New Procedures for the Diagnosis of Human Brucellosis in Mongolia

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Zandraa Jamba

aus Ulaanbaatar, Mongolei

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Aus dem Biochemischen Institut
des Fachbereichs Medizin der Justus-Liebig-Universität Giessen

Geschäftsführender Direktor: Prof. Dr. Klaus T. Preissner

Gutachter: Prof. Dr. Ewald Beck

Gutachter: Prof. Dr. Rolf Bauerfeind

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SUMMARY

The feasibility of developing immunological and molecular diagnostic tools for routine diagnosis of brucellosis under conditions of a less economically and scientifically advanced country in general and Mongolia in particular was analysed. Brucellosis is a major healthcare issue in Mongolia for both, humans as well as lifestock farming, leading to enormous economic losses every year. Diagnosis of the disease is demanding and insufficiently specific with the available tests. Using genomic sequences of the most important *Brucella* strains, new diagnostic procedures have been developed by means of molecular biotechnology. They include the production of several recombinant proteins as antigens in immunological assays such as ELISA. Combining of several of these antigens in a single recombinant fusion protein led to an unattained highly specific test for infections caused by *Brucella.* The genus *Brucella* includes different species (or biovars) carried by different primary animal hosts and being transmitted by different routes to humans. Furthermore, the severity of clinical manifestations differs among the species necessitating specific modalities of treatment and making differential diagnosis on the species level an important issue. Thus, another part of the study was focused on this topic. By means of multiplex nested PCR, a highly sensitive and specific differentiation between the major *Brucella* strains occurring in Mongolia was obtained. The immunological as well as the molecular diagnostic principles have been designed to be applicable in laboratory practice under limited economical conditions, including simple and inexpensive procedures for antigen production and DNA purification.

ZUSAMMENFASSUNG

Ziel der Arbeit war zu versuchen, neue immunologische und molekulare Methoden zur routinemäßigen Diagnose der *Brucellose* unter wirtschaftlich und wissenschaftlich weniger privilegierten Bedingung zu entwickeln, wie sie z. B. in der Mongolei vorherrschen. Die *Brucellose* ist in der Mongolei ein erhebliches Problem, das nicht nur die Gesundheit der Bevölkerung betrifft, sondern auch für die Tierzucht relevant ist und dort jedes Jahr zu großen ökonomischen Einbußen führt. Die Diagnose der Krankheit ist aufwendig und mit den vorhandenen Methoden wenig spezifisch. In der vorliegenden Arbeit wurden unter Zuhilfenahme der genomischen Sequenzen der wichtigsten Stämme von *Brucella* mittels molekularbiologischer Methoden neue Diagnoseverfahren entwickelt. Auf der einen Seite waren dies verschiedene rekombinante Antigene, die in immunologischen Tests wie dem ELISA zur Anwendung kamen. Die Verschmelzung mehrerer solcher rekombinanter Antigene in einem einzigen rekombinanten Fusionsprotein führte zu einer bislang unerreicht hohen Spezifität der Diagnose der Brucellose. Das Genus *Brucella* schließt mehrere Species (oder Biovare) ein, die bei verschiedenen Tierarten vorkommen und auf unterschiedliche Weisen auf den Menschen übertragen werden. Die Schwere der klinischen Manifestationen hängt von der Art des Erregers ab und bedingt unterschiedliche therapeutische Maßnahmen. Daher ist es wichtig, die Art der Erreger durch eine Differentialdiagnose zu identifizieren. Ein weiterer Teil der Arbeit befaßt sich daher mit diesem Problem. Mit Hilfe von Multiplex-PCR wurde eine sehr empfindliche und spezifische Differenzierung zwischen den wichtigsten in der Mongolei auftretenden *Brucella*-Stämmem erzielt. Sowohl das immunologische als auch das molekulare diagnostische Nachweisverfahren wurden so entwickelt, daß sie unter den bezüglich der Finanzierung und der wissenschaftlichen Laborausstattung limitierten Voraussetzungen der Mongolei durchführbar sind. Dies betrifft z. B. die Verwendung einfacher und kostengünstiger Methoden für die Produktion der Antigene für die Immundiagnose und für die Reinigung von DNA für PCR-Analysen.

CONTENTS

ABBREVIATIONS

Ab	Antibody
Amp	Ampicillin
AMOS	Abortus Melitensis Ovis Suis
AP	Alkaline Phosphatase
APS	Ammonium persulphate
BCIP	5-Bromo-4-Chloro-3-Indolylphosphate
BCV	*Brucella* containing vacuole
BSA	Bovine serum albumin
CDC	Center for disease control
CSF	Cerebrospinal fluid
CNS	Central nervous system
dNTP	Deoxyribonucleoside triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic reticulum
ERIC	Enterobacterial Repetitive Intergenic Consensus
ERES	Endoplasmic reticulum exit site
FPA	Fluorescence polarization assay
HRP	Horseradish peroxydase
HOOF	Hypervariable Octameric Oligonucleotide Fingerprints
IFA	Indirect immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IRS	Interspersed Repetitive Sequence
IS	Insertion sequence
LB-Medium	Luria-Broth-Medium
LPS	Lipopolysaccharide
MLVA	Multi Locus VNTR Analysis
NBT	Nitro blue tetrazolium
NCCD	National Center for Contagious Diseases
NK	Natural Killer
OD	Optical density

OMP	Outer membrane protein
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PO	Per os
RBC	Red blood cell
RBPT	Rose Bengal Plate Agglutination Test
RBT	Rose Bengal Agglutination Test
REP	Repetitive extragenic palindromic sequence
RFLP	Restriction Fragment Length Polymorphism
RNase A	Ribonuclease A
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
rpm	Rotations per minute
RT	Room temperature
RTD	Routine test dilution
S	Svedberg Unit
SARS	Severe Acute Respiratory Syndrome
SAT	Serum agglutination test
SDS	Sodium dodecylsulphate
SMZ	Sulfamethoxazole
S-LPS	Smooth-lipopolysaccharide
Taq	Thermus aquaticus
Th1	T helper cell 1
TMP	trimethoprim
T&S	Test and slaughter
U	Units
UV	Ultraviolet
VNTR	Variable Number Tandem Repeats
WHO	World Health Organisation

1 INTRODUCTION

1.1 General facts about brucellosis

Brucellosis is a group of closely related diseases caused by the members of the genus *Brucella* in animals and humans. It is a zoonosis transmittable to humans with a high degree of morbidity. More than 500,000 new cases of brucellosis are reported each year, and according to World Health Organization, this figure underestimates the magnitude of the problem. There were historically a number of synonyms for this infection: Malta fever, Mediterranean fever, Gibraltar fever, Cyprus fever, and undulant fever, but in the meantime all infections by species of *Brucella* are referred to as brucellosis. The organism was first isolated in 1887 by Sir David Bruce, who recovered a suspect organism from the spleens of British soldiers dying of Malta fever, hence the name for this illness. From this point on, it has progressively become clear that closely related bacteria caused all of these diseases. Thus, Meyer and Shaw created the genus *Brucella* in 1920 to accommodate these microorganisms.

Brucellosis is a complex disease and the range of primary hosts of *Brucella* includes several domestic or semi-domestic animals, cetaceans, pinnipeds and some wild rodents. In domestic animals the disease manifests by abortion and infertility. Humans usually acquire brucellosis from domestic animals through direct contact or consumption of their products and are not themselves source of contagion. The disease in humans is rarely deadly, but debilitating with possible severe consequences. The high degree of morbidity, for both animals and humans, is an important cause of financial loss and represents a serious public health problem in many developing countries (Corbel, 1997).

1.1.2 *Brucella* species: taxonomy, structure, biochemical characteristics and polymorphisms

Genus *Brucella* belongs to class I Alpha-2 Proteobacteria of phylum Proteobacteria. *Brucellae* are Gram-negative, facultative intracellular pleomorphic bacteria that can infect humans and many species of animals (Figure 1.1).

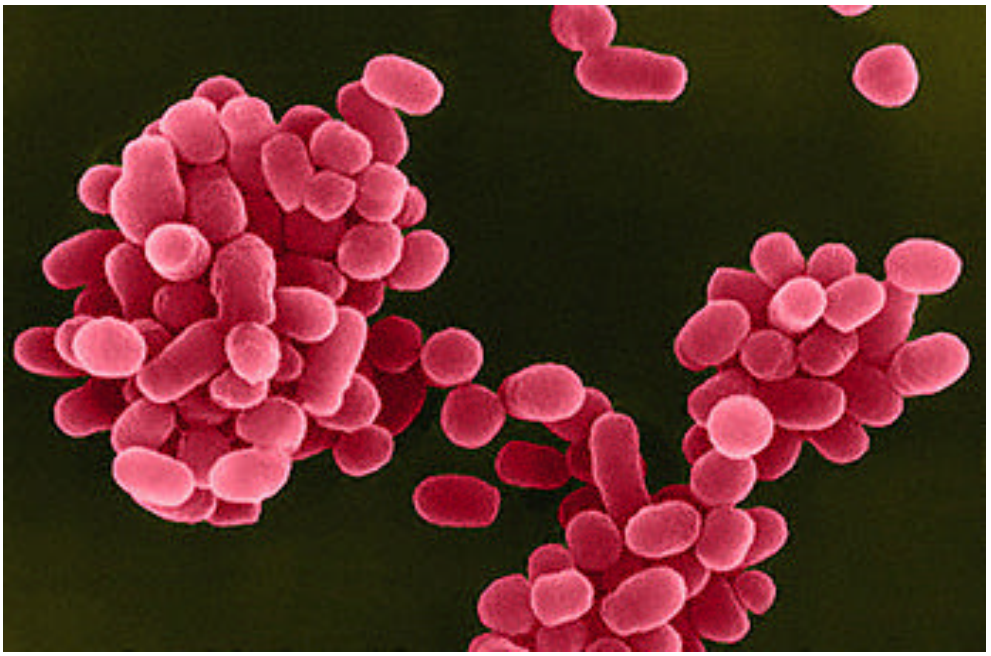

Figure 1.1 Electron microscopy of *B.abortus* (Dennis Kunkel Microscopy, Inc., 2004).

Six classical species were formerly recognized within the genus *Brucellae: B. melitensis, B. abortus, B. suis, B. neotomae, B. ovis and B. canis*. Two new species were isolated from marine animals *B. pinnipedialis and B. ceti* in mid 1990s (Foster et al., 2007), while recently, a novel species, *Brucella microti* has been detected and isolated from common vole (Scholz et al., 2008). This classification is mainly based on differences in pathogenicity and host preference. Although the six classical species can be differentiated by conventional phenotypic tests, they show a high degree of homology in their DNA-DNA hybridization assays (>90 % identity) suggesting that the *Brucella* genus should comprise a single species, *B. melitensis*, with the remaining species considered as biovars. However, molecular genotyping revealed that *Brucella* species display significant DNA inter-specific polymorphisms, justifying the current classification. Since the epidemiology and the severity of the diseases in humans is influenced by the *Brucella* type and its source (Corbel et al, 2000), the practical approach in classification is also of great importance. In general, *B. abortus* is associated with cattle, *B. melitensis* with sheep and goats, *B. suis* with swine, *B. ovis* causes infections specific for sheep and has not been implicated in human diseases, *B. canis* is usually associated with diseases in dogs but occasionally causes human brucellosis, and *B. neotomae* has been isolated on few occasions and has never been implicated in human diseases. The pathogenicity for humans of other *Brucella* species (*B. pinnipedialis, B. ceti,* and *B. microti*) still has to be clearly established.

The most common *Brucella* species to affect humans is *B. melitensis*, the most pathogenic species producing the most intense symptoms, the greatest tissue damage, and the most frequent incidence of localization in body organs, systems and tissue.

The taxonomic identity of the organisms in the genus can be discriminated based on their metabolic and antigenic properties. Further, these properties differentiate the species into a number of biotypes, primarily based on: their production of H_2S, CO_2 requirement for growth, phage typing, and the ability to grow in a medium containing dyes, such as basic fuchsin or thionin, and the agglutination by mono-specific antiserum (Table 1.1).

Species	Bio-type	CO_2 req't	H_2S prod'n	Growth on media containing		Agglutination with monospecific antisera			Lysis by phage† at RTD			
				thionin*	fuchsin*	A	M	R	Tb	Wb	Bk	Fz
B. abortus	1	(+)‡	+	-	+	+	-	-	L	L	L	L
	2	(+)	+	-	-	+	-	-	L	L	L	L
	3**	(+)	+	+	+	+	-	-	L	L	L	L
	4	(+)	+	-	+***	-	+	-	L	L	L	L
	5	-	-	+	+	-	-	-	L	L	L	L
	6**	-	(-)‡	+	+	+	-	-	L	L	L	L
	9	-	+	+	+	-	+	-	L	L	L	L
B. suis	1	-	+	+	-****	+	-	-	NL	L	L	PL
	2	-	-	+	-	+	-	-	NL	L	L	PL
	3	-	-	+	-	+	-	-	NL	L	L	PL
	4	-	-	+	(-)	+	+	-	NL	L	L	PL
	5	-	-	+	-	-	+	-	NL	L	L	PL
B. melitensis	1	-	-	+	+	-	+	-	NL	NL	L	NL
	2	-	-	+	+	+	-	-	NL	NL	L	NL
	3	-	-	+	+	+	+	-	NL	NL	L	NL
B.ovis		+	-	+	(+)	-	-	+	NL	NL	NL	NL
B.canis		-	-	+	-	-	-	+	NL	NL	NL	NL
B. neotomae		-	+	-	-	+	-	-	NL or PL	L	L	L

Table 1.1 Differentiation of *Brucella* species and biotypes (Stack JA and MacMillan AP, 1998).

L = Confluent lysis PL = Partial lysis NL = No lysis

* Concentration = 1/50 000 w/v

† Phage R will lyse non-smooth *Brucella abortus* at RTD
Phage R/O will also lyse *B. ovis* at RTD

‡ (+) = Most strains positive (-) = Most strains negative

** For more certain differentiation of *B. abortus* Type 3 and Type 6, thionin at 1/25 000 (w/v) is used in addition. Type 3 = + , Type 6 = - .

*** Some strains of this biovar are inhibited by basic fuchsin

**** Some isolates may be resistant to basic fuchsin, pyronin and safranin O

The three major species of *Brucella* affecting humans (*B. melitensis, B. abortus and B. suis*) contain two major surface antigens (designated as A and M), but the relative proportion of each antigen varies considerably from a species or a biotype to another. The antigen variations are due to the organisms' O-polysaccharide structure in the LPS, according to recent studies (Iriarte et al., 2004). The genomes of the classical *Brucella* species and their biotypes are comprised of two chromosomes, the only exception being *B. suis* biotype 3 strain 686 with a single chromosome. The larger chromosome is about 2.1 Mbp and contains the bacterial origin of replication. The smaller chromosome is approximately 1.2 Mbp and includes plasmid replicating functions (Halling et al., 2004).

Several molecular genotyping methods have been used to show that *Brucella* species display significant DNA polymorphisms, justifying current species classification (Cloeckaert, Vizcaino, 2004). The polymorphisms include: the variability in the genome organization, distribution of insertion sequences (IS) and the polymorphisms in genes encoding surface, cytoplasmic and periplasmic protein antigens. The studies also revealed intra-biotype DNA polymorphisms.

Studies of *Brucella* proteome (secretome and cellular proteome) have up to now uncovered a number of differences not only between *B. abortus* and *B. melitensis*, but also between the wild and attenuated strains of *B. melitensis* (Eschenbrenner et al., 2006).

1.1.3 Virulence and pathogenesis

Brucella infection primarily occurs through inhalation or ingestion of organisms via the nasal, oral, and pharyngeal cavities (Boschiroli et al., 2001). Bacteria also can invade the host organism directly into the bloodstream through wounds and mucosae. Following infection, the bacteria are transported, either free or within phagocytic cells, to the regional lymph nodes. The spread and multiplication of *Brucella* in lymph nodes, spleen, liver, bone marrow, mammary glands, and reproductive organs occurs via macrophages. *B. melitensis, B. abortus, B. suis*, and *B. canis* can infect humans and the pathological manifestations of brucellosis in humans are meningitis, endocarditis, spondylitis, and arthritis. The *Brucella* replication in the host is mainly due to their ability to avoid defence mechanisms of the host and proliferate within macrophages. Thus, these organisms not only resist killing by neutrophils following phagocytosis (Riley and Robertson, 1984) but also replicate inside the macrophages and non-phagocytic cells. In addition, survival in the macrophages is considered to be responsible for establishment of chronic infections and allows the bacteria to escape the extra-cellular mechanisms of host defence such as complement and antibodies.

Brucella preferentially infects macrophages, in which they modify phagocytosis, phagolysosome fusion, cytokine secretion, and apoptosis. This complex host-pathogen interaction is controlled by virulence genes which produce virulence factors. The most important of them are: LPS, cyclic glucans, OMPs, periplasmic enzymes, heat shock proteins, type IV secretion system (VirB operon), and the two-component regulatory system BvrS/R. Also important role in the virulence of this organism play some specific features as erythritol and iron metabolism which partly explains its tissue specific localisation in the case of animal infections.

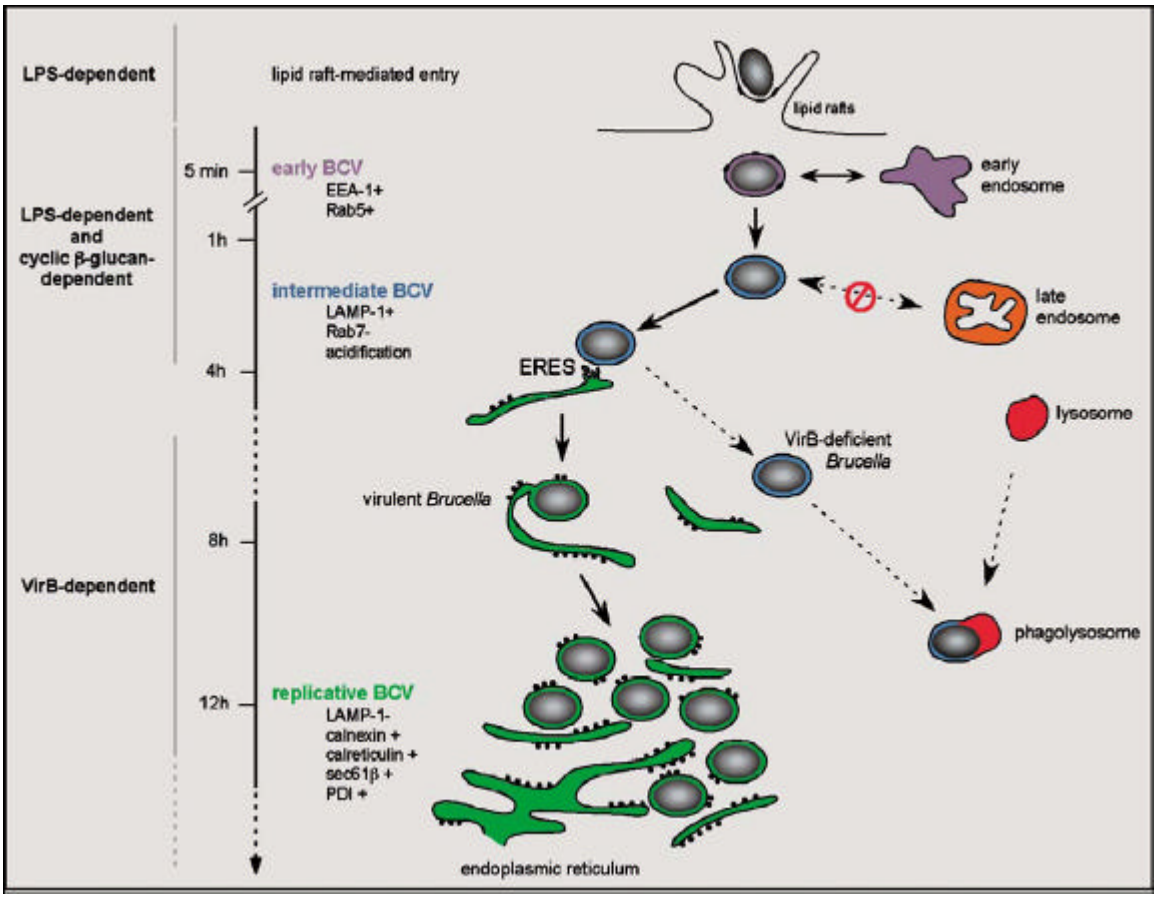

Figure 1.2 Intracellular trafficking of *Brucella* in macrophages. Following an LPS-dependent, lipid raft-mediated entry, *Brucella* is found in an early BCV (5–10 min p.i.) that interacts with early endosomes (purple), transiently acquiring Rab5 [13] and EEA-1 [11]. BCVs then mature into acidic intermediate vacuoles that accumulate LAMP-1, but not Rab7 [11], avoiding interactions with late endosomes and fusion with lysosomes via LPS and cyclic ß-glucan-dependent mechanisms [2,28]. Intermediate BCVs (blue) interact with ER exit sites (ERES; 2–8 h p.i.; [12]). Such interactions require the VirB type IV secretion system and lead to fusion between BCVs and the ER (8–12 h p.i.), generating an ER-derived organelle permissive for bacterial replication (12 h p.i. onwards; green [11]). Vacuoles containing VirB-deficient *Brucella* cannot sustain interactions and fuse with the ER. They ultimately fuse with lysosomes (red). Replicative BCVs exclude LAMP-1 and acquire various ER markers, (calnexin, calreticulin, sec61ß, PDI) as a result of membrane exchange with the ER. Bacterial replication is thought to occur through fission of the BCV into two daughter BCVs via further accretion of ER membranes (Celli J., 2005).

In the initial stage of infection, *Brucella*'s surface structures (LPS, proteins) bind to lipid rafts and several membrane receptors of macrophages. After phagocytosis, the cyclic-AMP/pkA pathway is activated in these cells, followed by phosphorylation of transcription factors. In epithelial cells, the bacterium recruits actin filaments in order to be vacuolized. In host cells, by preventing phagosome-lysosomal fusion, *Brucella* arrives at ER, the compartment that constitutes its replicating niche (Fig 1.2).

During intracellular trafficking, a cascade of genes is activated in *Brucella* in order to resist harsh environmental conditions of the phagosome. Finally, the accumulated bacteria are disseminated to other host cells.

1.1.4 Immune response

Brucella spp. are able to survive phagocytosis by several ways of intracellular life including the ability to prevent phagolysosomal fusion in specialized phagocytes such as macrophages and dendritic cells and by activation of a set of genes in response to the acidic environment (Cheers et al., 1979). Because these organisms are located intra-cellularly, an effective immune response includes mainly cell-mediated mechanisms. Immunity is largely based on production of interferon-?, which is controlled by IL-12, while its effective functioning for activation of macrophages depends on TNF-a. It is likely that depending on the stage of infection, both CD4 and CD8 cells make INF- ? in immune response. Both reactive oxygen intermediates and nitric oxyde contribute to control within macrophages and INF- ? serves to increase anti-*Brucella* activities.

Although many aspects of the immunobiology of brucellosis have become clear, this field still needs to be elucidated further.

Both innate and adaptive immune responses take part in *Brucella* infection. Innate immunity is induced in early stage of infection playing role to reduce the initial number of bacteria and to provide the environment for generating Th1 response in the host.

Complement is activated by classical and lectin pathways and results in deposition and complement-mediated killing of *Brucella*. The mechanism of activation mainly depends on the type of the infecting strain (smooth or rough). Rapid phagocytosis of bacteria by neutrophils occurs after opsonisation, however, most of *Brucella* survive. Using resistance mechanisms against bactericidal systems inside the cell, *Brucella* is transported to lymphoid tissues by neutrophils. A significant role in brucellosis play NK cells providing cytotoxity against infected cells. However, in some cases they fail to do so, promoting chronicity of infection. The bactericidal functions in macrophages are ROIs and RNIs (reactive oxygen/nitrogen

intermediates), which are induced by INF- ? and TNF-a. ROIs are suggested to be the main toxic agent. The macrophages have the ability to kill intracellular *Brucella* immediately after phagocytosis, but often intracellular bacteria survive and multiply by activating virulence genes and factors. Characteristic chronic granulomatous lesions (Figure 1.3) develop in infected tissues where macrophages, neutrophils and lymphocytes respond to proteins and saccharides of *Brucella*.

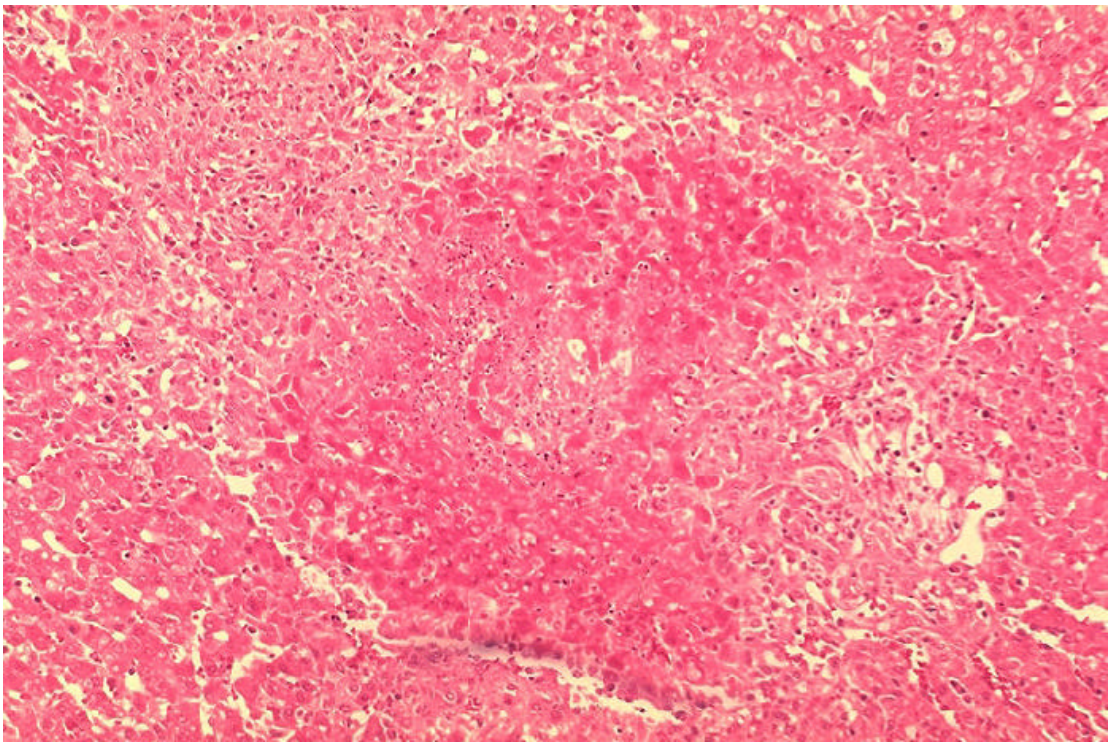

Figure 1.3 *Brucella* granuloma in liver.
(Image: http://upload.wikimedia.org/ Brucella_granuloma.jpg).

Sequentially, neutrophils appear first in response to *Brucella*, followed by macrophages and then lymphocytes.

Adaptive immune response to *Brucella* infection consists of antibody production, T cell activation, and cytokine activity. During infection, antibodies against LPS and a variety of bacterial proteins are detectable. The production of IgM or IgG antibodies at low concentrations in naturally infected species appears to promote lysis of *Brucella* by the classical complement pathway. Higher antibody levels of IgG present during active infection prevent bacterial lysis and likely promote phagocytosis of bacteria by macrophages enhancing bacterial intracellular localization (Ko and Splitter, 2003).

Optimal protection against intracellular bacteria is achieved by a coordinated interaction between different T cell subsets, CD4 and CD8, respectively. Another subset, ?d T cells,

activated by non-peptide antigens, control the increase in the number of intracellular *Brucella* organisms by secreting TNF-a and IFN-? to activate macrophage bactericidal function and by lysing the infected cells through cytotoxicity. Cytokines playing an essential role in brucellosis are IL-12, IFN-?, and TNF-a. IL-12 is a key cytokine produced by B cells and macrophages, and leads to Th1 immune responses in the host that will ultimately induce the secretion of IFN-? from T cells. The roles of other cytokines in infection need further studies.

1.1.5 Clinical spectrum of *Brucella* infection

The presentation of brucellosis is characteristically variable. The incubation period is often difficult to determine but is usually from 2 to 4 weeks. The onset may be insidious or abrupt. The sub-clinical infection is common.

In the simplest case, the onset is influenza-like, with fever reaching 38° to 40°C. Limb and back pains are unusually severe, however, and sweating and fatigue are marked. The leukocyte count tends to be normal or reduced, with a relative lymphocytosis. Hepatomegaly is reported in 20-60 %. On physical examination, splenomegaly may be the only finding. If the disease is not treated, the symptoms may continue for 2 to 4 weeks. Many patients will then recover spontaneously but others may suffer a series of exacerbations. These may produce an undulant fever in which the intensity of fever and symptoms recur and recede at about 10 day intervals. Anemia is often a feature. True relapses may occur months after the initial episode, even after apparently successful treatment.

Most affected persons recover entirely within 3 to 12 months but some will develop complications marked by involvement of various organs, and a few may enter an ill-defined chronic syndrome. Complications include arthritis, endocarditis, mycotic aneurysms, often sacroiliitis, and spondylitis (in about 10 percent of cases), central nervous system effects including meningitis (in about 5 %), uveitis, and, occasionally, epididymoorchitis. In contrast to animals, abortion is not a feature of brucellosis in pregnant women. Hypersensitivity reactions, which may mimic the symptoms of an infection, may occur in individuals who are exposed to infective material after previous, even sub-clinical, infection.

Organ systems involvement:

Skeletal System

Arthritis is said to be the most common localized complication of brucellosis. Osteoarticular manifestations of brucellosis are reported in 20-60 % of patients. In contrast to adults, in whom sacroiliitis predominates, childhood brucellosis most often affects large

peripheral joints and usually with monoarticular involvement (hips, knees, and ankles). Spondylitis is more common in elderly patients, and may result in paraspinal abscesses. Osteomyelitis involving the long bones have also has been reported but is less common in children than adults.

Gastrointestinal System

Brucellosis, like typhoid-fever, is an enteric fever in which systemic symptoms generally predominate over complaints localized to the gastrointestinal tract. Nausea, vomiting, anorexia, weight-loss, diarrhea or constipation followed with abdominal discomfort occurs in 30-60 % of the patients. A case of a child infected with *B.melitensis* in whom acute ileitis developed has been reported. The liver is probably always involved, but serum levels of hepatic enzymes are elevated only mildly. *B. suis* can cause suppurative abscesses involving the liver and spleen. Hepatic lesions are resolved with antibmicrobial therapy and cirrhosis does not occur. Splenomegaly occurs in 35 % of the paediatric patients.

Neurobrucellosis

Neurologic manifestations of brucellosis include meningitis, encephalitis, meningovascular complications, parenchymatous dysfunctions, peripheral neuropathy/ radiculopathy, Guillain-Barre's syndrome, brain abscess and psychosis. Central nervous system involvement occurs in less than 5 % of patients and usually presents as acute or chronic meningitis. Meningitis can be the presenting manifestation or it can occur late in the course of brucellosis. There is little to distinguish it clinically from meningitis caused by other bacteria, except for the lack of sings of meningeal irritation. Examination of cerebrospinal fluid (CSF) reveals lymphocytic pleocytosis with elevated protein content, and low to normal glucose level. Cultures of CSF are positive in less than one-half of cases, but antibodies are present in the majority of the cases in the CSF.

1.1.6 Treatment

The essential element in the treatment of all forms of brucellosis is the administration of effective antibiotics, and treatment should be implemented at an early stage. Treatment regimes usually consist of combination of at least two agents; however, the optimum antibiotic therapy is still disputed (1st International Conference on Emerging Zoonoses, 1997). The full treatment lasts 7 to 12 weeks. A shorter duration of treatment is associated with higher relapse rates. Additionally, severely ill patients should be treated in a hospital. In those patients with complications, additional treatment is necessary including, in some cases, surgical intervention.

The appropriate antibiotic therapy for brucellosis has been studied to some degree. Doxycycline (100 mg PO bid for 6 weeks) is the most appropriate monotherapy in simple infection; however, relapse rates approach 40 % for monotherapy treatment. Rifampin (600-900 mg/d) usually is added to doxycycline for a full 6-week course. In patients with spondylitis or sacroiliitis, doxycycline plus streptomycin (1 g/d IM for 3 weeks) was found to be more effective than the doxycycline/rifampin combination. Streptomycin currently is favoured over rifampin for combination therapy of any significant infection. In paediatric patients older than 8 years, doxycycline (5 mg/kg/d for 3 weeks) plus gentamicin (5 mg/kg/d IM for the first 5 d) was the recommended therapy. For children younger than 8 years, trimethoprim / sulfamethoxazole (TMP-SMZ) for 3 weeks and a 5-day course of gentamicin were most effective. TMP-SMZ also was effective in treating pregnant women, either as a single agent or in combination with rifampin or gentamicin.

Fluoroquinolones have a high relapse rate when used as monotherapy. Fluoroquinolones added to doxycycline have no advantage over the other regimens described, but may be preferred in an area where resistance to rifampin is high. No uniform recommendation exists for treatment of meningitis or endocarditis; however, TMP-SMZ plus rifampin remains the preferred combination. In endocarditis, early replacement of the infected valve is recommended, along with medical therapy. Corticosteroids are recommended in CNS infection, but data supporting their utility are lacking.

1.1.7 Control and prevention

Most human brucellosis originates from an infected animal. Human to human infection is rare. Prevention includes health education and pasteurization of milk.

However, education campaigns alone have never succeeded in fully eliminating these risks to humans (Robinson A, 2003). Attempts at vaccinating people at risk have resulted in effective protection, but also provoked severe reactions when given to sensitised individuals, or when administered incorrectly (Schurig et al, 2002). As a result, vaccination of humans is no longer routinely used; the ultimate prevention of human infection remains the elimination of the brucellosis in animals. Currently, methods used to prevent infections are test and slaughter (T&S) of sero-positive animals, vaccination, hygiene measures, and management. Various factors influence the choice of methods used such as husbandry system, climate, nomadic livestock breeding, prevalence of brucellosis among various animal species, and control programme resources available.

T&S is not realistic in the majority of places where *B. melitensis* is endemic (Banai, 2002). Therefore, until the disease prevalence is significantly reduced, whole herd immunisation should precede T&S activities. Experts advise a shifting away from immunisation to a T&S policy only after the individual prevalence rate is no greater than 2-3 % and the herd prevalence is 5-10 % (Food and Agriculture Organisation of the United Nations, Rome, Italy, 1992c).

One of the ultimate goals of *Brucella* research is to achieve its eradication or to prevent its expansion. Prevention of human brucellosis is dependent on control of the disease in domestic livestock, mainly through mass vaccination. In many countries, the use of *B. abortus* strain vaccines (S19, RB51) in cattle and *B. melitensis* strain Rev-1 vaccine in goats and sheep has resulted in the elimination or near-elimination of brucellosis in these animals. Studies are ongoing to develop an effective vaccine against *B. suis* and *B. ovis*. Since the treatment of animal brucellosis is very expensive, the mass vaccination of livestock should encouraged. Animal owners should be taught about the importance of vaccination of their animals. In spite of the clinical efficacy and cost effectiveness of vaccination, the limited availability of vaccines and lack of awareness has led to the persistence of brucellosis in most areas of developing countries. The lack of human vaccines and effective control measures make it necessary for the doctors and other health care workers to take protective measures. Protective clothing / barriers while handling stillbirths / products of conception and cultures can reduce occupation-related brucellosis. The avoidance of un-pasteurised dairy products prevents infection in the general population. Control and prevention of this disease needs not only coordinated activities of public health and veterinary institutions, but also a government control in order to establish and implement programmes for this purpose.

Vaccination of livestock along with proficient animal health services, good animal management, intensive breeding, control of movement and trade of animals are the key to eradication of brucellosis.

All vaccines used in vaccination of livestock are live attenuated rough strains. While vaccines for livestock are primarily aimed at interruption of transmission, the goal of human vaccination, which is under intensive investigation and development, is prevention of the disease. It is generally recognized that the prevention of human brucellosis is best achieved by control or eradication of the disease in animals. However, in some parts of the world this is not feasible and attempts have been made to control the disease by vaccination. Although several human vaccines have been tested to date, none is completely satisfactory. Attempts were made towards developing vaccines based on live-attenuated or mutant strains, protein subunits, and killed bacteria. While subunit vaccines contained different parts of bacterial cell (polysaccharide-protein, lipoprotein, intracellular enzymes etc.), the attenuated live vaccine strains were selected

on basis of virulence and LPS structure. These vaccines also differ in their routes of administration. The main disadvantage of these vaccines is the short-duration immunity, reactogenity and ability of some live strains to cause disease. Intensive investigations on effectiveness of DNA vaccines and live mutation-introduced strains are under way. The basic premise of DNA vaccines involves the introduction of gene(s) encoding protein antigens responsible for stimulating a protective immune response (Robinson, 1997). Use of mutants either lacking virulent properties or over-expressing immunogenic antigens are under study as well.

1.1.8 Epidemiology of brucellosis worldwide

Brucellosis is a zoonosis distributed worldwide. In developed countries, the animal disease has been brought under control, consequently reducing number of human cases. The human morbidity is usually high in areas where the disease is endemic in livestock, thus showing that the occurrence of human brucellosis mainly depends on animal reservoir. Humans are infected either by direct contact with infected animals and their products or by consumption of contaminated milk and dairy products. Direct contact implies that a person working or living in a place where the animal species susceptible to *Brucella* infection exist, and in close contact with animals potentially infected by brucellosis either by milking the animals or processing raw animal products like milk, meat or skin. In this case the actual route of infection is through skin abrasions and inhalation of aerosols.

In parts of the world where the *Brucella* infection is endemic in livestock, the incidence of human brucellosis remains high. However, in areas where *Brucella* spp. infection is present but the pasteurization of raw milk is practiced and dairy products are made from pasteurized milk, disease occurs at lesser frequencies. In these areas brucellosis is regarded as occupational hazard for persons who routinely work with animals and handle their raw products (Nicoletti, 1989). On the other hand, in areas where the quality control of dairy products is not enforced or the consumption of un-pasteurized milk and dairy products is a common nutritional habit, brucellosis occurs at relatively higher rate and causes a serious threat to public health. The route of infection in this case is mucosa of the digestive tract. Poor hygienic conditions in developing countries also contribute to the spread of the disease. However, due to growing international tourism, the numbers of reported cases of brucellosis in developed countries, where the disease was previously brought under control, is on the rise. Although the cases of human brucellosis are registered, the official figures do not fully reflect the number of people that are infected annually, underestimating the scope of the problem. Some cases often remain unrecognized due to inaccurate diagnosis and the consequent treatment as other diseases.

The epidemiology of human brucellosis, the most common zoonotic infection worldwide, has drastically changed over the past decade because of various sanitary, socioeconomic and political advances, as well as development of international travel. Several areas traditionally considered to be endemic - e.g. France, Israel, and most of Latin America - have achieved control of the disease. Nonetheless, new foci of human brucellosis have emerged, particularly in Central Asia, while the situation in certain countries of the Near-East (e.g. Syria) is rapidly worsening. Furthermore, the disease is still present, in varying trends, both in European countries and in the USA. Figure 1.4 depicts the incidence of human brucellosis worldwide. Table 1.2 shows the countries with the highest annual incidence of human brucellosis from 2000 onwards, as well as the incidence for selected other countries.

These changes in epidemiology of brucellosis reflect alterations in socioeconomic parameters, improvements in recognition and notification systems, outcomes of ongoing eradication programmes of animal brucellosis, and the evolution of the “global village” through international tourism (G.Pappas et al., 2006).

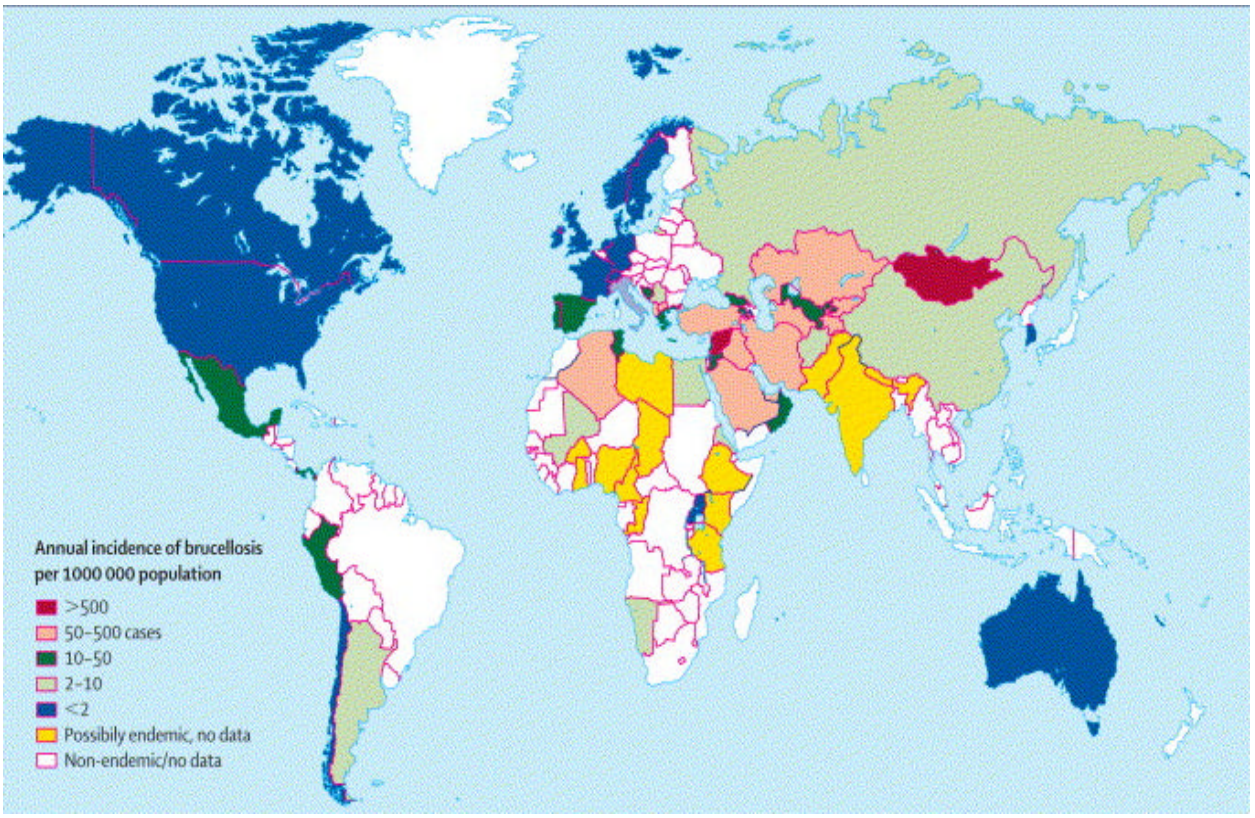

Figure 1.4 Worldwide incidence of human brucellosis (http://infection.thelancet.com).

Country and reference	Incidence (annual cases per million of population)
Europe	
Albania[6]	63·6
Bosnia and Herzegovina[7]	20·8
Denmark[7]	0·7
France[7]	0·5
Former Yugoslav Republic of Macedonia[8]	148
Georgia[7]	27·6
Germany[7]	0·3
Greece[9]	20·9
Ireland[7]	1·3
Italy[10]	9
Netherlands[7]	0·5
Norway[7]	0·7
Portugal[11]	13·9
Russia[7]	4·1
Serbia and Montenegro[7]	8·4
Spain[12]	15·1
Sweden[7]	0·3
Switzerland[13]	1·5
UK[14]	0·3
Africa	
Algeria[8]	84·3
Cameroon[7]	Endemic, no specific data available
Egypt[15]	2·95
Eritrea[16]	5·48
Ethiopia[7]	Endemic, no specific data available
Mali[8]	2
Namibia[8]	4·9
Tunisia[7]	35·4
Uganda[7]	0·9
North America	
Canada[7]	0·09
USA[17]	0·4
Mexico[18]	28·7
Central and South America	
Argentina[8]	8·4
Chile[7]	0·6
Colombia[7]	1·85
Guatemala[16]	15·7
Panama[7]	10·1
Peru[7]	34·9
Asia	
Afghanistan[7]	3·8
Armenia[16]	31·3
Azerbaijan[8]	52·6
China[19]	8
India	No data available, possibly endemic
Iraq[7]	278·4
Iran[8]	238·6
Israel[7]	9·2
Jordan[20]	23·4
Kazakhstan[21]	115·8
Korea, South[7]	1
Kuwait[7]	33·9
Kyrgyzstan[16]	362·2
Lebanon[22]	49·5
Mongolia[23]	605·9
Oman[7]	35·6
Pakistan	No data available, possibly endemic
Saudi Arabia[24]	214·4
Syria[7]	1603·4
Tajikistan[7]	211·9
Turkey[7]	262·2
Turkmenistan[7]	51·5
United Arab Emirates[25]	41
Uzbekistan[7]	18
Oceania	
Australia[26]	0·9

Table 1.2 List of countries with annual incidence of human brucellosis from 2000 onwards. Annual cases per million of population (Pappas et al., 2006).

1.1.9 Brucellosis in Mongolia

Mongolia, situated in Central Asia between Russia and China, has a population of 2.7 million people and an average population density of 1.5 persons per sqare kilometre Major political and healthcare changes began in 1990, when Mongolia ceased to be under the Soviet control and stopped receiving developmental aid as one of the Eastern Bloc satellites. Since that time, its economy has been changing from a centrally planned socialist system to a free market economy with healthcare delivery reflecting that transition.

Although progress is being made, Mongolia continues to struggle with poor transportation and communication, and limited material (including laboratory) facilities. Financial difficulties remain a major challenge as the country seeks to develop economic self-sufficiency and deliver modern health care to its people.

Approximately 23 % of Mongolia's population lives in rural areas and leads a nomadic or semi-nomadic way of life. In Mongolia, livestock rearing and milk production are important branches of the economy, employing approximately 50 % of the population. Their diet is heavily dependent on meat and dairy products, reflecting the importance that large domesticated animals have played in the country's history. In the past decade, the number of livestock has increased from 26 million to over 40 million, including 17.02 million sheep, 18.2 million goats, 2.4 million cattle, 2.1 million horses, and 350,500 camels (National Statistical Office of Mongolia, 2007, p.78). Not surprisingly, brucellosis remains one of the major veterinary and public health problems in Mongolia. The *Brucella* seroprevalence rate in cattle in 1987 ranged from 3.8 % to 35 % before a vaccination program (Denes B., 1997) but now appears to be approximately 5 % -10 % with some focal areas being close to 50 % (Andrea Mikolon, 2000). Seroprevalence in sheep and goats is less, approximately 2 %.

<u>Factors influencing transmission</u>

Traditional Mongolian practices have controlled food borne transmission of brucellosis from animal to human: milk was mostly not drunk in its natural state, but transformed to various typical drinks or foodstuffs, which have a preventive effect on the transmission of brucellosis. Naturally, animal husbandry has played an important role in the transmission of brucellosis between animals and from animal to humans. Even though modern husbandry methods have been introduced, such as dairy cattle farming, the bulk of animal production largely remains nomadic or semi-nomadic to this day. Crucial factors in this setting, from an epidemiological point of view, are the close contact between animal and human, some specific management practices and habits concerning hygiene (Roth, 2006).

Since the birthing season of most life stock falls on early spring in Mongolia, close human-animal contact usually occurs when herders bring the new born animals into their dwellings in order to protect from freezing. Furthermore, the winter shelters are crowded and the faeces become contaminated when abortions due to *Brucella* infection occur. These faeces are consequently transformed into fine dry dust, creating a dangerous source of airborne infection.

Specific management practices in Mongolia such as keeping various kinds of animals, especially goats and sheep, together in pastures and watering places also promotes transmission of the disease. Herders do not practice grazing control or fencing, thus making possible transmission of pathogens not only between domestic animals, but also between livestock and wildlife. Animal movement from various parts of the country to slaughterhouses in Ulaanbaatar is frequent, favouring the spread of animal diseases. The fact that animals are frequently mixed and regrouped further contributes to the spread of brucellosis.

Beside above factors, the situation is exacerbated by inadequate water resources in rural areas during the birthing season, when abortions and mass-parturition take place, leading to negligible hygienic measures. No adequate measures are practiced to protect against contact infections and to disinfect or clean equipment and shelters as well.

Routes of transmission

Early studies on transmisson of brucellosis (Dashdavaa, 1969) recognised the importance of animal and food borne transmission for human infection. Further studies confirmed that 94.3 % ± 0.6 % of human infections were caused by animal contact and 5.7 % ± 0.5 % by alimentation (Baldandorj, 1972). The importance of contact transmission has again been confirmed by later studies: 89.2 % of human infection was due to direct animal contact (62.1 % of these during animal birthing season), 4.3 % was due to contact with contaminated animal products and only 6.5 % of the human infection had been identified as food borne (Dashdavaa et al, 1981). Thus the main risk group has been identified: people having close contact to animals or working with animal products.

In the 1970s, scientific studies attested that 39 % of the herders were infected with brucellosis, 6.6 % of the students of Agriculture Institute and 2.2 % of the workers of the plants processing wool, leather or meat (Damdinsuren , 1972). Most of the herders were breeding small ruminants or cattle (Baldandorj , 1972). A survey in urban settings showed very high brucellosis prevalence among workers in plants processing animal products (50.8 % ± 6.2 % seropositive by allergic test; 20.3 % ± 3.0 % by standard tube agglutination test (SAT) and complex fixation test (CFT) and a relatively low prevalence of 1.9 % among unprofessional city residents (Baldandorj T, 1972). Surveys conducted in late 1980s reported that 13.5 % of the

herders were sero-positive (Ministry of Agriculture, 1991), and confirmed the severe contamination of persons working in high risk professions: 28.7 % of the persons working in slaughter houses were infected with brucellosis, 17.8 % working in dairy cattle farms, 19.6 % working in leather industry and 22.5 % working in the wool industry (Enkhbaatar et al, 2004). This situation seemed to become even worse in the late 1990s, when a survey conducted in 1996 among 42,000 members of the high risk group (herders, veterinarians etc.) showed that 30.9 % of them were infected (Ministry of Food and Agriculture, 1996).

Epidemiological characteristics

"The morbidity in human population clearly coincides with the lambing and kidding season, where 80 % of all cases of human infections are recorded" (Kolar J., 1970). It is concordant with other analyses showing that 70 % of new human infections occurred between March and July (Baldandorj, 1972). However, intense human infections have been documented in autumn as well (Tserendash, 1972). Thus apart from the birthing season, a further opportunity of humans to be infected is also associated with milking or slaughtering of infected animals, both more prevalent in the late summer and autumn.

The strains isolated from aborted foetuses and milk (Baldandorj, 1972) were biotypes I of *B. abortus* in cattle (Gombosuren, 1982) and all three biotypes (1,2, and 3) of *B. melitensis* in small ruminants (Tserendash, 1972) (Cvjetanovic et al, 1968). Human studies from 1964 to 1966 and 1975 to 1976 confirmed that most infections in humans in Mongolia were caused by *B. melitensis* (Dashdavaa et al, 1981) (Gombosuren, 1982). The analysis of patients diagnosed with brucellosis, between 1958 and 1969, showed that about 70 % had chronic brucellosis at the time of diagnosis (Baldandorj , 1972). By the early 1970s, 86.7 % of the brucellosis patients had chronic disease (Damdinsuren, 1972). Brucellosis was often diagnosed too late, probably due to limited access to healthcare facilities for diagnosis and treatment. More recently, between 1999 and 2001, 47.8 % of the brucellosis patients, treated at the Infectious Disease Centre in Ulaanbaatar (capital city), suffered from the chronic form of brucellosis, and a survey among 250 physicians resulted in 56.4 % of the cases being chronic in their consultancies (16 % acute, 20 % sub-acute) (Badarch, 2001). This high proportion in chronic cases reflected the poor quality of access to healthcare and diagnosis (Erdenchimeg et al, 2001).

As of 2001, approximately 8,000 human cases of chronic brucellosis were reported in Mongolia, and 1,000–1,500 new cases have been reported yearly since 1996 (National Statistical Office of Mongolia, 2007) as compared with approximately 100 cases annually in the United States.

Most chronic patients appear to have chronic skeletal disease diagnosed by clinical features, x-ray findings, and positive serologic results. Cultures are rarely done because of lack of appropriate safeguards for this level III pathogen but are performed occasionally.

Attempts to control this zoonotic infection have been unsuccessful because of an inconsistent strategy varying from vaccination of livestock to the destruction of infected animals. After numerous surveys in the 1960s, the World Health Organization (WHO) came to the conclusion that livestock vaccination was the only effective way to control brucellosis. The production of livestock vaccines was successfully established in 1970s and a country-wide mass-vaccination program of livestock planned for 11 years started in 1975. The vaccination of livestock successfully reduced human incidence of brucellosis to less than one case per 10,000 per year (Kolar, 1977). The vaccination program was interrupted in the early 1980s due to the end of the WHO assistance and democratic reforms followed by the discontinued economic dependence on the former Soviet Union in 1990. As a consequence, human brucellosis re-emerged. A large survey conducted during 1990–95 among herdsmen and other people who worked with animals showed that 16 % of the examined population were infected (Mongolia Health Sector Review, WHO, 1999). In 1999 the WHO conducted meetings with the Ministry of Health and the National Medical University to further assess the health impact of brucellosis in the country and make recommendations for its control. As a result, a whole-herd vaccination strategy covering 10 years was developed to start in 2000 (Mikolon, 1999).

According to recent reports (Health Sector Report, 2007, Mongolia), the human brucellosis level is presently about 60 cases per 100,000, and high incidence in animals continues to cause significant economic losses. Therefore, better control of brucellosis may have far-reaching effects for the Mongolian public health and economy by reducing morbidity and opening up new international trade opportunities for livestock.

1.2 Diagnostic methods for brucellosis

1.2.1 Clinical diagnosis

The clinical picture in human brucellosis can be misleading, and cases in which gastrointestinal, respiratory, dermal, or neurologic manifestations predominate are not uncommon. Thus, owing to its heterogeneous and poorly specific clinical symptomatology, the diagnosis of brucellosis always requires laboratory confirmation.

1.2.2 Laboratory diagnosis

For laboratory diagnosis of brucellosis specimens can be either of clinical or environmental origin. Clinical specimens best suited include: blood, sera, infected tissues and abscess material; bone marrow and tissue from spleen or liver, CSF, pleural fluid and even urine. Environmental samples can be milk, meat and other animal products.

Routine biochemical and hematological laboratory tests also overlap with those of many other diseases. Leukopenia or a normal white blood cell count is more common than leukocytosis. Normocytic anaemia is frequently present. Sometimes there is thrombocytopenia. Liver tests may be abnormal and a liver biopsy or bone marrow specimen can often (± 75 %) show granulomatous lesions. The cerebrospinal fluid can be abnormal with an increased lymphocyte count, raised CSF protein and normal glucose concentration.

Generally, laboratory tools include isolation and identification of *Brucellae* from clinical samples, detection of antigen, demonstration of genome and demonstration of *Brucella* specific antibodies. Following diagnostic methods are applied for diagnosis of human brucellosis:

Bacterial culture confirmed by light microscopy of Gram stained samples and urease test.

Immunoassay tests, which include: Rose Bengal test (RBT), Standard tube agglutination tests (SAT), Coombs test, Brucellacapt, radioimmunoassay, ELISA.

Nucleic acid detection tests which include mainly PCR based tests.

In Mongolia, the diagnosis of human brucellosis is primarily based on serological findings obtained from the Rose Bengal serum agglutination tests. Commercial ELISA tests, using LPS as an antigen, are also available. Veterinary laboratories are relatively more advanced in respect to culturing, typing and subtyping of *Brucella* species compared to human medical laboratories.

Definitive diagnosis of human brucellosis is made on the basis of medical history, physical examination and serological tests. Bacterial culture is not routine and no data are currently available on the use of the method in Mongolia. Imaging is also performed in order to detect involvement of internal organs such as spleen and liver. Electrocardiography is not a routine procedure for diagnosis of brucellosis, however is occasionally used for revealing possible endocarditis as complication of the disease. No recent data are available on predominance of *Brucella* species causing human brucellosis.

1.2.2.1 Bacterial culture

The isolation and identification of *Brucella* considered to be " golden standard " in diagnosis of brucellosis and is a proof of definite diagnosis.

From humans the organism is most commonly isolated from blood or bone marrow, but may be isolated from liver, spleen, cerebrospinal fluid or focal abscess.

Figure 1.5 *Brucella* spp. Colony Characteristics. The bacteria grow slowly on most standard laboratory media (e.g. sheep blood, chocolate and trypticase soy agars). The morphology of colonies is pinpoint, smooth, entire translucent, and non-hemolytic at 48h.
(Image: http://phil.cdc.gov/PHIL_Images/03182002/00014/PHIL_1902_lores.jpg).

Specimen are inoculated in media at 35-37°C and need 5 % CO_2. Standard blood media may be used for blood or bone marrow specimens, other specimens may use Trypticase soy agar with 5 % sheep blood agar (Figure 1.5), MacConkey agar, or Martin Lewis agar (Alton et al., 1988). Media for cultures are broth, solid, and 2 phase systems. Specific media include antibiotics for selectivity. Beside classical isolation procedures, there are newly developed

methods and growth systems such as lysis centrifugation method and automated blood culture systems.

After culturing the samples are stained with Gram's stain and observed using light microscopy (Figure 1.6); suspicious cultures are tested with further biochemical tests. These tests allow not only identification of *Brucella* as genus, but also further differentiation as biotypes.

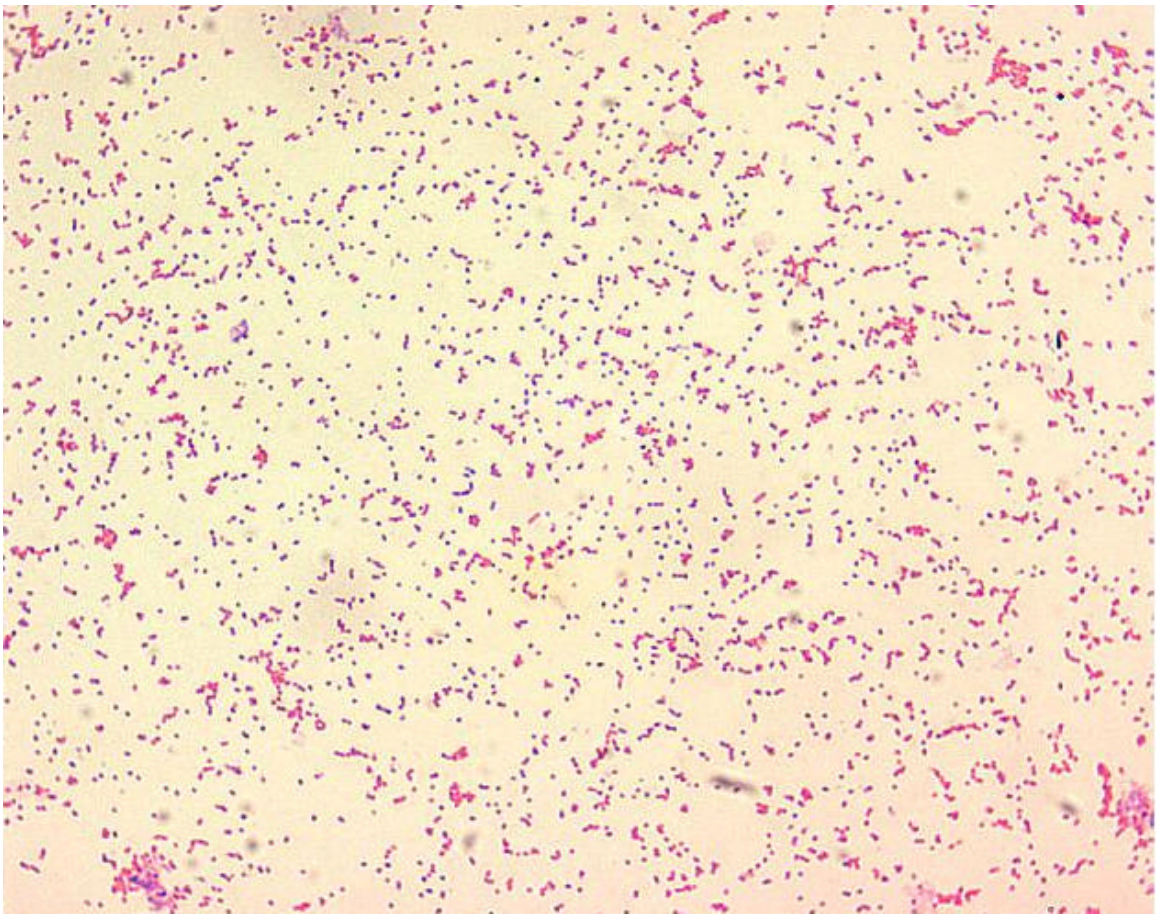

Figure 1.6 Gram-stained *Brucellae* under light microscopy.

(Image: http://phil.cdc.gov/PHIL_Images/03182002/00013/PHIL_1901_lores.jpg).

1.2.2.2 Immunological methods

Serological tests, such as Rose Bengal Plate Agglutination Test (RBPT) (Bercovich, 1998, Diaz-Aparicio et al., 1994), standard tube agglutination test (SAT), Coombs test (Orduna et al., 2000, Bercovich, 1998), immunocapture agglutination test (Brucellacapt) (Orduna et al., 2000), latex agglutination, complement fixation test, ELISA, lateral flow assay - a simplified version of ELISA, dipstick assay (Smits et al., 1999), fluorescence polarization assay (FPA) were mainstray of laboratory diagnosis for many years. Generally, all these tests use whole cell

or LPS as antigen and are applied for presumptive diagnosis. From above methods the first three are used frequently for diagnosis of human brucellosis, while others usually applied for diagnosis of animal disease, and differentiation of vaccinated from non-vaccinated animals.

Rose Bengal test that applies stained *B. abortus* antigen to detect serum antibodies is mainly used for screening purposes and is usually followed by one of more specific confirmatory assays.

Contamination of food also is matter of concern of public health sector. The milk ring test is a serological test for lacteal anti-*Brucella* IgM and IgA bound to milk fat globules in cow or goat milk . (Bercovic, 1998).

Antibodies usually begin to appear in the blood at the end of the first week of disease, IgM appearing first followed by IgG. Immunological methods used for diagnosis of brucellosis are based on antigen-antibody interaction, and antibodies can be either IgG or IgM, depending on the phase and stage of disease.

Generally immunoassays for diagnosis of brucellosis are suggested to lack specificity among population in endemic areas and individuals professionally exposed to *Brucella.* It is also known fact that these methods have limitations in the early phases of disease and in patients with relapses. One of serious drawbacks is the cross-reaction with other bacteria.

Antigens used in assay can also differ, representing whole killed cells, lysates or purified parts of bacteria. Most frequently used components are protein fractions of lysed bacteria, fractionated or complete LPS. The lipopolysaccharide O-side chain of smooth *Brucella* species seems to be the immunodominant antigen that elicits a long lasting serological response (Baldi et al., 1996). However, it is known fact that diagnostic tests based on lipopolysaccharide O-side chain antibodies can generate false-positives due to cross-reaction with antigens from other gram-negative bacteria (Weynants et al., 1996).

In recent years, several *Brucella* protein antigens have been genetically and antigenically characterized, and recombinant technology has been used for the development of novel immunoassays based on recombinant antigens for serological diagnosis of infections. Most of these diagnostic formats employed a single recombinant protein.

Single recombinant proteins such Omp31, Omp25, Aminopeptidase N, BP26, P15, P17 and P39 have been shown to carry immunodominant epitopes useful for serological diagnosis of animal brucellosis (see Results). They all proved to be specific, but general drawback was the lack of sensitivity. In order to increase sensitivity of test, steps were made towards the

development of a multiprotein diagnostic reagent (Letesson et al., 1997), suggesting that combination of several immunoreactive protein antigens would rather cover the spectrum of antibody response.

1.2.2.3 Molecular methods

The development of PCR has offered a new dimension in the diagnosis of different microorganisms, enabling to perform tests in just few hours. In principle, identification of *Brucella* at the genus level is sufficient to initiate therapy, however, further differential diagnosis at the species / biovar level is useful for elucidation of epidemiological aspects in order to take appropriate actions. Besides, molecular approach can be effectively used for disease follow-up, thus ensuring the recovery or monitor relapses.

The earliest assays were designed to exploit a single unique genetic locus that was highly conserved in *Brucella*. The advantage of these types of assays is that they tend to be simple and easy to perform, both sensitive and specific, rapid, and inexpensive. Such tests are useful for screening or for identification on the genus level, when species or biovar designations are not critical.

The first published PCR-based diagnostic assay was reported by Fekete et al. (1990). This assay was based on the amplification of a 635-bp sequence from a gene encoding a 43-kDa outer membrane protein of *B. abortus* strain S19. The authors were able to demonstrate that the assay was specific to *Brucella*, applicable to all species and biovars, and very sensitive (less than 100 bacteria). In the following years many genus-specific PCR assays were developed, targeting regions and genes such as 16SrRNA (Romero et al.,1995), BCSP31 (Serpe et. al., 1999), omp2a and omp2b (Leal-Klevezas et. Al., 1995), and IS711 (Halling et. al., 1993). One of this type of assays was later included in a more complex assay for detection and differentiation of four different bacterial pathogens: *C. burnetii*, *B. melitensis*, *B. anthracis*, and *Y. pestis* (McDonald et al., 2001).

As a result of applying adequate vaccines and consequent diagnosis, some countries have successfully eradicated some or all *Brucella* species from their livestock. The majority of other countries still endemic for brucellosis including Mongolia, have government-supported eradication or control programs. For reasons, such as differences in host preference, in the husbandry of host species, in modes of transmission, in pathogenicity to humans, in geographic distribution, and in the behaviour of reservoir hosts, the governmental regulatory policies for brucellosis are usually species-specific. By this way, correct identification of the species involved is essential for the initiation of appropriate action.

Epidemiological trace-back is an important component of any disease eradication or reduction program. In epizootic events, finding the source of infection and identifying possible points of transmission are key elements in preventing further spread of disease. Due to high genetic homogeneity among species of *Brucella,* strain identification is a difficult task. Classical bacteriology allows for identification of only a small number of subtypes below the species level. Furthermore, certain subtypes may dominate a geographic area. For example, when bovine brucellosis had a significant presence in the USA, about 85 % of infections were caused by *B. abortus* biovar1. Thus, differential PCR-based assays are particularly useful for epidemiological trace back, or for species-specific eradication programs.

PCR assays differentiating between *Brucella* species and/or biovars tend to be more complex and consequently more difficult to perform because appropriate target sites are rare in *Brucella* due to the remarkable homogeneity of the genus (Verger et al., 1985).

Discrimination of multiple species simultaneously utilises one of two approaches. The first approach includes complex reaction mixtures containing multiple primer pairs, each targeting a unique species-specific DNA sequence polymorphism. The second approach uses a single primer pair to amplify a DNA sequence containing internal species-specific polymorphism. Subsequently, the internal polymorphism is confirmed by some other method downstream.

Based on these two approaches, multiplex PCR assays for identification and differentiation of *Brucella* species and/or biovars such as AMOS (Bricker et al., 2003), and BaSS (Ewalt et al., 2003) were developed. PCR-RFLP assays, targeting omp2 (Ficht et al., 1990; Cloeckaert et al, 1995), omp25 (Cloeckaert et al., 1995), omp31 (Vizcaino et al., 1997), dnaK (Cloeckaert et al., 1996) genes were also successfully developed to differentiate the *Brucella* species.

Several alternative molecular approaches have also been developed in recent years exploiting regions of hypervariability for strain identification. Restriction mapping (McGillivery, 1998), pulsed gel electrophoresis (Allardet-Servent et al., 1988), ribotyping (Rijpens et al., 1996), IS-RFLP typing (Bricker et al., 2000) were all successful in identifying the variations to some degree. Additional methods such as arbitrary-primed PCR (Tcherneva et al., 2000), repetitive element PCR (REP-, ERIC-PCR) (Gillings et al., 1997), infrequent restriction site-PCR (IRS-PCR) (Cloeckert et al., 2003), ELISA-PCR were also developed and introduced in laboratory practice with certain success.

More recently, promising results in the typing of *Brucella* strains for epidemiological trace-back were obtained using variable number of tandem repeats analysis (VNTR), the

methods being multilocus VNTR (MLVA) analysis (Bricker et al., 2003) and the hypervariable octameric oligonucleotide finger-prints (HOOF-Prints) as its variant.

Recent improvements in PCR technology have made it possible to amplify and detect DNA targets simultaneously by real-time PCR (Redkar et al., 2001).

1.3 Goals and objectives of the study

The methods currently used in diagnosis of human brucellosis in Mongolia and other endemic countries are limited. The clinical picture of brucellosis is non-specific and may show great variability causing difficulties in diagnosis and consequent treatment.

Blood cultures are still the "gold standard" for microbiological diagnosis with good sensitivity for acute infections with *B. melitensis*. However, this sensitivity is markedly reduced in cases of long-term clinical courses or in patients infected by *B. abortus* and *B. suis* and it is a time-consuming process, which requires experience and skills for laboratory personnel (Yagupsky, 1999).

The sensitivity of serological tests using bacterial extracts as antigen is relatively high, but specificity is generally low in endemic areas due to high titre of antibodies in the healthy population (Ariza et al., 1992). In addition, most of these tests cross-react with other bacterial infections.

Molecular methods appear to be promising in the field of diagnosis, especially in the follow-up of patients and epidemiological trace-back. Analysis of the complete genome sequences of several *Brucella* species contributed tremendously in this respect. However, the assays described in the literature so far appear to be too complex and need to be modified to meet conditions of less wealthy countries.

The goal of this study was to develop and establish an appropriate procedure for definite diagnosis of human brucellosis in Mongolia and to elucidate some epidemiological aspects and routes of transmission of the disease. For this purpose the following objectives were targeted:

To develop pathogen DNA extraction and purification procedures from direct clinical samples in a simple and cost-effective way.

To choose specific sets of primers, and to establish conditions of nested PCR in order to increase both specificity and sensitivity of the assay.

To elucidate epidemiological factors and routes of transmission of the disease by developing species-specific PCR in order to determine the prevalent species of human brucellosis in Mongolia.

A second goal was to improve immunodiagnosis of the disease. Since immunological methods used for screening and diagnosing human brucellosis in Mongolia lack specificity, or are too expensive, it was attempted to search for new and better *Brucella*-specific recombinant antigens for use in immunoassays. In addition, a test system appropriate for conditions prevailing in Mongolia should be developed which had to meet such minimum criteria as:

To be simple and easy to perform.

To be both sensitive and specific.

To be rapid.

And to be inexpensive.

2 MATERIALS AND METHODS

2.1 Instruments

Electroporation system	
Electroporator Equibio Easyject Prima	Thermo Electron Corporation, Milford, MA, USA
Gel electrophoresis systems	
Horizontal minigel system (8 x 8 cm)	AGS, Heidelberg, Germany
Vertical minigel chamber (8 x 10 cm)	Keutz, Reiskirchen, Germany
Power supply EPS 500/400	Pharmacia, Freiburg, Germany
Shakers	
Horizontal shaker GFL 3020	Gesellschaft für Labortechnik, Burgwedel, Germany
Certomat R	Braun, Melsungen, Germany
Vortex Genie 2	Scientific Industries, Bohemia, NY, USA
Centrifuges	
Cooling centrifuge Beckman J2-21,	Beckman Instruments, Summerset, USA (with Rotors: JA 14 and JA 20)
Microfuge: Biofuge Pico	Heraeus Instruments, Hanau, Germany
Multifuge 3	Heraeus Instruments, Hanau, Germany
Waterbath	
GFL Wasserbad 1013	Gesellschaft für Labortechnik, Burgwedel, Germany
Thermocyclers	
T1 Thermocycler 96	Biometra GmbH, Göttingen, Germany
Primus 96	MWG Biotech AG, Ebersberg, Germany
Sonifier	
Sonoplus HD70	Bandelin, Berlin, Germany
Photographic equipment	
UV Transilluminator	Herolab GmbH, Wiesloch, Germany
Polaroid MP-4 Land Camera	Polaroid Corporation, Cambridge, MA, USA
Film: Polaroid 667 Professional	Polaroid Corporation, Cambridge, MA, USA
Microplate reader	
Bio Rad Model 3550-UV	Bio Rad, Munich, Germany

2.2 Materials

2.2.1 Chemicals

Acrylamid	Serva, Heidelberg, Germany
Agarose for gel electrophoresis	Sigma-Aldrich, Munich, Germany
Ammoniumpersulfate (APS)	Serva, Heidelberg, Germany
Ammoniumsulfate	Carl Roth GmbH, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich, Munich, Germany
Adenil Triphosphate (ATP)	Carl Roth GmbH, Karlsruhe, Germany
Biorex 70 resin	BIO-RAD Laboratories, Hercules, USA
Bovine serum albumin fraction V (BSA)	Carl Roth GmbH, Karlsruhe, Germany
5-Bromo-4-chloro-3-indolylphosphate (BCIP)	Gerbu, Wiesloch, Germany
Coomassie Brilliant Blue R250	Serva, Heidelberg, Germany
Dithiothreitol (DTT)	Biomol, Heidelberg, Germany
Ethidium bromide	Serva, Heidelberg, Germany
Ethylenediaminetetraacetic acid (EDTA)	Serva, Heidelberg, Germany
Guanidiniumhydrochlorid (GHC)	ICN Biomedicals, Germany
Heparin sepharose	Pharmacia-LKB, Upsala, Sweden
Hydrogen peroxide 30 % (w/v) (H_2O_2)	Sigma-Aldrich, Munich, Germany
Isopropyl-ß-D-thiogalactopyranose (IPTG)	Gerbu, Wiesloch, Germany
Kanamycin	Sigma-Aldrich, Munich, Germany
ß-Mercaptoethanol	Serva, Heidelberg, Germany
Nitro blue tetrazolium (NBT)	Gerbu, Wiesloch, Germany
N,N-dimethylacetamine (DMA)	Sigma-Aldrich, Munich, Germany
N,N,N‘,N‘- Tetramethylethylendiamine (TEMED)	Serva, Heidelberg, Germany
Phenylmethylsulphonylfluoride (PMSF)	Carl Roth GmbH, Karlsruhe, Germany
Polyethyleneimine	Sigma-Aldrich, Munich, Germany
Polyethylenglycol (Mr 3500-4500)	Carl Roth GmbH, Karlsruhe, Germany
Sodiumdodecylsulfate (SDS)	Carl Roth GmbH, Karlsruhe, Germany
Tetracycline	Sigma-Aldrich, Munich, Germany
Tetrabutylammonium borohydride (TBABH)	Sigma-Aldrich, Munich, Germany 3,3’,5,5’-Tetramethylbenzidine (TMB) Gerbu, Wiesloch, Germany
Tween-20 (polyoxyethylen sorbitan monolaureat)	Serva, Heidelberg, Germany
Triton X-100	Merck, Darmstadt, Germany
Tris (hydroxymethyl) aminomethane	Carl Roth GmbH, Karlsruhe, Germany
Urea	ICN Biomedicals, Germany

DNA size marker

Plasmid pSP 64 was cleaved a) with Hind III, b) with Dra I and c) with Hinf I. The individual cleavage assays were mixed in the relation 1:2:4 with respect to the amount of plasmid DNA, resulting in the following fragment sizes (base pairs):

Protein size marker

Protein marker I (14-116 kDa) AppliChem GmbH, Darmstadt, Germany

Silica suspension for purification of nucleic acids

50 g of silica (SiO_2) powder (Sigma No. S 5631) were suspended in 250 ml of water, centrifuged for 3 min at 5000 x g, the milky supernatant was discarded, the sediment resuspended in another 250 ml of water and centrifuged again. The procedure was repeated 3-5 times more until supernatant became clear. The sediment was suspended in one volume of water and kept in 2 ml aliquots at -20°C.

Nitrocellulose membrane

Protran BA 85, Schleicher & Schuell Bioscience, Dassel, Germany

Metal affinity resin

TALON® BD Biosciences, Palo Alto, CA, USA

2.2.2 Bacterial strains

Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB, lacIZ .M15* T*n10* (Tet)]
Escherichia coli XL1-Blue/pREP	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB, lacIZ .M15* T*n10* (Tet)]/pREP(Kan)

2.2.3 Bacterial DNAs (*Brucella* and other strains)

DNA samples from *Brucella* species type strains were kindly provided by professor G. Baljer, Institute for Hygiene and Infectious Diseases of Animals, University of Giessen. DNA from following type strains were used in experiments: *B. abortus* 544*, B. melitensis* 16M*, B. suis* 1330*, B. ovis* 63/290*.*

DNA-s from following additional pathogens were used for cross-reativity tests;

Haemophilus ducreyi (DSMZ, strain 8925, ATCC 33940)
Treponema pallidum (Prof. H-J. Wellensieck, University of Giessen, Germany)
Chlamydia trachomatis (Dr. E. Domann, University of Giessen, Germany)
Mycobacterium tuberculosis (Medical Microbiology Deptartment, University of Giessen)
Listeria monocytogenes (Prof. T. Chakraborty, University of Giessen)
Streptococcus pneumoniae (DSMZ strain 20566)
Streptococcus agalactiae (DSMZ strain 2134)
Bordetella pertussis (DSMZ strain 5571)
Bordetella parapertussis (DSMZ strain 13415)
Salmonella enteritidis (strain RKI 7271/03, Robert Koch Institut, Wernigerode)
Escherichia coli, EHEC strain (isolate from Dr. E. Domann, University of Giessen)

Yersinia enterocolytica (serovar O:9), (DSMZ strain 9499)

Neisseria gonorrhoeae (DNA from Dr. T. Meyer, MPI für biophysikalische Chemie, Göttingen)

2.2.4 Antisera

Sera from Brucellosis patients were obtained from the Brucellosis department of the National Center for Contagious Diseases (NCCD), Health Sciences University of Mongolia.

Additional sera were obtained from different departments of NCCD, including departments for Respiratory and Sexually Transmitted Diseases. 60 control sera were derived from German blood donors in the Academic Hospital at the University of Giessen, Germany.

Phosphatase- and peroxidase-conjugated goat anti-human IgG and IgM antibodies were bought from Dianova GmbH, Hamburg, Germany.

2.2.5 Enzymes

Restriction enzymes

Enzyme	U / µl	Buffer	Target sequence	Company
Bam HI	20	NEB 2 + BSA	G?GATCC	New England Biolabs®
EcoRI	20	NEB 2 + BSA	G?AATTC	New England Biolabs®
Hind III	20	NEB 2 + BSA	A?AGTCC	New England Biolabs®
Pst I	20	NEB 3 + BSA	CTGCA?G	New England Biolabs®
Kpn I	10	NEB 1 + BSA	GGTAC?C	New England Biolabs®
Xho I	20	NEB 2 + BSA	C?TCGAG	New England Biolabs®

Other enzymes

Enzyme	Activity	Company
T4 DNA ligase	1 Weiss-U / µl	New England Biolabs®
Taq polymerase	5 U / µl	Self-made (see below)
Proteinase K	0,3 U / µl	Sigma / Alldrich®
RNase A	0,5 Kunitz-U / µl	Sigma / Alldrich®
Lysozyme	100 U / µg	Sigma / Alldrich®

2.3 Buffers and solutions

2.3.1 Buffers and solutions for protein gel electrophoresis

Tris-glycin electrophoresis buffer (TG):

25 mM Tris; 192 mM glycin
0,1 % SDS

Sample buffer (SB)
50 mM Tris-HCl (pH 6.8)
2 % SDS
10 % glycerol
1 % ß-mercaptoethanol
12.5 mM EDTA
0.025 % bromphenol blue

6 x SB buffer (10 ml)

2 ml 1M Tris-HCl pH 6,8
0.8 g SDS
0.4 ml glycerol
0.4 ml ß-mercaptoethanol
1 ml 0.5 M EDTA
1 ml 0.1 % bromphenol blue
add H_2O to 10 ml

Coomassie Blue staining solution
50 % ethanol
10 % acetic acid
0.12 % Coomassie Brilliant Blue

Coomassie Blue staining solution
2.4 g Coomassie Blue
1 litre ethanol 99.6 %
200 ml acetic acid 96 %
add H_2O to 2 litres

Destaining solution
7.2 % acetic acid
5 % ethanol

Destaining solution
375 ml acetic acid 96 %
250 ml ethanol 99. 6 %
add H_2O to 5 litre

2.3.2 Buffers and solutions for DNA gel electrophoresis

Agarose gel electrophoresis buffer (E-buffer)

40 mM Tris/acetate (pH 8,0)
40 mM Na acetate
2.0 mM EDTA

20 x E-buffer
193.8 g Tris-OH
131.2 g Na acetate
160 ml 0.5 M EDTA
adjust pH 8.3 with acetic acid
add H_2O to 2 litre

Acrylamide gel electrophoresis buffer (TBE-buffer)

90 mM Tris/borate pH 8.3
2.5 mM EDTA

10 x TBE-buffer
108 g Tris-OH
55 g boric acid
40 ml 0.5M EDTA
adjust pH 8.0 with acetic acid
add H_2O to 1 litre

Loading buffer
20 mM Tris/HCl pH 7.5
50 % glycerol
0.02 % bromphenol blue
0.02 % xylene cyanol blue
20 mM EDTA

Loading buffer
0.2 ml 1 M Tris/HCl pH 7.5
5 ml glycerol
0.2 ml 10 mg/ml bromphenol blue
0.2 ml 10 mg/ml xylene cyanol blue

Ethidium bromide staining solution
1 µg ethidium bromide / 100 ml E-buffer

Staining solution
400 ml 1 x E-buffer
40 µl 10 mg/ml ethidium bromide solution

2.3.3 Buffers and solutions for methods of molecular biology

TE buffer
10 mM Tris/HCl pH 7.5
0.1 mM EDTA

TE buffer (100 ml)
1 ml 1 M Tris/HCl pH 7.5
20 µl 0.5 M EDTA
add H_2O to 100 ml

T4 Ligase buffer
30 mM Tris-HCl (pH 7,5)
10 mM $MgCl_2$
10 mM dithiothreitol (DTT)
10 % polyethyleneglycol (PEG)
1 mM ATP
100 µg /ml bovine serum albumin (BSA)

2 x T4 ligase buffer (1 ml)
30 µl 1 M Tris-HCl
10 µl 1 M $MgCl_2$
10 µl 1 M dithiothreitol
200 µl 50 % polyethyleneglycol
10 µl 0.1 M ATP
20 µl BSA (5 mg/ml)
720 µl H_2O

Restriction enzyme cleavage buffer NEB 1
10 mM BisTris Propane-HCl
10 mM $MgCl_2$
1 mM dithiothreitol (DTT)

10 x NEB 1
0.1 ml 1 M BisTris Propane/HCl pH 8.0
0.1 ml 1 M $MgCl_2$
0.1 ml 100 mM dithiothreitol
0.6 ml H_2O

Restriction enzyme cleavage buffer NEB 2
10 mM Tris-HCl
10 mM $MgCl_2$
50 mM NaCl
1 mM dithiothreitol (DTT)

10 x NEB 2
0.1 ml 1 M Tris/HCl pH 8.0
0.1 ml 1 M $MgCl_2$
0.1 ml 5 M NaCl
0.1 ml 100 mM dithiothreitol

Restriction enzyme cleavage buffer NEB 3
50 mM Tris-HCl
10 mM $MgCl_2$
100 mM NaCl
1 mM dithiothreitol (DTT)

10 x NEB 3
0.5 ml 1 M Tris/HCl pH 8.0
0.1 ml 1 M $MgCl_2$
0.2 ml 5 M NaCl
0.1 ml 100 mM dithiothreitol

2.3.4 Buffers for total DNA extraction

GuHCl lysis buffer (DNA extraction buffer)
5 M Guanidinum hydrochloride (GuHCl)
40 mM Tris/HCl pH 7.0
20 mM EDTA
1 % Tween 20

GuHCl lysis buffer (50 ml)
24 g GuHCl
2 ml 1 M Tris/HCl pH 7.0
2 ml of 0.5 M EDTA
0.5 ml Tween 20

Ethanol washing buffer
70 % ethanol
10 mM TrisHCl pH 7.0

Ethanol washing buffer
70 ml ethanol
1 ml 1M tris/HCl pH 7.0
29 ml H_2O

RBC lysis buffer:
300 mM NH_4Cl
30 mM NH_4HCO_3
30 mM KCl
0.1 mM EDTA

RBC lysis buffer (500 ml)
4.1 g NH_4Cl
0.6 g NH_4HCO_3
0.5 g KCl
0.1 ml 0.5 M EDTA

2.3.5 Buffers for alkaline lysis/silica method for plasmid preparation

Solution 1
100 mM Tris/HCl pH 7.5
10 mM EDTA

Solution 1
10 ml 1 M Tris/HCl pH 7.5
2 ml 0.5 M EDTA
88 ml H_2O

Solution 2
200 mM NaOH
1 % SDS

Solution 2
4 ml 5 N NaOH
10 ml 10 % SDS
86 ml H_2O

Solution 3
3 M Na acetate
2 M acetic acid

Solution 3
60 ml 5 M Na acetate
11.5 ml glacial acetic acid
28.5 ml H_2O

GuHCl lysis buffer, GuHCl washing buffer, and ethanol washing buffer needed for the consequent purification steps are shown above.

2.3.6 Buffers for purification of His-tagged proteins with TALON®

Protein lysis buffer

6 M guanidinium hydrochloride (GuHCl)
40 mM Tris/HCl pH 8.0
250 mM NaCl

Lysis buffer
57.3 g GuHCl
4 ml 1M Tris/HCl pH 8.0
5 ml 5 M NaCl
add H_2O to 100 ml
adjust to pH 8.0

Protein washing / equilibration buffer
8 M urea
40 mM Tris/HCl pH 8.0
250 mM NaCl

Washing/equilibration buffer
48 g urea
4 ml 1M Tris/HCl pH 8.0
5 ml 5 M NaCl
add H_2O to 100 ml
adjust pH to 8.0

Protein elution buffer
8 M urea
40 mM Tris/HCl pH 7.0
250 mM NaCl
250 mM imidazol

Elution buffer
48 g urea
4 ml 1M Tris/HCl pH 7.0
5 ml 5 M NaCl
1.7 g imidazol
add H_2O to 100 ml

2.3.7 Buffers for immunoassays

TBS-buffer

10 mM Tris-HCl pH 8.0
150 mM NaCl

20 × TBS buffer (2 litres)
200 ml 1 M Tris/HCl pH 8.0
175 g NaCl
add H_2O to 2 litres

TBST-buffer
10 mM Tris-HCl pH 8.0
150 mM NaCl
0.05 % Tween 20

20 × TBST buffer (2 litres)
200 ml 1 M Tris/HCl pH 8.0
175 g NaCl
10 ml Tween 20
add H_2O to 2 litres

Antigen dilution buffer
1 x TBS
0.2 % Tween 20
10 % glycerol
1 mM DTT

Antigen dilution buffer (100 ml)
5 ml 20 x TBS
2 ml 10 % Tween 20
10 ml glycerol
1 ml 1M DTT
82 ml H_2O

Ab dilution buffer
1 × TBST
1 % BSA
1 mM DTT

Ab dilution buffer (100 ml)
5 ml 20 x TBST
1 g BSA
1 ml 1M DTT
93 ml H_2O

AP buffer
100 mM Tris-HCl pH 9,5
100 mM NaCl
5 mM $MgCl_2$

AP buffer (200 ml)
20 ml 1M Tris-HCl pH 9,5
4 ml 5 M NaCl
1 ml 1 M $MgCl_2$
175 ml H_2O

NBT solution
50 mg/ml NBT (nitro blue tetrazolium)
in 70 % dimethylformamide, keep at -20°C

BCIP solution
50 mg/ml BCIP (5-Bromo-4-Chloro-3-Indolylphosphate)
in 100 % dimethylformamide, keep at -20°C

AP colour developing solution
60 µl BCIP solution and 60 µl NBT solution
dissolved in 10 ml of AP buffer (stable for 1 hour)

ELISA Washing buffer (1 x PBS):
0.1 M phosphate buffer (pH 7.2)
0.15 M NaCl
2.5 mM KCl

10 x PBS (1 litre)
90 g of NaCl
14.4 g of Na_2HPO_4
2.4 g of KH_2PO_4

Blocking buffer
1 % milk powder
1x PBS

Blocking buffer
1 g milk powder
100 ml 1 x PBS

Buffer A for ELISA assay
0.2 M potassium citrate pH 4.0
before use, add 25 µl of 30 % H_2O_2 to
8 ml of this stock solution

Buffer A (100 ml)
2.2 g citric acid
2.5 g tri-Na citrate (Mr = 294)
pH = 4.0 ± 0.1

Solution B for ELISA assay
41 mM tetramethylbenzidine (TMB)
8.2 mM TBABH in N, N-dimethylacetamide (DMA)
(absolute, H_2O content < 0.01 %)

Solution B:
100 mg TMB
20 mg TBABH
10 ml DMA

Substrate working solution for ELISA
Immediately before use,
add 200 µl solution B to 8 ml buffer A
with freshly added H_2O_2

Stop solution for ELISA assay
1 M H_2SO_4

Stop Solution:
5.4 ml 98 % H_2SO_4
add H_2O to 100 ml

2.4 METHODS

2.4.1 DNA purification

Isolation of nucleic acids using guanidinium-hydrochloride/silica

The following procedure is appropriate to isolate total DNA (1-5 µg) from a variety of bacteria, eukaryotic cells, or homogenised tissue (10 to 20 mg). 500 µl of guanidinum DNA extraction buffer (5 M GuHCl, 40 mM Tris/HCl pH 7.0, 20 mM EDTA, 1 % Tween 20) were added to 10-20 µl sedimented cells, vortexed and incubated for 10 min at 65°C. After 2 min centrifugation at 13.000 rpm in a minifuge, the supernatant was transferred to a new tube and 5 µl of silica suspension were added, mixed with vortex and incubated for 5 min at room temperature. The sample was centrifuged for 30 s at 13.000 rpm, the pellet resuspended in 300 µl 70 % ethanol washing buffer, centrifuged for 30 s at 13.000 rpm and the supernatant removed. The pellet was washed in the same way three more times with ethanol washing buffer (70 % ethanol, 10 mM TrisHCl pH 7.0). The last pellet was centrifuged again and residual ethanol was removed. 5 µl of TE buffer were added, incubated for 5 min at 55°C and centrifuged for 1 min at 13.000 rpm. The supernatant was collected and the pellet eluted with another 5 µl TE buffer. The supernatants were combined.

Alkaline lysis/silica method for plasmid preparation

The plasmid DNA purification procedure is a combination of the alkaline lysis protocol originally published by Ish-Horowicz and Burk (1981) and the above described silica adsorption method. It can be used for mini plasmid preparations (1.5 ml cultures) as well as for bigger preparations (50 ml cultures or more). In the following, the volumes of the different solutions needed in each step are given for mini plasmid preparations and for 50 ml cultures in brackets. 1.5 ml [50 ml] of over night culture were centrifuged for 3 min. The supernatant was removed by aspiration and the pellet resuspended in 100 µl [2 ml] of Solution 1. 10 µl [100 µl] of RNase A (5 mg/ml; kept at -20°C in 50 % glycerol) were added, and the sample was incubated at room temperature for 2 min. 200 µl [4 ml] of Solution 2 were added, quickly mixed (it is important not to vortex) and incubated for 2 min at room temperature (RT). Then, 150 µl [3 ml] of Solution 3 was added and again well mixed (without vortexing) and incubated for 1 min. The sample was centrifuged at 13.000 x g for 10 min at RT and the supernatant transferred to a new tube. Then, 400 µl [7 ml] of DNA extraction buffer and 7 µl [100 µl] of silica suspension were added.

The sample was mixed by vortexing, incubated at RT for 5 min, centrifuged for 1 min and the supernatant removed. To the pellet, 400 µl [7 ml] of ethanol washing buffer were added. The

sample was mixed by vortexing, centrifuged for 1 min and the supernatant discarded. The washing steps were repeated twice. Finally, the wet silica was centrifuged again for 1 min and the residual washing buffer removed completely. To elute the DNA, silica particles were resuspended in 20 µl [200 µl] TE buffer, incubated 10 min at 55°C, centrifuged for 2 min and the supernatant collected in a new tube. Silica was eluted once more with 20 µl [200 µl] TE buffer (only vortexed, not incubated at 55°C), centrifuged and the supernatants combined.

DNA purification from agarose gels

To purify DNA fragments from agarose gels the following protocol (Vogelstein et al., 1979) was used. The corresponding part of the gel was cut out under UV illumination, and incubated with 2-3 volumes of 4 M NaI at 60°C until the agarose was molten. 5-10 µl of silica suspension were added and incubated at room temperature for 5 min. After centrifugation for 30 s, the silica pellet was washed three times with ethanol washing buffer and the DNA eluted in the minimal volume of TE buffer as described above.

Buffy coat DNA extraction

2-3 ml of citrate or EDTA-blood were centrifuged for 15 minutes at 1000 x g. Then, supernatant plasma was collected and kept in the refrigerator. The yellow thin "buffy coat" layer on top of the sedimented erythrocytes was collected with a pipette (200 to 300 µl) and transferred to an Eppendorf tube. 1.2 ml of RBC lysis buffer were added, mixed shortly by vortexing, incubated 10 minutes at room temperatures, inverting the tube several times. Afterwards, the tube was centrifuged 1 minute at 2000 x g, and the supernatant removed. A pellet of 10-20 µl was obtained and mixed with DNA extraction buffer. DNA was extracted as described above.

2.4.2 Purification of recombinant Taq DNA polymerase

Taq DNA polymerase was purified from a recombinant strain of *E. coli* (*E. coli* XL-1 Blue/pQE-Taq) expressing an exonuclease-free mutant of the enzyme. The strain was constructed previously in the laboratory of Prof. E. Beck, University of Giessen (unpublished results). The thermostable enzyme can also be purified without the cooling steps described in the following protocol.

1 litre of LB medium containing 25 µg/ml kanamycin and 50 µg/ml ampicillin was inoculated with 100 ml of an over night culture of XL-1 Blue pQE-Taq cells. The cells were

grown at 37°C with optimal aeration for 1-2 hours. At an OD_{600} of 1.8, 1 ml of 1 M IPTG was added and then the culture vigorously shaked for another 4 hours. Cells were harvested by centrifugation (yield ~5 g) and frozen at -20°C. Freezing of the cells can be omitted, but is recommended if the enzyme preparation is not performed immediately. The cells were resuspended in 15 ml TMN buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 20 mM $(NH_4)_2SO_4$) together with 8 mg of lysozyme (from 10 mg/ml stock solution) and incubated for 15 min at 20-25°C. After adding 200 µl of 10 % Triton X-100 and 200 µl of 10 % Tween 20, the culture was mixed and incubated at 80°C for 20 min in a 50 ml screw-cap Falcon tube. After the heating step, 250 µl of 100 mM PMSF were added to prevent proteolytic degradation.

DNA was sheared by 1 min sonification (it is also possible to do it by repeated pressing through a syringe first with a 1 mm diameter needle and then with smaller needles) and centrifuged 15 min at 20.000 x g at 4°C. The supernatant (~15 ml) was collected and 0.6 ml of 5 M NaCl (0.2 M final concentration) were added.

The DNA was precipitated with polyethyleneimine (PEI) by adding dropwise 500 µl of 5 % PEI solution, mixing and incubating in ice for 10 min. The sample was centrifuged at 10.000 x g for 5 min. 4 aliquots of 500 µl of supernatant were transferred to Eppendorf tubes each and mixed with increasing amounts (2-8 µl) of 5 % PEI solution. They were incubated 5 min on ice, centrifuged and the amount of pellet compared. The minimal amount of PEI to precipitate DNA quantitatively was determinated and added to the bulk extract (usually 100-200 µl; use 1/100 volume of 5 % PEI solution in excess). The suspension was left 20 min on ice and centrifuged at 20.000 x g for 20 min. The supernatant was collected and diluted 6 fold with KTA buffer (20 mM Tris/HCl pH 8.5, 10 mM beta-mercaptoethanol, 10 % (w/v) glycerol, 0.1 mM EDTA, 0.05 % Triton X 100, 0.05 % Tween 20).

The excess of PEI was removed by passing the extract through a 2 ml BioRex 70 column equilibrated in KTA buffer + 30 mM ammonium sulphate. A disposable 4 ml plastic column (International Sorbent Technology, Hengoed, Mid Glamorgan, UK) was used. The column was rinsed with 2 ml KTA buffer and the flow-through loaded on another plastic column containing 2 ml heparin sepharose equilibrated with KTA buffer, 30 mM ammonium sulphate. The column was washed first with 50 ml KTA buffer, 40 mM ammonium sulphate. Thereafter, the column was washed with 20 ml KTA buffer, 40 mM ammonium sulphate, 50 % glycerol. Taq DNA polymerase was eluted with KTA buffer, 150 mM ammonium sulphate, 50 % glycerol. 0.5 ml fractions were collected and 3 µl aliquots analysed on a 12.5 % SDS polyacrylamide gel. The enzyme was found in fractions 4 to 7. The enzyme was stored at -20°C. For long term storage, Tween 20 to 1 % final concentration was added. The yield was approximately 30.000 units of enzyme at a concentration of 80 units/µl in the peak fraction.

2.4.3 Standard cleavage assay

Cleavage of DNA with BamHI and HindIII in the same assay was performed with NEB2 buffer, and 5 mg / ml BSA solution was added to final concentration 500 µg / ml and incubated for 1 to 2 hours at 37°C. For PstI, NEB3 as well NEB1 for KpnI buffers were used under the same conditions. In general, 20 U of each restriction enzyme were used to cleave approximately 5µg of plasmid DNA in a 50 µl assay.

2.4.4 Standard ligation assay

Ligation of fragments to be cloned in expression vectors was performed in 10 µl volume by T4 ligase (1 Weiss-U / µl) with ligation buffer containing 5 % PEG. To assay containing 1 µg (final concentration 0.1 µg / µl) of previously cleaved plasmid, 3 µg of fragment DNA was added to final concentration 0.3 µg / µl. Thus, 1:3 ratio of plasmid and fragment DNA amount was kept per 10 µl of assay. The ligation assay was incubated over night at 12ºC, and used the next day, after purification with silica, for the transformation of the electroporation competent cells.

2.4.5 Transformation of *E. coli* cells by electroporation

Transformation by electroporation was performed essentially as described by Sambrook et al. (1989). 5 ml of a fresh pre-culture (over night culture is not recommended) were taken to inoculate 200 ml pre-warmed LB medium. The cells were grown at 37°C until OD_{600} of 0.6 was reached. The culture was chilled on ice as quickly as possible and the cells centrifuged for 5 min at 10.000 x g. The pellet was resuspended in 25 ml ice-cold water. Centrifugation and resuspension were repeated two times and finally the pellet was resuspended in 400 µl ice-cold water and 10 % glycerol (final concentration) added. The cells were aliquoted in 40 µl portions and kept frozen at -70°C.

For transformation, 40 µl competent frozen cells were thawn on ice. 10 µl of silica purified ligase assay were added and the tube left in ice for 1 min. The cells were transferred in a pre-cooled 2 mm electroporation cuvette and electroporated at 2500 V. Immediately after, 1 ml of LB medium was added, the cells were transferred into an Eppendorf tube and incubated for 1 h at 37°C. 100 µl of the content of the Eppendorf tube were spread in one half of an agar plate containing selective antibiotics. The rest of the cells was centrifuged, resuspended in approx. 100 µl of medium and spread on the other half of the plate. The agar plate was incubated over night at 37°C.

2.4.6 Expression and purification of His-tagged proteins

Expression and purification of His-tagged proteins was performed as described by Hochuli et al. (1988). The recombinant protein antigens were cloned in pQE-30 (Qiagen, Hilden, Germany). The vector joins a histidine tag to the N-terminus of the recombinant protein (see Figure 2.4-1). Expression from the strong phage T5 promotor of this vector is regulated by a lac repressor provided in excess by the plasmid pREP (maintained in the cells by kanamycin selection).

Transformed with such vector *E. coli* XL-1Blue/pREP cells were grown in LB medium containing 50 µg/ml ampicillin and 25 µg/ml kanamycin up to density of 5 x 10^8 cells per ml (OD_{600} = 0.8-1.0). Then IPTG was added to a final concentration of 1 mM, and the cells (250 ml culture in a 1 litre Erlenmeyer flask) were further incubated under vigorous agitation on a shaker for 3-4 hours at 37°C. The sediment of cells after centrifugation were at -20°C. Next day, the cells were thawn, resuspended in 10 ml of Protein lysis buffer and incubated for 30 min at RT, shaking from time to time. The cell lysate was centrifuged for 20 min at 20.000 x g in a cooling centrifuge, the supernatant transferred to a 50 ml Falcon tube containing 1 ml suspension (0,5 ml packed volume) of TALON® (equilibrated in Protein washing buffer). The solution was mixed gently in a rotating wheel for 1 hour and centrifuged for 1 min at 3.000 x g in a swinging bucket centrifuge. The supernatant was removed carefully and 50 ml of Protein washing buffer were added. The solution was mixed gently in a rotating wheel again for 5 min, centrifuged for 1 min at 3.000 x g and the supernatant was removed carefully. The washing steps were repeated two times. The resin was then suspended in 4 ml of Protein washing buffer and loaded on a disposable plastic column.

The column was rinsed with 5 ml of washing solution and the proteins eluted by adding stepwise 0.5 ml of Protein elution buffer. 2 min pause were taken between each elution steps in order to concentrate the protein outflow. Aliquots of 3 µl and 15 µl of each of the 0.5 ml fractions were analysed with SDS PAGE. Protein concentration was determined by the Bradford assay (Bradford, 1976).

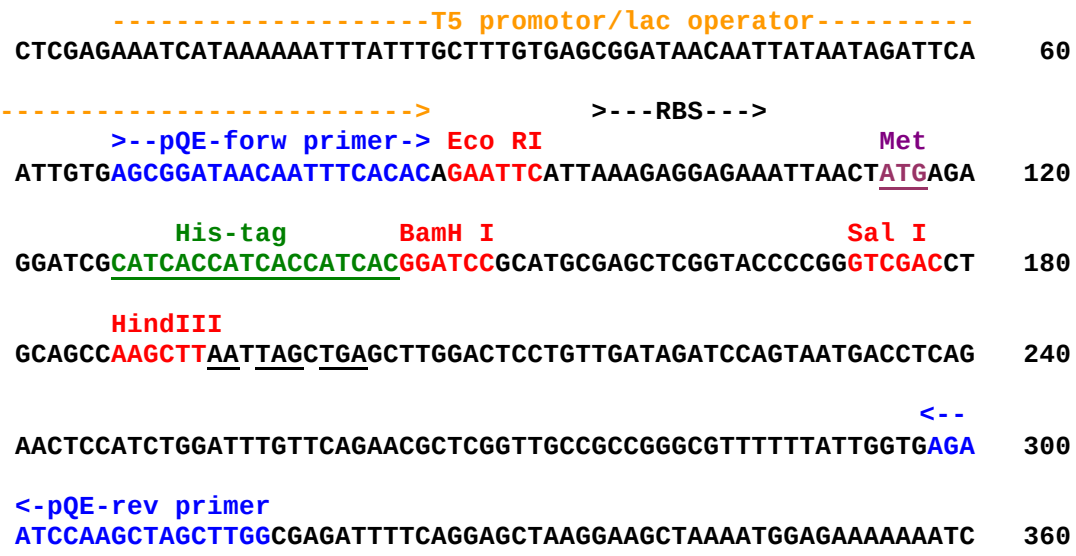

Figure 2.4-1 Multiple cloning site of the pQE-30 expression vector. Recognition sites of some restriction enzymes (red), histidine-tag (green), ribosome binding site (RBS), hybridation sites of pQE forward and reverse primers (blue), metionine codon (violet) and T5 promotor and lac operator (orange) at the beginning of the sequence are shown.

2.4.7 Line blot

The line blot is a simple immunoassay similar to the well known Western blot and was performed essentially as described by Klinkert *et al*., 1991. In contrast to the Western blot, antigens are not electrotransferred but applied manually to the nitrocellulose membrane.

Preparation of antigen test stripes

Antigen was applied to nitrocellulose sheets as a line with a 1.0 mm tip of an ink pen (Rotring Radiograph, Rotring GmbH, Hamburg, Germany), using a ruler as a guide. Alternatively, a 200 µl plastic pipette tip was used whose opening was squeezed with pliers to reduce the speed of flow. The antigen solution was prepared by mixing recombinant antigen (in 7 M urea as eluted from the TALON column) with different amounts of antigen dilution buffer (1xTBS, 0.2 % Tween 20, 10 % glycerol, 1 mM DTT). The concentration of protein is in the range of 0.1 - 0.01 mg/ml, however, the appropriate dilution has to be determined empirically for each batch of antigen by serial two-fold dilutions. The concentration just not yet reactive with negative control sera is correct. Lines of individual antigens were placed 0.5 cm apart. Non-specific binding sites were blocked by placing the air-dried nitrocellulose sheet in TBS buffer, 1 % Tween 20 for 1 hour. Sheets were then washed in TBST, cut perpendicularly to the antigen lines in 4 mm stripes, dried and stored at ambient temperature.

Immunodetection

The nitrocellulose stripes were re-hydrated in TBST and incubated in disposable incubation trays (Schleicher & Schuell, item No. 10448017) with 0.5 ml of a primary antibody solution (patient or control serum) diluted 1:100 - 1:200 in Ab dilution buffer (1 × TBST, 1 % BSA, 1 mM DTT) for 0.5-1 hours at room temperature or over night at 4°C on a shaker. The antibody was removed from the solution and saved at 4°C. The nitrocellulose stripes were washed three times for 10 minutes each with TBST and 0.5 M NaCl. Then, they were incubated with goat anti human IgG conjugated to alkaline phosphatase (Dianova, Hamburg, Germany; 1:7000 diluted in Ab dilution buffer) for 1-2 hours at room temperature on a shaker. Finally, the stripes were washed three times for 10 minutes in TBST and once with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The "disposable" incubation trays can be re-used many times when washed carefully with detergent using a brush.

AP staining protocol

To visualise the bound AP-conjugated antibodies, the following method was used. An AP colour developing solution was prepared just before use by adding 60 µl BCIP solution (50 mg/ml BCIP in 100 % dimethylformamide) and 60 µl NBT solution (50 mg/ml NBT in 70 % dimethylformamide) to 10 ml of AP buffer. The membrane stripes were added to the AP colour developing solution with the protein side up and it was waited for colour to develop on the membrane. When bands became clearly visible, the membranes were transferred to 10 mM Tris-HCl pH 8.0, 1 mM EDTA to stop the reaction. After drying at room temperature, the test stripes were photographed, or scanned into a computer.

2.4.8 ELISA

Flat-bottom 96 well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were incubated over night at 4°C with 0.1 ml of antigen diluted in PBS to a concentration of 1 ng/ml. The wells were washed three times with 0.35 ml PBS each and then incubated with 0.2 ml blocking buffer (1 % milk powder in PBS) for 1 hour at ambient temperature. The blocked plates were washed three times with 1 x PBS, dried and stored at room temperature. For long term storage (more than one month), the antigen-coated plates were sealed in plastic sheets. 100 µl of serum samples diluted 1:200 in blocking buffer were added to each well and incubated for 1 hour at room temperature. The plates were washed three times with 1 x PBS, the wells filled with a 1:5.000 dilution of goat anti human IgG-HRP (horseradish peroxidase) conjugate (Dianova, Hamburg, Germany) and incubated again for 1 hour at room temperature. The plates were washed three times with PBS as before, then 75 µl of HRP substrate working solution

were added to each well, incubated at room temperature, and the staining reaction stopped after 30 minutes with 100 µl of 1 M sulphuric acid and the results read at 450 nm. The HRP substrate working solution was prepared immediately before use as follows: 25 µl of a 30 % solution of H_2O_2 and 200 µl of 41 mM 3,3',5,5'-tetramethylbenzidine, 8.2 mM tetrabutylammonium borohydride in N,N-dimethylacetamide were added to 8 ml of 0.2 M potassium citrate buffer pH 4.0 (Frey et al., 2000).

2.4.9 PCR procedures

Standard PCR conditions

For all PCR assays, the same Taq DNA polymerase buffer was used. It is prepared as a 10 x buffer and has the following composition:

1 x Taq buffer
40 mM Tricine-KOH (pH 8.7 at 25°C)
15 mM K acetate
3.5 mM acetate
0.1 % gelatine
0.05 % Tween 20

10 x Taq buffer (10 ml)
4 ml 1 M Tricine-KOH
1.5 ml 1 M K acetate
350 µl 1 M acetate
2.5 ml 4 % heated gelatine solution
500 µl Tween 20
1.15 ml H_2O

Standard PCR reaction assay

Using efficient standard thermocyclers such as T1 thermocycler 96 from Biometra, or Primus 96 from MWG Biotech which are appropriate for 200 µl reaction tubes, have a heated lid and heating/cooling rates of at least 2-3°C/s, highly reproducible results were obtained with assays as small as 10 µl. A standard PCR reaction mixture had the following composition:

1 µl 10 x Taq buffer
1 µl mixture of 4 dNTPs (2.5 mM each)
0.5 µl 10 µM forward primer
0.5 µl 10 µM reverse primer
1 µl template DNA
5.8 µl H_2O
0.2 µl Taq DNA polymerase (5 units/µl)

The standard program of the thermocycler was set as follows:

step 1: 2 min at 94°C (denaturing of DNA template)

step 2: 35 cycles of [94°C 20 s, 60°C 1 min, 72°C 1 min]

step 3: 5 min 72°C (optional, to fill-in the DNA ends completely)

The same conditions were applied for nested PCR. 1 µl of a 1:10 dilution (with H_2O) of the result of first PCR assay was used as DNA template.

2.4.10 Gel electrophoresis

Agarose gels

Agarose gel electrophoresis was performed according to standard procedures (Sambrook et al. 1989). Plasmid DNA and PCR fragments were analysed on horizontal 1 % or 2 % agarose gels using a Tris/acetate electrophoresis buffer (E-buffer). To check migration of the DNA fragments, the samples were mixed with 20 % loading buffer containing 50 % glycerol and marker dyes. In 1 % agarose gels, bromphenol blue migrates at the position of 200 bp, whereas xylenecyanol blue migrates at the position of 300 bp. The gels were stained for 20 min with ethidium bromide staining solution. Then they were rinsed with tape water, the DNA bands visualised on an UV transilluminator and photographed by a Polaroid camera. Alternatively, a simple digital camera in combination with a yellow filter was used.

Polyacrylamide gels

For the analysis of proteins, discontinuous polyacrylamide gels (10 x 8 x 0.1 cm) containing SDS (Lämmli, 1970) were prepared in different concentrations according to the following recipe:

	Stacking gel	Separating gel	
	6 %	12,5 %	15 %
40 % Acrylamide / 1.3 % bisacrylamide solution	1,5 ml	5,0 ml	6,0 ml
4 x buffer	2,5 ml	4,0 ml	4,0 ml
H_2O	6,0 ml	7,0 ml	6,0 ml
Ammonium persulphate	10 µg	10 µg	10 µg
TEMED	15 µl	20 µl	20 µl
	10 ml	**16 ml**	**16 ml**

Stacking gel buffer:

125 mM Tris-HCl (pH 6.8)
0.1 % SDS

4 x Stacking gel buffer
12.14g Tris-OH
8 ml 10% SDS
adjust pH 6.8 with HCl (~8 ml HCl 37 %)
add H_2O to 200 ml

Separating gel buffer:

375 mM Tris-HCl (pH 8.8)
0.1 % SDS

4 x Separating gel buffer
45.43 g Tris-OH
10 ml 10% SDS
adjust pH 8.8 with HCl (~6 ml HCl 37 %)
add H_2O to 250 ml

2.4.11 Serological tests for brucellosis

RBT and the SAT were performed according to standard procedures. For the RBT, undiluted serum samples (30 µl) were mixed with an equal volume of Rose Bengal Slide Screening Test antigen (Biotech Laboratories, UK) on a white agglutination card. Results were rated negative when agglutination was absent and 1+ to 4+ positive according to the strength of the agglutination. The SAT was performed by preparing two-fold serial dilutions of the serum sample starting at a dilution of 1:20 in the wells of a microtiter plate and the addition of an equal volume of stained *Brucella abortus* antigen MM101 (Linear Chemicals, UK). The mixtures were incubated for 24 hours at 37°C and read by visual inspection. Positive results at dilutions ≥1:160 were considered consistent with brucellosis.

3 RESULTS

Brucellosis, an important disease worldwide, is preventable in principle and occurs in the parts of the world where animal and/or human health services are scarce or nonexistent. Mongolia is one of the countries where herds are large, animals breed extensively, and nomadic transhumance and other migration practices are economic necessities. Due to these key conditions, the control and eradication of brucellosis is a daunting task, necessarily requiring the development of inter-sectorial programs, including the routine screening of rural and professionally exposed risk groups for this disease in the population. Estimation of the actual incidences of brucellosis is an important issue of the country's health sector in order to control and prevent the disease.

For a disease that has been known for such a long time, several challenges still remain from the diagnostic point of view. Since the symptoms of brucellosis are non-specific, definite diagnosis cannot be established without help of laboratory tests. Although value of immunological tests cannot be questioned, they still need much refinement due to lack of specificity caused by cross-reactivity with other bacteria. The discrimination of false positives can also be ambiguous upon vaccination (in animals) and in diagnosing relapses (in humans), since the lipopolysaccharide O-chain of smooth *Brucella* species seems to be an immunodominant antigen that elicits a long lasting serological response (Baldi, 1996). Furthermore, these diagnostic tests also generate false positives due to cross-reaction with antigens from other Gram-negative bacteria (Weynants, 1996). Therefore, the identification of antigens that could potentially be useful for specific immuno-diagnoses is an important issue in brucellosis research.

It was attempted to address the above predicament by developing the following improved methods: First, PCR-based assays for diagnosing and differentiating brucellosis on the genus and/or species level as a direct indicator of infection, and second, immunodiagnostic tests using recombinant proteins as antigens, as an indirect indicator for infection and confirmatory test. At the same time, it was attempted to develop the new immunoassays for more specific screening by using recombinant proteins instead of bacterial lipopolysaccharide or whole bacterial cells as antigens.

3.1 Clinical specimens

As a prerequisite to establish new diagnostic procedures, peripheral blood samples of 500 suspected brucellosis patients were collected in disposable Sarstedt syringes containing EDTA. After centrifugation, both plasma and leukocytes purified from crude buffy coat collected from the top of the sedimented red blood cells were used.

Two groups of individuals were studied for PCR tests. The first group included patients with suspected disease on the basis of a clinically positive history, physical examination, positive in RBT and in confirmatory SAT, whereby a titre ≥1:160 was considered consistent with brucellosis. The discrimination between acute and chronic brucellosis was not made in this group. The samples were collected before hospitalization of the patients to the brucellosis department of National Center for Contagious Diseases (NCCD) of Mongolia. The second group consisted of 240 individuals that showed negative RBT and SAT. This group had neither clinical nor serological findings for brucellosis (negative control group).

DNA extracted from buffy coat of the seropositive samples was analysed with two different *Brucella* genus-specific nested PCR assays (detecting the genes of 16S rRNA and pcaC) as described in more detail below. Approximately 80 % of the samples positive by RBT and SAT were also positive in both of these PCR assays. These samples were considered as true *Brucella*-positive and further used for establishing new immunological tests.

Choosing positive history of disease, RBT, SAT, and PCR results as criteria, next 100 patient samples were selected as true positives for immunological assays with recombinant antigens. 50 additional true negative along with 50 ambiguous samples were included in these assays. True negative samples were selected on the basis of negative history, PCR, serological assay (RBT, SAT) results, while ambigouous sera derived from patients with no epidemiological evidence of brucellosis and negative PCR results, but positive for RBT and SAT.

3.2. Molecular methods: PCR

In order to establish a highly specific test for Brucellosis a number of genus- and/or species-specific PCR assays were designed. In view of fact that *Brucella* is found mainly inside of macrophages and monocytes in the circulating blood, genus-specific PCR assays were performed with DNA extracted from crude leukocyte preparations of all of the Mongolian patient samples described above. The DNA extraction procedure is described in the Materials and Methods section. In the following, the results for 100 from the 500 immunologically positive patient samples with seven different PCR assays developed in this work are described.

The sets of primers used were either specific to the genus *Brucella* as a whole or for individual species of *Brucella* known as the most frequent causative agents of human brucellosis: *B. melitensis, B. abortus* and *B. suis,* respectively. All primers were designed based on Genbank genomic sequence data (*Brucella melitensis M16* chromosomes I and II: NC 003317 and MC 003318; *Brucella abortus bv. 1 str. 9-941* chromosomes I and II: NC 006932 and NC 006933*; Brucella suis ATCC 23445* chromosomes I and II: NC 010169 and 010167). Optimal primer sequences within selected genomic regions were searched by the primer design software "Primer3" developed by the Whitehead Institute for Biomedical Research, Cambridge MA, USA (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). To facilitate performance of the assays, a uniform annealing temperature of 60°C was selected for all primers. Some of the primers were taken from the literature as indicated. Primer sequences, target genes and regions used in the PCR assays are shown in Table 3.1.

In order to check the reliability of the assays, different tests were used in parallel, two for general verification of the presence of *Brucella,* and three multiplex PCR assays for the discrimination of the species. To reach maximal sensitivity and specificity, all assays were designed as nested PCRs, employing an outer set of primers for the primary PCR cycles, and a different inner set of primers in secondary cycles. For initial screening, primers were selected to amplify part of the 16S rRNA gene which has the identical sequence in all *Brucella* strains. A second set of primers specific for the genus *Brucella* was designed using the carboxymuconolactone decarboxylase gene *pcaC* as template (Ratushna et al., 2006).

To identify the individual *Brucella* strains, three species-specific PCR assays were performed in parallel. In these assays, primers were used that recognised either genes occurring exclusively in one particular species each, or primers that led to species-specific differences in fragment size for the same gene. As species-specific targets, the following genes were selected: (i) the outer membrane transporter (OMT) gene (BruAb2_0168) for *B.*

Table 3.1 Diagnostic PCR primers

Primer	Sequence	Product size [b]	Specificity
Primers for genus specific PCRs			
16S-out-F	AGATTTATCGGCAAATGATCG	(809)	**all *Brucella* species**
16S-out-R	CCGGTCCAGCCTAACTGAA		
16S-inn-F	TTATCGGCAAATGATCGGC	**606**	
16S-inn-R	GTAAACACCCCGACGGCTA		
PcaC-out-F	TCAGGCGCTTATAACCGAAG[a]	(261)	**all *Brucella* species**
PcaC -out-R	ATCTGCGCATAGGTCTGCTT[a]		
PcaC -inn-F	TATAACCGAAGCGGCATGG	**247**	
PcaC -inn-R	TAGGTCTGCTTGGCGATCTT		
Primers for species-specific single PCRs			
OMT-out-F	TGCAGCTCACGGATAATTTG[a]	(783)	**B. abortus**
OMT-out-R	ACACCTTGTCCACGCTCAC[a]		
OMT-inn-F	AGCTCACGGATAATTTGACCAC	**715**	
OMT-inn-R	CCAGCATAATGGAACAGGTG		
GNTR-out-F	ATTCCCGAAAGCCGATAGAG	(182) **162**	**B. melitensis**
GNTR-out-R	GTCCTTTCAAACGCCGTCTA		
GNTR-inn-F	CCGAAAGCCGATAGAGTTTG[a]	(393) **373**	**B. abortus, B. suis, B. canis**
GNTR-inn-R	TCTACACCACGCTGAAGTCG[a]		
HlyD-out-F	GATGTTTTCCACTTGCGTCAC	(593)	**B. suis** (except of biovar 40)
HlyD-out-R	TCGGCCTGTGGATCTATTTC		
HlyD-inn-F	TTCCACTTGCGTCACTGTTC	**584**	
HlyD-inn-R	ATGTGAGCGAGGATGATTCC		
Primers for IS711-based multiplex PCR			
IS711-out-R	GAAAACATTGACCGCATTCAT		**common reverse primers**
IS711-inn-R	TTCATGGGTTTCGTCCATCT		
AlkB-out-F	TCACCAAATATATCCTGCACCA	(226)	
AlkB-inn-F	ATGGCGACGTGGTTGTCT	**189**	**B. abortus**
ChrI-112-out-F	TCATGACACCCAAACTTAGCC	(384)	**B. melitensis**
ChrI-112-inn-F	ACCCAAACTTAGCCATGGTG	**361**	
dUTPH-out-F	GCCCCACCAGATGAGAAA	(303)	**B. suis**
dUTPH-inn-F	CCGGGCTTTTCTCTATGACA	**254**	
Primers for EIBE-based multiplex PCR			
EIBE-out-F	TGTGCCAGCTTCGTTGTAAG[a]	(722) **480**	**B. suis**
EIBE-out-R	TGATAGCGCCAGACAACAAC[a]	(596) **354**	**B. abortus**
EIBE-inn-F	GCGCTGTTGATCTGACCTTG	(470) **228**	**B. melitensis**
EIBE-inn-R	GTCTGGGATCGCTGGTCTT		

[a] Ratushna et al., 2006.

[b] product size in base pairs. The product size of the first PCR is given in parantheses

abortus leading to a 715 bp product for *B. abortus* exclusively, (ii) the transcriptional regulator GNTR family gene (BMEII0204) leading to a 162 bp product for *B. melitensis* and a 343 bp product for the stains *B. abortus, B. suis* and *B. canis*, and (iii) the gene for HlyD family secretion protein (BRA0439) leading to a 585 bp product for *B. suis* exclusively (Ratushna et al., 2006).

In other discriminatory tests, a multiplex PCR targeting the insertion sequence IS711 and flanking regions was applied using three strain-specific forward primers and one common reverse primer (Bricker et al., 1994). All *Brucella* species contain at least 5 and as many as 35 copies of this element distributed throughout their genomes (in *B. abortus, B. canis* and *B. suis* 6-8, in *B. melitensis* 7-10, in *B. ovis* more than 28).

Finally, the species-specific polymorphism of the immunoglobulin-binding protein EIBE was used in a third discriminatory test, which led to a different sizes of amplification products with the different strains (Ratushna et al., 2006).

The PCR assays targeted the same genes as described in the above references, but in most cases different primer sequences were selected which appeared to be more appropriate according to the Primer3 software used. Furthermore, a second set of inner primers for nested PCR was designed for each test.

To facilitate performance of the assays, a uniform annealing temperature of 60°C was selected for all primers. Primers described in the literature fitting this condition were used without any changes. Nested primers ("inner" primers) were designed for each set of "outer" primers in order to improve sensitivity and specificity. The assays with the IS711-specific primers as well as with the EIBE gene-based primers were performed as multiplex PCRs in both, the primary amplification step with the "outer" primers as well as in the second amplification step with the inner primers. One microliter of the product of the primary PCR assays served as DNA template for the secondary nested PCR assays. The primary PCRs did normally not lead to visible bands upon gel electrophoresis after ethidium bromide staining, due to the low initial amount of the target DNA in the samples. However, after nested PCR, the resulting DNA bands became clearly visible. In case of the assays discriminating the different species *B. mellitensis, B. abortus* and *B. suis*, the inner primers had been selected in such a way that a clear assignment of fragment length to the bacterial strain could be made.

All these PCR assays were first established using *Brucella* reference strains *B. abortus 544, B. melitensis 16M and B. suis 1330* obtained from professor G. Baljer, Institute for Hygiene and Infectious Diseases of Animals, University of Giessen. Electrophoretic separation on 2 % agarose gels led to acceptable resolution of all of the resulting PCR products, even

though 6 % PAA gels allowed better discrimination of the smaller DNA bands. However, preparation of PAA gels for analysis of hundreds of PCR products appeared to be too demanding for routine assays. After it became clear that all PCR assays performed well with the reference strains, the same tests were performed with DNA samples from patients with clinical manifestations who had been positive in the immunological assays RBT and SAT.

Figure 3.1 shows exemplarily the results of six different assays with the same 18 patient samples (nested PCR only), a negative control with buffer alone and the positive controls using DNA from the three reference strains. The seventh experiment, the single species PCR with the *B. suis* specific primers HylD is not shown, because it was negative with all samples except of the *B. suis* positive control. In the same experiment, 100 patient samples were tested in parallel. It turned out that 80 out of the 100 patient samples (80 %) revealed detectable levels of *Brucella* DNA by applying nested PCR with 16S rRNA- and PCA-specific primers (Figure 3.1, panels A and B). All samples were processed for further PCR tests that were designed to differentiate *Brucella* at the species level. These tests revealed that 64 out of the 80 PCR-positive cases (80 %) were due to *B. abortus*, whereas the remaining 16 cases (20 %) were due to *B. melitensis*. No *B. suis*-specific DNA was detected amongst the samples analysed (Figure 3.1, panels C to F). All assays were repeated 3 times for each set of primers with identical results. The complete results with all 100 patient samples are listed in Table 3.2.

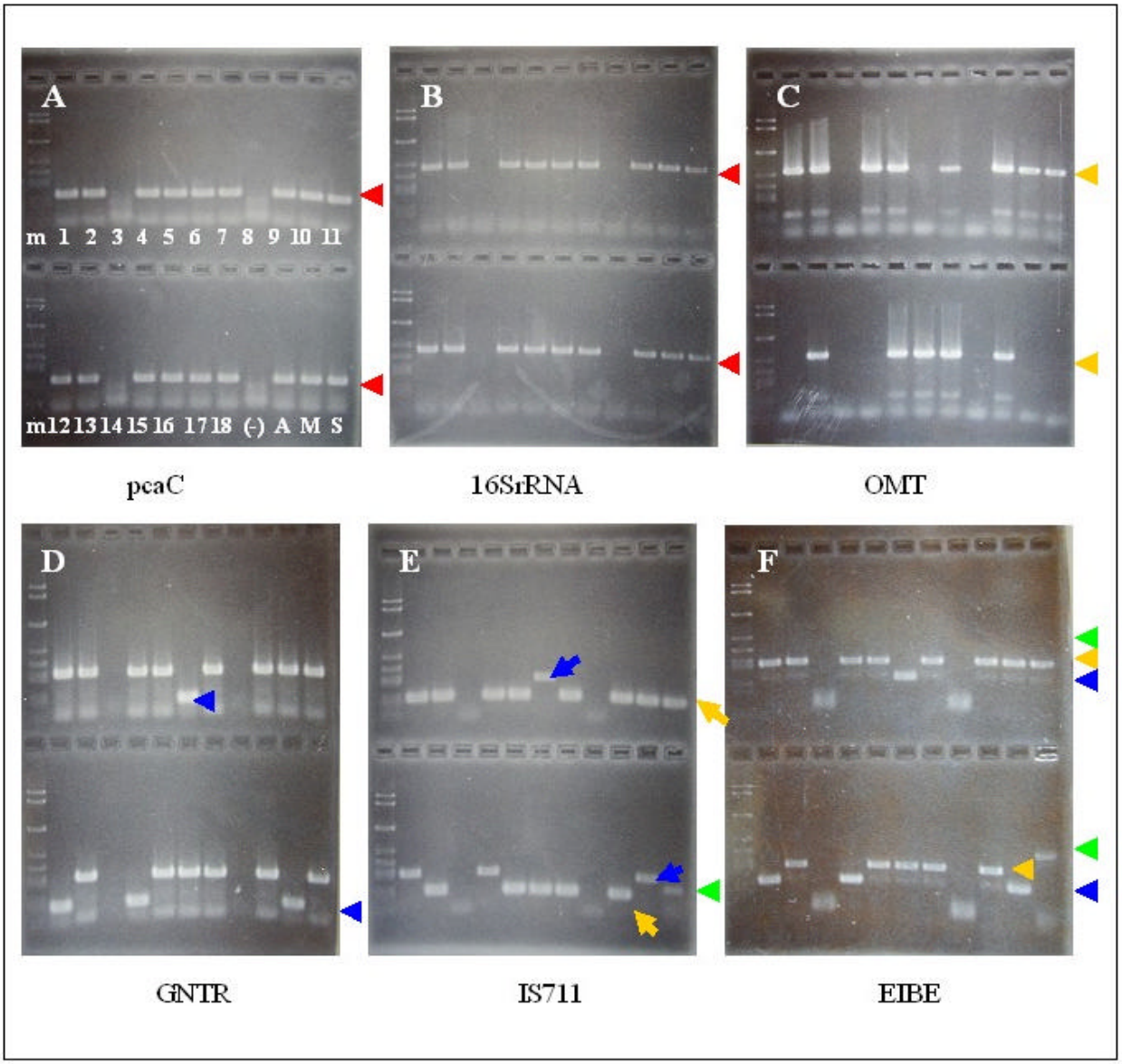

Figure 3.1 Comparison of the different *Brucella*-specific PCR assays. Each panel shows the products of nested PCR obtained with patient samples 1-18 after electrophoresis on 2% agarose gels. Lanes (-), A, M and S show products with the negative control, *B. abortus* 544, *B. melitensis* 16M, *B. suis* 1330 DNAs, respectively. **A**: carboxymuconolactone decarboxylase (pcaC)-specific primers, leading to a 247 bp fragment with all *Brucella* strains; **B**: 16S rRNS-specific primers leading to a 606 bp fragment with all *Brucella* strains; **C**: *B. abortus*-specific primers derived from the outer membrane transporter (OMT) gene, leading to a 715 bp fragment; **D**: *B. melitensis*-specific primers derived from the transcriptional regulator GNTR family gene, leading to a 162 bp fragment for *B. melitensis* and a 343 bp fragment for other *Brucella* strains. **E**: insertion sequence 711 (IS711)-specific primers, leading to a 361 bp fragment with *B. melitensis*, a 254 bp fragment with *B. suis*, and a 189 bp fragment with *B. abortus*; **F**: immunoglobulin-binding protein (EIBE)-specific primers, leading to a 480 bp fragment with *B. suis,* a 354 bp fragment with *B. abortus*, and a 228 bp fragment with *B. melitensis*. The expected positions of the DNA bands are marked in red for genus-specificity, Blue for *B. melitensis*, yellow for *B. abortus* and green for *B. suis.* Data for the *B. suis*-specific PCR are not shown because there were no positive results except for the *B. suis*-specific control.

Patient No	16S rRNA	pCA Bruegen	EIBE universal	IS711	B. melit. specific	B. abortus specific	B. suis specific
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	+	+	**m**	**m**	+	-	-
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
6	+	+	**a**	**a**	-	+	-
7	+	+	**a**	**a**	-	+	-
8	+	+	**a**	**a**	-	+	-
9	+	+	**a**	**a**	-	+	-
10	+	+	**a**	**a**	-	+	-
11	+	+	**m**	**m**	+	-	-
12	+	+	**a**	**a**	-	+	-
13	+	+	**a**	**a**	-	+	-
14	+	+	**a**	**a**	-	+	-
15	+	+	**a**	**a**	-	+	-
16	+	+	**a**	**a**	-	+	-
17	-	-	-	-	-	-	-
18	+	+	**m**	**m**	+	-	-
19	+	+	**a**	**a**	-	+	-
20	+	+	**m**	**m**	+	-	-
21	+	+	**a**	**a**	-	+	-
22	+	+	**a**	**a**	-	+	-
23	+	+	**m**	**m**	+	-	-
24	+	+	**m**	**m**	+	-	-
25	+	+	**m**	**m**	+	-	-
26	+	+	**a**	**a**	-	+	-
27	+	+	**a**	**a**	-	+	-
28	+	+	**a**	**a**	-	+	-
29	+	+	**a**	**a**	-	+	-
30	+	+	**m**	**m**	+	-	-
31	+	+	**a**	**a**	-	+	-
32	+	+	**a**	**a**	-	+	-
33	+	+	**a**	**a**	-	+	-
34	-	-	-	-	-	-	-
35	+	+	**a**	**a**	-	+	-
36	+	+	**a**	**a**	-	+	-
37	-	-	-	-	-	-	-
38	+	+	**m**	**m**	+	-	-
39	+	+	**m**	**m**	+	-	-
40	+	+	**a**	**a**	-	+	-
41	+	+	**a**	**a**	-	+	-
42	+	+	**a**	**a**	-	+	-
43	+	+	**a**	**a**	-	+	-
44	+	+	**a**	**a**	-	+	-
45	+	+	**a**	**a**	-	+	-
46	+	+	**a**	**a**	-	+	-
47	+	+	**a**	**a**	-	+	-
48	+	+	**a**	**a**	-	+	-
49	-	-	-	-	-	-	-
50	+	+	**a**	**a**	-	+	-
51	-	-	-	-	-	-	-
52	+	+	**a**	**a**	-	+	-
53	+	+	**a**	**a**	-	+	-
54	+	+	**a**	**a**	-	+	-
55	-	-	-	-	-	-	-
56	-	-	-	-	-	-	-
57	+	+	**a**	**a**	-	+	-
58	-	-	-	-	-	-	-
59	+	+	**a**	**a**	-	+	-
60	-	-	-	-	-	-	-
61	+	+	**a**	**a**	-	+	-
62	+	+	**a**	**a**	-	+	-
63	+	+	**a**	**a**	-	+	-
64	-	-	-	-	-	-	-
65	+	+	**m**	**m**	+	-	-
66	+	+	**a**	**a**	-	+	-
67	+	+	**m**	**m**	+	-	-
68	+	+	**a**	**a**	-	+	-
69	+	+	**a**	**a**	-	+	-
70	+	+	**m**	**m**	+	-	-
71	+	+	**a**	**a**	-	+	-
72	+	+	**a**	**a**	-	+	-
73	-	-	-	-	-	-	-
74	+	+	**a**	**a**	-	+	-
75	+	+	**a**	**a**	-	+	-
76	+	+	**a**	**a**	-	+	-
77	+	+	**a**	**a**	-	+	-
78	-	-	-	-	-	-	-
79	+	+	**m**	**m**	+	-	-
80	+	+	**a**	**a**	-	+	-
81	+	+	**m**	**m**	+	-	-
82	+	+	**a**	**a**	-	+	-
83	+	+	**a**	**a**	-	+	-
84	+	+	**a**	**a**	-	+	-
85	+	+	**m**	**m**	+	-	-
86	+	+	**a**	**a**	-	+	-
87	-	-	-	-	-	-	-
88	+	+	**a**	**a**	-	+	-
89	+	+	**a**	**a**	-	+	-
90	+	+	**a**	**a**	-	+	-
91	+	+	**a**	**a**	-	+	-
92	+	+	**a**	**a**	-	+	-
93	+	+	**a**	**a**	-	+	-
94	-	-	-	-	-	-	-
95	+	+	**a**	**a**	-	+	-
96	+	+	**a**	**a**	-	+	-
97	+	+	**a**	**a**	-	+	-
98	-	-	-	-	-	-	-
99	+	+	**a**	**a**	-	+	-
100	-	-	-	-	-	-	-
100	**80**	**80**	**16 m 64 a**	**16 m 64 a**	**16**	**64**	**0**

Table 3.2 Results of the different PCR assays. The results for the individual patients are marked as follows: +, = positive , - = negative, m = positive for *B. melitensis*, a = positive for *B. abortus*.

3.3 Establishing immunodiagnostics

Most commercial kits for diagnosis of brucellosis employ LPS or crude antigens, obtained by different methods of lysates of bacterial cultures. Serological assays for screening such as RBT and SAT use suspensions of killed whole bacteria. All assays have high sensitivity, which is needed for screening, but have the disadvantage of being not specific enough. The recombinant protein antigens, on the other hand, although showing high rates of specificity, fail to be sensitive enough and lead to false negative reactions. However, this disadvantage could be eliminated either by selecting more appropriate recombinant protein antigens, or combining several of such antigens in a single immunoassay. As individual recombinant antigens are usually not recognized by all patients sera due to the genetic heterogeinity of the immune system, it was decided to search for additional potential diagnostic antigens in the genomes of *Brucella* strains and to test their performance in immunological assays.

3.3.1 Selection of diagnostic antigens

The search of antigens was first based on published data of other groups, and since this approach did not lead to satisfactory results, exploration of available genomic data for potentially immuno-reactive pathogen proteins was performed in addition. The corresponding genomic sequences were obtained from different DNA data banks, preferentially from the NCBI collection of complete bacterial chromosomes (http://www.ncbi.nlm.nih.gov/Genbank). *Brucella* Bioinformatics Portal (BBP) (Xiang et al., 2006), a bioinformatics resource portal developed for *Brucella* research community was also of a great help in this study. Up to now, the genomes of following species and biovars have been released: *B. melitensis 16M, B. melitensis biovar abortus 2308, B. abortus biovar 1 strain 9-941, B. abortus S19, B. suis ATCC 23445,* and *B. suis 1330.* DNA sequences of other Brucella strains (*B. canis, B. ovis, B. neotomaea*) were not included in the search.

The genomic data of these *Brucella* strains were analysed giving preference to outer membrane proteins and taking an account their feasibility of expression as a recombinant protein in *E.coli*. Also the complexity of corresponding gene was analysed in order to detect cleavage sites and their interference with cleavage sites of expression vector. Besides, the sequences were checked for codons rarely used in *E.coli* and for their specificity to genus *Brucella*. The similarity of the sequences with other organisms was checked with the BLAST program (http://www.genedb.org/genedb/ blast.jsp).

The sequence information extracted from literature and different genomic data bases was used to clone the following 10 antigens. The abbreviations used for these antigens in the text are shown in bold face letters.

1. *B.abortus* bacterioferritin, **P15** (Denoel et al., 1995).
2. *B.abortus* 17-kDa protein, **P17** (Hemmen, F., et al., 1995).
3. *B.abortus* 39-kDa cytoplasmic protein, **P39** (Denoel et al., 1997).
4. *B.abortus* 26 kDa periplasmic protein, **BP26** (Rosetti et al., 1996).
5. *B.melitensis* Omp31outer membrane protein, **Omp31** (Cassatro et al., 2003)
6. *B.abortus* Omp25 outer membrane protein, **Omp25** (Cloeckaert, 1996).
7. *B.melitensis 16M* membrane alanine aminopeptidase, **AminopepN** (Contreras-Rodriguez et al., 2006).
8. *B.abortus* outer membrane lipoprotein-related protein, **LP**.
9. *B. abortus* hypothetical omp62289852 , **Baomp852**.
10. *B.abortus* outer membrane efflux protein, **Baompeff.**

The majority of the genes selected by this way had been described at a certain extent in the literature as indicated. However, most of these proteins were described as potential diagnostic antigens only, and only few of them had been tested extensively for immunodiagnosis of human brucellosis.

The general strategy for testing the listed antigens in immunoassays was to test them first individually and, depending on their immuno-reactivity, to combine individual selected antigens in a single chimeric fusion protein for two reasons: first to simplify the production and thereby optimise the purification of the recombinant antigen, and second, to increase both specificity and sensitivity of the assay. By this way, a fusion antigen was produced at the end. Its performance was experimentally tested in immunoassays and the results were compared with published data from existing diagnostic procedures. All protein antigens were well expressed in *E. coli* XL1-Blue/pREP, except LP which seems to be detrimental for *E. coli* cells, since it could be produced only in small quantities even using a synthetic gene containing optimised translational codons.

3.3.2 Synthesis of recombinant antigens: example of cloning by PCR amplification of P15 antigen

P15 was one of the antigens cloned first. The gene sequence, which encodes for 15 kDa bacterioferritin, was derived from *B. abortus* chromosomal sequence as shown in Figure 3.2. The protein coding sequence was amplified by PCR using primers derived from its genomic sequence (GeneBank NC_006932).

P-15 bacterioferritin

Coding sequence :

```
  1 cgcggccgtt gctgtggctg cttcccaaat gtcgtagaaa ccatcataag ggtgactgaa
 61 gaatactatc ttcgtcgcaa ccaaatggac gaaaacatta tccagttcat ggatcgtgtg
121 cgttctctac gagataaatt cgggagttca tggaatgaaa ggcgaaccaa aggtcatcga
181 gcggcttaac gaggcactgt ttcttgagct cggtgcggta aaccagtatt ggctgcacta
241 ccgtcttctc aacgattggg gttacacgcg ccttgcaaag aaggaacgcg aggaatccat
301 cgaggaaatg catcacgccg acaagctgat tgatcgcatt atcttccttg aaggctttcc
361 gaacctccag accgtttcgc cgttgcgcat tggccagaat gtgaaggaag ttctcgaagc
421 tgacctcaag ggtgaatatg acgctcgcgc ttcgtataag gaatcgcgcg aaatctgcga
481 caagctcggc gactatgtgt cgaagcagct tttcgacgaa cttctggccg atgaagaagg
541 ccatatcgac ttccttgaaa cccagcttga ccttctcgcc aagatcggcg gagaacgcta
601 tggccagctt aacgcggcgc ccgccgacga agctgagtaa gcctgtttca atctgtcttg
```

Primers:

BAp15-for: ATAGGATCCatgaaaggcgaaccaaaggt(BamHI)
BAp15-rev: TATAAGCTTtaagctggccatagcgttct(HindIII)

Translation:

```
  1 MKGEPKVIER LNEALFLELG AVNQYWLHYR LLNDWGYTRL AKKEREESIE EMHHADKLID
 61 RIIFLEGFPN LQTVSPLRIG QNVKEVLEAD LKGEYDARAS YKESREICDK LGDYVSKQLF
121 DELLADEEGH IDFLETQLDL LAKIGGERYG QLNAAPADEA E
```

Figure 3.2 Cloning of P-15 bacterioferritin of *B. abortus*. The coding sequence as extracted from GeneBank is shown above. Start and stop codons are marked in bold face, the location of the primers used for PCR amplification are underlined. The lower part of the figure shows the protein sequence. In the recombinant protein, the N-terminal sequence is fused to a hexameric histidine sequence which serves for purification by affinity chromatography. The last 9 amino acids of the original *B. abortus* protein are not contained due to suboptimal nucleotide sequences for efficient PCR priming.

In order to simplify cloning of different recombinant antigens, the same restriction sites were used at the beginning (BamHI) and at the end (HindIII) of the open reading frames for all constructions. To use this cloning strategy, the BamHI and HindIII restriction sites should not occur within the open reading frames. For this purpose, a search for these sites within the

genomic sequence was performed in each case using “The Sequence manipulation Suite” software (http://www.bioinformatics.org/sms2/, Stothard, 2000). The Baompefflux protein contained these two restriction sites in the N-terminal coding part and was therefore cloned as partial fragment including approximately 30 % of the C-terminal sequence only. However, in the other genes no such interfering BamHI and HindIII restriction sites were contained and the open reading frames of the proteins were cloned in most cases completely, omitting the start codon (AUG). This was done to prevent internal translation initiation at this site upon fusion with the histidine affinity tag sequence encoded by the vector plasmid. The stop codons at the C-terminal end were excised in most cases as well, to allow later fusion of different antigens to polyproteins. In some cases the HindIII restriction site at the end of the gene was substituted by a KpnI or PstI site, depending on the occurence of these sequences inside the open reading frame.

There is substantial experimental evidence that the presence of non-optimal codons (i.e., those translated by rare tRNAs) in a sequence can reduce the translation rate of proteins (Robinson et al., 1984; Bonekamp et al., 1985), which is one of the strategies for the regulation of protein expression in bacteria (Grantham et al., 1981). The codon frequencies in the *Escherichia coli* are shown in Figure 3.3.

TTT F 0.58	TCT S 0.17	TAT Y 0.59	TGT C 0.46
TTC F 0.42	TCC S 0.15	TAC Y 0.41	TGC C 0.54
TTA L 0.14	TCA S 0.14	TAA * 0.61	TGA * 0.30
TTG L 0.13	TCG S 0.14	TAG * 0.09	TGG W 1.00
CTT L 0.12	CCT P 0.18	CAT H 0.57	CGT R 0.36
CTC L 0.10	CCC P 0.13	CAC H 0.43	CGC R 0.36
CTA L 0.04	CCA P 0.20	CAA Q 0.34	CGA R 0.07
CTG L 0.47	CCG P 0.49	CAG Q 0.66	CGG R 0.11
ATT I 0.49	ACT T 0.19	AAT N 0.49	AGT S 0.16
ATC I 0.39	ACC T 0.40	AAC N 0.51	AGC S 0.25
ATA I 0.11	ACA T 0.17	AAA K 0.74	AGA R 0.07
ATG M 1.00	ACG T 0.25	AAG K 0.26	AGG R 0.04
GTT V 0.28	GCT A 0.18	GAT D 0.63	GGT G 0.35
GTC V 0.20	GCC A 0.26	GAC D 0.37	GGC G 0.37
GTA V 0.17	GCA A 0.23	GAA E 0.68	GGA G 0.13
GTG V 0.35	GCG A 0.33	GAG E 0.32	GGG G 0.15

Figure 3.3 Genetic standard code of *Escherichia coli*. Codons are followed by the amino acid they code for, and the corresponding usage frequencies. Codons marked in red present the lowest frequencies, and another group of condons with low frequencies in orange. Stop codons are indicated with an asterisc. Modified from Maloy et al., 1996

All genes cloned were checked for the presence of these rare *E. coli* codons. Luckily, all of them, with the exception of the LP gene, used preferentially abundant *E. coli* codons for translation. PCR primers for amplification of the corresponding coding sequences were designed first manually and their utility thereafter was checked according to the criteria of the Primer3 computer program (Rozen et al., 2000, http://frodo.wi.mit.edu/ primer3/input.htm). This helped to select appropriate annealing temperatures and to predict whether the selected primers were compatible with efficient priming.

Genes containing BamHI or HindIII restriction sites within the open reading frame were cloned in a way that only the largest possible fragments without these sites were included. PCR primers to amplify the largest possible fragment were designed by means of the Primer3 program. Restriction sites BamHI (g|gatcc) and HindIII (a|agctt) for cloning in the vector plasmid were added manually to the primers. The computer-predicted forward and reverse primers led usually to succfessfull amplification of the predicted DNA fragments using the selected annealing temperatures and the corresponding DNAs of either *B. melitensis* or *B. abortus* as templates.

The forward and reverse PCR primers designed for cloning of P15 consisted of 29 bp each with the following sequences (restriction sites are underlined).

BAp15-for: **ATAGGATCCatgaaaggcgaaccaaaggt** (BamHI)

BAp15-rev: **TATAAGCTTtaagctggccatagcgttct** (HindIII)

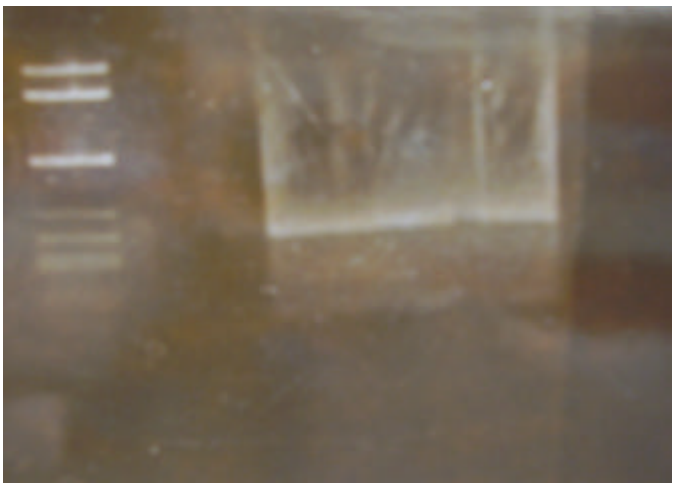

Figure 3.4 Amplification of P15 by PCR. A product of 457bp size was produced by BAp15-for and BAp15-rev primers.

3.3.3 Cloning in pQE-30 vector and protein expression

In order to insert the P15 gene into the pQE-30 expression vector, “sticky ends” were created by digesting the eluted PCR fragment with BamHI and HindIII restriction enzymes and subsequent ligation with the pQE vector cleaved with the same enzymes. After purification with silica to remove polyethyleneglycol and salts, the plasmid DNA was electroporated into competent *E. coli* cells that were thereafter selected on agar plates containing ampicillin and kanamicine. Resulting colonies were checked for the presence of the insert sequence by PCR, using the primers P15 forward and pQE reverse, the latter being located in the vector downstream from the insertion site. By this way, false positive results due to the presence of the non-ligated PCR fragment were avoided. One of the positive clones was selected for growth and protein purification.

The remaining recombinant antigens were cloned using basically the same procedure with minor differences such as using restriction enzymes other than HindIII at the end of open reading frame due to occurrence of this site inside of the coding sequence of the gene. In case of P17, P39 and Omp31, Omp25, and Baomp852, the HindIII cleavage site was replaced by either KpnI or PstI, respectively. The genes, primers and translation products of all recombinant antigens are listed in the Appendix section.

In case of the LP antigen, a very weak expression was observed only. Therefore it was decided to use a synthetic gene (ordered from GENEART, Regensburg) that substituted rare codons of *E. coli* by more frequently used ones. However, the new construct did not result in a better expression, the possible reason being therefore rather the toxicity of an excess of the protein to *E. coli*. Nevertheless it was possible to obtain sufficient amounts of this antigen for testing in immunological assays.

All recombinant proteins were expressed in *E. coli* XL1-Blue/pREP and purified by affinity chromatography in preparative scale as described in the Materials and Methods section. As an example, the elution profile from the TALON affinity purification of recombinant P-15 antigen analysed on an analytical SDS polyarcrylamide gel is depicted in Figure 3.5.

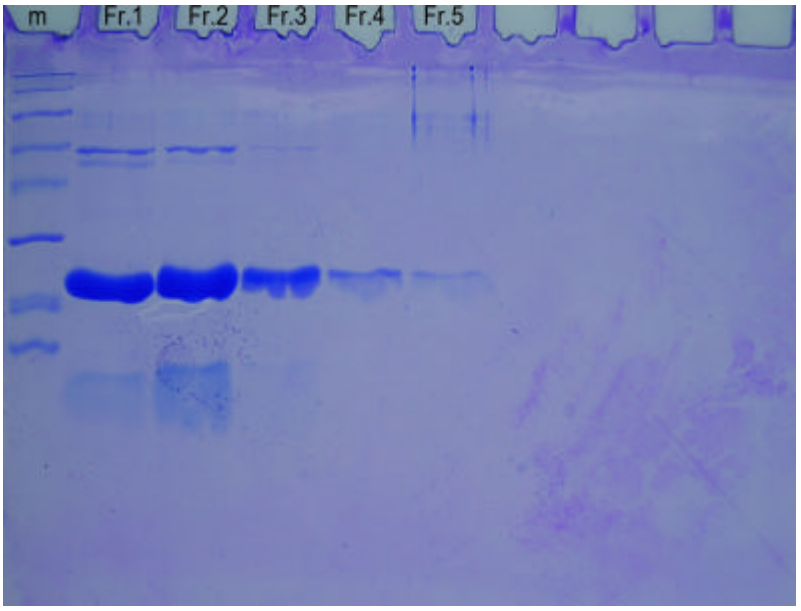

Figure 3.5 Analysis of P-15 purification by analytical SDS-PAGE gel electrophoresis. The first three fractions contain the highest concentration of the antigen.

The other recombinant antigens were expressed and purified in the same way. Aliquots of the purified proteins are shown in Figure 3.6.

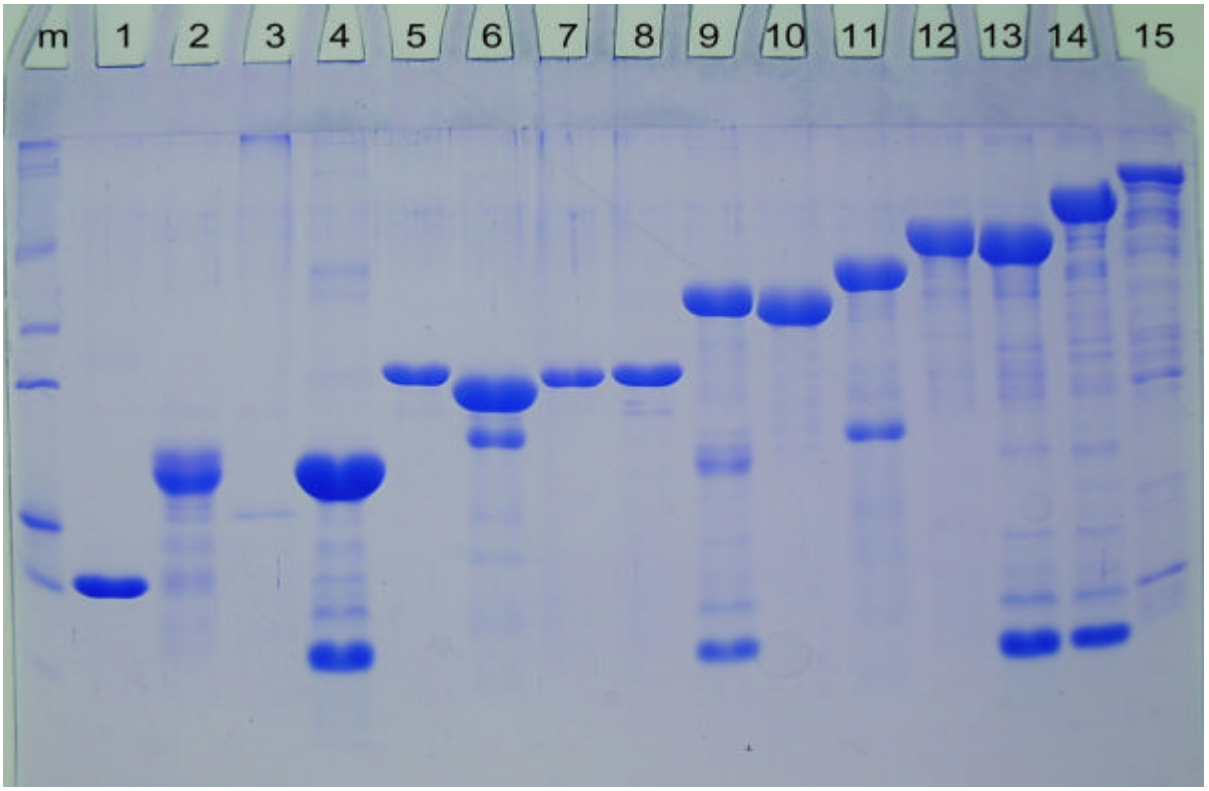

Figure 3.6 SDS PAGE analysis of all recombinant *Brucella* antigens. The left lane contains a protein size maker. Lanes 1 to 15 correspond to the following recombinant antigens: **1.** Baompefflux, **2.** P15, **3.** LP, **4.** P17, **5.** Omp31, **6.** Omp25, **7.** Baomp852, **8.** BP26, **9.** omp852-bp26, **10.** P39, **11.** Baomp852-BP26, **12.** BP26-P39, **13.** P15-P39, **14.** Triple fusion P15-bp26-P39, **15.** Aminopeptidase N.

3.3.4 Specificity and sensitivity of the antigens

The efficiency of the purified recombinant antigens for immunodiagnosis was tested using an immunoblot technique called "line blot" (described in the Materials and Methods section). Because the line blot compares the reactivity of different antigens and of different concentrations of these antigens in a single assay, this step allowed to reach two objectives: (1) to determine the optimal concentration of the antigens in immunoassays, and (2) to determine the reactivity with brucellosis patient sera and control sera, i.e. to detect possible cross-reactions. To determine the optimal concentration, the antigens were applied in serial dilutions on the membrane, then incubated with either brucellosis patient sera or with control sera. At the optimal concentration, an antigen leads to a clear reaction with specific sera, but not with control sera. An excessive concentration of the antigen leads to false positive reactions with control sera, and a sub-optimal concentration reduces sensitivity of the test.

The intensity of the detectable reaction depends rather on the affinity of an antigen to specific antibodies than on the concentration of these antibodies in patient sera. Some antigens had to be used at concentrations as high as 200 µg/ml, while others led to strong positive reactions at concentrations less than 1 µg/ml. Some antigens showed a strong background reaction with the negative control sera, mainly those that had to be used at high concentrations due to their low specific reactivity. As the affinity purification procedure does not remove bacterial components such as lipopolysaccharid completely, the high background reaction with the control sera may be due to antibodies directed against these components. *E. coli*-specific antibodies are present in all human sera and prevail even more in sera derived from people in developing countries. Bacterial contaminations in the antigen preparations cannot be removed completely without great effort. However, the more diluted an antigen can be used in immunoassays, the lower the cross-reaction with bacterial contaminations will be.

The line blot assay was also used to determine the specificity of the immuno-reaction of the recombinant antigens (Figure 3.7). Single test stripes were incubated either with individual sera derived from the mongolian brucellosis patients, or with sera from patients with other infectious diseases but without epidemiological, clinical, or serological evidence of brucellosis. In addition, antigens were tested with sera from healthy blood donors from Germany. As can be seen from the figure, not all of the brucellosis-positive serum samples reacted with all of the antigens, even though a clearly visible signal with selected antigens was detectable in most cases. The difference in reactivity of individual patients with specific antigens reflects most probably the genetic heterogeneity of individuals with respect to the immune system.

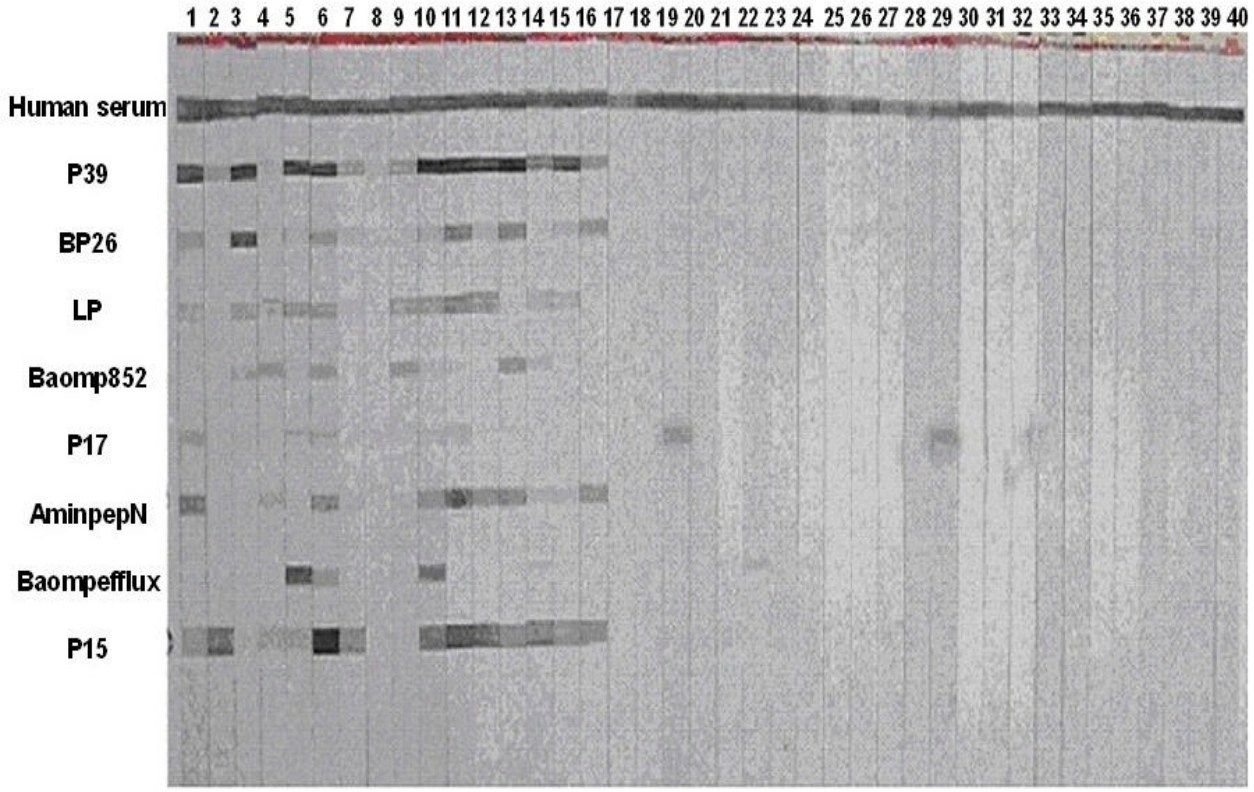

Figure 3.7 Specificity of the recombinant antigens determined in line blot. The individual antigens have been applied as a line on a nitrocellulose embrane, the membrane was then blocked in 1 % Tween 20, cut in 4 mm stripes, and incubated with sera from brucellosis patient (stripes 1 – 16), or negative control sera (stripes 17 – 40). Note that not each antigen reacts strongly positive with all patient sera, and some antigens lead to cross-reactions with negative control sera (P17 and Baompefflux). Human serum in the upper line serves as a control for the staining reaction of the alkaline phosphatase conjugated to the second antibody.

After the immuno-reaction of the recombinant antigens had been characterised in the line blot assays, they were tested in ELISA assays. Although ELISA assays depend on special equipment such as optical readers and washers, they are more appropriate for tests on large scale because they can be automated and are quantifiable. Figure 3.8 shows as an example the results of P15 in ELISA with 50 positive brucellosis patient sera and with 50 negative control sera.

Not all of the antigens turned out to be usefull for immonoassays in ELISA. Antigen P17 reacted with both negative and positive sera, thus rendering it useless for further utilization. The antigens Omp25 and Omp31 reacted with some *Brucella*-negative sera, while being not able to detect all positive samples. These antigens were therefore excluded from further experiments as well. The antigens P15, P39 and BP26 revealed reasonable reactivity with positive sera, but the intensity of signals produced was not so strong therefore the singular use for diagnosis appeared to be not efficient. Although they did not produce any false positives, there was a minor fraction of false negatives for each of the antigens. Such false negative reactions with

individual antigens were observed differently by true positive patient sera, thus reflecting rather the genetic heterogeneity of the immune system of the patients than a weakness of interaction with the corresponding antigen. In addtion recombinant proteins LP and Aminopeptidase N appeared to be usefull for specific diagnosis although the difference in OD values between positive and negative controls was not very high.

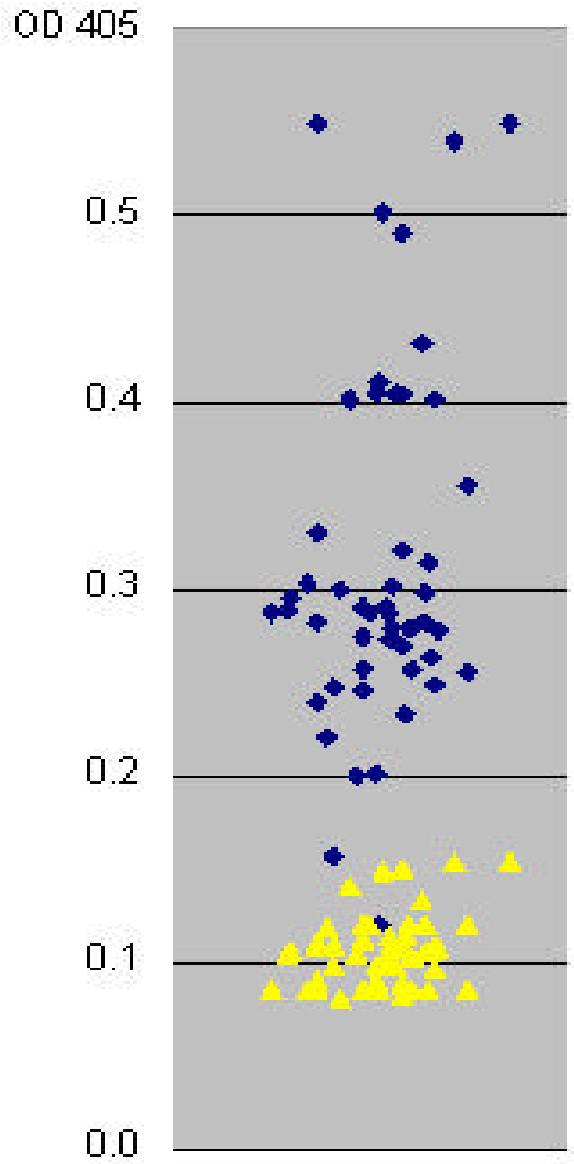

Figure 3.8 Antigen P15 ELISA with patient sera and control sera. P-15-coated microtitre plates were incubated with 50 PCR-positive and 50 negative control sera for one hour, washed, reacted with HRP conjugated second antibodies and stained as described in Materials and Methods section. Note that except of two of the positive sera, a clear discrimination between patients and controls can be observed

The results for all individual recombinant antigens including fusions (see further) in ELISA assays are compiled in Figure 3.9.

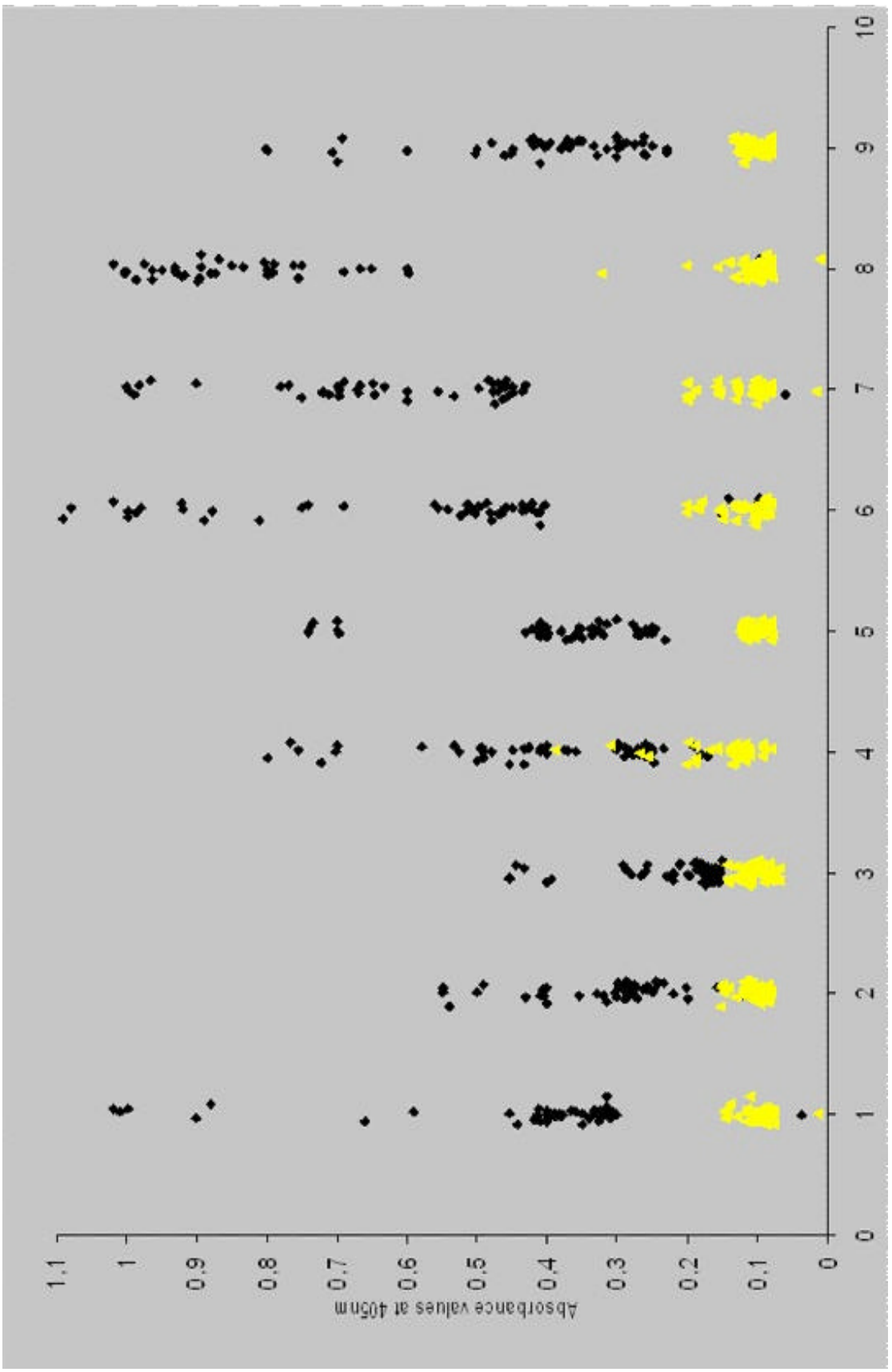

Figure 3.9 Results of ELISA assays of same 50 positive and 50 negative sera with recombinant diagnostic antigens. **1.** BP26, **2**. P15, **3**. Baomp852, **4.** Baompefflux, **5.** LP, **6.** P39, **7.** BP26-P39 fusion, **8.** P15-BP26-P39 fusion, **9.** AminopepN.

It turned out that some of the recombinant antigens showed low immuno-reactivity in immunoassays, especially in ELISAs. As shown on graphs although Baompefflux and Baomp852 proteins display reactivity to sera at certain extent, they fail to make a clear distinction between positives and negatives because of the weak reactivity. Antigen LP displays a much higher discriminatory power in this respect, but was still not at the desired level. The antigens P15, BP26 and P39 led to a much better distinction of positive and negative sera, making them candidates for further development strategy. A possible explanation of weak reactivity of individual antigens was either weak binding of the small peptides to the polysterene surface of the microtitre plate or / and were removal of them during the washing steps. To overcome these problems, it was attempted to combine the smaller peptide antigens to larger fusion proteins.

3.3.5 Fusion of selected antigens

Results on ELISA with individual antigens clearly showed that only the use of several, but not single antigens would lead to sufficient sensitivity in immunoassays. In order to safe effort in producing these antigens it was thought that combining of the most reactive recombinant antigens to larger fusion proteins could be a reasonable approach. In addition, it was possible that the synthetic proteins could adopt – at least in part - a three-dimensional conformation that would function as antigenic determinants better in the context of a polyprotein. The selected synthetic genes were fused with the help of “adapter” oligonucleotides. These adapters served several functions. First, they had to join the 3’ terminal HindIII, or PstI site of the anterior antigen in the correct reading frame with the BamHI site of the following antigen. The nucleotide sequence was designed in such way that both restriction sites were destroyed by the fusion. Second, the adapters served as spacers between the different antigens. In order to allow a maximum flexibility between the fused antigens, one or two proline residues were introduced in the spacer elements. Proline residues are frequently present in flexible areas of proteins (e.g. in the "hinge" regions of immunoglobulins). It was assumed that such flexible elements would prevent or reduce the interference of secondary structures of the individual antigenic regions with each other, and thus facilitate the individual antigenic motives to fold in more close to native confromation state. To avoid repeats of homologous sequences in the fused genes which could lead to elimination of parts of the construct by homologous recombination, different adaptor fragments were constructed. The adapter sequences used are shown in the following Figure 3.10.

Hind-Bam-AD1

```
         HindIII                              BamHI

5' NNN A       AG CTG CCG AGC CTG AGC A       GA TCC NNN 3'
3' NNN TTC GA       C GGC TCG GAC TCG TCT AG       G NNN 5'

    X   K          L   P   S   L   S   R          S   X
```

Hind-Bam-AD2

```
5' NNN A       A GCT ATT CCG CTG AGC A       GA TCC NNN 3'
3' NNN TTC GA        TAA GGC GAC TCG TCT AG       G NNN 5'

    X   K         A   I   P   L   S   R   S          S   X
```

Pst-Bam-AD1

```
        PstI

5' NNN CTG CA         T CTG CCG CTG AGC A       GA TCC NNN 3'
3' NNN G        AC GTA GAC GGC GAC TCG TCT AG       G NNN 5'

    X   L           H   L   P   L   S          R   S   X
```

Figure 3.10 Adapter sequences used to fuse antigen coding regions. Nucleotide sequences of the adapter oligonucleotides are depicted in complementary form. Residues providing "sticky ends" for the restriction site HindIII/PstI and BamHI are underlined. Amino acid sequences encoded by the different adaptors are shown below the nucleotide sequences.

3.3.6 Example of fusion: P15-bp26-P39

The antigens P15, BP26 and P39 were chosen first to construct a fusion protein since they had characteristics as useful antigens, reacting positively with patient sera and giving negative results with all negative control sera, by both IgM and IgG-specific ELISAs. In addition they showed significant difference in OD values between the negative and positive samples. Cloning of the fusions gene was performed in several steps. First, the coding regions of P15 and BP26 were fused with the adapter Hind-Bam-AD2. For this purpose, the plasmid pQE-P15 was cleaved by HindIII and XhoI. Cleavage products were separated by agarose gel electrophoresis and the 602 bp band with HindIII and XhoI ends was excised (Fig.3.11). The sequence contained the whole P15 gene along with a small fragment of the original pQE vector starting at XhoI site (see Figure 2.1). XhoI restriction was chosen for a better orientation and detection of fragments on the gel electrophoresis. Simultaneously, pQE-BP26 was cleaved by XhoI and BamHI resulting in a 3987 bp product containing the whole BP26 gene with the rest of plasmid pQE.

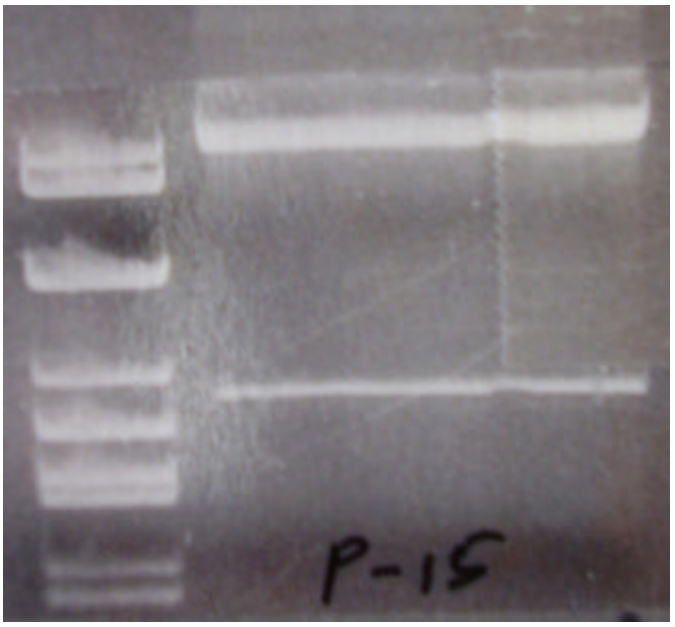

Figure 3.11 Cleavage of pQE 30 vector containing P15 by XhoI and HindIII.

In a second step these two fragments were ligated to a single plasmid. Whereas ligation of the XhoI sites was normal, the HindIII and BamHI sites were joined by means of the synthetic Hind-Bam-AD2 adapter (Figure 3.10). The resulting plasmid containing both genes was electroporated into *E. coli*. Upon antibiotic selection resulting colonies were characterised by analytical PCR using the primers BAp15-for and pQE-rev. Correct clones led to amplification of a 1213 bp product and were further analysed by restriction digest with BamHI and HindIII upon plasmid DNA preparation.

The next step was to include one more antigene (P39) into the new construct pQE-P15-AD2-BP26. Using the same strategy, the plasmid was cleaved by XhoI and HindIII again and the fragment containing both genes with XhoI and HindIII sites was isolated. Plasmid pQE-P39 was cut by XhoI and BamHI. The fragments containing P39 and P15-bp26 fusion genes were ligated as before, however using a different adapter, Hind-Bam-AD1.

Using the same strategy, combinations of the following genes were produced and consequently expressed in vector pQE-30: P15 and bp26, omp852 and bp26, P15 and P39, bp26 and P39, P15-bp26-P39. The resulting proteins were produced as described above. They were analysed by analytical SDS PAGE as shown in Figure 3.5 lanes 9 and 11-14.

3.3.7 Tests of the antigens with human sera samples in ELISA

A large number of serum samples had been tested in line blots to characterize the quality of the recombinant antigens. However, the line blot is not a very practical method for a routine diagnostic laboratory. In most laboratories, ELISA is the method of choice due to its capacity to be automated, and the possibility to test large amounts of samples. Furthermore, the results are more objective, since they are quantifiable by photometry.

Whereas alkaline phosphatase-conjugated second antibodies had been used in the line blots, HRP-conjugated second antibodies were used in the ELISA. Alkaline phosphatase assays are highly sensitive and the conjugates have the advantage of being very stable during storage. However, the tests can be hyper-sensitive leading occasionally to false positive results, most probably due to accidental contamination. Phosphatases are ubiquitous and very resistant enzymes that are also contained in body fluids such as sweat, saliva etc., thus being easily spread by handling reaction tubes and micro-plates even wearing gloves. For this reason, HRP-conjugated second antibodies were used in ELISA tests including the corresponding colour assays. Peroxidase assays are widely used and have the same sensitivity as alkaline phosphatase assays. However, they have the disadvantage that the enzymatic activity is not as stable as the activity of phosphatase, i.e. the conjugates have a limited halftime. At the other hand, there is a much lower risk of contamination since peroxidases occur less frequently in the nature than phosphatases, .

To obtain more exact values in the ELISA tests, double measurements were performed on each sample, with the mean value calculated thereafter thus reducing intra-assay variation. To discriminate positive versus negative results, a cut off value is established by using the following equation:

Cut off = average of negative controls + 2 to 5 x standard deviation (SD) of negative controls.

To determine how many SD have to be used in the formula, a test was performed with a large number of *Brucella* positive and negative sera and the cut off value was calculated at 2, 3, 4, or 5 x SD. Several false positives were found using 2 x SD while several false negative were observed when 4 x SD was used. In contrast, neither false positive nor false negative results were obtained using 2 x SD for the aminopeptidase N, and for Lp and 3 x SD for the triple fusion antigen P15-bp26-P39 indicating an optimal discrimination at these ranges. When the SAT negative patients' sera from Mongolia were used as negative controls, a slight increase in the

cut off values was observed as compared to the values obtained with the German negative control sera.

In order to find the optimal conditions to perform the ELISA with the antigens (LP, triple fusion P15-bp26-P39 and aminopeptidase N) a box titration was performed using a pool of positive sera as a control. The best results were obtained at serum dilutions of 1:100, and all three antigens were still reacting properly at dilutions 1:5000.

3.3.8 Comparison of antigens with existing serological methods

A total of 200 sera from different groups of patients were studied. The first 100 sera were from a group of the Mongolian patients with confirmed brucellosis. The case definition for initial brucellosis diagnosis was a history of exposure to domestic animals, or consumption of un-pasteurized milk or milk products, together with history of persisting fever, and symptoms and physical signs compatible with this infection, along with the positive results for RBT and SAT at dilutions =1/160. Only "true" positive serum samples were used in this ELISA, i.e. all samples had been tested before by PCR for the presence of *Brucella* DNA as described above. The second group of sera (50 samples) were derived from the individuals who had neither positive clinico-epidemiological nor laboratory evidence of the disease. The third group (50 samples) consisted of sera from individuals positive in serological tests (RBT and SAT), but negative in PCR and with no history of exposure to *Brucella* or any clinical signs of brucellosis. The results are summarised in Table 3.3.

Study group	**LP**		**P15-BP26-P39 fusion**		**Aminopeptidase N**	
	Positive	Negative	Positive	Negative	Positive	Negative
True positive (n=100)	97 (97%)	3 (3%)	99 (99%)	1 (1%)	90 (90%)	10 (10%)
True negative (n=50)	0 (0%)	50 (100%)	0 (0%)	50 (100%)	0 (0%)	50 (100%)
Ambiguous (n=50)	20 (40%)	30 (60%)	22 (44%)	28 (56%)	15 30%	35 (70%)

Table 3.3 Summary of results with the three new *Brucella*-specific ELISA tests. The table shows the number of positive and negative results obtained in ELISA with the three different antigens. The calculated percentage is given in parentheses.

All three recombinant antigens revealed a high specificity in the assay and did not lead to false positive results with the negative control sera. However, the sensitivity with the true positive samples was not 100 %. LP detected 97 out of the 100 positive samples and aminopeptidase N only 90. The best performance was seen with the P15-BP26-P39 fusion antigen reacting with 99 of the positive samples resulting in a sensitivity of 99 %.

The recombinant antigens led to different results with the third group of patients which is is positive in RBT and SAT, but negative in PCR. From the 50 samples, 20 were positive with LP, 22 with the P15-BP26-P39 fusion, and 15 with Aminopeptidase N. The absence of reactivity of the antigen with the negative control group shows that there is no crossreaction with other bacteria. Positive RBT and SAT results can be due to either crossreaction or past infection.

4 DISCUSSION

Brucellosis is a zoonosis transmittable to humans that shows a high degree of morbidity, both for animals and humans. Consequently, it causes significant financial loss and represents a serious public health problem in many countries. An appropriate diagnosis is the key for eradication and control of this disease. At present, several immunoassays are employed mainly to screen animal populations, but for humans such tests are usually applied when patients reveal clinical symptoms for confirmation of the disease.

Generally, the immunoassays for brucellosis applied in the endemic areas and individuals professionally exposed to *Brucella* have limited sensitivity and specificity. At the one hand, this is due to missing resources for health care sector allowing the use of simple and inexpensive screening procedures only. The other reason is that many healthy individuals in these groups of population have high titres of antibodies against *Brucella* due to a previous unnoticed infection and being recovered. These methods also have limitations due to low antibody titres in the early phases of the disease and in patients with relapsing infection since it is generally very difficult to observe and monitor disease dynamics by immunoassays. Another essential limitation of the commonly used immunoassays is their cross-reactivity with other bacteria since *Brucella* spp. share common antigenic structures with other pathogens at the level of the O-chain polysaccharide S-LPS. Thus, the diagnostic tests based on the lipopolysaccharide O-side chain antibodies can generate false-positives due to cross-reaction with antigens from other Gram-negative bacteria (Weynants et al., 1996). On the other hand, infections caused by the rough form of *B. canis* are under-diagnosed by tests that employ LPS from smooth-*Brucella* strains (Polt et al., 1982; Lucero et al., 2005).

One of the main goals of this work was to develop new diagnostic procedures that are not only more specific and sensitive compared to existing immunoassays, but also simple and cost-effective to perform in order to enable their routine use under the economically limited conditions of Mongolia. ELISA appeared to be the immunodiagnostic method of choice because in addition of being inexpensive and simple to perform it has the advantages of being fast and easily to be automated. Furthermore, it is applicable to different classes of immunoglobulins, allowing to monitor specific phases of the disease to a certain degree, at least.

Since ELISA tests using LPS as antigen lack specificity, the new assays were based on recombinant proteins. Using appropriate recombinant antigens, specificity of immuno-assays is in general high, although sensitivity is usually lower than in assays using whole cells or crude antigen preparations. Previous studies have shown that single recombinant proteins such as Omp31 (Cassatro et al., 2003), Omp25 (Cloeckaert, 1996), aminopeptidase N

(AminopeptidaseN), bp26 (Rosetti et al., 1996), P15 (Denoel et al., 1995), P17 (Hemmen, F., et al., 1995), and P39 (Denoel et al., 1997) carry immunogenic structures that could be useful for serological diagnosis of animal brucellosis. Higher sensitivities were obtained when several of these antigens were used in parallel, suggesting that their combination in a single-well test could further improve the performance of the assay. A first step towards the development of a multi-protein diagnostic reagent was attempted by Letesson et al., 1997, suggesting that a good diagnostic reagent should contain more than one immunoreactive protein to cover a broader spectrum of protein-antibody interactions.

During this work, more than ten different recombinant antigens have been produced and tested. In addition to the antigens described already, several new other immunoreactive proteins were detected by systematic screening of genomic data for potential surface antigens. The antigens omp852, Ompefflux, and LP found by this way turned out to react strongly with serum antibodies from brucellosis patients, thus representing new candidate antigens for ELISA. Optimal sensitivity was obtained by combining several of these antigens in fusion proteins.

4.1 Patient samples

The antisera used in this study were collected in Mongolia. Patients were first screened by RBT and positive results confirmed by SAT assays, taking 1/160 dilution as threshold value for positivity as recommended for endemic areas (Young, 2005; Corbel, 1989). While the use of a higher titre as a positivity threshold decreases the sensitivity of SAT (Orduña et al., 2000), the lower SAT titres do not always exclude a *Brucella* infection (Young, 1991) because some acute brucellosis patients and those with a chronic or prolonged disease evolution can show very low titres of specific antibodies. In latter two cases the use of Coombs test and Brucellacapt assays proved to be appropriate leading to positive results. (Orduña et al., 2000). Thus, the SAT assay is prone not only to the false positive results, but also to false negative results, although the percentage of such cases is not highly significant (Mantecón et al., 2006). The criteria for true positive serum samples used in this study were positive results with RBT, SAT and genus-specific PCR along with clinical manifestation.

4.2 Recombinant antigens

To date, complete genome sequences have been established for eight different *Brucella* strains. These sequences can be taken from the internet from the collection of complete bacterial chromosomes in the NCBI GenBank (http://www.ncbi.nlm.nih. gov/genbank). They include two strains of *B. melitensis* (*B. melitensis* 16M and *B. melitensis* biovar abortus 2308), two strains of *B. abortus* (*B. abortus* biovar 1 strain 9-941 and *B. abortus* S19), two strains of *B. suis* (*B. suis* ATCC 23445 and *B. suis* 1330*)* and the strains *B. ovis* ATCC 25840 and *B. canis* ATCC 23365*.* Most of these sequences have been finished only recently. For this work, mainly the sequences of *B. melitensis* biovar abortus 2308 and *B. abortus* biovar 1 strain 9-941 have been used to select candidates for recombinant test antigens.

Vector pQE-30 was used as an efficient expression system in *E. coli*. It allows to induce protein expression upon induction with IPTG, warranting by this way the production of large amounts of protein without interference with the viability of the bacterium. In addition, it provides a histidine affinity tag to the N-terminus of the recombinant proteins allowing simple purification by affinity chromatography. All recombinant proteins, except LP were expressed successfully. Since the low expression rate of LP was first assumed to be due to the presence of many "rare" *E. coli* codons, a synthetic gene was constructed avoiding these codons. However, the synthetic gene led to the same weak protein expression. Thus, the reason is rather a certain toxicity of the LP protein for *E. coli.*

In standard protein purifications, approximately 1 to 2 milligrams of protein were obtained from one litre of culture. All single antigens as well as fusion antigens were successfully expressed and purified and all stages of the process were easily reproducible. The purified protein contained in average 1 mg per ml. Optimal dilutions of antigens for ELISA were determined empirically before testing them on a large number of samples.

Protein purification using His-tag affinity chromatography results in relatively pure antigens. However, it was not to be expected that the purified proteins would fold in native conformation. This depends mainly on the extreme over-expression leaving little time and/or space to gain the native three-dimensional structure. And this was also due to the denaturing conditions of the highly concentrated urea solution. The lack of native conformation to certain extent affects the reactivity with antibodies, and means to circumvent this problem are still to be studied. Recombinant proteins owing low antigenicity have to be used in relatively high concentrations, however the specificity of the assays could be also affected by parallel increase of residual bacterial lipopolysaccharide content which cannot be completely removed during simple affinity chromatography. This is an important issue in view of fact that the healthy

individuals have antibodies against lipopolysaccharide and other carbohydrate structures of many bacterial species colonizing the intestinal tract.

It remains to be emphasized that cloning and expression of recombinant diagnostic antigens is feasible with reasonable effort and can lead to improvement of existing diagnostic systems. Inadequate diagnosis exists for many infectious diseases which are listed under the category "neglected diseases" according to the nomenclature of WHO. Except for the newly emerging zoonoses such as SARS and highly pathogenic avian influenza H5N1, the vast majority are not prioritized by health systems at national and international levels and are therefore labelled as neglected. The availability of the complete genomic sequences of most of these pathogens combined with useful computer programs for handling this large amount of structural information makes new diagnostic approaches based on recombinant antigens feasible not only in developed countries, but also under less privileged conditions of developing ones. The production and running costs of such assays are relatively low, and basic laboratory equipment such as centrifuges and ELISA readers are the sole investment, and which can be obtained also as a donation from a sponsor. Preparing missing specific diagnostic tests by this approach, many widespread diseases could be better recognized and controlled in the near future.

4.3 Specificity

Using recombinant proteins in immunoassays warrants relatively high specificity in general. High sequence homology of some of the cloned proteins has also been observed with two related bacteria of the class *Alphaproteobacteria*, *O. anthropi and R. tropici.* Rare cases of cross-reaction of *Brucella*-specific recombinant antigens with *Orchobactrum anthropi* have been described (Velasco et al., 1997). However, since very few human cases of infection by these bacteria were reported and they normally affect only individuals with immune deficiency leading to nosocomial infections (Ezzedine et al., 1994), this homology appears not to be relevant to affect diagnosis. Other taxonomically unrelated pathogens, such as *Y. enterocoltitica* 0:9 and *E. coli* O157:H7, also cause cross-reactions, however, only in case of using lipopolysacchaide or whole bacteria as antigens. A very weak background reactivity observed in our assays - only when high concentrations of recombinant antigen had to be used - derives mainly from O-polysaccaride side chains of *E. coli* LPS which is present in low concentration in the antigen preparation (Wakelin et al., 2006). This problem can be circumvented either by using more diluted antigens, or by additional purification steps removing the residual (mainly LPS) contamination from the preparation (Colangeli et al., 1998). The latter step makes the preparation of the assays more demanding and expensive, thus the better way is to search for alternative more immunoreactive antigens.

The recombinant antigens LP and the P15-bp26-P39 triple fusion were tested in ELISA with sera of German blood donors and RBT as well as SAT-negative sera from Mongolian individuals. No false positive results were obtained in these assays. The antigens were also tested against the sera from the Mongolian patients with diagnosed bacterial pneumonia (*H. influenzae, M. pneumoniae, S pneumoniae, N. meningitidis*) and syphilis (*T. pallidum).* Few cases amongst this group of individuals were found to be positive not only by the recombinant antigen ELISA but also by SAT assays. This can be interpreted as suspected *Brucella* infection cases which need further confirmation by PCR. In brucellosis endemic areas such as Mongolia, due to its non-specific clinical manifestation *Brucella* infections can be overlooked on the background of another disease with either more severe or/and intense symptoms.

4.4 Antibodies during the course of *Brucella* infection

Recent studies on reactivity of cytosolic proteins (Mantecón et al., 2006) have shown differences in the evolution of the specific antibodies. A gradual decrease of IgM and IgA was observed, while IgG levels became elevated and persisted through 10 months of infection. Another study (Kwaasi et al., 2005) analysed the reactivity of different fractions of proteins with these antibodies during a course of infection. It is believed that the presence of the IgM generally indicates a recent infection, and it acts mainly against the LPS and the bacterial surface structures. IgG as an indicator of a past infection, also can show high titres in individuals in endemic areas. To our experience, both IgM and IgG antibodies display more or less similar behaviour independent of a phase (acute / chronic) of the disease, however, presence of higher levels of IgM was observed in majority of cases of recent infection (data not shown). It should be mentioned that complete history, i.e. determination of phase of the disease was not done for all patients. Our experiments also indicated that immunoassays with recombinant antigens using either only anti-IgM or anti-IgG antibody-conjugates in tests for human brucellosis could cause false negative results suggesting the use of both of them in immunological tests. Thus, the detection and a subsequent determination of the antibody profile on per case basis could be of clinical importance.

4.5 ELISA

Compared to alternative immunoassays, ELISA is a most versatile method, and results being available within in short time. It also has the advantage of being readily automated, thus enabling its use as a screening test with the results numerically quantifiable.

Weak reactivity of antigens such as Ompefflux and omp852 in ELISA can be explained by their small size and consequently, by weak binding to the plastic surface of microtiterplates.

One of the ways to overcome it is to increase the size of protein either through fusion with another antigen, or chemical cross linking to a carrier protein. There are many other techniques that increase either the specificity or sensitivity of the ELISA including the use of various enzymes and washing methods. An important source of non-specific background is the use of phosphatase-conjugated second antibodies in ELISA. This non-specific background could be drastically reduced by using peroxidase-conjugated antibodies and including skim milk as a blocking reagent. Background with phosphatase assays easily arises from small contaminations because phosphatases are ubiquitous enzymes occurring in all body fluids including sweat on the finger tips.

Even though performance of ELISA with *Brucella* specific antigens can still be improved, both LP and P15-bp26-P39 triple fusion from our initial experiments seem to have qualities of antigens highly useful for diagnostic purposes. The other new antigens Ompefflux and Omp852 need to be improved further and are considered to express in full length (Ompefflux) or to be included in fusion protein designs (Omp852) in future studies. In any case, it is apparent that the new ELISA tests can serve as an extension of the generally used SAT assay in Mongolia, or even as a the only single method to diagnose *Brucella* infections.

4.6 PCR

Since serologically positive responses can occur in convalescent hosts or may arise from antigenically cross-reactive bacteria, characterization of cultured bacteria is suggested to be the “gold standard” for diagnosis of *Brucella* infections at present. However, beside the low yield due to fastidious nature of the organism, identification by culturing has many drawbacks such as time needed to culture, the bio-safety requirements, training of the personnel for complex procedures and the limited number of well-defined *Brucella* subtypes. PCR-based methods as a diagnostic tool for brucellosis have been proven to be faster and more sensitive than traditional methods. So far, the acceptance of molecular diagnostics has been slow, especially in developing countries. One of the purposes of this work was to develop a PCR assay that has the advantage of being rapid, specific, sensitive and inexpensive as compared to the currently practiced methods in Mongolia.

4.7 Specimens and DNA extraction

We have found that buffy coat isolated from minute amounts of blood is a good source for *Brucella*-specific DNA for PCR. The simple method for leukocyte isolation developed in this work appears to be the preferable method in clinical laboratory practice. Neither the extraction of DNA from whole blood nor the extraction of DNA from serum results in better yields.

Moreover, very little DNA was found in serum and corresponding few positive results were obtained with this DNA, which, according to some authors, is recommended specimen for the PCR assays (Zerva, 2001). Although the leukocytes are the target and the main place of localization of *Brucella* upon infection, their presence in plasma, especially in the acute phase of brucellosis, can not be ruled out. Whole blood specimen would be better, in principle, because they contain all possible forms of the bacteria, but they are not the optimal source of DNA. It is very demanding to extract pure DNA from the large amount of tissue represented by the erythrocytes. 2 ml volumes of coagulation-inhibited blood turned out to be ideal because it can be used in parallel for both, immunological tests as well as for harvesting of leukocytes for DNA extraction. The quantity of leukocytes obtained from this volume of blood was sufficient to detect *Brucella* DNA with high sensitivity, while lower volumes of 0-5-1.0 ml recommended by some authors (Queipo-Ortuño et al., 1997) led to reduced performance of PCR.

In order to efficiently separate leukocytes it was tried to use a 2.5 % glutaraldehyde solution to clot and isolate the yellow layer of leukocytes and platelets (buffy coat) from the top of the sedimented erythrocytes. The method proved to be effective, but because it is a time-consuming procedure it was substituted by a method including red blood cell lysis buffer as described in the Materials and Methods section. In cases of focal complications such as neurobrucellosis, the specimen for the PCR assays can be cerebrospinal fluid, synovial fluid and purulent material as well. Both the leukocyte pellets and the whole blood specimens can be stored for long time at -20° C. An alternative method includes guanidinium hydrochloride to extract and conserve the DNA at the same time. In this case, the samples can be stored at 4°C. DNA extracted from our specimens was stable at -20° C for at least three years when dissolved in a neutral buffer.

The method for extraction and isloation of DNA used in this work combines leukocyte isolation procedure from whole blood by RBC lysis buffer (Kawasaki, 1990) and a newly developed guanidine-silica extraction protocol. This method turned out to be fast, inexpensive, and safer as compared to phenol-chloroform extraction, which employs hazardous organic solvents. Extraction can be carried out in a single tube, which is important in terms of avoiding cross-contamination, and can also be applied for the routine clinical diagnosis. Pre-treatment of samples by proteinase K as recommended by many authors, did not influence the DNA yield, but could be useful for extractions from other tissues, e.g. biopsy materials in focal complications of brucellosis.

It is important to avoid contaminations with potential PCR-inhibitory substances, such as hemin and its derivatives. Other compounds such as the EDTA and heparin do not influence the PCR efficiency by applying the guanidinium-silica purification procedure. Thus, the various

washing steps are essential: the first wash with erythrocyte lysis solution to remove most of the hemoglobin derivatives and various other washings thereafter. On the other hand, the diagnostic PCR signal can be inhibited if the amount of DNA exceeds a certain threshold value (Cogswell et al., 1996). Accordingly, this work included evaluation of the effects of washing and empirical study of acceptable concentrations of the total DNA (bacterial plus host leukocytic) to obtain an optimal yield and to ensure the reliability of PCR signals for the diagnosis of *Brucella* spp. in human peripheral-blood samples.

4.8 Primers. Design, PCR conditions, reaction mix, ready-to-use mixes

Primers for PCR diagnosis were selected using DNA sequences available from Gene Bank Database and other resources. All computer programs needed for DNA manipulation were freely obtained from the internet. In order to increase specificity as well as sensitivity, nested primers were designed for each specific target sequence. The annealing temperatures were chosen universally at 60°C for convenience. The genus-specific primers were developed and tested first . Two sets of nested primers, first specific for 16SrRNA gene (Herman et al., 1992; Cetinkaya et al., 1999; Romero et al., 1995) and another for pcaC 4-carboximuconolactone decarboxylase gene (PCA) (Ratushna et al., 2006) were designed and successfully tested. Although primers specific for 16SrRNA gene had a slight sequence homology to that of *O. anthropi,* this fact was judged insignificant because this bacterium rarely causes disease in humans and the homology was not of a high degree. This set of four primers proved to be both sensitive and specific. The assays with these sets of primers were designed to result in different band sizes (247 bp for PCA and 606 bp for 16SrRNA primers), which is important in case of combining these primers in multiplex PCR assays.

Species-specific primers were designed using a different approach. First, we designed set of primers where the reverse IS*711* primer is located at 3 end of the IS*711* element, whereas the reverse primers are derived from species-specific loci from *B. abortus*, *B. melitensis* and *B. suis*. This multiplex approach has the advantage of enabling a simultaneous detection of several species, but establishing was more demanding than with normal PCR assay. It was required to adjust the concentrations for all three sets of primers very precisely to obtain reliable results for all species in a single reaction. Analogous approaches were described by several authors for *B. suis* (Bricker et al., 1994; Redkar et al., 2001). The published multiplex PCR does not detect all biovars of *B. suis*, covering only the biovar 1. By this reason, other sets of primers were designed in this work.

To detect all potential *Brucella* species occurring in Mongolia, species-specific primers were designed that amplified only one specific region of each species at a time and resulted in a

particular size of the amplicon for each species. Species-specific primer sequences were selected targeting the following genes and regions: outer membrane transporter for *B. abortus*, transcriptional regulator, GNTR family gene encoding sequence for *B. melitensis*, and HlyD family secretion protein for *B. suis* (Ratushna et al., 2006). We attempted to combine these assays into a single multiplex reaction, but it turned out to be not feasible to adjust the concentration of all primers in a way that high sensitivity for all three species was warranted. The primers were therefore used in single species-specific PCR reactions.

Thirdly, based on a size-specific polymorphism of immunoglobulin-binding protein EIBE, we selected primers which amplify different size products in each species, allowing differentiation of the species by the size of the amplified products (Ratushna et al., 2006). Reasonable results were obtained using these primers, but the coverage of all strains occurring worldwide should be questioned and has still to be confirmed for Mongolia, since the primer designs were based on the strains detected in the USA.

In addition to *Brucella*-specific PCR, all samples were screened with *Coxiella burnetii*–specific primers in order to reveal the presence of this pathogen in our samples (not shown in the Results section). The pathogen has the same route of transmission and similar pathogenetic features, necessitating a differential diagnosis. Multiplex PCRs were performed, with the *C. burnetii*- and each genus-specific *Brucella* primer pairs. The16SrRNA primers proved to be useful for the assay, since they had the same annealing temperatures. Amongst 20 patients being negative in *Brucella*-specific PCR but showing typical clinical signs of disease 5 cases of *C. burnetii*-infections were detected by this way. This shows that this disease is an important issue of the health sector of Mongolia in animal-human transmission of zoonoses and the disease caused by this agent needs further study and investigation.

A frequently discussed disadvantage of the nested PCR assays is that they have a certain risk of carry-over contaminations which, however, can be avoided by careful handling. In order to adapt the PCR assays for laboratories with a limited experience or limited facilities for PCR, we have developed a method of preparation of off-the-shelf, pre-optimized, pre-mixed, pre-dispensed PCR reaction mixes using trehalose as a presevative. A reconstitution of the mix by water and the template DNA addition are the only things required. Previous experiments were made with sugars such as betaine and arabinose, but trehalose turned out to be of best for this purpose. According to our experience, this mixture is stable at least for three months at room temperature and helps enormously to prevent any kind of contamination. This method was already described (Klatser et al., 1998; Spiess et al., 2004) for detection of *Mycobacteria* and in the meantime such ready-to-use mixes are already commercially available for several pathogens.

4.9 Brucellosis in Mongolia

Opposite to other published data and studies on epidemiology of brucellosis in Mongolia, results in this work show prevalence of *B. abortus* infection in patients. The apparent prevalence of *B. abortus* infection in Mongolian brucellosis patients, despite sheep and goat being a dominant livestock throughout the country is unexpected. It is in conflict with some statistical data and epidemiological transmission models developed by other researchers (Zinsstag et al., 2005, Ebright et al., 2003), but agrees with data from other countries (Pappaset al., 2006; Reid, 2005), where *B. abortus* is prevalent. It can be explained by change in the epidemiological pattern of brucellosis during the last decades like in some other regions of the world (Fosgate et al., 2002) such in California, due to immigration and altered socioeconomic structure of population.

It is known that the main route of transmission of *B. abortus* from cattle to humans is the consumption of raw milk or its products. Mongolia, where traditional practices prevent this route of transmission (heating, transformation to foodstuff with low pH etc.) has experience of controlling and preventing the disease, however, a switch to the market economy along with reduction in the governmental regulatory policy influenced the re-emergence of the disease. Brucellosis in Mongolia in the last decades is probably widespread among the privately owned animals and the chances of the home-made milk products sold on the markets or bought from neighbours of not being adequately pasteurized are high. The spread of brucellosis in farm animals might be attributable to the privatization of collective (state-owned) farms as a result of the changed political and economic situation in the country. Collective farm animals were distributed to small private farms; families who own these animals might disregard or not be aware of sanitary and health requirements necessary to prevent transmission of brucellosis to humans. A probable consequence of privatization of the animal sector is an increased volume of home-made animal food products with inadequate sanitary control over production (Kozukeev, 2006). Referring to this fact, the increase in brucellosis transmission via this route in Mongolia cannot be ruled out although the cases of infection of cattle by *B. melitensis* seem to be emerging as an important problem in some southern European countries, e.g. in Israel, Kuwait and Saudi Arabia (Corbel, 1997).

Another possibility is that the cases of *B. abortus* infection in Mongolia could be under-reported, since infections with *B. melitensis* are generally considered more likely to be diagnosed than infection with other *Brucella* species due to its greater severity of the disease. Therefore, in most cases patients with only severe manifestation and complications of brucellosis seek medical help. It can be explained by some specific features or factors in Mongolia as the distance to the nearest health facility: Some households are far from hospitals

and poor infrastructure make accessibility to health care difficult. Even in areas where there is transportation, affordability of the costs of transport makes patients unable to present to hospitals in time as well. Due to these factors and conditions the percentage of reported *B. melitensis* infections could be higher than it is in reality.

Studies carried out in the late 70s and 80s in Mongolia on the blood cultures for isolation and differentiation of *Brucella* species have shown the prevalence of *B. melitensis* in human brucellosis. Although blood cultures with further typing are “gold standard” for definite diagnosis of the disease, they have some drawbacks. In acute forms of infection produced by *B. melitensis*, the yield of blood cultures is usually high, reaching 70-80 % of cases (Ariza et al., 1995). However, this figure is notably reduced in infections caused by *B. abortus* and *B. suis,* where the positive results rarely surpasses 30-50 % (Colmenero et al., 1996). These facts should not be ignored and could be a reason of under-diagnosing of the cases of *B*. *abortus* infections.

Finally, this work was carried out with a set of samples collected in a short span of time from ambulatory outpatients coming mainly from Ulaanbaatar, the capital city. It is possible that the majority of them had a common source of infection caused by local outbreak on a milk farm near the city.

Another possibility lays in different vaccination practices of or efficiencies of vaccinations on different kinds of livestock in Mongolia, causing the varied clearance of animals from *Brucella*, e.g. small livestock versus cattle.

5 CONCLUSION

During this work two mayor principles for the diagnosis of Brucellosis, one of the major challenges of health in Mongolia, have been approached. First, a highly specific procedure for general immunodiagnosis was established. A set of protein antigens was selected to develop an indirect ELISA test for serological diagnosis of human brucellosis. The antigens were tested on various serum samples in order to evaluate their specificity and sensitivity in the diagnosis of this disease. Two of the generated antigens, namely the LP and a fusion polyprotein including three immunodominant proteins have proven to be useful. To our experience, the fusion protein antigen had much more enhanced specificity and sensitivity than single antigens. In principle, this fusion antigen is a great step forward to highly specific and sensitive immunodiagnosis leading to far more precise results than the diagnostic procedures applied to date. However including additional reactive antigen candidates could still enhance the quality of the assay.

Attempts were also made to develop fast, specific and sensitive PCR assays for diagnosis of *Brucella* spp. on the genus and species level. The PCR assays proved to be specific and sensitive enough to be a method of choice for definite diagnosis of *Brucella* infections in Mongolia and could be applicable in the routine clinical diagnosis. The genus-specific PCR assays were confirmed to be appropriate in terms of simplicity and sensitivity. The 16SrRNA as well as the PCA primers combined with other primers can be used for diagnosis of other pathogen with similar pathogenesis, e.g. *C. burnetii*. Contaminations can be drastically reduced by preparing ready-to-use mixes, enabling use by the laboratory personnel of limited training. Introduction of the PCR-based assays will be of great importance for comprehensive epidemiological analyses of brucellosis in Mongolia.

6 REFERENCES

Allardet-Servent A, Bourg G, Ramuz M, Pages M, Bellis M, Roizes G. 1988. DNA polymorphism in strains of the genus *Brucella*. J Bacteriol. 170(10):4603-7.

Alton GG, Jones LM, Angus RD, Verger JM. 1988. Techniques for the brucellosis laboratory. Institut National De La Recherche Agronomique, Paris.

Araj GF, Kaufmann AF. 1989. Determination by enzyme-linked immunosorbent assay of immunoglobulin G (IgG), IgM, and IgA to *Brucella melitensis* major outer membrane proteins and whole-cell heat-killed antigens in sera of patients with brucellosis. J Clin Microbiol. 27(8):1909-12.

Ariza J, Pellicer T, Pallares R, Foz A, Gudiol F. 1992. Specific antibody profile in human brucellosis. Clin Infect Dis. 14(1):131-40.

Ariza J, Corredoira J, Pallares R, Viladrich PF, Rufi G, Pujol M, Gudiol F. 1995. Characteristics of and risk factors for relapse of brucellosis in humans. Clin Infect Dis. 20(5):1241-9.

Badarch U. 2001. Population Knowledge level on Brucellosis. Dissertation. Mongolian Agricultural University, Ulaanbaatar, Mongolia.

Baldandorj T. 1972. Epidemiology and Prevention of Brucellosis in Mongolia. Dissertation. Mongolian Agricultural University, Ulaanbaatar, Mongolia.

Baldi PC, Giambartolomei GH, Goldbaum FA, Abdon LF, Velikovsky CA, Kittelberger R, Fossati CA. 1996. Humoral immune response against lipopolysaccharide and cytoplasmic proteins of *Brucella abortus* in cattle vaccinated with *B. abortus S19* or experimentally infected with *Yersinia enterocolitica* serotype 0:9. Clin Diagn Lab Immunol. 3(4):472-6.

Banai M. 2002. Control of small ruminant brucellosis by use of *Brucella melitensis Ref. 1* vaccine: laboratory aspects and field observations. Veterinary Microbiology. 90:497-519.Bercovich Z. 1998. Maintenance of Brucella abortus free herds: A review with emphasis on epidemiology and the problems of diagnosing brucellosis in areas of low prevalence. Veterinary Quarterly. 20(3): 81 - 88.

Bonekamp F, Andersen HD, Christensen T, Jensen KF. 1985. Codon-defined ribosomal pausing in Escherichia coli detected by using the pyrE attenuator to probe the coupling between transcription and translation. Nucleic Acids Res. 13:4113-4123.

Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. 1990. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 28: 495-503.

Boschiroli ML, Foulongne V, O'Callaghan D. 2001. Brucellosis: a worldwide zoonosis. Current Opinion in Microbiology. 4(1): 58 - 64.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 7;72:248-54.

Bricker BJ, Halling SM. 1994. Differentiation of *Brucella abortus bv. 1, 2,* and *4, Brucella melitensis, Brucella ovis*, and *Brucella suis bv. 1* by PCR. J Clin Microbiol. 32(11):2660-6.

Bricker BJ, Ewalt DR, MacMillan AP, Foster G, Brew S. 2000. Molecular characterization of *Brucella* strains isolated from marine mammals. J Clin Microbiol. 38(3):1258-62.

Bricker BJ, Ewalt DR, Olsen SC, Jensen AE. 2003. Evaluation of the *Brucella abortus* species-specific polymerase chain reaction assay, an improved version of the *Brucella* AMOS polymerase chain reaction assay for cattle. J Vet Diagn Invest. 15(4):374-8.

Bricker BJ, Ewalt DR, Halling SM. 2003. *Brucella* HOOF-Prints: strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). BMC Microbiol. 11;3:15.

Cetinkaya B, Ongör H, Muz A, Ertas HB, Kalender H, Erdogan HM. 1999. Detection of *Brucella* species DNA in the stomach content of aborted sheep fetuses by PCR. Vet Rec. 27;144(9):239-40.

Cheers C, Pagram F. 1979 . Macrophage activation during experimental murine brucellosis: a basis for chronic infection. Infect Immun. 23(2):197-205

Cloeckaert A, Verger JM, Grayon M, Grepinet O. 1995. Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. Microbiology. 141 (Pt 9):2111-21.

Cloeckaert A, Verger JM, Grayon M, Grépinet O. 1996. Polymorphism at the dnaK locus of *Brucella* species and identification of a *Brucella melitensis* species-specific marker. J Med Microbiol. 45(3):200-5.

Cloeckaert A, Baucheron S, Vizcaino N, Zygmunt MS. 2001. Use of recombinant BP26 protein in serological diagnosis of *Brucella melitensis* infection in sheep. Clin Diagn Lab Immunol. 8(4):772-5.

Cloeckaert A, Grayon M, Grépinet O, Boumedine KS. 2003. Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site-PCR and development of specific PCR identification tests. Microbes Infect. 5(7):593-602.

Cloeckaert A, Vizcaino N. 2004. DNA polymorphism and taxonomy of *Brucella* species. Chapter 1 in *Brucella* Molecular and Cellular Biology. Horizon Scientific Press, Norfolk, UK.

Cogswell FB, Bantar CE, Hughes TG, Gu Y and Philipp MT. 1996. Host DNA can interfere with detection of *Borrelia burgdorferi* in skin biopsy specimens by PCR. J. Clin. Microbiol. 34:980-982.

Colangeli R, Heijbel A, Williams AM, Manca C, Chan J, Lyashchenko K, Gennaro ML. 1998. Three-step purification of lipopolysaccharide-free, polyhistidine-tagged recombinant antigens of Mycobacterium tuberculosis. J Chromatogr B Biomed Sci Appl. 714(2):223-35.

Colmenero JD, Reguera JM, Martos F, Sanchez-De-Mora D, Delgado M, Causse M, Martin-Farfan A, Juarez C. 1996. Complications associated with *Brucella melitensis* infection: a study of 530 cases. Medicine (Baltimore). 75(4):195-211. Erratum in: Medicine (Baltimore) 1997. 76(2):139.

Contreras-Rodriguez A, Ramirez-Zavala B, Contreras A, Schurig GG, Sriranganathan N, Lopez-Merino A. 2003. Purification and characterization of an immunogenic aminopeptidase of *Brucella melitensis*. Infect Immun. 71(9):5238-44.

Corbel MJ. 1989. Microbiology of genus *Brucella*. Pp 53-72 in Brucellosis: Clinical and laboratory aspects., edited by Young EJ and Corbel MJ. Boca raton, Florida: CRC Press, Inc.

Corbel MJ. 1997. Brucellosis: an overview. Emerging infectious diseases. 3: 313-321.

Corbel M, Cosivi O, Elberg S. 2000. Guiedelines for the management of human brucellosis or Guidelines for the Diagnosis, Treatment and Prevention of Brucellosis in Humans and Animals. Ref type: Unpublished Work.

Cvjetanovic V, Batmonk B. 1968. Assignment report on Brucellosis in farm animals strengthening of health services (Epidemiology) (WHO Project: Mongolia 0001). SEA/EPID/20, SEA/VPH/3, SEA- 68/1049, 1-19. Regional office for South-East Asia, World Health Organization. WHO Project : Mongolia 0001. Ref Type: Report

Damdinsuren L. 1972. Several clinical problem and treatment of Brucellosis in Mongolia. Dissertation, Mongolian Agricultural University, Ulaanbaatar, Mongolia.

Dashdavaa J. 1969. Clinical and Epidemiological Character of Brucellosis in Mongolia. Dissertation, Medical Research Institute, Ministry of Health of Mongolia; Scientific Research Institute of Pathogen, Ministry of Health of Kazakhstan, Soviet Union; 1969a.

Dashdavaa J. 1969. Clinical and Epidemiological Character of Brucellosis in Mongolia. Dissertation. Medical Research Institute, Ministry of Health of Mongolia; Scientific Research Institute of Pathogen, Ministry of Health of Kazakhstan, Soviet Union; 1969b.

Dashdavaa J, Tobuugiin G. 1981. Scientific Conference for 20-th Anniversary of the establishment of Organisations against Brucellosis. Ulaanbaatar, Mongolia: Ministry of Health, State Brucellosis Dispensary, Medical Research Institute, Infection - Epidemiological Sector.

Denes B. 1997. Serological findings obtained in cattle herds immunised with the *Brucella melitensis Rev.1* and the *B. abortus B19* vaccine in Mongolia. Acta Veterinaria Hungarica. 45: 33-43.

Enkhbaatar L, Dondog N, Tsetsegmaa J. 2004. Brucellosis. Ulaanbaatar: Infectious Research National Centre.

Erdenchimeg G, Bandi M. 2001. National surveillance strategy of human Brucellosis. June 4; Ulaanbaatar; 2001.

Ewalt DR, Bricker BJ. 2003. Identification and differentiation of *Brucella abortus* field and vaccine strains by BaSS-PCR. Methods Mol Biol. 216:97-108.

Ezzedine H, Mourad M, Van Ossel C, Logghe C, Squifflet JP, Renault F, Wauters G, Gigi J, Wilmotte L, Haxhe JJ. 1994. Ezzedine H, Mourad M, Van Ossel C, Logghe C, Squifflet JP, Renault F, Wauters G, Gigi J, Wilmotte L, Haxhe JJ. An outbreak of Ochrobactrum anthropi bacteraemia in five organ transplant patients. J Hosp Infect. 27(1):35-42.

Fekete A, Bantle JA, Halling SM, Sanborn MR. 1990. Preliminary development of a diagnostic test for *Brucella* using polymerase chain reaction. J Appl Bacteriol. 69(2):216-27.

Ficht TA, Bearden SW, Sowa BA, Marquis H. 1990. Genetic variation at the omp2 porin locus of the *Brucellae*: species-specific markers. Mol Microbiol. 4(7):1135-42.

1st International Conference on Emerging Zoonoses. 1997. Brucellosis: an Overview. Emerging Infectious Diseases. 3(2):213-20.

Food and Agriculture Organisation of the United Nations Rome, Italy. 9.10.92a. Expert Consultation on Strategies in Diagnosis and Control of Brucellosis in Asia. 1-25. Beijing, China, Food and Agriculture Organisation of the United Nations Rome, Italy. Ref Type: Report.

Fosgate GT, Carpenter TE, Chomel BE, Case JT, DeBess EE, and Reilly KF. 2002. Time-Space Clustering of Human Brucellosis, California, 1973-1992. Emerging Infectious Diseases.

Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckaert A. 2007. Brucella ceti sp. nov. and Brucella pinnipedialis sp. nov. for Brucella strains with cetaceans and seals as their preferred hosts. Int J Syst Evol Microbiol. 57: 2688-93.

Frey A, Meckelein B, Externest D, Schmidt MA. 2000. A stable and highly sensitive 3,3',5,5'-tetramethylbenzidine-based substrate reagent for enzyme-linked immunosorbent assays. J Immunol Methods. 13;233(1-2):47-56.

Gillings M, Holley M. 1997. Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. Lett Appl Microbiol. 25(1):17-21.

Government of Mongolia, World Health Organization. 1999. Mongolia Health Sector Review. Ulaanbaatar: World Health Organizatino. p.52.

Grantham R, Gautier C, Gouy M, Jacobzone M, Mercier R. 1981. Codon catalog usage is a genome strategy modulated for gene expressivity. Nucleic Acids Res. 9: r43-74.

Halling SM, Gibas C, Boyle S. Comparative genomics of *Brucella melitensis, B. suis*, and *B.abortus.* 2004. Chapter 5 in *Brucella* Molecular and Cellular Biology. Horizon Scientific Press, Norfolk, UK.

Herman L, De Ridder H. 1992. Identification of Brucella spp. by using the polymerase chain reaction. Appl Environ Microbiol. 58(6):2099-101.

Hertz-Fowler C, Peacock CS, Wood V, Aslett M, Kerhornou A, Mooney P, Tivey A, Berriman M, Hall N, Rutherford K, Parkhill J, Ivens AC, Rajandream MA, Barrell B. 2004. GeneDB: a resource for prokaryotic and eukaryotic organisms. Nucleic Acids Res. 32.

Hochuli E, Bannwarth W, Döbeli H, Gentz R, Stüber D. 1988. Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent. Bio/Technology. 6: 1321 – 1325.

Iriarte M, Gonzalez D, Delrue RM, Monreal D, Conde R, Lopez-Goni I, Letesson JJ, Moriyon I. 2004. *Brucella* Lipopolysaccharide: Structure, Biosynthesis and Genetics. Chapter 7 in *Brucella* Molecular and Cellular Biology. Horizon Scientific Press, Norfolk, UK.

Ish-Horowicz D, Burke JF. Rapid and efficient cosmid cloning. 1981. Nucleic Acids Res. 10;9(13):2989-98.

Kawasaki ES. 1990. Sample preparation from blood, cells and other fluids. Pp. 146–152 in PCR Protocols: a Guide to Methods and Applications, 1st edn., edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press.

Klatser PR, Kuijper S, van Ingen CW, Kolk AH. 1998. Stabilized, freeze-dried PCR mix for detection of mycobacteria. J Clin Microbiol. 36(6):1798-800.

Klinkert MQ, Bommert K, Moser D, Felleisen R, Link G, Doumbo O, Beck E. 1991. Immunological analysis of cloned *Schistosoma mansoni* antigens Sm31 and Sm32 with sera of schistosomiasis patients. Trop Med Parasitol. 42(4):319-24.Ko J, Splitter GA. 2003. Molecular Host-Pathogen Interaction in Brucellosis: Current Understanding and Future Approaches to Vaccine Development for Mice and Humans. Clinical Microbiology Reviews. 16(1): 65-78.

Kolar J. 1970. Assignment report on strengthening of health services (epidemiology) (WHO project: Mongolia 0001) October 1965 - June 1969. SEA-70/2572. World Health Organization. SEA/VPH/8 Rev.1*, SEA/EPID/32 Rev.1. Ref Type: Report

Kolar J. 1977. *Brucella* vaccines production in Mongolia. World Health Organization, Assignment Report on WHO Project MOG BLG 001, SEA/Vaccine/89, 40 pp.

Kozukeev TB, Ajeilat S, Maes E, Favorov M 2006. Risk factors for brucellosis--Leylek and Kadamjay districts, Batken Oblast, Kyrgyzstan, January-November, 2003. Centers for Disease Control and Prevention (CDC). MMWR Morb Mortal Wkly Rep. 28;55 Suppl 1:31-4.

Kwaasi AA, Al-Mohanna FA, Nakeeb SM, Roberts GT, Al-Thawadi S, Hassan AY, Al-Hokail A 2005. Correlation of antigenic expression with progress in antibiotic therapy of acute human brucellosis. J Med Microbiol. 54(Pt 6):533-8.

Leal-Klevezas DS, López-Merino A, Martínez-Soriano JP. 1995. Molecular detection of *Brucella spp*.: rapid identification of *B. abortus biovar I* using PCR. Arch Med Res. 26(3):263.

Letesson JJ, Tibor A, van Eynde G, Wansard V, Weynants V, Denoel P, Saman E 1997. Humoral immune responses of *Brucella*-infected cattle, sheep, and goats to eight purified recombinant *Brucella* proteins in an indirect enzyme-linked immunosorbent assay. Clin Diagn Lab Immunol. 4(5):556-64.

Lucero NE, Escobar GI, Ayala SM, Jacob N. 2005. Diagnosis of human brucellosis caused by *Brucella canis*. J Med Microbiol. 54(Pt 5):457-61.

Mantecon MA, Gutierrez P, del Pilar Zarzosa M, Duenas AI, Solera J, Fernandez-Lago L, Vizcaino N, Almaraz A, Bratos MA, Rodriguez Torres A, Orduna-Domingo A. 2006. Utility of an immunocapture-agglutination test and an enzyme-linked immunosorbent assay test against cytosolic proteins from *Brucella melitensis B115* in the diagnosis and follow-up of human acute brucellosis. Diagn Microbiol Infect Dis. 55(1):27-35. Epub 2006 Feb 23.

McDonald R, Cao T, Borschel R. 2001. Multiplexing for the detection of multiple biowarfare agents shows promise in the field. Mil Med. 166(3):237-9.

Mongolian Statistical Yearbook. 2007. P.78 in National statistical office of Mongolia.

Morgan B, Corbel WJ and MJ. 1990. *Brucella* infections in man and animals: contagious equine metritis. Pp. 547–570 in Topley and Wilson's principles of bacteriology, virology and immunology, 8th ed., edited by Parker MT and Collier LM. Edward Arnold, London, England.

Nicoletti P. 1989. Relationship between animal and human brucellosis. Pp. 97–126 in Brucellosis: clinical and laboratory aspects., edited by Young EJ and Corbel MJ. CRC Press, Boca Raton, FL.

Orduna A, Almaraz A, Prado A, Gutierrez MP, Garcia-Pascual A, Duenas A, Cuervo M, Abad R, Hernandez B, Lorenzo B, Bratos MA, Torres AR. 2000. Evaluation of an immunocapture-agglutination test (Brucellacapt) for serodiagnosis of human brucellosis. J Clin Microbiol. 38(11):4000-5.

Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. 2006. The new global map of human brucellosis. The Lancet Infectious Diseases. 6(2): 91 – 99.

Polt SS, Dismukes WE, Flint A, Schaefer J. 1982. Human brucellosis caused by *Brucella canis*: clinical features and immune response. Ann Intern Med. 97(5):717-9.

Queipo-Ortuño MI, Morata P, Ocón P, Manchado P, and Colmenero JD. 1997. Rapid diagnosis of human brucellosis by peripheral blood PCR assay. J. Clin. Microbiol. 35:2927-2930.

Ratushna VG, Sturgill DM, Ramamoorthy S, Reichow SA, He Y, Lathigra R, Sriranganathan N, Halling SM, Boyle SM and Gibas CJ. 2006. Molecular targets for rapid identification of *Brucella spp*. BMC Microbiol 6: 13.

Redkar R, Rose S, Bricker B, DelVecchio V. 2001. Real-time detection of *Brucella abortus*, *Brucella melitensis* and *Brucella suis*. Mol Cell Probes. 15(1):43-52.

Reid AJ. 2005. Brucellosis--a persistent occupational hazard in Ireland. Int J Occup Environ Health. 11(3):302-4.

Riley LK and Robertson DC. 1984. Ingestion and intracellular survival of *Brucella abortus* in human and bovine polymorphonuclear leukocytes. Infect Immun. 46(1): 224-230.

Rijpens NP, Jannes G, Van Asbroeck M, Rossau R, Herman LM. 1996. Direct detection of *Brucella spp*. in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. Appl Environ Microbiol. 62(5):1683-8.

Robinson A., 2003. Guiedelines for coordinated human and animal brucellosis surveillance. 1-46. Rome, FAO. FAO animal production and health paper. FAO. Ref Type: Serial (Book, monograph).

Robinson HL."Nucleic acid vaccines: an overview. 1997. Vaccine. 15: 785-787.

Romero C, Gamazo C, Pardo M, Lopez-Goni I. 1995. Specific detection of *Brucella* DNA by PCR. J Clin Microbiol. 33(3):615-7.

Robinson M, Lilley R, Little S, Emtage JS, Yarranton G, Stephens P, Millican A, Eaton M, Humphreys G. 1984. Codon usage can affect efficiency of translation of genes in Escherichia coli. Nucleic Acids Res. 12:6663-6671.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning a laboratory manual., vol. 1, edited by C. S. Harbor: Cold Spring Harbor laboratory Press.

Scholz HC, Hubalek Z, Sedlácek I, Vergnaud G, Tomaso H, Al Dahouk S, Melzer F, Kämpfer P, Neubauer H, Cloeckaert A, Maquart M, Zygmunt MS, Whatmore AM, Falsen E, Bahn P, Göllner C, Pfeffer M, Huber B, Busse HJ, Nöckler K. 2008. Brucella microti sp. nov., isolated from the common vole Microtus arvalis. Int J Syst Evol Microbiol. 58 Pt 2):375-82.

Schurig G G, Sriranganathan N., Corbel MJ. 2002. Brucellosis vaccines: past present and future. Veterinary Microbiology. 90:479-96.

Serpe L, Gallo P, Fidanza N, Scaramuzzo A, Fenizia D. 1999. Single-step method for rapid detection of *Brucella spp*. in soft cheese by gene-specific polymerase chain reaction. J Dairy Res. 66(2):313-7.

Smits HL, Basahi MA, Diaz R, Marrodan T, Douglas JT, Rocha A, Veerman J, Zheludkov MM, Witte OW, de Jong J, Gussenhoven GC, Goris MG, van Der Hoorn MA. 1999. Development and evaluation of a rapid dipstick assay for serodiagnosis of acute human brucellosis. Journal of Clinical Microbiology. 37(12): 4179 - 4182.

Spiess AN, Mueller N, Ivell R. 2004. Trehalose is a potent PCR enhancer: lowering of DNA melting temperature and thermal stabilization of taq polymerase by the disaccharide trehalose. Clin Chem. 50(7):1256-9.

Tcherneva E, Rijpens N, Jersek B, Herman LM. 2000. Differentiation of *Brucella* species by random amplified polymorphic DNA analysis. J Appl Microbiol. 88(1):69-80.

Tovuugiin G. 1982. Epidemiological Situation of Brucellosis in Mongolia and its Issues on Prevention. Dissertation, Department of Epidemiology and Infectious Diseases, Medical Research Institute, Ministry of Health, Mongolia and Sate Dispanseriat of Infectious Diseases.

Troy SB, Rickman LS, Davis CE. 2005. Brucellosis in San Diego: epidemiology and species-related differences in acute clinical presentations. Medicine (Baltimore). 84(3):174-87.

Tserendash C. 1972. Animal Brucellosis in Mongolia. Dissertation, Experimantal veterinary institute of the all-union; Academy of agricultural science named by the all-union.

Velasco J, Diaz R, Grillo MJ, Barberan M, Marin C, Blasco JM, Moriyon I. 1997. Antibody and delayed-type hypersensitivity responses to *Ochrobactrum anthropi* cytosolic and outer membrane antigens in infections by smooth and rough *Brucella spp.* Clin Diagn Lab Immunol. 4(3):279-84.

Verger J-M, Grimont F, Grayson M. 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Sys. Bacteriol. 35: 292-295.

Vizcaíno N, Verger JM, Grayon M, Zygmunt MS, Cloeckaert A. 1997. DNA polymorphism at the omp-31 locus of *Brucella spp*.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. Microbiology. 143 (Pt 9):2913-21.

Vogelstein B, Gillespie D. 1979. Preparative and analytical purification of DNA from agarose. Proc Natl Acad Sci U S A. 76(2):615-9.

Wakelin SJ, Sabroe I, Gregory CD, Poxton IR, Forsythe JL, Garden OJ, Howie SE. 2006. "Dirty little secrets"--endotoxin contamination of recombinant proteins. Immunol Lett. 106(1):1-7

Weynants V, Tibor A, Denoel PA, Saegerman C, Godfroid J, Thiange P, Letesson JJ. 1996. Infection of cattle with *Yersinia enterocolitica O:9* a cause of the false positive serological reactions in bovine brucellosis diagnostic tests. Vet Microbiol. 48(1-2):101-12.

Xiang Z, Zheng W, He Y. 2006. BBP: *Brucella* genome annotation with literature mining and curation. BMC Bioinformatics. 16;7:347.

Yagupsky P. 1999. Detection of *Brucellae* in blood cultures. J Clin Microbiol. 37(11):3437-42.

Young EJ. 1991. Serologic diagnosis of human brucellosis: analysis of 214 cases by agglutination tests and review of the literature. Rev Infect Dis. 13(3):359-72.

Young EJ. 2005. *Brucella* species. Pp 2669-2674 in Mandell, Douglas, and Bennett's principles and practice of infectious diseases., edited by Mandell GL, Bennett JE, Dolin R. Philadelphia: Chirchill Livingstone.

Zang GQ, Hotta A, Mizutani M, Ho T, Yamaguchi T, Fukushi H, Hirai K 1998. Direct identification of *Coxiella burnetii* plasmids in human sera by nested PCR. J Clin Microbiol 36: 2210-2213.

Zerva L, Bourantas K, Mitka S, Kansouzidou A, Legakis NJ. 2001. Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. J Clin Microbiol. 39(4):1661-4.

7 APPENDIX

DNA, primers, and protein sequences of the recombinant antigens

Recombinant proteins cloned and expressed in *E.coli* :

B.abortus bacterioferritin, **P15**

B.abortus 17-kDa protein, **P17**

B.abortus 39-kDa cytoplasmic protein, **P39**

B.abortus 26 kDa periplasmic protein, **bp26**

B.melitensis Omp31outer membrane protein, **omp31**

B.abortus Omp25 outer membrane protein, **omp25**

B.melitensis 16M membrane alanine aminopeptidase, **AminopepN**

B.abortus outer membrane lipoprotein-related protein, **LP**

B. abortus hypothetical omp62289852 , **Baomp852**

B.abortus outer membrane efflux protein, **Baompeff**

P15-bp26 fusion

P15-P39 fusion

Bp26-Baomp852 fusion

Bp26-P39 fusion

P15-bp26-P39 fusion

P15

DNA sequence (gi|29423750|gb|AY229988.1| *Brucella melitensis biovar Abortus* bacterioferritin (bfr) gene):

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  1 cgcggccgtt gctgtggctg cttcccaaat gtcgtagaaa ccatcataag ggtgactgaa
 61 gaatactatc ttcgtcgcaa ccaaatggac gaaaacatta tccagttcat ggatcgtgtg
121 cgttctctac gagataaatt cgggagttca tggaatgaaa ggcgaaccaa aggtcatcga
181 gcggcttaac gaggcactgt ttcttgagct cggtgcggta aaccagtatt ggctgcacta
241 ccgtcttctc aacgattggg gttacacgcg ccttgcaaag aaggaacgcg aggaatccat
301 cgaggaaatg catcacgccg acaagctgat tgatcgcatt atcttccttg aaggctttcc
361 gaacctccag accgtttcgc cgttgcgcat tggccagaat gtgaaggaag ttctcgaagc
421 tgacctcaag ggtgaatatg acgctcgcgc ttcgtataag gaatcgcgcg aaatctgcga
481 caagctcggc gactatgtgt cgaagcagct tttcgacgaa cttctggccg atgaagaagg
541 ccatatcgac ttccttgaaa cccagcttga ccttctcgcc aagatcggcg gagaacgcta
601 tggccagctt aacgcggcgc ccgccgacga agctgagtaa gcctgtttca atctgtcttg
661 aaagccgggg cgcatcgctc cggcttttc tttgg
```

Primers:
BAp15-for: ATAGGATCCatgaaaggcgaaccaaaggt(BamHI)
BAp15-rev: TATAAGCTTtaagctggccatagcgttct(HindIII)

Sequence size: 457 bp.
Protein sequence including His-tag:

MRGSHHHHHHGSMKGEPKVIERLNEALFLELGAVNQYWLHYRLLNDWGYTRLAKKEREES
IEEMHHADKLIDRIIFLEGFPNLQTVSPLRIGQNVKEVLEADLKGEYDARASYKESREIC
DKLGDYVSKQLFDELLADEEGHIDFLETQLDLLAKIGGERYGQL

Molecular weight of recombinant protein: 19.1 kD.

P17

DNA sequence (gi|599653|emb|Z46864.1|BAORFP17 *B.abortus* gene for open reading frame):

```
   1 gaattccgat cagtgcatag tttccgcgtg ctcgcgcaat ggtgcgcggg cttgttctcg
  61 gggcggggtg aaactcccca ccggcggtat gaaaagcaat tttcaagccc gcgagcgcct
 121 gaaatggaag ccgattcgca tgccatttca gggtcagcag atccggtgag atgccggagc
 181 cgacggttaa agtccggatg gaagagagcg aatgagcgtc acgattgcgc cttccggcgt
 241 cgttcttgcg ttcttttgtg cgccctgatt ctagtttcgt gaggaacct**a tg**aaccaaag
 301 ctgtccgaac aagacatcct ttaaaatcgc attcattcag gcccgctggc acgccgacat
 361 cgttgacgaa gcgcgcaaaa gctttgtcgc cgaactggcc gcaaagacgg gtggcagcgt
 421 cgaggtagag atattcgacg tgccgggtgc atatgaaatt ccccttcacg ccaagacatt
 481 ggccagaacc gggcgctatg cagccatcgt cggtgcggcc ttcgtgatcg acggcggcat
 541 ctatcgtcat gatttcgtgg cgacggccgt tatcaacggc atgatgcagg tgcagcttga
 601 aacggaagtg ccggtgctga gcgtcgtgct gacgccgcac catttccatg aaagcaagga
 661 gcatcacgac ttcttccatg ctcatttcaa ggtgaagggc gtggaagcgg cccatgccgc
 721 cttgcagatc gtgagcgagc gcagccgcat cgccgcgctt gtc**tga**ctaa ccctctataa
 781 tacgcccgca atgggtataa atgtcgaatt c
```

Primers:
BAp17-for: ATAGGATCCcaaagctgtccgaacaagaca(BamHI)
BAp17-rev: ATAGGTACCttgcgggcgtattatagagg (KpnI)

Sequence size: **496 bp**.

Protein sequence, including His-tag:
MRGSHHHHHHGSQSCPNKTSFKIAFIQARWHADIVDEARKSFVAELAAKTGGSVEVEIFDVPGAYEIPLH
AKTLARTGRYAAIVGAAFVIDGGIYRHDFVATAVINGMMQVQLETEVPVLSVVLTPHHFHESKEHHDFFH
AHFKVKGVEAAHAALQIVSERSRIAALV

Molecular weight of recombinant protein: 18.51 kD.

P39

DNA sequence (gi|13936894|gb|AF360361.1|AF360361 *Brucella melitensis biovar Abortus* immunogenic 39-kDa protein(p39)and39UgpA1(39ugpa1)genes):

```
   1 **atg**ggcgcct gttgccaatg cgcaggaaaa gcagaatgtc gaggttctgc actggtgacg
  61 tccggcggcg aagcgtccgc gcttgaagtt ttgaaaaaag atcttgaaag caagggcatt
 121 agctggaccg atatgccggt tgcaggtggc ggcggcacgg aagccatgac cgttttgcgc
 181 gcgcgcgtta ccgcaggcaa tgcgccaacc gcggtgcaga tgctgggttt tgacattcgc
 241 gactgggcgg agcagggcgc actcggcaat ctcgatacgg ttgcttccaa ggaaggctgg
 301 gaaaaggtta ttccggctcc cttgcaggaa tttgccaaat atgacggcca ctggattcgt
 361 gcgcccgtca atattcactc caccaactgg atgtggatca acaaggctgc tctcgacaag
 421 gctggcggca aggagccgac caattgggat gagctgattg cgcttctcga caatttcaag
 481 gcgcagggca ttacgccgat cgcgcatggc ggccagccgt ggcaggatgc aaccattttc
```

```
 541 gatgcggttg ttctttcatt cggcccggat ttctacaaga aggccttcat cgatctcgac
 601 ccggaagcac tgggcagcga taccatgaag caggccttcg accgcatgtc caagcttcgc
 661 acctatgttg atgacaactt ctccggccgt gactggaacc ttgcttcggc catggttatc
 721 gaaggcaagg ccggtgtcca gttcatgggc gactgggcga agggcgagtt cctcaaggcg
 781 ggcaagaagc cgggtgagga tttcgtctgc atgcgttatc cgggcacgca gggtgctgtc
 841 actttcaatt ccgacatgtt cgccatgttc aaggtttcgg aagacaaggt tcccgcacag
 901 cttgaaatgg cttcggcgat tgaaagccct gccttccagt ctgcctttaa tgtggtgaag
 961 gggtcggccc cggcacgcac ggatgtgccc gataccgctt tcgatgcctg tggcaagaag
1021 gccattgccg atgtcaagga agcaaacagc aagggcactc tgcttggctc catggcgcat
1081 ggctatgcca atccggctgc cgtgaagaat gcgatctacg acgtcgtgac ccgccagttc
1141 aacggccagc tttcttcgga agatgccgtc aaggaactcg ttgtggcggt tgaagccgca
1201 aaataa
```

Primers:
BAp39-for:ATAGGATCCgaaagcaagggcattagctg(BamHI)
Bap39-rev: ATAGGTACCcttattttgcggcttcaacc(KpnI)

Sequence size: 1102 bp.

Protein sequence, including His-tag:
MRGSHHHHHHGSESKGISWTDMPVAGGGGTEAMTVLRARVTAGNAPTAVQMLGFDIRDWAEQGALGNLDT
VASKEGWEKVIPAPLQEFAKYDGHWIRAPVNIHSTNWMWINKAALDKAGGKEPTNWDELIALLDNFKAQG
ITPIAHGGQPWQDATIFDAVVLSFGPDFYKKAFIDLDPEALGSDTMKQAFDRMSKLRTYVDDNFSGRDWN
LASAMVIEGKAGVQFMGDWAKGEFLKAGKKPGEDFVCMRYPGTQGAVTFNSDMFAMFKVSEDKVPAQLEM
ASAIESPAFQSAFNVVKGSAPARTDVPDTAFDACGKKAIADVKEANSKGTLLGSMAHGYANPAAVKNAIY
DVVTRQFNGQLSSEDAVKELVVAVEAAK

Molecular weight of recombinant protein: 40.87 kD.

BP26

DNA sequence (gi|32699289|gb|AY166764.1| Brucella melitensis biovar Abortus strain 870 BP26 (bp26) gene):

```
   1 atgaacactc gtgctagcaa tttctcgca gcctcatttt ccacaatcat gctcgtcggc
  61 gctttcagcc tgcccgcttt cgcacaggag aatcagatga cgacgcagcc cgcgcgcatc
 121 gccgtcaccg gggaaggcat gatgacggcc tcgcccgata tggccattct caatctctcg
 181 gtgctacgcc aggcaaagac cgcgcgcgaa gccatgaccg cgaataatga agccatgaca
 241 aaagtgctcg atgccatgaa gaaggccggc atcgaagatc gcgatctcca gacaggcggc
 301 atcaatatcc agccgattta tgtctatcct gacgacaaga acaacctgaa agagcctacc
 361 atcaccggct attctgtatc caccagtctc acggttcgcg tgcgcgaact ggccaatgtt
 421 ggaaaaattt tggatgaatc cgtcacgctc ggtgttaatc agggcggtga tttgaacctg
 481 gtcaatgata atccctccgc cgtgatcaac gaggcgcgca agcgcgcagt ggccaatgcc
 541 attgccaagg cgaagacgct tgccgacgct gcaggcgtgg ggcttggccg tgtggtggaa
 601 atcagtgaac tgagccgccc gcccatgccg atgccaattg cgcgcggaca gttcagaacc
 661 atgctagcag ccgcaccgga caattccgtg ccgattgccg caggcgaaaa cagctataac
 721 gtatcggtca atgtcgtttt tgaaatcaag taa
```

Primers:
BMbp26-for: ATAGGATCCatgaacactcgtgctagcaattt (BamHI)
BMbp26-rev: TATAAGCTTagctgctagcatggttctgaactg(HindIII)

Sequence size: 671 bp.

Protein sequence, including His-tag:

MRGSHHHHHHGSIGSMNTRASNFLAASFSTIMLVGAFSLPAFAQENQMTTQPARIAVTGEGMMTASPDMA
ILNLSVLRQAKTAREAMTANNEAMTKVLDAMKKAGIEDRDLQTGGINIQPIYVYPDDKNNLKEPTITGYS
VSTSLTVRVRELANVGKILDESVTLGVNQGGDLNLVNDNPSAVINEARKRAVANAIAKAKTLADAAGVGL
GRVVEISELSRPPMPMPIARGQFRTMLA

Molecular weight of recombinant protein: 25.42 kD.

Omp31

DNA sequence (gi|17982305|gb|AE009483.1| *Brucella melitensis 16M* chromosome I, section 40 of 195):

```
   1 atgtttagct taaaagggac tgttatgaaa accgcacttc ttgcatccgt cgcaatgttg
  61 ttcacaagct cggctatggc tgccgacatc atcgttgctg aaccggcacc cgttgcagtc
 121 gacacgttct cttggactgg cggctatatt ggtatcaatg ctggttacgc tggcggcaag
 181 ttcaagcatc cgttctcagg catcgagcag gatggggccc aagatttttc aggttcgctc
 241 gacgtcacgg ccagcggctt tgttggcggc gttcaggccg gttataactg gcagcttgcc
 301 aacggcctcg tgcttggtgg cgaagctgac ttccagggct cgacggttaa gagcaagctt
 361 gttgacaacg gtgacctctc cgatatcggc gttgcaggca acctcagcgg cgacgaaagc
 421 ttcggcctcg agaccaaggt tcagtggttt ggaacggtgc gtgcgcgcct cggcttcacc
 481 ccgactgaac gcctgatggt ctatggtacc ggtggtttgg cctatggtaa ggtcaagacg
 541 tcgcttagcg cctatgacga tggtgaatcg ttcagcgccg gaaactccaa gaccaaggct
 601 ggctggacgc ttggtgcagg tgtagaatac gccgtcacca acaattggac cctgaagtcg
 661 gaatacctct acaccgacct cggcaagcgt tccttcaatt acattgatga agaaaacgtc
 721 aatattaaca tggaaaacaa ggtgaacttc cacaccgtcc gcctcggtct gaactacaag
 781 ttc taa
```

Primers:
omp31-for: ATAGGATCCatgtttagcttaaaagggactgttat(BamHI)
omp31-rev: ATACTGCAGttagaacttgtagttcagaccgag (PstI)

Sequence size: 786 bp.

Protein sequence, including His-tag:
MRGSHHHHHHGSMFSLKGTVMKTALLASVAMLFTSSAMAADIIVAEPAPVAVDTFSWTGGYIGINAGYAG
GKFKHPFSGIEQDGAQDFSGSLDVTASGFVGGVQAGYNWQLANGLVLGGEADFQGSTVKSKLVDNGDLSD
IGVAGNLSGDESFVLETKVQWFGTVRARLGFTPTERLMVYGTGGLAYGKVKTSLSAYDDGESFSAGNSKT
KAGWTLGAGVEYAVTNNWTLKSEYLYTDLGKRSFNYIDEENV
NINMENKVNFHTVRLGLNYKF

Molecular weight of recombinant protein: 29.18 kD.

Omp25

DNA sequence (gi|157310308|emb|AM712382.1| Brucella sp.CCM 4916 omp25 gene for outer membrane protein 25):

```
   1 atgcgcactc ttaagtctct cgtaatcgtc tcggctgcgc tgctgccgtt ctctgcgacc
  61 gcttttgctg ccgacgccat ccaggaacag cctccggttc cggctccggt tgaagtagct
 121 ccccagtata gctgggctgg tggctatacc ggtctttacc ttggctacgg ctggaacaag
 181 gccaagacca gcaccgttgg cagcatcaag cctgacgatt ggaaggctgg cgcctttgct
 241 ggctggaact tccagcagga ccagatcgta tacggtgttg aaggtgatgc aggttattcc
 301 tgggccaaga agtccaagga cggcctggaa gtcaagcagg gctttgaagg ctcgctgcgt
 361 gcccgcgtcg gctacgacct gaacccggtt atgccgtacc tcacggctgg tattgccggt
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```
 421 tcgcagatca agcttaacaa cggcttggac gacgaaagca agttccgcgt gggttggacg
 481 gctggtgccg gtctcgaagc caagctgacg gacaacatcc tcggccgcgt tgagtaccgt
 541 tacacccagt acggcaacaa gaactatgat ctggccggta cgactgttcg caacaagctg
 601 gacacgcagg atatccgcgt cggcatcggc tacaagttct aa
```

Primers:
omp25-for: ATAGGATCCatgcgcactcttaagtctctcg(BamHI)
omp25-rev: ATACTGCAGttagaacttgtagccgatgcc (PstI)

Sequence size: 642 bp.

Protein sequence, including His-tag:
MRGSHHHHHHGSMRTLKSLVIVSAALLPFSATAFAADAIQEQPPVPAPVEVAPQYSWAGGYTGLYLGYGW
NKAKTSTVGSIKPDDWKAGAFAGWNFQQDQIVYGVEGDAGYSWAKKSKDGLEVKQGFEGSLRARVGYDLN
PVMPYLTAGIAGSQIKLNNGLDDESKFRVGWTAGAGLEAKLTDNILGRVEYRYTQYGNKNYDLAGTTVRN
KLDTQDIRVGIGYKF

Molecular weight of recombinant protein: 24.55 kD.

AminopepN

DNA sequence (gi|17983309|gb|AE009570.1| *Brucella melitensis 16M* chromosome I, section 127 of 195):

```
   1 atgcgtactg aaaccggcca tactttccgt ctcgaagatt atcgccagac accttacgcc
  61 atacccgaaa cgaaactcga cttcacactg gagccggaaa aaaccatcgt gcgcgcaacg
 121 ctcaccatag agcgccgctc cgatacgccc gccggtacgc cgctcgttct ccacggtgac
 181 gaattgaagc tcgtgagcct tgccatcgac ggcaaggcgc tttccgacaa cagcttttcg
 241 gccacgcccg accagttgac catcagcgat cttccgaaag atgtgcgctt caccttgcag
 301 atcgtgaccg aggtgaaccc aacagccaat cgccagcttt ccggccttta ccgctccagc
 361 ggcgtctatt gcacccaatg cgaggcggaa ggctttcgtc gcatcaccta tttttacgac
 421 cgcccggacg tgctgtcggt ctatacggtg cgtgtcgatg ccgaccgcaa agccgctccc
 481 atcctgcttt caaacggcaa ccctgtcgaa aacggcatgg tggagggcca gccggaacgg
 541 cattttgccg tctggcacga cccgcatcca aaaccctcct atcttttcgc gctcgtcgcc
 601 ggttcgctcg gcgtggtgaa agaccacttt acaacccgat ctggacggcc cgtcgatctc
 661 gccatccatg tggaacatgg caaggagggc cgcgcgcttt atgcgatgga cgcgctgaaa
 721 cgctccatga aatgggacga ggaaaaattc ggccgcgaat atgaccttaa cgttttcaat
 781 atcgtcgccg tctccgattt caacatgggc gcgatggaga acaagggcct caatatcttc
 841 aacgacaaat atgtgctggc cgatcctgaa accgtgaccg atgcggatta tgccggcatc
 901 gaagccgtta tcgcgcatga atatttccac aactggaccg gcaaccgcat cacctgccgc
 961 gactggttcc agctatgcct caaggaaggc ctgacggttt atcgcgatca cgaattttcc
1021 gccgaccagc gctcgcgccc tgtcaagcgc attgcggagg tgaaaatcct gaaagcgcag
1081 caattcccgg aggatgcagg ctcgcttgcc catccggtgc gccctcgcga atatcgcgag
1141 atcaataatt tctatacggc aaccgtctat gaaaaaggtt cggaagtcgt tcgcatgatc
1201 cgcaccatca tcgggccgga gctgttccgc aagggcatgg acctctattt cgagcgccat
1261 gacggcgatg cggcgaccat cgagaatttc atccaggttt ttgccgatgt ttccgggcag
1321 gatttctcgc aattcgcgct ctggtacgat caggccggta caccgaaggt ggaagccggg
1381 ttccatcatg acgcagccgc gaagacattc acgatcaagc tggaacagtc acttgcgccg
1441 acacccggcc agtcgatcag gaagcccatg catataccca ttgccttcgg cctgatcggg
1501 ccggacggca aggacatgca gccctcgtcg gtggaaggcg gcgaggtgcg cgacggcgta
1561 atccatttgc gccgcccatc cgaaaccatc gtcttccatg gcatcgaggc ccggcccgtg
1621 ccgtcgctgc tgcgcggctt ttcggcgcca gtcaatctcg ccgcgcctct cacggcggaa
1681 gaccgggttt tccttgccct gaacgatagc gaccccgttg cgcgctggca ggcgatgaac
1741 agcattttct ctgcgaccct tctggatggc gccaagcgtg tgcgcggcgg gcatcagccg
1801 gaaaccgatc cgaagatcgt cgcgctggcc ggaaaggtcg ccttcgatga aatgctggac
1861 ccggctttcc gggcgctttg cctgacgctg ccgagcgaaa gcgatatcgc gcgcgaaatg
```

```
1921 ggtaacaatg tcgatccaga cgcaatcctc gccagccgca accatctgat tgcagcaatc
1981 gcttcaggct atgccgatgg atttgccggg ctctatgaca cgctgaagca ggaaggggcg
2041 ttttcacccg atgcggcccc ggcgggaaag cgtgccttgc gtagcgccct tctcgattat
2101 ctcagcgttc aggaaaagag ccctgaacgt gcagaaaggc aatttgtcga agccgacaac
2161 atgacggacc gcgccacggc gctggccgtt ctggtccatc gttttggcga tagcggcgaa
2221 gcccgtcagg cgctcgcaac cttcgagcaa acgttcggcc aggatgcgct cgtgatggac
2281 aaatggttca tcgtgcaggc gacacgcccc ggcgaaacgg cccttgaagc agtgagggaa
2341 ctgacccgcc atccgctctt ttctctcgac aatccaaatc gcgtgcgcgc gctcatcggc
2401 gcatttacgg cttccaaccc gaccgggttc aaccgccaag atggtgcagc ctatggtttc
2461 ctcgccgata cgcttctgac cattgatccg aaaaacccgc agctttccgc acggcttttg
2521 acggcaatgc gctcatggcg gtcgctggaa gaggtgcggc gcgaacatgc ccgcgcggca
2581 ctggcgcgca ttgcaggcgc aggcaaactc tccaccgatc tgcgcgacat catcgaccga
2641 acgctcgcct ga
```

Primers:
AminP-for: ATAGGATCCatgcgtactgaaaccggccatac(BamHI)
AminP-rev: TATAAGCTTtcaggcgagcgttcggtc (HindII)

Sequence size: 2652 bp.

Protein sequence, including His-tag:
MRGSHHHHHHGSMRTETGHTFRLEDYRQTPYAIPETKLDFTLEPEKTIVRATLTIERRSDTPAGTPLVLH
GDELKLVSLAIDGKALSDNSFSATPDQLTISDLPKDVRFTLQIVTEVNPTANRQLSGLYRSSGVYCTQCE
AEGFRRITYFYDRPDVLSVYTVRVDADRKAAPILLSNGNPVENGMVEGQPERHFAVWHDPHPKPSYLFAL
VAGSLGVVKDHFTTRSGRPVDLAIHVEHGKEGRALYAMDALKRSMKWDEEKFGREYDLNVFNIVAVSDFN
MGAMENKGLNIFNDKYVLADPETVTDADYAGIEAVIAHEYFHNWTGNRITCRDWFQLCLKEGLTVYRDHE
FSADQRSRPVKRIAEVKILKAQQFPEDAGSLAHPVRPREYREINNFYTATVYEKGSEVVRMIRTIIGPEL
FRKGMDLYFERHDGDAATIENFIQVFADVSGQDFSQFALWYDQAGTPKVEAGFHHDAAAKTFTIKLEQSL
APTPGQSIRKPMHIPIAFGLIGPDGKDMQPSSVEGGEVRDGVIHLRRPSETIVFHGIEARPVPSLLRGFS
APVNLAAPLTAEDRVFLALNDSDPVARWQAMNSIFSATLLDGAKRVRGGHQPETDPKIVALAGKVAFDEM
LDPAFRALCLTLPSESDIAREMGNNVDPDAILASRNHLIAAIASGYADGFAGLYDTLKQEGAFSPDAAPA
GKRALRSALLDYLSVQEKSPERAERQFVEADNMTDRATALAVLVHRFGDSGEARQALATFEQTFGQDALV
MDKWFIVQATRPGETALEAVRELTRHPLFSLDNPNRVRALIGAFTASNPTGFNRQDGAAYGFLADTLLTI
DPKNPQLSARLLTAMRSWRSLEEVRREHARAALARIAGAGKLSTDLRDIIDRTLA

Molecular weight of recombinant protein: 99.46 kD.

LP

DNA sequence (gi|62289568|ref|YP_221361.1| Brucella abortus biovar 1 str. 9-941outer membrane lipoprotein-related protein):

```
   1 atgcgtgctg attgttatga aggccggttt gttatgatga tagtttccag gttttctcgc
  61 cctgttccag ttatatcgat gatgtttgtc gtgtcgctgg cactgtcggc ctgcggcacg
 121 acgggcggcg gcaaggggag cggttttcca tcgctgggcg gctcatcaca gaagccggaa
 181 acgaacctgc tcgcttcgct tggcaacggc ctgcttggca attcggccag tcagttgagt
 241 gcggctgatc gcaggaaggc gctggaagct gaatatcgcg cgcttgaata ttcgccagcg
 301 gggaaatcgg ttttgtggag cggagccgga tcaaacgctg gcgacgtgac ggcggcgcaa
 361 ccctatcagg tcggctcgca gaactgccgc caatattcgc atagtttcac cattggcggg
 421 gatcagcaga cggtgcgtgg cacggcttgc cgcaatcccg acggtagctg gacaccgctg
 481 acctga
```

Primers:
LP-for: ATAGGATCCatgcgtgctgattgttatgaag(BamHI)
LP-rev: TATAAGCTTtcaggtcagcggtgtcca (HindIII)

Sequence size: 486 bp.
Protein sequence, including His-tag:

MRGSHHHHHHGSMRADCYEGRFVMMIVSRFSRPVPVISMMFVVSLALSACGTTGGGKGSGFPSLGGSSQK
PETNLLASLGNGLLGNSASQLSAADRRKALEAEYRALEYSPAGKSVLWSGAGSNAGDVTAAQPYQVGSQN
CRQYSHSFTIGGDQQTVRGTACRNPDGSWTPLT

Molecular weight of recombinant protein: 18.21 kD.

Baomp852

DNA sequence (gi|62289852|ref|YP_221645.1| Brucella abortus biovar 1 str. 9-941 outer membrane protein, hypothetical):

```
   1 atgaaaaatg cggcttccat tacgatagcg agcggtatta tcggcttggt tgcgcatggt
  61 atcggatatt gtgccggtac ggcgcgacag gcctccaagc ctgtacaggc tgcaacctcc
 121 accgttgcct ttaacccgaa ggccgtcgaa aacatcgtgc gcaattatct gcttcagaac
 181 ccggaactga tgctggaagt gcagaccgcg cttgaaacca aacaggctca tgcggcacag
 241 gaacaggtga agcaggtact ggccgccaat cagagcgttc ttttcgaccc caagcatgat
 301 gctgtctttg gcaacccgaa tggcgacgtg acggtctatg aattcttcga ttacaattgc
 361 ggctattgca agcgcgcgct tcccgatatg gaagcgatcc tgaaaaaaga tccgaatgtt
 421 cgttatgtcc tcaaggagtt cccgatcctg gggccggatt ccatgcgtgc gcatgtggtc
 481 tcccaggctt tcaaggcgct gatgccggag aagtacccgg aatttcatga aatgctgctt
 541 ggcgggcatg ggcgcgcaac ggaggaatcc gcgattgccg acgccgtaaa gctcggcgcc
 601 gatgaagcta agcttcgtga aaaaatgaag gacccggcca tcaccggcgc tttccagcgg
 661 acctaccagc ttgcgcaaca gctcaacatc accggcactc cgtcctatgt catcggcgac
 721 gaactggtgc ccggcgctat tggtatcgat ggattgcggc agaggatcgc ggccgcccgg
 781 gacgctgcaa agaagtaa
```

Primers:
Baomp852-for: ATAGGATCCTtacaggctgcaacctccac (BamHI)
Baomp852-rev: ATACTGCAGcaatccatcgataccaatagc(PstI)
Sequence size: 654 bp.

Protein sequence, including His-tag:
MRGSHHHHHHGSLQAATSTVAFNPKAVENIVRNYLLQNPELMLEVQTALETKQAHAAQEQVKQVLAANQS
VLFDPKHDAVFGNPNGDVTVYEFFDYNCGYCKRALPDMEAILKKDPNVRYVLKEFPILGPDSMRAHVVSQ
AFKALMPEKYPEFHEMLLGGHGRATEESAIADAVKLGADEAKLREKMKDPAITGAFQRTYQLAQQLNITG
TPSYVIGDELVPGAIGIDGL

Molecular weight of recombinant protein: 25.39 kD.

Baompeff

DNA sequence (gi|62289884|ref|YP_221677.1| *Brucella abortus biovar 1 str. 9-941*outer membrane efflux family protein):

```
   1 atgaggtaca cggtgttcaa agcgtgcaag gaactggtag cggccgcagt attgttgtct
  61 ggcaccgttt taacggggca ggccgctctg tcggagacgc tgactggcgc tctcgtcaag
 121 gcttacaaga ataatgcttc cctgaattcc tcgcgggcag gggtgcgcat tcaggacgaa
 181 aacgtggcga ttgccaaatc tgcctaccgg ccacagatta ccggttctta taatatatcg
 241 agaggcaaga cgccggccac cgattatcgt acgactggta cggttggcat ccagttgaac
 301 cagatgcttt tcgacggttt tcagaccagg aacaatgttg ccgccgctga aacgcaagtc
 361 ttcgcgcagc gcgaaaacct ccgcaatgat gagcagaaca cgctctatca ggcggtagcc
 421 gcctatatgg atgtttacca gcttcgccag attgctgcac tgcgcgagaa gaaccttgcc
```

```
 481 gccatgaacg agcaggtgcg tgccgcacgc gctcgccttg atgtgggtga ggggacgcgc
 541 accgacgttg ctcaggcaga ggccagccgc tccacagcca tagccgctct caacgccgcg
 601 cgtgcagacg tgaaaacggc ggaagccacc tatatgcagg tcgttggatc gctgccggac
 661 aagcttaccc cggcttctgc ggccaggcat cttccccagt cgccgagcca ggcctatgcg
 721 tctgcgctcg cttctcatcc cggcatcctc gccacgaaat atgccgtcaa tgccgccggt
 781 tataatgtca aggccaagga aggcgcactg cttccgacca ttggcctgac ggcaagcgcc
 841 agccagcttg acactatcgc agggacggat atgggcgacg gtaacacggc atcgatcggc
 901 gttggcgtca gcattccgat ctacacgggc ggccgcacgt cagcgcagat ccgccagtcc
 961 aaggaacagc ttggtcaggc gcgaatcgag gttgatgtcg tgcaggacaa ggttcgtcag
1021 gccatcagtt cagcctggtc gcagctggaa gctgcccgtg cctcggtcgc agcaaaccgt
1081 gatggtattg ccgccgcaca gcttgccctc gatggcgtca ttgaagaacg caaggttggc
1141 cagcgcacga cgcttgacgt gttgaatgcc cagaacgatc tcgtcgcagt gcagatcgct
1201 ctagttcagg ccgaacatga tgttgttgtg gcgagctatg ctcttctgaa tgccacgggc
1261 cgcatgactg ccgaccagct tggtttgcag gtggcccagt ataagccgga agagcactac
1321 aaggcggtga aagacaagtg gttcggcctg cgtacgcctg atggccgcta atcggctctg
1381 caaacaatat ttcgatggcg cctgtgggcg ccatttctgt ttgtggcgat tcgcatcgtc
1441 caatttctta ataaatccat aaagctgtga gtttggatga tgcgttatc
```

Primers:
BAompeff-for: ATAGGATCCatcgaggttgatgtcgtgcag (BamHI)
BAompeff-rev: TATAAGCTTacgcatcatccaaactcaca (HindIII)

Sequence size: 510 bp.

Protein sequence, including His-tag:
MRGSHHHHHHGSIEVDVVQDKVRQAISSAWSQLEAARASVAANRDGIAAAQLALDGVIEERKVGQRTTLD
VLNAQNDLVAVQIALVQAEHDVVVASYALLNATGRMTADQLGLQVAQYKPEEHYKAVKDKWFGLRTPDGR

Molecular weight of recombinant protein: 15.31 kD.

P15-bp26 fusion

DNA sequence:

```
   1 atgaaaggcg aaccaaaggt catcgagcgg cttaacgagg cactgtttct tgagctcggt
  61 gcggtaaacc agtattggct gcactaccgt cttctcaacg attggggtta cacgcgcctt
 121 gcaaagaagg aacgcgagga atccatcgag gaaatgcatc acgccgacaa gctgattgat
 181 cgcattatct tccttgaagg ctttccgaac ctccagaccg tttcgccgtt gcgcattggc
 241 cagaatgtga aggaagttct cgaagctgac ctcaagggtg aatatgacgc tcgcgcttcg
 301 tataaggaat cgcgcgaaat ctgcgacaag ctcggcgact atgtgtcgaa gcagcttttc
 361 gacgaacttc tggccgatga agaaggccat atcgacttcc ttgaaaccca gcttgacctt
 421 ctcgccaaga tcggcggaga acgctatggc cagcttaaag ctattccgct gagcagatcc
 481 atgaacactc gtgctagcaa ttttctcgca gcctcatttt ccacaatcat gctcgtcggc
 541 gctttcagcc tgcccgcttt cgcacaggag aatcagatga cgacgcagcc cgcgcgcatc
 601 gccgtcaccg gggaaggcat gatgacggcc tcgcccgata tggccattct caatctctcg
 661 gtgctacgcc aggcaaagac cgcgcgcgaa gccatgaccg cgaataatga agccatgaca
 721 aaagtgctcg atgccatgaa gaaggccggc atcgaagatc gcgatctcca gacaggcggc
 781 atcaatatcc agccgattta tgtctatcct gacgacaaga acaacctgaa agagcctacc
 841 atcaccggct attctgtatc caccagtctc acggttcgcg tgcgcgaact ggccaatgtt
 901 ggaaaaattt tggatgaatc cgtcacgctc ggtgttaatc agggcggtga tttgaacctg
 961 gtcaatgata atccctccgc cgtgatcaac gaggcgcgca agcgcgcagt ggccaatgcc
1021 attgccaagg cgaagacgct tgccgacgct gcaggcgtgg ggcttggccg tgtggtggaa
1081 atcagtgaac tgagccgccc gcccatgccg atgccaattg cgcgcggaca gttcagaacc
1141 atgctagcag c
```

Sequence size: 1153 bp
Adapter sequence, HindIII-BamHI-AD2 is underlined (see the sequence in RESULTS)
Protein sequence, including His-tag:

MRGSHHHHHHMKGEPKVIERLNEALFLELGAVNQYWLHYRLLNDWGYTRLAKKEREESIEEMHHADKLIDRIIFLEG
FPNLQTVSPLRIGQNVKEVLEADLKGEYDARASYKESREICDKLGDYVSKQLFDELLADEEGHIDFLETQLDLLAKI
GGERYGQLKAIPLSRSMNTRASNFLAASFSTIMLVGAFSLPAFAQENQMTTQPARIAVTGEGMMTASPDMAILNLSV
LRQAKTAREAMTANNEAMTKVLDAMKKAGIEDRDLQTGGINIQPIYVYPDDKNNLKEPTITGYSVSTSLTVRVRELA
NVGKILDESVTLGVNQGGDLNLVNDNPSAVINEARKRAVANAIAKAKTLADAAGVGLGRVVEISELSRPPMPMPIAR
GQFRTMLA

Molecular weight of recombinant protein: 43.01 kDa

P15-P39 fusion

DNA sequence:

```
   1 atgaaaggcg aaccaaaggt catcgagcgg cttaacgagg cactgtttct tgagctcggt
  61 gcggtaaacc agtattggct gcactaccgt cttctcaacg attggggtta cacgcgcctt
 121 gcaaagaagg aacgcgagga atccatcgag gaaatgcatc acgccgacaa gctgattgat
 181 cgcattatct tccttgaagg ctttccgaac ctccagaccg tttcgccgtt gcgcattggc
 241 cagaatgtga aggaagttct cgaagctgac ctcaagggtg aatatgacgc tcgcgcttcg
 301 tataaggaat cgcgcgaaat ctgcgacaag ctcggcgact atgtgtcgaa gcagcttttc
 361 gacgaacttc tggccgatga agaaggccat atcgacttcc ttgaaaccca gcttgacctt
 421 ctcgccaaga tcggcggaga acgctatggc cagcttaaag ctattccgct gagcagatcc
 481 gaaagcaagg gcattagctg gaccgatatg ccggttgcag gtggcggcgg cacggaagcc
 541 atgaccgttt tgcgcgcgcg cgttaccgca ggcaatgcgc caaccgcggt gcagatgctg
 601 ggttttgaca ttcgcgactg ggcggagcag ggcgcactcg gcaatctcga tacggttgct
 661 tccaaggaag gctgggaaaa ggttattccg gctcccttgc aggaatttgc caaatatgac
 721 ggccactgga ttcgtgcgcc cgtcaatatt cactccacca actggatgtg gatcaacaag
 781 gctgctctcg acaaggctgg cggcaaggag ccgaccaatt gggatgagct gattgcgctt
 841 ctcgacaatt tcaaggcgca gggcattacg ccgatcgcgc atggcggcca gccgtggcag
 901 gatgcaacca ttttcgatgc ggttgttctt tcattcggcc cggatttcta caagaaggcc
 961 ttcatcgatc tcgacccgga agcactgggc agcgatacca tgaagcaggc cttcgaccgc
1021 atgtccaagc ttcgcaccta tgttgatgac aacttctccg gccgtgactg gaaccttgct
1081 tcggccatgg ttatcgaagg caaggccggt gtccagttca tgggcgactg ggcgaagggc
1141 gagttcctca aggcgggcaa gaagccgggt gaggatttcg tctgcatgcg ttatccgggc
1201 acgcagggtg ctgtcacttt caattccgac atgttcgcca tgttcaaggt ttcggaagac
1261 aaggttcccg cacagcttga aatggcttcg gcgattgaaa gccctgcctt ccagtctgcc
1321 tttaatgtgg tgaaggggtc ggccccggca cgcacggatg tgcccgatac cgctttcgat
1381 gcctgtggca agaaggccat tgccgatgtc aaggaagcaa acagcaaggg cactctgctt
1441 ggctccatgg cgcatggcta tgccaatccg gctgccgtga agaatgcgat ctacgacgtc
1501 gtgacccgcc agttcaacgg ccagctttct tcggaagatg ccgtcaagga actcgttgtg
1561 gcggttgaag ccgcaaaata a
```

Sequence size: 1581 bp

Adapter sequence, HindIII-BamHI – AD2 is underlined (see the sequence in RESULTS)

Protein sequence, including His-tag:
MRGSHHHHHHGSMKGEPKVIERLNEALFLELGAVNQYWLHYRLLNDWGYTRLAKKEREESIEEMHHADKL
IDRIIFLEGFPNLQTVSPLRIGQNVKEVLEADLKGEYDARASYKESREICDKLGDYVSKQLFDELLADEE
GHIDFLETQLDLLAKIGGERYGQLKAIPLSRSESKGISWTDMPVAGGGGTEAMTVLRARVTAGNAPTAVQ
MLGFDIRDWAEQGALGNLDTVASKEGWEKVIPAPLQEFAKYDGHWIRAPVNIHSTNWMWINKAALDKAGG
KEPTNWDELIALLDNFKAQGITPIAHGGQPWQDATIFDAVVLSFGPDFYKKAFIDLDPEALGSDTMKQAF
DRMSKLRTYVDDNFSGRDWNLASAMVIEGKAGVQFMGDWAKGEFLKAGKKPGEDFVCMRYPGTQGAVTFN
SDMFAMFKVSEDKVPAQLEMASAIESPAFQSAFNVVKGSAPARTDVPDTAFDACGKKAIADVKEANSKGT
LLGSMAHGYANPAAVKNAIYDVVTRQFNGQLSSEDAVKELVVAVEAAK

Molecular weight of recombinant protein: 59.4 kDa

BP26-P39 fusion

DNA sequence:

```
   1 atgaacactc gtgctagcaa ttttctcgca gcctcatttt ccacaatcat gctcgtcggc
  61 gctttcagcc tgcccgcttt cgcacaggag aatcagatga cgacgcagcc cgcgcgcatc
 121 gccgtcaccg gggaaggcat gatgacggcc tcgcccgata tggccattct caatctctcg
 181 gtgctacgcc aggcaaagac cgcgcgcgaa gccatgaccg cgaataatga agccatgaca
 241 aaagtgctcg atgccatgaa gaaggccggc atcgaagatc gcgatctcca gacaggcggc
 301 atcaatatcc agccgattta tgtctatcct gacgacaaga acaacctgaa agagcctacc
 361 atcaccggct attctgtatc caccagtctc acggttcgcg tgcgcgaact ggccaatgtt
 421 ggaaaaattt tggatgaatc cgtcacgctc ggtgttaatc agggcggtga tttgaacctg
 481 gtcaatgata atccctccgc cgtgatcaac gaggcgcgca agcgcgcagt ggccaatgcc
 541 attgccaagg cgaagacgct tgccgacgct gcaggcgtgg ggcttggccg tgtggtggaa
 601 atcagtgaac tgagccgccc gcccatgccg atgccaattg cgcgcggaca gttcagaacc
 661 atgctagcag ctaagctgcc gagcctgagc agatccgaaa gcaagggcat tagctggacc
 721 gatatgccgg ttgcaggtgg cggcggcacg gaagccatga ccgttttgcg cgcgcgcgtt
 781 accgcaggca atgcgccaac cgcggtgcag atgctgggtt ttgacattcg cgactgggcg
 841 gagcagggcg cactcggcaa tctcgatacg gttgcttcca aggaaggctg ggaaaaggtt
 901 attccggctc ccttgcagga atttgccaaa tatgacggcc actggattcg tgcgcccgtc
 961 aatattcact ccaccaactg gatgtggatc aacaaggctg ctctcgacaa ggctggcggc
1021 aaggagccga ccaattggga tgagctgatt gcgcttctcg acaatttcaa ggcgcagggc
1081 attacgccga tcgcgcatgg cggccagccg tggcaggatg caaccatttt cgatgcggtt
1141 gttctttcat tcggcccgga tttctacaag aaggccttca tcgatctcga cccggaagca
1201 ctgggcagcg ataccatgaa gcaggccttc gaccgcatgt ccaagcttcg cacctatgtt
1261 gatgacaact tctccggccg tgactggaac cttgcttcgg ccatggttat cgaaggcaag
1321 gccggtgtcc agttcatggg cgactgggcg aagggcgagt tcctcaaggc gggcaagaag
1381 ccgggtgagg atttcgtctg catgcgttat ccgggcacgc agggtgctgt cactttcaat
1441 tccgacatgt tcgccatgtt caaggtttcg gaagacaagg ttcccgcaca gcttgaaatg
1501 gcttcggcga ttgaaagccc tgccttccag tctgccttta atgtggtgaa ggggtcggcc
1561 ccggcacgca cggatgtgcc cgataccgct ttcgatgcct gtggcaagaa ggccattgcc
1621 gatgtcaagg aagcaaacag caagggcact ctgcttggct ccatggcgca tggctatgcc
1681 aatccggctg ccgtgaagaa tgcgatctac gacgtcgtga cccgccagtt caacggccag
1741 ctttcttcgg aagatgccgt caaggaactc gttgtggcgg ttgaagccgc aaaataa
```

Sequence size: 1797 bp

Adapter sequence, HindIII-BamHI – AD1 is underlined (see the sequence in RESULTS)

Protein sequence, including His-tag:

MRGSHHHHHHGSMNTRASNFLAASFSTIMLVGAFSLPAFAQENQMTTQPARIAVTGEGMMTASPDMAILN
LSVLRQAKTAREAMTANNEAMTKVLDAMKKAGIEDRDLQTGGINIQPIYVYPDDKNNLKEPTITGYSVST
SLTVRVRELANVGKILDESVTLGVNQGGDLNLVNDNPSAVINEARKRAVANAIAKAKTLADAAGVGLGRV
VEISELSRPPMPMPIARGQFRTMLAAKLPSLSRSESKGISWTDMPVAGGGGTEAMTVLRARVTAGNAPTA
VQMLGFDIRDWAEQGALGNLDTVASKEGWEKVIPAPLQEFAKYDGHWIRAPVNIHSTNWMWINKAALDKA
GGKEPTNWDELIALLDNFKAQGITPIAHGGQPWQDATIFDAVVLSFGPDFYKKAFIDLDPEALGSDTMKQ
AFDRMSKLRTYVDDNFSGRDWNLASAMVIEGKAGVQFMGDWAKGEFLKAGKKPGEDFVCMRYPGTQGAVT
FNSDMFAMFKVSEDKVPAQLEMASAIESPAFQSAFNVVKGSAPARTDVPDTAFDACGKKAIADVKEANSK
GTLLGSMAHGYANPAAVKNAIYDVVTRQFNGQLSSEDAVKELVVAVEAAK

Molecular weight of recombinant protein: 64.16 kDa

Baomp852-bp26 fusion

DNA sequence:

```
   1 ttacaggctg caacctccac cgttgccttt aacccgaagg ccgtcgaaaa catcgtgcgc
  61 aattatctgc ttcagaaccc ggaactgatg ctggaagtgc agaccgcgct tgaaaccaaa
 121 caggctcatg cggcacagga acaggtgaag caggtactgg ccgccaatca gagcgttctt
 181 ttcgacccca agcatgatgc tgtctttggc aacccgaatg gcgacgtgac ggtctatgaa
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 241 ttcttcgatt acaattgcgg ctattgcaag cgcgcgcttc ccgatatgga agcgatcctg
 301 aaaaaagatc cgaatgttcg ttatgtcctc aaggagttcc cgatcctggg gccggattcc
 361 atgcgtgcgc atgtggtctc ccaggctttc aaggcgctga tgccggagaa gtacccggaa
 421 tttcatgaaa tgctgcttgg cgggcatggg cgcgcaacgg aggaatccgc gattgccgac
 481 gccgtaaagc tcggcgccga tgaagctaag cttcgtgaaa aaatgaagga cccggccatc
 541 accggcgctt tccagcggac ctaccagctt gcgcaacagc tcaacatcac cggcactccg
 601 tcctatgtca tcggcgacga actggtgccc ggcgctattg gtatcgatgg attgctgcat
 661 ctgccgctga gcagatccat gaacactcgt gctagcaatt ttctcgcagc ctcatttcc
 721 acaatcatgc tcgtcggcgc tttcagcctg cccgctttcg cacaggagaa tcagatgacg
 781 acgcagcccg cgcgcatcgc cgtcaccggg gaaggcatga tgacggcctc gcccgatatg
 841 gccattctca atctctcggt gctacgccag gcaaagaccg cgcgcgaagc catgaccgcg
 901 aataatgaag ccatgacaaa agtgctcgat gccatgaaga aggccggcat cgaagatcgc
 961 gatctccaga caggcggcat caatatccag ccgatttatg tctatcctga cgacaagaac
1021 aacctgaaag agcctaccat caccggctat tctgtatcca ccagtctcac ggttcgcgtg
1081 cgcgaactgg ccaatgttgg aaaaattttg gatgaatccg tcacgctcgg tgttaatcag
1141 ggcggtgatt tgaacctggt caatgataat ccctccgccg tgatcaacga ggcgcgcaag
1201 cgcgcagtgg ccaatgccat tgccaaggcg aagacgcttg ccgacgctgc aggcgtgggg
1261 cttggccgtg tggtggaaat cagtgaactg agccgcccgc ccatgccgat gccaattgcg
1321 cgcggacagt tcagaaccat gctagcagct a
```

Sequence size: 1351 bp

Adapter sequence, PstI -BamHI – AD1 is underlined (see the sequence in RESULTS)

Protein sequence, including His-tag:

MRGSHHHHHHGSLQAATSTVAFNPKAVENIVRNYLLQNPELMLEVQTALETKQAHAAQEQVKQVLAANQS
VLFDPKHDAVFGNPNGDVTVYEFFDYNCGYCKRALPDMEAILKKDPNVRYVLKEFPILGPDSMRAHVVSQ
AFKALMPEKYPEFHEMLLGGHGRATEESAIADAVKLGADEAKLREKMKDPAITGAFQRTYQLAQQLNITG
TPSYVIGDELVPGAIGIDGLLHLPLSRSMNTRASNFLAASFSTIMLVGAFSLPAFAQENQMTTQPARIAV
TGEGMMTASPDMAILNLSVLRQAKTAREAMTANNEAMTKVLDAMKKAGIEDRDLQTGGINIQPIYVYPDD
KNNLKEPTITGYSVSTSLTVRVRELANVGKILDESVTLGVNQGGDLNLVNDNPSAVINEARKRAVANAIA
KAKTLADAAGVGLGRVVEISELSRPPMPMPIARGQFRTMLAA

Molecular weight of recombinant protein: 50.12 kDa

P15-bp26-P39 fusion

DNA sequence:

```
   1 atgaaaggcg aaccaaaggt catcgagcgg cttaacgagg cactgtttct tgagctcggt
  61 gcggtaaacc agtattggct gcactaccgt cttctcaacg attggggtta cacgcgcctt
 121 gcaaagaagg aacgcgagga atccatcgag gaaatgcatc acgccgacaa gctgattgat
 181 cgcattatct tccttgaagg ctttccgaac ctccagaccg tttcgccgtt gcgcattggc
 241 cagaatgtga aggaagttct cgaagctgac ctcaagggtg aatatgacgc tcgcgcttcg
 301 tataaggaat cgcgcgaaat ctgcgacaag ctcggcgact atgtgtcgaa gcagcttttc
 361 gacgaacttc tggccgatga agaaggccat atcgacttcc ttgaaaccca gcttgacctt
 421 ctcgccaaga tcggcggaga acgctatggc cagcttaaag ctattccgct gagcagatcc
 481 atgaacactc gtgctagcaa ttttctcgca gcctcatttt ccacaatcat gctcgtcggc
 541 gctttcagcc tgcccgcttt cgcacaggag aatcagatga cgacgcagcc cgcgcgcatc
 601 gccgtcaccg gggaaggcat gatgacggcc tcgcccgata tggccattct caatctctcg
 661 gtgctacgcc aggcaaagac cgcgcgcgaa gccatgaccg cgaataatga agccatgaca
 721 aaagtgctcg atgccatgaa gaaggccggc atcgaagatc gcgatctcca gacaggcggc
 781 atcaatatcc agccgattta tgtctatcct gacgacaaga acaacctgaa agagcctacc
 841 atcaccggct attctgtatc caccagtctc acggttcgcg tgcgcgaact ggccaatgtt
 901 ggaaaaattt tggatgaatc cgtcacgctc ggtgttaatc agggcggtga tttgaacctg
 961 gtcaatgata atccctccgc cgtgatcaac gaggcgcgca agcgcgcagt ggccaatgcc
1021 attgccaagg cgaagacgct tgccgacgct gcaggcgtgg ggcttggccg tgtggtggaa
1081 atcagtgaac tgagccgccc gcccatgccg atgccaattg cgcgcggaca gttcagaacc
1141 atgctagcag ctaagctgcc gagcctgagc agatccgaaa gcaagggcat tagctggacc
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1201 gatatgccgg ttgcaggtgg cggcggcacg gaagccatga ccgttttgcg cgcgcgcgtt
1261 accgcaggca atgcgccaac cgcggtgcag atgctgggtt ttgacattcg cgactgggcg
1321 gagcagggcg cactcggcaa tctcgatacg gttgcttcca aggaaggctg ggaaaaggtt
1381 attccggctc ccttgcagga atttgccaaa tatgacggcc actggattcg tgcgcccgtc
1441 aatattcact ccaccaactg gatgtggatc aacaaggctg ctctcgacaa ggctggcggc
1501 aaggagccga ccaattggga tgagctgatt gcgcttctcg acaatttcaa ggcgcagggc
1561 attacgccga tcgcgcatgg cggccagccg tggcaggatg caaccatttt cgatgcggtt
1621 gttctttcat tcggcccgga tttctacaag aaggccttca tcgatctcga cccggaagca
1681 ctgggcagcg ataccatgaa gcaggccttc gaccgcatgt ccaagcttcg cacctatgtt
1741 gatgacaact tctccggccg tgactggaac cttgcttcgg ccatggttat cgaaggcaag
1801 gccggtgtcc agttcatggg cgactgggcg aagggcgagt tcctcaaggc gggcaagaag
1861 ccgggtgagg atttcgtctg catgcgttat ccgggcacgc agggtgctgt cactttcaat
1921 tccgacatgt tcgccatgtt caaggtttcg gaagacaagg ttcccgcaca gcttgaaatg
1981 gcttcggcga ttgaaagccc tgccttccag tctgccttta atgtggtgaa ggggtcggcc
2041 ccggcacgca cggatgtgcc cgataccgct ttcgatgcct gtggcaagaa ggccattgcc
2101 gatgtcaagg aagcaaacag caagggcact ctgcttggct ccatggcgca tggctatgcc
2161 aatccggctg ccgtgaagaa tgcgatctac gacgtcgtga cccgccagtt caacggccag
2221 ctttcttcgg aagatgccgt caaggaactc gttgtggcgg ttgaagccgc aaaataa
```

Sequence size: 2277 bp

Adapter sequences, HindIII-BamHI – AD1 is underlined and HindIII-BamHI – AD2 marked by bold face and underlined (see the sequence in RESULTS)

Protein sequence, including His-tag:

```
MRGSHHHHHHGSMKGEPKVIERLNEALFLELGAVNQYWLHYRLLNDWGYTRLAKKEREESIEEMHHADKL
IDRIIFLEGFPNLQTVSPLRIGQNVKEVLEADLKGEYDARASYKESREICDKLGDYVSKQLFDELLADEE
GHIDFLETQLDLLAKIGGERYGQLKAIPLSRSMNTRASNFLAASFSTIMLVGAFSLPAFAQENQMTTQPA
RIAVTGEGMMTASPDMAILNLSVLRQAKTAREAMTANNEAMTKVLDAMKKAGIEDRDLQTGGINIQPIYV
YPDDKNNLKEPTITGYSVSTSLTVRVRELANVGKILDESVTLGVNQGGDLNLVNDNPSAVINEARKRAVA
NAIAKAKTLADAAGVGLGRVVEISELSRPPMPMPIARGQFRTMLAAKLPSLSRSESKGISWTDMPVAGGG
GTEAMTVLRARVTAGNAPTAVQMLGFDIRDWAEQGALGNLDTVASKEGWEKVIPAPLQEFAKYDGHWIRA
PVNIHSTNWMWINKAALDKAGGKEPTNWDELIALLDNFKAQGITPIAHGGQPWQDATIFDAVVLSFGPDF
YKKAFIDLDPEALGSDTMKQAFDRMSKLRTYVDDNFSGRDWNLASAMVIEGKAGVQFMGDWAKGEFLKAG
KKPGEDFVCMRYPGTQGAVTFNSDMFAMFKVSEDKVPAQLEMASAIESPAFQSAFNVVKGSAPARTDVPD
TAFDACGKKAIADVKEANSKGTLLGSMAHGYANPAAVKNAIYDVVTRQFNGQLSSEDAVKELVVAVEAAK
```

Molecular weight of recombinant protein: 84.09 kDa

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CURRICULUM VITAE

Surname:	Jamba
Name:	Zandraa
Birth date and place:	24 March, 1969, Ulaanbaatar, Mongolia
Nationality:	Mongolian
Sex:	male
Marital status:	married
Mailing addtess:	lmgjamba@magicnet.mn
Telephone:	+976-11-456267 (home)
	+976-11-324041 (office)

Education:

1978-1988	High school
1988-1991	Biological faculty of the University of Szeged, Hungary
1991-1997	Health Sciences University of Mongolia
1997-1999	Residency in Graduate school of the same University
Degree obtained:	Medical doctor

Work experience:

1999-2002	Researcher in the Laboratory of National Center for Contagious Diseases, Ulaanbaatar, Mongolia
2002-2004	Lecturer in Health Sciences University of Mongolia
2003 up to present	Doctoral student in Institute of Molecular Biology, University of Giessen, Germany

Erklärung:

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Giessen, im Juni 2008

Zandraa Jamba