Monoclonal antibodies as tools in antigen detection assay and vaccine development:

design of a sensitive detection test for *Brucella* bacteria and profiling of the malaria vaccine candidate antigen reticulocyte-binding homolog 2 (PfRH2)

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Dekan

Dedicated to my family

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Acknowledgements

Summary

We aimed at identifying immunodominant *Brucella* antigens for implementation in new detection tools or for subunit vaccine development. In particular, our strategy was to produce *Brucella* cell surface antigen-specific monoclonal antibodies (mAbs) for the development of an antigen capture assay for the detection of *Brucella* cells as potential bio threat agents in complex samples. We generated a panel of *Brucella* lipopolysaccharide (LPS)-specific mAbs by immunising mice with inactivated *B. melitensis* and *B. abortus* cells. The mAbs recognised *Brucella* species with 'smooth' LPS independently of the way how the bacterial cells were inactivated. Two mAbs were implemented into a bead-based Luminex assay detecting 'smooth' *Brucella* spp. with species-dependent detection limits of 2×10^2 to 8×10^4 cells per mL. Integration of the Luminex assay into a multiplex format enabled simultaneous detection of *Brucella* spp. and three other bio threat agents within a single sample. The developed Luminex assay may be applied for the detection of whole *Brucella* cells both in natural *Brucella* outbreak and in bioterrorism attack scenarios.

We also tried to generate mAbs against *Brucella* cell surface proteins from mice immunised with inactivated whole *Brucella* cells. While serum antibody responses against both LPS and protein antigens were seen in Western blotting analyses, attempts to generate protein-specific mAbs failed, most likely due to the immunodominant nature of the LPS. Western blot analyses with *Brucella* lysate also identified antibodies against some immunodominant *Brucella* proteins in the serum of cattle naturally infected with *Brucella* spp., however, identification of the recognised proteins with a *Brucella*-specific peptide microarray failed.

In a second part of the thesis we aimed at evaluating the potential of the *Plasmodium falciparum* reticulocyte-binding homolog 2 (PfRH2) present in the rhoptries as a malaria blood stage vaccine antigen. We produced PfRH2-specific mAbs by immunising mice with the 40kDa receptor-binding domain of PfRH2. The PfRH2-specific mAbs cross-reacted with the natural PfRH2 protein present in schizont stage parasites and showed a rhoptry-characteristic staining pattern in immunofluorescence microscopy. However when evaluated in functional *in vitro* and *in vivo* assays PfRH2-specific mAbs showed no inhibitory effect on erythrocyte invasion. Furthermore, the invasion-inhibitory effect of mAbs specific for the cysteine-rich protective antigen (PfCyRPA) was not enhanced by PfRH2-specific mAbs.

Zusammenfassung

Unser Ziel war es immundominante *Bruzellen* Antigene zu identifizieren, welche in neuen Detektionsverfahren oder bei der Impfstoffentwicklung Anwendung finden. Wir verfolgten im speziellen die Strategie, spezifische monoklonale Antikörper (mAk) gegen Oberflächenantigene von *Bruzellen* herzustellen und diese für die Entwicklung von Antigen Detektionstests zu verwenden um *Bruzellen* als potenzielle biologische Kampfstoffe in komplexen Proben nachzuweisen. Wir produzierten Lipopolysaccharid (LPS)-spezifische mAk indem Mäuse mit inaktivierten *B. melitensis* und *B. abortus* Bakterien immunisiert wurden. Die mAk erkannten *Bruzellen* Arten mit "glattem" LPS unabhängig davon, wie die Bakterien inaktiviert wurden. Zwei mAk wurden in einem Luminex Verfahren, welches auf magnetischen Mikrokugeln basiert, eingesetzt und "glatte" *Bruzellen* spp. konnten je nach Art bis zu einem spezifischen Detektionslimit von 2×10^2 bis 8×10^4 Zellen pro mL nachgewiesen werden. Die Umsetzung des Luminex Testverfahrens in eine Multiplex Anwendung ermöglichte den gleichzeitigen Nachweis von *Bruzellen* spp. sowie drei weiteren biologischen Kampfstoffen in einer einzigen Probe. Im Falle eines natürlichen oder bioterroristischen Ausbruchsszenarios, könnte das hier entwickelte Luminex Testverfahren für den Nachweis von ganzen *Bruzellen* eingesetzt werden.

Des Weiteren strebten wir an mAk gegen Oberflächenproteine von *Bruzellen* herzustellen indem Mäuse mit inaktivierten ganzen *Bruzellen* immunisiert wurden. Obwohl wir in Western Blot Analysen Serumantikörper Reaktionen gegen LPS und Protein Antigene beobachten konnten, war es uns, sehr wahrscheinlich aufgrund der Immundominanz des LPS, nicht möglich, Protein-spezifische mAk herzustellen. Ferner zeigten Western Blot Analysen auf *Bruzellen* Lysat, dass Antikörper gegen immundominante *Bruzellen* Proteine im Serum von natürlich infizierten Rindern vorhanden sind. Die Bestimmung dieser detektierten Proteine mit Hilfe eines *Bruzellen*-spezifischen Peptid-Mikroarrays war jedoch nicht möglich.

Der zweite Teil der Arbeit hatte zum Ziel das "reticulocyte-binding homolog 2" Protein (PfRH2), welches in den Rhoptrien von *Plasmodium falciparum* zu finden ist, auf seine Eignung als potenzielles Vakzin Antigen für Malaria Blutstadien zu untersuchen. Nachdem Mäuse mit der rekombinanten 40kDa Rezeptorbindungsdomäne von PfRH2 immunisiert wurden, konnten PfRH2-spezifische mAk hergestellt werden. Diese PfRH2-spezifischen mAk zeigten eine Kreuzreaktivität mit dem endogenen PfRH2 Protein im Schizontenstadium der Parasiten sowie eine Rhoptrien-charakteristische Färbung in der Immunfluoreszenzmikroskopie. Die Evaluierung der PfRH2-spezifischen mAk in funktionellen *in vitro* und *in vivo* Verfahren zeigte jedoch keine Inhibierung der Erythrozyten Invasion. Des Weiteren konnte der wachstumsinhibierende Effekt von PfCyRPA- (cysteine-rich protective antigen) spezifischen mAk durch die PfRH2-spezifischen mAk nicht gesteigert werden.

1. Introduction

1.1 Brucellosis

1.1.1 Epidemiology

Brucellosis, also known as 'Malta fever', 'undulant fever' or 'Bang's disease' represents the most common bacterial zoonosis worldwide. The disease has been named after David Bruce who discovered the genus *Brucella* in 1887 in a soldier who had died from a then unknown febrile illness [1]. Although brucellosis is mainly associated with animals, especially domestic livestock, wildlife and marine mammals, it accounts for more than 500,000 new human infections annually [2]. Brucellosis shows a worldwide distribution and is prevalent in more than sixty countries in almost all continents (Fig. 1) with the highest incidences in Central Asia including Mongolia, the Middle East and North Africa [3–5]. Recent foodborne outbreaks have been reported from Peru and Mongolia threatening thousands of people [6].
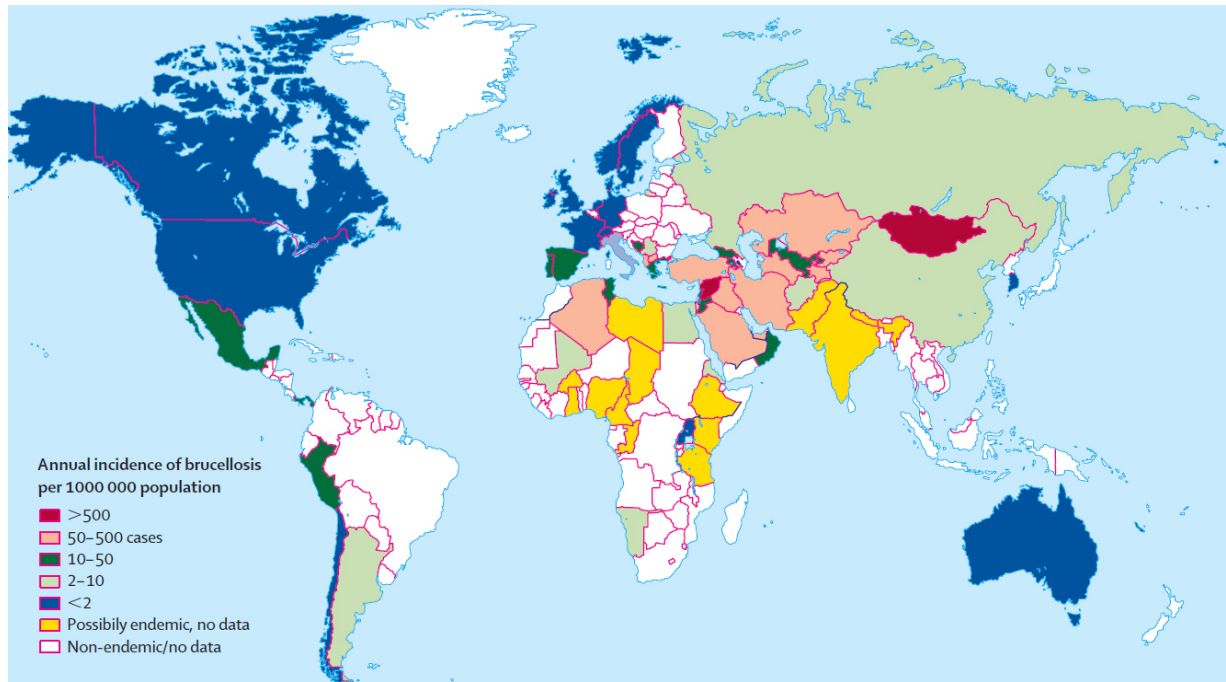
Figure 1. Worldwide incidence of human brucellosis since 2000 [4]

1.1.2 **Causative organism**

The Brucellosis is caused by an infection with gram-negative, aerobe, facultative intracellular and non-motile *Brucella* species (spp.). Genome analyses revealed high similarities between almost all members of the *Brucella* genus. Therefore, there was a debate whether *B. melitensis* could be the ancestor of all other *Brucella* spp. [2, 7–9]. Although, classical virulence genes are absent in *Brucella*, some factors such as the lipopolysaccharide (LPS), the VirB type IV secretion system and flagella genes are somehow associated with virulent functions [1, 10]. Upon invasion of the host via the respiratory tract, the digestive system or small lesions in the skin, *Brucella* spp. are taken up by phagocytic cells such as macrophages or dendritic cells. Within these cells *Brucella* spp. persist and multiply and can systemically spread within the host body [2]. Infections occur in various animals (Table 1) where different *Brucella* spp. show specific host preferences. In shared facilities or pasture however, when different livestock species are in close contact transient infections with other *Brucella* spp. might occur [1, 2]. The six classical species of *Brucella* mainly infect domestic animals and rodents with, *B. melitensis* (biovars 1-3) preferentially infecting sheep and goats, *B. abortus* (1-6, 9) cattle, *B. suis* (1, 3) pigs, *B. canis* dogs, *B. ovis* sheep and *B. neotomae* preferentially infecting rodents. Yet, some newer species such as *B. ceti* and *B. pinnipedialis*, *B. microti* and *B. inopinata* have been identified also affecting wildlife animals including marine mammals [2, 7]. According to new observations from a recent study it is hypothesised that amphibians might also be affected by brucellosis since potentially novel *Brucella* spp. were isolated and identified from African bullfrogs [11]. A brucellosis infection in animals leads to hygroma formation and abscesses, infertility in males but mainly to abortions or still births in pregnant females. Furthermore transmission of *Brucella* spp. between animals occurs via abortions, during mating or via bacteria released into the milk. Resulting losses in food-producing animals are of great economic importance [2, 12].

Species	Host preference
Brucella melitensis	Sheep, goat (*Ovis* spp. and *Capra* spp.)
Brucella abortus	Cattle (*Bos taurus* and *Bos indicus*)
Brucella suis	Pig (*Sus scrofa*)
Brucella canis	Dog (*Canis lupus familiaris*)
Brucella ceti	Dolphin, porpoise, whale (Cetacea)
Brucella pinnipedialis	Seal (Pinnipedia)
Brucella inopinata	Unknown
Brucella ovis	Sheep (*Ovis* spp.)
Brucella neotomae	Desert woodrat (*Neotoma lepida*)
Brucella microti	Common vole (*Microtus arvalis*)
Brucella sp. (baboon isolate)	Baboon (*Papio* spp.)

Table 1. Host preference of *Brucella* species [2]

Humans who are accidental hosts for *Brucella* spp. get infected through direct contact e.g. with body fluids or aerosolised *Brucella* spp. from infected animals, mainly domestic livestock but mostly through the consumption of uncooked or unpasteurised animal products and through laboratory contacts [1, 9]. Human to human transmission is rare which is why humans are suspected to be dead-end hosts [9, 13]. Five *Brucella* spp., *B. melitensis, B. abortus, B. suis, B. canis and B. pinnipedialis*, are pathogenic for humans [3]. However, *B. melitensis* and *B. abortus* are the most virulent species in humans and responsible for the majority of infections and severe diseases [2, 14]. Brucellosis in humans usually presents as an acute febrile illness with flu-like symptoms (chills, headache, joint and back pain and weakness) with an incubation time of two to four weeks, which can, however, last up to two months and more. The disease can cause a systemic infection involving almost any organ or tissue and, may persist for weeks or months, especially in the absence of adequate treatment. While human infections with brucellosis are rarely fatal (1-2% of cases, mainly due to infective endocarditis), persistence, chronification and relapses of the disease cause high morbidities [2, 9, 12].

1.1.3 **Diagnosis**

Diagnosis of human brucellosis

Due to the unspecific and variable signs and symptoms of human brucellosis, a clinical diagnosis alone is unreliable. Although the clinical history and some background information on working activities and eating habits can deliver important clues in the anamnesis [12], a suspected brucellosis case should always be confirmed by laboratory diagnosis. The gold standard diagnosis for *Brucella* infections consists of bacteriological cultures where the causative agent is isolated and cultivated from blood, bone marrow or other tissues and body fluids. Since the bacterial load in the blood is dependent on the infection stage, cultures from blood samples are best performed during the symptomatic phase of the disease were bacteraemia is highest. Microbiological assays may be applied on cultured bacteria to rapidly identify possible *Brucella* colonies. The slide agglutination test is recommended for this purpose using 'smooth' *Brucella* antiserum to agglutinate with *Brucella* spp. from a positive culture. However, since the cultivation of *Brucella* bacteria takes time (approximately from 7 up to 30 days or more), is work extensive and hazardous due to the high risk of infection, serological diagnostic tests are frequently employed to confirm a first suspicion [15]. Such serological testing is fast, non-hazardous, more sensitive and can be applied for routine diagnosis. Yet, since all serological assays measure anti-*Brucella* antibodies, the diagnosis is only indirectly linked to an active *Brucella* infection and diagnosis relies on high or rising antibody titres (standard agglutination titres >1:160). Thus, antibodies are present for a long time after an acute infection and persisting antibodies due to frequent exposure might interfere with the tests and should receive attention. The reference serological method is the serum agglutination test (SAT) with a sensitivity of 84.6% to 91.7%. However, additional agglutination tests such as the slide, plate or card agglutination are also routinely used. The Rose Bengal test (RBT) is a card agglutination test recommended by the WHO with a sensitivity of over 99%. In endemic areas the RBT is used for rapid screening but the test is prone to cross-reactions with the 'smooth' lipopolysaccharide (LPS) of other gram-negative bacteria such as *Yersinia enterocolitica* O9, *Francisella tularensis*, *Vibrio cholerae* O1 and *Escherichia coli* O157 and positive results need to be confirmed with other more specific

methods. Indeed, the 'smooth' LPS, the immune dominant antigen of 'smooth' *Brucella* spp., is frequently used as the capturing antigen in a variety of standard serological assay including RBT, SAT, immunocapture agglutination test (Brucellacapt) and enzyme-linked immunosorbent assay (ELISA). Since 'rough' *Brucella* spp. such as *B. canis* and *B. ovis* do not express a 'smooth' LPS, diagnosis with standard serological assays are not possible. The Coombs' test (CT) can be used to complement the SAT but is quite complex. The same is true for the complement fixation test (CFT) which is not recommended for routine use in small laboratories due to the technical complexity but has a high sensitivity of 91.7%. In addition, indirect ELISAs are recommended for smaller laboratories and frequently used in routine clinical diagnosis mainly in non-endemic areas. The sensitivity of the SAT and ELISA are similar in acute cases but the ELISA is more sensitive in chronic cases. The Brucellacapt assay showed a high sensitivity and specificity of 98% and 96%. SAT, Coombs' test, CF, ELISA and Brucellacapt require proper-equipped laboratories. On the other hand the lateral flow assay (LFA) with the principle of a capture ELISA needing only a single drop of blood as well as the fluorescent polarisation immunoassay (FPA) where antibodies are bound to a fluorescent-labelled *Brucella* O-polysaccharide antigen, are both rapid point-of-care assays suitable for fast and simple testing in poor areas. Sensitivities and specificities of these tests are 95% and 95% for the LFA and 96% and 98% for the FPA, respectively [12, 14, 15].

Diagnosis of animal brucellosis

Testing of livestock is not performed on a regular basis and is rather implemented within control and surveillance programs according to country-specific guidelines [1]. For a defined diagnosis of animal brucellosis, *Brucella* spp. have to be cultured and identified from infected tissues or organs (e.g. placenta), foetal stomach contents, body fluids or milk. The slide agglutination test may be used for a provisional yet fast identification of *Brucella* spp.. Serological assays detecting specific antibodies in serum or milk are widely used for practical reasons. The RBT and the buffered plate agglutination test (BPAT) are used worldwide for serological diagnosis of *Brucella*-infected herds. Indirect ELISA, CFT and the RBT are recommended for the screening of herds and individual

animals. The FPA is highly sensitive (96%) and specific (98%) with human samples but evaluations on animals have been limited and need further investigation. The milk/*Brucella* ring test (BRT), which detects IgM and IgA antibodies that agglutinate when bound by a pre-stained, inactivated whole *B. abortus* cell and the indirect ELISA, are both used primarily for the detection of antibodies in milk from dairy livestock collected from the bulk tank where milk is pooled from different animals. All serological assays perform best for the diagnosis of bovine brucellosis (*B. abortus*) for which they are mainly intended. Some modifications are required on these tools in order to test for other *Brucella* spp.. Overall, care has to be taken since antibodies elicited after vaccination or infections with other bacteria expressing 'smooth' LPS can affect test results of serological assays [1, 7, 12].

Molecular detection tools

Various PCR-based identification methods have been developed in recent years to detect *Brucella* DNA in cultures or clinical samples including blood, infected tissue or organs, body fluids (cerebrospinal and vaginal fluids, semen) and milk of infected humans and animals. However, low numbers of bacteria present in some clinical samples as well as inhibitory effects from different sample components, interfere with the tests. Various sample preparation methods are available commercially for the extraction of *Brucella* DNA and to obtain better purity. Overall, PCR-based assays are more sensitive than culture and more specific than serology [15, 16]. Several molecular targets are commonly used in conventional and/ or real-time PCR assays to confirm *Brucella* infections including 43kDa, *omp2*, *omp31*, 16S rRNA, 16S-23S rRNA spacer, *bcsp*31, IS711, *virB2*, BMEI1162-IS711 and *B. ovis* islands. *Bcsp*31, the gene encoding the 31kDa *Brucella* surface protein, is conserved among all *Brucella* spp. and used most frequently in PCR assays [16]. PCR methods based on a deletion in the *ery*CD operon or mutations in the *wbo*A gene enable the distinction of the vaccine strains *B. abortus* S19 or *B. abortus* RB51, respectively. The vaccine Rev1 of *B. melitensis* can be detected by PCR methods based on single nucleotide polymorphism (SNP) in the *rps*L or *omp2* gene [16, 17]. *In situ* hybridisation assays using labelled *Brucella* DNA or the loop-mediated amplification (LAMP) method working at a constant temperature were

able to detect *Brucella* spp. and might be alternative molecular approaches to PCR. Furthermore, various molecular approaches identifying *Brucella* spp. to a species or biovar level are available for a differential detection/diagnosis or as epidemiological tools [16]. However, molecular assays are not yet routinely implemented in the standard laboratory diagnosis of brucellosis [17].

1.1.4   **Treatment and Prevention**

The recommended treatment against brucellosis consists of a combination therapy with two antibiotics: doxycycline (200 mg/day) for six weeks together with either 2-3 weeks of streptomycin (1 g/day) or with six weeks of rifampicin (600-900 mg/day). Another accepted alternative is the combination of 6 weeks of doxycycline (200 mg/day) with seven days of gentamicin (5mg/kg/day). Fluoroquinolone or trimethoprim/sulfamethoxazole (TMZ/SMZ) antibiotics are likewise used [1, 12]. In case of complications such as spondylitis, neurobrucellosis or *Brucella* endocarditis, longer antibiotic therapy, the use of alternative drugs and/or surgery are required. No standard treatment has been defined for children below the age of eight years. Overall, an early implementation of the treatment using effective drugs as well as an adequate treatment length are important to reduce and prevent complications and relapses [12]. Currently, no effective and safe vaccine is available for human prophylaxis. However, several antigenic proteins have shown a promising protection potential in *Brucella* mouse models [18] and are evaluated for further testing. Brucellosis control and elimination in animals is an efficient way to reduce or prevent human infections; but enormous financial resources would be necessary to achieve this goal [6, 12]. Therefore, education and training on, and provision of, safe food, personal hygiene measures, safe working conditions and proper personal and environmental protection are important methods supporting vaccinations strategies with life attenuated vaccines (Rev1, S19 and RB51) to control brucellosis in livestock and prevent human infections [12, 19, 20].

1.1.5 **Potential of *Brucella* spp. in bioterrorism**

The use of biological reagents in warfare can be dated back to the 14th century BC [21]. Since then, a vast number of bacteria and viruses have been used for military purposes mainly during the First and Second World War [22]. In 1972, the Biological Weapons Convention <<*on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction*>> [23] was signed with the consent of international leaders from all over the world. However, several attacks for example with *Bacillus anthracis* (Anthrax) [24] and *Salmonella typhimurium* [25] have been reported after the signing of the convention giving rise to fears of bioterrorist attacks by individuals or private organisations. Although, there is no evidence that *Brucella* has ever been used for bioterrorist purposes [22], *Brucella* spp. are classified as category B organisms with the potential for application in biological threat situations [26–28]. This classification is explained by the facts that *Brucella* spp. can be easily gained and cultured from natural sources such as infected or dead animals, can be efficiently transmitted via aerosols with an infectious dose of only ten bacteria, are stable under various conditions and are infectious to animals and humans likewise [1, 12]. Although, *Brucella* spp. are not the first choice for a bioterrorism attack, widespread dissemination via the food chain through domestic livestock and corn or contaminations during food manufacturing and storage possess a major threat to humans especially in areas where brucellosis has been eliminated and clinicians, health worker and veterinarians are not aware of the disease. Such an altered distribution would affect both the human and animal population on a social and economic basis [29–31]. Furthermore, although human brucellosis is rarely lethal [32], delayed diagnosis and treatment might lead to severe disease with increased mortality [33].

1.2 Malaria

1.2.1 Distribution

Malaria, one of 'the big three' infectious diseases besides HIV/AIDS and tuberculosis [34], affects almost half of the world's population in 97 countries (Fig. 2). 3.3 billion people are at risk for infection with the malaria parasite and for developing the disease. Although, a decrease in malaria incidence (30%) and mortality (47%) has been reported since 2000, almost 200 million people worldwide are still suffering from malaria with an estimated 584'000 deaths in 2013. Countries in sub-Saharan Africa face the highest malaria burden accounting for 90% of all malaria deaths of which 87% occur in children below the age of five [35, 36].
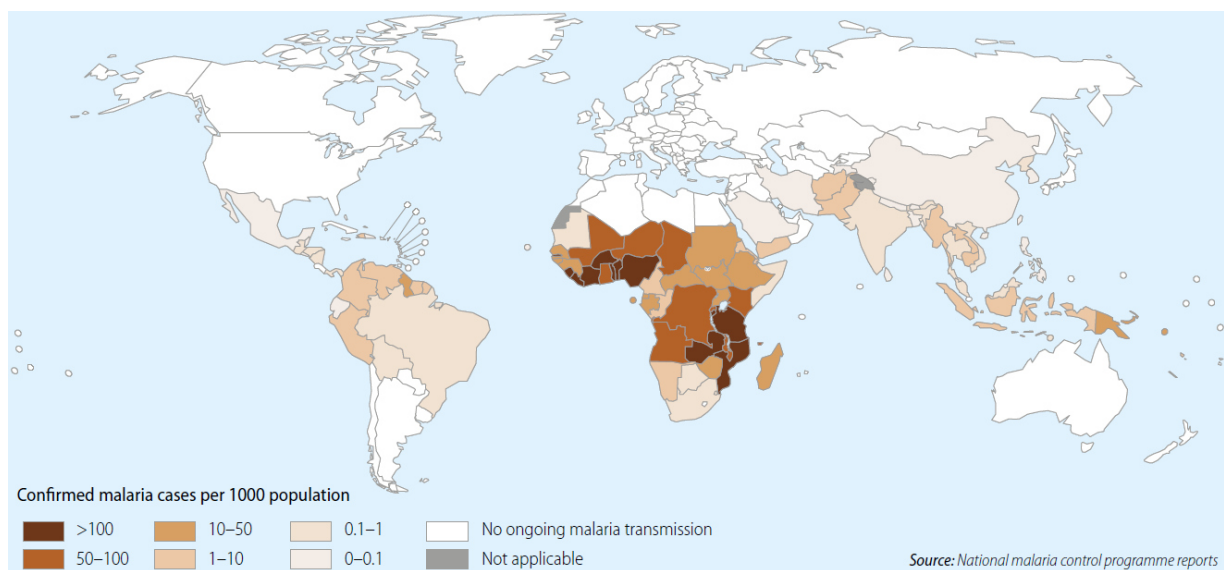
Figure 2: Countries with on-going transmission of malaria, 2013 [36]

1.2.2 **Causative organism**

Malaria in humans is caused by five protozoan *Plasmodium* parasite species, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. *P. falciparum* is responsible for 98% of the malaria-related deaths [37]. Malaria is transmitted to humans exclusively via infected female *Anopheles* mosquito vectors and depends to varying degrees also on climate conditions, seasonality and human immunity. Worldwide, 20 different *Anopheles* species are important for malaria transmission all of which preferentially bite during dusk and night-time [35].

1.2.3 **P. falciparum life cycle**

The life cycle of *Plasmodium* spp. requires a mosquito vector and the human host to undergo repeated asexual and sexual cycles (Fig. 3). During a mosquito bite, infectious sporozoites enter the human body and infect hepatocytes where the parasite develops into merozoites within 6-15 days. Upon rupture of the infected hepatocytes, merozoites enter the bloodstream and actively invade erythrocyte cells through the binding of specific receptors at the erythrocyte surface. Within a parasitophorous vacuole, malaria parasites undergo distinct development steps, from the so-called ring-stage to the trophozoite stage and the schizont stage. On average, 16 merozoites develop asexually from each schizont and are released into the bloodstream once the infected erythrocyte ruptures. Every merozoite is capable of infecting a new erythrocyte. Each asexual cycle of *P. falciparum* takes 48h and the malaria parasites undergo several such cycles before some parasites develop into gendered, sexual gametocytes. These gametocytes persist in the blood where they can be taken up by a new mosquito host during a blood meal. Within the mosquitos parasites mate and undergo further development steps until, after approximately 12 days, infectious sporozoites are formed, able to start a new cycle in humans [37–39].
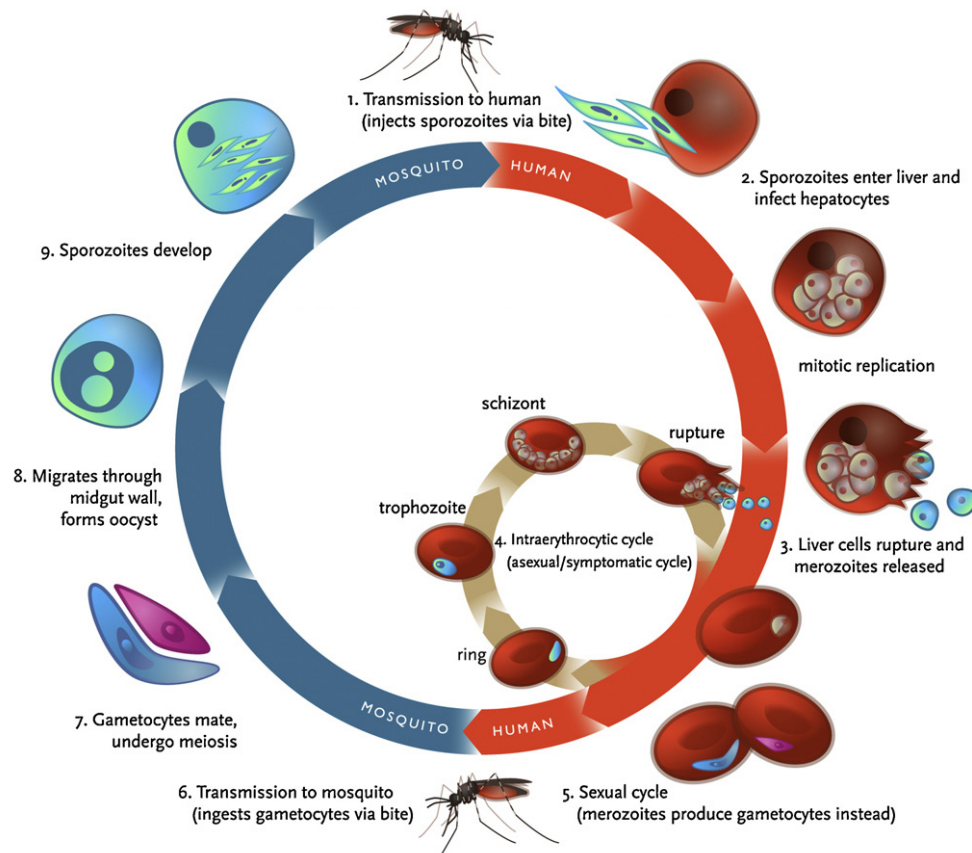
Figure 3: Life cycle of *Plasmodium* parasites [39]

The asexual stages of *P. falciparum* are the ones mainly responsible for clinical symptoms like fever, headache, chills and nausea which develop approximately 7 days after an infectious bite. Depending on the parasite species and immunological status of the person, the disease can progress to a critical illness with severe anaemia, general shock-like syndrome, lactic acidosis and inflammation of the brain which often results in the death of the patient [35, 40]. Young children, pregnant women, individuals with HIV/AIDS, travellers and immigrants are at highest risk of developing malaria due to insufficient or absent protective immunity [35]. Partial immunity acquired naturally through repeated exposure to malaria in highly endemic areas may result in an asymptomatic or milder disease and can protect an individual from death. Relapses of clinical symptoms are reported for *P. vivax* and *P. ovale* infections because the parasites latently persist in the liver [35, 40].

1.2.4 **Treatment and Prevention**

An early treatment of malaria is important to reduce transmission, severe disease and to prevent death. All fever cases with a suspected malaria infection should be confirmed with microscopy or rapid diagnostic tests before treatment is applied. To date, artemisinin-based combination therapy is the most efficient available treatment, especially for malaria caused by *P. falciparum* [35]. For *P. vivax* infections, chloroquine is the first-line drug of choice which can prevent relapses in combination with artemisinin. For areas with reported artemisinin resistance as well as in low-transmission areas, a single primaquine dose should be given at day one of the artemisinin-based treatment [35, 36]. Another crucial part of malaria control are measures to prevent malaria disease and/ or reduce transmission. Important tools to this avail are vector control (e.g. indoor spraying of houses with insecticides and eliminating potential breeding sites by covering open water around the house), personal protection against mosquito bites (insecticide-treated mosquito nets (ITN), protective clothing and repellents) and chemoprevention with antimalarial drugs in high transmission areas (e.g. for pregnant women and children below 5 years) [35, 36]. However, emerging resistance against insecticides and anti-malarial drugs are a major concern that weaken frontline malaria control interventions [36].

1.2.5 **Vaccines**

To support and maintain malaria control interventions and to follow the Global Malaria Action Plan towards elimination and eradication of malaria [41], an efficient vaccine against malaria is needed especially in view of emerging insecticide and drug resistance. Already in the 1970s vaccination trials with attenuated sporozoites showed a protective effect in humans [42, 43]. To date, the RTS,S/AS01 (RTS,S), a pre-erythrocytic subunit vaccine targeting the circumsporozoite protein at the surface of sporozoites, is the most advanced vaccine candidate. A large phase III trial has been completed recently showing that the RTS,S efficiently protects 36% of young children (5-17 month) and 26% of infants (6-12 weeks) from clinical malaria in sub-Saharan Africa, provided the child received all 4 doses of the vaccine [44, 45]. In October 2015, the WHO's Strategic Advisory Group of Experts on Immunisation (SAGE) recommended to perform pilot studies to assess whether it is achievable to administer 4 doses of the RTS,S over the course of 18 months, whether deaths can be prevented and whether the vaccine is safe [46–48]. However, as of today, no fully licensed vaccine is freely available on the market [35, 41, 44].

Due to the complexity of the parasites life cycle and several potential antigen targets present in each parasite stage, vaccine approaches are quite diverse. Pre-erythrocytic vaccines aim at preventing infections through sporozoites from an infectious mosquito bite or at blood-stage disease by targeting liver stages. This approach requires an antibody response against sporozoites or the stimulation of T cells to target intracellular hepatocytic stages [49]. The predominant candidate vaccine antigens for this purpose are the circumsporozoite protein (CSP [50]) and the thrombospondin-related adhesion protein (TRAP [51, 52]). The initial idea for a pre-erythrocytic vaccine evolved when mice immunised with irradiated sporozoites showed protection and the same result could also be achieved when only the CSP was used for immunisation [53, 54]. Additional studies revealed that antibodies against CSP inhibited parasite invasion of hepatocytes and reduced the risk of clinical malaria [55, 56]. The CSP is predominantly expressed at the surface of sporozoites [57] and is currently the most frequently used antigen for different candidate vaccines [58, 59] including the most advanced vaccine

RTS,S [44, 45]. TRAP is expressed on the surface of sporozoites and on infected hepatocytes [51, 52]. A TRAP-based vaccine achieved a partial protection in *P. falciparum*-naïve adults through the induction of specific IFN-γ producing T cells but failed to induce protection in children [60, 61]. However, upon modifications of the viral vector the vaccine induced high levels of long-lasting IFN-γ producing T cells [62, 63], proved to be safe and immunogenic and has shown to partially protected Kenyan adults against *P. falciparum* infection [64, 65]. A study has recently been conducted to access the efficacy of TRAP- and CSP-based vaccines using identical vector systems [66]. Further, pre-erythrocytic vaccine antigens in clinical development include the liver stage antigens (LSA1 and 3) and the exported protein 1 (Exp-1) [49]. New approaches in the clinical development stage use attenuated sporozoites [67–72] which achieved protection in more than 90% of volunteers which were immunised with attenuated sporozoites through >1000 mosquito bites before challenged [73].

The aim of asexual blood stage vaccines is to eliminate or reduce parasites in the blood by preventing merozoite invasion or replication in erythrocytes which is directly affecting mortality and morbidity [37, 40]. The rationale behind is that partial protection against symptomatic disease and severe malaria in adults can be acquired through natural immune responses against blood stage parasite antigens [59]. Few antigens are targeted by blood stage vaccines including apical membrane antigen 1 (AMA1 [74–78]), erythrocyte-binding antigen-175 (EBA-175 [79]), glutamate-rich protein (GLURP [80–82]), merozoite surface protein 1 (MSP1 [83]), MSP2 [84, 85], MSP3 [80, 82, 86–89] and serine-repeat antigen 5 (SERA5 [90, 91]), all present on the surface of merozoites or secreted from apical organelles upon invasion. Naturally acquired immunity against all these merozoite antigens have been associated with a certain protection from clinical disease [92, 93].

Overall, only few blood stage candidate vaccines have been tested in recent years in phase II clinical trials to access their protective efficacy in humans. The AMA1-combination 1 vaccine adjuvanted with Alhydrogel showed no protective effect on clinical malaria or *P. falciparum* density in young vaccinated children in Mali [78]. In malaria naïve adults, receiving a vaccine based on the recombinant AMA1 protein with

adjuvant system AS01B or AS02A, no protective effect was seen in challenge experiments with *P. falciparum*-infected mosquitoes but parasitemia was significantly reduced in the AMA1/AS02A group [77]. The same vaccine, FMP2.1/AS02A (AMA1/AS02A), showed only minor protection (9.9%) against clinical episodes in children in Mali within 24 month post vaccination but significantly higher anti-AMA1 antibody titres [76]. A plasmid DNA vaccine based on AMA1 and CSP in a prime-boost regiment with recombinant human serotype 5 adenovirus vectors sterilely protected 27% of naïve adults when challenged with bites of infected mosquitoes. Protection was mainly associated with cell-mediated immunity to AMA1 and CSP [94]. The vaccine FMP1/AS02 based on 42-kDa fragment of MSP1 in AS02 induced high anti-MSP1-42 antibody titres but failed to induce protective efficacy (5.1%) in young Kenyan children [83]. A vaccine based on MSP1, MSP2 and the *P. falciparum* ring-infected erythrocyte surface antigen (combination B) in montanide ISA720 reduced parasite density by 62% in vaccinated children in Papua New Guinea who were not treated with sulfadoxine-pyrimethamine (SP). However, in children receiving SP to clear infections prior to vaccination the vaccine had no effect. Furthermore, in both groups no effect on clinical malaria episodes were observed [84].

Transmission-blocking vaccines aim for targeting the sexual gametocyte stages such as the surface antigens Pfs48/45 [95–97], Pfs230 [95, 96, 98] and HAP2 [99, 100] or early parasite stages within the mosquito such as the 25kDa surface antigen (Pfs25 [101]) by preventing the formation of infectious sporozoites to interrupt parasite transmission from an infected to an uninfected person [40]. The presence of transmission-blocking antibodies in humans from endemic areas drives the idea for such a vaccine [102, 103]. Transmission-blocking vaccines do not protect the vaccinated individual from disease and therefore need to be used in the entire population to enable overall protective coverage employing herd immunity [49]. The leading transmission-blocking candidate vaccine is Pfs25 which is the only one tested in human clinical trials so far [104]. A vaccine combining the recombinant Pfs25 and Pvs25 of *P. falciparum* and *P. vivax* in montanide ISA 51 has recently been tested in a phase I trial observing an unexpected reactogenic feature and systemic adverse events [101].

Overall, more than 40 pre-clinical and clinical vaccine projects, mainly against *P. falciparum*, evaluate candidate vaccines targeting pre-erythroytic, blood stage and sexual stage antigens together with different adjuvants or delivering systems. Furthermore, different antigen combinations are included either as stage-specific or as multicomponent vaccine [58, 59].

1.3 **Hybridoma technology and monoclonal antibodies**

A natural immune response after infection with, or immunisation against, a pathogen, stimulates different B lymphocytes (B cell) to produce antibodies. Such a pool of antibodies directed against several different epitopes of the antigen is called polyclonal and antibodies bind their targets with different specificity and affinity. Next to polyclonal antibodies, monoclonal antibodies are identical copies of each other produced by a single B cell using the hybridoma technology. Monoclonal antibodies are monospecific, binding to only one specific epitope of the antigen [105].

The discovery of the hybridoma technology by Georges J. F. Köhler and César Milstein in 1975 revolutionised the wide field of health care and research by providing an unlimited supply of specific monoclonal antibodies [106, 107]. In 1984, Köhler, Milstein and Niels K. Jerne were awarded the Nobel Prize in Physiology or Medicine *<<for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies>>* [108]. Hybridomas are cell hybrids of an antibody-producing B cell with a fast-growing cancerous cell which enables the production of large quantities of identical monoclonal antibodies. For the production of monoclonal antibodies, mice are immunised with the antigen of interest (e.g. whole cells, crude lysate, recombinant proteins or peptides) formulated with or without an adjuvant or delivery system (Fig. 4). During the immune reaction of the mouse against the antigen, B cells are differentiated in the spleen. B cells are antibody-producing cells with a short lifespan of a few days. Repeated immunisations are necessary to reach a sufficient antibody titre and to generate high-affinity antibodies [109, 110].
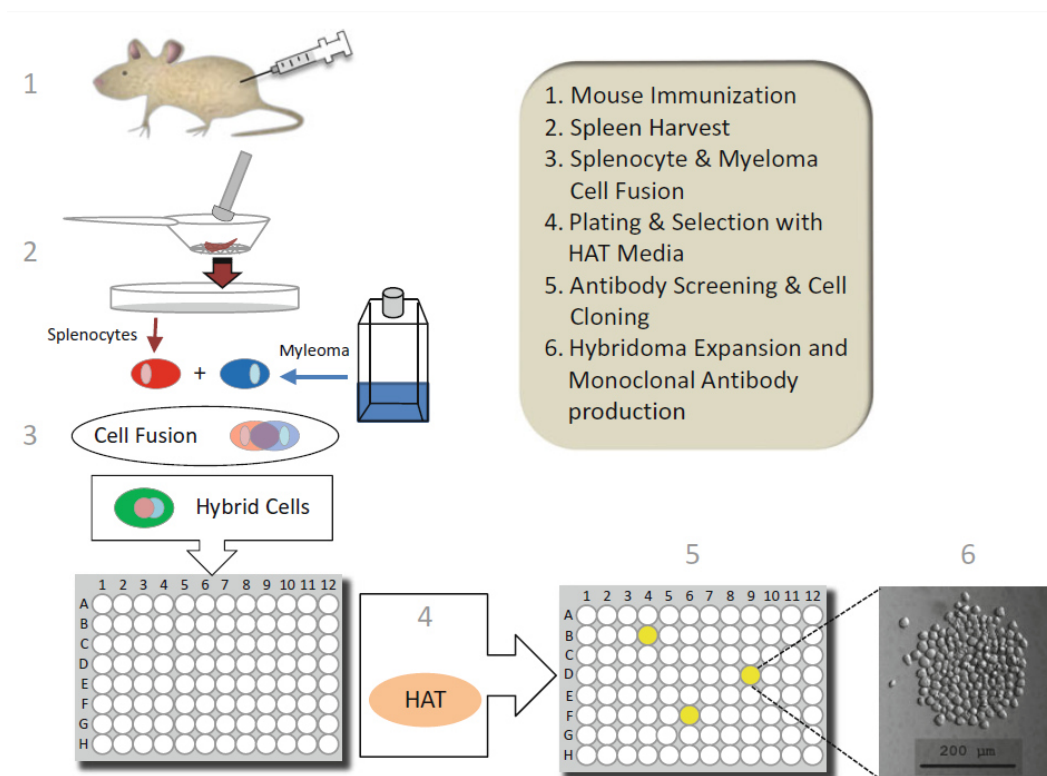
Figure 4: Production of monoclonal antibodies [109]

A hybridoma is then produced by fusing a splenocyte-derived B cell to a cancerous myeloma cell in the presence of polyethylene glycol (PEG) which mediates the fusion process by permeableising the cell membranes. Myeloma cells (e.g. P3X63-Ag8, NS1, SP2/0 [109]) are immortal. Furthermore, they lack the enzyme hypoxanthine guanidine phosphoribosyltransferase (HGPRT) and are thus sensitive to the enzyme inhibitor aminopterin which blocks the de novo synthesis of nucleotides. Normal cells have two different pathways for the biosynthesis of nucleotides, the de novo pathway and the salvage pathway. For the salvage pathway, the HGPRT enzyme must be present to synthesise nucleotides. Cells which are lacking HGPRT, such as myeloma cells, are thus killed in the presence of aminopterin. This feature is exploited to select suitable hybridomas. Hybridised cells are grown in a hypoxanthine-aminopterin-thymidine (HAT) selective medium with splenocyte-myeloma hybrids being the only surviving and growing cells. This stems from the fact that correctly fused hybridomas survive in the presence of aminopterin due to the ability for eternal life (coming from the myeloma cell)

and the presence of the HGPRT enzyme enabling salvage-dependent DNA synthesis (coming from the splenocyte counterpart). Incorrectly fused splenocyte-splenocyte hybrids are inherently short-lived and die fast whereas myeloma-myeloma hybrids die because they cannot use the salvage pathway for DNA synthesis due to the HGPRT-deficiency and the fact that the de novo pathway is blocked by aminopterin presence in the HAT medium.

Culture supernatant has to be tested for the presence of antibodies secreted by hybridoma cells. Therefore an appropriate screening assay has to be defined and special care is required regarding the antigen used in the assay to avoid selection of unspecific antibodies. Selected hybridoma lines are cloned by limiting dilutions to ensure monoclonal growth. Before expanding a certain hybridoma line for large-scale production of antibodies, the antibody isotype is determined. This is crucial to select which antibody purification technique is appropriate and also serves as control to guarantee monoclonal growth. After antibody purification, further characterisation steps are required such as specificity testing, epitope mapping and affinity measurements according to the field of application [109, 110].

For the use in scientific or therapeutic purposes, poly or monoclonal antibodies are selected primarily according to the intended use. However, other factors concerning the production and purpose can influence the decision. For the production of polyclonal antibodies rabbits, sheep, goats and chicken are used with the goal to obtain high quantities of antibodies. The production is fast, inexpensive and quite easy to perform. The heterogeneity of polyclonal antibodies in the binding of different antigen epitopes is the reason for their high overall specificity and renders polyclonal antibodies more stable against antigenic changes. However, the supply of polyclonal antibodies is limited by the production capacity of immunised animals (depending on size and lifespan) and antibodies obtained from different animals are slightly diverse due to individual immune responses [105, 110]. Monoclonal antibodies are completely homogeneous which enables different epitope-specific evaluations and characterisations of target molecules. Due to their monospecificity, monoclonal antibody-based assays may be susceptible to epitope alterations which affect the antibody binding site. Several monoclonal antibodies

with different defined specificities can be combined in one assay to overcome this problematic. Although the selection of appropriate B cells and the production of monoclonal antibodies using the hybridoma technology is time consuming, expensive and requires trained personnel, once a hybridoma is generated, the supply of identical, highly pure and epitope-specific monoclonal antibodies is consistent and unlimited [105].

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2.   Goals and Objectives

2.1 Goals

The goals of the thesis were:

i.   to identify immunodominant antigens of *Brucella* spp. and to generate specific monoclonal antibodies against them for implementation in new detection tools for *Brucella* cells or for subunit vaccine development

ii.   to evaluate the potential of PfRH2 as a *P. falciparum* malaria blood stage vaccine candidate antigen

2.2 Objectives

1.   To identify immunodominant *Brucella* antigens by analysing sera from mice immunised with whole *Brucella* cells and cattle naturally infected with *Brucella* spp.

2.   To generate monoclonal antibodies against *Brucella* cell surface antigens with spleen cells of mice immunised with whole *Brucella* cells or with recombinant *Brucella* proteins

3.   To develop an antigen capture assay for the detection of *Brucella* bacteria

4.   To establish a multiplex assay for the simultaneous detection of *Brucella* spp., *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* in complex samples

5.   To generate *Plasmodium falciparum* reticulocyte-binding homolog 2 (PfRH2)-specific monoclonal antibodies from mice immunised with the 40kDa receptor-binding domain of PfRH2

6.   To evaluate the potential of PfRH2-specific monoclonal antibodies to inhibit erythrocyte invasion by *P. falciparum* merozoites *in vitro* and *in vivo*

3. Article 1. Development of a bead-based Luminex assay using lipopolysaccharide specific monoclonal antibodies to detect biological threats from *Brucella* species

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3.1 **Abstract**

Background

Brucella, a Gram-negative bacterium, is classified as a potential bioterrorism agent mainly due to the low dose needed to cause infection and the ability to transmit the bacteria via aerosols. Goats/ sheep, cattle, pigs, dogs, sheep and rodents are infected by *B. melitensis, B. abortus, B. suis, B. canis, B. ovis* and *B. neotomae*, respectively, the six classical *Brucella* species. Most human cases are caused by *B. melitensis* and *B. abortus*. Our aim was to specifically detect *Brucellae* with 'smooth' lipopolysaccharide (LPS) using a highly sensitive monoclonal antibody (mAb) based immunological assay.

Methods

To complement molecular detection systems for potential bioterror agents, as required by international biodefense regulations, sets of mAbs were generated by B cell hybridoma technology and used to develop immunological assays. The combination of mAbs most suitable for an antigen capture assay format was identified and an immunoassay using the Luminex xMAP technology was developed.

Results

MAbs specific for the LPS O-antigen of *Brucella* spp*.* were generated by immunising mice with inactivated *B. melitensis* or *B. abortus* cells. Most mAbs recognised both *B. melitensis* and *B. abortus* and antigen binding was not impeded by inactivation of the bacterial cells by γ irradiation, formalin or heat treatment, a step required to analyse the samples immunologically under biosafety level two conditions. The Luminex assay recognised all tested *Brucella* species with 'smooth' LPS with detection limits of 2×10^2 to 8×10^4 cells per mL, depending on the species tested. Milk samples spiked with *Brucella* spp*.* cells were identified successfully using the Luminex assay. In addition, the bead-based immunoassay was integrated into a multiplex format, allowing for simultaneous, rapid and specific detection of *Brucella* spp*., Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* within a single sample.

Conclusion

Overall, the robust Luminex assay should allow detection of *Brucella* spp. in both natural outbreak and bio threat situations.

Keywords

Brucellosis, Luminex, antigen capture assay, monoclonal antibodies, multiplex

3.2 **Introduction**

Brucellosis, a zoonotic bacterial disease caused by Gram-negative *Brucellae* and classified as a potential bioterrorism disease [1], leads to abortions in animals and flu-like symptoms with periodic bouts of fever in humans. *B. melitensis, B. abortus, B. suis, B. canis, B. ovis* and *B. neotomae* are the six classical species that infect mainly goats/ sheep, cattle, pigs, dogs, sheep and rodents, respectively, while *B. melitensis* and *B. abortus* cause most of the human infections [2–4]. Like other Gram-negative bacteria, *Brucellae* express lipopolysaccharide (LPS), a major component of the outer membrane. The three structural components of LPS are the lipid A, the core oligosaccharide and the O-polysaccharide (O-antigen). In 'smooth' *Brucella* species, the O-polysaccharide is a linear polymer of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues, whereas 'rough' strains have a truncated version without the O-antigen [5, 6]. *Brucella* LPS is able to induce protective antibodies [7–9], which are potentially important for serological diagnosis [10–16]. Because of the threat posed by natural outbreaks or by a deliberate release of the bacteria as a bioterror agent [17], there is a need for rapid and reliable identification systems, preferably based on multiplex formats covering a range of relevant species. This is especially important for fastidious agents such as *Brucella* or *Francisella* species where tracing by cultivation is hampered by long cultivation time.

The aim of this study was to develop a rapid and sensitive immunological assay to detect all *Brucellae* with 'smooth' LPS, particularly *B. melitensis* and *B. abortus*. To this end, monoclonal antibodies (mAbs) specific for *Brucella* LPS were generated and used to design a highly specific and sensitive antigen capture assay. An optimal combination of mAbs was identified and a *Brucella* LPS-specific Luminex xMAP assay [18, 19] was developed, capable of detecting four of the major *Brucella* species (*B. melitensis, B. abortus, B. suis, B. neotomae*) with high sensitivity. Additionally, the Luminex assay works in a multiplex format, simultaneously detecting four category A and B bacterial bioterrorism agents and suitable for detecting *Brucella* in complex samples.

3.3 **Materials and Methods**

Ethics Statement

This study was carried out in strict accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzverordnung) of the Swiss Federal Food Safety and Veterinary Office. The protocol was granted ethical approval by the Veterinary Office of the county of Basel-Stadt, Switzerland (Permit Number: 2375).

Production and inactivation of bacteria

Bacterial strains used in this study are listed in Table 1. *Brucella* spp. were cultured on Columbia blood agar plates supplemented with 5% goat blood [20]. Bacteria were inactivated by 3% formalin (55 °C for 15 min), heat (60 °C for >20 h) or gamma (γ) irradiation at 30-40 kGy (Leoni Studer Hard AG, Däniken, Switzerland). Sterility was checked by incubating bacteria for three days on agar plates and no growth was observed.

Production of anti-LPS mAbs

To produce *Brucella* LPS-specific mAbs, mice carrying human immunoglobulin Cγ1 heavy and Cκ light chain gene segments [21] were immunised four times subcutaneously with a dose of 10^8 CFU of differentially inactivated *Brucella* species, either adjuvant-free or as adjuvanted formulation, in combination with the Sigma Adjuvant System® (SAS, Sigma Aldrich). Mice received either gamma (γ) irradiated *B. melitensis* in sterile Phosphate buffered saline (PBS, Sigma Aldrich), γ irradiated *B. melitensis* with SAS, formalin inactivated *B. melitensis* in PBS or formalin inactivated *B. abortus* in PBS.

Three days before cell fusion, two selected mice received an intravenous booster injection with 10^8 *Brucella* cells in PBS. Myeloma cells (PAI) were mixed 1:3 (fusion 1) and 1:1 (fusion 2) with spleen cells from the corresponding mouse in Iscove's Modified Dulbecco's Medium (IMDM, Sigma Aldrich). Cells were fused with 1 mL of pre-warmed (37 °C) Polyethylene glycol (PEG 800, Roche), dissolved in 150 mL HAT selective medium (IMDM 1% 200 mM L-Glutamine (100X), 1% Pen/Strep (100X, [+] 10,000 Units/mL Penicillin [+] 10,000 µg/mL Streptomycin, Gibco), 20% FBS, HAT

media supplement 50X Hybri-Max™, Sigma Aldrich) and cultured in 96-well tissue culture plates. Cells secreting *Brucella*-specific IgG were identified by ELISA coated with γ irradiated *B. melitensis* cells (16 M). From the two independent fusions, eleven hybridoma cell lines producing LPS-specific mAbs were identified and cloned by limiting dilution. MAbs were purified from spent culture supernatant of the hybridoma clones by protein A affinity chromatography (HiTrap rProtein A FF, Amersham Biosciences). Purified mAbs were dialysed against PBS, aliquoted, and stored at -80 °C.

Enzyme-linked immunosorbent assay (ELISA)

In indirect ELISA (iELISA), Maxisorp™ microtitre plates (Nunc, Thermo Scientific) were coated for 36 hours at 4 °C with 50 µL of a 10 µg/mL solution of extracted LPS or with 50 µL of a bacterial suspension containing 10^7 inactivated *Brucella* cells per mL. Wells were then blocked with 5% milk powder in PBS for two hours, followed by three washings with PBS containing 0.25% Tween-20. Plates were incubated with appropriate dilutions of mouse sera or anti-LPS mAbs in PBS for 1-2 hours at room temperature. After washing, plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (γ-chain specific) antibodies (Southern Biotech) for one hour. TMB (TMB Microwell Peroxidase Substrate System (2-C), KPL) or ABTS substrate (ABTS® Peroxidase Substrate System, KPL) was added and incubated at room temperature until appropriate colour intensity was reached (five to 30 min). The optical density (OD) of the reaction product was recorded after five to 30 minutes at 570 nm or 405 nm using a microplate reader.

In antigen capture ELISA (cELISA), microtitre plates were coated with 50 µL of a 10 µg/mL solution of unlabelled mAbs in PBS. After being blocked and washed, wells were incubated with dilutions of inactivated *Brucella* cells in PBS. Biotinylated detection mAbs (10 µg/mL) were added and incubated for one hour. After repeated washing, streptavidin-peroxidase polymer conjugate (1 µg/mL, Sigma Aldrich) was added and developed with the ABTS substrate.

Isotypes of anti-LPS mAbs were determined by detecting mAbs bound to anti-mouse lambda light chain antibody-coated plates with alkaline phosphatase-conjugated antibodies specific for mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Aliquots of extracted LPS from *B. melitensis* and *B. abortus* were mixed with sample buffer (Laemmli buffer, Invitrogen) and heated for 15 min at 96 °C before loading on 4-12% Bis-Tris gels. SeeBlue® pre-stained protein standard (Invitrogen) was used as a molecular weight marker. Following gel electrophoresis, LPS was transferred electrophoretically to nitrocellulose membranes. Blots were blocked for two hours with 5% milk powder in PBS, cut into strips and then incubated with purified mAbs (10 µg/mL) for one hour. The strips were washed four times for 15 minutes with PBS containing 0.05% Tween-20 and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG heavy-chain antibodies (Sigma Aldrich) for one hour. Strips were treated with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium to visualise bands.

Immunofluorescence assay (IFA)

30 µL droplets of a fixing solution containing 4% paraformaldehyde and 10% PBS were placed in each well of a pre-coated Poly-L-Lysin microscope glass slide (Diagnostic Microscope Slides ES-242B-AD-CE24, Thermo Scientific). Ten µL of a bacterial suspension containing 10^8 γ irradiated *B. melitensis* (16 M) or *B. abortus* (544) cells were added to each well and incubated for 30 min at room temperature. Wells were washed five times with PBS and then incubated for 15 min with 50 µL of blocking buffer containing 1% fatty acid-free bovine serum albumin (BSA) in PBS. Afterwards, 30 µL of 10 µg/mL mAbs diluted in blocking buffer were added and incubated for one hour. Wells were washed five times with blocking buffer before 30 µL of detection antibody (Alexa Fluor 568 (2 mg/mL, Invitrogen) conjugated donkey anti-mouse IgG (H+L), 1:400 in blocking buffer) was added for an additional hour. Finally, wells were washed five times, mounted with ProLong® Gold antifade reagent with 4',6-diamidino-2-phenylindole

(DAPI, Invitrogen) and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy.

Luminex assay

Anti-LPS mAbs were coupled to magnetic beads (Bio-Plex Pro Magnetic COOH Beads, Biorad) according to the manufacturer's instructions and adjusted to a working concentration of 40 beads/µL in blocking buffer (1% BSA in PBS). In the coupling reaction, 6 µg of antibody was applied to 5×10^5 beads. Fifty µL of working bead mixture was used per microtitre well. Fifty µL bacterial samples were then added to each bead-containing well and incubated for two hours on a microplate shaker at 37 °C in the dark. After incubation, the plates were washed with PBS containing 0.05% Tween-20 and the beads were resuspended in 50 µL of biotinylated detection antibody at a concentration of 10 µg/mL in blocking buffer and incubated for 1 hour. After repeated washing, 50 µL of a streptavidin-R phycoerythrin (ProZyme Inc.) solution was added and incubated for 30 min. The plate was then washed and the beads resuspended in 125 µL of blocking buffer before loading onto the BioPlex 200 instrument (Bio-Rad Laboratories). Reporter fluorescence was measured and expressed as mean fluorescence intensity of at least 100 beads per region. Multiplexed assays were performed in a single well format with mAb pairs 3D12/10G1 (*Brucellae*), MTA1/MTD6 [19], YPF19/YPF19 [22] and T14/FB11 [23].

Statistical analysis

All data were obtained from experiments performed in duplicate (at a minimum). Antigen-free controls consisted of PBS (instead of sample suspended in PBS) and were further diluted with the diluent used for the particular assay. These controls were included in each experiment to determine the cut-off. Mean value, standard deviation and LOD (limit of detection) were calculated in Excel. Figure assembly, data transformation and non-linear regression (sigmoidal curve, dose-response variable slope) were done with GraphPad Prism.

3.4 **Results**

Generation and characterisation of *Brucella* LPS-specific mAbs

Two mice exhibiting high ELISA IgG titres against *B. melitensis* (16 M) or *B. abortus* (NCTC 10093 544) cells after immunisation with inactivated bacterial cells were chosen for the generation of *Brucella* LPS-specific mAbs. Eleven hybridoma cell clones were obtained by screening with a *B. melitensis* (16 M, γ irradiated, 5 x 10^7 CFU/mL) whole cell ELISA. Two mAbs (3A10 and 4F11) were generated from a mouse immunised with γ irradiated *B. melitensis*, and nine (1A3, 10G1, 3D12, 2G12, 2G2, 1B6, 2E3, 5B10, 1E2) from a mouse immunised with formalin inactivated *B. abortus* cells. Determination of the mouse IgG subclass of the produced LPS-specific mAbs showed a predominance of the IgG2b(λ) isotype; only mAbs 4F11 and 1E2 were of the IgG3(λ) isotype. While all 11 mAbs recognised extracted LPS from *B. abortus* (type A O-antigen), mAbs 1E2 and 4F11 showed a markedly weaker reactivity with *B. melitensis* LPS (type M O-antigen) than did the others in ELISA (Fig. 1A) and Western blotting (Fig. 1C). The Western blot profiles (Fig. 1C) were typical for 'smooth' LPS of *Brucella* spp. [24]. In immunofluorescence analysis with inactivated *B. melitensis* and *B. abortus* cells, all anti-LPS mAbs yielded a homogenous circular surface staining. Figure 1B shows a representative staining for mAbs 3D12 and 10G1 with *B. melitensis* cells. The differences in the fine-specificities of the mAbs observed in ELISA correlated with differences in immunofluorescence analysis, where surface staining by mAbs 1E2 and 4F11 with *B. melitensis* cells was weak (data not shown). For the analysis of the samples under biosafety level two conditions, inactivation is required. Different methods, γ irradiation, formalin inactivation and heat treatment, are available for that. Irrespective of the inactivation method, the anti-LPS mAbs reacted with *B. melitensis* and *B. abortus* cells in ELISA (Fig. 1D).

To develop a highly sensitive antigen capture assay, a suitable combination of a capturing and a biotinylated-detecting mAb was selected from the pool of 11 mAbs. In a sandwich ELISA format, the majority of mAb combinations tested were suitable for detecting *B. melitensis* cells (Fig. 2). Despite its weak reactivity with *B. melitensis* LPS, mAb 1E2 could effectively be used as a capture antibody but it failed to interact with

B. melitensis cells when used as a detection antibody. A differentiation between *B. melitensis* and *B. abortus* cells was thus only observed with mAb 1E2 as a detection antibody (data not shown). MAb 3D12 performed best as an antigen capture antibody while mAb 10G1 was selected as the detection antibody as it gave the highest read out in combination with mAb 3D12 as the capture antibody. Hence, further development focussed on the mAb pair 3D12/10G1.

Developing a Luminex assay for rapid and sensitive detection of *Brucella* spp.

The mAb pair 3D12/10G1 was used to develop an antigen capture assay based on Luminex xMAP technology. While similar to ELISA in overall assay format, the Luminex technology combines advanced fluidics, optics, and digital signal processing with up to 500 color-coded microspheres to provide an accurate measurement of multiple analytes from a single sample [25]. Each bead set can be conjugated to a specific biomolecule (such as an antibody) to capture analytes of interest using a very small sample volume. Here, the mAb 3D12 was coupled to magnetic beads and used as the capture antibody, and the biotinylated mAb 10G1 was used as the detection antibody. The sensitivity of this Luminex assay was determined by analysing serial dilutions of inactivated *B. melitensis*, *B. abortus* and *B. suis* cells. The limit of detection (LOD) was calculated as the mean fluorescence intensity of the blank plus three times the standard deviation (SD) and set as the threshold (dashed line in Fig. 3A). The detection limits in a sample volume of 50 µL were 2×10^2 cells per mL for *B. melitensis*, 5×10^3 cells per mL for *B. abortus* and 8×10^4 cells per mL for *B. suis*. Depending on the species tested, the sensitivity of the Luminex assay was 4 to 50 times higher than that of a corresponding antigen capture ELISA (Fig. 3B), where at least 10^4, 2×10^4 and 3×10^6 cells per mL, respectively, were required for accurate detection.

The specificity of the bead-based assay was tested with several biotypes of *B. melitensis* (1-3), *B. abortus* (1,3), *B. suis* (1,2), *B. canis, B. ovis and B. neotomae,* as well as with other potential bioterror agents (*F. tularensis, B. anthracis, S. typhimurium, Y. pestis, B. mallei, B. pseudomallei* [1]) and bacteria (*Y. enterocolitica* O9*, E. coli* O157 and *V. cholera* O1 [26–29]) with structurally similar O-antigens of α1,2-linked 4-amino-

4,6-dideoxy-α-D-mannopyranosyl subunits and *O. anthropi*, the closest relative of *Brucellae* [30]. The Luminex assay detected all 'smooth' *Brucella* species (*B. melitensis, B. abortus, B. suis* and *B. neotomae*) independently of their biotype (Table 2). Overall, *Brucella* species expressing the M O-antigen were detected with higher sensitivity compared to A or AM O-antigen expressing *Brucella*. Cross-reactivity with *Y. enterocolitica* O9 was found, as predicted by the structural identity of the type A O-antigen [27, 28]. Neither *B. canis* nor *B. ovis* cells expressing a 'rough' LPS nor any of the other bacterial species tested gave positive signals.

The newly developed singleplex assay for *Brucella* spp. was integrated into a previously established multiplex assay to allow for simultaneous detection of the four potential bioterror agents, *B. melitensis, B. anthracis, F. tularensis* and *Y. pestis*, in a single run of the assay. Mixed samples containing combinations of the four bacterial species were prepared in PBS and tested in the multiplexed immunoassay format (Fig. 4A). All four bio threat agents tested were accurately detected and no cross-reactivities between individual singleplex assays were observed. The specificity of the Luminex assay for *B. anthracis, F. tularensis* and *Y. pestis* had been tested prior to the multiplex testing (Additional file 1 and 2).

In addition, the multiplex assay specifically identified all four bacterial species from a spiked milk sample, indicating that the newly developed Luminex assay is also suitable for detecting *Brucella* spp. in complex biological samples (Fig. 4B).

3.5 **Discussion**

Brucellosis is one of the most common bacterial zoonosis worldwide and an important cause of economic losses and human suffering [2, 4]. Moreover, *B. abortus*, *B. melitensis* and *B. suis* could be developed as bioterrorism agents due to their ability to undergo aerosolisation [31]. Isolation by cultivation is the standard method for identifying *Brucella* bacteria in biological samples, but may take up to four weeks to complete. Methods based on the polymerase chain reaction that identifies nucleic acid fragments from bacteria are becoming more practical for detecting *Brucella* spp. [32, 33]. However, according to international biodefense regulations, immunological detection methods for potential bioterror agents are required in addition to molecular detection and identification assays.

In this study, we showed that *Brucella* O-antigen-specifc mAbs represent potent immuno-capturing components for a highly sensitive detection system for *Brucella* cells in complex samples. Immunisation of laboratory mice with inactivated *Brucella* bacteria combined with a *B. melitensis* whole cell ELISA for selecting B cell hybridoma lines that produce *Brucella*-specific antibodies yielded exclusively LPS-specific mAbs, although anti-protein IgG antibodies could also be detected in the serum of the immunised mice (data not shown). This observation might be explained by the fact that in 'smooth' *Brucella* species, outer membrane proteins and other membrane components are masked by O-polysaccharide chains of LPS [34, 35]. All of the mAbs generated recognised LPS from *B. abortus* (type A O-antigen) and from *B. melitensis* (type M O-antigen). However, mAbs 1E2 and 4F11 differed from the other mAbs in fine-specificity in that they showed a markedly reduced reactivity with *B. melitensis* LPS. None of the sample inactivation methods tested (γ irradiation, formalin and heat treatment) affected the interaction between the mAbs produced and the bacterial cells, corroborating the suitability of *Brucella* LPS as a stable target antigen for detection. Dependent on infrastructural constraints (i.e. availability of gamma irradiation) and application, laboratories may have different preferences concerning the inactivation method.

As expected from the comparative binding studies, the majority of the mAb combinations tested were suitable for detecting *Brucella* cells in a sandwich capture ELISA format. A differentiation between *B. melitensis* and other *Brucella* species expressing 'smooth' LPS was achieved with mAb 1E2 in a suitable test format. The Luminex immunoassay with the selected mAb pair, 3D12 and 10G1, captured and detected cells of all 'smooth' *Brucella* species and biotypes tested but also showed cross-reactivity with *Y. enterocolitica* serotype O9. The O-antigens of *Y. enterocolitica* O9*, E. coli* O157*, V. cholera* O1 and *B. abortus* all consist of a linear polymer of α1,2-linked 4-amino-4,6-dideoxy-α-D-mannopyranosyl residues (perosamine). However, they differ in the *N*-acylation of the perosamine sugar [26]. While *B. abortus* and *Y. enterocolitica* O9 are *N*-acylated with formic acid, *V. cholera* O1 are substituted with (S)-2,4-dihydroxybutanoic acid [36]. These derivatisations can have major effects on antibody binding, which may explain why our mAbs only showed cross-reactivity with *Y. enterocolitica* O9. PCR can verify whether a result obtained with mAb pair 3D12/10D1 is true or false positive due to *Y. enterocolitica* O9 contamination. To conclusively analyse environmental samples a combination of molecular and immunological methods is recommended [37]. Our mAbs are specific to *Brucella* carrying 'smooth' LPS, hence a detection of 'rough' *Brucella* species is not possible. A LPS-independent detection based on surface-exposed structures might solve this problem.

Depending on the *Brucella* species tested, the assay was able to detect 10 to 4,000 cells in a sample volume of 50 µL. Currently available molecular identification assays for *Brucella* spp*.* offer comparable or even lower detection limits [32, 33, 38].
Recently, a capture ELISA for diagnostic purposes was developed using LPS-specific monoclonal antibodies to detect LPS antigens in the blood [39]. Both, our approach for generating LPS-specific monoclonal antibodies, as well as the overall purpose of our test development were different. We developed a highly sensitive Luminex multiplex assay for the detection of bio threat agents both in natural outbreak and bio threat situations.

The conversion of the ELISA into the Luminex bead-based assay markedly increased the sensitivity for detecting *Brucella* and allowed integration of the *Brucella* assay into a multiplex assay to simultaneously detect a range of relevant bio-threat species. The multiplexed immunodetection assay accurately detected *Brucella* spp., *B. anthracis*, *F. tularensis* and *Y. pestis* cells within a single mixed sample. Brucellosis is transmitted to humans through consumption of unpasteurised dairy products or through direct contact with infected animals. Although detecting *Brucella* cells in milk is complicated [40], the Luminex multiplex assay specifically identified all tested bacterial species from spiked milk samples, demonstrating that the developed assay is a suitable tool for detecting *Brucella* cells in complex samples.

3.6 Conclusion

The Luminex assay described here is a suitable tool for specifically detecting *Brucella* spp. even in complex samples such as milk. Four bio-threat agents can be detected in the multiplex format, quickly and specifically. Overall, using the Luminex assay together with common molecular and cultivation methods is crucial to fulfilling international biodefense regulations for rapidly and reliably identifying biological threat agents. In the future, the Luminex assay may also be considered for detecting *Brucella* in clinical samples.

3.7 Acknowledgements

3.8 **Tables and Figures**

Table 1. Bacterial strains

Bacterial species	Strain	Inactivation method	Source
Brucella spp. (biotype)			
B. abortus (1)	NCTC 10093 544	γ, formalin, heat	SL
	19S	formalin	SL
	A146-10	formalin	RKI
B. abortus (3)	A104-10 Uckermark	γ	RKI
B. canis	NCTC 10854 RM-666	formalin	SL
B. ovis	CNCTC 6741	heat	RKI
B. melitensis (1)	NCTC 10094 16 M	γ, formalin, heat	SL
	ICM 3.33	formalin	SL
	ICM 583/2003	formalin	SL
	ICM 91/2004	formalin	SL
	102A01C2F	formalin	SL
	A146-13	formalin	RKI
	A104-11 Tgb. Nr. 117518	γ	RKI
B. melitensis (2)	A104-12 799/97, B3898	γ	RKI
B. melitensis (3)	A104-13 210739, Mainz	γ	RKI
B. suis (1)	NCTC 10316 1330	formalin	SL
B. suis (2)	A 104–14 Rostock	heat	RKI
B. neotomae	A148-7 5 K33	γ	RKI
Other bacteria			
F. tularensis tularensis	ATCC 6223	formalin	SL
F. tularensis holarctica	LVS, NCTC 10857	formalin	SL
B. anthracis (spores)	Böhm 73202.2000 (PX02+)	formalin	SL
	Böhm A1 (PX01+)	formalin	SL
S. typhimurium	ATCC 14028	formalin	SL
Y. enterocolitica	310 (IT2, ST9) O9	formalin	SL
Y. pestis	CO92	γ, formalin	SL
O. anthropi	ATCC 49188	formalin	SL
E. coli	O:157, 15326	formalin	SL
B. mallei	NCTC 03709 106	formalin	SL
B. pseudomallei	H05410-0490	formalin	SL
V. cholera	O1, ATCC 14734	formalin	SL

Bacteria were inactivated by 3% formalin, heat (60 °C for >20 h) or gamma (γ) irradiation (30-40 kGy). SL = Spiez Laboratory (Federal Office for Civil Protection, Spiez, Switzerland). RKI = Robert Koch Institute (Berlin, Germany).

Table 2. Specificity of the developed bead-based Luminex assay

| Bacterial species	Strain	O-Antigen	Luminex assay (mean fluorescence intensity)
Brucella spp. (biotype)			
B. abortus (1)	NCTC 10093 544	A[1,3]	**13107**
	19S	A[1,3]	**11300**
	A146-10	A[1,3]	**5607**
B. abortus (3)	A104-10 Uckermark	A[1,3]	**3172**
B. canis	NCTC 10854 RM-666	R[2,3]	33
B. ovis	CNCTC 6741	R[2,3]	23
B. melitensis (1)	NCTC 10094 16 M	M[1,3]	**25860**
	ICM 3.33	M[1,3]	**13129**
	ICM 583/2003	M[1,3]	**16972**
	ICM 91/2004	M[1,3]	**15030**
	102A01C2F	M[1,3]	**22285**
	A146-13	M[1,3]	**11407**
	A104-11 Tgb. Nr. 117518	M[1,3]	**5977**
B. melitensis (2)	A104-12 799/97, B3898	A[1,3]	**2958**
B. melitensis (3)	A104-13 210739, Mainz	AM[1,3]	**1884**
B. suis (1)	NCTC 10316 1330	A[1,3]	**5167**
B. suis (2)	A 104–14 Rostock	A[1,3]	**10316**
B. neotomae	A148-7 5 K33	A[1,3]	**1875**
Other bacteria			
F. tularensis tularensis	ATCC 6223	D[4]	18
F. tularensis holarctica	NCTC 10857	D[4]	22
B. anthracis (spores)	Böhm 73202.2000 (PX02)	D[5]	19
S. typhimurium	ATCC 14028	D[6]	19
Y. enterocolitica	310 (IT2, ST9) O9	A[7,8,9]	**1291**
Y. pestis	CO92	R[10]	20
O. anthropi	ATCC 49188	D[11]	20
E. coli	O157, 15326	A[7,12]	22
B. mallei	NCTC 03709 106	D[13]	18
B. pseudomallei	H05410-0490	D[14]	20
V. cholera	O1, ATCC 14734	A[15]	24

Luminex LOD was defined as two times the mean fluorescence intensity of the blank (mean blank = 20) and used as the threshold for positive results. Values in bold indicate positive results. Classification of O-antigens [4, 26–29, 41–50]: A = α1,2-linked 4-amino-4,6-dideoxy-α-D-mannopyranosyl subunits, M = α1,3-linked and α1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues, D = different O-antigen structure compared to *Brucella*, R = 'rough' LPS (no O-antigen). Meikle et al. 1989[1], Adone et al. 2011[2], Corbel 2006[3], Wang et al. 2011[4], Crich and Vinogradova 2007[5], Watson et al. 1992[6], Perry et al. 1986[7], Caroff et al. 1984[8], Bundle et al. 1984[9], Skurnik et al. 2000[10], Velasco et al. 1996[11], Perry and Bundle 1990[12], Burtnick et al. 2002[13], Perry et al. 1995[14], Kenne et al. 1982[15].
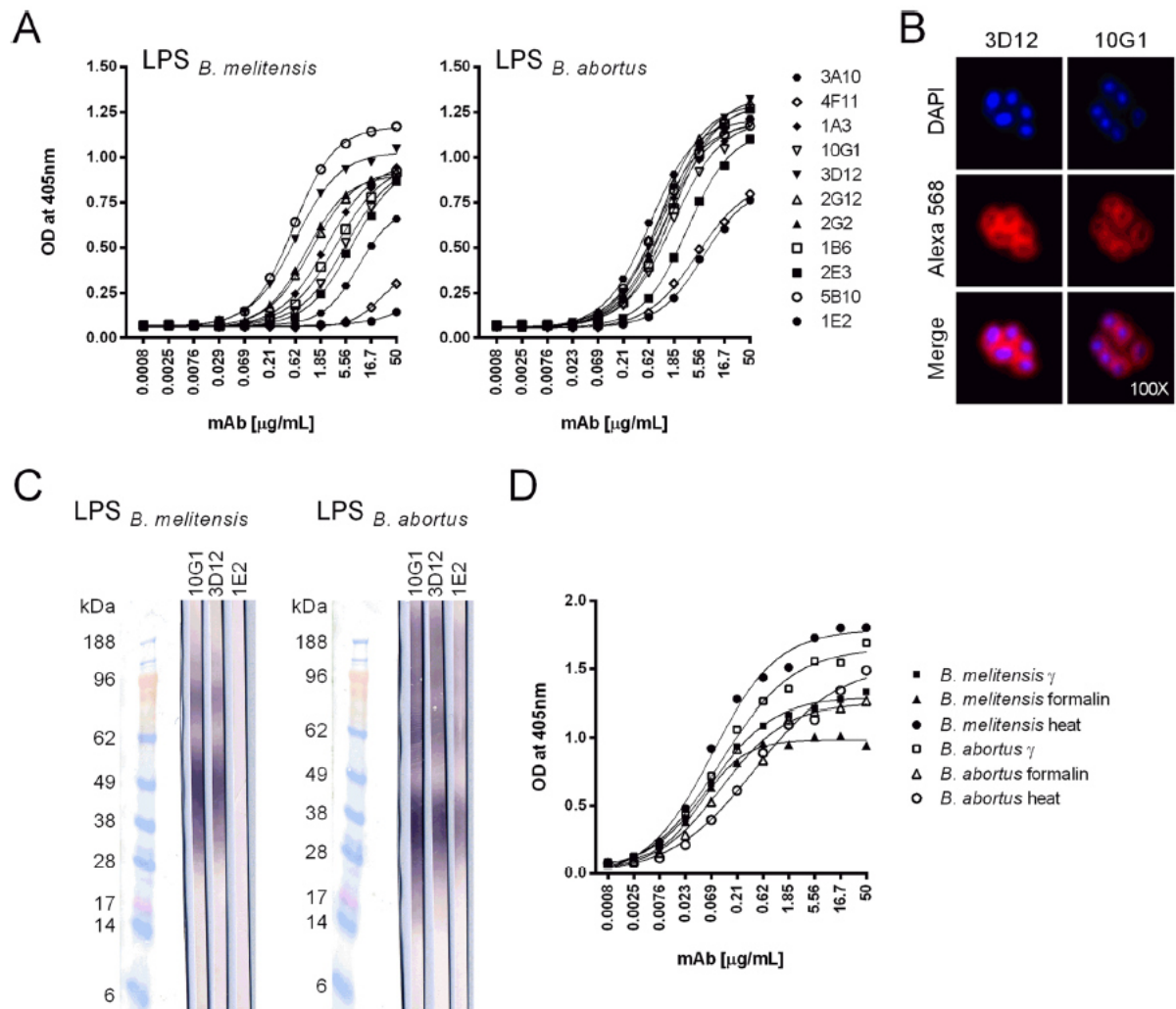
Figure 1. Antigen-binding properties of the generated *Brucella* LPS-specific mAbs

(A) Reactivity of the produced mAbs with extracted *B. melitensis* or *B. abortus* LPS in ELISA. **(C)** Western blot staining-patterns obtained with mAbs 10G1, 3D12 and 1E2 after SDS-PAGE of extracted *B. melitensis* and *B. abortus* LPS. **(B)** Indirect immunofluorescent staining of inactivated *B. melitensis* (16 M) cells by mAbs 3D12 and 10G1. The upper panel shows DNA staining with DAPI, the middle panel Alexa 568-specific immunofluorescence staining and the lower panel merged pictures of both stainings. **(D)** Reactivity of mAb 3D12 with gamma, formalin and heat inactivated *B. melitensis* (16 M) and *B. abortus* (544) cells in ELISA.
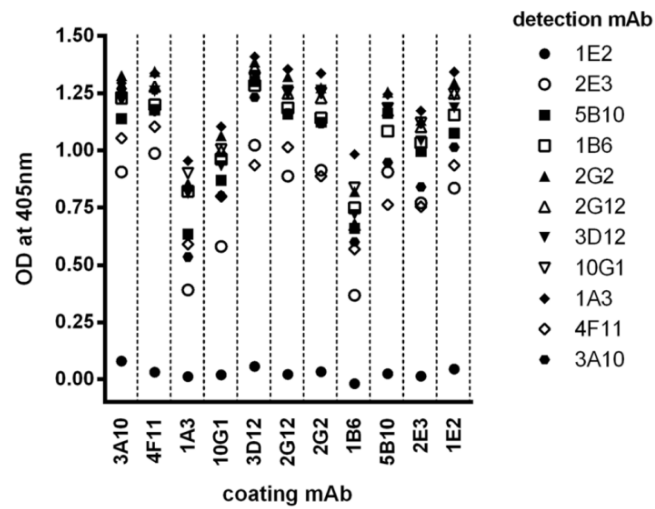
Figure 2. Comparative testing of mAb pairs in an antigen capture ELISA

To evaluate optimal antibody combinations, each of the 11 mAbs was used as a capture or detection (biotin-conjugate) antibody at a concentration of 10 µg/mL. Optical densities were measured for each antibody combination using gamma-irradiated *B. melitensis* (16 M) cells at a concentration of 10^7 cells/mL.
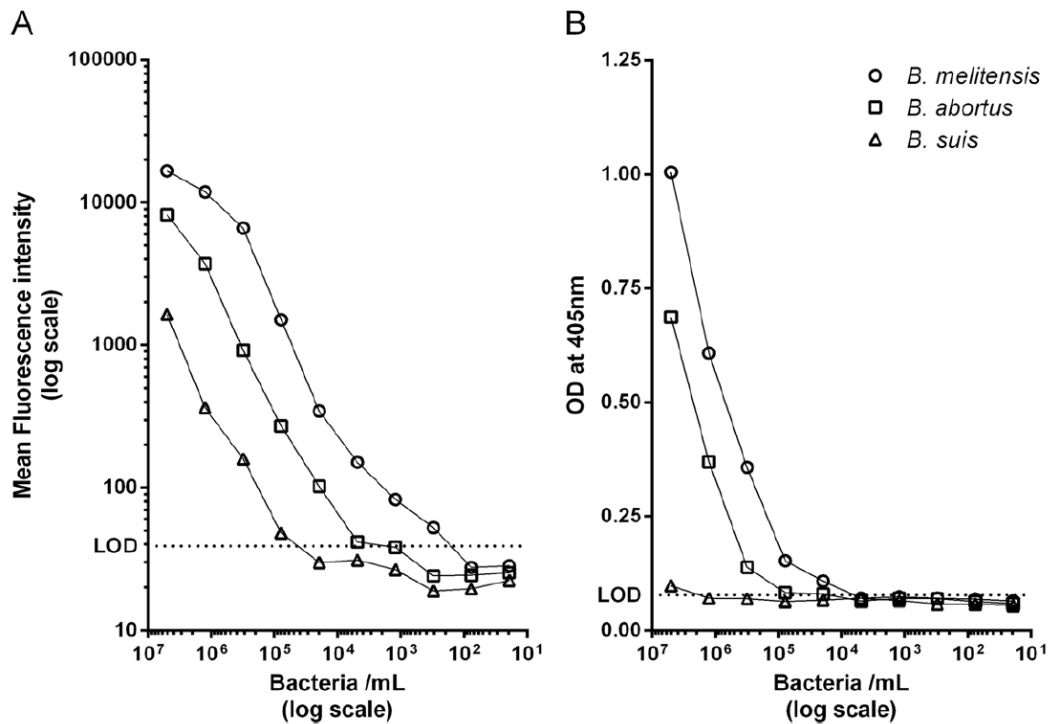
Figure 3. Comparison of the sensitivity of the bead-based Luminex immunoassay (A) and the corresponding antigen capture ELISA (B)

Assay sensitivities were determined by analysing serial dilutions of inactivated *B. melitensis* (16 M, γ), *B. abortus* (544, γ) and *B. suis* (1330, formalin) cells. Dashed lines indicate the assay dependent limit of detection (LOD) defined as mean blank (i.e., the no-antigen control) plus three times the standard deviation (SD).
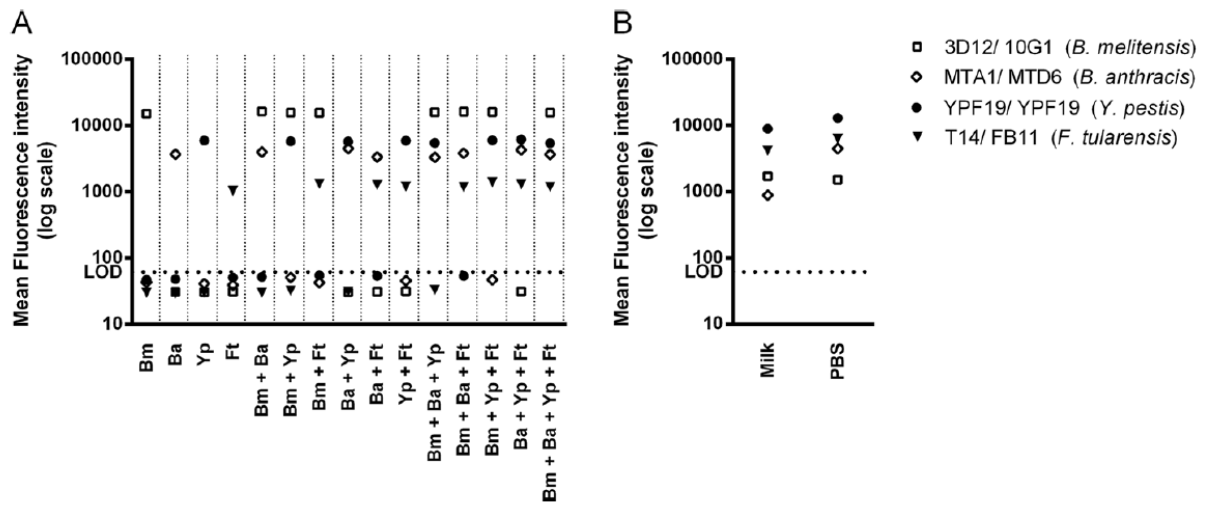
Figure 4. Multiplexed Luminex immunoassay for detecting potential bioterror agents, *B. melitensis*, *B. anthracis*, *F. tularensis* and *Y. pestis*

(A) Test samples contained *B. melitensis* 16 M (Bm, 5×10^5 cells/mL), *B. anthracis* PXO1+ (Ba, 5×10^5 cells/mL), *F. tularensis* 6223 (Ft, 5×10^5 cells/mL) and *Y. pestis* CO92 (Yp, 5×10^4 cells/mL) cells in PBS either alone or in combination. In **(B)**, PBS and milk samples were spiked with all four bacterial species and used at a concentration of 2.5×10^6 cells/mL. MAbs 3D12, MTA1, T14 and YPF19, coupled to distinct magnetic beads, were used as capture antibodies and the biotinylated mAbs 10G1, MTD6, FB11 and YPF19 were used for detection. Reporter dye fluorescence intensities measured for each bead set are shown. Dashed lines indicate the limit of detection (LOD) defined as mean blank plus three times the standard deviation.

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Additional file 1. Additional bacterial strains

Bacterial species	Strain/Isolate	Inactivation method	Source
Bacillus spp. spores			
B. anthracis	ames	γ-irradiated	USCRP
	Böhm A58	10% formaldehyde	IVB,B
	Böhm A1	10% formaldehyde	IVB,B
	Böhm A 73202.2000	10% formaldehyde	IVB,B
	NCTC 8234	10% formaldehyde	NCTC
	NCTC 10340	10% formaldehyde	NCTC
	NCTC 01328	10% formaldehyde	NCTC
	NCTC 02620	10% formaldehyde	NCTC
	W514 Bern	10% formaldehyde	IVB,B
B. cereus	ATCC10876	10% formaldehyde	ATCC
	ATCC11778	10% formaldehyde	ATCC
	ATCC13061	10% formaldehyde	ATCC
	ATCC14579	10% formaldehyde	ATCC
	ATCC33019	10% formaldehyde	ATCC
B. subtilis	ATCC6051	none	ATCC
	ATCC6633	none	ATCC
	ATCC11774	none	ATCC
B. thuringiensis ssp. kurstaki	SP09	10% formaldehyde	SIBA
	ATCC10792	10% formaldehyde	ATCC
B. athropheaeus	ATCC9372	none	ATCC
B. circulans	ATCC61	none	ATCC
B. licheniformis	ATCC12759	none	ATCC
	ATCC14580	none	ATCC
B. megaterium	ATCC9885	none	ATCC
	ATCC14581	none	ATCC

B. sphaericus	ATCC4525	none	ATCC
B. pumilus	ATCC14884	none	ATCC
Yersinia spp. (biotype)			
Y. pestis	ICM 1/41	3%formalin	ICM
	ICM 1/48	3%formalin	ICM
	EV 76	3%formalin	RKI
	CO92	3%formalin	DRDC
	NCTC (0)2868	3%formalin	NCTC
	NCTC 10030 (13927/58)	3%formalin	NCTC
Y. pseudotuberculosis	BV1, cattle	3%formalin	ICM
Y. pseudotuberculosis	BV2, cattle	3%formalin	ICM
Y. pseudotuberculosis	BV3, cattle	3%formalin	ICM
Y. pseudotuberculosis	BV4, cattle	3%formalin	ICM
Y. pseudotuberculosis	1.5, Lausanne 2931	3%formalin	ICM
Y. enterocolytica (1)	309, human	3%formalin	ICM
Y. enterocolytica (2)	382, human	3%formalin	ICM
Y. enterocolytica (3)	371, human	3%formalin	ICM
Y. enterocolytica (4)	28, water	3%formalin	ICM
Francisella spp.			
F. philomiragia	ATCC25015	3% formalin	ATCC
F. tularensis ssp. tularensis	ATCC 6223	3% formalin	IVB,Z
	Schu 4	3% formalin	RKI
F. tularensis ssp. holarctica	NCTC 10857	3% formalin	NCTC
	JF 3824, human	3% formalin	IVB,B
	JF 3825, monkey	3% formalin	IVB,B
	human, (Ft5)	3% formalin	KSBL
	human, (Ft6)	3% formalin	KSA
	human, (Ft7)	3% formalin	KSA
	human, (Ft8)	3% formalin	KSA
	human, (Ft9)	3% formalin	KSA
	JF 3820, hare	3% formalin	IVB,B
	JF 3821, hare	3% formalin	IVB,B

	JF 3822, hare	3% formalin	IVB,B
	JF 3826, monkey	3% formalin	IVB,B
	JF 3829, monkey	3% formalin	IVB,B
	JF 3859, hare	3% formalin	IVB,B
	JF 4092, hare	3% formalin	IVB,B
	JF 4128, human	3% formalin	IVB,B
	JF 4212, human	3% formalin	IVB,B
	JF 4242, hare	3% formalin	IVB,B
	JF 4429, human	3% formalin	IVB,B
	JF 4455, hare	3% formalin	IVB,B
	JF 4456, human	3% formalin	IVB,B

Bacteria were inactivated by 3% formalin, 10% formaldehyde or not. NCTC = National Collection of Type Cultures (London, UK), ICM = Instituto cantonale die microbiologica, Bellinzona (Bellinzona, Switzerland), DRDC = Defence Research and Development Canada (Ottawa, Canada), IVB,Z = Institut für Veterinärbakteriologie der Universität Zürich (Zurich, Switzerland), IVB,B = Institut für Veterinärbakteriologie der Universität Bern (Bern, Switzerland), KSBL = Kantonsspital Liestal (Liestal, Switzerland), KSA = Kantonsspital Aarau (Aarau, Switzerland), USCRP = U.S. Critical Reagents Program, SIBA = Sampling and Identification of Biological Agents, Isolate from Profiency Test 2009, RKI = Robert Koch Institute, Isolates from Profiency Tests (EQADeBa, QUANDHIP, Berlin, Germany), ATCC = American Type Culture Collection.
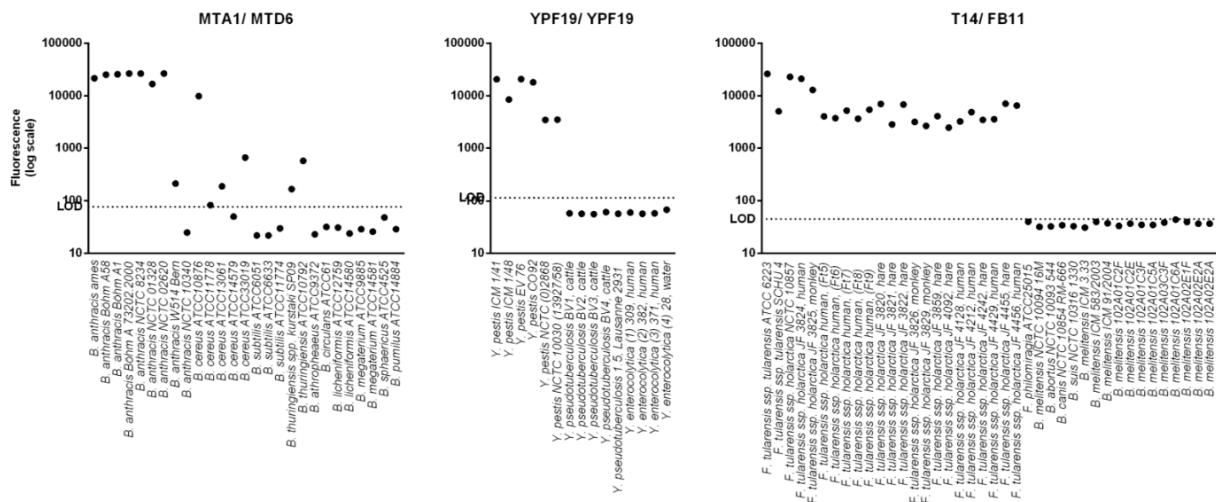
Additional file 2: Specificity of the developed bead-based Luminex immunoassay for _B. anthracis, F. tularensis_ and _Y. pestis_

MAb pairs MTA1/MTD6 [19], YPF19/YPF19 [22] and T14/FB11 [23] were analysed for their specific detection of _B. anthracis_, _Y. pestis_ and _F. tularensis_, respectively. Coating mAbs (6 µg) were coupled to 5 x 10^{5} magnetic beads (Bio-Plex Pro Magnetic COOH Beads, Biorad). The working concentration was 40 beads/µL in blocking buffer (1% BSA in PBS) with 50 µL per well. Each bead containing well was incubated for 2 hours with 50 µL bacterial samples (_Bacillus_ and _Francisella_ spp., 5 x 10^{6} cells/ml, _Yersinia_ spp., 5 x 10^{5} cells/mL) on a microplate shaker at 37 °C in the dark. Plates were washed with PBS containing 0.05% Tween-20 and the beads incubated in 50 µL of biotinylated detection antibody (10 µg/mL in blocking buffer) for 1 hour. After repeated washing, 50 µL of a streptavidin-R phycoerythrin (ProZyme Inc.) solution was added and incubated for 30 min. The plate was washed and the beads resuspended in 125 µL of blocking buffer before loading onto the BioPlex 200 instrument (Bio-Rad Laboratories). Reporter fluorescence was measured and expressed as mean fluorescence intensity of at least 100 beads per region. Dashed lines indicate the assay dependent limit of detection (LOD) defined as mean blank (i.e., the no-antigen control) plus three times the standard deviation (SD). Data were collected from three independent experiments in duplicates.

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4. Article 2. Approaches to generate monoclonal antibodies against *Brucella* cell surface proteins

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Working chapter

4.1 **Abstract**

Background

Brucella spp., the causative agent of brucellosis, leads to infections predominantly in livestock and dogs and accidentally in humans with *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae* representing the six classical species. In the absence of a safe and effective vaccine for humans the disease is mainly treated with a combination of antibiotics. However, the diagnosis of a brucellosis infection is difficult and all methods (cultivation, serology and molecular detection tools) have certain drawbacks. Our aim was to identify protein targets for the use in antigen detection assays or as subunit vaccine components. Our strategy was in particular aiming at the generation of monoclonal antibodies (mAbs) against cell surface structures for detailed antigen characterisation and the development of immunological antigen detection systems. For the generation of mAbs, we used an approach, immunisation with whole bacterial cells, which has been successful in our hands with a range of different pathogens yielding mAbs against a variety of cell surface antigens.

Results

For the identification and characterisation of cell surface proteins, sets of mice were immunised with inactivated whole *Brucella* cells and animals showing reactivity with protein antigens in Western blotting were used for the generation of B cell hybridomas. However, all hybridomas selected based on reactivity with whole *Brucella* cells in ELISA produced antibodies specific for the O-antigen of lipopolysaccharide (LPS), but not for surface proteins. Therefore a peptide microarray covering 40 selected *Brucella* proteins was evaluated as alternative screening tool. However, neither sera from mice immunised with whole *Brucella* cells, nor sera from cattle naturally infected by *Brucella* bacteria showed significant reactivity with the sets of 15 amino acid long overlapping linear peptides of the array.

Conclusion

Our Western blotting analyses showed that both whole cell immunisation of mice and natural infections of cattle with *Brucella* bacteria lead to the generation of serum antibodies against LPS, but also against a range of *Brucella* protein antigens. However, our approach to select hybridomas secreting mAbs against these proteins by whole cell ELISA failed. Using a peptide microarray covering 40 selected *Brucella* proteins did not turn out to be a viable alternative screening method. These results indicate that the immunogenicity of *Brucella* cell surface proteins is low and that immunisation with recombinantly expressed cell surface proteins may therefore be the best approach to generate mAbs against them.

Keywords

Brucellosis, immunogenic proteins, monoclonal antibodies, peptide microarray

4.2 **Introduction**

Brucellosis, one of the leading bacterial zoonosis worldwide, is causing abortions and infertility in animals [1, 2]. A wide range of animals are affected, with *B. melitensis* (sheep/goats), *B. abortus* (cattle), *B. suis* (pigs), *B. canis* (dogs), *B. ovis* (sheep) and *B. neotomae* (desert wood rat) being the six major *Brucella* species [3]. Humans, as accidental hosts, present with an acute febrile illness with the potential for chronification of the disease [1]. Human infections are acquired through direct or indirect contact with domestic livestock (sheep, goats, cattle, pigs) and dogs or through animal products (unpasteurised milk, raw meat). Each year half a million new human cases are estimated, causing high morbidity and economic losses [2, 3].

Three life attenuated vaccines, S19, RB51 and Rev1 [4–6] are used in livestock to control the disease but are infectious for humans [7–9]. For treating brucellosis infections in humans, antibiotics are used but relapses due to persistence of *Brucella* in macrophages and the resulting chronification requires extended treatment regimens [10]. A large number of serological assays e.g. agglutination tests, ELISAs, rapid point-of-care assays are available for diagnostics together with molecular detection tools. However, cultivation methods to isolate and identify *Brucella* spp*.* are still the gold standard in laboratory diagnosis [11–13]. Since, all tests have certain drawbacks e.g. long cultivation times, risk of infection, inefficiency, low specificity, inconsistency and costs [12, 13] and difficulties in detecting 'rough' *Brucella* [14, 15], new antigen detection tools have to be developed. Furthermore, no protective and safe vaccine is available for humans [16]. The identification of immunodominant cell surface antigens represents a promising approach for the development of both protein-based detection systems and a subunit vaccine [16–22].

The major aim of this study was to identify *Brucella* species-specific immunodominant cell surface proteins through serum analyses and the generation of mAbs from mice immunised with whole *Brucella* cells.

4.3 **Materials and Methods**

Ethics statement

This study was carried out in strict accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzverordnung) of the Swiss Bundesamt für Veterinärwesen. The protocol was ethically approved by the kantonale Veterinäramt Basel-Stadt, Switzerland (Permit Number: 2375).

Bacterial strains

B. melitensis (NCTC 10094 16 M) and *B. abortus* (NCTC 10093 544) strains inactivated by 3% formalin, heat (60 °C for >20 h), or through gamma (γ) irradiation (30-40kGy) were used [23].

Cattle serum

Serum samples of cattle were collected in 2011 during a serological brucellosis study in livestock in Northern Togo [24]. Briefly, sera were tested by the Rose Bengal test (RBT) and by indirect ELISA (iELISA, CHEKIT Brucellose Serum ELISA Test Kit, IDEXX Laboratories, ME, USA) for classification of positive and negative serum. Twenty-five bovine sera were selected to analyse their specific immunological profile by ELISA and Western blotting. Nine sera were tested positive by iELISA and the RBT (double positive), five tested positive only by iELISA (single positive) and eleven tested negative in both assays (double negative). All sera were filtered through 0.22 μm filters prior to use.

Generation of recombinant BQ and BP proteins

Two *Brucella* proteins BQ (putrescine-binding periplasmic protein) and BP (spermidine/putrescine-binding periplasmic protein) were produced recombinantly by Lionex (Braunschweig, Germany). Protein sequences are listed below.

BQ (BMEI0411, PotF truncated, 342 aa)

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPTLQERVVNIYNWSDYIDDSILKD
FTKETGIKVVYDVYDSNEILETKLLAGGSGYDLVVPSGEFLGRQIPAGVFLKLDKDKLPN
LKNMWDEISTRAATYDPGNEYSVNYMWGTTGIGYNKAKIKEALGTDTIDSWDVLFDPE
KTAKLKDCGIYLLDSASEMLRPALNYLGLDPNSPSPDDLQKAQDLYLKIRPNIRKFHSS
EYINALANGDICMAVGYSGDIFQARDRAEKAKQGVEIGYSIPKEGALIWFDQMAIPADA
KHVPEALEFMNYMMRPEVAAKASNYVFYANGNKASQKFIDKEILDDPEIYPSDEVMKK
LFVPTPYDTKTQRVVTRAWTKIVTGQ

BP (BMEII0923, PotD truncated, 323 aa)

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPTLRDLTVASWGGNYQDAQREI
YFKPFAEKTGKPLLDESWDGGYGVIQSKVKAGSPNWDVVQVEAEELALGCADGLYEK
IDWDKVGGKDKFLDSAVNDCGVGAIVWSTAIAYNGDKLKDGPKSWADFWDVKKFPGK
RSLRKSAKYTLEFALMADGVDKDDVYDVLSTPEGVDRAFKKLDELKPHIVWWEAGAQ
PLQLLASDEVVMASAYNGRITGINRSEGKNFKVVWPGSIYAVDSWVILKGAENKDAGL
DFIAFASEPEHQVKLPKYVAYGLPNKEAAAKVPEEYAADLPTAKANMKDALALDVDFWI
DHSEELTKRFNAWLAQ

Immunisation and generation of monoclonal antibodies

Immunisation of mice and the production of monoclonal antibodies were performed as described elsewhere [23]. Briefly, twelve transgenic Ig-tg mice [25] were immunised three times subcutaneously (s.c.) with 10^9 inactivated whole *B. melitensis* and *B. abortus* cells per mL adjuvanted with the Sigma Adjuvant System® (SAS, Sigma Aldrich) or PBS. Mouse B3 (2.3) and D2 (4.2) immunised with γ irradiated *B. melitensis* and formalin inactivated *B. abortus*, respectively, were chosen for monoclonal antibody (mAb) production [23].

A second set of ten transgenic Ig-tg mice (2 per group) was immunised three times s.c. with 10^9 heat inactivated *B. melitensis* cells per mL in SAS (group A) or PBS (group B), heat inactivated *B. abortus* in SAS (group C) or PBS (group D) and γ irradiated *B. melitensis* in SAS (group E). Two additional booster injections were administered, using the same bacteria formulations. The first injection was given s.c. in SAS for

mouse A1-E1 and intravenously in PBS for mouse A2-E2, the second for all mice s.c. in SAS. Mouse A1 and E1 were chosen for the production of mAbs. An additional mouse was included to collect serum before immunisation (pre immune serum).

A third set of four mice (2 per group) were immunised three times s.c. with 20 μg recombinant protein BQ (group A) and BP (group B) per mouse both adjuvanted in SAS.

Serum was taken always at the day of immunisation (pre immune serum) and three weeks after each immunisation (immune serum).

ELISA

An indirect ELISA (iELISA) on whole *B. melitensis* and *B. abortus* cells was performed as described elsewhere [23]. Briefly, 10^7 heat treated or γ irradiated whole *B. melitensis* and *B. abortus* cells per mL in PBS were coated overnight at 4 °C in Maxisorp™ microtitre plates (Nunc, Thermo Scientific). Wells were then blocked with 5% milk powder in PBS or Superblock buffer (SuperBlock™ (PBS) Blocking Buffer, Thermo Scientific) before applying appropriate dilutions of mouse or bovine serum, respectively. A horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific, 1:4,000, Sigma Aldrich) or alkaline phosphatase (AP) conjugated goat anti-mouse IgG (γ chain specific, 1:10,000, Sigma Aldrich) antibody was used for detection of mouse serum, an alkaline phosphatase (AP) conjugated rabbit anti-bovine IgG (Fc fragment specific, 1:10,000, Jackson ImmunoResearch) antibody for the detection of bovine serum. ABTS substrate (ABTS® Peroxidase Substrate System, KPL) or 1 mg/mL p-Nitrophenyl phosphate (pNPP, Sigma Aldrich) in substrate buffer was applied and the optical density (OD) of the reaction product was recorded at 405 or 570 nm using a microplate reader. Serum was tested on recombinant proteins as described above by applying mouse serum on 10, 5, 2.5 and 1 μg/mL pre-coated BQ and BP proteins.

Immunofluorescence assay

As described before [23], the immunofluorescence assay (IFA) was performed using 10 μL heat inactivated or γ irradiated *B. melitensis* and *B. abortus* cells fixed by 4% paraformaldehyde (Sigma Aldrich) and 10% PBS diluted in distilled water to pre-coated Poly-L-Lysin IFA slides (Diagnostic Microscope Slides ES-242B-AD-CE24, Thermo Scientific). Diluted serum (1:100) was applied for 1 hour.

SDS-PAGE gel electrophoresis, Western blotting and silver staining

Western blot analysis was done as described elsewhere [23]. Briefly, mouse serum (before and after immunisation) was tested in a 1:100, 1:200 and/ or 1:1,000 dilution in PBS on inactivated (formalin, heat or γ) *B. melitensis* and *B. abortus* lysate (10^8 or 5×10^8 bacteria/mL) or 10 μg recombinant BQ and BP proteins per gel (200 μL). Bovine serum diluted 1:100 and 1:1,000 in Superblock buffer (SuperBlock™ (PBS) Blocking Buffer, Thermo Scientific) containing 0.05% Tween-20 was tested on *B. melitensis* (γ) and *B. abortus* (γ) lysate (5×10^8 bacteria/mL) after blocking with Superblock buffer. For detecting mouse serum a Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific, 1:4,000, Sigma Aldrich) or alkaline phosphatase (AP) conjugated goat anti-mouse IgG (γ chain specific), 1:10,000, Sigma Aldrich) antibody was used. An alkaline phosphatase (AP) conjugated rabbit anti-bovine IgG (Fc fragment specific, 1:10,000, Jackson ImmunoResearch) antibody was used to detect bovine serum. For the development of the assay the ECL (Pierce® ECL Western blotting substrate, Thermo Scientific) or the NBT/BCIP (BCIP 100X (5-bromo-4-chloro-3-indolyl phosphate) and NBT 100X (nitroblue tetrazolium, Biorad) system was used.

After separating proteins from *B. melitensis* (γ) lysate on a SDS-PAGE gel, the gel was stained with silver ions (SilverQuest™ Silver Staining Kit, Invitrogen) for protein visualisation. Stained protein bands of interest were excised, destained and sent for nano-LC mass spectrometry analysis (PMSCF, University Bern, Switzerland).

PEPperCHIP® design and performance of a peptide microarray

A new peptide microarray technology called PEPperCHIP® (PEPperPRINT, Heidelberg, Germany) was used to identify immunodominant antigens of *Brucella* species. Therefore 40 *Brucella* proteins (mainly membrane proteins listed in Table 1) were selected using GeneOntology and Patric databases together with a literature research for outer membrane proteins (OMPs) of *Brucella* [17, 26–34]. Same proteins from different *Brucella* spp*.* were included to cover sequence alterations. All proteins were translated into 15 amino acid (aa) peptides with a peptide-peptide overlap of 11 aa resulting in 3,521 different peptides. The C- and N-terminus of the antigens were thereby elongated by neutral GS linkers to avoid truncated peptides. In a next step all peptides were spotted as duplicates onto a polymer coated surface (10 nm PEGMA) with 2 x β-alanine and 1 x aspatic acid as linker. FLAG and HA peptides were spotted as control peptides. The unique PEPperCHIP® microarrays were produced by PEPperPRINT and the assay was performed according to their protocol from 2012. Briefly, for analyses of mouse serum, slides were pre stained with 1 mL Cy5 (DyLight 649) conjugated goat anti-mouse IgG (H&L, Rockland) antibody diluted 1:5,000 in staining buffer (PBS, pH7.4 with 0.05% Tween 20 and 0.1% BSA) for 30 min and blocked afterwards with 1 mL PBS containing 0.05% Tween-20, 1% BSA for 1 h. PBS with 0.05% Tween-20 was used as washing buffer. Pre immune and immune serum of mouse A1 (*B. melitensis,* heat) was used in a 1:200 dilution in staining buffer and applied after each other (1. pre immune, 2. immune serum) on the same slide over night at 4 °C. The slide was washed and the fluorescence intensity was measured in between. For detection the same Cy5 antibody was used and fluorescence intensities were measured using a microarray scanner (GenePix 4000B, Molecular Devices). The parameters were set as following: gain = 600, power = 100%, pixel size = 20 μm, lines to average = 5, focus = 30 μm. For the staining of the FLAG and HA control peptides, Cy5 conjugated monoclonal anti-HA (12CA5) and Cy3 conjugated anti-FLAG (M2, both 1:1,000) antibodies were used, both provided by PEPperPRINT. Data measured by the GenePix microarray scanner was further analysed using the PepSlide® Analyzer Software (Sicasys, Heidelberg, Germany) to obtain fluorescence intensities of each spotted peptide.

Bovine serum D (double positive) and V (double negative) were tested as mentioned above in a 1:500 dilution on individual microarrays. Superblock buffer (SuperBlock™ (PBS) Blocking Buffer, Thermo Scientific) containing 0.1% Tween-20 was used for blocking, Superblock buffer containing 0.05% Tween-20 for washing and staining. For the pre staining and detection step a Cy3 (DyLight 549) conjugated goat anti-bovine IgG (H&L, 1:100, Jackson ImmunoResearch) antibody was used.

Statistical analysis

Basic calculations (mean values, differences in signal intensities) were performed in Microsoft Excel. Figure assembly, data transformation and linear/ non-linear regression were performed with GraphPad Prism whereas figures containing Western blot and immunofluorescence assay data were generated with Adobe Photoshop CS.

4.4 **Results**

Analysis of immune responses after whole cell immunisation

Immunisation of mice with inactivated whole *Brucella* cells induced the production of Abs against LPS and *Brucella* proteins [23]. All immune sera tested showed dose dependent development of IgG antibodies reactive with inactivated whole *B. melitensis* cells in ELISA (Fig. 1A) and produced a circular surface staining with fixed *B. melitensis* cells in immunofluorescence microscopy (Fig. 1B). In Western blotting analyses with *B. melitensis* and *B. abortus* lysate all immune sera stained multiple protein bands (Fig. 1C). However, marked differences in staining patterns were observed. In general immunisation with γ irradiated bacteria (immunisation groups A and B) induced primarily antibodies reacting with protein antigens with a molecular weight >24kDa. In comparison, sera from mice immunised with formalin inactivated bacteria (immunisation groups C and D) reacted also strongly with some smaller antigens. Reactivity with *B. abortus* lysate was for all immune sera much weaker than with *B. melitensis* lysate.

Two fusions performed with spleen cells of mice immunised with γ irradiated bacteria (B3) or formalin inactivated bacteria (D2) and screened by whole cell ELISA resulted in the generation of LPS- but not protein-specific mAbs [23].

In a next step new sets of mice were immunised with inactivated whole *Brucella* cells with or without adjuvants. Individual animals tested positive in whole cell ELISA, Western blotting and immunofluorescence assay (Fig. 2) were selected for the generation of mAbs employing a different screening strategy.

In this alternative approach for the identification of immunodominant cell surface proteins, Western blot analyses (Fig. 3A) were used to identify proteins reactive with the immune sera. Nine protein bands corresponding in size to the stained bands were cut out from a silver stained SDS-PAGE gel (Fig. 3B). Nano-LC mass spectrometry (LC-MS) analyses identified in eight of the nine excised gel blocks a predominant protein with a sequence coverage of 24 to 77% and a protein abundance above 50 (Table 2). The calculated molecular weights of these proteins correlated with those observed in the Western blot analyses. The identified proteins are known to be located

in various compartments of the bacteria (ER, mitochondria, ribosome, cytoplasm, inner membrane and periplasm), but are most likely not associated with the cell surface.

Sera from mice belonging to the immunisation groups A, B, C, D, E (Fig. 2) were also tested for reactivity with the two recombinant *Brucella* proteins BQ and BP in ELISA and Western blotting (Fig. 4). Six mouse sera reacted with BQ (A1, A2, C1, C2, D1, D2) and one with BP (E1) in both assays with a specific staining for BQ and BP at 42kDa and 40kDa, respectively, in Western blot analysis (Fig. 4B). Based on these results mouse A1 and E1 were selected for the generation of mAbs. However, when screened by ELISA with recombinant BQ and BP, none of the culture supernatants yielded a positive signal.

Immunisation with recombinant *Brucella* antigens

Mice immunised with the recombinant *Brucella* proteins BP and BQ and sera were tested for reactivity with recombinant BQ and BP in ELISA and Western blot analysis. While strong and specific IgG responses against the recombinant proteins were observed in ELISA (Fig. 5A) and Western blotting (data not shown), no binding to *Brucella* cells was found in the cell ELISA (Fig. 5B). Also in Western blot analyses with *Brucella* lysate, no reactivity was observed (Fig. 5C).

Characterisation of the immunological profile of bovine serum

Bovine sera from a serological brucellosis study in northern Togo were tested for protein-specific immune responses in a whole cell ELISA. Sera had been tested before both by the Rose Bengal test and with a commercial brucellosis serum ELISA Test Kit. Nine double positive sera (A-I), five single positive sera (J-N) and eleven double negative sera (O-Y) were tested in ELISA on whole *Brucella* cells (Fig. 6). All double positive sera (A-I) and one single positive serum (K) showed high antibody titre against *B. melitensis* and *B. abortus* cells above a threshold of OD = 0.6.

When the bovine sera were tested in Western blot analysis on *B. melitensis* and *B. abortus* lysate (Fig. 7), the strongest signals were seen with the double and single positive sera A-N. While most of these tracings were characteristic for a LPS-specific

staining [23], some protein bands were also stained. Certain proteins seemed to be recognised by all single- and double-positive sera, but not by double negative sera, which showed only sporadic staining of a few proteins.

Evaluation of the PEPperCHIP® peptide microarray technology for screening

To avoid signals associated with the immunodominant LPS, a microarray (PEPperCHIP®) covering peptides derived from 40 selected *Brucella* proteins was evaluated as screening tool. Serum of mice immunised with whole *Brucella* cells (serum A1) and serum of naturally infected cattle (positive serum D and negative serum V) were selected. Although, all immune sera were reactive with *Brucella* proteins in ELISA or Western blotting (Fig. 2, 5, 7) only marginal reactivities with the *Brucella* protein-derived peptides were observed (Suppl. Fig. 1-2).

4.5 **Discussion**

Brucellosis is affecting a variety of domestic animals and wildlife with humans being infected accidentally. With 500,000 new cases every year, brucellosis is one of the major zoonoses occurring worldwide [1, 3]. Since early and intensive antibiotic treatment is crucial for efficiently treating human infections in the absence of a safe and effective vaccine [10, 16], a fast and precise diagnosis of brucellosis is important. Cultivation and the identification of the causative agent are required beside serological assays used to identify *Brucella*-specific antibodies. Mostly, whole cells, lipopolysaccharide (LPS) or the O-antigen of LPS are used as target antigen [11–13]. Since *B. canis,* capable of infecting humans [3], and *B. ovis* are both expressing the 'rough' version of LPS [14, 15] and cross-reactivities are observed, especially with *Y. enterocolitica* O9, serological assays based on 'smooth' LPS can lead to false negative or false positive results, respectively [35–38].

Primary aim of our project was to contribute to attempts to identify *Brucella* spp*.* specific immunodominant proteins [16–22] to support development of new tools for brucellosis control. Definition of new protein targets can in particular accelerate development of new antigen detection assays, candidate vaccines and molecular fine typing methods for epidemiological studies.

In this context, we were mainly interested in protein antigens at the surface of *Brucella* spp*.* that are accessible to the humoral immune system. Therefore, we have immunised mice with inactivated whole *Brucella* cells. The generated immune sera were highly reactive with whole *Brucella* cells and as expected, strong antibody responses against the O-polysaccharide of 'smooth' LPS were observed [39]. However, in addition, Western blotting analyses with *Brucella* cell lysates revealed that also protein-specific serum antibodies were generated. Using this immunisation approach for other bacterial pathogens, such as *Mycobacterium ulcerans* and *Neisseria meningitidis* (Diaz et al. [40], Ispasanie et al. (unpublished work)), has allowed us to generate panels of mAbs against ranges of cell surface proteins. In contrast, we obtained, in the case of the

Brucella immunised mice, only B cell hybridoma cell lines producing LPS-specific mAbs [23]. It might be that the screening on immobilised whole cells hampered the selection of cell lines producing anti-protein antibodies, since LPS might mask very efficiently the accessibility of protein antigens at the surface of *Brucella* cells [27, 41]. On the other hand, immune sera showed reactivities with *Brucella* proteins in Western blotting analyses. However, when we identified protein bands stained in Western blot analysis with *Brucella* lysates by mass spectrometry, we found that immune responses were directed against proteins located in various compartments of the bacterium (Table 2), but most likely not associated with the bacterial surface. This might be explained by processing of the bacterial cells after immunisation making immunodominant proteins, located inside the cell, accessible to the immune system.

In another approach we tested sera of whole cell immunised mice against the two recombinantly expressed periplasmic *Brucella* proteins BQ and BP. Some of the sera were indeed showing reactivities against BQ or BP in ELISA and Western blot analysis (Fig. 4) and two mice were selected for the production of mAbs by B cell hybridoma technology. However, when screened in ELISA on recombinant proteins, no B cell hybridoma line secreting BQ- or BP-specific mAbs could be identified, although, the immune sera of the mice used for hybridoma generation were positive in the same ELISA. We do not have an explanation for this, since microscopic analyses revealed that the fusion process per se worked and large numbers of hybridomas were generated.

Since the attempts to obtain protein-specific mAbs through whole cell immunisation had failed, we immunised mice in a next step directly with the two recombinant proteins BQ and BP, resulting in high IgG serum titres against both proteins in ELISA and protein-specific signals in Western blot analysis. However, no cross-reactivities could be observed with whole cells in ELISA or with *Brucella* lysate in Western blotting (Fig. 5). ELISA negativity might be related to the periplasmic localisation of the two proteins. Lack of cross-reactivity in Western blotting is more difficult to explain but it might be that

mainly conformational epitopes were recognised by serum antibodies, prohibiting the binding of the denatured protein in Western blot analysis.

To escape from the dominance of LPS reactivities in the hybridoma screening process, we evaluated the use of peptide microarrays [42] containing peptides derived from a series of *Brucella* proteins, mainly membrane proteins and outer membrane proteins. However, sera of mice immunised with whole *Brucella* cells showed only marginal binding to a few peptides, most likely representing unspecific background reactivities. It might be that the serum antibodies were recognising primarily conformational and discontinuous epitopes, not mirrored by the relatively short (15 amino acids) linear peptides. Furthermore, as indicated by LC-mass spectrometry analysis whole cell immunisation may, in the case of *Brucella* bacteria, not trigger preferentially antibody response against cell surface proteins. Serum of mice immunised with recombinant BQ or BP proteins was not tested since no BQ- or BP-derived peptides were present on the microarray. Since the microarray assay did not yield strong signals with the immune sera, no attempts were made to screen hybridoma supernatants with this method.

In a last step, we screened bovine serum antibody responses in the course of natural *Brucella* infections. Sera had been categorised before according to their infection status determined by the Rose Bengal test and a commercial serological ELISA test. All double positive and one single positive sera recognised whole *Brucella* cells in ELISA. When tested on *Brucella* lysate in Western blotting analyses, strong signals were observed both with LPS and a range of protein antigens. It is remarkable that some proteins were stained by practically all sera from *Brucella* infected animals, but not from uninfected controls. While the identification of these proteins by mass spectrometry would represent a promising approach for lining up immunodominant proteins, we did not follow this up, since we became aware of similar comprehensive studies performed by Wareth et al. [43]. Lack of reactivity of the cattle sera with the microarray may indicate that cell surface proteins are in the case of *Brucella* bacteria not immunodominant.

4.6 **Acknowledgements**

4.7 Tables and Figures

Table 1. List of *Brucella* proteins

No.	Protein	Region	AC[1] (GenBank)
1	membrane protein	permease of the drug/metabolite transporter (DMT) superfamily	WP_004686021
2	transporter, dme family	permease of the drug/metabolite transporter (DMT) superfamily	AAL51352
3	cytochrome C biogenesis protein	cytochrome c biogenesis protein CcdA	WP_002966050
4	transporter, dme family	permease of the drug/metabolite transporter (DMT) superfamily	AAL53681
5	preprotein translocase subunit TatC	sec-independent protein secretion pathway component TatC	WP_002964014
6	transporter, dme family	permease of the drug/metabolite transporter (DMT) superfamily	AAL51687
7	cadmium transporter	divalent metal cation (Fe/Co/Zn/Cd) transporter	WP_004685799
8	cadmium transporter	divalent metal cation (Fe/Co/Zn/Cd) transporter	WP_002964670
9+10	sodium alanine symporter	Na+/alanine symporter	WP_004684997
11	cobalt-zinc-cadmium resistance protein czcd	Co/Zn/Cd efflux system component	AAL52619
12+13	transporter	permease of the drug/metabolite transporter (DMT) superfamily	WP_004684405
14	MATE efflux family protein	Na+-driven multidrug efflux pump	AIJ86325
15	MATE efflux family protein	Na+-driven multidrug efflux pump	AIJ55733
16	biopolymer transport protein ExbB	TonB-system energizer ExbB	WP_002964760
17	MATE family efflux transporter	subfamily of the multidrug and toxic compound extrusion (MATE)-like proteins	WP_004685435
18	membrane protein	permease of the drug/metabolite transporter (DMT) superfamily	WP_002966348
19	C4-dicarboxylate ABC transporter permease	TRAP transporter, 4TM/12TM fusion protein	WP_004685691
20	C4-dicarboxylate ABC transporter permease	TRAP transporter, 4TM/12TM fusion protein	WP_004685691
21	OmpA-like transmembrane domain protein	opacity protein and related surface antigens	ACD73018
22	31 kDa outer-membrane immunogenic protein precursor	opacity protein and related surface antigens	AAL51583
23	outer-membrane immunogenic protein	opacity protein and related surface antigens	AEW14628
24	outer-membrane immunogenic protein	opacity protein and related surface antigens	EFG36419
25	outer membrane protein Omp31	opacity protein and related surface antigens	ADZ67646
26	OMP31	opacity protein and related surface antigens	ACV07678

|			
27	pETOmp	opacity protein and related surface antigens	CCJ65172
28	outer membrane lipoprotein omp19	protease inhibitor Inh	EFG36760
29	outer membrane protein 16	outer membrane protein OmpA and related peptidoglycan-associated (lipo)proteins	AGI97133
30	Porin Omp2b	Porin subfamily	AEQ08269
31	Omp2b	Porin subfamily	AAX83996
32	Omp2b	Porin subfamily	AHN46277
33	TonB-dependent receptor protein	TonB-dependent copper receptor	EEW87781
34	Bp26	uncharacterized conserved protein YggE	ABC17803
35+36	Dnak protein	molecular chaperone DnaK	AAL53183
37	molecular chaperone Hsp70	molecular chaperone DnaK (HSP70)	WP_019443743
38	molecular chaperone Dnak	RNA polymerase-binding protein DksA	WP_002964154
39	chaperone protein Dnak	molecular chaperone DnaK	EEH15197
40	flagellar motor protein MotB	flagellar motor protein MotB	WP_004685912

Proteins 1-20 represent membrane proteins identified by GeneOntology and PATRIC databases (GO term: 0016020), proteins 21-40 represent immunogenic proteins identified by Connolly et al. and He et al. [16, 17]. [1]Accession number.

**Table 2. Identification of unknown proteins by LC mass spectrometry**

Protein	PMSS[1]	Peptides[2]	Coverage[3] [%]	Weight[4] [kDa]	Description	Predicted localisation	AC[5] (Swiss-Prot, GenBank)
a	126.9	16	39.9	68.2	chaperone protein DnaK	ER, mitochondria, ribosome	Q8YE76
b	368.6	30	68.5	57.5	60 kDa chaperonin	cytoplasm	Q8YB53
c	139.0	18	46.6	54.8	ATP synthase subunit beta	inner membrane	Q8YJ35
d	223.4	19	65.2	42.6	probable sugar-binding periplasmic protein	periplasm	Q8YCE2
e	50.7	8	26.4	37.1	Leu/Ile/Val-binding protein homolog 1	periplasm	Q8YJ23
f	102.3	10	50.2	31.5	31 kDa immunogenic protein	periplasm	P0A3T2
g	89.5	10	53.0	23.4	probable transaldolase	cytoplasm	Q8YJ42
h	66.7	8	77.0	18.7	bacterioferritin	cell	P49944
i	16.2	2	23.7	10.5	50S ribosomal protein L23	ribosome	Q8YHN8

Nine unknown proteins (a-i) cross-reactive with immune serum of mice immunised with inactivated whole *Brucella* cells in Western blot analysis were identified by nano-LC mass spectrometry. Following parameters were measured: [1]protein abundance (semi quantitative measure), [2]protein unique peptides, [3]percent coverage of the entire protein sequence and [4]molecular weight. [5]Accession number.
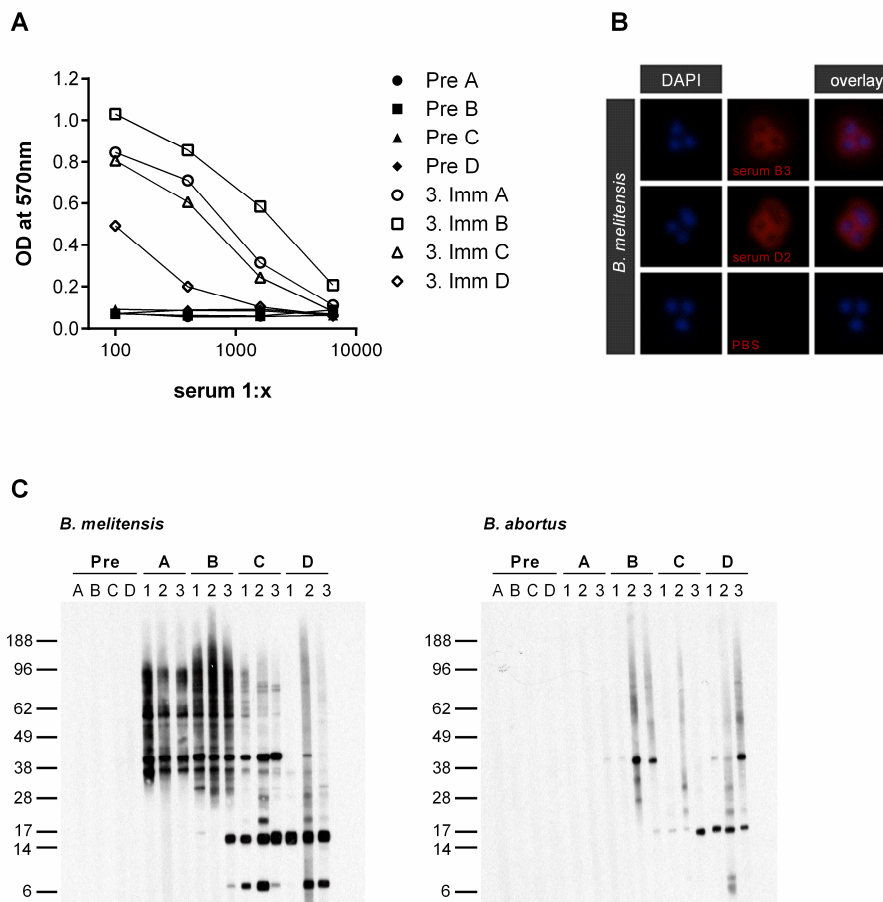
Figure 1. Serum reactivity of mice immunised with whole *Brucella* cells inactivated by formalin treatment or gamma irradiation
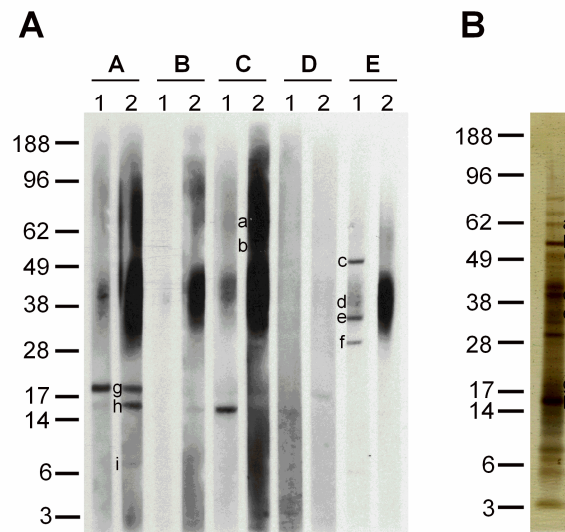
Four groups of mice were immunised three times with inactivated whole *Brucella* cells and serum was tested in ELISA, Western blot and immunofluorescence analysis (IFA). Group 1 (A) received gamma (γ) irradiated *B. melitensis* in PBS, group 2 (B) γ irradiated *B. melitensis* with Sigma Adjuvant System, group 3 (C) formalin inactivated *B. melitensis* in PBS and group 4 (D) formalin inactivated *B. abortus* in PBS. **(A)** Pre immune (Pre) and immune serum after the third immunisation (3. Imm) were pooled (3 mice each) and serial dilutions (4-fold, starting from 1:100) tested in ELISA on γ irradiated *B. melitensis.* **(B)** Immune serum (1:100) of mouse B3 and D2 and PBS as negative control (red) were tested in IFA on 2.5 x 10^8 fixed *B. melitensis* (γ) per mL. Nuclei were stained with DAPI (blue). Merged pictures of both stainings are shown in the right panel. Original magnification X160. **(C)** Pooled pre immune (Pre) and individual immune sera (1:200) after the third immunisation (group A-D) were tested on formalin inactivated *B. melitensis* and *B. abortus* (both 5 x 10^8 bacteria/mL) lysate.
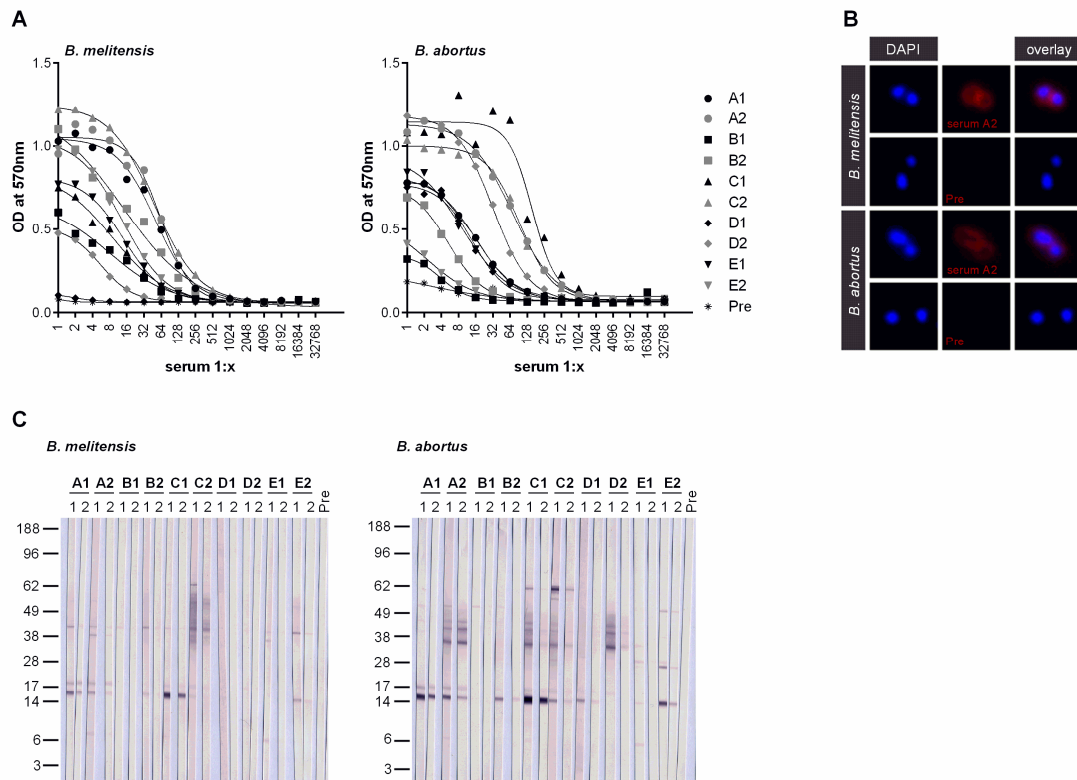
Figure 2. Serum reactivity of mice immunised with whole *Brucella* cells inactivated by heat treatment or gamma irradiation

Five groups of mice were immunised with inactivated whole *Brucella* cells and serum was tested with ELISA, Western blot and immunofluorescence analysis (IFA). Group 1 (A) received heat inactivated *B. melitensis* with Sigma Adjuvant System (SAS), group 2 (B) heat inactivated *B. melitensis* in PBS, group 3 (C) heat inactivated *B. abortus* with SAS, group 4 (D) heat inactivated *B. abortus* in PBS and group 5 (E) γ irradiated *B. melitensis* with SAS for three times. Two additional booster injections with the same bacteria formulation were given, first subcutaneously (s.c.) in SAS (A1-E1) or intravenously in PBS (A2-E2), second for all mice (A1-E2) s.c. in SAS. **(A)** Individual immune sera after the fifth immunisation and pre immune serum before immunisation (Pre) were diluted serially (2-fold, starting from 1:50) and tested in ELISA on γ irradiated whole *B. melitensis* and *B. abortus* cells. **(B)** Pre immune serum (Pre) and immune serum (1:100) of mouse A2 (red) was tested in IFA on 4% paraformaldehyde fixed *B. melitensis* (heat) and *B. abortus* (heat) cells (both 5 x 10^7 bacteria/mL). Nuclei were stained with DAPI (blue). Merged pictures of both stainings are shown in the right panel. Original magnification X160. **(C)** Pre immune (Pre) and individual immune sera after the fifth immunisation (1=1:100, 2=1:1,000) were tested on γ irradiated *B. melitensis* and *B. abortus* (both 5 x 10^8 bacteria/mL) lysate.

Figure 3. Identification of immune reactive proteins from mice immunised with whole *Brucella* cells

(A) Immune sera of mice (1:100, 2 mice per group) immunised three times with heat inactivated *B. melitensis* with Sigma Adjuvant System (SAS, A), heat inactivated *B. melitensis* in PBS (B), heat inactivated *B. abortus* with SAS (C), heat inactivated *B. abortus* in PBS (D) and γ irradiated *B. melitensis* with SAS (E) followed by additional booster injections with the same bacteria formulation first in SAS (A1-E1) or in PBS (A2-E2), second in SAS (all mice), were tested in Western blot analysis on γ irradiated *B. melitensis* (10^9 bacteria/mL) lysate. **(B)** A silver stained (SilverQuest™ Silver Staining Kit, Invitrogen) SDS-Page gel loaded with the same *B. melitensis* (γ) lysate was done in parallel to visualise separated proteins. Bands (a-i), immune reactive with mouse serum in Western blot analysis were excised from the stained gel and destained according to product guidelines. Unknown proteins were identified by nano-LC mass spectrometry (PMSCF, University Bern, Switzerland).
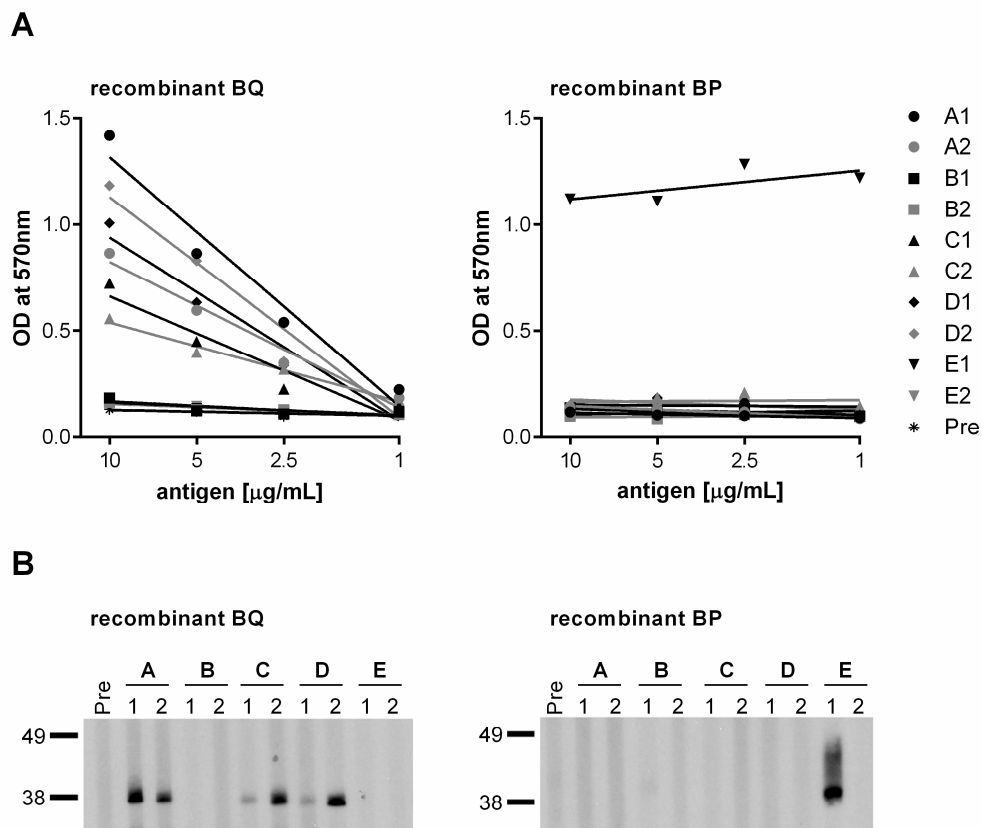
Figure 4. Reactivity of whole cell immunised mouse serum with recombinant BQ and BP antigens

Serum of mice (2 per group) immunised three times with heat inactivated *B. melitensis* with Sigma Adjuvant System (SAS, A), heat inactivated *B. melitensis* in PBS (B), heat inactivated *B. abortus* with SAS (C), heat inactivated *B. abortus* in PBS (D) and γ irradiated *B. melitensis* with SAS (E), followed by additional booster injections with the same bacteria formulation first in SAS (A1-E1) or in PBS (A2-E2), second in SAS (all mice), were tested in ELISA and Western blot analysis on two recombinant proteins (BQ and BP) of *Brucella* species. Pre immune serum (Pre) and individual immune sera (1:100) after the fifth immunisation were tested on 10, 5, 2.5 and 1 μg recombinant BQ or BP per mL in ELISA **(A)** and 10 μg recombinant BQ or BP per gel in Western blot analysis **(B)**.
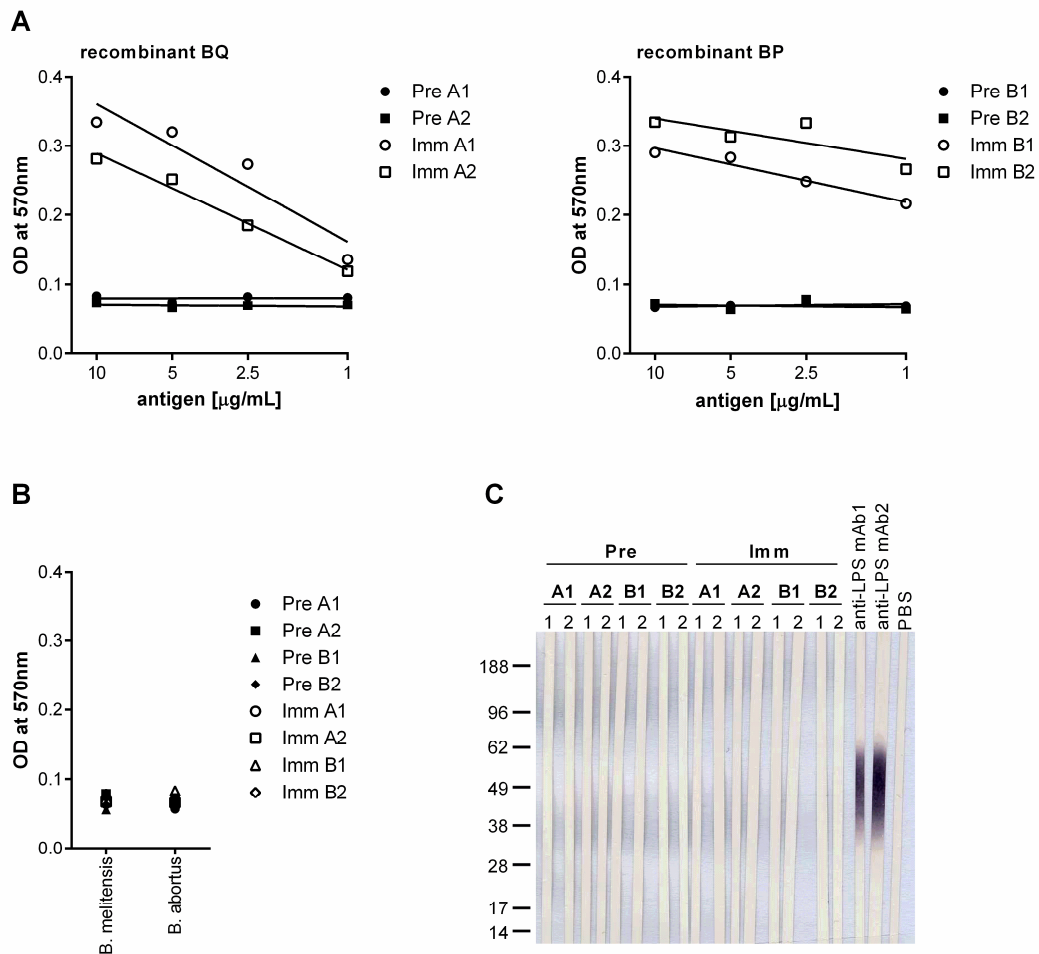
Figure 5. Serum reactivity of mice immunised with recombinant BQ and BP

Two groups of mice were immunised with recombinant BQ or BP proteins as adjuvanted formulation, in combination with the Sigma Adjuvant System (SAS). Two mice per group received three subcutaneous injections with 20 μg recombinant BQ (A) or BP (B) in combination with SAS. Pre immune (Pre) and immune serum after the third immunisation (Imm) were diluted 1:100 and tested separately in ELISA on 10, 5, 2.5 and 1 μg recombinant BQ or BP per mL **(A)** and heat inactivated whole *B. melitensis* and *B. abortus* cells **(B)**. Same sera (1=1:100, 2=1:1,000) were tested in Western blot analysis on *B. melitensis* (heat, 10^8 bacteria/mL) lysate **(C)**. Anti-LPS monoclonal antibodies (mAbs 3D12 (1) and 10G1 (2), [23]) and PBS served as controls.
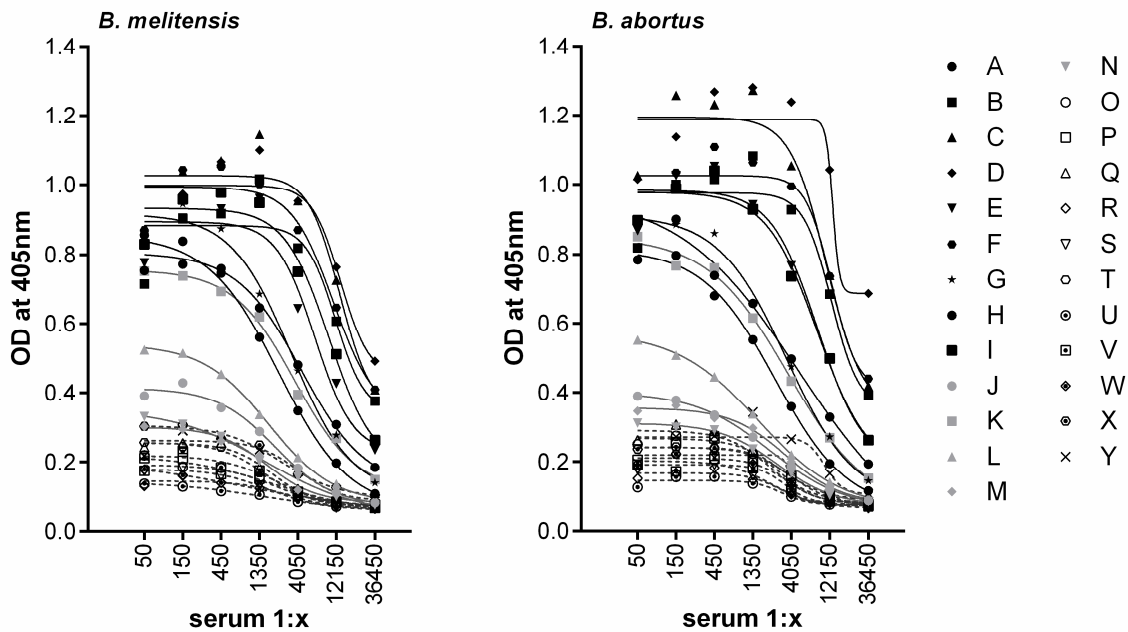
Figure 6. Reactivity of bovine serum with inactivated whole *Brucella* cells in ELISA

Serum from cattle (livestock in Northern Togo) tested positive for *Brucella* species by iELISA and the Rose Bengal test (RBT, serum A-I), by iELISA alone (J-N) and iELISA and RBT negative control serum (O-Y) was serially diluted (3-fold, starting from 1:50) and analysed in ELISA on γ irradiated whole *B. melitensis* and *B. abortus* cells.
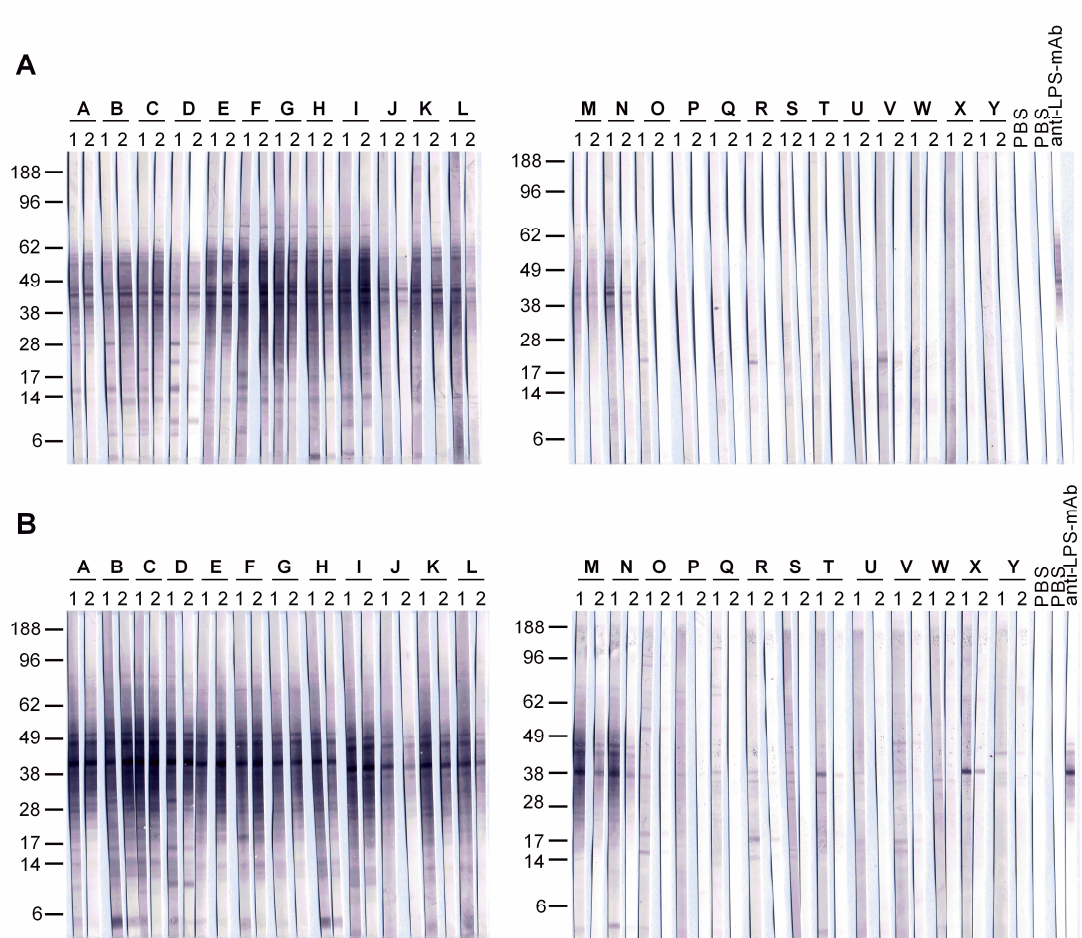
Figure 7. Reactivity of bovine serum with *Brucella* lysate

Serum from cattle (livestock in Northern Togo) tested positive for *Brucella* species by iELISA and the Rose Bengal test (RBT, serum A-I), by iELISA alone (J-N) and iELISA and RBT negative control serum (O-Y) was tested in Western blot analysis on *B. melitensis* (γ, **A**) and *B. abortus* (γ, **B**) lysate (both 5 x 10^8 bacteria/mL). All sera were diluted 1:100 (1) and 1:1,000 (2). An anti-LPS monoclonal antibody (mAb 3D12, [23]) and PBS were included as controls.
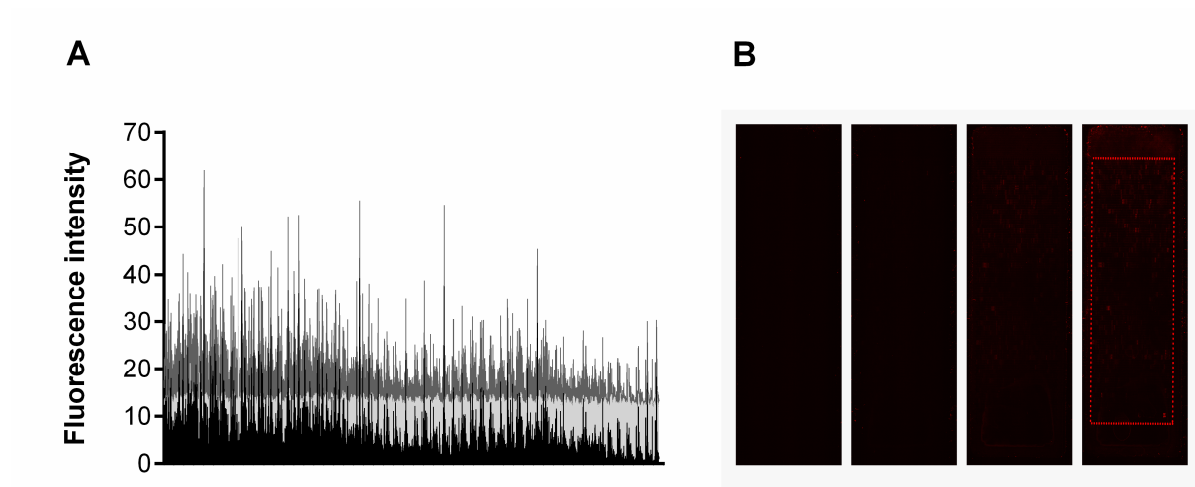
Supplementary Figure 1. Screening of serum from mice immunised with inactivated whole *Brucella* cells with peptide microarrays

Serum of mice immunised with heat inactivated *B. melitensis* was tested on microarrays (PEPperCHIP®) covering protein-derived peptides of *Brucella* bacteria. **(A)** Fluorescence intensities of immune serum after the fifth immunisation (dark grey) and pre immune serum before immunisation (light grey) are shown for all peptides. Differences in signal intensity are shown in black. **(B)** Original microarray scans are showing (from left to right) the array before applying serum (negative control), after applying pre immune serum, after applying immune serum and after applying Cy5-conjugated monoclonal anti-HA (12CA5) antibodies to stain HA control peptides surrounding the array. Serum was tested in a 1:200 dilution and Cy5-conjugated goat anti-mouse IgG antibodies were used for detection. Fluorescence intensity was measured using a microarray scanner (GenePix 4000B, Molecular Devices) with parameters set as following: gain = 600, power = 100%, pixel size = 20 μm, lines to average = 5, focus = 30 μm.
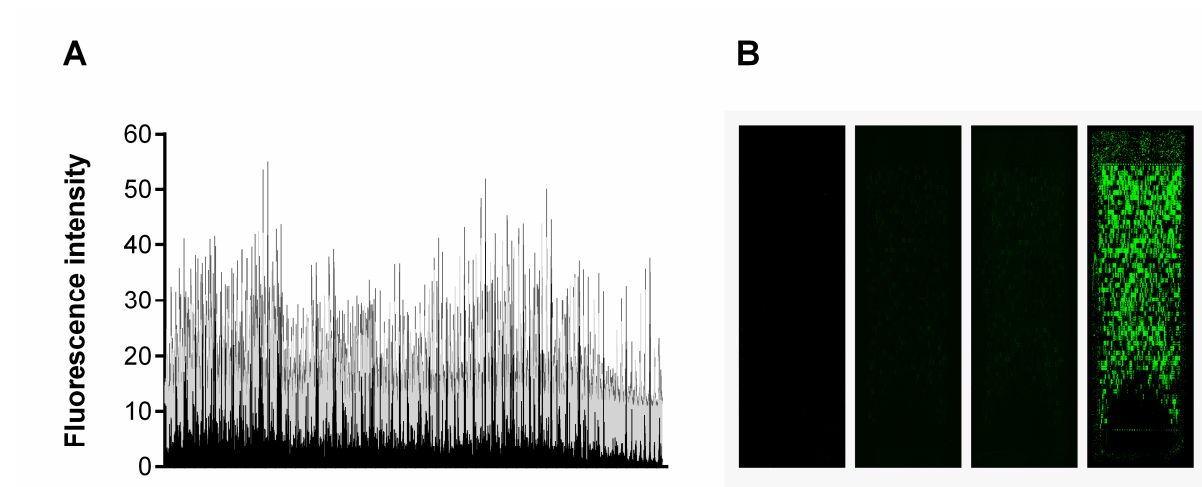
Supplementary Figure 2. Screening of bovine serum with peptide microarrays

Bovine serum of naturally infected cattle, classified as double positive or double negative serum by the Rose Bengal test and a commercial iELISA was tested individually on microarrays (PEPperCHIP®) covering protein-derived peptides of *Brucella*. **(A)** Fluorescence intensities of double positive (dark grey) and double negative (light grey) serum are shown for all peptides. Differences in signal intensity are shown in black. **(B)** Original microarray scans are showing (from left to right) the array before applying serum (negative control), after applying double negative serum, after applying double positive serum and after applying Cy3-conjugated monoclonal anti-FLAG (M2) antibodies to stain FLAG control peptides surrounding the array. Serum was tested in a 1:500 dilution and Cy3-conjugated goat anti-bovine IgG antibodies were used for detection. Fluorescence intensity was measured using a microarray scanner (GenePix 4000B, Molecular Devices) with parameters set as following: gain = 600, power = 100%, pixel size = 20 μm, lines to average = 5, focus = 30 μm.

4.8 **References**

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5. Article 3. Lack of parasite growth inhibitory activity of *P. falciparum* RH2-specific monoclonal antibodies

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Working chapter

5.1 **Abstract**

We have recombinantly expressed the 40kDa receptor-binding domain of the *Plasmodium falciparum* reticulocyte-binding homolog 2 (PfRH2$_{40}$) in *Escherichia coli* and used the adjuvanted purified protein to immunise mice. Using spleen cells of an immunised mouse we subsequently generated a set of anti-PfRH2 monoclonal antibodies (mAbs) by B cell hybridoma technology. All five mAbs obtained cross-reacted with the erythrocyte invasion ligand PfRH2, produced by schizont stage parasites, and yielded in immunofluorescence microscopy the expected dotted staining pattern characteristic for rhoptries. Nevertheless, none of the mAbs was active in an *in vitro* growth inhibition assay on its own. Furthermore, the anti-PfRH2 mAbs did not enhance the parasite growth inhibitory activity of mAbs specific for the cysteine-rich protective antigen (PfCyRPA). Also, in a *P. falciparum* animal infection model based on NOD-scid IL2Rγ^{null} mice engrafted with human erythrocytes, anti-PfRH2 mAbs did not show parasite inhibitory activity.

Keywords

PfRH2, malaria, *Plasmodium*, blood stage, vaccine, growth inhibition

5.2 **Introduction**

In 2013, the WHO estimated 200 million malaria-infected people and 500,000 deaths worldwide [1] with *Plasmodium falciparum* causing most of the severe malaria cases in humans [2]. An effective malaria vaccine could be an important tool towards malaria control and elimination [3]. Since the most advanced vaccine candidate, RTS,S, showed only partial protection against malaria, second generation vaccines are of great need [4, 5]. Clinical symptoms in malaria are primarily caused by toxic substances released into the blood stream during rupture of erythrocytes [1], hence a disease-preventing vaccine needs to block merozoite-erythrocyte interactions during erythrocyte invasion. Single antigens from blood stage parasites, including MSP1 and AMA1 tested in clinical trials, were not blocking erythrocyte invasion efficiently [6, 7]. However, approaches that targeted multiple merozoite antigens yielded promising invasion-inhibitory effects [8–10].

Previous studies indicated that rabbits immunised with different fragments of the *P. falciparum* reticulocyte-binding homolog 2 (PfRH2) elicited inhibitory serum antibodies that efficiently block *P. falciparum* parasite invasion *in vitro* [2, 8, 11–16]. PfRH2, a member of the PfRH protein family, is a blood stage merozoite adhesin expressed during erythrocytic schizogony and located in the rhoptry neck of *P. falciparum* apical organelles [11–13, 17]. During merozoite invasion, PfRH2 is relocated from the rhoptry neck both to the moving junctions and the merozoite surface to bind human erythrocytes through sialic acid independent receptors [13, 18, 19]. The receptor-binding domain is a conserved region of 40kDa [14], identical in the two homologs of PfRH2 (PfRH2a and PfRH2b).

In this study, the receptor-binding domain of PfRH2 (PfRH2$_{40}$) was recombinantly expressed in *E. coli* and used to immunise mice to generate a set of anti-PfRH2 monoclonal antibodies (mAbs). However, none of the produced mAbs showed any activity in *in vitro* or *in vivo* growth inhibition assays.

5.3 **Materials and Methods**

Ethics statement

This study was carried out in strict accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzverordnung) of the Swiss Federal Food Safety and Veterinary Office. The protocol was granted ethical approval by the Veterinary Office of the county of Basel-Stadt, Switzerland (Permit Number: 2375).

Cultivation of *P. falciparum* parasites

P. falciparum (strain 3D7) was cultivated as published elsewhere [20, 21]. Medium was supplemented with 0.5% AlbuMax™ (Life Technologies) and parasites were cultivated with 5% human erythrocytes received from the Swiss Red Cross (Basel, Switzerland). Cultures were synchronised by sorbitol [22] or percoll treatment [23] to obtain ring-stage parasites or schizonts, respectively.

Generation of recombinant PfRH2$_{40}$

The 40kDa receptor-binding domain of PfRH2, consisting of amino acids 495-860, was expressed recombinantly in *E. coli* together with a hexahistidine (His) tag as described elsewhere [15].

Production of anti-PfRH2 mAbs

Mouse immunisation and generation of mAbs were performed as described previously [24]. Briefly, NMRI mice (Harlan) were immunised three times subcutaneously with 25 μg of recombinant PfRH2$_{40}$ (rPfRH2$_{40}$) per mouse adjuvanted with the Sigma Adjuvant System® (SAS, Sigma Aldrich). The mouse selected for cell fusion received an additional intravenous booster injection with 25 μg rPfRH2$_{40}$ in PBS. Myeloma cells (PAI) were mixed 1:5 with spleen cells in Iscove's Modified Dulbecco's Medium (IMDM, Sigma Aldrich) to seed about 0.2 million cells per well of ten 96-well culture plates. Five PfRH2-specific IgG antibody containing wells were identified by ELISA.

ELISA

Maxisorp™ microtitre plates (Nunc, Thermo Scientific) were coated overnight (ON) at 4 °C with 50 µL of a 4 µg/mL solution of $rPfRH2_{40}$. Wells were then blocked with 5% skim milk powder in PBS before applying serial dilutions of mouse serum or purified mAbs in PBS. An alkaline phosphatase-conjugated goat anti-mouse IgG (γ-chain specific) antibody (Sigma-Aldrich) was added for detection of bound IgG. Hundred µl/well of 1 mg/mL p-Nitrophenyl phosphate (pNPP, Sigma Aldrich) in substrate buffer were finally added and the optical density (OD) of the reaction product was recorded at 405 nm.

Isotypes of anti-PfRH2 mAbs were determined by detecting mAbs bound to $rPfRH2_{40}$-coated plates with alkaline phosphatase-conjugated antibodies specific for mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Parasite lysates were prepared by saponin lysis of *P. falciparum* 3D7-infected erythrocytes as described elsewhere [20]. Briefly, percoll-synchronised parasite cultures were pelleted and erythrocytes lysed in 20-30 volumes of 0.06% (w/v) saponin in PBS for 20 min on ice. Parasites were washed once with PBS and the pellet dissolved in 3-5 volumes of PBS.

Hundred µL $rPfRH2_{40}$ (0.5 µg/gel) or parasite lysate was mixed equally with sample buffer (Laemmli buffer, Invitrogen) and heated for 15 min at 70 °C before loading on 4-12% Bis-Tris gels (Life technologies). SeeBlue® pre-stained protein standard (Invitrogen) was used as a molecular weight marker. Following gel electrophoresis, proteins were transferred electrophoretically to nitrocellulose membranes. Blots were blocked with 3% milk powder in PBS containing 0.05% Tween-20, cut into strips and then incubated with 500 µL mouse serum (1:100) or purified mAbs (10, 1, 0.1 µg/mL). The strips were washed with 0.5% milk, 0.05% Tween in PBS, incubated with alkaline phosphatase-conjugated goat anti-mouse IgG heavy-chain antibodies (Sigma Aldrich) and afterwards treated with ECL Western blotting detection reagent (ECL Western Blotting Substrate, Pierce) to visualise bands.

Immunofluorescence assay (IFA)

For immunofluorescence microscopy, parasite cultures were pelleted and diluted 1:5 in PBS to prepare smears. Slides were fixed in ice cold 60% methanol and 40% acetone for 2 min and blocked with 3% BSA in PBS. Fifty μL of 1:100 diluted mouse serum and 10 μg/mL mAbs diluted in blocking buffer were added and incubated for one hour. Wells, painted on the slide with an oil-based water repellent pen, were washed five times with blocking buffer before 50 μL of detection antibody was added for an additional hour. Alexa 488-labeled goat anti-mouse IgG (H+L) antibodies (Invitrogen) were used to detect bound anti-PfRH2 mAbs or mouse serum antibodies and a directly Alexa 488-labeled mouse anti-RAP-1 5-2 mAb was used to stain the RAP-1 antigen [25]. Blocking buffer alone served as negative control. Finally, wells were washed five times, mounted with ProLong® Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) and covered with a coverslip. Antibody binding and DNA staining were assessed by fluorescence microscopy (magnification X63).

In vitro growth inhibition assays

In vitro growth inhibition assays were performed as described elsewhere [26]. Briefly, 50 μL *P. falciparum* 3D7 cultures were set up in triplicate in 96-well culture plates with 50 μL purified anti-PfRH2 mAbs in final concentrations of 500, 250 and 125 μg/mL for 96 hours (two cycles of erythrocyte invasion). For combination tests, 250 or 125 μg/mL finally concentrated anti-PfRH2 mAbs were mixed with same concentrations of the parasite growth-inhibitory anti-CyRPA mAb c12 [27] in 50 μL PBS. Hydroethidine™ (0.15 μg/mL) was used to stain viable parasites and the assay was analysed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest software. For each sample a total of 30,000 cells were analysed and the percent growth inhibition calculated from the mean parasitemia of triplicate test and control wells as follows: percent inhibition = (control-test)/(control/100). Anti-CyRPA mAb c12 and an isotype-matched control (anti-mycolactone) mAb JD5.2 were included as positive and negative control, respectively.

In vivo growth inhibition assays

MAbs were tested in the *P. falciparum* SCID murine model [28] employing non-myelodepleted NOD-scid *IL2Rγ*[null] mice engrafted with human erythrocytes essentially as described [27, 28]. Human blood (0.75 mL) was administered daily by the intravenous (i.v.) or intraperitoneal route. Three mice per group received a single dose of 0.5 or 2.5 mg anti-CyRPA mAb c12 and one mouse a single dose of 2.5 mg anti-PfRH2 mAb AS2 by i.v. injection one day before infection with 3×10^7 parasitised erythrocytes. The control group received the same volume of PBS. Parasitemia was monitored daily by flow cytometry over six days (day four to nine after mAb injection).

Statistical analysis

All data were obtained from experiments performed in duplicate (at a minimum). In each experiment antigen-free controls (PBS instead of sample) were included to determine the cut-off. Mean value, standard deviation and percent growth inhibition was calculated in Microsoft Excel. Figure assembly, data transformation and non-linear regression (sigmoidal curve, dose-response variable slope) were done with GraphPad Prism and Adobe Photoshop.

5.4 **Results**

Generation of PfRH2-specific mAbs

All mice, immunised three times with 25 μg of adjuvanted rPfRH2$_{40}$, developed high anti-PfRH2 IgG titres in ELISA (Fig. 1A). In Western blotting analysis with rPfRH2$_{40}$, serum antibodies stained, as expected, a band with a molecular mass of about 40kDa (Fig. 1B). When tested with *P. falciparum* 3D7 schizont lysate, the immune sera stained predominantly a 80kDa band (Fig. 1B), one of the fragments (80, 140, 220kDa [14]) expected for the endogenous PfRH2. In immunofluorescence microscopy with fixed *in vitro* cultivated schizonts, a dotted staining was observed with all sera (Fig. 1C) consistent with the localisation of the endogenous protein in the parasite rhoptries [11–13, 17].

Five hybridoma cell clones (AS1-AS5) producing anti-PfRH2 mAbs of the IgG1(λ) isotype were generated by conventional B cell hybridoma technology with spleen cells of one of the immunised mice and screening with rPfRH2$_{40}$ coated ELISA plates.

Characterisation of PfRH2-specific mAbs

All five generated mAbs recognised rPfRH2$_{40}$ both in ELISA (Fig. 2A) and in Western blotting analysis with comparable efficiency. On fixed parasites, they all yielded the expected dotted staining pattern (Fig. 2C). Anti-RAP-1 mAb [25] was included as positive control staining of the rhoptry bulb of *P. falciparum* (Fig. 2C). However, in Western blot analysis with schizont lysate, staining was only observed with mAb AS2, showing specific signals at 80 and 220kDa (Fig. 2B).

Performance of anti-PfRH2 mAbs in *in vitro* growth inhibition assays

Even at a concentration as high as 500 μg/mL, none of the five mAbs generated showed any *in vitro* parasite growth inhibitory activity (Fig. 3A). Also in combination with the parasite growth inhibitory anti-CyRPA mAb c12 [27], anti-PfRH2 mAbs caused no significant enhancement of the inhibitory activity (Fig. 3B).

Performance of anti-PfRH2 mAbs in an *in vivo* growth inhibition assay

The Western blotting positive mAb AS2 was selected for testing in the *P. falciparum* mouse infection model, employing non-myelodepleted NOD-scid *IL2Rγ^null^* mice engrafted with human erythrocytes [28]. In the PBS control group parasitemia reached 19.4 ± 0.8% by the end of the experiment at day nine (Fig. 4). Anti-PfRH2 mAb AS2 showed no parasite inhibitory effect *in vivo*. In contrast, parasite growth in the presence of the anti-CyRPA control mAb c12 was inhibited in a dose dependent manner as expected with a parasitemia at day nine of 2.3 ± 0.6% (2.5 mg) and 10.1 ± 2.3% (0.5 mg).

5.5 **Discussion**

PfRH2 is a protein located in rhoptries of blood stage merozoites [2, 13, 29] that is one of the parasite ligands translocated to the moving junctions and the parasite surface during invasion to bind erythrocytes through sialic acid independent receptors [13, 18, 19]. Our study was conducted to evaluate the potential of antibodies raised against $rPfRH2_{40}$ to inhibit erythrocyte invasion.

We generated a set of five mAbs specific for the 40kDa receptor-binding domain of PfRH2 that showed cross-reactivity with PfRH2, associated with the rhoptries of schizont stage parasites. Although specific binding of the mAbs to their target structure could thus be demonstrated, they showed no biological activity in an *in vitro* growth inhibition assay. Furthermore, none of the five anti-PfRH2 mAbs enhanced the growth inhibitory activity of the anti-CyRPA mAb c12 [27]. The anti-PfRH2 mAb AS2, tested in addition in the *in vivo P. falciparum* SCID-murine model [28], showed no inhibitory effect on parasite growth. In contrast, a parasite *in vitro* growth inhibitory activity has been described for polyclonal anti-PfRH2 antibodies [2, 8, 11–16]; in two of these studies rabbits had been immunised with the 40kDa receptor-binding domain of PfRH2 ($PfRH2_{40}$) also used here. Our results show, that binding of antibodies to this receptor-binding domain does not inevitably lead to inhibition of erythrocyte invasion by merozoites. Possibly, binding of antibodies to several epitopes of PfRH2 is required to achieve the relatively limited inhibitory activity (25% [14] and about 30% [15]) observed at a high end final concentration (5 mg/mL) of polyclonal anti-$PfRH2_{40}$ rabbit IgG.

5.6 **Conclusion**

PfRH2 is regarded as a potential malaria blood stage candidate vaccine antigen located in the apical organelles of merozoites and interacting with receptors on the surface of erythrocytes. While several studies have shown that polyclonal anti-PfRH2 antibodies can partly inhibit merozoite invasion, *in vitro* we have not found such an activity for parasite-binding mAbs generated against the PfRH2 receptor-binding domain.

5.7 **Acknowledgements**

5.8 **Figures**

A

B

C
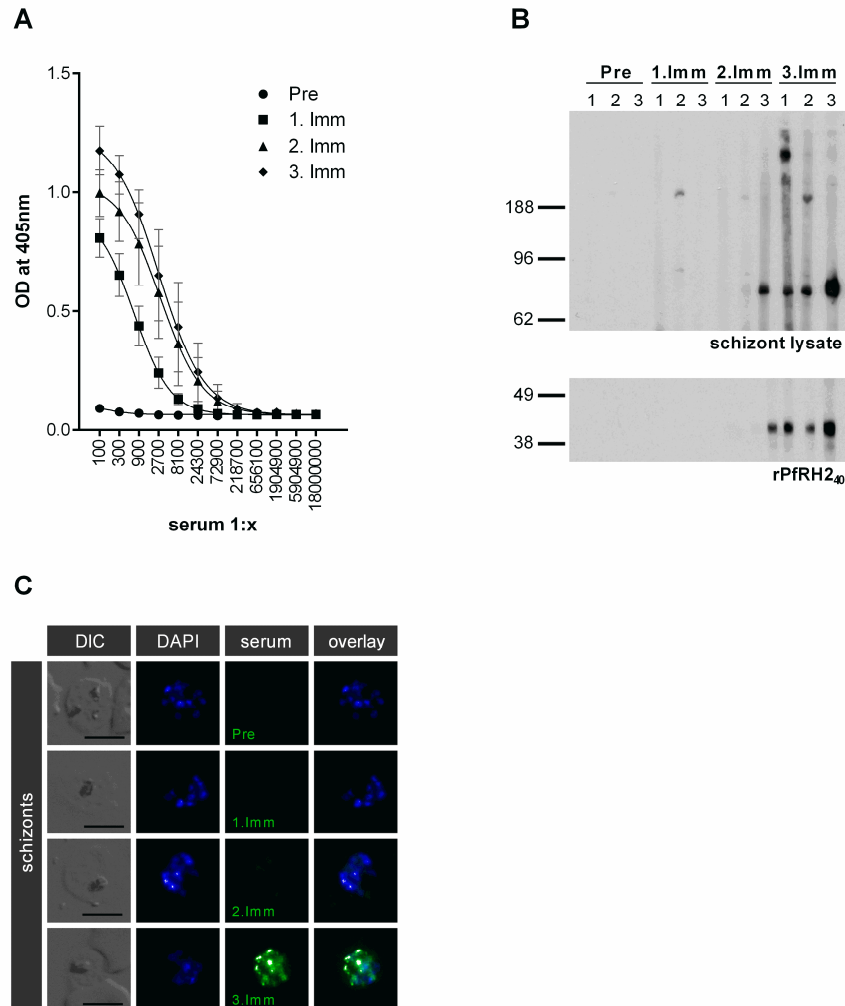
Figure 1. Immunogenicity of rPfRH2$_{40}$ in mice

Serum samples, taken before immunisation (Pre), after the first (1. Imm), the second (2. Imm) and the third immunisation (3. Imm) of mice with adjuvanted rPfRH2$_{40}$, were tested for the induction of anti-PfRH2 IgG antibodies. **(A)** Titration (1:3 dilution steps) of sera tested in ELISA on plates coated with rPfRH2$_{40}$. **(B)** Western blot profiles obtained with 1:100 diluted mouse sera after SDS-PAGE separation of *P. falciparum* strain 3D7 schizont lysate or rPfRH2$_{40}$. **(C)** Staining of methanol/acetone fixed schizont parasites with 1:100 diluted mouse serum (green) by immunofluorescence microscopy. Nuclei were stained with DAPI (blue). Merged pictures of both stainings are shown in the right panel. Scale bars represent 5 μm. Original magnification X63.
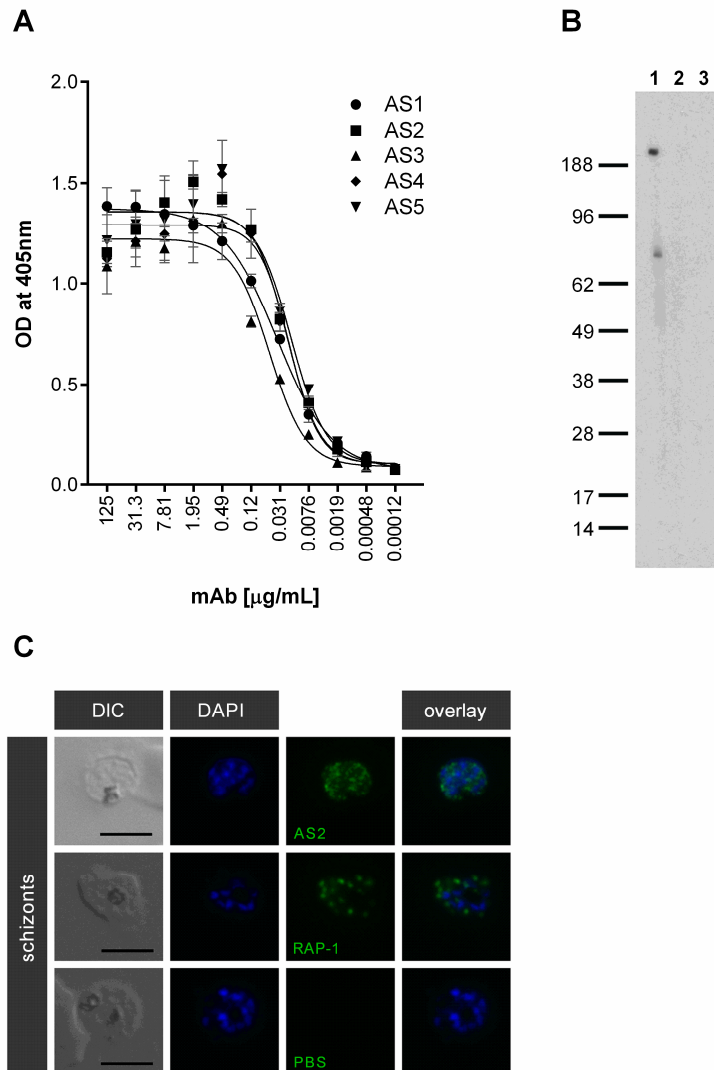
Figure 2. Antigen-binding activities of anti-PfRH2 mAbs

(A) Titration of mAbs (1:4 dilution steps starting from a concentration of 125 μg/mL) in ELISA using plates coated with rPfRH2$_{40}$. (B) Western blot staining pattern obtained with mAb AS2 (concentration: 1: 10 μg/mL, 2: 1 μg/mL and 3: 0.1 μg/mL) on *P. falciparum* strain 3D7 schizont lysate containing PfRH2 derived proteins of 80, 140, 220kDa. (C) Immunofluorescence staining of methanol/acetone fixed schizont parasites with mAb AS2 (concentration: 10 μg/mL). Nuclei were stained with DAPI (blue). Anti-RAP-1 mAb 5-2 and PBS were included as controls. The right panel shows merged pictures. Scale bars represent 5 μm. Original magnification X63.
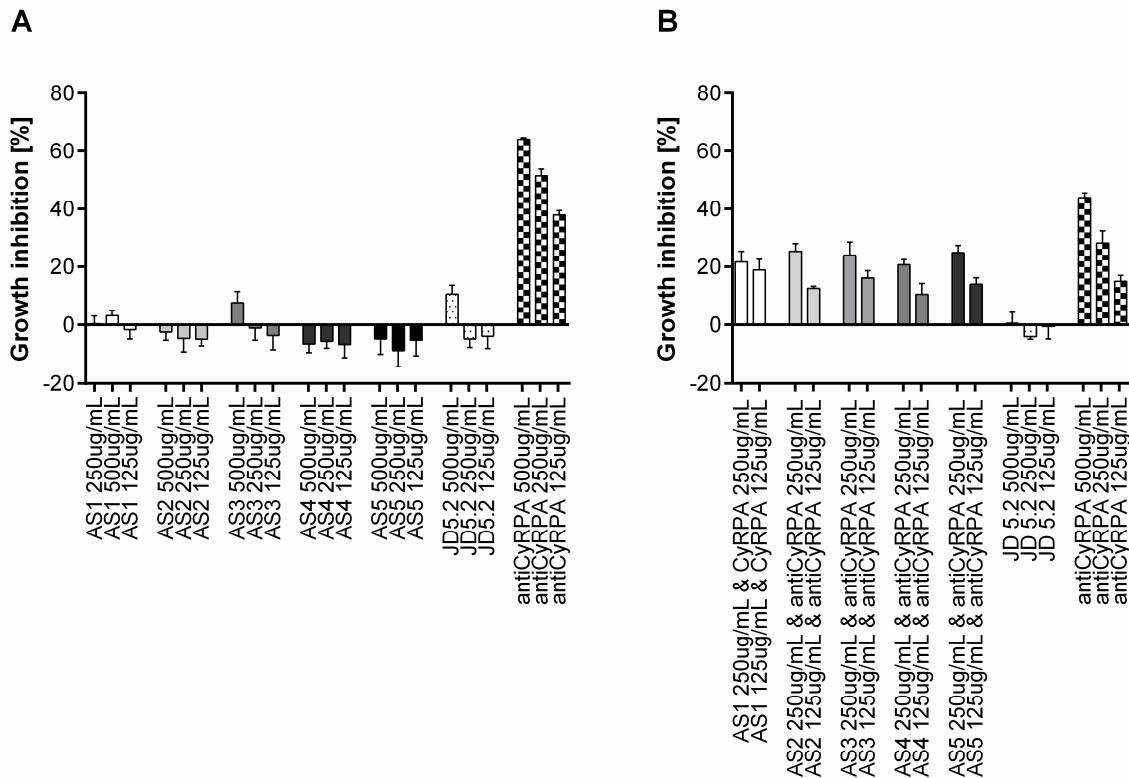
Figure 3. *In vitro* growth inhibitory effect of anti-PfRH2 mAbs

(A) Single mAbs (500, 250 and 125 µg/mL) and (B) mAb combinantions of 250, 125 µg/mL anti-PfRH2 mAbs and 250, 125 µg/mL anti-CyRPA mAbs, respectively, were incubated with synchronised *P. falciparum* 3D7 parasites for 96 hours to access their effect on merozoite invasion. Assays were performed in 96-well culture plates with parasite cultures adjusted to a final haematocrit and parasitemia of 0.5%. The parasite growth inhibitory anti-CyRPA mAb c12 and the unrelated isotype-matched anti-mycolactone mAbs JD5.2 were included as positive and negative control, respectively. Parasite growth inhibition was calculated against the parasitemia of PBS controls and expressed as percent growth inhibition. MAbs were tested in triplicate with single bars representing the mean value. Error bars indicate the SD.
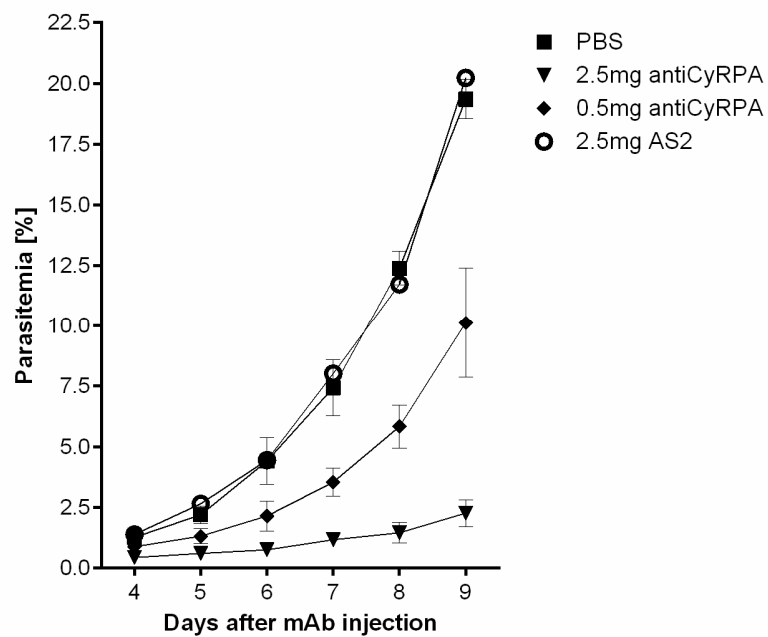
Figure 4. *In vivo* growth inhibitory effect of anti-PfRH2 mAbs

NOD-*scid IL2Rγ^{null}* mice were infected with *P. falciparum* 3D7 one day after receiving 0.5 or 2.5 mg anti-CyRPA mAb c12 or 2.5 mg anti-PfRH2 mAb AS2 by i.v. injection. Parasitemia was monitored over six days. The mean parasitemia in human erythrocytes in the peripheral blood of three mice per group is depicted with error bars indicating the SD. Only one mouse received anti-PfRH2 mAb AS2. PBS served as negative control, the anti-CyRPA mAb c12 as positive control.

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6. General Discussion and Conclusions

6.1 Monoclonal antibodies as multifunctional tools

Since the discovery of the hybridoma technology in 1975 by Georges J. F. Köhler and César Milstein, monoclonal antibodies (mAbs) have been used for various applications including research, diagnostics and therapy [1]. One current focus is the clinical use of mAbs as immune suppressor after transplantations (e.g. Muromonab-CD3) as well as therapeutics to treat autoimmune diseases, cancer and other diseases such as allergic asthma or cardiovascular problems [2]. The use of mAbs against infectious diseases is yet uncommon with only two mAbs approved for the treatment of respiratory syncytial virus (Palivizumab) and *Bacillus anthracis* infections (Axibacumab) [3]. Although several mAbs for the treatment of different viral, bacterial and fungal infections are currently tested in clinical trials, none of them target parasitic diseases [4]. In addition to their therapeutic use, mAbs are suitable to determine HLA and tumour markers, pregnancy and blood groups as well as for the detection of pathogens and the diagnosis of diseases. Especially in the nuclear medicine, mAbs became an important diagnostic tool for various infectious and inflammatory diseases and cancer [3, 5]. Furthermore, mAbs are widely used in basic laboratory research for the identification, localisation, quantification and characterisation of target molecules by standard assays such as Western blotting, microscopy/ immunofluorescence microscopy, ELISA, flow cytometry, immunohistochemistry and immunoprecipitation [1, 6].

Within the framework of this thesis, we generated mAbs against surface antigens of *Brucella* species for implementation in new antigen detection tools as well as mAbs against the reticulocyte-binding homolog 2 (PfRH2) of *Plasmodium falciparum* to evaluate their potential to inhibit erythrocyte invasion as an important criterion for the qualification of the target antigen as vaccine component.

6.2 **A sensitive mAb-based Luminex assay for *Brucella* spp. detection**

With the production of *Brucella* lipopolysaccharide (LPS)-specific mAbs we were able to develop an antigen detection assay for *Brucella* species (spp.). This assay has proven favourable for various applications due to three main features: 1) the detection of *Brucella* spp. was possible in complex samples such as milk, 2) four potential bio-threat agents including *Brucella* spp. could simultaneously be detected in a multiplex format and 3) with 2×10^2 to 8×10^4 bacteria per mL the test shows a high sensitivity for the detection of *Brucella* spp. (Article 1).

Detection of *Brucella* spp. in natural outbreaks

Natural outbreaks of human brucellosis related to the consumption of unpasteurised dairy products have been reported from several countries, especially due to *Brucella melitensis* contaminations of sheep and goat milk [7–9]. In livestock (cattle, sheep, goats and camel) it was reported that up to 80% of infected animals constantly shed *Brucella* spp. into the milk due to persisting bacteria in the mammary glands and lymph nodes [10]. Furthermore, studies showed that *B. melitensis* could be isolated from sheep more than three years after initial infection and that one cow, infected with *B. abortus*, even shed bacteria into the milk for up to nine years post infection [11]. Such a continuous contamination of milk with viable *Brucella* spp. poses a major threat for animals and humans. In order to screen dairy livestock for *Brucella* infections, milk would be an optimal sample, since it is cheap and easily obtained either from bulk tanks where the milk of all animals is collected, or from individual animals. Collecting milk samples can be achieved without invasive methods and testing can be performed regularly and followed up with individual blood testing in case of a positive test result on milk [12, 13].

The here developed Luminex assay is a promising new tool for the implementation in milk testing if further studies confirm the performance of the assay on raw milk samples of infected animals. So far, the detection of *B. melitensis* with the Luminex assay was comparable between spiked PBS and milk samples and was not impaired by other milk

contents. These findings indicate, that *B. melitensis*, *B. abortus* and *B. suis* could be detectable in milk with the same limits of 2×10^2, 5×10^3 and 8×10^4 bacteria per mL, respectively, as observed in experimental tests with PBS (Article 1).

To date, bacterial culture is the gold standard for brucellosis diagnosis, since it reflects the current infection state by identifying viable *Brucella* spp., but serological assays are frequently performed to save time [10, 12, 14]. Two serological assays are commonly applied for detecting *Brucella* antibodies in milk, the milk/ *Brucella* ring test (BRT) and the indirect milk ELISA (iELISA). The advantages of the BRT are its simple execution and the fact that it can be read by eye. Additionally, it takes only one hour or, for increased sensitivity, overnight. The BRT shows a sensitivity of 85-89% and a specificity of 95% [15–17]. Despite these advantages, the World Organisation for Animal Health (OIE) stated that the BRT is not effective for detecting *B. melitensis* in milk [18]. In comparison, the iELISA assay, using 'smooth' *B. melitensis* LPS as coating antigen, revealed a sensitivity of 100% for *B. abortus* (23 out of 23) and 97% for *B. melitensis* (30 out of 31) in testing milk from infected cattle when compared to bacterial culture [19]. Several tests (ID Screen® Brucellosis Milk Indirect, SVANOVIR®*Brucella*-Ab I-ELISA) are also commercially available but mainly for testing bovine milk [20, 21]. Both serological assays are simple, portable, sensitive and commercial assays enable standardised procedures. However, since the diagnosis relies on antibodies it rather reflects exposure than active disease. Also, antibodies elicited by vaccination or induced by bacteria sharing similar LPS, lead to false positive test results [14, 22].

Furthermore, several molecular diagnostic methods have been evaluating milk to detect *Brucella* DNA. A study using an *omp*2-based PCR assay identified 65% of *B. melitensis* infected goats without cross-reactivities with closely related bacteria or *Y. enterocolitica* O3, O9, *V. cholera* O1 and *E. coli*. Furthermore, as few as ten *Brucella* bacteria (*B. melitensis* and *B. abortus*) could be detected in 1 mL of spiked milk [23]. A PCR assay using the 16S rRNA sequence of *B. abortus* had a detection limit of 170 *B. abortus* cfu/mL and 1,700 *B. melitensis* cfu/mL in spiked milk samples. Direct comparison of the PCR assay and a *B. melitensis* LPS-based iELISA revealed sensitivities of 87.5% and 98.2%, respectively, compared to culture positivity of milk

samples from infected cows. Polymerase inhibitors in the milk are thought to interfere with the PCR [19]. A *bcsp*31-based multiplex PCR detected as few as 800-2,000 *B. abortus* cfu/mL in spiked milk samples. Sensitivities were 85% with PCR and 75% with the BRT when milk was tested from *B. abortus* infected cows. On pooled bulk milk, PCR only identified four out of six samples (67%) correctly [24]. A study using an IS711-based PCR assay revealed a detection limit of 100 *B. abortus* cfu/mL and 1,000 *B. melitensis* cfu/mL [25]. Molecular methods are complex and performed primarily in reference laboratories. Overall, sensitivities were comparable to serological assays available for milk testing. Newer approaches to detect *Brucella* spp. in milk such as the loop-mediated isothermal amplification (LAMP) assay are currently being developed. This assay was used to amplify DNA of several *Brucella* spp. in a single tube and *Brucella* spp. could be specifically detected in milk samples of infected animals [26]. Furthermore, micro-Raman spectroscopy was evaluated to identify *Brucella* spp. in spiked milk samples and sensitivities between 93.6 and 100% were observed for this technique [27].

All serological assays and molecular methods described here have certain advantages and disadvantages, need standardisations, improved sample preparation or further evaluation. The Luminex assay developed during the scope of this thesis proofed to have *B. melitensis* and *B. abortus* detection limits comparable with those of PCR-based methods. Furthermore, the assay is able to detect *B. suis*, is not negatively influenced by complex samples and can detect intact bacteria present in acute brucellosis infections. Despite these promising advantages, the Luminex assay is most likely too complex for testing milk samples on-site since it requires expensive machinery and trained personnel which makes the assay more suitable for applications in reference laboratories. Although lower sensitivities were reached with the corresponding capture ELISA (Article 1), it might still be possible to detect bacteria in milk. Additionally, ELISA applications are easier to perform and therefore more suitable for the implementation in decentralised control activities. Overall, the testing of individual and bulk milk samples is a suitable approach to detect infections in animals and the combination of several test methods might strengthen the diagnostic power.

Detection of bio threat agents including *Brucella* spp. in bioterrorism attacks

Beside the application of the Luminex assay for detecting natural infections in livestock, the assay could also be implemented for the detection of bio threat agents in case of a bioterrorism attack. A possible scenario could be the contamination of the food chain. Since *Brucella* spp. can be killed by pasteurisation at 72-75 °C for 15 to 30 seconds [28], milk or other milk products would have to be contaminated after such procedures or during storage. The survival time of *Brucella* spp. in milk products is 87 days in UHT milk at 20 °C, up to four days in raw unpasteurised milk at 5 °C and less than a week in yogurt at 5 °C [28]. In areas where pasteurisation is not at all or insufficiently applied, infection of dairy livestock might lead to *Brucella* contaminations of milk products. Since the Luminex assay proved able to detect *Brucella* spp. in milk samples, the assay could also be applicable for testing other milk products. Appropriate preparation methods to homogenise or dissolve the sample in buffer have to be developed.

Another possible infection scenario concerns the distribution of *Brucella* spp. via aerosols, with ten bacteria sufficient for the establishment of an infection [10, 12]. Such an aerosol distribution could also lead to a contamination of the environment since *Brucella* spp. are quite robust with survival times of 4 to 66 days in soil and 1 to >57 days in open water [12]. In a potential bio threat situation, performing bacterial cultures is of great importance since this is the only method to investigate whether bacteria are still viable [12]. However, cultivation takes time and fast detection is required to access the dimensions and to implement appropriate measures and decontamination procedures [29]. Apart from this, multiplex assays are able to simultaneously test for different bio threat agents next to *Brucella* spp. as shown by different study groups [30–32]: i) A multiplex PCR-based reverse line blot hybridisation assay (mPCR/RLB) was developed and simultaneously detected *Bacillus anthracis*, *Yersinia pestis*, *B. melitensis* and *Burkholderia pseudomallei* through hybridisation of species-specific biotin-labelled PCR products to membrane-bound probes [30]. In two other studies, the Luminex technology was used to detect amplified multiplex PCR products specific to ii) *B. anthracis*, *Y. pestis*, *Francisella tularensis* and *B. melitensis* [31] or iii) *B. anthracis*, *Y. pestis*, *F. tularensis*, *Brucella* spp. and *B. pseudomallei* [32] through hybridisation

with fluorescent-bead-labelled probes. All multiplex assays mentioned here were species-specific without cross-reactivities or interferences of different primer or target DNA samples. Detection limits of 0.3 pg (i), 0.1 pg (ii) and 22.5 pg (iii) were observed for *Brucella* template DNA with 1 pg corresponding to 200 bacteria.

The developed Luminex assay is also capable of detecting four bio threat agents simultaneously. Since the detection of all four bacteria, *Brucella* spp., *B. anthracis*, *F. tularensis* and *Y. pestis* could be performed in a single sample without interferences or cross-reactivities, the assay represents a powerful tool for a simultaneous and high throughput detection of these organisms, especially if the source of the biological threat is unknown. The shown ability to detect all four bio threat agents in complex samples such as milk hints towards the probable applicability of the test for the detection of bacteria in environmental samples including soil or water. We could likewise show that inactivation methods such as gamma-irradiation, formalin inactivation or heat treatment did not affect the specific binding of the monoclonal antibodies to the *Brucella* surface (Article 1). Hence, the inactivation of a sample prior to testing would reduce the risk of accidental infection and therefore simplify further test procedures without interference with the antibody-antigen binding.

Results of immunological assays such as the Luminex assay should be reconfirmed by PCR-based methods since cross-reactivities, mainly due to the structural similarities between O-antigens, are reported [14]. For the Luminex assay this applies for the *Y. enterocolitica* O9 O-antigen (Article 1). As mentioned before, several PCR assays are valid for detecting *Brucella* DNA in milk which could be applied for the detection of *Brucella* spp.. In addition, further conventional and real-time PCR assays are available [15]. However multiplex PCR-based approaches which target several bio threat agents [30–32] might be more suitable for this venture, especially if the biological threat agent is unknown and the possibility of an attack with multiple agents exists. PCR assays such as the 'Bruceladder' multiplex PCR [11] are able to differentiate all *Brucella* spp., including vaccine strains, to the species level within the same reaction. This species-level distinction is of great importance since not all *Brucella* spp. pose the same threat to human and animal hosts [14, 33, 34]. A combination of bacterial cultures,

sensitive antigen detection assays (Luminex assay) and appropriate PCR-based methods could allow fast and specific identification of biological threats for a detailed risk assessment and implementation of appropriate procedures.

The Luminex assay as potential diagnostic tool

Currently, the diagnosis of human and animal brucellosis is mainly based on cultivation and isolation of the causative organism supported by different serological diagnostic assays [10, 12, 14, 22, 35].

Although no clinical samples have been tested so far, the Luminex assay might enable the detection of intact bacteria or LPS present in blood, serum, organs/ tissue or body fluids of infected humans and animals. Especially, since low detection limits of 2×10^{2}, 5×10^{3} and 8×10^{4} bacteria per mL for *B. melitensis*, *B. abortus* and *B. suis*, respectively, have been observed (Article 1). While antigen detection would be a strong indication for an ongoing infection, serological assays rather provide a retrospective picture of disease exposure. Recently, a similar approach was pursued using a mAb-based capture ELISA which is able to detect 'smooth' LPS in i) serum samples spiked with *B. melitensis* LPS, ii) serum of mice challenged with *B. melitensis* cells and iii) serum of humans with blood cultures positive for *B. melitensis*. The capture ELISA has proven to be a feasible tool for *Brucella* LPS detection in clinical samples [36]. The Luminex assay showed fewer cross-reactivities than serological assays [14, 22], which in addition to cross-reactivity with *Y. enterocolitica* O9 also seen with the Luminex assay, show also false-positive reactions with other similar O-antigens of *Vibrio cholera* O1 and *Escherichia coli* O157 and with *Ochrobactrum anthropi,* the closest relative of *Brucella* spp.. Furthermore, apart from *B. canis* and *B. ovis*, expressing a 'rough' LPS, all other classical *Brucella* spp., *B. melitensis* (biovar 1-3), *B. abortus* (1,3), *B. suis* (1,2) and *B. neotomae* can be detected by the Luminex assay, independently of the biovar. In addition, test results can be obtained within one day, saving time compared to bacterial culture and since the assay is performed in 96-well plates, a large number of samples can be tested in parallel.

6.3 Immunodominant *Brucella* protein antigens

For 'smooth' *Brucella* spp*.* it is known that the LPS predominantly mediates the antibody response upon infection and therefore almost all serological assays are based on 'smooth' LPS as target antigen [22, 37, 38]. However, analyses of sera from naturally or experimentally infected animals and humans with confirmed brucellosis revealed that *Brucella* protein antigens also stimulate the immune system. Identified antigens include enzymes (superoxide dismutase, transaldolase), heat shock proteins (chaperone DnaK, GroEL), binding proteins (ABC amino acid transporter periplasmic binding protein), ribosomal proteins, proteins for energy production and reduction/oxidation reactions (ATP synthase, fumarate reductase) and membrane associated proteins (OMP2b, OMP16, OMP31b, OMP25, Bp26) [39–43]. Such immunodominant proteins are of great interest for various applications such as serological assays, antigen detection systems, vaccine development and epidemiological studies. Furthermore, since 'smooth' LPS is inadequate for testing for exposure to 'rough' *Brucella* spp. such as *B. canis* and *B. ovis* [22, 35], protein antigen-based tests might offer better prospects.

Our Western blotting analyses on *Brucella* lysate identified both LPS and protein-specific antibodies in the serum of naturally infected cattle and mice immunised with whole *Brucella* cells (Article 2). For antigen characterisation and applications in antigen detection assays, monoclonal antibodies were produced from the mice immunised with whole *Brucella* bacteria. However, only LPS-specific mAbs were obtained, although comparable studies on *Mycobacterium ulcerans* and *Neisseria meningitidis* in our laboratory had shown that cell surface protein-specific mAbs can be produced by mouse immunisation with and hybridoma screening on bacterial cells [44]. However, our mass spectrometry analysis, performed with *Brucella* proteins reactive with mouse immune serum, revealed no *Brucella* surface-exposed proteins. Since *Brucella* spp. are intracellular pathogens, immune responses against almost any antigen might be elicited by natural infection upon processing of the bacteria by macrophages [45]. While for antigen detection assays surface exposed antigens are required to recognise whole cells, the identified non-surface exposed immunodominant

proteins of *Brucella* spp. might be suitable for serological testing. With proteins instead of 'smooth' LPS as target antigens it may be possible to develop serological tests that differentiate between vaccination, exposure to different *Brucella* spp. and exposure to other gram negative bacteria with cross-reactive O-antigens [14, 22]. To evaluate whether identified immunodominant proteins are suitable antigens for serological approaches, series of sera from exposed humans and animals need to be screened to select antigens with the best performance for particular hosts.

Towards a safe and effective vaccine against human brucellosis different approaches are on the way. Subunit vaccines based on recombinant proteins or plasmid DNA are promising vaccine candidates since they are more safe than live-attenuated vaccines [46]. For potential inclusion into subunit vaccines several proteins have been tested alone or in combination with other antigens or delivery systems in mouse models for brucellosis [47]. Some of these antigens such as OMP16 [48, 49], OMP31 [50–53], Cu-Zn superoxide dismutase [54–56] and Bp26 [57], formulated as protein or DNA vaccine, showed good protective efficacies similar to live attenuated animal vaccines [47]. Since these protective protein antigens were identified by serum analyses for immunodominant antigens [39–43], testing of sera from humans and animals infected with *Brucella* spp. seems to be a suitable and powerful tool to identify new proteins with potential for subunit vaccines. While some of the vaccine candidates showed promising results in mouse models, none of them has been tested in clinical trials.

6.4 **New malaria blood stage candidate vaccines**

Currently, malaria is threatening almost half of the world's population with recent estimates of 200 million infected people and more than half a million deaths per year. Within the *Plasmodium* species, *P. falciparum* is the most virulent responsible for 98% of malaria-related deaths, primarily among children below the age of five in sub-Saharan Africa [58, 59]. Upon an infectious mosquito bite parasites enter the human body where they first invade hepatocytes to undergo initial development steps before they enter the blood stream to invade and replicate in erythrocytes. Clinical symptoms of malaria and severe disease are directly linked to the erythrocytic phase due to the release of parasite-derived toxic substances into the blood upon rupture of the infected erythrocyte, the release of cytokines by the host and sequestration of the infected erythrocytes [60, 61]. Although, the development and replication of *P. falciparum* parasites is primarily intracellular, i) sporozoites entering the human body through an infectious bite and ii) merozoites released from infected hepatocytes and erythrocytes are exposed to the extracellular environment and to the humoral immune system. To allow invasion, these extracellular forms need close cell-cell interactions with host cells which is mediated by surface molecules and molecules secreted from apical organelles of the parasite. Such proteins are potential targets for protective antibodies and therefore candidate antigens for the development of a blood stage vaccine [60]. Evidences that humans can be vaccinated against malaria were obtained by individuals living in endemic areas with frequent exposure to malaria who became first resistant to severe malaria and later on to clinical disease [62]. In addition, the fact that immunoglobulin transferred from semi-immune adults to *P. falciparum*-infected children reduced parasitaemia, showed that antibodies are relevant for immune protection against *P. falciparum* blood stages [63]. Therefore, attempts are made to develop a blood stage component for a malaria vaccine that elicits antibodies which either block erythrocyte invasion or inhibit subsequent replication of the parasites [64].

Currently, vaccine development against blood stage parasites relies on a handful of merozoite antigens including AMA1, EBA-175, GLURP, MSP1, MSP2, MSP3 and

SERA5. Most of them are evaluated in phase I trials to assess safety and immunogenicity aspects [65–77]. Phase II trials with the leading candidate antigens AMA1 and MSP1 failed to effectively protect from clinical malaria [78–81]. Also a vaccine combining MSP1, MSP2 and the ring-infected erythrocyte surface antigen (combination B) did not effectively protect children from clinical malaria episodes [82]. However, an AMA1 and CSP-based DNA vaccine, boosted with adenovirus vectors, induced sterile protection in 27% of naïve adults when challenged with bites of infected mosquitoes due to cell-mediated immunity to AMA1 and CSP [83]. Furthermore, it was reported that AMA1 and CSP-based peptidomimetics, formulated in virosomes, reduced the rate of clinical malaria episodes in vaccinated children by 50% [84]. These multistage candidate vaccines combine an anti-infection component (CSP) against sporozoites and an anti-invasion as well as an anti-disease component (AMA1) against merozoites [85]. In comparison, RTS,S/AS01, a pre-erythrocytic vaccine based on CSP and the leading candidate in *P. falciparum* malaria vaccine development, efficiently protects 36% of young children and 26% of infants from clinical malaria in sub-Saharan Africa [86, 87].

To contribute to the development of new blood stage candidate vaccines, we evaluated the *P. falciparum* reticulocyte-binding homolog 2 (PfRH2) in functional *in vitro* assays and in an *in vivo* mouse model (Article 3). PfRH2 is a member of the reticulocyte-binding homolog (PfRH) family including PfRH1-5, expressed in schizonts and merozoites where it is located in the rhoptry neck of apical organelles interacting with receptors on the surface of erythrocytes. Previous studies revealed that serum antibodies induced upon immunisation of rabbits with different PfRH2 fragments were able to efficiently block erythrocyte invasion by *P. falciparum* merozoites *in vitro* [88–95]. To confirm these observations, we immunised mice with the 40kDa receptor-binding domain of PfRH2 and generated PfRH2-specific mAbs by B cell hybridoma technology. These mAbs cross-reacted with the endogenous PfRH2, produced by schizont stage parasites and showed a rhoptry-characteristic staining in immunofluorescence microscopy but failed to inhibit erythrocyte invasion by *P. falciparum* merozoites *in vitro* (Article 3). While binding of the produced mAbs to the receptor-binding domain of

PfRH2 was not enough to inhibit invasion, it is possible that different epitopes of PfRH2 have to be targeted to achieve an invasion inhibitory effect.

Functional *in vitro* assays such as the growth inhibition assay (GIA) are frequently used to assess the functionality of vaccine-induced antibodies, both in pre-clinical studies and in clinical trials by measuring antibody-dependent inhibition of parasite replication and erythrocyte invasion [96]. These assays offer a rapid and simple tool to evaluate new blood stage candidate vaccine antigens potentially involved in the invasion of or the replication in erythrocytes. However, concerns exist about the significance of such assays, especially regarding a future potential protective effect in humans against clinical malaria. Alternatively, the growth inhibitory effect of antibodies can be studied *in vivo* in nonhuman primates or rodent models such as SCID-mice engrafted with human erythrocytes. Recently, the *P. falciparum* NOD-scid *IL2Rγ^null* mouse model, engrafted with human erythrocytes, was used to evaluate a new blood stage candidate vaccine antigen of *P. falciparum*, the cysteine-rich protective antigen (PfCyRPA) [97, 98]. It has recently been reported that a complex, which is essential for the invasion of erythrocytes by *P. falciparum*, is formed including PfCyRPA, the reticulocyte-binding homolog 5 (PfRH5) and the PfRH5-interacting protein (PfRipr) [99]. Furthermore, PfRH5 is another promising blood stage candidate vaccine inducing inhibitory antibodies and capable of efficiently protecting *Aotus* monkeys against *P. falciparum* challenge [100–104]. PfCyRPA-specific mAbs, which showed already strong parasite growth inhibitory activity *in vitro*, did also protect mice by passive immunisation. Parasite multiplication in this SCID-mouse model is very reproducible and a determination of the concentration of antibodies, required for parasite growth inhibition, is possible by analysing the dose-dependency of the inhibitory activity (Article 3). As already indicated by the negative *in vitro* results, no parasite growth inhibitory activity was observed when PfRH2-specific mAbs where tested in the SCID-mouse model. Furthermore, the PfRH2-specific mAbs did not enhance the *in vitro* growth inhibitory effect of PfCyRPA-specific mAbs.

6.5 **Conclusions**

In this thesis the generation of monoclonal antibodies against immunodominant *Brucella* cell surface antigens and the *P. falciparum* reticulocyte-binding homolog 2 (PfRH2) is described. The monoclonal antibodies were used for the development of a sensitive antigen detection assay specific for *Brucella* spp. and contributed to the evaluation of PfRH2 as potential new *P. falciparum* blood stage candidate vaccine antigen.
Major findings are:

1. Serum of mice immunised with whole *Brucella* cells and cattle naturally infected with *Brucella* spp. were used to identify immunodominant *Brucella* antigens. Western blot analyses on *Brucella* lysate revealed the generation of serum antibodies against both LPS and a range of *Brucella* proteins. However, mass spectrometry analyses showed that none of the proteins reactive with the mouse immune sera was located on the bacterial cell surface and thus suitable as target for an antigen capture assay for the detection of *Brucella* cells.

2. When spleen cells of mice immunised with whole *Brucella* cells were used for the generation of monoclonal antibodies (mAbs) by B cell hybridoma technology, exclusively mAbs specific for the O-antigen of lipopolysaccharide (LPS) were obtained. Attempts to produce *Brucella* protein-specific mAbs by this immunisation approach and screening by whole cell ELISA were not successful.

3. For the development of an antigen capture assay detecting *Brucella* spp. bacteria, two LPS-specific mAbs were selected. While mAb 1 was coupled to magnetic beads to capture the antigen, a biotinylated mAb 2 was used for detection. Implemented in the Luminex xMAP technology, the mAbs specifically detected *Brucella* spp. expressing 'smooth' LPS with high sensitivities.

4. The developed Luminex assay was integrated in a multiplex format and allowed for the simultaneous and specific detection of the four bio threat agents *Brucella* spp. *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* in complex samples.

5. Mice were immunised with the 40kDa receptor-binding domain of the *P. falciparum* reticulocyte-binding homolog 2 (PfRH2) and PfRH2-specific mAbs were generated by B cell hybridoma technology. All mAbs recognised the erythrocyte invasion ligand PfRH2, produced by schizont stage parasites, and showed a rhoptry-characteristic staining in immunofluorescence microscopy.

6. To access whether PfRH2-specific mAbs are able to inhibit erythrocyte invasion by *P. falciparum* merozoites, mAbs were tested in an *in vitro* growth inhibition assay. None of the PfRH2-specific mAbs showed any activity on its own and the parasite growth inhibitory activity of cysteine-rich protective antigen (PfCyRPA)-specific mAbs was not enhanced by PfRH2-specific mAbs. Furthermore, when tested in a *P. falciparum* experimental infection model based on NOD-scid IL2Rγ[null] mice engrafted with human erythrocytes, PfRH2-specific mAbs did not show parasite inhibitory activity.

6.6 **References**

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7. Curriculum Vitae

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Education

08/2011 - 12/2015	**PhD in Microbiology** *Monoclonal antibodies as tools in antigen detection assay and vaccine development: design of a sensitive detection test for Brucella bacteria and profiling of the malaria vaccine candidate antigen reticulocyte-binding homolog 2 (PfRH2)* Swiss Tropical and Public Health Institute (Swiss TPH), University of Basel, Switzerland
09/2009 - 01/2011	**Master of Science in Infection Biology and Epidemiology** *In vitro studies to compare the efficacy of marketed and new drugs against Trichuris muris* Swiss Tropical and Public Health Institute (Swiss TPH), University of Basel, Switzerland
10/2006 - 06/2009	**Bachelor of Science in Biology** Major in Integrative Biology University of Basel, Switzerland
09/2003 - 07/2006	**High school** Biotechnologisches Gymnasium, Mathilde Planck Schule, Lörrach, Germany

Work experience

08/2011 - 12/2015	**PhD Project (Prof. G. Pluschke)** Department of Medical Parasitology and Infection Biology, Molecular Immunology Unit, Swiss TPH, Switzerland
02/2011 - 05/2011	**Research assistant for the development of novel drugs against helminthic diseases (Prof. J. Keiser)** Department of Medical Parasitology and Infection Biology, Helminth Drug Development Unit, Swiss TPH, Switzerland
09/2009 - 01/2011	**MSc Project (Prof. J. Keiser)** Department of Medical Parasitology and Infection Biology, Helminth Drug Development Unit, Swiss TPH, Switzerland

Skills

Laboratory	**Cell culture** (monoclonal antibody production) **Parasite culture** (cultivation of *Plasmodium falciparum*) **Immunological techniques** (ELISA, Western blotting, Luminex, Immunofluorescence assay, flow cytometry) **Molecular techniques** (PCR, Cloning, Protein expression and purification) **Peptide microarray** **Drug screening** (Alamar Blue, Motility assay)
Computer	Microsoft Office, Graph Pad Prism, Adobe Photoshop, PepSlide Analyzer
Language	**German** (native speaker) **English** (fluent) **French** (basic knowledge)

Presentations

06/09 - 10/09/2015	**Poster** (Development of a bead-based Luminex assay using lipopolysaccharide specific monoclonal antibodies to detect biological threats from *Brucella* species) **9th European Congress on Tropical Medicine and International Heath**, Basel, Switzerland
07/12 - 08/12/2013	**Poster** (Development of a mAb-based Luminex assay for the detection of *Brucellae* in complex biological samples) **66th Annual Brucellosis Research Conference**, Chicago, Illinois

Publications

2015   <u>**Silbereisen A.**</u>**, Tamborrini T., Wittwer M., Schürch N., Pluschke G.**
Development of a bead-based Luminex assay using lipopolysaccharide specific monoclonal antibodies to detect biological threats from *Brucella* species
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2013   **Keiser J., Tritten L.,** <u>**Silbereisen A.**</u>**, Speich B., Adelfino R., Vargas M.**
Activity of Oxantel Pamoate Monotherapy and Combination Chemotherapy against *Trichuris muris* and Hookworms: Revival of an Old Drug
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Exploration of novel *in vitro* assays to study drugs against *Trichuris* spp.
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2011   **Tritten L.,** <u>**Silbereisen A.**</u>**, Keiser J.**
In Vitro and *In Vivo* Efficacy of Monepantel (AAD1566) against Laboratory Models of Human Intestinal Nematode Infections
PLoS Negl Trop Dis. 2011 5; 12