 1:BRUME sugar-binding periplasmic protein

Protein 2: Competence Lipoprotein

PROTEIN 1 RESULTS

SP='YES' Cleavage site between pos. 27 and 28: ANA-QE D=0.821 D-cutoff=0.570 Networks=SignalP-noTM

-Prediction of the possibility that a peptide binds to mouse MHCI (all alleles, 11aa) http://tools.immuneepitope.org/mhci/result/

Allele	#	Start	End	Length	Peptide	Method used		Percentile rank
H-2-Db	1	299	309	11	AVTFNSDMFAM	Consensus (ann/smm)	13	0.35
H-2-Db	1	378	388	11	MAHGYANPAAV	Consensus (ann/smm)	7	1.45
H-2-Db	1	140	150	11	AAPVNIHSTNW	Consensus (ann/smm)	10	1.45
H-2-Db	1	106	116	11	GALGNLDTVAS	Consensus (ann/smm)	6+CD4	1.85
H-2-Db	1	360	370	11	KAIADVKEANS	Consensus (ann/smm)	11	2.9
H-2-Db	1	307	317	11	FAMFKVSEDKV	Consensus (ann/smm)	21	2.95
H-2-Db	1	26	36	11	NAQEKQNVEVL	Consensus (ann/smm)	17	3.05
H-2-Db	1	22	32	11	APVANAQEKQN	Consensus (ann/smm)	20	3.1
H-2-Db	1	382	392	11	YANPAAVKNAI	Consensus (ann/smm)	16	4.35
H-2-Db	1	325	335	11	SAIESPAFQSA	Consensus (ann/smm)	15	4.75
H-2-Db	1	256	266	11	SAMVIEGKAGV	Consensus (ann/smm)	8+CD4	4.8
H-2-Db	1	141	151	11	APVNIHSTNWM	Consensus (ann/smm)	3	5.6
H-2-Db	1	390	400	11	NAIYDVVTRQF	Consensus (ann/smm)	5	5.85
H-2-Db	1	84	94	11	TAGNAPTAVQM	Consensus (ann/smm)	9+CD4	6.05
H-2-Db	1	352	362	11	TAFDACGKKAI	Consensus (ann/smm)	22	6.95
H-2-Db	1	400	410	11	FNGQLSSEDAV	Consensus (ann/smm)	23	7.05
H-2-Db	1	87	97	11	NAPTAVQMLGF	Consensus (ann/smm)	19+CD4	7.7
H-2-Db	1	298	308	11	GAVTFNSDMFA	Consensus (ann/smm)	14	8.3
H-2-Db	1	131	141	11	FAKYDGHWIAA	Consensus (ann/smm)	1	8.6
H-2-Db	1	174	184	11	ALLDNFKAQGI	Consensus (ann/smm)	18	8.85
H-2-Db	1	248	258	11	SGRDWNLASAM	Consensus (ann/smm)	4+CD4	8.85
H-2-Db	1	214	224	11	KAFIDLDPEAL	Consensus (ann/smm)	2+CD4	9.15

NOTE: If the query includes all the alleles and all the lengths, the result can not be interpreted (too much information). Reducing the aa number to 11 , and maintaining all the alleles, the table is analyzed, although all the compatible percentile rank values affect the H-2-Db allele.

-Prediction of the relative ability of a peptide/MHC complex to elicit an immune response. (http://tools.iedb.org/immunogenicity)

Immunogenicity predictions - Prediction Results

Masking: default
Masked variables: [1, 2, 'cterm']
Predictions:

Peptide	Length	Score	
FAKYDGHWIAA	11	0.35607	1
KAFIDLDPEAL	11	0.30124	2
APVNIHSTNWM	11	0.16739	3
SGRDWNLASAM	11	0.15779	4
NAIYDVVTRQF	11	0.15402	5
GALGNLDTVAS	11	0.12924	6
MAHGYANPAAV	11	0.11824	7
SAMVIEGKAGV	11	0.09446	8
TAGNAPTAVQM	11	0.07358	9
AAPVNIHSTNW	11	0.06599	10
KAIADVKEANS	11	0.05786	11
AAVKNAIYDVV	11	-0.00284	12
AVTFNSDMFAM	11	-0.05904	13
GAVTFNSDMFA	11	-0.05986	14
SAIESPAFQSA	11	-0.07407	15
YANPAAVKNAI	11	-0.08246	16
NAQEKQNVEVL	11	-0.11847	17
ALLDNFKAQGI	11	-0.12471	18
NAPTAVQMLGF	11	-0.1539	19
APVANAQEKQN	11	-0.18366	20
FAMFKVSEDKV	11	-0.28313	21
TAFDACGKKAI	11	-0.28322	22
FNGQLSSEDAV	11	-0.30273	23

PROTEIN2 RESULTS

Networks=noSignalP-noTM

Allele	#	Start	End	Length	Peptide	Method used		Percentile rank
H-2-Ld	1	139	149	11	SPESAYAYYII	ann	19	0.2
H-2-Dd	1	138	148	11	TSPESAYAYYI	ann	21	0.4
H-2-Ld	1	99	109	11	HPYTEWARKAL	ann	3+CD4	0.4
H-2-Kd	1	100	110	11	PYTEWARKALV	Consensus (ann/smm)	4+CD4	0.75
H-2-	1	287	297	11	LQPRENGNSWL	ann	9+CD4	0.9

Dd								
H-2-Kd	1	61	71	11	KYVETIDPADK	Consensus (ann/smm)	1	0.95
H-2-Dd	1	18	28	11	RAGDRMTSFKF	ann	46	1.2
H-2-Dd	1	202	212	11	AGKEMQIGRYY	ann	27	1.5
H-2-Ld	1	144	154	11	YAYYIIGLSYF	ann	10+CD4	1.7
H-2-Ld	1	269	279	11	FPDSQWYKDSY	ann	48	1.7
H-2-Kk	1	85	95	11	LDEAAKKFAAI	Consensus (ann/smm)	38	1.8
H-2-Db	1	164	174	11	QAASRRAIAAM	Consensus (ann/smm)	8+CD4	1.9
H-2-Kd	1	274	284	11	WYKDSYKLLQS	Consensus (ann/smm)	51+CD4	2.1
H-2-Dd	1	144	154	11	YAYYIIGLSYF	ann		2.1
H-2-Dd	1	67	77	11	DPADKLYNEGL	ann	32	2.2
H-2-Ld	1	244	254	11	RLVEAYYALGL	ann	5	2.2
H-2-Db	1	250	260	11	YALGLTSEAQM	Consensus (ann/smm)	26+CD4+++	2.35
H-2-Kd	1	26	36	11	FKFTGVTKTAL	Consensus (ann/smm)	14	2.4
H-2-Kk	1	184	194	11	SEYTDDAKTKI	Consensus (ann/smm)	42+43	2.4
H-2-Ld	1	136	146	11	YPTSPESAYAY	ann	41	2.6
H-2-Dd	1	265	275	11	LGKNFPDSQWY	ann	30	2.8
H-2-Db	1	112	122	11	AAFTNYRKGNY	Consensus(ann/smm)	30	2.85
H-2-Dd	1	127	137	11	SMAKRYNTLYP	ann	37	2.9
H-2-Kk	1	31	41	11	VTKTALLSGTI	Consensus (ann/smm)	36	3.05
H-2-Kd	1	211	221	11	YYLERKEYLAA	Consensus (ann/smm)	13	3.15
H-2-Dd	1	135	145	11	LYPTSPESAYA	ann	39+40	3.2
H-2-Kd	1	184	194	11	SEYTDDAKTKI	Consensus (ann/smm)		3.3
H-2-Kd	1	121	131	11	NYEEAISMAKR	Consensus (ann/smm)	35	3.35
H-2-Dd	1	98	108	11	QHPYTEWARKA	ann	2	3.4
H-2-Dd	1	259	269	11	QMAASVLGKNF	ann	47	3.4
H-2-Kb	1	277	287	11	DSYKLLQSGGL	Consensus (ann/smm)	49+50+CD4+++	3.55
H-2-	1	50	60	11	CASKNDDIDLT	Consensus	33	3.6

Db						(ann/smm)		
H-2-Kd	1	135	145	11	LYPTSPESAYA	Consensus (ann/smm)		3.75
H-2-Kk	1	294	304	11	NSWLAKAGALI	Consensus (ann/smm)	25	3.75
H-2-Dd	1	277	287	11	DSYKLLQSGGL	ann		3.8
H-2-Dd	1	292	302	11	NGNSWLAKAGA	ann	29	4.1
H-2-Dd	1	82	92	11	AGRLDEAAKKF	ann	34	4.2
H-2-Dd	1	156	166	11	QIPDVTRDQAA	ann	12+CD4	4.3
H-2-Kd	1	15	25	11	RYQRAGDRMTS	Consensus (ann/smm)	16	4.6
H-2-Dd	1	268	278	11	NFPDSQWYKDS	ann	45	4.7
H-2-Kd	1	152	162	11	SYFRQIPDVTR	Consensus (ann/smm)	7	4.75
H-2-Kb	1	149	159	11	IGLSYFRQIPD	Consensus (ann/smm)	24	5.05
H-2-Dd	1	209	219	11	GRYYLERKEYL	ann	18	5.2
H-2-Dd	1	171	181	11	IAAMQEVIDRF	ann	15	5.3
H-2-Dd	1	201	211	11	LAGKEMQIGRY	ann	44	5.3
H-2-Kk	1	161	171	11	TRDQAASRRAI	Consensus (ann/smm)	31+CD4++	5.4
H-2-Ld	1	147	157	11	YIIGLSYFRQI	ann	23+CD4	5.5
H-2-Kb	1	115	125	11	TNYRKGNYEEA	Consensus (ann/smm)	20	5.65
H-2-Kk	1	212	222	11	YLERKEYLAAI	Consensus (ann/smm)	17	5.7
H-2-Kd	1	143	153	11	AYAYYIIGLSY	Consensus (ann/smm)	6+CD4	6.0

NOTE: also in this case the query involves all the alleles, but there is more variability

-Prediction of the relative ability of a peptide/MHC complex to elicit an immune response.
(http://tools.iedb.org/immunogenicity)

Immunogenicity predictions - Prediction Results
Masking: default
Masked variables: [1, 2, 'cterm']
Predictions:

Peptide	Length	Score	
KYVETIDPADK	11	0.33869	1
QHPYTEWARKA	11	0.29819	2
HPYTEWARKAL	11	0.27874	3
PYTEWARKALV	11	0.24109	4
RLVEAYYALGL	11	0.19232	5

AYAYYIIGLSY	11	0.19046	6
SYFRQIPDVTR	11	0.17448	7
QAASRRAIAAM	11	0.16629	8
LQPRENGNSWL	11	0.15639	9
YAYYIIGLSYF	11	0.13506	10
YAYYIIGLSYF	11	0.13506	11
QIPDVTRDQAA	11	0.0931	12
YYLERKEYLAA	11	0.04507	13
FKFTGVTKTAL	11	0.04068	14
IAAMQEVIDRF	11	0.03514	15
RYQRAGDRMTS	11	0.03038	16
YLERKEYLAAI	11	0.01392	17
GRYYLERKEYL	11	0.01152	18
SPESAYAYYII	11	0.00979	19
TNYRKGNYEEA	11	0.0071	20
TSPESAYAYYI	11	0.0021	21
YIIGLSYFRQI	11	-0.012	23
IGLSYFRQIPD	11	-0.01247	24
NSWLAKAGALI	11	-0.01462	25
YALGLTSEAQM	11	-0.04401	26
AGKEMQIGRYY	11	-0.05003	27
AAFTNYRKGNY	11	-0.06062	28
NGNSWLAKAGA	11	-0.07575	29
LGKNFPDSQWY	11	-0.07578	30
TRDQAASRRAI	11	-0.079	31
DPADKLYNEGL	11	-0.09117	32
CASKNDDIDLT	11	-0.09628	33
AGRLDEAAKKF	11	-0.10833	34
NYEEAISMAKR	11	-0.11843	35
VTKTALLSGTI	11	-0.11889	36
SMAKRYNTLYP	11	-0.13878	37
LDEAAKKFAAI	11	-0.14395	38
LYPTSPESAYA	11	-0.16381	39
LYPTSPESAYA	11	-0.16381	40
YPTSPESAYAY	11	-0.1717	41
SEYTDDAKTKI	11	-0.17708	42
SEYTDDAKTKI	11	-0.17708	43
LAGKEMQIGRY	11	-0.20818	44
NFPDSQWYKDS	11	-0.212	45
RAGDRMTSFKF	11	-0.23241	46
QMAASVLGKNF	11	-0.23351	47
FPDSQWYKDSY	11	-0.34091	48
DSYKLLQSGGL	11	-0.45993	49
DSYKLLQSGGL	11	-0.45993	50
WYKDSYKLLQS	11	-0.50986	51

6.2.3 MHCII BINDING PREDICTION RESULTS

PROTEIN 1 RESULTS

http://tools.immuneepitope.org/mhcii/

#	Start	End	Peptide	Method used		Percentile rank
1	406	420	SEDAVKELVVAVEAA	Consensus (comb.lib./smm/nn)	21	0.09
1	407	421	EDAVKELVVAVEAAK	Consensus (comb.lib./smm/nn)	19	0.10
1	70	84	GGGTEAMTVLRARVT	Consensus (comb.lib./smm/nn)	20	0.31
1	69	83	GGGGTEAMTVLRARV	Consensus (comb.lib./smm/nn)	25	0.33
1	71	85	GGTEAMTVLRARVTA	Consensus (comb.lib./smm/nn)	18	0.39
1	1	15	MHKLLKLAAMGTAAC	Consensus (comb.lib./smm/nn)		0.42
1	2	16	HKLLKLAAMGTAACA	Consensus (comb.lib./smm/nn)		0.42
1	68	82	AGGGGTEAMTVLRAR	Consensus (comb.lib./smm/nn)	28	0.42
1	197	211	ATIFDAVVLSFGPDF	Consensus (smm/nn/sturniolo)	15	0.44
1	201	215	DAVVLSFGPDFYKKA	Consensus (smm/nn/sturniolo)		0.48
1	199	213	IFDAVVLSFGPDFYK	Consensus (smm/nn/sturniolo)	30	0.51
1	200	214	FDAVVLSFGPDFYKK	Consensus (smm/nn/sturniolo)	39	0.51
1	198	212	TIFDAVVLSFGPDFY	Consensus (smm/nn/sturniolo)	21	0.52
1	9	23	AMGTAACALLAGMAP	Consensus (comb.lib./smm/nn)	40	0.67
1	192	206	QPWQDATIFDAVVLS	Consensus (comb.lib./smm/nn)	8	0.86
1	191	205	GQPWQDATIFDAVVL	Consensus (comb.lib./smm/nn)	3	0.87
1	8	22	AAMGTAACALLAGMA	Consensus (comb.lib./smm/nn)		1.05
1	214	228	KAFIDLDPEALGSDT	Consensus (smm/nn/sturniolo)		1.16
1	5	19	LKLAAMGTAACALLA	Consensus (comb.lib./smm/nn)		1.22
1	165	179	EPTNWDELIALLDNF	Consensus (comb.lib./smm/nn)	6	1.23
1	202	216	AVVLSFGPDFYKKAF	Consensus (smm/nn/sturniolo)		1.24
1	10	24	MGTAACALLAGMAPV	Consensus (comb.lib./smm/nn)		1.25
1	193	207	PWQDATIFDAVVLSF	Consensus (comb.lib./smm/nn)	12	1.33
1	166	180	PTNWDELIALLDNFK	Consensus (comb.lib./smm/nn)	4	1.36
1	194	208	WQDATIFDAVVLSFG	Consensus (comb.lib./smm/nn)	9	1.38
1	167	181	TNWDELIALLDNFKA	Consensus (comb.lib./smm/nn)	13	1.43

1	7	21	LAAMGTAACALLAGM	Consensus (comb.lib./smm/nn)		1.46
1	6	20	KLAAMGTAACALLAG	Consensus (comb.lib./smm/nn)		1.56
1	67	81	VAGGGGTEAMTVLRA	Consensus (comb.lib./smm/nn)	29	1.62
1	203	217	VVLSFGPDFYKKAFI	Consensus (smm/nn/sturniolo)		1.63
1	164	178	KEPTNWDELIALLDN	Consensus (comb.lib./smm/nn)	5	1.68
1	250	264	RDWNLASAMVIEGKA	Consensus (comb.lib./smm/nn)		1.73
1	213	227	KKAFIDLDPEALGSD	Consensus (smm/nn/sturniolo)		1.73
1	249	263	GRDWNLASAMVIEGK	Consensus (comb.lib./smm/nn)	23+24	1.75
1	251	265	DWNLASAMVIEGKAG	Consensus (comb.lib./smm/nn)		1.79
1	215	229	AFIDLDPEALGSDTM	Consensus (smm/nn/sturniolo)	36	1.99
1	72	86	GTEAMTVLRARVTAG	Consensus (comb.lib./smm/nn)	31	2.01
1	3	17	KLLKLAAMGTAACAL	Consensus (comb.lib./smm/nn)		2.05
1	4	18	LLKLAAMGTAACALL	Consensus (comb.lib./smm/nn)		2.05
1	73	87	TEAMTVLRARVTAGN	Consensus (comb.lib./smm/nn)	34	2.06
1	405	419	SSEDAVKELVVAVEA	Consensus (comb.lib./smm/nn)	27	2.11
1	330	344	PAFQSAFNVVKGSAP	Consensus (smm/nn/sturniolo)		2.15
1	329	343	SPAFQSAFNVVKGSA	Consensus (smm/nn/sturniolo)		2.21
1	328	342	ESPAFQSAFNVVKGS	Consensus (smm/nn/sturniolo)		2.26
1	327	341	IESPAFQSAFNVVKG	Consensus (smm/nn/sturniolo)		2.28
1	118	132	EGWEKVIPAPLQEFA	Consensus (comb.lib./smm/nn)	33	2.28
1	326	340	AIESPAFQSAFNVVK	Consensus (smm/nn/sturniolo)		2.37
1	117	131	KEGWEKVIPAPLQEF	Consensus (comb.lib./smm/nn)	16	2.39
1	2	16	HKLLKLAAMGTAACA	Consensus (comb.lib./smm/nn)		2.40
1	83	97	VTAGNAPTAVQMLGF	Consensus (comb.lib./smm/nn)		2.40
1	84	98	TAGNAPTAVQMLGFD	Consensus (comb.lib./smm/nn)		2.40
1	3	17	KLLKLAAMGTAACAL	Consensus (comb.lib./smm/nn)		2.43
1	252	266	WNLASAMVIEGKAGV	Consensus (comb.lib./smm/nn)		2.43

1	4	18	LLKLAAMGTAACALL	Consensus (comb.lib./smm/nn)		2.45
1	248	262	SGRDWNLASAMVIEG	Consensus (comb.lib./smm/nn)		2.51
1	249	263	GRDWNLASAMVIEGK	Consensus (comb.lib./smm/nn)	23+24	2.51
1	105	119	QGALGNLDTVASKEG	Consensus (smm/nn/sturniolo)		2.54
1	335	349	AFNVVKGSAPARTDV	Consensus (smm/nn/sturniolo)		2.55
1	106	120	GALGNLDTVASKEGW	Consensus (smm/nn/sturniolo)		2.59
1	336	350	FNVVKGSAPARTDVP	Consensus (smm/nn/sturniolo)		2.61
1	104	118	EQGALGNLDTVASKE	Consensus (smm/nn/sturniolo)		2.66
1	334	348	SAFNVVKGSAPARTD	Consensus (smm/nn/sturniolo)		2.69
1	103	117	AEQGALGNLDTVASK	Consensus (smm/nn/sturniolo)		2.70
1	333	347	QSAFNVVKGSAPART	Consensus (smm/nn/sturniolo)		2.70
1	1	15	MHKLLKLAAMGTAAC	Consensus (smm/nn/sturniolo)		2.76
1	2	16	HKLLKLAAMGTAACA	Consensus (smm/nn/sturniolo)		2.76
1	3	17	KLLKLAAMGTAACAL	Consensus (smm/nn/sturniolo)		2.81
1	102	116	WAEQGALGNLDTVAS	Consensus (smm/nn/sturniolo)		2.81
1	212	226	YKKAFIDLDPEALGS	Consensus (smm/nn/sturniolo)		2.81
1	4	18	LLKLAAMGTAACALL	Consensus (smm/nn/sturniolo)		2.87
1	92	106	VQMLGFDIRDWAEQG	Consensus (smm/nn/sturniolo)	2	2.87
1	91	105	AVQMLGFDIRDWAEQ	Consensus (smm/nn/sturniolo)	7	2.88
1	90	104	TAVQMLGFDIRDWAE	Consensus (smm/nn/sturniolo)	14	2.89
1	89	103	PTAVQMLGFDIRDWA	Consensus (smm/nn/sturniolo)	17	2.90
1	93	107	QMLGFDIRDWAEQGA	Consensus (smm/nn/sturniolo)	1	2.93
1	247	261	FSGRDWNLASAMVIE	Consensus (comb.lib./smm/nn)		3
1	1	15	MHKLLKLAAMGTAAC	Consensus (comb.lib./smm/nn)		3

Immunogenicity prediction (Class I peptide)

-Prediction of the relative ability of a peptide/MHC complex to elicit an immune response.
(http://tools.iedb.org/immunogenicity)

Masking: default
Masked variables: [1, 2, 'cterm']

Predictions:

Peptide	Length	Score	
QMLGFDIRDWAEQGA	15	0.63779	1
VQMLGFDIRDWAEQG	15	0.57139	2
GQPWQDATIFDAVVL	15	0.56498	3
PTNWDELIALLDNFK	15	0.55901	4
KEPTNWDELIALLDN	15	0.51402	5
EPTNWDELIALLDNF	15	0.49149	6
AVQMLGFDIRDWAEQ	15	0.44513	7
QPWQDATIFDAVVLS	15	0.42336	8
WQDATIFDAVVLSFG	15	0.38641	9
YKKAFIDLDPEALGS	15	0.37934	10
KKAFIDLDPEALGSD	15	0.3388	11
PWQDATIFDAVVLSF	15	0.33696	12
TNWDELIALLDNFKA	15	0.31533	13
TAVQMLGFDIRDWAE	15	0.29432	14
ATIFDAVVLSFGPDF	15	0.2787	15
KEGWEKVIPAPLQEF	15	0.25799	16
PTAVQMLGFDIRDWA	15	0.2547	17
GGTEAMTVLRARVTA	15	0.24239	18
EDAVKELVVAVEAAK	15	0.23977	19
GGGTEAMTVLRARVT	15	0.23689	20
TIFDAVVLSFGPDFY	15	0.2306	21
SEDAVKELVVAVEAA	15	0.22819	22
GRDWNLASAMVIEGK	15	0.22687	23
GRDWNLASAMVIEGK	15	0.22687	24
GGGGTEAMTVLRARV	15	0.22668	25
KAFIDLDPEALGSDT	15	0.22356	26
SSEDAVKELVVAVEA	15	0.22039	27
AGGGGTEAMTVLRAR	15	0.2146	28
VAGGGGTEAMTVLRA	15	0.21104	29
IFDAVVLSFGPDFYK	15	0.20879	30
GTEAMTVLRARVTAG	15	0.20145	31
SGRDWNLASAMVIEG	15	0.1915	32
EGWEKVIPAPLQEFA	15	0.18201	33
TEAMTVLRARVTAGN	15	0.17166	34
FSGRDWNLASAMVIE	15	0.14568	35
AFIDLDPEALGSDTM	15	0.10899	36
WAEQGALGNLDTVAS	15	0.10798	37
AEQGALGNLDTVASK	15	0.07432	38
FDAVVLSFGPDFYKK	15	0.06142	39
AMGTAACALLAGMAP	15	0.03492	40
LKLAAMGTAACALLA	15	0.01836	41
AAMGTAACALLAGMA	15	0.01793	42
KLAAMGTAACALLAG	15	0.01673	43
LAAMGTAACALLAGM	15	0.00018	44
MGTAACALLAGMAPV	15	-0.01359	45
RDWNLASAMVIEGKA	15	-0.03466	46
QSAFNVVKGSAPART	15	-0.04258	47
TAGNAPTAVQMLGFD	15	-0.04935	48
LLKLAAMGTAACALL	15	-0.05317	49
LLKLAAMGTAACALL	15	-0.05317	50

LLKLAAMGTAACALL	15	-0.05317	51
ESPAFQSAFNVVKGS	15	-0.06147	52
AIESPAFQSAFNVVK	15	-0.0616	53
EQGALGNLDTVASKE	15	-0.07372	54
VTAGNAPTAVQMLGF	15	-0.08376	55
FNVVKGSAPARTDVP	15	-0.08798	56
IESPAFQSAFNVVKG	15	-0.09316	57
SAFNVVKGSAPARTD	15	-0.09572	58
AFNVVKGSAPARTDV	15	-0.09862	59
WNLASAMVIEGKAGV	15	-0.10811	60
GALGNLDTVASKEGW	15	-0.1087	61
QGALGNLDTVASKEG	15	-0.12409	62
DWNLASAMVIEGKAG	15	-0.1291	63
DAVVLSFGPDFYKKA	15	-0.15664	64
SPAFQSAFNVVKGSA	15	-0.18826	65
KLLKLAAMGTAACAL	15	-0.20311	66
KLLKLAAMGTAACAL	15	-0.20311	67
KLLKLAAMGTAACAL	15	-0.20311	68
VVLSFGPDFYKKAFI	15	-0.21345	69
HKLLKLAAMGTAACA	15	-0.22121	70
HKLLKLAAMGTAACA	15	-0.22121	71
HKLLKLAAMGTAACA	15	-0.22121	72
AVVLSFGPDFYKKAF	15	-0.2528	73
MHKLLKLAAMGTAAC	15	-0.28214	74
MHKLLKLAAMGTAAC	15	-0.28214	75
MHKLLKLAAMGTAAC	15	-0.28214	76
PAFQSAFNVVKGSAP	15	-0.30832	77

PROTEIN 2 RESULTS

http://tools.immuneepitope.org/mhcii/

#	Start	End	Peptide	Method used		Percentile rank
1	251	265	ALGLTSEAQMAASVL	Consensus (comb.lib./smm/nn)		0.77
1	252	266	LGLTSEAQMAASVLG	Consensus (comb.lib./smm/nn)		0.85
1	276	290	KDSYKLLQSGGLQPR	Consensus (comb.lib./smm/nn)		0.96
1	100	114	PYTEWARKALVMAAF	Consensus (comb.lib./smm/nn)		1.19
1	101	115	YTEWARKALVMAAFT	Consensus (comb.lib./smm/nn)	13	1.19
1	102	116	TEWARKALVMAAFTN	Consensus (comb.lib./smm/nn)		1.19
1	103	117	EWARKALVMAAFTNY	Consensus (comb.lib./smm/nn)		1.21
1	274	288	WYKDSYKLLQSGGLQ	Consensus (comb.lib./smm/nn)		1.33
1	277	291	DSYKLLQSGGLQPRE	Consensus		1.33

				(comb.lib./smm/nn)		
1	235	249	TRQVEEALARLVEAY	Consensus (comb.lib./smm/nn)	2	1.33
1	157	171	IPDVTRDQAASRRAI	Consensus (smm/nn/sturniolo)		1.40
1	236	250	RQVEEALARLVEAYY	Consensus (comb.lib./smm/nn)	1	1.42
1	253	267	GLTSEAQMAASVLGK	Consensus (comb.lib./smm/nn)		1.47
1	275	289	YKDSYKLLQSGGLQP	Consensus (comb.lib./smm/nn)		1.53
1	154	168	FRQIPDVTRDQAASR	Consensus (smm/nn/sturniolo)	19	1.59
1	155	169	RQIPDVTRDQAASRR	Consensus (smm/nn/sturniolo)		1.59
1	156	170	QIPDVTRDQAASRRA	Consensus (smm/nn/sturniolo)		1.59
1	158	172	PDVTRDQAASRRAIA	Consensus (smm/nn/sturniolo)	17	1.59
1	159	173	DVTRDQAASRRAIAA	Consensus (smm/nn/sturniolo)	16	1.59
1	160	174	VTRDQAASRRAIAAM	Consensus (smm/nn/sturniolo)		1.59
1	148	162	IIGLSYFRQIPDVTR	Consensus (smm/nn/sturniolo)	20	1.89
1	237	251	QVEEALARLVEAYYA	Consensus (comb.lib./smm/nn)	3	1.92
1	149	163	IGLSYFRQIPDVTRD	Consensus (smm/nn/sturniolo)	18	1.93
1	99	113	HPYTEWARKALVMAA	Consensus (comb.lib./smm/nn)		1.94
1	151	165	LSYFRQIPDVTRDQA	Consensus (smm/nn/sturniolo)	6	1.97
1	150	164	GLSYFRQIPDVTRDQ	Consensus (smm/nn/sturniolo)	7	2.03
1	254	268	LTSEAQMAASVLGKN	Consensus (comb.lib./smm/nn)		2.28
1	152	166	SYFRQIPDVTRDQAA	Consensus (smm/nn/sturniolo)	11	2.46
1	273	287	QWYKDSYKLLQSGGL	Consensus (comb.lib./smm/nn)		2.46
1	238	252	VEEALARLVEAYYAL	Consensus (comb.lib./smm/nn)	5	2.49
1	246	260	VEAYYALGLTSEAQM	Consensus (smm/nn/sturniolo)		2.56
1	247	261	EAYYALGLTSEAQMA	Consensus (smm/nn/sturniolo)		2.61
1	34	48	TALLSGTIAVLIPLA	Consensus (comb.lib./smm/nn)	10	2.66
1	248	262	AYYALGLTSEAQMAA	Consensus (smm/nn/sturniolo)		2.68
1	190	204	AKTKIRVARDQLAGK	Consensus (smm/nn/sturniolo)		2.71
1	191	205	KTKIRVARDQLAGKE	Consensus		2.71

				(smm/nn/sturniolo)		
1	192	206	TKIRVARDQLAGKEM	Consensus (smm/nn/sturniolo)		2.71
1	193	207	KIRVARDQLAGKEMQ	Consensus (smm/nn/sturniolo)		2.71
1	194	208	IRVARDQLAGKEMQI	Consensus (smm/nn/sturniolo)		2.71
1	163	177	DQAASRRAIAAMQEV	Consensus (comb.lib./smm/nn)		2.74
1	250	264	YALGLTSEAQMAASV	Consensus (comb.lib./smm/nn)		2.76
1	234	248	NTRQVEEALARLVEA	Consensus (comb.lib./smm/nn)	4	2.90
1	143	157	AYAYYIIGLSYFRQI	Consensus (smm/nn/sturniolo)		2.92
1	249	263	YYALGLTSEAQMAAS	Consensus (comb.lib./smm/nn)		2.94
1	164	178	QAASRRAIAAMQEVI	Consensus (comb.lib./smm/nn)		2.96
1	35	49	ALLSGTIAVLIPLAG	Consensus (comb.lib./smm/nn)	8	3

Immunogenicity prediction (Class I peptide)

-Prediction of the relative ability of a peptide/MHC complex to elicit an immune response.
(http://tools.iedb.org/immunogenicity)

Masking: default
Masked variables: [1, 2, 'cterm']

Predictions:

Peptide	Length	Score	
RQVEEALARLVEAYY	15	0.48196	1
TRQVEEALARLVEAY	15	0.45016	2
QVEEALARLVEAYYA	15	0.4075	3
NTRQVEEALARLVEA	15	0.33414	4
VEEALARLVEAYYAL	15	0.33083	5
LSYFRQIPDVTRDQA	15	0.27236	6
GLSYFRQIPDVTRDQ	15	0.26796	7
ALLSGTIAVLIPLAG	15	0.23049	8
AYAYYIIGLSYFRQI	15	0.20828	9
TALLSGTIAVLIPLA	15	0.20148	10
SYFRQIPDVTRDQAA	15	0.19206	11
HPYTEWARKALVMAA	15	0.17038	12
YTEWARKALVMAAFT	15	0.16864	13
PYTEWARKALVMAAF	15	0.16563	14
VTRDQAASRRAIAAM	15	0.16148	15
DVTRDQAASRRAIAA	15	0.16139	16
PDVTRDQAASRRAIA	15	0.13666	17
IGLSYFRQIPDVTRD	15	0.12019	18
FRQIPDVTRDQAASR	15	0.11751	19
IIGLSYFRQIPDVTR	15	0.08394	20
KTKIRVARDQLAGKE	15	0.08045	21
IPDVTRDQAASRRAI	15	0.0761	22
TKIRVARDQLAGKEM	15	0.06848	23

RQIPDVTRDQAASRR	15	0.05639	24
AKTKIRVARDQLAGK	15	0.05548	25
QIPDVTRDQAASRRA	15	0.05031	26
TEWARKALVMAAFTN	15	0.04458	27
DQAASRRAIAAMQEV	15	0.03111	28
QAASRRAIAAMQEVI	15	0.01951	29
VEAYYALGLTSEAQM	15	-0.00883	30
EWARKALVMAAFTNY	15	-0.03334	31
KIRVARDQLAGKEMQ	15	-0.10516	32
EAYYALGLTSEAQMA	15	-0.14475	33
AYYALGLTSEAQMAA	15	-0.17251	34
YYALGLTSEAQMAAS	15	-0.18618	35
IRVARDQLAGKEMQI	15	-0.25936	36
YALGLTSEAQMAASV	15	-0.27861	37
ALGLTSEAQMAASVL	15	-0.31223	38
LGLTSEAQMAASVLG	15	-0.32501	39
GLTSEAQMAASVLGK	15	-0.33767	40
LTSEAQMAASVLGKN	15	-0.35119	41
DSYKLLQSGGLQPRE	15	-0.54666	42
KDSYKLLQSGGLQPR	15	-0.6116	43
WYKDSYKLLQSGGLQ	15	-0.66386	44
YKDSYKLLQSGGLQP	15	-0.68051	45
QWYKDSYKLLQSGGL	15	-0.81303	46

6.2.4 ANTIBODY EPITOPE PREDICTION RESULTS

Bepipred Linear Epitope Prediction

Average:0.152 **Minimum:**-1.618 **Maximum:**1.742 **Threshold:** 0.350

PROTEIN 1 RESULTS

Predicted epitopes:

No.	Start Position	End Position	Peptide	Peptide Length
1	23	31	PVANAQEKQ	9
2	41	46	SGGEAS	6
3	55	58	LESK	4
4	60	62	ISW	3
5	64	74	DMPVAGGGGTE	11
6	84	90	TAGNAPT	7
7	102	109	WAEQGALG	8
8	113	123	TVASKEGWEKV	11
9	125	126	PA	2
10	129	129	Q	1
11	131	133	FAK	3
12	145	145	I	1
13	158	169	LDKAGGKEPTNW	12
14	181	182	AQ	2
15	184	197	ITPIAHGGQPWQDA	14
16	210	210	D	1
17	221	231	PEALGSDTMKQ	11
18	244	250	DDNFSGR	7

19	252	252	W	1
20	278	286	LKAGKKPGE	9
21	294	301	PGTQGAVT	8
22	314	319	EDKVPA	6
23	326	332	AIESPAF	7
24	340	356	KGSAPARTDVPDTAFDA	17
25	363	371	ADVKEANSK	9
26	380	388	HGYANPAAV	9
27	402	409	GQLSSEDA	8

PROTEIN 2 RESULTS

Predicted epitopes:

No.	Start Position	End Position	Peptide	Peptide Length
1	1	3	MRS	3
2	15	22	RYQRAGDR	8
3	50	58	CASKNDDID	9
4	64	73	ETIDPADKLY	10
5	83	88	GRLDEA	6
6	97	104	RQHPYTEW	8
7	117	124	YRKGNYEE	8
8	134	143	TLYPTSPESA	10
9	159	167	DVTRDQAAS	9
10	180	192	RFPNSEYTDDAKT	13
11	201	203	LAG	3
12	230	238	EEYSNTRQV	9
13	259	259	Q	1
14	267	276	KNFPDSQWYK	10
15	286	297	GLQPRENGNSWL	12

6.3 EXPERIMENTAL INFECTIONS RESULTS

The Mann-Whitney test performed considering the differences of the ratios of the percentages of IFN-γ^+ cells in response to *Yersinia* and *Brucella* antigens between the animals immunized with *Yersinia* vs those infected with *Brucella*, showed that the difference between the two set of values is statistically significant ($p<0.05$). The statistical analysis was also performed considering the ratios of the percentages of IFN-γ^+ cells, $CD4^+$ IFN-γ^+ T cells and $CD8^+$ IFN-γ^+ T cells in response to *Yersinia* and *Brucella* antigens.

Results obtained from animals immunized with *Y. enterocolitica* (Fig.1) showed an expansion of IFN-γ^+ lymphocytes, following the re-exposure to YeO:9 (2.36% in immunized versus 0.36% in uninfected controls) but not to *Brucella* antigen (Fig.1A). The difference of IFN-γ production by cells stimulated by the two antigens is statistically significant. In details, the production of IFN-γ by $CD4^+$ and $CD8^+$ T cell subsets is similar, even if the role of $CD4^+$ T cells in producing this cytokine is slightly higher (Fig.1B,C). This is the first evidence of a different behavior of T lymphocytes when stimulated with different antigens, that are normally cross-reactive.Results showed that $\gamma\delta^+$ T-lymphocytes display a minor contribution to IFN-γ production, reaching 0.1% in response to YeO:9 and 0.01% in seronegative animals (Fig. 1D).

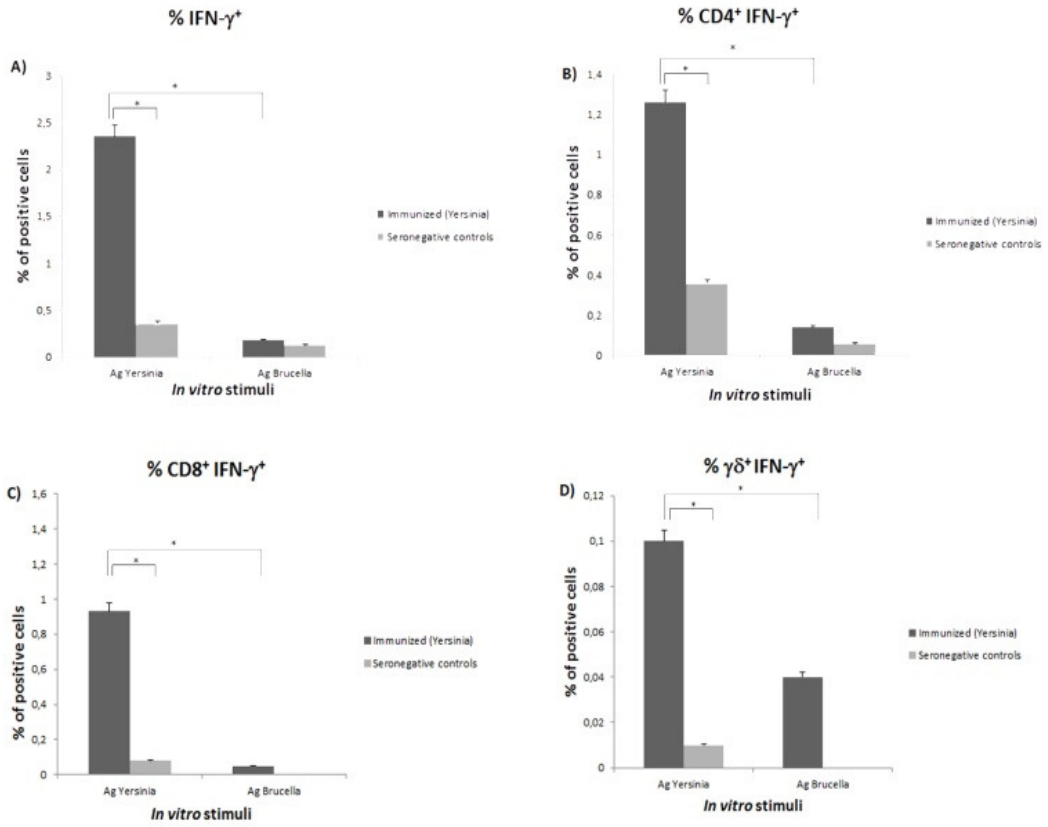


Fig. 1. Flow cytometric analysis in *Yersinia*-immunized animals: the antigen-specific stimulation resulted in a higher percentage of IFN-γ^+ T-lymphocytes (2.36%), compared with the same cells but stimulated with *Brucella* antigen (0.18% - Panel A). The detection of $CD4^+$ IFN- γ^+ T-lymphocytes (Panel B) and CD8+ IFN-γ^+ T-lymphocytes (Panel C) 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. $\gamma\delta^+$ IFN-γ^+ T cells detection (Panel D) amounted to 0.1 % in response to YeO:9 and to 0.04% in seronegative animals. Experiments were repeated three times and data showed are the mean of different experiments.*p<0.05

The results obtained by cattle naturally infected by *Brucella* (Fig.2), showeda good production of IFN-γ when cells were stimulated both by *Brucella* and *Yersinia* antigens (Fig.2A). Data showed that in the same animals IFN-γ^+ lymphocytes are similarly present when *in vitro* exposed to *Yersinia* (5.99%) or *Brucella* stimulus (5.70%). The analysis of T-cell subsets showed that $CD4^+$ T cells produce a similar percentage of IFN-γ^+ in response to both antigens, thus confirming a cross-reactivity in cell-mediated immunity (Fig. 2B). The analysis of T cell subsets showed a higher production of IFN-γ by $CD4^+$ T cells rather than $CD8^+$ lymphocytes (Fig.2B,C). 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* antigen) and *Brucella* (2.77% *in vitro* stimulated with *Brucella* antigen). We did not observe any significant statistic differences when $CD8^+$ T cells (Fig.2C) were analyzed in response to the two antigens. Data regarding the analysis of $\gamma\delta^+$ T cells revealed that the difference of IFN-γ production is not statistically significant between animals positive for *Brucella* and *in vitro* stimulated with the two antigens, but a statistically significant difference was detected when PBMC from infected and seronegative cattle were exposed to *Yersinia.* (Fig.2D).

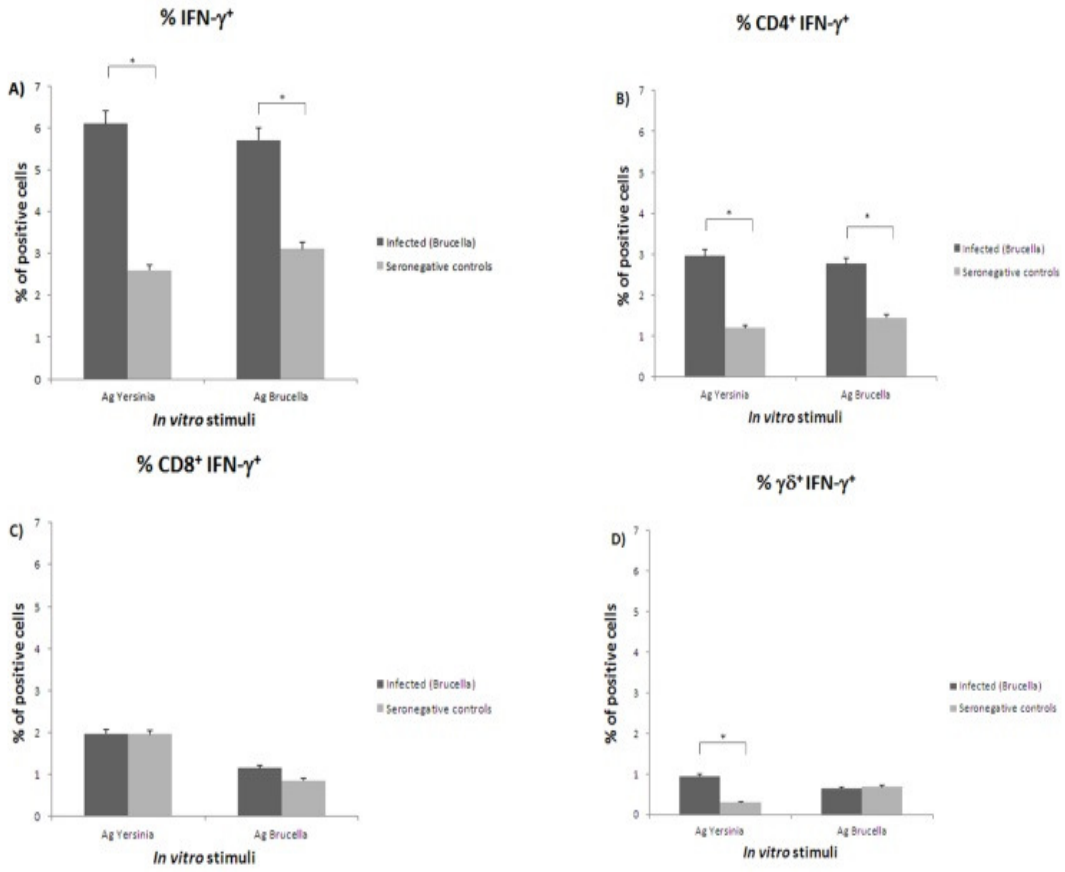


Fig. 2. Flow cytometric analysis in animals naturally infected by *Brucella*: IFN-γ^+ T-cells detection is similar when cells are *in vitro*exposed to *Yersinia* (6.09%) or *Brucella* antigens (5.7%) (Panel A). $CD4^+$ T cells (Fig. 2B) produce IFN-γ when exposed to both antigens (2,96% to *Yersinia* and 2,77% to *Brucella*). Statistical significances were assessed and percentages of IFN-γ^+ and $CD4^+$ IFN-γ^+ T cells were compared to seronegative controls (1,20% when exposed to *Yersinia* and 1,45% to *Brucella*) (*p<0.05). No significant expansion of $CD8^+$ (Panel C) and $\gamma\delta^+$ IFN-γ^+ T was observed in response to both the antigens (Panel D). Data shown are the mean of the percentages obtained in different experiments.

The immune response mounted by $CD4^+$- and $CD8^+$- IFN-γ^+ $CD45R0^+$ T cells in *Yersinia*-immunized animals was analyzed but no statistically significant expansions were found (data not shown).

The analysis of memory cells producing IFN-γ in animals naturally infected with *Brucella* revealed that there is a statistically significant difference in the expansion of $CD4^+$ IFN-γ^+ $CD45R0^+$ T cells when animals are stimulated with the two antigens: the amount of these cells is higher when stimulated with *Yersinia* than with *Brucella* antigens (Fig. 3A). $CD8^+$ IFN-γ^+ $CD45R0^+$ T cells detection showed statistically significant difference after stimulation with both antigens (Fig. 3B)

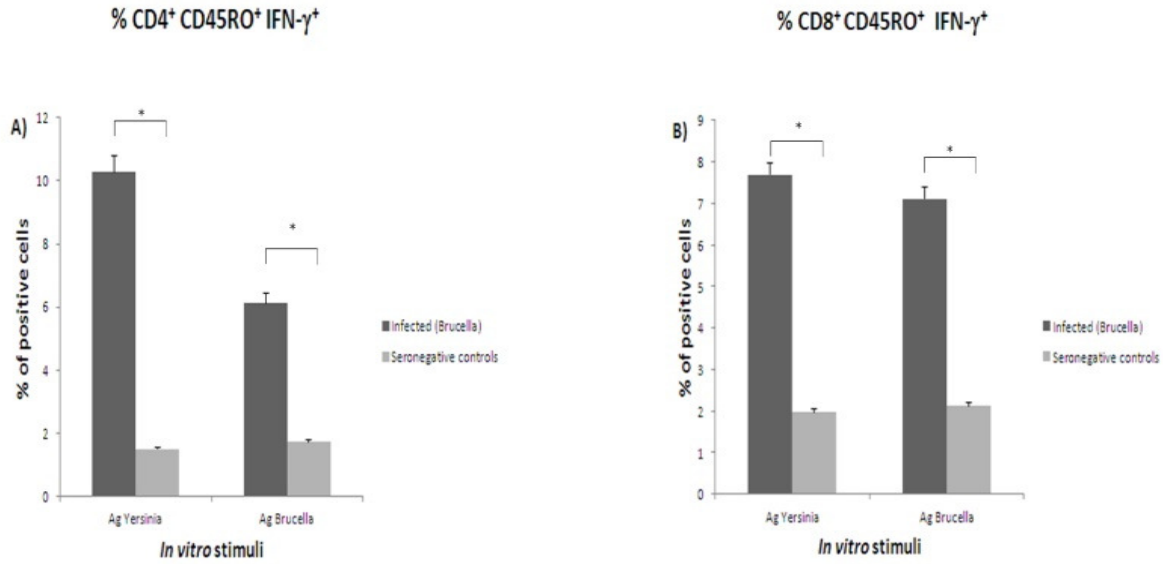


Fig. 3. **Analysis of IFN-γ-producing memory cells in animals naturally infected with *Brucella***: the amount of $CD4^+$ IFN-γ^+ $CD45R0^+$ T cells detected is statistically higher when in vitro stimulated with *Yersinia* antigen (10,27%) compared to *Brucella* antigen (6,13%) (panel A) (*p<0.05). $CD8^+$ IFN-γ^+ $CD45R0^+$T cells detection shows there is no statistically significant difference after stimulation with *Yersinia* (7,685%) or *Brucella* antigens (7,11%), although it is higher in the *Brucella* seropositive than in the seronegative animals (panel B).

The percentage of IFN-γ^+ cells and the different pattern of response, exclusive for *Yersinia* antigen in animals immunized with *Ye*O:9, could open a new possibility for the diagnostic determination of the two pathogens.

The bioinformatics studies allowed the selection of two proteins, based on different criteria including:

- lenght

- in silicostudies

- nature and numerosity of B cell/Tcellepitopes

- nature and abundance of epitopes for T cells

We are evaluating to produce them in vitro or produce only some epitopesselected according to the following criteria:

a. Best likelihood to be loaded onto MHCI or MHCII

b. Best immunogenicity

c. UNICITY : avoiding overlap so that each epitope didn't stimulate both CD4 and / or CD8

d. NO OVERLAPPING with linear B cell epitopes

e. Exclusion of signal peptides

For each protein we selected four epitopes, two loaded onto MHCI and two onto MHCII

Sugar-binding periplasmic protein:

- MHC I peptids

H-2-Db H-2-Db	1	378	388	11	MAHGYANPAAV	Consensus (ann/smm)	7	1.45
H-2-Db	1	140	150	11	AAPVNIHSTNW	Consensus (ann/smm)	10	1.45

- MHC II peptids

1	192	206	QPWQDATIFDAVVLS	Consensus (comb.lib./smm/nn)	8	0.86
1	236	250	RQVEEALARLVEAYY	Consensus (comb.lib./smm/nn)	1	1.42

Competence Lipoprotein

- MHC I peptids

H-2-Kd	1	61	71	11	KYVETIDPADK	Consensus (ann/smm)	1	0.95
H-2-Ld	1	244	254	11	RLVEAYYALGL	ann	5	2.2

- MHC II peptids

1	192	206	QPWQDATIFDAVVLS	Consensus (comb.lib./smm/nn)	8	0.86

1	191	205	GQPWQDATIFDAVVL	Consensus (comb.lib./smm/nn)	3	0.87

On the other side, the experimental work carried out allowed the detection, for the first time in cattle, of lymphocyte populations, $CD4^+$ 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* antigen. Even if the correspondent behavior cannot be detected in *Brucella*-infected animals, due to the persistent cross-reactivity between the two antigens, the analysis of the expansion of IFN-γ^+ cells in response to *Brucella* and *Y. enterocolitica* antigens could be useful to distinguish between *Yersinia* and *Brucella* infection in cattle. Non-LPS *Brucella* proteins could be of paramount importance for the specific diagnosis of *Brucella* infection, since they allow to avoid cross-reactivity issues.

Further research is needed on the identification, isolation, characterization and cloning of both inner and outer membrane proteins which could be used as diagnostic antigens that are more sensitive and specific. This should be followed by the development of subunit or live antigen-deleted vaccines, able to protect animals without interfering with diagnostic tests, and should be a major goal of research in the near future. The antigens obtained from the comparative analysis of bacterial protein patterns will be synthesized (whole protein or its peptides) and used for in vitro stimulation of lymphocytes obtained from Balb-c mice experimentally infected. These antigens will also be retested on the mouse model to verify their properties specific immunogenic. For this purpose, we will infect two groups of mice with *Brucella melitensis* and *Yersinia enterocolitica* strains respectively , according to Wang *et al*., 2013, and after scarification, we will withdraw the sera and the spleens. The sera will be used for the determination and eventual quantization of cytokine production; from spleens we will isolate lymphocytes, which will be cultured and *in vitro* stimulated with the antigens selected, in order to test their specificity. They will be treated by surface and intracytoplasmic markers (CD3, CD4, CD8, IFN-γ, CD69, CCR5) in order to compare, by flow cytometric analyses, the cell activation state and determine, for comparison, which cytokine (and/or which cell population) is specifically activated in samples from mice infected with *Brucella,* but not in samples from mice infected with *Yersinia.*

The future research aims to verify, on one side, whether there are diagnostic protein antigens able to discriminate *Brucella* infection from other diseases, on the other hand, a pattern of lymphocyte activation, with relative cytokines production, which can be useful to increase the panel of available laboratory diagnostic tests.

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