Epidemiology and Diagnosis of Brucellosis in Mongolian Bactrian Camels

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Prof. Dr. Martin Spiess,

Dekan

Dedicated to my beloved father for his measureless love and his appreciation from Heaven.

I also want to dedicate this PhD to my mother, my sister, my brother and my husband for their eternal love and support.

Table of Contents

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Summary

Brucellosis is among the most important zoonoses globally, and particularly in Mongolia. Mainly *Brucella abortus and Brucella melitensis* are transmitted to people from different livestock species and where they cause great economic losses. Camels are susceptible to both *Brucella* spp., but camel brucellosis has not received proper attention from researchers and authorities. We do not know if camels are primary hosts of *Brucella* spp. More information on the epidemiology of brucellosis in Mongolian Bactrian camels is needed given their growing economic and livelihood importance for herders and the renewed efforts to eliminate brucellosis from Mongolia through mass livestock vaccination that does not include camels. Despite decreasing camel populations, brucellosis cases in camels increased in the past two decades. Close monitoring of the situation in camels, and a better understanding of the epidemiology became central of assessing progress towards brucellosis elimination.

The aim of this PhD study was to contribute to the understanding of effective and long-lasting control of brucellosis in Mongolia. The objectives were the following: i) to understand the epidemiology of camel brucellosis in Mongolia, ii) to identify the *Brucella* species involved before and after implementation of vaccination campaigns, iii) to assess the performance of serological tests in Mongolian Bactrian camels, iv) to contribute to a better understanding of the transmission of brucellosis between camels and other animal species. Addressing these objectives should lead to recommendations to the government on diagnosis and priority actions. This PhD tested the following hypotheses: i) the seroprevalence of camel brucellosis is below 5% and the most important risk factor is herding together with cattle, ii) *Brucella abortus* is the main causative species, iii) there is more variance of camel brucellosis at district than at provincial levels.

During two consecutive years, repeated random multi-stage cluster surveys were done in the Eastern provinces of Dornod and Sukhbaatar in 2013 and 2014 and in the Southern & Western provinces of Dornogobi, Umnogobi and Khovd in 2014 and 2015. In each province, 6 districts were selected proportional to the size of their camel populations. A total of 977 camel sera were tested with the RBT, CFT, I-ELISA, C-ELISA and FPA. In view of comparison to other livestock, cattle and small ruminant sera were also enrolled.

The overall apparent brucellosis seroprevalence in 1822 randomly selected camels (considering clustering within herds) was 2.3% (95% CI 1.6-3.3), but ranged from 0.3% to 6.1% in provinces and was significantly higher in the East than in the South and West. Camel seropositivity was associated to herding camels with cattle. The results confirm that brucellosis exists up to important (endemic) seroprevalences in Mongolian camels. A repeated epidemiological survey did not find a drop in camel seropositivity after one year of

introduction of vaccination. Further monitoring is needed to assess if camel seroprevalances decrease with ongoing ruminant vaccination. Past monitoring of vaccination campaigns showed that achieved coverage was critical for cattle due to difficulties of veterinarians to restrain the animals. This should be coupled with more confirmation that only *B. abortus* exists in camels.

Sensitivity (Se) and specificity (Sp) were assessed for camel and cattle sera using as positive reference culture positive and as negative sera from herds with no animal tested RBT positive and no reported clinical brucellosis signs during the past five years. The use of RBT in camels showed low sensitivity. We recommend either the I-ELISA or FPA with very high Se for monitoring of camels. Another confirmatory test such as the CFT can be added – or both tests combined to further increase Sp. The higher costs of these tests than the RBT seem justified by the need of a sensitive monitoring test in camels. The brucellosis reference strain and sera bank in Mongolia has to acquire also true positive and true negative samples from camels.

This study detected mixed *B. ovis* and *B. abortus* in randomly selected serologically positive and negative sera of camels, cattle, goats and sheep by qPCR. *B. ovis* is less pathogenic for small ruminants than other *Brucella* species and therefore, samples collected based on brucellosis symptoms in ruminants would likely not be collected for slight symptoms caused by *B. ovis* alone. *B. ovis* has so far not been reported for Mongolia. Bruce ladder *Brucella* spp PCR that is used on cultures from clinical material can hide *B. ovis* results and other diagnostic species identification methods should be evaluated.

We have fitted a demographic model for camels and cattle of Eastern provinces. No other livestock species were added because we only found epidemiological linkages between camels and cattle. Transmission within and between cattle and camels were added and the model with all transmission pathways had the best pay-off. Unexpectantly, the model fitted camel to cattle transmission stronger than that of cattle to camel. Inter-institutional veterinary and human health collaborations in Mongolia need to be fostered to further assess if camel seropositivity decreases in parallel to vaccination of cattle and to jointly define knowledge gaps for brucellosis elimination.

Zusammenfassung

Die Brucellose gehört zu den wichtigsten Zoonosen weltweit und insbesondere in der Mongolei. Die Menschen infizieren sich hauptsächlich mit Brucella abortus und Brucella melitensis von verschiedenen Nutztierarten, wo die Krankheit auch grosse wirtschaftliche Verluste verursacht. Kamele sind empfänglich für beide Brucella spp., hingegen wurde Kamelbrucellose bis anhin kaum erforscht und in Kontrollprogrammen berücksichtigt. Wir wissen nicht, ob Kamele auch Primärwirte von Brucella spp. sein können. Wegen der zunehmenden Bedeutung der Haltung vom Kamelen (Camelus bactrianus) für die Wirtschaft und den Lebensunterhalt in der Mongolei, braucht es mehr Information über die Epidemiologie der Kamelbrucellose. Dies auch hinsichtlich der erneuten Kontrollmassnahmen mit der Massenimpfung der Wiederkäuer, wo aber die Kamele ausgeschlossen sind trotz steigender Zahlen von Kamelbrucellose der letzten 20 Jahre. Die Überwachung der Kamelbrucellose und ein besseres Verständnis der Epidemiologie sind somit zentral um die Fortschritte der Brucellosebekämpfung in der Mongolei zu bemessen.

Die Hauptzielsetzung dieser PhD Arbeit war ein Beitrag zu einem besseren Verständnis für eine effektive und langfristige Kontrolle der Brucellose in der Mongolei. Die Ziele waren die Folgenden: i) die Epidemiologie der Kamelbrucellose zu verstehen; ii) Die *Brucella* Spezies, die Kamele infizieren, vor und nach der Einführung der Impfungen bei Rindern und Kleinwiederkäuer zu identifizieren; iii) die Leistungsfähigkeit der serologischen Tests für Kamele zu bestimmen; und iv) einen entscheidenden Beitrag zum Beschrieb der Übertragung der Brucellose zwischen Kamelen und anderen Spezies zu leisten. Basierend auf den Resultaten dieser Arbeiten sollen Empfehlungen für die Regierungsämter über Diagnose und prioritäre Handlungen erfasst werden. Die folgenden Hypothesen wurden getestet: i) die Seroprävalenz der Kamelbrucellose ist kleiner als 5% und der Hauptrisikofaktor ist das Halten zusammen mit Rindern; ii) *Brucella abortus* ist der Haupterreger; iii) die Varianz vom Vorkommen der Kamelbrucellose ist grösser zwischen den Distrikten als zwischen den Provinzen.

Während zwei nachfolgenden Jahren wurden wiederholte “multi-stage cluster surveys” in je zwei östlichen Provinzen (Dornod und Sukhbaatar) in 2013 und 2014 durchgeführt, sowie in drei südwestlichen Provinzen (Dornogobi, Umnogobi und Khovd) in 2014 und 2015. In jeder Provinz wurden 6 Distrikte proportional zu ihrer Anzahl von Kamelen ausgewählt. Insgesamt wurden 977 Kamelseren mit dem Rose Bengal Test (RBT), Komplementärfixationstest (CFT,) I-ELISA, C-ELISA und dem Fluoresenzpolarisations Test (FPA) getestet. Damit wir den Status der Kamele mit dem anderer Nutztierarten vergleichen konnten, wurden ebenfalls Rinder und Kleinwiederkäuer in die Studie aufgenommen.

Die scheinbare Seroprävalenz von 1822 zufällig ausgewählten Kamelen – mit Berücksichtigung der Klumpung innerhalb von Herden – war 2.3% (95% KI 1.6-3.3), aber mit einer Bandbreite zwischen den Provinzen von 0.3% bis 6.1% und war signifikant höher in den östlichen als in den südwestlichen Provinzen. Kamelseropositivität war assoziiert mit dem Halten von Kamelen zusammen mit Rindern. Diese Resultate bestätigen, dass die Kamelbrucellose in hohen (endemischen) Seroprävalenzen in der Mongolei vorkommt. Die wiederholten Studien vor und nach der Einführung der Impfung bei Wiederkäuern fanden keinen Abfall der Seroprävalenzen bei Kamelen. Weiteres Monitoring ist nötig um zu sehen ob mit den fortschreitenden Impfkampagnen die Positivität der Kamele abnimmt. Die Impfungen der Rinder erreicht möglicherweise nicht die gewünschte Impfdichte wegen den Schwierigkeiten die Tiere zu handhaben, wie in anderen Studien gezeigt wurde. Weiter soll abgeklärt werden ob, wie in dieser Studie gefunden, nur *B. abortus* die Kamele infiziert.

Die Sensitivität (Se) und die Spezifizität (Sp) wurden für verschiedene Tests für Kamel- und Rinderseren anhand von kulturpositiven Seren und Seren von negativen Herden ohne klinische Anzeichen während der letzten 5 Jahre evaluiert. Der RBT zeigte eine tiefe Sensitivität für Kamelseren. Wir empfehlen für Kamele entweder den I-ELISA oder FPA, welche beide eine fürs Monitoring erforderliche hohe Sensitivität aufweisen, wobei dann ein weiterer Bestätigungstest wie der CFT angefügt werden kann, um die Sp zu erhöhen. Die höheren Kosten dieser Tests können mit der gewonnen Se im Vergleich zum RBT gerechtfertigt werden. Die nationale Brucellose-Serum- und Stamm Bank in der Mongolei muss unbedingt wahr-positive und -negative Proben von Kamelen aufnehmen.

Zufällig ausgewählte RBT positiven und negativen Seren von Kamelen, Rinder, Schafe und Ziegen wurden mit qPCR ein Mix von *B. ovis* und *B. abortus* entdeckt. *B. ovis* ist weniger pathogen für Kleinwiederkäuer als andere *Brucella* Stämme und somit sind die Brucellose-Symptome, worauf gewisse Proben gesammelt wurden, wahrscheinlich nicht verursacht durch *B. ovis* alleine. *B. ovis* wurde noch nie für die Mongolei berichtet. Die Bruce ladder *Brucella* spp. PCR, welche zur Differenzierung der Stämme anhand von Kulturen benutzt wird, kann *B. ovis* Positivität nicht gut aufzeigen und somit sollen andere Nachweismethoden evaluiert werden.

Wir haben ein demografisches Model für Kamel- und Rinderpopulation in den östlichen Provinzen angepasst. Andere Spezies wurden nicht ins Model aufgenommen, weil wir vorerst die epidemiologischen Beziehungen zwischen Kamelen und Rindern klären wollten. Die Übertragung innerhalb und zwischen Rindern und Kamelen wurde untersucht. Das Model mit allen möglichen Übertragungswege hatte die beste Abdeckung (‘pay-off’). Unerwartet war, dass die Passung der Daten stärker für die Kamel zu Rind Übertragung als

die Rind zu Kamel Übertragung war. Inter-institutionelle Zusammenarbeiten zwischen der Veterinär- und der Humangesundheit müssen in der Mongolei gestärkt werden, um weiter die Kamelbrucellose zu verfolgen und um gemeinsam soweit fehlendes Wissen für die Elimination der Brucellose in der Mongolei zu erarbeiten.

List of Tables

List of Figures

List of Abbreviations

AHP	Animal Health Project
APHA	Animal and Plant Health Agency
C-ELISA	Competitive Enzyme Linked Immunosorbent Assay
CFT	Complement Fixation Test
CI	Confidence Interval
FPA	Fluorescence Polarization Assay
I-ELISA	Indirect Enzyme Linked Immunosorbent Assay
ICC	Intraclass correlation coefficient
GEE	Generalized estimating equation
MoFA	Ministry of Food and Agriculture
MoFALI	Ministry of Food, Agriculture and Light Industry
mRBT	Modified Rose Bengal Test
NCCD	National Centre for Communicable Diseases
NSO	National Statistical Office of Mongolia
NZD	Neglected Zoonotic Diseases
OR	Odds ratio
RBT	Rose Bengal Test
SCVL	State Central Veterinary Laboratory
SVM	School of Veterinary Medicine
SVM-MULS	School of Veterinary Medicine, Mongolian University of Life Sciences
Swiss TPH	Swiss Tropical and Public Health Institute
UNDP	United Nations Development Programme
VABA	Veterinary and Animal Breeding Agency

1. Introduction

Brucellosis is still endemic in Mediterranean countries, Eastern Europe, the Middle East, Africa, South and Central America, and Asia (Herrick *et al.*, 2014). Brucellosis in livestock causes enormous economic losses in developing countries and poses a severe health risk to consumers of dairy products and people in close contact to infected livestock (Kansiime *et al.*, 2014; Shimol *et al.*, 2012; Zinsstag *et al.*, 2015). It is considered nowadays as one of the neglected zoonotic diseases (NZD) (WHO).

Brucellosis is one of the most widespread zoonoses world-wide (Dean *et al.*, 2012). *Brucella abortus, B. melitensis* or *B. suis* strains affect people and domesticated animals including cattle, sheep, goat, camels and pigs, but also related wild animal species are susceptible. Therefore, the disease can thus transmitted from various animal species (Megersa *et al.*, 2012) to people, but transmissions from cattle and small ruminants directly or indirectly via their products to people are most important.

Little information is available on the epidemiology of camel brucellosis and its impact on human health (M. Gwida *et al.*, 2012). Camels belong to the even-toed ungulates (order of Artiodactyla) and are also ruminants as are cattle and small ruminants, but at the level of the taxonomic family are not Bovidae (with a four chambered stomache), they are Camelidae (with a three-chambered stomache). However, we will refer to ruminants in this thesis when we actually meaning domesticated Bovidae including cattle and small ruminants.

Camelidae include new world camelidae (llamas and alpacas) and domesticated old world camels that are either one-humped camels (dromedaries, *Camelus dromedarius*) or two-humped camels (Bactrian camels, *Camelus bactrianus*). The two old world species are closely related and can be cross-bred with fertile descendants. Next to having one or two humps, an interesting differentiating feature is that dromedaries are not susceptible to Foot and Mouth Disease (FMD) whereas two-Bactrian camels are (Larska *et al.*, 2009). Camels are adapted to a dry environment and heat: Dromedaries are kept in warm arid and semi-arid regions and Bactrian camels in cold arid regions such as in Mongolia. Camels have fewer sweat glands and they can close their nostrils. Physiological adaptations include oval-shaped red blood cells that flow quicker in a dehydrated state; concentrated urine and dry dung. Kidneys and intestines are efficient in retaining water. Camels can tolerate water losses up to 30% of their body weight (Franklin, 2011). Reasons for keeping camels are manifold: milk, hides, meat, transportation means and highly valued camel wool of Bactrian camels. Owing to degradation and desertification of formerly more productive pastures, numbers of camels are increasing worldwide.

Camel brucellosis has not received proper attention from researchers and authorities (M. M. Gwida *et al.*, 2011). Brucellosis was reported in camels as the first time in 1931 (Abbas & Agab, 2002; M. Gwida *et al.*, 2012). Since then, brucellosis has been reported from virtually all camel-keeping countries. Camels are not known to be a primary host of *Brucella* spp, but they are susceptible to both *B. abortus* and *B. melitensis* (M. Gwida *et al.*, 2012). Their epidemiological role in spread and maintenance of brucellosis in mixed livestock keeping systems is hardly understood - and this in the face of rapidly growing camel populations.

1.1. History of brucellosis in Mongolia

In the 1960^{ies} in Mongolia, **test and slaughter** campaigns of cattle and small ruminants were carried out. These have decreased the overall brucellosis prevalence in livestock (and subsequently human brucellosis incidences), but were not well aligned with the socio-cultural context in Mongolia, i.e. the mobile livestock husbandry system. The first **livestock vaccination campaign** for small ruminants took place between 1975 and 1986 with financial and technical assistance from WHO and UNDP. It led to a dramatic decrease of human brucellosis incidence. The prevalence of animal brucellosis was down to below 1%. However, the remaining prevalence was high enough for the disease to spread again as soon as the vaccinations stopped. The change of government in the 1990s affected greatly the health and veterinary systems. A next planned mass vaccination campaign 1991-1995 was not implemented due to the end of the Soviet period in Mongolia in 1991. The government-funded surveillance system lacked funding and new private veterinarians were less interested in disease surveillance and control work. Subsequently, brucellosis started to increase sharply in 1993. Mongolia recorded the second highest incidence worldwide and the highest in the WHO SEA region. A next mass vaccination was then implemented between 2000 and 2009. However, this campaign did not achieve the needed immunisation coverage due to an un-anticipated fast growing livestock population (denominator) with the use of the same annual vaccine doses (numerator) that were calculated in 2000 (Roth, 2007; Shabb *et al.*, 2013)

In 2006, there were only two countries with estimated **human brucellosis incidences** > 500 cases / 100’000 and year – these were Syria and Mongolia (Pappas *et al.*, 2006). According to the Mongolian National Centre for Contagious Disease (NCCD), most new human infections occurred during the lambing season between March and May and during the main slaughtering season from October to end of November. The main sources of human infection were contact with aborted foetus, manual removal of retained placenta and traditional home slaughter of animals by cutting the abdomen to manually rupture the aorta. High risk groups (whereby one person could belong to more than one group) among the

diagnosed cases were herders (51%), abattoir workers (21%), leather and wool factory workers (36%) and veterinarians (MOFALI statistics, 2000). Sixty-six per cent of patients were women (Baljinnyam *et al.*, 2014; Roth *et al.*, 2012). In addition to the above cited high risk groups, consumers of raw milk products can be at risk since the bacteria can multiply in the mammary lymph nodes and bacteria shed into the livestock milk (Alton & Forsyth, 1996; Dagnaw, 2015).

Representative multi-stage cluster sampling surveys were conducted to assess the seroprevalence of brucellosis in sheep, goats, cattle, yaks, camels and dogs in Zavkhan and Sukhbaatar Aimags (provinces) in 2010. In Sukhbaatar, the found seroprevalences were 5% for goats, 7% for sheep, 8% for cattle and 3% for camels – and all were significantly higher than those reported from 1990 to 2008 (Baljinnyam *et al.*, 2011). A new national vaccination campaign started in 2010 in one Western Province – Zavkhan. Table 1.1 shows the vaccination scheme of the ongoing vaccination in cattle, sheep and goats (sheep and goats are referred to small ruminants in the following). Note that the initially proposed scheme is currently being re-discussed in view of insufficient production of vaccine doses in Mongolia and if young stock alone could be vaccinated for three years in a row.

Table 1. 1 Vaccination scheme of the Mongolian

Number of provinces (location)	Years of vaccination starting 2010											
	10	11	12	13	14	15	16	17	18	19	20	21
1 (Zavkhan Aimag)	W	Y	Y	W	Y	Y	W	Y	Y	W	Y	Y
8 (Western Aimags)		W	Y	Y	W	Y	Y	W	Y	Y	W	Y
9 (Central Aimags)			W	Y	Y	W	Y	Y	W	Y	Y	W
3 (Eastern Aimags)				W	Y	Y	W	Y	Y	W	Y	Y

Vaccination scheme of the Mongolian cattle and small ruminant vaccination programme 2010 – 2021, W: whole herd; Y: young stock alone

1.2. Current control efforts and epidemiological knowledge

The creation of a National Reference laboratory for Brucellosis is ongoing in Mongolia. The main objectives of this Reference Laboratory are to have a bank with positive and negative gold standard sera from Mongolian livestock and people (confirmed by culture) and reference *Brucella* strains to standardize diagnostic procedures and reagents, and to assess vaccine quality (Blasco & Roth, 2012).

Currently, many gaps exist in the knowledge of the epidemiology of brucellosis in camels in Mongolia. Mass livestock vaccination campaign never covered camels (nor horses), and is also the case in the ongoing campaigns. Reports from veterinary laboratories have indicated that the prevalence of brucellosis in camels in some localities in Mongolian is increasing

(unpublished data). A notable 3% seroprevalence have been found in a population-based survey in Sukhbaatar province in 2010 (Baljinnyam *et al.*, 2011). A large screening survey for 8 livestock diseases (brucellosis, bovine tuberculosis, glanders, Maedi Visna, tick-borne encephalitis, West Nile Fever, Infectious Anaemia and Enzootic Leucosis) in 2011 showed high seropositivity in camels in Dornod and Sukhbaatar provinces: 37% of 260 tested camels in Dornod and 1% of 469 camels in Sukhbaatar (Unpublished results, 2011). The difference between the two neighbouring Eastern provinces is difficult to explain but could include a systematic error in the laboratory assessment.

For surveillance and control of brucellosis, sensitive and reliable detection methods are needed. This is true for both epidemiological studies and monitoring of vaccination campaigns. Although serological tests are the mainstay of diagnosis in livestock brucellosis including camels, these tests have been directly transposed from cattle without adequate validation for camels (M. M. Gwida *et al.*, 2011).

Infected (or vaccinated) animals are most commonly detected using serological tests based on the detection of antibodies against lipopolysaccharide (LPS), the dominant antigen of the outer membrane of the organism (Sanogo *et al.*, 2013). However, the gold standard for the diagnosis of brucellosis remains the isolation and identification of the organism (Rahman *et al.*, 2013).

As to brucellosis serology, the Rose Bengal test (RBT) and complement fixation test (CFT) are commonly used for the routine serological diagnosis of ovine/caprine and bovine as well as human brucellosis (Portanti *et al.*, 2006). A positive result with the RBT is usually confirmed by some other more specific serological tests like CFT or the indirect or competitive Enzyme–Linked Immunosorbent Assays (ELISAs) (Ghanem *et al.*, 2009; Schelling *et al.*, 2003). During recent years different indirect-ELISAs using smooth Lipopolysaccharides (S-LPS) as the antigen have been reported to be at least as sensitive and specific as the combination of both RBT and CFT for the diagnosis of brucellosis in ruminants (Ghanem *et al.*, 2009). Note that sensitivity of the RBT is an issue for sera from small ruminants and thus it is recommended to use 3 parts of sera and 1 part of reactive. This increases sensitivity but reduces specificity (OIE, 2009). In contrast for human sera the low specificity of active brucellosis is rather the issue. The modified RBT looks at titres obtained at different dilutions ofhuman serum (Diaz *et al.*, 2011). The more diluted a serum still gives a positive result, the more specific the result, particularly for active brucellosis.

In addition, the detection of antibodies does not always mean there is an active case of brucellosis. Vaccinated animals can yield persistent post-vaccine immune responses still months after vaccination (and the persistence is longer after sub-cutaneous vaccination than

after conjunctival vaccination). Other gram-negative bacteria such as *Yersinia enterocolotica* may cross-react with smooth *Brucella* spp. (Hamdy & Amin, 2002). In general, these tests are validated with sera from the manufacturers' regions – mainly in industrialised countries. Therefore, they need to be critically reviewed for their use in other regions. Camel antibodies have special features (single domain antibodies) and tests designed for cattle and small ruminants cannot be used uncritically. Only few studies have assessed the performance of diagnostic tools for use in camels.

1.3. A review of camels in Mongolia

In Mongolia, a Bactrian camel herd yields many different kinds of profits. Its milk yield is comparable to a cow and transports as much as a horse. An adult camel produces in a life-time 300 - 480 kilograms of wool, 7000- 8000 litres of milk and 8 - 10 calves. They can travel 800 - 1000 kilometres in one go and carry 180 - 200 kilograms on their back or 250-300 kilograms harnessed to their sides. One camel equals the meat of 7-8 sheep, the wool of 5 sheep and soft wool of 10 - 12 goats (Buyankhishig, 2011).

In Mongolia, the camel population was 228'700 in 1910. It's peak was in 1954 with 895'300 camels and since 1955 decreased continuously to 559'000 in 1985, 537'500 in 1990, 476'000 in 1992, 367'500 in 1995, 315'500 in 2000 and 254'200 in 2005 (Buyankhishig, 2011; Fukuda, 2013; Namshir & Yondondorj, 1993). The numbers then slightly increased to 277'100 in 2009 (MoFA, 2010). The organisation of the camel husbandry and livestock production as well as access to markets have strongly influenced the total number of camels kept in Mongolia. For example, with the introduction of the communist 5 years planning system and production in kolkhozes (collective farms), the camel population dropped by 20.3% (174'400 heads) in 1960 - 1965. After breakdown of the planning economy and cooperatives, camels were distributed equally to families. This led to imbalanced structures within breeding herds. For camel breeding, male animals should rotate between different herds. Also, some families had now 3-4 camels due to privatization, but they did not have the experience to herd camels. Many camels were subsequently sold or slaughtered already at 1 to 2 years of age. There was a large reduction of 31.4% (about one-third of the total population and representing 170'000 heads) when privatisation and free market were introduced between 1990 and1995. The total then represented 94% of the current camel population in Mongolia (Baljinnyam, 2016; Buyankhishig, 2011).

In the 1950ies, more male camels were castrated for their use in caravans, for cart pulling, to ride and for transportation in general. Nowadays, transportation is much less important, whereas milk, wool and racing became more important according the demands of the free market economy (Table 1. 2).

Table 1. 2 Productivity parameters of camels in Mongolia (1970 – 2008)

	Unit	1970	1975	1980	1985	1990	1995	2001	2005	2006	2007	2008
Meat	Ton	11.5	18.8	19.4	19.5	23.2	14.8	20.1	14.0	12.1	5.3	6.8
Milk	Ton	1.2	1.5	1.1	1.0	1.0	0.8	2.0	2.4	4.1	3.8	4.4
Wool	Ton	3314	3013	3089	2846	2431	1794	1572	1002	969	975	1002
Calves	Per 100 female camels	34	43	33	39	38	40	32	45	44	45	42

Although the number of calves per 100 females increased, the productivity declined lately, mainly because the products could not be sold well in the market. The amount of sold wool per camel decreased not due to lower production, but rather due to poor processing and marketing (Table 2). Camel meat represents 2.4 - 2.8% of the national meat supply. An average of 8'000 tonnes of meat is produced each year from 30'700 slaughtered animals. Milk and dairy products of camels are important and in dry and desert regions cow milk cannot replace the needed supply. A lactating camel produces 0.4 to 1.7 (Buyankhishig, 2011; Indra *et al.*, 2003), and about 2 litres per day in August (Ishii & Samejima, 2006). The milk yield of the Bactrian camel is lower than that of the dromedary (Lensch, 1999).

If the negative trend in camel numbers continues, this might lead to a reduced genetic variability in Mongolian camels, which, in return, could affect production traits as well as the potential for adaptation. Authors have stressed the importance of preserving the current variation in the Mongolian camel population as a highly valuable, desert livestock species (Chuluunbat *et al.*, 2014).

1.4. Brucellosis in Mongolian camels

Camel brucellosis seropositivity was estimated at 4.9% in 1964 (Baljinnyam, 2016). Shumilav tested Mongolian camels in 1974, and he determined that CFT was four times more sensitive than the SAT. He examined two camel herds with 3'751 and 54'673 animals using both tests and determined a prevalence of 4.3% and 0.6% in herd 1, and 3.7% and 1.0% in herd 2 with CFT and SAT, respectively (Wernery, 2014).

Brucellosis re-spread after the mass vaccination campaigns between 1975 and 1986. It was estimated that the camel brucellosis prevalence was between 20 and 30% (a total of 100'000 – 150'000 affected camels) in 1987-1990. Test and slaughter campaigns were then implemented for camels and other livestock in Mongolia. In Sainshand district (of the Dornogobi province), in 1988 the clinical signs of 10 affected camels were described as a severe disease with limping, lying down or death. In the same district, also abortions in

camels were reported. The sero-prevalence in three tested herds was 48.2% (103/214), 53.6% (80/151), and 53.2% (91/171) (Namshir & Yondondorj, 1993). In the 1980ies, it was estimated that 25'000 female camels each year were infertile due to brucellosis, and that of infected camels 12% aborted. In addition, 65'000 of camels were lost due to the test and slaughter programme (Namshir & Yondondorj, 1993).

In 1991, Mocalov tested 29'300 camels with the RBT, CFT and SAT and an overall seroprevalence of 9.7% was found (Wernery, 2014). In 2003, 17 camels were tested with the RBT and the prevalence of this small sample was 23.5% (Erdenebaatar *et al.*, 2003; Wernery, 2014). More recently in 2010, serological surveys just before the implementation of the mass vaccination campaigns (in cattle and small ruminants) found notable 3% brucellosis seropositivity in camels in Eastern Mongolia (Baljinnyam *et al.*, 2011; Bataa *et al.*, 2010). Indeed, brucellosis seroprevalences were found high (>3%) in camels in the multi-disease screening survey in Dornod in 2011 (Unpublished results, 2011) We could analyse the brucellosis serology data of this screening survey. There were between 6 and 3590 camel sera from the 22 Mongolian provinces. We found a moderate correlation (Spearman's rho of 0.26) between camel and cattle seropositivity at district level, however, sheep were only very weakly correlated and goats not at all (unpublished data).

Genetic analysis using PCR on *Brucella* spp. isolates of camels from different countries – including an isolate from a Mongolian camel – showed that they all belonged to *Brucella abortus* biovar 3 and were grouped with the Chinese *B. abortus* bv.3 (Ji-Yeon Kim, 2016).

1.5. Goal, Objectives and Hypotheses

This PhD work was set-up to start to bridge the most prevailing knowledge gaps on camel brucellosis in view of ongoing mass vaccination against brucellosis in cattle, sheep and goats in Mongolia, but also on the impact of brucellosis on the Mongolian camel population and its related livelihoods. The main knowledge gaps identified were the availability of validated diagnostic tools for camels, the knowledge on the epidemiology of brucellosis in camels (including its impact on human health), and also if camels need to be targeted in future control efforts or if camels will not pose a threat to vaccination efforts in other ruminants once the mass vaccinations take no longer place. Can the disease be maintained in camels or are they only spill-over hosts? Recommendations should be validated with authorities and communities.

Goal

The overall goal was to contribute to the understanding of effective and long-term control of brucellosis in Mongolia.

Objectives

The specific objectives were the following:

1. To describe the epidemiology of camel brucellosis in Mongolia
2. To validate serological diagnostic tests for camels
3. To identify the causative agent of camel brucellosis in Mongolia
4. To make recommendations on diagnosis and on priority interventions in view of ongoing ruminant vaccination and propose next steps with authorities and communities

Hypotheses

The following hypotheses were defined at the very beginning as null hypotheses and have directed the study design

1. The seroprevalence of camel brucellosis is below 5% in Mongolia and the most important risk factor for camel seropositivity is herding together with cattle
2. The main causative agent of prevalence of brucellosis in the Mongolian camel population is *Brucella abortus*
3. There is more variance of camel brucellosis seropositivity at district level than at provincial level
4. Camel seroprevalences decrease as mass vaccination of cattle, sheep and goats goes on
5. Brucellosis control in small ruminants and cattle alone will not lead to stop *Brucella* spp. circulation in Mongolia because camels can maintain the infection

2. General Methodology

2.1. Epidemiology of camel brucellosis in Mongolia

2.1.1. Target population

The target populations were camels and camel owners in Mongolia. Camels in Mongolia are almost exclusively kept with other livestock, mainly sheep and goats, but also cattle. Only very rarely camel owners in Mongolia keep camels alone. Camel herds in spring, when the pastures are generally not good, are continuously on the move to find new pastures and are further away from urban centres than other livestock and when herders want to keep the new-born animals together with their mothers. Note that during this period it is rather difficult to collect milk samples given the protective behaviour of female camels (who can be rather aggressive towards people after calving). The density of camels in Mongolia is shown in Figure 2. 1. The density is highest in the South, including the Gobi desert.

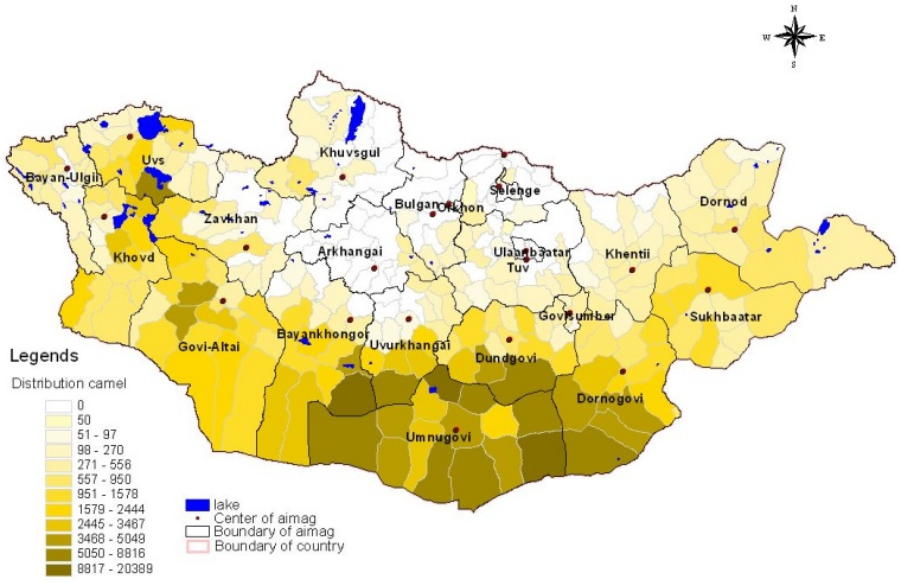

Figure 2. 1 Camel populations are in Southern Mongolia

Camel populations are mainly present in Southern Mongolia, the drier areas of Mongolia including the desert Gobi. Mongolia borders with China in the South and Russia in the North (NSO, 2015)

2.1.2. Study design

A repeated cross-sectional study was conducted to assess the epidemiology of camel brucellosis in Mongolia. The study included in its first year (2013) two provinces (Aimags) in Eastern Mongolia - the in 2013 last two provinces without introduction of mass ruminant vaccination and that had important camel populations. These were re-sampled in the

following year 2 (2014). In years 2 and 3 (2015), three more Aimags in the other parts of Mongolia were enrolled based on proportional to size selection according to their respective camel populations (Figure 2. 2, Table 2. 3).

Figure 2. 2 The selected provinces (Aimags**)** Sukhbaatar, Dornod, Umnogobi, Dornogobi and Khovd

Sampling started in the lighter shaded Eastern provinces in 2013 and were re-sampled in 2014. The darker grey three Southern & Western provinces were sampled in 2014 and 2015.

Table 2. 1 The sampling plan

	2013 and 2014		2014 and 2015		
Province / District	Sukhbaatar	Dornod	Umnogobi	Dornogobi	Khovd
1	Baruun-Urt	Gurvanzagal	Dalanzadgad	Ikhkhet	Bulgan
2	Dariganga	Choibalsan	Bulgan	Airag	Dorgon
3	Ongon	Tsagaan-Ovoo	Khankhongor	Delgerekh	Duut
4	Sukhbaatar	Khalkhgol	Gurvantes	Sainshand	Zereg
5	Tumentsogt	Sergelen	Tsogt-Ovoo	Saikhandulaan	Mankhan
6	Uulbayan	Bayantumen	Khurmen	Khuvsgul	Munkhkhairkhan

The sampling plan indicating the provinces and the six proportionally to size selected districts

Herds selected in a first year were revisited a following year. There were no vaccination campaigns in Sukhbaatar and Dornod in 2013, but ruminant vaccination started in 2014. Umnogobi was in 2014 and 2015 the only Mongolian province not covered by livestock brucellosis vaccination; however, there were vaccinations in 2014 and 2015In Dornogobi and Khovd provinces. Sampling took place 5.5 – 6 months after a vaccination campaign (Figure 2. 3).

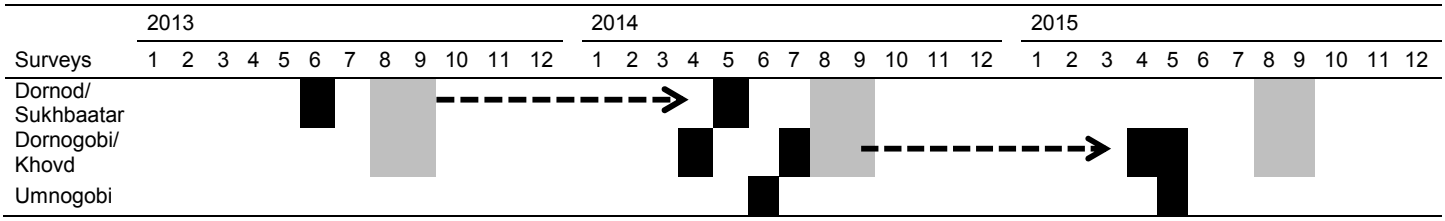

Figure 2. 3 The chronology of the surveys in the five provinces

The chronology of the surveys in the five provinces (months coloured in black) and the livestock vaccination campaigns indicated as light grey bars. The months between a past campaign and a survey is shown as dotted flash: the periods between vaccination and sampling were 5.5 – 6 months.

Same herds were to be re-visited, if possible, because in Dornod and Sukhbaatar we wanted to assess if serological status, health and probability of getting isolates from camels changed between the surveys before and after introduction of vaccination of other ruminants. In other Aimags, we wanted to see if serological status changed between years with ongoing vaccination. We expected that seropositivity of camels would decrease because the infection pressure of brucellosis transmission from cattle and small ruminants to camels was decreased.

2.1.3. Sample size calculation

The cluster sample size calculation considered an intraclass correlation coefficient (ICC, also called ‘rho’ [p]) which is the ratio ‘variance between cluster / total variance’ (Bennet *et al.*, 1991). An ICC of 0.1 was taken for all livestock species, indicating that livestock within clusters (herds) were slightly more alike than livestock in other clusters. An ICC of 0.1 had been reported for a range of endemic zoonosis (Otte & Gumm, 1997) and is in addition based on previous livestock brucellosis serological surveys in Mongolia (Baljinnyam *et al.*, 2014). An ICC of 0.1 led to a design effect D of 1.2 and 1.4 when 3 and 5 animals, respectively, were sampled per cluster.

The sample size calculation for one province further considered that the maximal standard error should be maximal +/-2.5% (which would give a precision of +/-5% at a 95% level of confidence). We assumed seroprevalences of the different livestock species as were reported for Sukhbaatar in 2010 (3% for camels, 5% for goats, 7% for sheep and 8% for cattle). The total calculated sample size for one province was to sample in 30 herds at least 3-5 camels, 3-5 cattle, 5 sheep and 5 goats (Table 4).

Table 2. 2 The sample sizes for each livestock species

	Assumed seroprevalence	Total sample	N herds	N individuals per herd	Precision of the estimate
Camels	3%	≥90	30	≥3 (up to 5)	+/- 2%
Goat	5%	90	30	3	+/- 5%
Sheep	7%	150	30	5	+/- 5%
Cattle	8%	160	32	5	+/- 5%

The sample sizes for each livestock species calculated for one province together with the estimated precision at a confidence level of 95%. The total of calculated livestock in one province and year was 490 animals in 32 herds. We have planned to include more than 5 camels where other livestock species were not present. To account for none-participation in the study and absence of a species in a herd, an additional 4 herder families were to be included in the initial selection of herds – thus 36 herds to be selected in one province.

2.1.4. Random multi-stage cluster sampling

We went from province (Aimag) – district (Soum) – household (and respective hot ail of a selected household) and randomly selected livestock - and thus had a four stage cluster sampling (WHO, 2015). The unit in the sampling frame list were the households registered in 2012 and in 2013 in the districts. One household with its animals is rarely alone. Households rather stay within a hot ail: several families who pasture their livestock together and share watering places. A hot ail was the ‘epidemiological unit’. All animals of a hot ail were considered as the herd belonging to the household. Interviews were done with the selected livestock owner (if resampled in a following year, commonly only one interview was done at the first encounter).

Given the expected variance at different levels and to best use available logistical field resources, we concluded that sufficient but not too many Aimags should be included, but rather more herds in one province. Indeed, we expected relative high variance between herds as has been found in previous studies, e.g. the baseline study on brucellosis in Sukhbaatar. The rational to include 5 Aimags was based on expected moderate variance between Aimags – and also that Aimags were at different stages of implementation of the vaccination campaigns.

First (1st) stage sampling: Provinces (Aimags)

In the Eastern provinces Sukhbaatar and Dornod, sero-prevalence of brucellosis were high in camels during the eight disease screening survey in Dornod (2011) and during an epidemiological survey (2010). Both Aimags have substantial numbers of camels, although not as high as in the Southern Aimags (Table 6). The main reason to enrol these two Aimags was that the livestock vaccination campaigns only started in September 2013 and therefore we could sample before and after introduction of the vaccination campaigns.

The other 3 provinces (Umnogobi, Dornogobi and Khovd Aimags) were selected randomly proportional to the size of the camel populations (NSO, 2012). People and livestock were unevenly distributed in the 5 selected provinces. In Umnogobi province, the proportion of sheep and cattle per camel were much smaller than in other provinces. The 5 provinces were divided in 73 districts and total of 7.7 million cattle, sheep, goats and camels (Table 2.3).

Table 2. 3 Basic demographics of the 5 selected provinces (Aimags) in 2012

#	Province	N District	Human pop.	%pop. Aimag/ Soumcentre	Camel	Sheep	Goats	Cattle	Total livestock	Ratio[a] / ha
1	Sukhbaatar	13	55,648	51	9,752	1,090,831	845,297	151,132	2,097,012	37.6
2	Dornod	14	74,723	75.4	5,007	584,778	346,886	119,737	1,056,408	14.1
3	Umnogobi	15	56,585	66.4	97,317	299,816	961,258	10,762	1,369,153	24.2
4	Dornogobi	14	60,935	48.1	31,446	531,494	619,137	50,298	1,232,375	20.2
5	Khovd	17	78,449	50.4	18,634	758,206	1,085,371	100,742	1,962,953	25
	Total selected Aimags	73	326,340		162,156	3,265,125	3,857,949	432,671	7,717,901	

[a]Ratio animal (camel, sheep, goats and cattle) / human population; (NSO, 2012)

Second (2nd) stage of sampling: Districts (Soums)

The selection of each 6 districts in the provinces was also proportional to size of camels. The selected districts are shown in Table 3. The field team visited the district veterinarians to inform them about the purpose of the study.

Third (3rd) stage of sampling: Hot ails and households

The district authorities (Governor and officers) provided the most recent updated (about 6 months prior to a survey) list with all registered households in the district. This list also included the number of different livestock species kept. Non-camel owners and those having less than 3 camels were excluded from selection. Eligible households were randomly selected from the list in Excel using the rand() command. The initial selection was done for a total of 36 camel-keeping households (6 for each of the 6 districts) considering that not all households would be found.

With the help of the district veterinarians, who knew about the approximate whereabouts and itineraries of the selected families, a rough map of zones to be visited was drawn and a travel plan prepared. All selected families found and who have agreed to participate in the study were enrolled. As the families stayed within a hot ail - where animals of 2-3 families are herded together - the sampling unit was the hot ail herd. During a visit in a hot ail, the field team introduced in a standardised way to all hot ail members the goals, purpose, implications, their time needed as well as what happens with the sera and reporting of the results. A signed informed consent of the selected livestock owner was sought. Provided that the household/hot ail agreed that their animals were blood sampled, livestock owners were asked to drive sheep and goats into sheep fences.

When an initially selected family could not be found in the expected zone, or did not consent to participate, or did not have time to participate due to immediate moving, or also when the camel herd was far away after watering, a replacement selection of the nearest hot ail nearest in Northern direction was made. A replacement of a hot ail for the first contact was made in less than 6% of cases in Sukhbaatar and Dornod provinces, while it went up to 30% of cases in Khovd, Dornogobi and Umnogobi provinces.

Each province was to be re-sampled in the following year and, if ever possible, same hot ails enrolled. We could contact some previous participants per mobile phone and ask their position and availability of the camel herd. Where telephone contact was not possible, the team went to the same zone of last year's place of encounter and searched the family with the help of herders in a radius up to 30 kilometres. If the family was not found, the nearest to last year's place was enrolled. Where we knew from the beginning that a next visit was not possible, an initially selected but not yet enrolled family was searched and asked for participation. A replacement hot ail was enrolled in 10% in the second year in Dornod and Sukhbaatar. This proportion was higher at 40% for the second year in Umnogobi, Dornogobi and Khovd due to having the camel herds further away from the household and more frequent movements of families in these provinces.

A questionnaire was filled in with one member of the selected herder family. The interview included questions on the i) knowledge on epidemiology of brucellosis ii) herd risk factors (including buying/selling of animals, sharing of pastures, cross-border movements), iii) herd demographics, iv) herd health, and v) family health. The coordinates of the hot ail at time of visited was recorded with a GPS and North and East coordinates filled in the herder questionnaire. The mobile phone number of each participant was recorded for feed-back on the results and to make appointment for a re-visit one year later. The questionnaire was

pretested and a translation from Mongolian to English and back translation was done for verification of translation errors.

Fourth (4th) stage of sampling: Individual animals

The field team members selected with a random number a first goat and sheep while they were coming out of the fence area. Then every i^{th} sheep and goat was sampled according to the sampling interval. Camels and cattle were selected by spinning a bottle and a random number to tell which animals were to be included in the direction of the bottle head. Species, sex, age of animals , breed, and main use were recorded for each animal on a data sheet that also included date and hot ail id and if there were any clinical symptoms (e.g. abortions) in the herd in the past couple of years.

Samples: Eight (8) mL of blood were collected from the jugular vein of each livestock using a Vacutainer with disposable needle. Each animal's identification number was labelled on the corresponding Vacutainer tube whichwere put 2-3 hours in a box before centrifugation with a portable centrifuge during 5 to 10 minutes for 1000-1500 rpm. The serum was transferred into two 2mL tubes. The red blood coagulate was destroyed according to biosafety guidelines.

Vaginal swabs and milk samples were taken for bacteriology from camels, cattle and small ruminants with history of abortionin the herd. The swabs were placed in transport medium tube (BD BBL^{TM}Culture swab plus, Amies without Characoal, Becton Dickinson, France). As to milk samples 10–20 mL of milk were taken from each teat. The first streams were discarded and the sample was milked directly into a sterile vessel (OIE, 2008).

The sera for serology and swabs and milks samples for bacteriology were stored on ice in a cool box and transported regularly to the Veterinary Laboratory at the province centre where they were kept at –20°C until transported to the School of Veterinary Medicine and to the State Central Veterinary Laboratory (SCVL) in cool boxes.

2.1.5. Field team

The field team was composed of a driver, the PhD candidate and one master student, one local assistant knowing the roads and the whereabouts of the hot ails and who could assist in the laboratory work. Since the team was composed by a local veterinarian, sick livestock could be examined and treated on the spot. Herders were also encouraged to inform the veterinarian or the PhD student by mobile phone in case of observed abortion for further sampling of material to be used for culturing.

2.1.6. Data management and analysis

A database was be maintained in MS Access, and analysed using Stata 14. Double data entry tables in Access were done for all questionnaires and livestock data sheets as well as laboratory results and the double entered data sets were compared and cleaned (by cross-checking the original questionnaires/forms) in Epi-Info 3.5.

2.1.7. Ethical considerations

The study was in the framework of previous studies including both people and livestock (but without camels) that have obtained formal ethical clearance by the ethical committee of the Ministry of Health in 2012. In this study, confidentiality was guaranteed. All information was analysed with anonymous data sets. All questionnaires and data were stored safely. Samples and data were only used for the purpose stated in the information for participants and the project information. The following ethical issues were further considered:

- Safety was very important and all potential risks were minimised with application of best practices and professional handling
- Best practices were applied to assess livestock brucellosis in a herd
- Interviews were conducted in a private environment
- The sample size was well justified
- Animal owners with positive serological results in their livestock were contacted on their mobile phone by the study team to report on the finding. They were informed that they should protect themselves during obstetric work/slaughtering, boil the milk before consumption and do not consume the fresh blood and raw livestock products. Also they were advised that all ruminants should be vaccinated and all camels re-tested. They were also advised how they can best prevent that a potentially infected animal enters their herd.

2.2. Assessment of serological diagnostic tests for camels in Mongolia

2.2.1. Serological tests

The majority of studies on camelid brucellosis are performed based on serological methods for diagnosis, but none of the serological tests are yet validated on camel brucellosis, as acknowledged by the World Organisation of Animal Health (OIE) (Wernery, 2014). Infected animals are detected using serological tests based on the detection of antibodies against lipopolysaccharide (LPS), the dominant antigen of the outer membrane of the organism (Nielsen, 2002). Classical serological tests include the Rose Bengal Test (RBT), the complement fixation test (CFT) and serum agglutination test (SAT) all of which employ a

whole cell antigens the key diagnostic reagent. More recently tests are such as the I-ELSIA, C-ELISA, and the FPA employ purified LPS or O-antigen is the basis for the generally good sensitivity of these assays. However, this use of this antigen can lead to false positive results when animals are infected with bacteria possessing O-antigen of similar structure such as Yersinia enterocolotica O:9. From prior studies we know that most of these tests of have been used for camel sera and have provided reasonable results, although there were few attempts to determine the actual concentration of antibodies needed to make a diagnostic test positive. The IgM response is followed almost immediately by production of IgG1 antibodies and, inconsistently, by smaller amounts of IgG2 and IgA. The main isotype for serological testing is IgG1. Serological tests that measure IgM are not desirable as false positive results occur, leading to low assay specificity (Nielsen, 2002). The CFT detects IgG1 antibodies but not IgG2, in excess can cause prozoning (in an agglutination or precipitation reaction, the zone of relatively high antibody concentrations within which no reaction occurs) or even false-negative reaction in IgG1-containing sera. Few animals may be negative to the RBT but positive to the CFT. It can be that these results could be expected if there is some serum IgG, antibody, but very little IgM antibodies. This may be the situation in a chronically infected animal, in which continued exposure to antigen has reduced the level of IgM (**Table 6**) (Chappel, 1989; Nielsen, 2002). Therefore, most assays predominantly measure IgG1 which is the most useful.

Table 2. 4 Concentration of antibodies of different isotypes

	Concentration of antibody ug/ml		
	IgG_1	IgG_2	IgM
CFT	10	-	5
SAT	100	100	10
RBT	50	50	5

Concentration of antibodies of different isotypes required to generate a minimal positive reaction in three serological tests.

This study was used the following serological tests: Rose Bengal test (RBT), modified Rose Bengal test (mRBT), complement fixation test (CFT), indirect enzyme linked immunosorbent assay and competitive enzyme linked immunosorbent assay (I-ELISA and C-ELISA) as well as the fluorescence polarization assay (FPA). These serological tests were validated for brucellosis in cattle sera.

2.2.1.1. Rose Bengal Test

The Rose Bengal test (RBT) is the most widely used serological test for brucellosis in all livestock species. The test is recommended as a suitable screening test for brucellosis with high sensitivity to befollowed by confirmatory test (OIE, 2009). The RBT is technically simple

to perform; it is a rapid result and less costly for epidemiological studies at local and national levels. Antigen for the Rose Bengal test is prepared from killed standard strain of *B. abortus* and stained with Rose Bengal dye, which is suspended in acid buffer pH 3.65. Equal volume (30 µL) of stained antigen and test serum is mixed. After thorough mixing on a white glossy tile, the mixture is rotated gently for up to four minutes before reading. The result is read and recorded as positive and negative based on the absence and presence of agglutination due to an antigen and antibody complex (Getachew *et al.*, 2016; OIE, 2009). If incubated for more than 4 minutes, sometimes false reactions occur due to the formation of fibrin clots (Poester *et al.*, 2010).

2.2.1.2. Complement Fixation Test

The Complement Fixation Test (CFT) is the recommended confirmatory test for brucellosis seropositivity given its high specificity (but lower sensitivity) (OIE, 2009). The CFT is complex and time-consuming to perform and requires numerous preparatory steps and well trained laboratory staff. An important number of reagents and their controls must be titrated daily. Most conveniently these are carried out in microtitre well-plates. The basic test consists of *B. abortus* antigen, usually in form of whole bacteria and that is available commercially. Sheep erythrocytes are washed and concentrated (OIE, 2009). A lyophilisation complement (usually guinea pig sera - that is also available commercially) is reconstituted with distillation water and titrated with a haemolytic system (so that equal volume of the sheep erythrocytes and heamolysin is achieved). A haemolysin (rabbit anti-sheep erythrocytes antibodies) is also titrated with the sheep erythrocytes and complement. Test sera are diluted (1 : 2.5) and incubated for heat inactivation (to destroy any indigenous complement) in a water bath at 56°C for 30 minutes. Buffer is added to all wells of a 96-well-plate with round (U) bottoms. Positive control is added in first well, followed by a negative control (these can be bought commercially). In the remaining wells of the same colon prepared test sera are added. The amount is the same as the buffer, thus sera are diluted 1 : 5. Half of these mixtures are pipetted from one colon to the next until a dilution of 1 : 40 is present (in the last, 4^{th}, colon, half of the mixture is discarded). Antigen (predetermined by titration) is added in each well. Also, the antigen, complement, haemolysin controls are tested on the same plate. Dilution complement (predetermined by titration) is added in each well. The well-plate is covered with a sealing tape and is incubated in a water bath at 37°C for an hour. Prepared haemolysin solution (equal volume of 2.5% of prepared sheep red blood cells (RBCs) and dilution haemolysin) is added in each well. The late is again sealed, gently shaken and is incubated at 37°C for 30 minutes. Before reading of the results, for the plates are stored for 18-22 hours at 2-8^{0}C in a refrigerator. No lysis (non-haemolysis) of

sheep RBCs indicates the presence of antibodies in the test serum, while lysis of sheep RBCs indicates the absence of antibodies in the test serum. Results are interpreted as negative if 100% haemolysis of sheep RBCs, while positive if 75%, 50%, 25%, 0% of sheep RBCs haemolysis. Results are recorded as "-" "+", "++", "+++", "++++",(Getachew *et al.*, 2016; OIE, 2009, 2016; Poester *et al.*, 2010; Staak *et al.*, 2000).

2.2.1.3. Indirect Enzyme Linked Immunosorbent Assays

Several commercial indirect enzyme linked immunusorbent assay (I-ELISA), using different antigen preparations, antiglobulin-enzyme conjugates (usually horseradish peroxidase), and different substrate are available. Washing procedures are used between each stage of the assay. The most commonly used system depends on enzymes for detection and consists of smooth LPS (S-LPS) preparation attached to a polystyrene matrix in 96 well plates. I-ELISAs have high sensitivity, but the specificity can be rather low. Commercial kits using whole cell, S-LPS or the O-polysaccharide (OPS) as antigens have been validated and results obtained from different assays are not always comparable. I-ELISA for diagnosing anti-*Brucella* antibodies in small ruminants and pigs are essentially the same as those described for cattle, but the cut-offs should be properly established for these species using appropriate validation techniques (OIE, 2016; Poester *et al.*, 2010).

In this study, I-ELISA commercial Brucella abortus Antibody Test kit using short incubation method provided by IDEXX was used. A wash solution was dispensed into each well in 96-well-plate pre-coated inactivated antigen *B. abortus*. Undiluted positive, negative controls and test serums were added into the plate and thus the final dilution of the sera was 1 : 10. This mixture was gently shaken, covered with plate sealing tape and incubated in a water bath at 37°C for 30 minutes. Each well was washed with the wash solution three times. Then the conjugate was added into each well, covered with plate sealing tape and incubated in a water bath at 37°C for 30 minutes. The plate with all its wells was re-washed three times. The substrate was added into each well at room temperature (18-26°C) for 15 minutes. Finally, the stop solution was added and the plate was read using ELISA reader machine. Optical density (OD) was measured at a wavelength of 450 nm. To assess the quality of a plate, the OD of the positive control was not exceed 2.00 and the OD of the negative control not 0.500

Results were calculated as percentage of the ratio between the corrected sample OD and positive control OD (S/P-ratio). S was the OD of the test sample (Sample A_{450}) minus the the OD of the negative control (NCx), over P: the OD of the positive control (PCx) minus the OD of the NCx. S/P %= 100x (Sample A_{450} –NCx) / (PCx-NCx). A cut-off of ≥ 80 % according to the manufacturer was to be considered for positive test samples.

2.2.1.4. Competative Enzyme Linked Immunosorbent Assays

Several variations of the competitive-ELISA, using S-LPS or OPS as antigens, are used for cattle, small ruminants and pig brucellosis serology. Different antiglobulin-enzyme conjugates, substrate/ chromogens and antigens are prepared from different smooth *Brucella* strains. The C-ELISA uses a monoclonal antibody specific for one of the epitopes of the *Brucella* spp. OPS antigens have often been shown for cattle, sheep and swine to have higher specificity, but slightly lower sensitivity than the I-ELISA. This assay is an excellent confirmatory assay for the diagnosis of brucellosis in most mammalian species. Kits are commercially available from different manufacturers (OIE, 2016; Poester *et al.*, 2010).

Commercial Comp-ELSIA kit by APHA (Animal and Plant Health Agency) Scientific was used in this study. The diluting buffer, wash solution, conjugate, substrate (OPD), stop solution and reconstituted positive and negative controls were prepared according to the manufacturer's protocol. Test sera were added into 80 wells of the 96-well-plate pre-coated with *B. melitensis* LPS antigen. In the last two colons each 6 wells were used for positive and negative controls and 4 well for conjugate alone. The (shortly before use) prepared conjugate solution was dispensed in all wells. The final serum dilution was 1 : 6. Plates were shaken for 2 minutes in order to mix the sera and conjugate solution. Then plate were covered with a plate sealer and incubated at room temperature (21°C ± 6°C) by hand shaking every 10 minutes during 60 minutes. A plate was washed 5 times. Substrate was added into all wells and incubated at room temperature (21°C ± 6°C) for 15 minutes. Stop solution was added into all wells and the plate was read using ELISA reader machine at a wavelength of 450 nm.

Results were considered if the OD of the mean of the 6 negative control wells was greater than 0.7; the 4 conjugate control wells was greater than 0.7, the mean of the 6 positive control wells was less than 0.1. Finally, the binding ratio (mean of positive controls / mean of negative controls) was greater than 10. The results of the test sera were more positive the lower the OD. A positive/negative cut-off can be calculated as 60% of the mean of the optical density (OD) of the 4 conjugate control wells. Any test sample giving an OD equal to or below this value should be regarded as being positive.

2.2.1.5. Fluorescence polarization assay

The Fluorescence Polarization Assay (FPA) (Brucella FPA®, Diachemix, LLC) is simple to use for detecting antibodies against *Brucella spp.* and has been more recently developed based on physical principle a molecule spins in liquid medium (Godfroid *et al.*, 2010) and improvement of the Perrin's theory (Dandliker & de Saussure, 1970). The rate of rotation

molecule in solution relates with its mass. By attaching a fluorescing molecule to an antigen molecule, the rate of rotation can be measured using polarized light. Thus, the rotation rate of the specific antigen molecule that extracted from *Brucella* O-polysaccharide, labelled with fluoroscein isothiocyanide (FITC) changes if anti-*Brucella* lipopolysaccharide (LPS) antibodies binds to it, which increases size of the molecule. FPA measure the degree of depolarization in milli-polarization units (mP). In the presence of antibodies against *Brucella* spp., large fluorescent complexes are formed, while negative samples remain uncomplexed.. These smaller molecules spin more quickly and therefore cause greater depolarisation of the light than do positive samples for *Brucella* spp. (Godfroid *et al.*, 2010; Minas *et al.*, 2007; OIE, 2016; Poester *et al.*, 2010).

Sample dilution liquid (1:10) was added in borosilicate glass test tubes in a rack. One positive, three negative controls and test sera were added in tubes with sample dilution. They were incubated at room temperature for 5 minutes. The Conjugate tracer was added and mixed in each tube. A second incubation at room temperature was done for 3 minutes. Tubes were placed in the FPA reader (that was initialised with blank readings). The results in mP unit wer recorded. Controls are rerun after every 50 samples or every 60 minutes. Results were calculated as the sample mP minus the mean of the three negative controls (sample mP – mean negative control mP). Negative results were below 10 mP, doubtful results is between 10 and 20 mP, while positive sample results were above 20 mP.

2.2.2. Assessment of serological tests for camels and test comparisons

Assessing a diagnostic test procedure with binary (yes/no) outcome entails determining the operating characteristics of the test with respect to the disease of interest. The intrinsic characteristics of the test are sensitivity and specificity. Sensitivity (Se) is the probability that the test outcome is positive in an individual with the disease, and is estimated by the proportion of positive test results among a sample of an individual with the disease (cases). Specificity (Sp) is the probability that the test outcome is negative in a subject who is free from the disease of interest, and is estimated by the proportion of negative results in a sample of disease-free subjects. The positive (or negative) predictive value of the test in a given population is the probability that a test positive (or negative) subject has (or does not have) the disease. Although predictive values are of obvious clinical and epidemiological relevance, they are not intrinsic to the test, insofar as they also depend on the prevalence of the disease in the population under study (Flahault *et al.*, 2005)

The needed sample size of positive and negative sera to assess Se and Sp of a test is calculated according to the OIE guidelines. The lower the expected Se and Sp of the test, the higher the sample size. Published If we expect a Se of 0.9 and a Sp of 0.95 of a

diagnostic test, and we allow an error of 0.035 and a level of confidence of 95% (α / Type I error = 0.05), we obtain with the following equation the sample size, where Π is the expected Se / Sp; d the error, $z_{1-\alpha/2}$ the quantile of the standard normal distribution.

$$n \geq \frac{(z_{1-\alpha/2})^2 \pi(1-\pi)}{d^2}$$

We calculate a total of 282 needed cases to estimate the Se and 149 controls to calculate the Sp with precision of +/- 3.5%. The exact binomial CIs are for Se 90 (95% CI 86.0 – 93.3) and for Sp 95 (95% 90.6 – 98.1).

We have cross-checked these numbers of needed negative and positive sera with our sample size in the epidemiological study.

The RBT test for a first triage of positive and negative samples and then compare to herd history was used. Positive sera samples are from positive culture animals of this study (see below Objective 3) and Research Veterinarian Institute that had positive *Brucella* spp. culture and positive serological test results from same herds with *Brucella* spp. cultured camel, and their sera were considered as positive camel sera for test validation. Negative sera are from camel herds in Aimags/Soum with absent camel seropositivity (and certainly absent from any other animal in the herd), no history of abortion and where other sampled livestock where seronegative (when sampled without vaccination campaign), and that were all negative in the applied tests (RBT, ELISAs, CFT and FPA) and we were consider as negative sera.

In addition, serum standards and other reagents, available from OIE, WHO, FAO, or other international organizations, can be used to harmonize the assay with expected results gained from reference reagents of known activity. We could not import to Mongolia *Brucella abortus* Positive Serum and *Brucella melitensis* Positive Serum by Animal Health and Veterinary Laboratories Agency, UK.

We have assumed that we need at least 4 times more samples of potentially positive samples and 2 times the numbers of potentially negative samples totalling in a minimum of 1500 sera samples from camels (of which we also have material for bacteriology). All camel sera should be tested with all serological tests presented above.

Overall prevalences with 95% confidence intervals (95% CI) were estimated for each species for the RBT, CFT, I-ELISA, C-ELISA and FPA results.

The cut-off values of the I-ELISA and FPA as manufacturer's recommendation were set at 80 and 20 mP (millipolarisation level), respectively. A combination of statistical approaches was used to select an optimum cut-off for tests. This was accomplished by insertion of a cut-

off point on the continuous scale of test results (I-ELISA or FPA) then plotted on frequency histograms. Likewise, the cut-point value, the Se and Sp and the area under curve (AUC) and their 95% CI were determined with comparison of pairwise ROC curves.

2.2.2.1. ROC curve and Likelihood ratio

The sensitivity and specificity can be computed across all the possible and different threshold values. Then, the plot of sensitivity versus 1-Specificity is called receiver operator characteristic (ROC) curve and the area under the curve, as an effective measure of accuracy has been considered with a meaningful interpretations (Hajain-Tilaki, 2013). The area under curve (AUC) summarizes the entire location of the ROC curve, and it helps us estimate how high that is the discriminative power of test. This area equals the probability that a random individual with disease has a higher value of the test variable than a random healthy individual (if the variable is raised in sick individuals). A perfect test thus yields AUC of 1, whereas a non-discriminating test gives a value of 0.5 (Thrusfield, 2005). Also, cut-off values were set that optimized using receiver operating characteristics (ROC) analysis (Greiner *et al.*, 2000). The cut-off PP values were approximated by visual inspection of the frequency distribution graphs as the point that gives maximum distinction between positive and negative samples (Jacobson, 1996).

The slope of smooth ROC curve can be interpreted in terms of the likelihood ratio (LR) of the test. The likelihood ratio provides a suitable useful measure of diagnostic accuracy, which is independent of prevalence. It compares the proportion of animals diseased and non-diseased, in relation to their test results. The likelihood ratio of a positive test result (LR+) is the ratio of the proportion of affected individuals that test positive, and the proportion of healthy individuals that test positive. The LR+ is therefore a quantitative indication of the strength of a positive result. The perfect diagnostic test would be have an LR+ equal to infinity (detecting all true positives, and generating no false positives), and the best test for ruling in a disease is therefore the one with the highest LR+. The likelihood ratio of a negative test result (LR-) is vice-versa. Perfect diagnostic test would have an LR- equal to zero (producing no false negatives, but detecting all true negatives), and the best test for ruling out a disease is therefore the one with the lowest LR- (Hajain-Tilaki, 2013; Thrusfield, 2005).

2.2.2.2. Kappa statistic

The agreement between used serological tests was calculated using Kappa analysis (when Kappa=1 indicates perfect agreement, whereas Kappa=0 indicates that there is no agreement). The methods of calculation were determined the level of agreement among all

pairs of tests as percentage and using the kappa statistics. The percentage of samples which yielded the same result on each test was determined. Kappa is a measure of agreement that is adjusted for agreement due to chance. Altman (1991) suggested that >0.80: very good agreement; 0.61-0.80: good agreement; 0.41-0.60: moderate agreement; 0.21-0.40 fair agreement; and ≤0.2 poor agreement (Thrusfield, 2005). Fleiss et al. (2003) suggests ≥0.75 indicates excellent agreement, whereas ≤0.40 indicates poor agreement. Everitt (1989) suggests ≥0.81: almost perfect agreement; 0.61-0.80: substantial agreement; 0.41-0.60: moderate agreement; 0-0.20: slight agreement; 0: poor.

Visual best cut-off values will also be shown in frequency graphs in Ms excel. All positive and negative results were compared to information on individual and herd health including also the other species. (Nielsen *et al.*, 2008). Published sensitivity and specificity ranges for the commonly used serological tests are presented (Table 2.5). These are values obtained from the literatures. The Performance Index provides an overall estimate of the accuracy of the test by adding the sensitivity and specificity values. Min and Max values represent the lowest and highest indexes.

Table 2. 5 The Sensitivities (Se) and Specificities (Sp) values

	Se (%)	Sp (%)	Species	References
RBT	91.8-92.5	100	S	Blasco *et al.* (1994)
	34.4-47.8		SG	Abuharfeil & Abo-Shehada (1998)
	21.0-98.3	68.8-100	C	Nielsen *et al*. (2002) Poester *et al.* (2010)
	64.7-85.3	99.0-99.9	SG	Nielsen *et al*. (2004)
	67.0-74.1	99.3-100	S	Minas et al. (2005)
	91.6-93.4	99.8-100	SG	EFSA-Q (2006)
	75.8	99.7	SG	Minas *et al.* (2007)
	75.8	99.7	SG	Minas *et al*. (2008)
	54.9	97.7	C	Sanogo *et al.* (2013)
CFT	80.6	99.1	C	Minas *et al*. (2007)
	80.6	99.1	SG	Minas *et al*. (2008)
	23.0-97.0	30.6-100	C	Nielsen *et al*. (2002) Poester *et al.* (2010)
	90.0-91.8	99.7-99.9	C	Godfroid *et al*.(2010)
I-ELISA	100	100	SG	Blasco *et al.* (1994)
	66.5-78.7		S	Abuharfeil & Abo-Shehada (1998)
	88.1-96.7	94.7-99.2	SG	Burriel *et al*. (2004)
	82.1-96.6	96.4-98.4	SG	Nielsen *et al*. (2004)
	94.5-97.5	99.3-99.9	G	Nielsen *et al*.(2005)
	92.7-96.3	100	S	Minas et al. (2005)
	94.5-95.8	99.1-99.3	SG	EFSA-Q (2006)
	98.2	00.5	C	Minas et al. (2007)
	98.2	99.5	SG	Minas *et al*. (2008)
	97.6-98.8	99.8-100	SG	Ramirez-Pfeiffer *et al*. (2008)
	97.2	97.1-99.8	C	Godfroid *et al*.(2010)
	92.0-100	90.6-100		Nielsen *et al*. (2002) Poester *et al.* (2010)
	0.96	0.94	C	Durr et al.(2013)
	0.95	0.99	S	Durr et al.(2013)

	0.80	0.99	G	Durr et al.(2013)
	96.1	95	C	Sanogo *et al.* (2013)
C-ELISA	95.2	99.7	C	Godfroid *et al*.(2010)
	95.7-100	99.7-99.8		Nielsen *et al*. (2002) Poester *et al.* (2010)
FPA	95.9	97.9	SG	Minas et al. (2007)
	95.9	97.9	SG	Minas *et al*. (2008)
	96.6	99.1	C	Godfroid *et al*.(2010)
	99.0	99.3	C	Nielsen *et al*. (2002) Poester *et al.* (2010)
	0.95	0.96	C	Durr et al.(2013)
	0.93	0.98	SG	Durr et al.(2013)

Compiles the Sensitivities (Se) and Specificities (Sp) values for common used serological tests of Brucellosis and summarised for sheep and goat sera, SG- sheep and goats, S-sheep, G-goats, C-cattle

2.2.2.3. Classification of positive and negative sera

Classification of truly positive camel and cattle sera was based on positive culture. Brucella spp. isolates from camels and cattle were all *B. abortus*. Small ruminant sera were not further considered due to small number of available culture positive sera and because epidemiologically camel brucellosis is closer correlated to cattle brucellosis than that of small ruminants (Bayasgalan *et al.*, forthcoming). As to truly negative sera, we have considered field sera from herds with – by the herd owner - no reported past 5 years brucellosis testing or typical signs of brucellosis. In addition, only sera from herds with no livestock (camel, cattle and small ruminants) in both samplings that was positive with the RBT.

Field sera (those tested with all 5 serological tests) were compared pairwise and a test result also compared to the following three combinations of results of the four other tests. In combination 1 positive sera were those with all positive results and negative sera all others independent if they had a positive result in one or two tests. Combination 2 considered as positive sera that were positive in any one test and negative if negative in all tests. Finally, combination 3, only considered all positive and all negative (and sera with inconsistent results were dropped).

2.3. Identification of the agent of camel brucellosis in Mongolia

2.3.1. Samples

During the field surveys, swabs were taken from vaginal discharge of female camel and other animals (cattle, sheep and goats) from same herds with abortion history. However, good timing of herd visit dates was rarely given. The milk samples were taken from lactating animals, if ever possible, during the lambing season then until July and from all ruminant animals with reported ill-health. The choice of samples usually depended on the abortion history. Indeed, if possible we wanted to take swabs and milk samples from all herds.

To identify *Brucella* spp. from swab and milk samples, samples were shipped for culturing to the State Central Veterinary Laboratory (SCVL) with its bacteriology department due biosafety measures and trained bacteriologists. Handling of potentially *Brucella* spp contaminated samples and cultures are considered as hazardous in the laboratory. Culture was attempted for 250 milk samples and 195 swabs of camel, cattle, sheep and goats.

2.3.2. *Brucella* spp. characterization of the culture

Most *Brucella* strains, particularly *B.abortus* biovar 2 and *B.ovis*, grow better in media containing 5-10% of sterile (equine or bovine) serum and AB supplement (antibiotics) free from *Brucella* antibodies. Growth normally appears after 3–4 days. Briefly, vaginal swabs or milk samples (after centrifugation and concentration) were streaked both on petri dishes with Farrell's medium and CITA medium. The inoculated plates were incubated at 37°C in absence and presence of 10% CO_2 for up to 2 weeks (Junior *et al.*, 2015; OIE, 2008). Colonies appears pinpoint, smooth, glistening, bluish translucent on selective serum agar. As they age, the colonies become opaque and about 2-3 mm in diameter. Strains of *B. abortus*, *B. suis*, *B. melitensis* and *B. neotomae* are usually in the smooth form when first isolated. Colonies of rough morphology occur in each of these species on subculture. *B.ovis* and *B. canis* are always in the rough form (OIE, 2009).

Biochemical characterization: The main biochemical characteristics of Gram negative bacteria such as Brucella are the Oxidase and Urease tests (Table 2.6). Note that camels are described in literature of being susceptible to both, *B. abortus* and *B. melitensis.*

Table 2. 6 Differential characteristics of *Brucella* species

Species	Colony morphology	Serum requirement	Oxidase	Urease	Preferred host	Susceptible host
B. abortus	S	-[a]	+[b]	+[c]	Cattle & other Bovidae	Camels
B. melitensis	S	-	+	+[d]	Sheep & goats	Camels

a- *Brucella abortus* biovar 2 generally requires serum for growth on primary isolation
b- Some African isolates of *B. abortus* biovar 3 are negative
c- Intermediate rate, except strain 544 and some field strains that are negative
d- Slow rate, expect some strains that are rapid

A bacteriologist picked colonies based on Brucella colony morphology. These were stained by Gram Stain and modified Ziehl-Neelsen stain. In addition, the reactivity to oxidase strips was tested and both urea agar and urea broth were used for urease tests. Colonies positive to these tests were passaged to obtain pure cultures, from which DNA was extracted.

2.3.3. Molecular methods

PCR methods were extended beyond genus and species identification but also to improve diagnostic tests and a diversity of methods has been developed. Applications for PCR methods range from the diagnosis of the disease to characterization of field isolates for epidemiological purposes including taxonomic studies (Santis *et al.*, 2011).

2.3.3.1. Samples

Control DNA samples of cultures were obtained *B. abortus, B. melitensis, B. ovis and B. suis* from Spiez Laboratory, Spiez, Switzerland. Extracted DNA from livestock *Brucella* spp. cultures were either from the epidemiological study 2013 – 2015 (1 camel and 3 cattle) or DNA from 17 previously analyzed *Brucella* spp. cultures 2012 - 2013 (8 sheep, 2 goats, 2 cattle and 5 humans) from the Veterinary Research Institute (VRI) of Mongolia (Baljinnyam, 2016). In addition, we had extracted DNA from both vaccine strains S19 (*B. abortus*) and Rev1 (*B. melitensis*) from the State Central Veterinary Laboratory (SCVL), Mongolia. All samples (with the exception of the vaccine strains) were collected before introduction of the mass livestock vaccination campaigns.

A total of 240 sera were from randomly selected Mongolian livestock. The multi-stage cluster sampling is described for epidemiological study on camel brucellosis in Mongolia (Bayasgalan Chultemdorj Roth *et al.*, Forthcoming). Each 30 randomly selected seropositive and 30 seronegative sera were from camels, cattle, sheep and goats. These sera were handled at the School of Veterinary Medicine, Ulaanbaatar, where never PCR for *Brucella* spp. has been done. In addition, negative control sera were from 10 cattle, 5 goats and 5 sheep from Switzerland. Also, 23 *Brucella* spp. DNA samples were extracted from RBT positive sera of culture positive animals. Nine of these sera came from the Veterinary Research Institute (3 camels, 3 sheep and 3 goats) and 14 samples (8 cattle, 5 sheep and 1 goat) came from the Central Veterinary Laboratory of Mongolia.

2.3.3.2. DNA extractions

DNA was extracted from bacterial culture using G-Dex^{tm}IIc Genomic DNA Extraction kit (iNtRoN Biotechnology, Inc) according to the manufacturer's instructions in Mongolia.

Brucella spp. DNA was attempted to be extracted from 283 sera (240 randomly selected Mongolian livestock sera, 20 negative controls from Switzerland by using the QIAamp Mini kit (Qiagen, France) according to the manufacturer's instructions (Protocols for Bacteria), and 23 positive controls from Mongolia. Shortly, if available, 40 uL of serum were mixed with 140 µL buffer ATL and 20 µL proteinase K and incubated at 56°C for one hour. Afterwards

200 µL buffer AL was added, followed by a second incubation for 10 min at 70°C. Then, together with 200 uL ethanol (100%) the tubes were subjected to the spin column. After two washing steps, the elution was done with 50 µL buffer AE and eluted DNA was stored at –80°C until further processing. In order to control contamination during the extraction process, only filter tips were used and a reagent control was used in parallel with the samples.

2.3.3.3. Bruce-ladder multiplex PCR

INgene Bruce-ladder V is a fast method for the molecular typification of *Brucella* spp., from purified DNA or DNA from an isolated colony. Tubes for the amplification of samples were prepared in addition to three tubes for positive controls amplification, and one for the negative control. Equal volumes A and B directly from the freezer were mixed in crushed ice. An appropriate amount of amplification mixture for the number of samples to be processed and an excess amount of 10% (to compensate for possible volume losses during pipetting) was prepared. The tubes used for mixing were kept in crushed ice at all times. The prepared mixtures were then homogenized correctly. The content of all tubes was carefully mixed and it was ensured that all liquid was well deposited at the bottom of the tube. If not, the tubes were lightly centrifuged.

The thermocycler conditions were set as followings: 1 cycle of denaturation at 97°C for 7 min, 25 cycles of amplification at 95°C for 35 sec, 64°C for 45 sec and 73°C for 3 min, 1 cycle of final extension at 72°C for 6 min. Samples were maintained at 4°C until subjecting them to the thermocycler.

The PCR products were analyzed by 1.5% agarose gel electrophoresis (GelRed reagent, GelRed™ Nucleic Acid Gel Stain Biotium, was used instead of Etidiumbromid,), and fragment sizes were estimated using the 1 kb plus DNA ladder as molecular size marker (Invitrogen). Gel images were captured with a G-Box equipment (G:Box F3 Syngene, USA).

Results were interpretated in a way that the negative control showed no band; positive sample bands were visualized for *B. suis* at 1682, 1071, 587 and 272 bp; for *B. ovis* at 1683 and 587 bp; for vaccine strains Rev 1- 1682, 587 and 218 bp; while S19 showed a band at1682; *B. abortus* at 1682 and 587 bp, and *B. melitensis* at 1682, 1071, 587 bp. Positive controls Rev1, RB51 and *B. suis* were included in Bruce-ladder multiplex PCR kit.

2.3.3.4. Quantitative real-time PCR (qPCR)

All sera were tested by quantitative real-time PCR (TaqMan assay). Quantitative real-time PCR was performed using the TaqMan® method. Briefly, one reaction mixture consisted of 26 µL containing 7.75 µL of ultrapure water, 12.5 µL of Kappa Probe Fast (ROX) Master Mix, 1.25 uL of each primer (18 µM), 1.25 µL of TaqMan® probe (5 µM), and 2 µL of DNA

product The amplification program employed was the StepOnePlus™ Real-Time PCR System (Applied Biosystems) using 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 15 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

The primers and probes were tested using the DNA of strains of *B. abortus, B. melitensis, B. ovis and B. suis* from the Spiez Laboratory. The DNA of each *Brucella spp.* was diluted as follows: non-dilution; 1:100; 1:1000; 1:10000 and 1:100000. A total of DNA extracted from 23 sera from culture positive animals, 2 vaccine strains and 283 sera were tested by qPCR.

Results were analyzed using the StepOne™ Software v2.3. For all steps, nuclease free filter tips and nuclease free water was used. Non-template controls were used as negative controls and reconfirmed *Brucella* spp. DNA was used as positive control.

The CT values of qPCR were considered as weak when CT values of 35-40 cycles (≥35), strong when below a CT value of 35 cycles (<35) and very strong when below a CT value of 30 cycles (≤30). All sera were tested for detection of species and sub-species 5 - 6 times by qPCR.

2.4. The role of camels in the ongoing ruminant mass vaccination in Mongolia

The camel plays an important socio-economic role within the pastoral and agricultural systems. Camel populations grow (over-proportionally) mainly due to desertification of past more productive pastures. *Camelus bactrianus* are kept in cold arid regions such as in Mongolia and *Camelus dromedarius* are kept in warm arid and semi-arid regions. Camel milk and meat are main food resources, and wool and hides additionally increase income of mobile pastoralists (nomads) in arid regions (M. Gwida *et al.*, 2012; Kudi *et al.*, 1997).

An understanding of multiple livestock population dynamics is important to understand brucellosis transmission in Mongolia and elsewhere (Shabb *et al.*, 2013; Zinsstag *et al.*, 2005).

2.4.1. Data collection

Data was obtained from several sources and for 4 years for camels and cattle. Only cattle were considered because there were no previous indications that small ruminants play a role of brucellosis transmission to camels. Annual livestock census data for 2011-2014 were provided by the National Statistical Office of Mongolia (NSO, 2015). Initial data on animal brucellosis seroprevalences in 2011 were provided from the mass screening survey with Rose Bengal Test (RBT) by the State Central Veterinary Laboratory. Data on camel and cattle brucellosis seroprevalences by RBT were provided by the provincial veterinary laboratory in Dornod province in 2012.

The missing data in Sukhbaatar province for 2012 was extrapolated by using the average of 2011 and 2013. Regarding 2013 and 2014, camel and cattle brucellosis seroprevalences were from a repeated epidemiological survey on camel brucellosis in Mongolia, also using the RBT. All data for Sukhbaatar and Dornod provinces were fitted in the model without intervention both in. In these two Eastern provinces, livestock vaccination in cattle, sheep and goats was not yet implemented in 2012 and only started in September 2013. In 2014, sampling was more than 5-6 months after campaigns and therefore ruminants would have lost their seropositivity due to vaccination. Still, the repeated epidemiological survey was found a stable seropositivity in camels one year of introduction of vaccination (Bayasgalan Chultemdorj Roth *et al.*, Forthcoming).

2.4.2. Model descriptions

It was developed a deterministic model with stochastic parameter specification of cattle-cattle, camel-camel, cattle to camel and camel to cattle brucellosis transmissions in steps of one (1) year (t), which is adapted to the availability of data and for validation. Because only data on seropositive animals were available, we used only one seropositive compartment (instead of two compartments: “infectious” and “recovered” compartments) and have retained for each species a compartment S for susceptible and I for infected/seropositive. We considered transmission within cattle and within camels; and between cattle to camels. We have omitted to conceptualize the the transmission from camels to cattle because camels are not known to be a primary host of *Brucella* spp. *(M. M. Gwida et al., 2011)*. We have assigned the state variables at time t for both species compartments “cattle” (subscript c) and “camel” (subscript b for Bactrian) as S_c and S_b and I_c and I_b, respectively. The model was run for 20 years.

3. Collaborations and Responsibilities

The following institutions and people were involved in this PhD (Table 9). This study was undertaken within the framework of Animal Health Project (AHP) and was largely funded by the Swiss Agency for Development and Cooperation (SDC) in Mongolia. The Animal Health project (2012 – 2016) aimed to improve Mongolian animal health systems including the veterinary curricula and education, obtaining freedom of foot and mouth disease status and working towards elimination of brucellosis in Mongolia. Next to the scientific collaborators presented in Table 3.1, important partners where the district deputies governors providing the initial household and livestock lists and district veterinarians who have strongly facilitated the field work and have provided technical assistance.

Table 3. 1 Scientific collaborations

Institution	Role/Domain of Support	Names
SVM-MULS	PhD student	Chimedtseren Bayasgalan
Swiss TPH	Brucellosis epidemiology and main supervisor	Esther Schelling
Swiss TPH	Statistical support	Jan Hattendorf
Swiss TPH	Modelling of transmission between livestock species	Jakob Zinsstag
Swiss TPH	Faculty representative	Marcel Tanner
Swiss TPH	RT-PCR	Gerd Pluschke
Swiss TPH	RT-PCR and Bruce-ladder PCR	Theresa Ruf
Swiss TPH	Luminex assay	Angelika Silbereisen
SVM- MULS	Culture and serology, co-supervisor	Tungalag Chultemdorj
SVM-MULS	Serology and data entry	Bayanzul Argamjav
SVM-MULS	Serology and data entry	Badmaa Battsetseg
SVM-MULS	Serology and data entry	Ganbaatar Otgontuya
SVM-MULS	Field work	Erkhebmyar
SCVL	Culture and strain dentification	Munkhgerel Jantsandorj
VRI	Epidemiology and culture	Erdenebaatar Janchivdorj
VRI	Culture and serological test validation	Batbaatar Vanabaatar
AHP-SDC	Epidemiology and *Brucella* spp comparison strains	Zolzaya Baljinnyam

4. Risk factors of brucellosis seroposotivity in Bactrian camels of Mongolia

Chimedtseren Bayasgalan[1,3,4 *], Tungalag Chultemdorj [1], Felix Roth [2], Jakob Zinsstag [3,4], Jan Hattendorf [3,4], Battsetseg Badmaa [1], Bayanzul Argamjav [1], Esther Schelling [3,4]

[1] School of Veterinary Medicine, Mongolian University of Life Sciences, PO Box 17024 Zaisan, Ulaanbaatar, Mongolia,

[2] Health Economist and Public Health Expert, Consultant, PO Box 4054 Basel, Switzerland

[3] Swiss Tropical and Public Health Institute, PO Box, CH-4002 Basel, Switzerland,

[4] University of Basel, Basel, Switzerland

* corresponding author, chimee_vet@muls.edu.mn

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4.1. Abstract

Background: More information on brucellosis epidemiology in Bactrian camels is needed due to their growing economic and livelihood importance for herders and renewed efforts in Mongolia to eliminate brucellosis through mass vaccination of ruminants excluding camels. Brucellosis prevalence in camels increased over the past two decades. Random multi-stage cluster surveys were done in the Eastern provinces of Dornod and Sukhbaatar in 2013 and 2014 and in the Southern & Western provinces of Dornogobi, Umnogobi and Khovd in 2014 and 2015. A total of 1,822 camels, 1,155 cattle, and 3023 small ruminant sera were collected and tested with the Rose Bengal Test. In addition, 195 vaginal swabs and 250 milk samples for bacteriological culture were taken from livestock with history of abortion.

Results: The overall apparent seroprevalence in camels was 2.3% (95% confidence interval 1.6-3.3). The main risk factor for camel seropositivity was being in an Eastern province when compared to Southern & Western provinces (odds ratio 13.2, 95% CI 5.3-32.4). Camel seroprevalences were stable over the two consecutive survey years, despite introduction of ruminant vaccination: 5.7% (95% CI 3.1-10.2%) and 5.8% (3.3-10.1%) in Eastern provinces and 0.4% (0.2-1.2%) and 0.5% (0.1-2.0%) in Southern & Western provinces. We isolated *Brucella abortus* from camels and cattle. Camel seropositivity was associated to keeping cattle together with camels. Monitoring of vaccination campaigns showed that coverage in cattle was insufficient because animals could not be adequately restrained.

Conclusions: The present study reveals that brucellosis is present with important seroprevalence in Mongolian camels and was endemic in Eastern provinces. Camel herd seropositivity was most closely associated to infection in cattle.

Longer term monitoring is needed to assess whether camel seroprevalance decreases with ongoing vaccination in Mongolia. This should be coupled with further confirmation on *Brucella* spp. isolates. To date, only *Brucella abortus* was isolated, but camels are also susceptible to *Brucella melitensis*. Clear verbal and written information on disease prevention in livestock and household members is important, particularly for remote camel herders who had only moderate knowledge on brucellosis epidemiology and preventive measures.

Keywords Bactrian camel, brucellosis, epidemiology, Mongolia, seroprevalence, *Brucella* spp., risk factors

4.2. Background

The Bactrian camel (two humped) and the dromedary (one humped Arabian camel) represent the old-world domesticated camel species and are closely related [1, 2]. The Bactrian camel inhabits cold deserts in the southern areas of Russia, Mongolia, East-Central Asia and China [3].

Camel husbandry in Mongolia is practiced primarily by pastoralists in the Gobi Desert. Camels produce milk, wool and meat and are also used for racing and, less commonly now, for transportation of people and goods. In 2014, it was estimated that there were 367,900 camels in Mongolia [4]. The camel population resides in close contact with cattle, sheep, goats and occasionally horses, particularly at watering places (wells, branch-water, ditch-water, rivers, and lakes) and during calving and wool shearing periods. Camels, unlike other domestic large animals, often travel up to 16 km daily in search of food [5]. They are less susceptible to some highly contagious livestock diseases, such as foot-and-mouth disease [6].

Brucellosis is a zoonosis caused by the intracellular, Gram negative bacteria of the genus *Brucella*. Sheep and goats are the main hosts for *Brucella melitensis*, while cattle are the main host for *Brucella abortus* and pigs are the main host for *Brucella suis*. These three species cause the majority of the disease burden in animals and are also the most important *Brucella* pathogens in people. However, other species (e.g. *Brucella canis*) are also potentially infectious to humans [7, 8].

Brucellosis is thought to be the most economically important zoonosis worldwide because it is endemic in many countries and impacts both human and livestock health [9-11]. Brucellosis is transmitted from animals to people often through consumption of unpasteurized milk and dairy products [12-15], but direct contact, particularly with livestock abortion material, is more important among livestock-keeping communities. The disease is rarely fatal in people but causes high morbidity in both animals and humans [16, 17].

Camels are susceptible to both *B. abortus* and *B. melitensis* [18-20]; however, camels are considered to be secondary hosts of *Brucella* spp. [3, 14]. Brucellosis was reported in camels as early as in 1931 by Solonitsiun in Russia [18, 21]. Since then, serological evidence of brucellosis has been reported from the most important camel-keeping countries [3, 18, 21-23]. Camels infected with brucellosis show fewer clinical signs than other livestock species, in particular less than domesticated cattle, sheep and goats [24]. This may be a reason why little information is available on epidemiology of brucellosis in camels and its impact on human health, notably in Mongolia [3, 25].

Brucellosis serological tests have rarely been validated for camels. Empirically, the Rose Bengal test is commonly used for diagnosis in camels and seems to give accurate results [24, 26].

Camels were included in mass screening surveys in Mongolia, but risk factors for exposure were not further evaluated. A screening survey in 2011 [27], which sampled between 6 and 3,590 camel sera from each of the 22 Mongolian provinces, found a moderate correlation (Spearman's rho = 0.26) between camel and cattle brucellosis seropositivity at district level; however, sheep were very weakly correlated while goats were not at all correlated (unpublished data). There is almost no information on which *Brucella* spp. cause seropositivity in Mongolian camels due to a lack of strain isolation and characterization. Past and current mass livestock vaccination campaigns in Mongolia did not include camels or horses. Older reports from veterinary laboratories indicated that the serological prevalence of brucellosis in camels in different Mongolian localities was increasing [28]. Notably, in 2010 a 3% seroprevalence in camels was found in a population-based survey in Sukhbaatar province [29].

Camels may be a reservoir for *Brucella* spp., and other livestock are at risk for reinfection when vaccination campaigns are discontinued because they are kept together. However, effective control of brucellosis could be achieved by establishing diagnostic and surveillance systems, by estimating the cost-benefits of control measures to guide policy makers, by rigorously implementing control programs, and by policies to connect human health and veterinary services at demographic, socioeconomic and political levels. Ruminant (Bovidae) mass vaccination was estimated to be highly cost effective for Mongolia [30]. In a mobile context, test and slaughter is hardly feasible. Instead, vaccination of cattle and small ruminants over several years is the viable control measure for mobile livestock husbandry systems, where there is also no feasible individual animal tracking system. The required vaccination coverage to interrupt transmission, in cattle (minimum 60% truly immunized animals) and in small ruminants (minimum 40%), must be monitored [31]. Post-vaccination campaign monitoring in cattle and small ruminants is now undertaken. However, the role of Bactrian camels in brucellosis epidemiology must be more clearly understood for successful elimination efforts in Mongolia, in particular, the ability of camels to maintain an own infection cycle and reintroduce brucellosis to domesticated Bovidae.

The objectives of this study were to contribute to understanding the epidemiology of camel brucellosis in Mongolia and to identify the *Brucella* species involved before and after implementation of vaccination campaigns in cattle and small ruminants. We tested the

hypotheses that the seroprevalence of camel brucellosis is below 5% in Mongolia and the most important risk factor of camel seropositivity was herding together with cattle.

4.3. Results

A total of 6000 serum samples (1822 camels, 1155 cattle, 1531 sheep, 1492 goats) were collected from 365 herds in five provinces over three years. In addition, 195 vaginal swabs (72 from camels, 51 from cattle, 29 from sheep, 43 from goats) and 250 milk samples (104 from camels, 68 from cattle, 46 from sheep, 32 from goats) were collected for bacteriological culture. In total, 310 out of the 365 herds sampled completed a questionnaire during the study, with 240 being completed at the first visit of a herd. No camels were sampled in 9 herds, so the total camel herds was 356 (Table 4.1).

Table 4. 1 Distribution of the camel herds sampled in 5 provinces

	Year 1 (2013)	Year 2 (2014)	Year 3 (2015)
Dornod	32	24 + 8	
Sukhbaatar	37	34 + 4	
Dornogobi		36	22 + 14
Umnogobi		37	24 + 12
Khovd		36	19 + 17

Distribution of the 356 camel herds sampled in 5 provinces over three years of sampling.

In a second year the number of re-sampled herds and (+) the number of newly sampled herds is shown. The selected districts within the 5 provinces and the sites of sampling for the first and second years are depicted in Figures 4.1 and 4. 2.

A total of 1822 camel sera were tested, of which 17, 10 and 10 sera showed +, ++ and +++ postive agglutination, respectively. The 37 seropositive camels were in 29 of the 356 camel herds, and in herds with more than one positive camel different strengths of agglutination were seen. The overall apparent brucellosis seroprevalence in camels was 2.3% (95% CI 1.6–3.3). The estimated true seroprevalence was 1.8%. About one fifth of the camel sera collected originated from male animals. The majority of camels sampled were adults (13.9% young vs. 86.1% of adult camels), and seroprevalences were comparable across age groups (Table 4. 2).

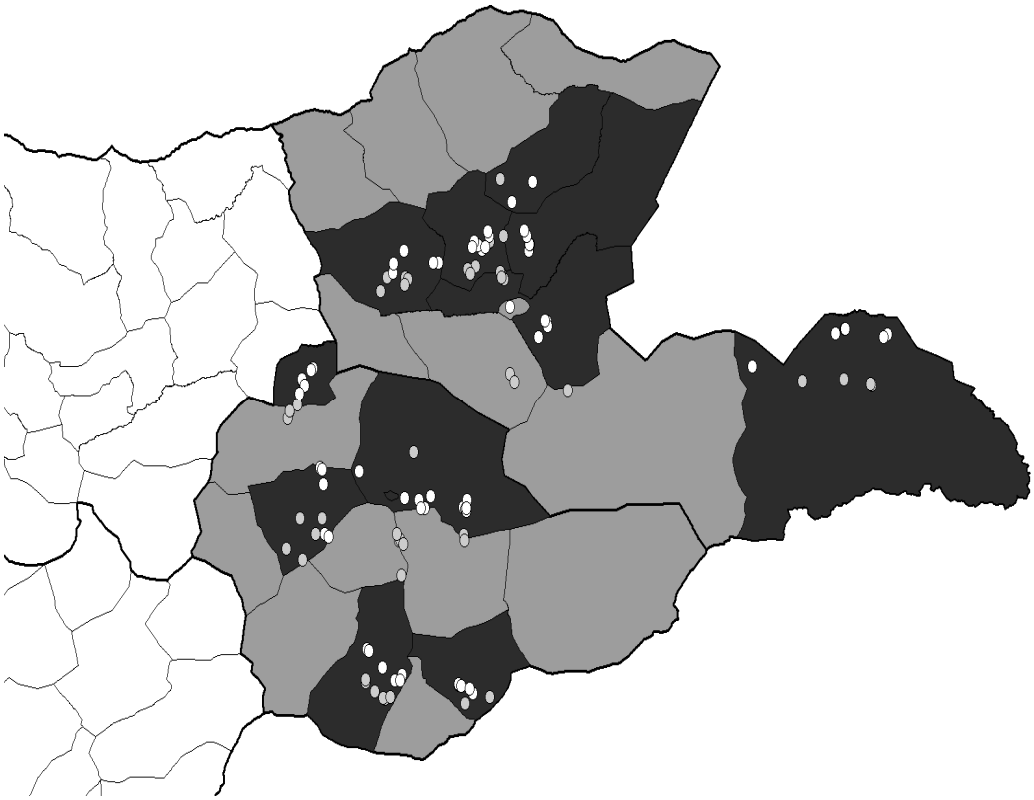

Figure 4. 1 Map of Eastern provinces Dornod and Sukhbaatar

Map of Eastern provinces Dornod and Sukhbaatar (shaded in grey) and showing the selected districts (in black). The location of camel herds at time of sampling in 2013 (white dots) and 2014 (grey dots) are shown.

Camel brucellosis seropositivity was highest in Sukhbaatar (6.1%, 95% CI 3.5-10.1%) followed by Dornod (5.3%, 2.9.-9.6%), Dornogobi (0.8%, 0.3-2.3%), Umnogobi (0.4%, 0.1-1.4%), and Khovd (0.3%, 0.04-1.9%). The camel seroprevalences remained steady between the first and second years of sampling with 5.7% (95% CI 3.1-10.2%), and 5.8% (95% CI 3.3-10.1%) in Eastern provinces, and, at much lower levels, in the Southern & Western provinces with 0.4% (0.2-1.2%) in 2014 and 0.5% (0.1-2.0%) in 2015 (Table 4. 2).

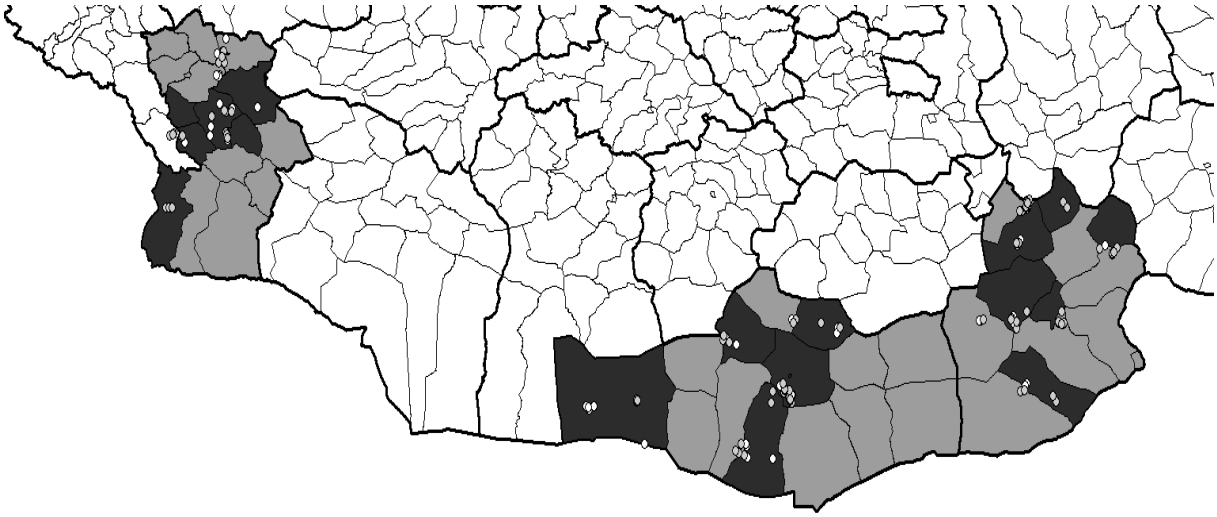

Figure 4. 2 Map of Southern & Western provinces Dornogobi, Umnogobi and Khovd

Map of Southern & Western provinces Dornogobi, Umnogobi and Khovd (shaded in grey) and showing the selected districts (in black).

The location of camel herds at time of sampling in 2014 (with dots) and 2015 (grey dots) are shown. Due to movement of *hot ails* not all herds were found in the district they have been registered few months earlier.

Table 4. 2 Results of camel seroprevalences by the Rose Bengal Test

Variable	Category	n	n pos	Seroprevalence[b]	95% CI[b]
Aimag	Dornod	241	13	5.3	2.9-9.6
	Sukhbaatar	298	18	6.1	3.5-10.6
	Dornogobi	388	3	0.8	0.3-2.3
	Umnogobi	526	2	0.4	0.1-1.4
	Khovd	369	1	0.3	0.04-1.9
Sex	Female	1429	26	2.2	1.4-3.4
	Males	332	10	3.0	1.6-5.5
Age	≤ 4 years	253	5	2.0	0.8-4.7
	> 4 years	1569	32	2.3	1.6-3.4
Year	Eastern provinces 2013	237	13	5.7	3.1-10.2
	Eastern provinces 2014	302	18	5.8	3.3-10.1
	Southern & Western provinces 2014	897	4	0.4	0.2-1.2
	Southern & Western provinces 2015	386	2	0.5	0.1-2.0

Legend Table 4: Results of camel seroprevalences by the Rose Bengal Test (RBT) stratified by sex, age class, province and sampling year. [a]Positive with RBT, [b]95% confidence interval (CI) calculated with the panel variable on the level of herd to consider potential clustering within herds; Eastern provinces: Sukhbaatar and Dornod Southern & Western provinces: Dornogobi, Umnogobi and Khovd

Regarding risk factors, camel age and sex were not significantly associated with seropositivity. The Eastern provinces had significantly higher seropositive proportions than the Southern & Western provinces (Table 4. 3). Keeping camels together with cattle was significantly associated to brucellosis seropositivity in camels, whereas the presence of small ruminants was not. Out of all camels sampled, 86.9%, 93.1%, and 94.4% were kept together with cattle, sheep and goats, respectively (Table 4. 3).

Table 4. 3 Analysis of risk factors for camel seropositivity, multivariable analysis

		n neg	% neg	n pos	% pos	OR	95% CI	p-value
Province	Dornogobi	385	99.2	3	0.8	ref		
	Dornod	228	94.6	13	5.4	7.9	2.1-30.1	0.003
	Khovd	368	99.7	1	0.3	0.4	0.05-3.2	0.4
	Sukhbaatar	280	94.0	18	6.0	10.2	2.7-38.6	0.001
	Umnogobi	524	99.6	2	0.4	0.5	0.1-2.4	0.4
Age class	≤ 4years	248	98.2	5	1.8	ref		
	> 4years	1537	98.0	32	2.0	1.2	0.4-3.2	0.7
Sex	Female	1403	99.2	26	1.8	ref		
	Male	322	97.0	10	3.0	0.8	0.3-1.8	0.5
Year	2013	224	94.5	13	5.5	ref		
	2014	1177	98.2	22	1.8	1.0	0.4-2.4	1.0
	2015	384	99.5	2	0.5	1.0	0.2-5.6	1.0
Cattle present	no	238	100	0	0.0	ref		
	yes	1547	97.7	37	2.3	8.1	1.5-inf	0.01[ŧ]
Sheep present	no	126	100	0	0.0	ref		
	yes	1659	97.8	37	2.2	4.0	0.7- inf	0.1[ŧ]
Goats present	no	102	100	0	0.0	ref		
	yes	1683	97.8	37	2.2	3.2	0.6- inf	0.2[ŧ]

Analysis of risk factors for camel seropositivity, multivariable analysis showing odds ratios using a generalized estimating equation (GEE) model considering the panel variable at herd level [†] -exact logistic regression, * $p \leq 0.05$

We found no association between camel seropositivity and history of abortion or preventive biosafety measures such as destroying abortion material (Table 3). None of the biosafety measure (e.g., buying of live animals, safely disposing of abortion material) question outcomes were associated with seropositivity in camels, nor was the variable with distances of camel herds to the closest district centre (mean distance was 55 kilometers).

Owners of seropositive camels had significantly more sources of information on brucellosis, indicating that they were informed about brucellosis in their herd. However, knowledge of herders on brucellosis can only be judged as moderate. From a total of 38 possible scores of the three knowledge themes with 19 questions, the median score achieved by participants was 23.

At the herd level, no significant correlations were found between camel and ruminant seroprevalences with the regression model using bootstrapping, regardless of considering all herds or considering only herds in provinces with no ruminant vaccination to ensure that seropositivity in ruminants was not a result of vaccination (even though there is little possibility of seropositivity persisting from previous vaccination) (Table 4). Goats in provinces and years with on vaccination were negatively correlated.

Table 4. 4 Regression coefficients using bootstrap re-sampling technique

		N herds	Intercept (95% confidence interval [CI])	Slope (95% CI)
Ruminants	All herds	348	0.02 (-0.01 - 0.03)	0.06 (-0.07 - 0.21)
	No vaccination	137	0.03 (0.01 - 0.05)	0.08 (-0.14 - 0.5)
Cattle	All herds	292	0.03 (0.01 - 0.04)	0.06 (-0.02 - 0.2)
	No vaccination	103	0.04 (0.01 - 0.06)	0.06 (-0.06 - 0.3)
Sheep	All herds	333	0.03 (0.02 - 0.04)	0.03 (-0.04 - 0.1)
	No vaccination	127	0.03 (0.01 - 0.05)	0.1 (-0.2 - 0.7)
Goats	All herds	341	0.03 (0.02 - 0.04)	-0.003 (-0.07 - 0.1)
	No vaccination	133	0.03 (0.01 - 0.05)	-0.1 (-0.27 - -0.04)*

Regression coefficients using bootstrap re-sampling technique for camel herd seropositivity and within herd seropositivity of cattle, sheep and goats (all herds) and only for herds in a province without vaccination (no vaccination) * significant negative correlation

Variances of camel serpositivity were higher at herd level than province and district levels (Table 4. 5). The ICC was estimated at herd level. For the cluster sample size calculation we assumed an ICC of 0.1 at herd level. In the Eastern provinces, this was nearly the case; however, the ICC was much lower in the Southern & Western provinces, where there were rarely seropositive camels. Clustering in herds is higher than in provinces or districts, therefore, correlation within herds (as the ecological unit) was accounted for in the statistical analysis.

Table 4. 5 The variances and intraclass correlation coefficient (ICC) of camel seropositivity

Eastern provinces	2013	2014	Both years
Variance at herd level	2.1	2.0	1.2
Variance at district level	0.16	0.48	0.4
Variance at province level	<0.01	<0.01	<0.01
Calculated ICC for camel herds	0.2	0.12	0.06
Southern & Western provinces	2014	2015	Both years
Variance at herd level	5.6	2.85	2.2
Variance at district level	2.0	1.3	1.1
Variance at province level	<0.01	<0.01	<0.01
Calculated ICC for camel herds	<0.001	0.04	<0.001

The variances and intraclass correlation coefficient (ICC) of camel seropositivity at different levels. The greater the variances between herds compared to the overall total variance, the higher the ICC.

Brucella spp. were isolated from the milk of one camel and from three vaginal swabs of cattle. The four isolated *Brucella* strains were identified as *B. abortus* (Figure 4. 3).

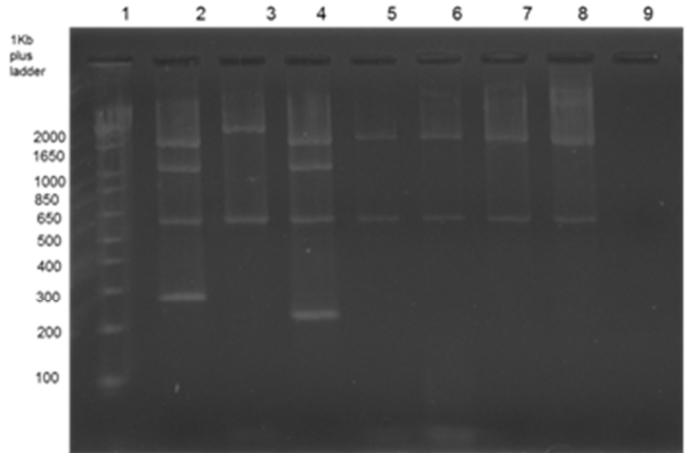

Figure 4. 3 Agarose gel electrophoresis PCR products

Leigend Figure 4.3 Lane 1: DNA ladder; Lane 2: positive control *Brucella suis;* Lane 3: positive control *Brucella abortus* (vaccine strain RB51) with two bands at 2524 and 587 bp; Lane 4: positive control *Brucella melitensis* (vaccine strain Rev1); Lane 5: the isolate from a camel; Lanes 6-8: isolates from cattle; Lane 9: negative control

4.4. Discussion

A mass screening survey in all 22 provinces of Mongolia in 2011 reported seroprevalences of brucellosis in camels between 0.2% and 5.9% [28]. A previous (2010) population-based randomized survey in Sukhbaatar of Eastern Mongolia found a seroprevalence of 3% in camels [29]. In this study, we assessed seroprevalence and risk factors of camel seropositivity, in consideration of previous exposure to *Brucella* spp. There are shortcomings of using a serological test to define an outcome, as there will be false seronegative and false seropositive results, particularly when specificity of the test is low. Results subsequently need to be interpreted cautiously. In consideration of false positives, many authors set the cut-off for a seropositive herd as having at least two positive animals. In a complementary study on serological test characteristics comparing five different tests for use in camels, we concluded that the RBT is valid to assess brucellosis exposure status of Mongolian camels given its high specificity. However, due to lower sensitivity in camels when compared to other livestock species, we do not recommend it as a screening test for brucellosis monitoring in camels [26].

Between 2013 and 2015, seroprevalences in provinces showed high variation ranging from 0.3% to 6.1%, but the prevalences in regions were stable between the two sampling years. The ICC depends on the degree of clustering and also on the prevalence. The ICC used for the sample size calculation (0.1) was appropriate for the Eastern provinces; however, since a much lower ICC at herd level was calculated for the Southern & Western provinces, we may have slightly oversampled there [32, 33].

Brucellosis seroprevalences above 5% in livestock species are important, indicating endemic status [34]. Eastern provinces had significantly higher seroprevalences than Southern & Western provinces. Being in an Eastern province was the most important risk factor of camel brucellosis seropositivity, with an OR of 13.2 when compared to the Southern & Western provinces. The same result was seen when the cut-off value of camel seropositivity was set at higher agglutination (++ positivity). The majority of serological studies on brucellosis report higher seroprevalences in older animals [20, 35], which we did not see among camels. Nonetheless, another study reported that brucellosis infection began early in life, probably through suckling, and persisted into adulthood [14].

Public health education campaigns should continue among herders to inform them about brucellosis prevention practices and herd and human health management. Past surveys in the framework of monitoring vaccination outcomes coupled with human brucellosis prevalence found that all information sources (veterinarians, radio/TV, newsletters to herders, information brochures and newspapers) significantly improved herder knowledge on brucellosis epidemiology, prevention and clinical signs in both people and livestock. Since Mongolian herders are literate, both oral and written information material is appropriate.

Musa et al. [36] reported that cattle were a possible source of infection for camels because all small ruminants tested in their study were negative. Hadush et al. [20] reported that camel herds with close contact in pastures with cattle and small ruminants were 3.6 and 2.3 times, respectively, more at risk to be brucellosis seropositive than those with no contact. We found an association between camel seropositivity and cattle, but not small ruminant, keeping. The fact that our camel isolate was *B. abortus* further supports a linkage of brucellosis in cattle and in camels. This finding is consistent with the screening in all Mongolian provinces with a correlation of camel and cattle seropositivity at district level, as well as previous reports of identification on *Brucella* spp. from camels in Asia, where another isolate from a Mongolian camel also was *B. abortus* [37]. Monitoring surveys of achieved vaccination coverage from 2012-2015 indicate that sufficient coverage was achieved in small ruminants, but coverage was critically low in cattle. Veterinarians reported that cattle were difficult to restrain adequately to administer conjunctival vaccination. Achieving insufficient vaccination coverage in cattle in the first year of newly introduced ruminant vaccination campaigns could explain why camel seropositivity remained stable between the years, both without and with cattle vaccination.

4.5. Conclusions

The results of this survey confirm the presence of *Brucella* spp. in camel herds in Mongolia. Camel seropositivity was significantly higher in Eastern than in Southern & Western provinces and was associated with keeping cattle together with camels. Decrease of camel brucellosis seropositivity was not observed despite ongoing ruminant vaccination. Repeated studies are needed to see if seroprevalences in camels drop over time with ongoing vaccination in other livestock species. Close attention should be given to achieve and monitor sufficient vaccination coverage in cattle in Mongolia. More isolates are needed to confirm that seropositivity in camels is limited to infection with *B. abortus*.

4.6. Materials and methods

4.6.1. Study design and selection of herds

We purposely selected the two Eastern provinces Sukhbaatar and Dornod for the first year of the study in 2013. The seroprevalence of brucellosis was high (>3%) in camels in the multi-disease screening survey in Dornod in 2011 [27] and in Sukhbaatar during an epidemiological survey in 2010 [29]. Both provinces had a substantial number of camels and had not yet been included in the livestock (cattle, sheep and goats) brucellosis vaccination campaigns initiated in September 2013. Therefore, it was possible to sample before and after introduction of vaccination in 2014 in both Eastern provinces. The selection of three additional provinces in 2014 (second year of the study) was proportional to the size of their respective camel population as available from the annual livestock census [38]. Selection of provinces and districts proportional to size better ensured equal probability of camels to be enrolled in the study. The selected provinces had on average 32,500 camels per province. The Southern and Eastern provinces (Umnogobi, Dornogobi and Khovd) were surveyed in year 2 (2014) and year 3 (2015) (Figure 4.4 and Table 4.1). During the study period, Umnogobi was the only province (out of 22) with no livestock brucellosis vaccination due to the large proportion of camels and vast size of the province. In areas using conjunctival vaccination of cattle and small ruminants, sampling was more than 5 months after vaccination campaigns, so the animals would no longer be seropositive due to vaccination [39, 40]. In each province, six districts were selected proportional to size of the camel population.

Repeated surveys using multi-stage cluster sampling were done in all provinces. Households with camels were randomly selected from lists of families registered with the district governor’s office.

Figure 4. 4 Map of Mongolia showing the surveyed provinces.
The light grey provinces of Dornod and Sukhbaatar (Eastern provinces) were sampled in 2013, and a second survey was done in 2014. Surveys in the darker grey provinces of Khovd, Umnogobi and Dornogobi (Southern & Western provinces) started in 2014 and were repeated in 2015.

The epidemiological sampling unit in rural zones was the *hot ail*, typically 2-3 families which pasture their livestock together and share watering places during certain times of the year. The entire *hot ail* herd of a selected family was included. District veterinarians indicated the zone where a selected *hot ail* was at the time of sampling, then the study team would travel to the zone and ask encountered herders about precise locations for the selected *hot ail*. Six and thirty percent of initially selected *hot ails* could not be sampled in Eastern and Southern & Western provinces, respectively. Reasons for non-participation were family moved too far away, family was preparing to move and did not have time or family's camel herd was located too far from the *hot ail*. In such cases of non-participation, a replacement *hot ail* was enrolled which was either additionally selected from the district family list (the initial selection assumed that not all families would be found in a district) or from the nearest *hot ail* located in a northern direction from where the team determined that a selected *hot ail* could not be enrolled. For second year sampling in the same province, herders selected the previous year were contacted by mobile phone to establish their location and schedule the sampling. Reasons for non-participation were the same as for the first enrollment, and revisits were not possible in 10% and 40% in Eastern and Southern & Western provinces, respectively, so replacements were enrolled (Table 1).

4.6.2. Sample size

The sample size calculation assumed an intraclass correlation coefficient (ICC) of 0.1 for all livestock species. The ICC is the ratio of the variance between clusters over the total variance [41]. An ICC of 0.1 was reported for a range of endemic zoonoses [42] and was assumed, based on previous livestock brucellosis serological surveys in Mongolia [43]. This led to a design effect D of 1.2 and 1.4, when 3 and 5 animals, respectively, were sampled per cluster (herd).

The sample size calculation aimed to estimate the prevalence in each province with a precision, defined as one half-length of the 95% confidence interval, of 5%-points. We assumed seroprevalences of the different livestock species as were reported by Sukhbaatar in 2010 (3% for camels, 5% for goats, 7% for sheep, 8% for cattle). The calculated sample size for a province was to sample 30 herds each with at least 3 camels, 3 cattle, 5 sheep and 5 goats.

4.6.3. Selection of animals and sampling

In a selected herd, sheep and goats were selected when exiting an enclosure using the sampling interval i: total number of animals divided by 5. The first animal was selected with a random number and then every i^{th} sheep and goat was sampled. Camels and cattle were selected in the direction of the bottle head after the bottle was spun and a random number to tell which animals were to be included in that direction. Species, sex, age, breed, and main use for each animal were recorded on a data sheet, where any noted clinical symptoms (e.g. abortions) in the herd within the past months were also registered.

Blood samples were collected from the jugular vein using a Vacutainer® tube with disposable needle. Tubes were centrifuged for 5-10 minutes at 1000-1500 rpm, then serum was aliquoted into two 2 mL Eppendorf tubes®, which were stored on ice in a cool box and transported regularly to the Veterinary Laboratory at the Province Center, where they were kept at -20 °C until transported to the School of Veterinary Medicine (SVM), Ulaanbaatar, and again stored at -20 °C until further processing.

Vaginal swabs and/or milk samples for bacteriology were collected from individual animals with history of abortion. Swabs were placed in transport medium tube (BD BBL™Culture swab plus, Amies without Characoal, Becton Dickinson, France) and transported to the State Central Veterinary Laboratory (SCVL) in cool boxes. Milk samples consisted of 10–20 mL of milk taken from each teat. The first streams were discarded and then the milk sample was collected into a sterile vessel [44].

4.6.4. Serological testing

All serum samples were tested by the Rose Bengal Test (RBT) using RBT antigen (Biocombinate, Mongolia). Camel and cattle sera were tested with a serum:RBT reactive ratio of 1:1 (25µL 25µL) and small ruminant sera were tested with a serum:RBT reactive ratio of 3:1 (75µL : 25µL), all for 4 minutes, as recommended by the World Organization for Animal Health [45] and according to Mongolian national standards [46]. Results were recorded as agglutination negative (-), doubtful (+/-), or positive (+, ++ or +++) according to the strength and time to reaction. All tests and readings were performed by the same person. The serological test results were transformed to a binary outcome with the cut-off of seropositivity set at positive + agglutination. The RBT test with camel sera performed with 99% specificity, which is comparable to other livestock, however, with a rather low sensitivity of 75% [47].

4.6.5. Bacteriological examination

Milk samples were centrifuged to concentrate bacteria, at 6000–7000 g for 15 minutes in sealed tubes to avoid potential for aerosolization [7, 44]. A mixture of cream and deposit was streaked both on petri dishes with Farrell's medium (*Brucella* medium base, CM0169; antibiotic supplement, SR0083, Oxoid™) and with CITA medium (blood agar base number 2, CM0271, Oxoid™; and antibiotic supplements vancomycin, colistin, nystatin, nitrofurantoin, amphotericin B, Sigma™, as well as containing 5–10%, inactivated horse serum, SR0035, Oxoid™). The inoculated plates were incubated at 37 °C in absence and presence of 10% CO_2 for up to 2 weeks [44, 48]. A bacteriologist selected colonies based on *Brucella* colony morphology. These were stained by Gram Stain (K001, Himedia) and modified Ziehl-Neelsen stain (21820 Sigma™). In addition, reactivity to oxidase strips (MB0266A, Oxoid) was tested and both urea agar and urea broth were used for urease tests (urea agar 211795, BD BBL™; Bacto agar 214010 BD and Urease Test Broth 221719, BBL™). Colonies positive for these tests were passaged to obtain pure cultures, from which DNA was extracted using G-Dex™IIc Genomic DNA Extraction kit (iNtRoN Biotechnology, Inc).

To identify *Brucella* species, the Bruce-Ladder multiplex PCR, using INgene Bruce-ladder (V R.10.BRU.k5) kits, was used. The PCR products were analyzed by 1.5% agarose gel electrophoresis (GelRed reagent used in place of Etidiumbromid, GelRed™ Nucleic Acid Gel Stain Biotium), and fragment sizes were estimated using the 1 kb plus DNA ladder as molecular size marker (Invitrogen). Gel images were captured with G-Box (G:Box F3 Syngene, USA).

4.6.6. Questionnaires

The questionnaires were written in English and translated to Mongolian before pre-testing with 3 herder families in the vicinity of Ulaan Bator. The member of each selected camel-keeping family with the best knowledge on management of the camel herd was interviewed to obtain information about the herd, household and individual risk factors for brucellosis (Additional file 1). The interview included questions on i) knowledge of epidemiology of brucellosis ii) history of brucellosis in the household, iii) herd risk factors (including buying/selling of animals, sharing of pasture and watering places), iv) herd and human health management (including disposal of aborted fetuses/placentas), v) vaccination of cattle and small ruminants (Additional file 2). Questionnaires were not filled in on second visits to the same household. The coordinates of the household (*hot ail)* at time of the visit was recorded with a GPS. The mobile phone number of each participant was recorded for dissemination of results and to establish contact for second visits.

4.6.7. Data management and analysis

Questionnaire and sample data were double entered in Microsoft Access® and compared and corrected using Epi-Info 3.5.3 (Centers for Disease Control and Prevention, USA). An identification system was used to uniquely identify all samples and individuals and facilitated merging data sets at province, district, household/herd and individual levels. Data analyses were done using Stata 14 (StataCorp IC, USA).

We calculated seroprevalences for brucellosis in camels using generalized estimating equations (GEE, Stata command xtgee) to account for clustering at herd level, which expands the confidence interval compared to simple binary confidence intervals (CI). The apparent seroprevelance was converted to an estimated true seroprevalence using the formula developed by Rogan and Gladen [49] to account for the fact that the apparent seroprevalence might be over- or underestimated. A multivariable GEE model accounting for clustering was used to assess the association of biologically plausible risk factors to the serological outcome. Since vaccination of other livestock was highly linked to province and year it was not included in the multivariable analysis. Exact logistic regression was used for explanatory variables, with zero cell counts in two-by-two tables. Other variables, such as knowledge of herders or preventive measures, were not tested as risk factors in the multivariable model in order to keep the model simple. Other variables were tested with univariable GEE models. Age categorization of camels was based on breeding maturity: young camels were ≤ 4 years and adult camels were > 4 years. The variance components at different sampling levels were determined with the generalised linear latent and mixed models (gllamm) command in Stata for hierarchical models. The ICC at the *hot ail* level was

estimated with ANOVA. The ICC was estimated at the herd level because the variance components indicated that correlation within clusters was highest at this level, so it was used for the sample size calculation. Correlations between camel and other livestock herd seropositivity was done with linear regression models in R version 3.3.2. The 95% confidence intervals of the intercepts and slopes of the regressions were constructed using bootstrap re-sampling technique and the information on total number of livestock per herd and species.

We assigned scores to the questions on knowledge within three themes: transmission of brucellosis between herds, transmission from livestock to people, and clinical signs of livestock brucellosis. Correct answers were scored as 2, 'Do not know' as 0, and wrong answers as -1. All scores within a knowledge theme were summed and the median taken to classify those with lower and higher scores.

Abbreviations

RBT: Rose Bengal Test; ICC: intraclass correlation coefficient; CI: Confidence Interval; OR: Odds ratio; GEE: generalized estimating equation; SVM: School of Veterinary Medicine; SCVL: State Central Veterinary Laboratory

4.7. Declarations

Ethics approval and consent to participate

The study continued the framework of previous studies, including both people and livestock, which obtained formal ethical clearance from the ethical committee of the Mongolian Ministry of Health in 2012. In this study, confidentiality was guaranteed. All information was analyzed using anonymous data sets. All questionnaires and data were stored confidentially. Samples and data were only used for the purpose stated in the information for participants and the project information, where livestock owners also gave written consent to participate. Further ethical considerations were i) Safety was very important and all potential risks were minimized with application of best practices and professional handling; ii) Best practices were applied to assess livestock brucellosis in a herd; iii) Interviews were conducted in a private environment; iv) The sample size was well justified and v) Animal owners with positive serological results in their livestock were contacted on their mobile phone by the study team to report the finding. They were informed that they should protect themselves during obstetric work/slaughtering, boil the milk before consumption and not consume fresh blood or raw livestock products. Also they were advised that all ruminants should be vaccinated and all camels re-tested. They were also advised on how to best prevent potentially infected animals from entering their herds.

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Consent for publication

Not applicable

Availability of data and materials

The data set(s) supporting the results of this article are included in the manuscript. The datasets used and/or analyzed during the current study are available from the corresponding author on substantiated request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CB performed most of the research work. BB and BA assisted in serological and bacteriological analysis and data management. CB, ES and TC drafted the manuscript. FR, JZ, JH, ES and TC contributed to study conception and design, data analysis and interpretation of data. ES contributed to manuscript editing. All authors read and approved the final version of the manuscript.

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5. Serological tests for brucellosis in Mongolian Bactrian camels

Bayasgalan, C.[123], Chultemdorj, T.[1], Vanabaatar, B.[4], Jantsandorj, M.[5], Felix, R.[6], Argamjav, B.[1], Badmaa, B.[1], Zinsstag, J.[23], & Schelling, E.[23]*

[1]School of Veterinary Medicine, Mongolian University of Life Sciences, Ulaanbaatar, Mongolia

[2]Swiss Tropical and Public Health Institute, 4002 Basel, Switzerland

[3]University of Basel

[4]Veterinary Research Institute, Ulaanbaatar, Mongolia

[5]State Central Veterinary Laboratory, Ulaanbaatar, Mongolia

[6]Health Economist and Public Health Expert, Consultant, 4054 Basel, Switzerland

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5.1. Abstract

With ongoing efforts of brucellosis elimination in Mongolia through mass vaccination of cattle and small ruminants, close monitoring of the situation in camels, who are not vaccinated, becomes increasingly important. Camels are susceptible to both *Brucella abortus* and *B. melitensis.* In Mongolia only *B. abortus* was isolated from camels. A repeated epidemiological survey did not find a drop in camel seropositivity after one year of introduction of vaccination. However, brucellosis serological test characteristics for use in camels were not known. The aim of this study was to assess the performance of five serological tests in Mongolian Bactrian camels: the Rose Bengal Test (RBT), the Complement Fixation Test (CFT), the indirect and complement ELISA (I-ELISA and C-ELISA) and the Fluorescence Polarization Assay (FPA). A total of 977 camel sera from the epidemiological study were tested with all five tests. In view of comparison to other livestock, cattle and small ruminant sera were also enrolled. Among the field sera, one camel and three cattle sera were from culture positive animals Additional 10 camel and 9 cattle culture positive sera were from the veterinary institutes in Mongolia. Test agreement using Kappa statistics and test characteristics using ROC curves were done test pairwise and compared to three combinations of classified positive and negative sera (either all positive or negative in all other tests). Sensitivity (Se) and specificity (Sp) were assessed for camel and cattle sera using as positive reference culture positive and as negative sera from herds with no animal tested RBT positive and no reported clinical brucellosis signs during the past five years. The use of the RBT in camel showed low sensitivity. We recommend either the I-ELISA or FPA with very high Se for monitoring of camels. Another confirmatory test such as the CFT can be added – or both tests combined to further increase Sp. The higher costs of these tests than the RBT seem justified by the need of sensitive monitoring test in camels. The brucellosis reference strain and sera bank in Mongolia has to acquire also true positive and true negative samples from camels.

Keywords: Bactrian camel, brucellosis, Mongolia, Rose Bengal Test, Complementary fixation test, indirect and complement ELISA, fluorescence polarization assay, area under the curve, kappa statistics, gold standard, sensitivity, specificity

5.2. Introduction

Domesticated old-world camels are either one-humped camels (dromedaries) or two-humped camels (Bactrian camels). Camels are adapted to dry environments (Franklin, 2011). Camels are less susceptible to some livestock diseases such as foot-and-mouth disease (FMD) but are more susceptible to infection than other animals to other diseases

such as paratuberculosis (caused by *Mycobacterium avium* ssp *paratuberculosis*), clostridial infecions, entertoxiaemia, and, presuming also to brucellosis (Abbas & Agab, 2002). For the latter disease, brucellosis, camels, however, show seemingly less clinical signs. This may be a reason why there is up to date little knowledge on the epidemiology of brucellosis in camels and its impact on human health.

Brucellosis is endemic in the Mediterranean, in Eastern Europe, the Middle East, Africa, South and Central America, and Asia and the most economically important zoonosis over worldwide (Ciocchini *et al.*, 2013; Herrick *et al.*, 2014; J. McDermott *et al.*, 2013). The bacteria can be transmitted from animals to humans, most often via unpasteurized milk (Dean *et al.*, 2012; Megersa *et al.*, 2012) and contact with infected animals (Lindahl, 2014). Worldwide, 500,000 new human cases of brucellosis in human occur annually (Ciocchini *et al.*, 2013; Pappas *et al.*, 2006). In livestock, it causes enormous losses for economies of developing countries and poses a severe health risk to consumers of dairy products (Kansiime *et al.*, 2014; Shimol *et al.*, 2012).

Brucellosis was reported in camels as early as 1931 (Abbas & Agab, 2002; M. Gwida *et al.*, 2012; Mustafa, 1987). Since then, brucellosis has been reported from virtually all camel-keeping countries. Camels are not known to be a primary host of *Brucella* spp., but they are susceptible to the two main zoonotic pathogens: *B. abortus* and *B. melitensis* (M. Gwida *et al.*, 2012).

Generally, clinical diagnosis of brucellosis is challenging in people (Ciocchini *et al.*, 2013) and livestock. The clinical signs of brucellosis in camels are not sufficient to differentiate brucellosis from many other diseases. Serology of bacterial diseases in general and for brucellosis specifically, greatly help to establish the diagnosis. The disease is typically confirmed through laboratory diagnosis (Poester *et al.*, 2010). Serological tests for brucellosis, however, are not yet evaluated for camels (M. M. Gwida *et al.*, 2011) and/or acknowledged by the World Organization of Animal Health (OIE) (Corbel, 2006; M. M. Gwida *et al.*, 2011; Wernery, 2014).

A general problem with brucellosis serology (in people and livestock) is its possible cross-reactions to other Gram-negative bacteria and that serology cannot differentiate between *Brucella* spp. that have caused the infection. Therefore, a screening positive result RBT is commonly confirmed by another more specific serological test such as the indirect Enzyme–Linked Immunosorbent Assays (I-ELISA) (Ghanem *et al.*, 2009; Schelling *et al.*, 2003). A combination of different serological tests can increase diagnostic efficacy in camels, (Wernery, 2014). The complement fixation test (CFT), which was often used as a confirmatory test due to its specificity, is now progressively being replaced by ELISAs and

the more recently developed Fluorescence polarization assay (FPA) (Corbel, 2006; Gul & Khan, 2007; M. M. Gwida *et al.*, 2011; Wernery, 2014).

We should not only consider that serological tests were not validated for camels, but also more generally, that commercially available test kits were assessed with sera from the manufacturers' places, thus mainly in industrialized countries. Therefore, they need to be critically reviewed for their use in other regions. This is the main reason why Mongolia has started to invest in a brucellosis strain and sera bank with samples from all susceptible domesticated animals (and – less advanced – also such a human bank).

Camel antibodies have special features and tests designed for domesticated Bovidae (cattle, sheep and goats) should not be used uncritically. Camelidae's antibody consists (only) of a single monomeric variable antibody domain. Like a whole antibody, it is able to bind selectively to a specific antigen. With a molecular weight of 12–15 kDa, single-domain antibodies are smaller than common mammal antibodies (150–160 kDa), which are composed of two heavy protein chains and two light chains. Given the smaller size of camelidae antibodies, they are more heat resistant than typical mammal antibodies. Researchers have made use of this special feature of camel antibodies in the development of diagnostic tests or clinical products for other species (Deffar *et al.*, 2009; Koenig, 2007; Lawrence, 2004). Only few studies have validated the performance of diagnostic tools for use in camels. Although serological tests are the mainstay of brucellosis diagnosis in livestock including camels, these tests have been directly transposed from cattle without adequate validation for camels (Melzer *et al.*, 2011). From prior epidemiological studies coupled with clinics, we know that the RBT and the I-ELISA have been used for camel sera and have provided reasonably good results, but were not further assessed (see for example Schelling *et al.*, 2003).

More validated serological tests for camels are important for Mongolia with its important -for livelihoods of Mongolian pastoralists and for economy making use of the dry grassland-Bactrian camel population. Given the ongoing brucellosis elimination efforts in Mongolia though mass vaccination of cattle, sheep and goats- thus that do not include camels – camels need to be monitored closely to further define if they are only spill-over hosts from domesticated Bovidae (and thus their seroprevalences will decrease in parallel to achieved vaccination coverage over years - or if they could maintain brucellosis infection and thus can represent a risk of re-infection in 5 to 6 years to come when livestock vaccination campaigns are stopped). Such a monitoring of camel brucellosis seropostivity will need the most sensitive test for screening and a good specific test for confirmation (actually, in allusion to HIV serological testing).

The aim of this study was to assess test characteristics for use of a range of brucellosis serological tests in Bactrian camel sera from Mongolia. We use here a sub-set of samples that were collected in an epidemiological study with random cluster surveys. The results of the epidemiological study are published elsewhere (Bayasgalan *et al*, forthcoming). We wanted to formulate recommendations for use of serological tests on camel sera and their interpretation in Mongolia.

5.3. Materials and Methods

5.3.1. Samples

Sera and biological samples were collected in two Eastern Mongolian provinces (Dornod and Sukhbaatar) in 2013 and 2014 and in three Southern & Western provinces (Dornogobi, Umnubobi and Khovd) in 2014 and 2015. The selected of households (*hot ails*) with their herds were enrolled in a multi-stage random cluster sampling (Bayasgalan Chultemdorj Roth *et al.*, Forthcoming) Serum samples and additional vaginal swabs and milk samples were collected from camels, cattle, sheep and goats. Samples were stored on ice in a cool box and transported regularly to the Veterinary Laboratory at the province centesr and were kept in aliquots at –20°C until transported to the School of Veterinary Medicine, Ulaanbaatar.

The Central Veterinary Laboratory made available samples from their brucellosis strain and sera bank. All obtained sera were *Brucella* spp. positive cultures (*B. melitensis* isolated from 1 goat and 2 sheep and *B. abortus* from 5 cattle). All of these sera from *Brucella* spp. isolation positive animals were tested with the same five tests and protocols used in this study.

In addition, we have obtained information on 49 RBT positive sera samples from the Veterinary Research Institute in Mongolia. These sera were tested upon brucellosis clinical signs (at individual or herd level). There were 10 camel, 20 cattle, 10 sheep and 9 goat Rose Bengal Test (RBT) positive sera. All of these sera were further tested with the indirect enzyme linked immunosorbent assay (I-ELISA) and competitive enzyme linked immunosorbent assay (C-ELISA) according to this study. However, only a sub-sample was tested with the CFT and the FPA due to lack of sera. These sera – collected based on clinical signs of individual animals or herd health (such as storm of abortions in a herd) – were for camels and cattle accompanied partially by biological samples for culture.

The needed sample size of positive and negative sera was calculated according to the OIE guidelines (OIE, 2013). We calculated a total of 282 positive and 149 controls to estimate an expected sensitivity (Se) of 90 (95% CI 86.0-93.3) and specificity (Sp) of 95 (95% 90.6-98.1).

5.3.2. Serological testing

The Rose Bengal test (RBT) is usually used as rapid screening test, but may be less specific due to *Brucella* spp. cross-reactivity with other bacteria (Manishimwe *et al.*, 2014). However, further serial testing with more specific tests increases specificity, but also increases the chances of misdiagnosing true-positive cases (Racloz *et al.*, 2013; Schelling *et al.*, 2003). The complement fixation test (CFT) known as good specific test is more and more replaced by easier to handle, and also specific tests such as indirect and competitive enzyme linked imunosorbent assay (I- and C-ELISA), and, most recently, the fluorescence polarisation assay (FPA).

All field sera (1822 camels, 1155 cattle, 1531 sheep and 1492 goats) were initially screened using the RBT (antigen from Biocombinate, Mongolia). This reactive is commonly used for both human and livestock samples on brucellosis diagnosis in Mongolia. Sera were tested for 4 minutes using a serum (25µL) : RBT reactive (25µL), ratio of 1:1 for camels and cattle, and a serum (75µL) : RBT reactive (25µL) ratio of 3:1 for small ruminants as recommended by the World Organization for Animal Health (OIE, 2009) and according to Mongolian national standards (Animal brucellosis, serological diagnostic method, (MASM, 2002). Results were recorded as negative "-", doubtful "+/-", or positive "+", "++" or "+++"according to the strength and time to reaction.

All RBT positive field camel sera plus a random selection of negative samples (selected by the random sampling command in Stata 12) were further tested with CFT, the I-ELSIA, the C-ELISA, and the FPA. Table 1 shows the testing of camel sera. Clearly, those field samples tested with all tests were not based on a random selection, and therefore, we do not refer to seroprevalences but rather to seropositivity or proportions of seropositive samples. The distribution of sera of cattle, sheep and goats is shown in Table S1.

Table 5. 1 Camel sera collected during an epidemiological study on camel brucellosis

N camel sera tested with RBT	CFT	I-ELISA	C-ELISA	FPA
1822	1106	1690	1623	1275

Camel sera collected during an epidemiological study on camel brucellosis in Mongolia for 2013-2015 (Bayasgalan et el. forthcoming). All sera were tested with the RBT and all RBT seropositive samples and a random sub-sample were tested with other serology tests.

As to the reagents of the CFT, the complement, hemolysin, antigen positive and negative controls were supplied by Biocombinate, Mongolia. The reagents were titrated and performed according to recommendation by the World Organization for Animal Health (OIE, 2009) and national standards for Animal brucellosis serological diagnostic method (MASM,

2002). The 1:2.5 diluted sera was inactivated at 56°C for 30 minutes. Sera with strong reaction, more than 75% fixation of complement (3+) at a dilution of 1:5 or at least with 50% fixation of the complement (2+) at a dilution of 1:10 and above were classified as positive. Absence of fixation (seen as complete haemolysis) was considered as negative. Serological test results were interpreted according to the manufactures' recommendations.

Other serological tests, namely the I-ELISA for ruminant brucellosis (IDEXX, Switzerland AG), the C-ELISA (compELISA, APHA, UK) and the FPA (Diachemix , LLC, USA) were used and results were interpreted according to the manufacturer's recommendations (Bayasgalan *et al.*, forthcoming). The cut-off values of the I-ELISA, C-ELISA and FPA were initially set as the manufacturer's recommendations at ≥80% of % S/P-ratio), 60% of the mean of the optical density (OD) of the 4 conjugate control wells, and ≥20 mP (millipolarisation level), respectively. We restricted all serological results to those field samples that were tested with all five serological tests.

5.3.3. Bacteriology

The standard bacteriology and characterization used in Mongolia is described elsewhere (Bayasgalan *et al*., forthcoming). Briefly, vaginal swabs or milk samples (after centrifugation and concentration) were streaked both on petri dishes with Farrell's medium and CITA medium. The inoculated plates were incubated at 37°C in absence and presence of 10% CO_2 for up to 2 weeks. A bacteriologist picked colonies based on *Brucella* colony morphology. These were stained by Gram Stain and modified Ziehl-Neelsen stain. In addition, the reactivity to oxidase strips was tested and both urea agar and urea broth were used for urease tests. Colonies positive to these tests were passaged to obtain pure cultures, from which DNA was extracted. For the identification of *Brucella* species, the Bruce-Ladder multiplex PCR using INgene Bruce-ladder kit was used.

Classification of positive and negative sera

Classification of truly positive camel and cattle sera was based on positive culture. Brucella spp. isolates from camels and cattle were all *B. abortus*. Small ruminant sera were not further considered due to small number of available culture positive sera and because epidemiologically camel brucellosis is closer correlated to cattle brucellosis than that of small ruminants (Bayasgalan *et al.*, forthcoming). As to truly negative sera, we have considered field sera from herds with – by the herd owner - no reported past 5 years brucellosis testing or typical signs of brucellosis. In addition, only sera from herds with no livestock (camel, cattle and small ruminants) in both samplings that was positive with the RBT.

Field sera (those tested with all 5 serological tests) were compared pairwise and a test result also compared to the following three combinations of results of the four other tests. In combination 1 positive sera were those with all positive results and negative sera all others independent if they had a positive result in one or two tests. Combination 2 considered as positive sera that were positive in any one test and negative if negative in all tests. Finally, combination 3, only considered all positive and all negative (and sera with inconsistent results were dropped).

5.4. Data analyses

Data were double entered MS Access and compared and cleaned with the Data Compare utility of in Epi-Info 3.5. Analyses were carried out with the cleaned data sets in STATA 14 (StataCorp IC 14 USA).

Se (that the test is positive given a livestock was exposed) and Sp (the test is negative given a livestock was not exposed) probabilities were calculated as percentage of the number true positives over all tested positive in the sample and Sp the percentage of the number of all true negatives among all negatives tested. Binary confidence intervals were constructed at 95% level of confidence.

The agreement between serological tests was calculated using Kappa analysis. Kappa is a measure of agreement that is adjusted for agreement due to chance. It was suggested that a Kappa > 0.80 indicates very good agreement; 0.61-0.80: good agreement; 0.41-0.60 moderate agreement; 0.21-0.40 fair agreement; and ≤ 0.2 poor agreement (Thrusfield, 2005). However, other authors such as (Fleiss *et al.*, 2003) have suggested that a Kappa ≥ 0.75 indicates excellent agreement, whereas ≤ 0.40 indicates poor agreement. The level of agreement was determined among all pairs of tests and the three combinations of other tests as described above. Confidence intervals were obtained the STATA command kapci that uses bootstrapping approach.

The sensitivity and specificity can be computed across different threshold values in comparison to a reference standard. The plot of sensitivity versus 1 - Specificity is called receiver operator characteristic (ROC) curve and the area under the curve (AUC), is an effective measure of accuracy, describes how much a chosen cut-off explains of the discriminative power of test (Hajain-Tilaki, 2013). Essentially, the area equals the probability that a random individual with true disease has a higher value of the test variable than a random healthy individual. A perfect test yields AUC of 1 when compared to the gold standard, whereas a non-discriminating test gives a value of 0.5 (Thrusfield, 2005). The likelihood ratio (LR) of the ROC provides a suitable useful measure of diagnostic accuracy,

which is independent of prevalence. It compares the proportion of animals diseased and non-diseased, in relation to their test results. The likelihood ratio of a positive test result (LR+) is the ratio of the proportion of affected individuals that test positive, and the proportion of healthy individuals that test positive. A perfect diagnostic test would have an LR+ equal to infinity (detecting all true positives, and generating no false positives). A good diagnostic test has an LR+ > 10. The likelihood ratio of a negative test result (LR-) is vice-versa. Perfect diagnostic test would have an LR- equal to zero (producing no false negatives, but detecting all true negatives). The best test for ruling out a disease is therefore the one with the lowest LR- (Hajain-Tilaki, 2013; Thrusfield, 2005). A good diagnostic test has an LR- < 0.1.

We have used ROC curve analyses for the two tests with standardised continuous test result outcomes (I-ELISA or FPA). The C-ELISA does not produce comparable outcomes between plates since a cut-off sera is added on each plate and the distribution of OD values of positive samples is not given because the majority reaches the threshold of an OD of zero.

5.5. Results

A total of 977 field camel sera were tested for brucellosis using the RBT, CFT, I-ELISA, C-ELISA, and FPA and 13 sera were positive in all 5 tests. In addition, 94 cattle sera, 112 sheep and 132 goat sera were submitted to the same serological tests and 22 cattle and 8 small ruminants were positive in all 5 tests. From 1 camel and from 3 cattle, *B. abortus* was isolated.

The overall proportion of brucellosis seropositivity among the 977 camel sera was in the order from highest to lowest 8.0% (95% CI 6.3-9.7), 6.6% (5.1-8.2), 4.2% (2.9-5.5), 3.7% (2.5-4.9), and 3.2% (2.1-4.3) obtained with the I-ELISA, CFT, FPA, C-ELISA, and RBT, respectively. The lowest seropositivity was thus obtained with the RBT. The order of tests giving seropositivity from highest to lowest was different in other livestock species. In cattle it was from 61.7% with the RBT to 59.6% with the I-ELISA to 55.3% with the FPA to 41.5% the C-ELISA and 41.5% with the CFT. This reflects what is often stated in literature: the RBT as sensitive screening test and the CFT as less sensitive but specific brucellosis serology test. In sheep, the order was similar to cattle with the exception that the I-ELISA gave lowest seropositivity: RBT (28.6%), FPA (21.4%), C-ELISA (19.6%), CFT (12.5%), and I-ELISA (5.4%). The most sensitive test in goats was the C-ELISA (20.0%), then the FPA (16.7%), CFT (10.3%), RBT (3.2%), and I-ELISA (2.7%).

Comparisons of I-ELISA and FPA Test combinations (as described) vs. I-ELISA in camel (Table 5.9) and other ruminants were fair agreements (STable 5.9), while test combinations

(I-ELISA, C-ELISA, CFT and RBT) vs. FPA in camel were moderate agreements (Table 10), and in cattle were fair agreements, and in small ruminants were poor agreements (STable 10).

The pair-wise test comparisons of camel sera results on RBT vs. I-ELISA, CFT vs. FPA, and I-ELSIA vs. FPA showed only fair agreements (Kappa value 0.21-0.40), while other test comparisons had moderate agreements (Kappa 0.41-0.60). The highest agreement was between the RBT and the C-ELISA (Kappa value of 0.55) followed by RBT and CFT (0.52) (Table 5. 2). The fact that all agreements for camel were significant at $p<0.05$ indicates sufficient sample size, which was not the case for cattle. In cattle, all comparisons of serological tests had moderate agreements (Table S5.2). Note that this was not the case for small ruminants with either fair or poor agreements. Highest agreement in cattle was between the RBT and FPA (Kappa value of 56) followed by I-ELISA and FPA (0.52).

Table 5. 2 The cross-table values and Kappa statistic (K value)

		CFT		I-ELISA		C-ELISA		FPA	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
RBT	Pos	26	5	21	10	19	12	17	14
	Neg	39	907	57	889	17	929	24	922
K value		0.52 (0.40-0.64)		0.36 (0.24-0.47)		0.55 (0.41-0.70)		0.45 (0.31-0.60)	
CFT	Pos			34	31	25	40	22	43
	Neg			44	868	11	901	19	893
K value				0.43 (0.33-0.54)		0.47 (0.35-0.60)		0.38 (0.26-0.51)	
I-ELISA	Pos					24	54	23	55
	Neg					12	887	18	881
K value						0.40 (0.27-0.51)		0.35 (0.24-0.47)	
C-ELISA	Pos							19	17
	Neg							22	919
K value								0.47 (0.33-0.61)	

The cross-table values and Kappa statistic (K value) of pairwise test result comparisons of camel sera Next to the pairwise test comparisons, the I-ELISA and FPA (because these were also further evaluated with ROC analysis) were compared to classification of a reference using three combinations. A good agreement was seen between the FPA and combination 3 (Kappa value of 0.6). Given that the I-ELISA has produced highest seropositivity among all tests in camels, the best agreement (but only moderate) was obtained with combination 2, when any positive result in another test was taken (Table 5. 3). In cattle, a very good agreement (0.83) was found between the FPA and the binary classification of combination three with all positive and all negative in the other tests (Table S3).

Table 5. 3 Test comparison of camel sera

		Combination 1		Combination 2		Combination 3	
		Pos	Neg	Pos	Neg	Pos	Neg
I-ELISA	Pos	13	0	40	54	13	0
	Neg	65	899	38	845	38	845
K value		0.27 (0.15-0.39)		0.41 (0.32-0.51)		0.39 (0.24-0.54)	
FPA	Pos	13	2	26	91	13	2
	Neg	28	934	15	845	15	845
K value		0.45 (0.30-0.61)		0.29 (0.20-0.38)		0.60 (0.42-0.70)	

Test comparison of camel sera between results obtained with the I-ELISA and FPA and three combinations of classification: Combination 1 considering as positive those that were all positive in other 4 tests, Combination 2 considering as positive if positive in any other test, but as negative if negative in all others, and Combination 3 all positive and all negative.

As to ROC curve analyses in camel sera, the highest AUCs were achieved when taking as reference the classification of positives when all other tests were positive and as negative when all other four tests were negative (Combination 3) (Tables 5.4 and 5.5).. At the manufacturer's recommended cut-off, the AUC for the I-ELISA was at 0.98 (LR+ 22.6; LR- < 0.01) and for the FPA it was 0.96 (LR+ 373 and LR- 0.1). These are high AUCs – An AUC of 1 means perfect test yields. Clearly, the reference here is not a gold standard with positive culture as outcome. The results for the I-ELISA were slightly lower than seen for cattle (I-ELISA AUC at 0.99) (Table S5.4), however higher than in the FPA (0.93) (Table S5.5). But the number of cattle samples was also much lower than the number of camel samples. Optimisation (with the highest correctly classified sera when LR+ and LR- are maximised) increased the specificities of the test, but a rather high cost of the sensitivity. For example for I ELISA in cattle and taking Combination 3 as reference, the Se was at 100% and the Sp at 95.6% and at the point of highest correct classification the Se dropped to low 15.4% while the Sp was at thigh 99.9%.

The pairwise test comparisons showed moderate discriminatory ability since their AUC values were between 0.69 and 0.84 in camels. An example of a ROC curve graph is shown in Figure 5.1.

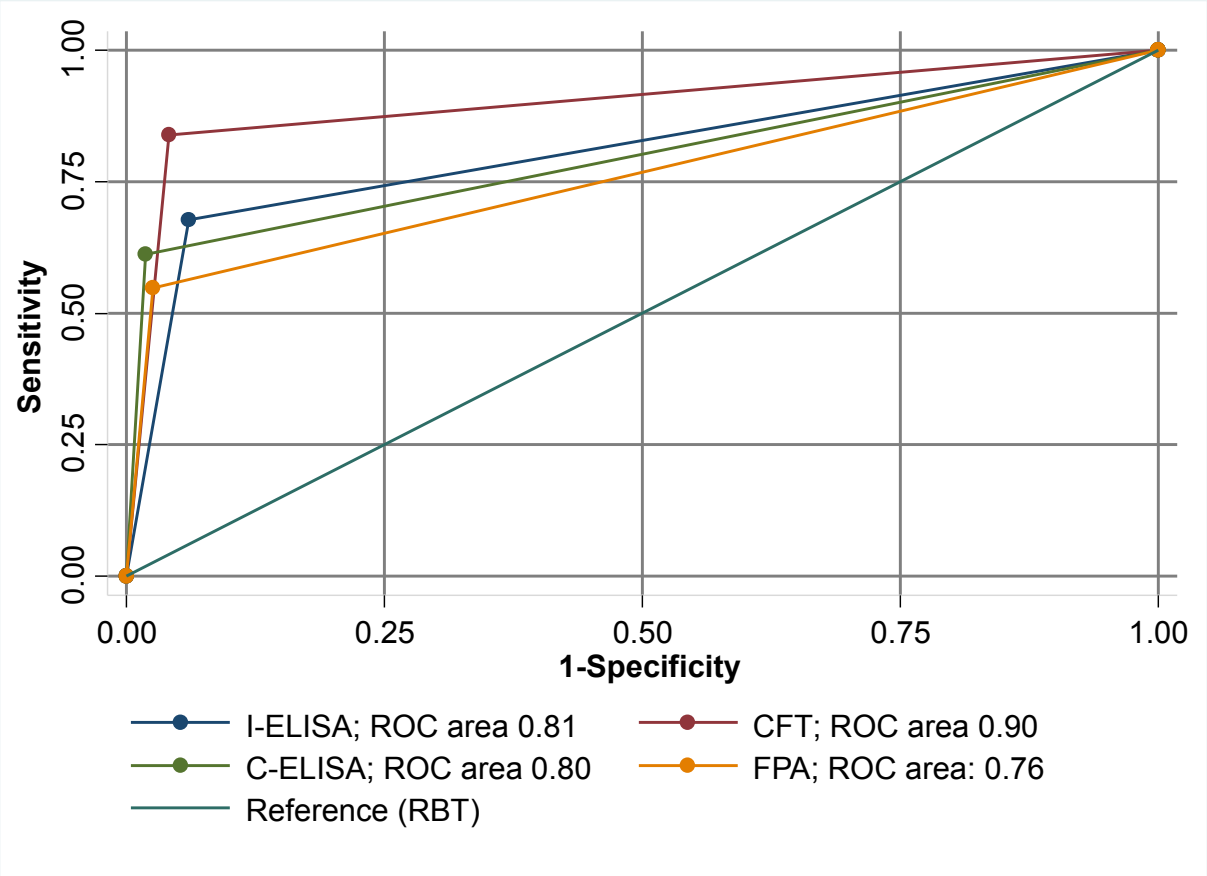

Figure 5. 1 ROC curves of serological tests

ROC curves of I-ELISA, C-ELISA, CFT and FPA performed on camel sera when taking the RBT as reference.

The estimation of Se and Sp taking culture positive sera as positive and sera from presumptive infection free herds as negative reference, We have not estimated values of the RBT since RBT results were used for definition of the negative samples. All culture positive sera (11 camels and 12 cattle) were RBT positive. We observe high Se of 100% (95% CI 71.5-100), 100% (29.2-100) and 100% (39.8-100) of the I-ELISA, FPA and CFT for camels, respectively. We note the large confidence intervals of these estimates. The Sp remain good at 94.4% (91.8-96.4), 98.6% (97.0-99.5) and 95.8% (93.4-97.5), respectively.

Table 5. 4 The % S/P ratio of the I-ELISA outcomes of camel sera analysed with ROC curve statistics

	Cut-off	Se %	Sp %	LR+	LR-	AUC				Cut-off	Se %	Sp %	LR+	LR-
						Area	95% CI		P value					
RBT	≥80.2	67.7	93.9	11.0	0.3	0.81	0.71	0.91	0.05	≥208.0	6.5	99.8	30.5	0.9
CFT	≥80.2	52.3	95.1	10.6	0.5	0.69	0.60	0.78	0.05	≥208.0	4.6	99.9	42.1	1.0
C-ELISA	≥80.2	66.7	94.2	11.4	0.4	0.79	0.69	0.90	0.05	≥208.0	8.3	99.9	78.4	0.9
FPA	≥80.2	56.1	94.0	9.4	0.5	0.72	0.61	0.83	0.06	≥208.0	7.3	99.9	96.0	0.9
Test combination 1	≥80.2	100	93.2	14.6	0.0	0.97	0.96	0.99	0.007	≥208.0	15.4	99.8	74.2	0.8
Test combination 2	≥80.2	42.6	95.6	9.6	9.6	0.65	0.6	0.7	0.04	≥208.0	3.2	99.9	28.2	0.97
Test combination 3	≥80.2	100	95.6	22.6	0.0	0.98	0.98	0.99	0.005	≥208.0	15.4	99.9	135.8	0.8

The % S/P ratio of the I-ELISA outcomes of camel sera analysed with ROC curve statistics while taking other test outcomes and combinations hereof as references. The manufacturer's recommended cut-off is at ≥ 80%.

Table 5. 5 The millipolarisation level (mP) of the FPA outcomes of camel sera analysed with ROC curve statistics

	Cut-off	Se %	Sp %	LR+	LR-	AUC				Cut-off	Se %	Sp %	LR+	LR-
						Area	95% CI		P value					
RBT	≥20.4	51.6	99.3	69.8	0.5	0.84	0.74	0.94	0.05	≥54.5	38.7	99.7	122.1	0.6
CFT	≥20.4	26.2	99.3	39.8	0.7	0.73	0.65	0.80	0.04	≥65.6	15.4	99.9	140	0.8
C-ELISA	≥20.4	50.0	99.5	94.1	0.5	0.83	0.74	0.92	0.05	≥105.4	16.7	99.9	156.8	0.8
I-ELISA	≥20.4	25.6	99.7	93.8	0.7	0.70	0.62	0.76	0.04	≥46.8	19.2	99.9	172.9	0.8
Test combination 1	≥20.4	86.7	99.0	83.4	0.1	0.96	0.89	1.0	0.04	≥123.9	20.0	99.9	192.4	0.8
Test combination 2	≥20.4	18.0	99.8	77.2	0.8	0.67	0.61	0.73	0.03	≥46.8	12.8	99.9	110.3	0.8
Test combination 3	≥20.4	86.7	99.8	372.7	0.1	0.96	0.89	1.0		≥54.5	66.7	99.9	573.3	0.3

The millipolarisation level (mP) of the FPA outcomes of camel sera analysed with ROC curve statistics while taking other test outcomes and combinations hereof as references. The manufacturer's recommended cut-off is at ≥ 20 mP.

Table 5. 6 Estimation of Se and Sp for camel and cattle sera

Test	Species	Test result	Reference		Se (95% CI)	Sp (95% CI)
			Pos	Neg		
I-ELISA	Camel	Pos	405	0		
		Neg	24	11	100 (71.5-100)	94.4 (91.8-96.4)
	Cattle	Pos	12	2		
		Neg	4	10	83.3 (51.6-97.9)	75.0 (47.6-92.7)
C-ELISA	Camel	Pos	423	2		
		Neg	6	9	80 (44.4 – 97.5)	98.6 (97.0-99.5)
	Cattle	Pos	15	1		
		Neg	1	11	91.7 (61.5-99.8)	93.8 (69.8-99.8)
FPA	Camel	Pos	423	0		
		Neg	6	3	100 (29.2-100)	98.6 (97.0-99.5)
	Cattle	Pos	15	1		
		Neg	1	9	90 (55.5-98.7)	93.8 (69.8-99.8)
CFT	Camel	Pos	411	0		
		Neg	18	4	100 (39.8-100)	95.8 (93.4-97.5)
	Cattle	Pos	14	1		
		Neg	2	9	90.0 (55.5-99.7)	87.5 (61.7-98.4)

Estimation of Se and Sp for camel and cattle sera using as reference culture positive sera and presumptive brucellosis free herds as negative reference.

5.6. Discussion

In literature it is described that single diagnostic tests for brucellosis are insufficient to identify all brucellosis infected animals. Therefore, a combination of serological tests seems useful to reduce false positive and false negative results (Racloz *et al.*, 2013). This study was conducted for comparison between RBT, CFT, I-ELISA, C-ELISA, and FPA in detection of *Brucella* spp. antibodies in camels, cattle, sheep and goats collected from five provinces in Mongolia. Serological tests used have been validated by studies and manufacturers for their use with cattle, sheep and goats. However, a commercial test used in another setting than that of the manufacturer should be critically assessed with sera from a region. For this purpose, currently a brucellosis sera and strain bank is being established in Mongolia. This bank, so far, includes hardly samples from camels. Still, we wanted to have a better idea on the performance of these serological tests in camels. With ongoing vaccination campaigns in cattle and small ruminant, it becomes increasingly important to know the most sensitive test for screening of camels during monitoring surveys and a specific test for confirmation. At the time of sampling of field sera, camels were free of clinical signs of brucellosis and they were not vaccinated.

The results indicate that seropositivity in field camel sera was highest with the I-ELISA (8.0%), while it was lowest at 3.2% with the RBT. RBT seropositivity did not increase

sensitivity when we have used a ratio of 2 serum : 1 reactive or 3 serum : 1 reactive (unpublished data), as is recommended for small ruminants to increase sensitivity of the RBT (which comes at a certain cost of specificity). The advantage of the RBT is that it can be rather easily best performed, does not require expensive laboratory equipment and is a cheap test. RBT reactive is produced in Mongolia.

In our epidemiological study, we have used the RBT to test all camel sera. Acknowledging the RBT has the lowest sensitivitiy of all serological test used, the estimated seroprevalences may be significantly lower than if another test such as the I-ELISA or FPA had been used. A study in Iraq revealed a significant difference between estimated seroprevalences of brucellosis in sheep by RBP and C-ELISA, despite that the agreement between the two tests was good (Kappa value of 0.71) (Manishimwe *et al.*, 2014).

The Complement Fixation Test (CFT) is the recommended confirmatory test for brucellosis seropositivity given its high specificity (but lower sensitivity) (OIE, 2009). However, the CFT is complex and time-consuming to perform and requires numerous preparatory steps and well trained laboratory staff. An important number of reagents and their controls must be titrated daily.

Several evaluation studies for cattle and small ruminants determined that among serological test assays, the FPA had a higher sensitivity and specificity when compared to ELISA and CFT (Gall & Nielsen, 2004; Nielsen, 2002). But this is also contrasted by a study that reported pairwise comparison of ROC curves of FPA vs. ELISA, FPA vs. RBT and ELISA vs. RBT and did not find significant differences between the tests (Konstantinidis *et al.*, 2007). Indeed, for camel sera we found the highest agreement of Kappa 0.6 and the highest AUC between the FPA compared to classification of all positive with the other results and negative with all other tests. In addition, we have estimated the combined highes Se and Sp when reference sera were used. There seems to be little doubt that the FPA performs well with camel sera.

As to the C-ELISA, among 11 sera with positive culture, two sera were negative in the C-ELISA. This may be due to generally lower sensitivity of the C-ELISA when compared to the I-ELISA. Other studies in Sudanese and Indian cattle also reported Kappa of 0.86 and 0.72 agreement, respectively, between RBPT and C-ELISA (Manishimwe *et al.*, 2014). However, these agreements are not always good. In Iraqi cattle, an agreement between RBP and C-ELISA, had a Kappa value of 0.35 (Iraqi *et al.*, 2009). We also found good agreement between the RBT and C-ELISA in camel sera, but both tests have a lower Se than others. Still, initially we have thought that the C-ELISA would perform better than the I-ELiSA given that its conjugate is species-independent. It seems that the I-ELISA conjugate, however, has

a good affinity also to camel antibodies. It detected all reference positive sera at a reasonable specificity.

Comparison of camel sera to other species was useful. Actually, the performance was best for cattle sera followed by camels and lowest for small ruminant sera (not all data shown). The sensitivity and specificity of the diagnostic serological tests for *Brucella* spp. depend mainly on the antigen used (Azwai *et al.*, 2001). Commercially available test seem to have a higher affinity for antibodies to *B. abortus* than *B. melitensis*. The former was isolated from Mongolian cattle and camels and the latter is the major pathogen in small ruminants. By far we did not reach the sample size of true positive sera. This was due to poor outcome in the laboratory and the fact that no camels were yet in the reference sera and culture bank. Additional sera of culture positive camels were provided by the veterinary research institute. However, sample size calculation was also conservative for expected lower Se and Sp (of 90% and 95%, respectively) then we actually have found. Still, the precision of our sensitivity estimates with up to +/- 20 % for Se (for the C-ELISA) is not sufficient and should be re-estimated as more camel reference sera become available.

Where three and more test results are available, Bayesian modelling to estimate test characteristics has become popular. Since we have now a better idea on prior estimates for test characteristics when used on camel sera (not that so far there was no literature), in a next step we primarily want more precision of these estimates with more reference sera and then consider further possible modelling.

5.7. Conclusions

Due to lower sensitivity of the RBT in camels when compared to other livestock species, we do not recommend it as a screening test for brucellosis monitoring in camels, despite that it is the cheapest and most easily to handle test among the five tests assessed. We recommend either the I-ELISA or FPA for screening. Another confirmatory test such as the CFT can added – or both tests combined, however, the former two tests also have good specificity. The combination of these serological tests, although more expensive, time consuming, and require more specialized laboratories, will reduce false positive and particularly false negative results, which is needed as brucellosis elimination efforts with mass vaccination continue in Mongolia and the situation in camels (that are not vaccinated) needs to be closely monitored. In parallel, the brucellosis reference strain and sera bank in Mongolia finally needs also true positive and true negative samples from camels.

5.8. Acknowledgements

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6. Real time PCR for detection of *Brucella* spp. from DNA of cultures and livestock sera of Mongolia

6.1. Introduction

Brucellosis is the most important zoonosis disease affecting public and livestock health. It remains endemic in many parts of the world (Hassanain & Ahmed, 2012; Kaltungo *et al.*, 2014; Lindahl, 2014; Zinsstag *et al.*, 2011). Brucellosis is caused by *Brucella* species that infect a wide range of animal hosts including all domestic large animals and even marine mammals (Moreno, 2014; Newby *et al.*, 2003). Currently, 11 species are recognized within the genus *Brucella,* but the most six important "classical species" are, *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella ovis*, *Brucella canis*, and *Brucella neotomae* (Awwad et al., 2016). Brucellosis is transmitted from livestock to humans usually thorough the consumption of unpasteurized milk and dairy products (Baljinnyam *et al.*, 2014). Brucellosis is ranked among the livestock diseases causing most economic losses which include decrease productivity as a result of abortions, weak offspring, reduced milk production and losses of trade opportunities (Arasoğlu *et al.*, 2013; Awwad *et al.*, 2016; Dean *et al.*, 2012; Iraqi *et al.*, 2009; J. McDermott *et al.*, 2013; Romero *et al.*, 1995).

The diagnosis of brucellosis is usually done by a combination methods, but is mainly based on the detection of specific antibodies against *Brucella* spp. in sera using serological tests (Montasser *et al.*, 2011). Although several serological tests are commonly used for laboratory diagnosis of brucellosis, there is no single test that is appropriate in all epidemiological situations due to problems with cross-reactivity with other Gram-negative bacteria, and sensitivity and specificity of the diagnostic tests (M. M. Gwida *et al.*, 2011; Matope *et al.*, 2011; Pappas *et al.*, 2006; Poester *et al.*, 2010; Weiner *et al.*, 2010).

While Isolation and culture of *Brucella* spp. remains is the gold standard test for diagnosis of brucellosis, its sensitivity is rather low and cultures can be easily contaminated with other bacteria (Matope *et al.*, 2011). Most *Brucella* spp. strains are slowly growing organisms on primary isolation; some of them requirie serum enriched culture media. Culture success depends on the number of *Brucella* in a sample, of the nature of the sample which is commonly contaminated with other bacteria. Also culture is commonly time-consuming and even experienced laboratories report only isolation rates between 20 and 50% (Awwad *et al.*, 2016; Poester *et al.*, 2010; Romero *et al.*, 1995).

To overcome some of identification complication, efforts have been made on the development of molecular diagnostic assays based on the amplification of genomic targets through different polymerase chain reaction (PCR) approaches (Arasoğlu *et al.*, 2013; Poester *et al.*, 2010). Molecular diagnostic techniques represent an important breakthrough in the diagnostic practice. Maher (2012) suggested that PCR is considered as the golden

test for diagnosis of brucellosis: it is more sensitive and specific than culture and serology. Also, PCR is a very useful tool not only for the diagnosis of acute brucellosis, but also as a predictive marker for the course of the disease and valuable for the early detection of relapses (Hassanain & Ahmed, 2012).

A variety of PCR assays have been formulated that all can differentiate *Brucella* species, such an ampliocn size differentiation, the size discrimination combined with restriction fragment length polymorphism (RFLP) or randomly amplified polymorphic analysis. More recently, real time PCR assays for the detection of *Brucella* spp. have been developed.

Real time PCR offers improved sensitivity, specificity speed of performance when compared to conventional PCR (M. M. Gwida *et al.*, 2011). In contrast to standard PCR, quantitative real-time PCR (RT-PCR) has several advantages: i) quantification of the starting material is possible, ii) high sensitivity and specificity, iii) fast since no post-PCR processing is necessary and lastly iv) there is a reduced possibility to contaminate the surroundings since it is a “one tube” process. However it is an expensive method which requires highly sophisticated laboratory material that is often not available in resource low and middle-income countries. At beginning of this study, the aim was to assess the performance of a recently developed Luminex assay for brucellosis (Silbereisen *et al.*, 2015) with field sera from Mongolia. Since we did not an agreement, sera were further tested the livestock sera with RT-PCR. The aim of this paper is to follow-up on the unexpected results obtained and to make recommendations on the usefulness of RT-PCR in Mongolia.

6.2. Materials and methods

6.2.1. DNA from positive *Brucella* spp. cultures

Control DNA samples of cultures were obtained *B. abortus, B. melitensis, B. ovis and B. suis* from Spiez Laboratory, Spiez, Switzerland. These control DNA samples were used to test primers and probes. Extracted DNA from livestock *Brucella* spp. cultures were either from the epidemiological study 2013 – 2015 (1 camel and 3 cattle) or DNA from 17 previously analyzed *Brucella* spp. cultures 2012 - 2013 (8 sheep, 2 goats, 2 cattle and 5 humans) from the Veterinary Research Institute (VRI) of Mongolia (Baljinnyam, 2016). In addition, we had extracted DNA from both vaccine strains S19 (*B. abortus*) and Rev1 (*B. melitensis*) from the State Central Veterinary Laboratory (SCVL), Mongolia. All samples (with the exception of the vaccine strains) were collected before introduction of the mass livestock vaccination campaigns.

6.2.2. Sera samples for DNA extraction

A total of 240 serawere from randomly selected Mongolian livestock The multi-stage cluster sampling is described for epidemiological study on camel brucellosis in Mongolia (Bayasgalan Chultemdorj Roth *et al.*, Forthcoming). Each 30 randomly selected seropostive and 30 seronegative sera were from camels, cattle, sheep and goats. These sera were handled at the School of Veterinary Medicine, Ulaanbaatar, where never PCR for *Brucella* spp. has been done. In addition, negative control sera were from 10 cattle, 5 goats and 5 sheep from Switzerland. Also, a 23 *Brucella* spp. DNA samples were extracted from RBT positive sera of culture positive animals. Nine of these sera came from the Veterinary Research Institute (3 camels, 3 sheep and 3 goats) and 14 samples (8 cattle, 5 sheep and 1 goat) came from the Central Veterinary Laboratory of Mongolia.

6.2.3. Serological tests

The sera collected in the random multi-stage epidemiological study were tested with the Rose Bengal Test (RBT, Biocombinate, Mongolia), the Complement Fixation Test (CFT, Biocombinate, Mongolia), the indirect enzyme linked immunosorbent assay (I-ELISA, IDEXX AG), the competitive enzyme linked immunosorbent assay (compELISA, APHA, UK) and the fluorescence polarization assay (FPA, Diachemix, LLC, USA). Serological test results were interpreted and classified according to the manufacturer's recommendations.

DNA extraction

Brucella spp. DNA was attempted to be extracted from 283 sera (240 randomly selected Mongolian livestock sera, 20 negative controls from Switzerland by using the QIAamp Mini kit (Qiagen, France) according to the manufacturer's instructions (Protocols for Bacteria), and 23 positive controls from Mongolia using G-Dex^{tm}llc Genomic DNA Extraction kit (iNtRoN Biotechnology, Inc)). Shortly, if available, 40 uL of serum were mixed with 140 µL buffer ATL and 20 µL proteinase K and incubated at 56°C for one hour. Afterwards 200 µL buffer AL was added, followed by a second incubation for 10 min at 70°C. Then, together with 200 uL ethanol (100%) the tubes were subjected to the spin column. After two washing steps, the elution was done with 50 µL buffer AE and eluted DNA was stored at –80°C until further processing. In order to control contamination during the extraction process, only filter tips were used and a reagent control was used in parallel with the samples.

6.2.4. Bruce-Ladder multiplex PCR

INgene Bruce-ladder V is a fast method for the molecular typification of *Brucella* spp., from purified DNA or DNA from an isolated colony. The assay consists of three steps: i) extraction of the genetic material from the sample, ii) amplification of a specific DNA region of the

bacteria, and iii) evaluation of the amplification product. The kit allows detecting and differentiating *Brucella* spp. affecting livestock: *B. melitensis*, *B. abortus*, *B. suis* and *B. ovis* as well as the RB51, B19 and Rev1 vaccine strains.

Tubes for the amplification of samples were prepared in addition to three tubes for positive controls amplification, and one for the negative control. Equal volumes A and B directly from the freezer were mixed in crushed ice. After thorough homogenization, the required volumes were taken for the assay. An appropriate amount of amplification mixture for the number of samples to be processed and an excess amount of 10% (to compensate for possible volume losses during pipetting) was prepared. The tubes used for mixing were kept in crushed ice at all times. The prepared mixtures were then homogenized correctly. Back in the crushed ice, 50 µL of the mixture was added to labeled tubes. Next, 1 µL of previously extracted DNA samples was added to tubes, 1 µL of positive control A1 (B. suis), A2 (RB51) and A3 (Rev1). Amplification controls was added to the corresponding tube and 1 µL of water to the tube labelled as negative control. The content of all tubes was carefully mixed and it was ensured that all liquid was well deposited at the bottom of the tube. If not, the tubes were lightly centrifuged.

The thermocycler conditions were set as followins: 1 cycle of denaturation at 97°C for 7 min, 25 cycles of amplification at 95°C for 35 sec, 64°C for 45 sec and 73°C for 3 min, 1 cycle of final extension at 72°C for 6 min. Samples were maintained at 4°C until subjecting them to the thermocycler.

The PCR products were analyzed by 1.5% agarose gel electrophoresis (GelRed reagent, GelRed™ Nucleic Acid Gel Stain Biotium, was used instead of Etidiumbromid,), and fragment sizes were estimated using the 1 kb plus DNA ladder as molecular size marker (Invitrogen). Gel images were captured with a G-Box equipment (G:Box F3 Syngene, USA).

Results were interpretated in a way that the negative control showed no band; positive sample bands were visualized for *B. suis* at 1682, 1071, 587 and 272 bp; for *B. ovis* at 1683 and 587 bp; for vaccine strains Rev 1- 1682, 587 and 218 bp; while S19 showed a band at1682; *B. abortus* at 1682 and 587 bp, and *B. melitensis* at 1682, 1071, 587 bp. Positive controls Rev1, RB51 and *B. suis* were included in Bruce-ladder multiplex PCR kit.

6.2.5. Quantitative real-time PCR (qPCR)

All sera were tested by quantitative real-time PCR (TaqMan assay). Quantitative real-time PCR was performed using the TaqMan® method. Briefly, one reaction mixture consisted of 26 µL containing 7.75 µL of ultrapure water, 12.5 µL of Kappa Probe Fast (ROX) Master Mix,

1.25 uL of each primer (18 µM), 1.25 µL of TaqMan® probe (5 µM), and 2 µL of DNA product The amplification program employed was the StepOnePlus™ Real-Time PCR System (Applied Biosystems) using 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 15 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Table 6. 1 *Brucella* primers and probes sequences used for amplification by real-time PCR

	Forward Primer	Reverse Primer	Probe	5'Flurophor/ 3'quencher
Brucella spp. (bcsp31)	GCTCGGTTGCCAA TATCAATGC	GGGTAAAGCGTCGCC AGAAG	AAATCTTCCACCTTGC CCTTGCCATCA	6-FAM/ BHQ1
B. abortus (IS711)	GCGGCTTTTCTAT CACGGTATTC	CATGCGCTATGATCTG GTTACG	CGCTCATGCTCGCCA GACTTCAATG	6-FAM/ BHQ1
B. melitensis (IS711)	AACAAGCGGCAC CCCTAAAA	CATGCGCTATGATCTG GTTACG	CAGGAGTGTTTCGGC TCAGAATAATCCACA	6-FAM/ BHQ1
B. ovis (BBOV_A0504)	CGCTATCGATGGC GTAGTTG	CCCTGATTTCAAGCCA TTCC	TGGCCTGACGGACGC GCTTATC	6-FAM/ BHQ1

The primers used to identify *Brucella spp.* target the *Brucella* cell surface 31kDA protein (*bcsp31)* involved in O-chain biosynthesis, which is a highly conserved in the genus *Brucella* (Table 6. 1). The insertion sequence IS*711* is highly conserved in the genus *Brucella,* but the insertion location as well as the copy number varies from species to species. Because of this variance, primers and probes designed to detect the species *B. abortus* and *B. melitensis* target the IS*711*. The reverse primer is for both species is the same since its corresponding binding site is inside the IS*711* gene. However, the forward primer targets specific insertion sites, for *B. abortus* it is an IS*711* element downstream of the alkB gene and for *B. melitensis* an element downstream of BMEI1162 (Hinic *et al.*, 2008; Probert *et al.*, 2004).

The primers and probes were tested using the DNA of strains of *B. abortus, B. melitensis, B. ovis and B. suis* from the Spiez Laboratory. The DNA of each *Brucella spp.* was diluted as follows: non-dilution; 1:100; 1:1000; 1:10000 and 1:100000. A total of DNA extracted from 23 sera from culture positive animals, 2 vaccine strains and 283 sera were tested by qPCR.

Results were analyzed using the StepOne™ Software v2.3. For all steps, nuclease free filter tips and nuclease free water was used. Non-template controls were used as negative controls and reconfirmed *Brucella* spp. DNA was used as positive control.

The CT values of qPCR were considered as weak when CT values of 35-40 cycles (≥35), strong when below a CT value of 35 cycles (<35) and very strong when below a CT value of

30 cycles (≤30). All sera were tested for detection of species and sub-species 5 - 6 times by qPCR.

6.3. Results

Positive controls of 12 DNA culture positive DNA including 2 vaccine strains (Rev1 and S19) were tested by the Bruce-Ladder multiplex PCR. Eight samples were classified as *B. abortus*, and 2 samples as *B. melitensis*.CR.

To positive probes for *B. ovis* were detected in all dilutions and had a high correlation between species and sub-species results. The probe for *B. abortus* cross-reacted weakly with *B. suis* in non-diluted sample, but CT values were low (e.g. strong positive result). The probe for *B. melitensis* cross-reacted with *B. abortus* on both non-diluted and 1:100 diluted samples, while *B. melitensis* weakly cross-reacted with *B. suis* and *B. ovis* in non-diluted DNA samples. We have only used the *B. suis* probe to test the control DNA of *B. suis* culture in this study. As to the extracted DNA obtained from 27 cultures in Mongolia, they were all positive in the genus PCR. However, species could not be assigned from 3 and 1 samples from camels and cattle, respectively (Table 6. 2). The remaining DNA samples from SCVL were classified as vaccine strains S19 and Rev 1 (Table 6. 2). 3 samples were classified as mixture between *B. abortus* and *B. melitensis*. No *B. ovis* was detected in these DNA samples from culture.

Table 6. 2 DNAs of positive cultures *Brucella* and vaccine strains

qPCR	Samples (SCVL)			Samples (VRI)			
	Camel	Cattle	Vac strains	Cattle	Goat	Sheep	Human
No identified *Brucella* species	3	1					
B. abortus			1 (S19)			9	
B. melitensis			1 (Rev 1)	1	1		4
B. abortus / B. melitensis			2 (Rev1), 1 (S19)	1			1
Total	3	1	6	2	1	9	5

DNAs of positive cultures *Brucella* and vaccine strains from State Central Veterinary Laboratory (SCVL) and Veterinary Research Institute (VRI)

Previously, 158 out of the 240 randomly selected sera were tested with RBT, CFT, I-ELISA, C-ELISA and FPA. All 240 sera were tested with the RBT. From these, 77 DNA samples (37 camels, 17 cattle, 14 goats and 9 sheep) were negative, whereas 20 DNA samples (3

camels, 6 cattle, 7 goats and 4 sheep) were identified as *Brucella spp*. but could not be sub classified to a *Brucella* spp. (Table 2). Another 126 samples (19 camels, 24 cattle, 37 goats and 46 sheep) were identified as *B. abortus,* while 3 (1 camel and 2 cattle) were identified as *B. ovis.* A mix of two sub-species (*B. abortus and B. ovis*) was detected in 14 samples (11 cattle, 2 goats and 1 sheep) DNA samples (Table 6. 3).

Table 6. 3 Results of qPCR on the 240 serum samples from the randomized epidemiological study

qPCR	Species				Total
	Camel	Cattle	Goat	Sheep	
Negative	37	17	14	9	77
Brucella spp.	3	6	7	4	20
B. abortus	19	24	37	46	126
B. ovis	1	2	0	0	3
B. abortus / B. ovis	0	11	2	1	14
B. melitensis	0	0	0	0	0
Total	60	60	60	60	240

Brucella spp. included all samples with positive genus PCR, but these samples could not be assigned to a *Brucella* species.

CT values of detection for *B. ovis* were between 35.0 - 39.6 (thus weak positivity). Whereas taking a cut-off of positive and negative DNA samples for *B. abortus* at a CT value of 35 in 56 DNA samples (7 camels, 20 cattle, 15 goats and 14 sheep) we found strong positivity (CT value of <35) (Table 3). In 11 samples (1 camel, 4 cattle, 3 goats and 3 sheep CT values were even below 30 (Table 6. 4).

Table 6. 4 Results of qPCR

qPCR	Species				Total
	Camel	Cattle	Goat	Sheep	
Negative;≥ 35 CT	53	40	45	46	184
B. abortus < 35 CT	6	16	12	11	45
B. abortus <30 CT	1	4	3	3	11
Total	60	60	60	60	240

Results of qPCR considering as strong and very strong reactions of *B. abortus* when CT values of below 35 cycles and below 30, respectively

We found no difference between binary outcome of seropositivity with the five serological tests and strong qPCR results (Table 5). In addition, considering continuous outcomes of serological tests, we only found very weak correlation.

Table 6. 5 From results of qPCR

		RBT		CFT		I-ELISA		C-ELISA		FPA	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
qPCR	Pos	2	9	0	7	1	9	0	7	1	6
	Neg	59	170	31	131	37	170	42	151	39	127

From results of qPCR, 11 samples very strong positive reveal with results five serological tests

As to DNA extracted from negative sera from Switzerland, none of the *Brucella* primers yielded a positive result. Positive control sera (from animals with *Brucella* culture) showed that 21 / 23 samples were genus PCR positive. The remaining two samples were, however, *B. abortus* species PCR positive. Further two samples could not be assigned to a species, and, as was seen with DNA extracted directly from cultures, there were also samples where both *B. abortus* and *B. melitensis* were detected (Table 6). No *B. ovis* was detected in these samples.

Table 6. 6 DNA extracted from of positive sera

qPCR	Samples (SCVL)			Total	Samples (VRI)			Total
	Cattle	Goat	Sheep		Camel	Goat	Sheep	
No sub-species identified	1			1		1		1
Negative	4		3	7	3		1	4
B.abortus		1 (no *Bruclla* genus indentification)		1		2	1 (no *Bruclla* genus indentification)	3
B.melitensis	2		1	3				
B.abortus/B.melitensis	1		1	2			1	1
Total	8	1	5	14	3	3	3	9

DNA extracted from of positive sera that had a corresponding *Brucella* culture. Sera samples were provided by the State Central Veterinary Laboratory (SCVL) and Veterinary Research Institute (VRI).

6.4. Discussion

Mongolian randomly selected sera samples were initially shipped to Switzerland to assess the Luminex assay (Silbereisen *et al.*, 2015) with field sera. We found no correlation between the Luminex assay and the sera samples (and their serology results), which was

lessastonishing given that the former detects *Brucella* spp. antigen and the latter antibodies. However, this led us to attempt DNA extraction from sera samples and do highly sensitive RT-PCR using *Brucella* genus and species premiers.

The Bruce Ladder kit was used for cultures based on the fact that different band sizes and band patterns are shown for different *Brucella* spp. and the kit differentiates between vaccine and field strains. However *B. ovis and B. abortus* will not be detected in case of additional (mixed) presence of *B. suis, B. melitensis* or Rev-1 DNA in a sample because these will add an additional band and thus hide the fewer bands of *B. ovis* and *B. abortus*. Also, *B. melitensis* will not be detected if *B. suis* or Rev-1 DNA are present. Studies with artificially mixed cultures should be performed. We have compared our results on cultures with the species classifications of researchers at the Veterinary Research Institute and State Central Veterinary Laboratory and we did not find 100% correlation between the two sets of results.

This study detected mixed *B. ovis* and *B. abortus* in randomly selected serologically positive and negative sera of camels, cattle, goats and sheep by qPCR. *B. ovis* is less pathogenic for small ruminants than other *Brucella* species and therefore, samples collected based on brucellosis symptoms in ruminants would likely not be collected due to slight symptoms caused by *B. ovis* alone. However, *B. ovis* has never been reported for Mongolia. In addition, there is no report on *B. ovis* in cattle. These are thus highly unexpected results that should be followed up, because B. ovis PCR was positive for the *B. ovis* positive DNA control, but negative in all other Brucella spp. DNA and sera samples. It was only detected in the sera with RT-PCR. For mixed DNA samples that used Brucel-ladder PCR, *B. ovis* could not have seen in mixed infections.

B. melitensis was not detected in randomly selected 240 field sera by qPCR, despite that *B. melitensis* was detected in control DNA of *B. melitensis* cultures.

qPCR showed a very good correlation between genus and species probes and has detected both vaccine strains S19 and Rev 1. However interestingly, the qPCR probes did not detect *B. abortus* from culture DNA collected earlier and that were classified as B. abortus by Bruce-ladder PCR. But B. abortus was detected *Brucella* DNA from field sera.

Brucella has been found to be present in the lymph nodes. It has a high affinity to the placenta (causing abortiions). Therefore, numbers of bacteria in the blood at different stages of infection (acute infection to chronic and later stages) and levels of antibody titers seemingly have a negative correlation (Awwad *et al.*, 2016). Indeed, samples collected based on clinical symptoms of livestock (i.e. abortion) were seropositive and cultures were obtained from clinical abortion material. However, these sera were often bacteria DNA

negative due to absence of still circulating bacteria in the blood. Therefore, one also needs to be careful to compare Bruce ladder *Brucella* spp PCR that is used on culture from clinical material vs. highly sensitive qPCR that looks at circulating bacteria in the blood (sera).

6.5. Conclusions and outlook

The results obtained here on high proportions of *B. abortus* detected by qPCR in randomly selected sera, the absence of any *B. melitensis* detection in recent samples as well as the fact of *B. ovis* and mixed infections in sera of Mongolian livestock were not due to chance, because we could cross-check and assess the approach with different positive and negative samples. We excluded possible contamination of sera that were only handled in an institute that does not do any brucellosis PCR. Therefore, we recommend to further evaluate the threshold of detection, but primarily follow-up on further detection of *B. ovis* with direct qPCR detection in samples (milk and swabs) that have been classified as *B. suis, B. melitensis* or Rev-1 by Bruce-ladder. The confirmation of *B. ovis* and the absence of *B. meilitensis* in more recent samples would have important implications for samples needed in the new brucellosis sera and culture reference bank in Mongolia, detection methods to be used and on the assessment of serological tests.

7. The role of camels in the ongoing ruminant mass vaccination in Mongolia

7.1. Introduction

Brucellosis is a highly contagious zoonosis affecting humans and all domestic animals including camels. It is also considered to be an important public health problem in countries where the disease is endemic such as Mongolia (M. Gwida *et al.*, 2012; J. McDermott *et al.*, 2013; Muma *et al.*, 2013; Racloz *et al.*, 2013; Zinsstag *et al.*, 2005). Brucellosis is a bacterial disease caused by various species of the genus *Brucella* (Baljinnyam *et al.*, 2014; Montasser *et al.*, 2011). Currently, 11 species are recognized within the genus *Brucella*, but the most pathogenic species in terms of public health impact and economics are *Brucella abortus*, *Brucella melitensis* and *Brucella suis,* despite other species also potential human pathogens (Awwad et al., 2016; Poester et al., 2010). Brucellosis is cross-transmitted between animals through contaminated pastures, feed and water sources, and direct contact via contaminated secrets (O. Mohammed *et al.*, 2011; Tesfaye *et al.*, 2011), while it is mainly transmission to humans via consumption of raw dairy products and by direct contact during assistance in livestock delivery and abortion materials (Bonfoh *et al.*, 2012).

The camel play an important socio-economic role within the pastoral and agricultural systemsCamel populations grow (over-proportionally) mainly due to desertification of past more productive pastures. *Camelus bactrianus* are kept in cold arid regions such as in Mongolia and *Camelus dromedarius* are kept in warm arid and semi-arid regions. Camel milk and meat are main food resources, and wool and hides additionally increase income of mobile pastoralists (nomads) in arid regions (M. Gwida *et al.*, 2012; Kudi *et al.*, 1997).

Both *C. bactrianus* and *C. dromedarius* are frequently infected both *Brucella abortus* and *Brucella melitensis* and cross transmission with other livestock species, particularly with infected large and small ruminants occurs when they are in close contact (M. Gwida *et al.*, 2012; Musa *et al.*, 2008). The camel population lives in close contact with cattle, sheep, goats and occasionally horses, particularly during the calving and wool shearing periods and at watering places (wells, branch-water, ditch-water, rivers, and lakes) in Mongolia.

There are no complete studies on vaccination or elimination strategies of brucellosis camels (M. Gwida *et al.*, 2012; Tibary *et al.*, 2006), and none in Mongolia with an important population of Bactrian camels.

In Mongolia, camels have been included in mass screening surveys, but their risk factors of exposure were not further evaluated. We could further analyse the data from a mass screening survey in 2011 (Unpublished results, 2011). There were between 6 and 3590 camel sera from the 22 Mongolian provinces. We found a moderate correlation (Spearman's rho of 0.26) between camel and cattle seropositivity at district level.Sheep were only very

weakly correlated and goats not at all (unpublished data). Indeed, we have found the same correlations between brucellosis seropositivity of livestock species at herd level (correlation between herding of cattle and camels together) in an epidemiological study. The repeated surveys in this same study did not find a drop in camel seropositivity after one year of introduction of vaccination. However, the study found a highly significant difference of camel brucellosis seropositivity of 5.7% in Eastern provinces vs. 0.5% in Southern & Western provinces. In Mongolia, only once *B. abortus* was isolated from a camel and there is no isolate of *B. melitensis* (Bayasgalan Chultemdorj Roth *et al.*, Forthcoming).

The cost-effectiveness of ruminant (Bovidae) mass vaccination has been shown for Mongolia. Vaccination of cattle and small ruminants over several years is the viable control measure in the mobile livestock husbandry system, where, in addition, there is no individual animal tracking system. In this mobile context, test and slaughter is hardly feasible. The minimally needed vaccination coverages in cattle is 60% (of truly immunized animals) and 40% in small ruminants to interrupt transmission (Zinsstag *et al.*, 2005). It is crucial to monitor achieved coverages and such a monitoring in cattle and small ruminants after vaccination campaigns is now in place in Mongolia. However, the role played by Bactrian camels in the epidemiology of brucellosis, particularly if possible maintaining of the infection cycle alone is possible once brucellosis is eliminated in other livetock species, and thus possible re-introduction to domesticated Bovidae must also be understood in view of elimination efforts of brucellosis in Mongolia.

Camels need to be monitored closely also in future to further define if they are only spill-over hosts from domesticated Bovidae. An understanding of multiple livestock population dynamics is important to understand brucellosis transmission in Mongolia and elsewhere (Shabb *et al.*, 2013; Zinsstag *et al.*, 2005). In this study we have attempted to model camel brucellosis in Mongolian Eastern provinces regarding the likelihood of possible own maintanence vs. being a spill-over host alone. Only camels and cattle were considered because only a correlation between camel and cattle seropositivity was found in the epidemiological study. The goal was to contribute to a better understanding of the transmission of brucellosis between camels and other animal species.

7.2. Materials and Methods

7.2.1. Data collection

Data was obtained from several sources and for 4 years for camels and cattle. Only cattle were considered because there were no previous indications that small ruminants play a role of brucellosis transmission to camels. Annual livestock census data for 2011-2014were

provided by the National Statistical Office of Mongolia (NSO, 2015). Initial data on animal brucellosis seroprevalences in 2011 were provided from the mass screening survey with Rose Bengal Test (RBT) by the State Central Veterinary Laboratory. Data on camel and cattle brucellosis seroprevalences by RBT in 2012 were provided by the provincial veterinary laboratory in Dornod province.

The missing data in Sukhbaatar province for 2012 was extrapolated by using the average of 2011 and 2013. Regarding 2013 and 2014, camel and cattle brucellosis seroprevalences wer from a repeated epidemiological survey on camel brucellosis in Mongolia, also using the RBT. All data for Sukhbaatar and Dornod provinces were fitted in the model without intervention both in. In these two Eastern provinces, livestock vaccination in cattle, sheep and goats was not yet implemented in 2012 and only started in September 2013. In 2014, sampling was more than 5-6 months after campaigns and therefore ruminants would have lost their seropositivity due to vaccination., Still, the repeated epidemiological survey was found a stable seropositivity in camels one year of introduction of vaccination (Bayasgalan Chultemdorj Roth *et al.*, Forthcoming).

Rarely, brucellosis serological tests have been validated for camels. The advantage of the RBT is that it can be rather easily performed, does not require expensive laboratory equipment and is a cheap test. RBT reactive is produced in Mongolia (Zinsstag *et al.*, 2015). The RBT is also commonly used in camels Gwida *et al.*, 2011. However, we have seen, unlike in cattle, that the RBT in camels had the lowest sensitivity of serological tests (with comparable specificity) and therefore, seroprevalences are likely under-estimated (Bayasgalan Chultemdorj Felix *et al.*, Forthcoming; M. M. Gwida *et al.*, 2011).

7.2.2. Model description

We have developed a deterministic model with stochastic parameter specification of cattle-cattle, camel-camel, cattle to camel and camel to cattle transmissions in steps of one (1) year (t) (Table 1 and Fig 1), which is adapted to the availability of data and for validation. Because only data on seropositive animals were available, we used only one seropositive compartment (instead of two compartments: “infectious” and “recovered” compartments) and have retained for each species a compartment S for susceptible and I for infected/seropositive. We considered transmission within cattle and within camels; and between cattle to camels. We have omitted to conceptualize the the transmission from camels to cattle because camels are not known to be a primary host of *Brucella* spp. *(M. M. Gwida et al., 2011)*. We have assigned the state variables at time t for both species compartments “cattle” (subscript c) and “camel” (subscript b for Bactrian) as S_c and S_b and I_c and I_b, respectively. The model was run for 20 years.

The fitted values against the weighted compartments of susceptible and seropositive camels and cattle are shown in Table 1 for the years 2011 - 2014.

Table 7. 1 The baseline year estimates of brucellosis in camels and cattle summarized for the two provinces Dornod and Sukhbaatar

Years	N camels	N Susceptible camels (S_b)	N Seropositive camels (I_b)	Ncattle	N Susceptible cattle (S_c)	N Seropositive cattle (I_c)
2011	16‘235	15‘908	327	246‘338	241‘167	5‘171
2012	14‘759	14‘156	603	270‘869	262‘105	8‘764
2013	13‘617	12‘896	721	296‘729	273‘807	22‘922
2014	13‘206	12‘376	830	338‘396	299‘554	38‘842
Weighting		0.00064	0.00462		0.00004	0.00007

7.2.2.1. Compartments and flows

Compartments S_c is the susceptible cattle population Compartment I_c is the brucellosis-seropositive cattle population. The size of I_c was obtained by multiplying the two provincial cattle population with the seroprevalence estimated in the epidemiological study. Compartment S_b is the susceptible camel population. Compartment I_b is the brucellosis-seropositive camel population. The initial size of I_c was obtained by multiplying the two provincial cattle population with the prevalence estimated in during years of surveys. Table 2 shows the parameters units, and estimates used in the transmission model of cattle and camels in Eastern provinces of Mongolia (Table 2).

Table 7. 2 Parameters, estimates and units used in the brucellosis transmission model

Parameters	Description	Estimate	Unit (remarks)
Cattle			
b_c	cattle birth rate	0.15	Year $^{-1}$
S_c	susceptible cattle	241167	(cattle*year) $^{-1}$
I_c	seropositive cattle	5171	(cattle*year) $^{-1}$
β_{cc}	cattle contact rate	0	(cattle*year) $^{-1}$
μ_c	mortality rate of cattle	0.046	Year $^{-1}$
Camel			
b_b	camel birth rate	0.02	Year $^{-1}$
S_b	susceptible camel	15908	(camel*year) $^{-1}$
I_b	seropositive camel	327	(camel*year) $^{-1}$
B_{bb}	camel contact rate	3.23	(camel*year) $^{-1}$

μ_b	mortality rate of camel	0.002	Year $^{-1}$

Parameters, estimates and units used in the brucellosis transmission model among camels and cattle and between cattle and camels

The descriptions of flows are visualized in Figure 1 and are as follows: flows into the susceptible cattle compartment (S_c) are newborn cattle; cattle birth rate (unit: cattle/year) = $\alpha_c(S_c + I_c)$, where α_c is the (same) birth rate of susceptible and infected cattle (Table 1)Flows out of compartment S_c are mortality of susceptible cattle, seropositive (infected) cattle: the mortality rate (unit: cattle/year) of susceptible cattle = - $\mu_c S_c$, where μ_c is the mortality rate of cattle. Seropositive cattle (=infected in cattle), (unit: cattle/year) is = - $\beta_{cc} S_c I_c$ in analogy to equation (2). Flows into compartment S_c appear as positive terms and flows out of compartment S_c as negative terms in equation (2). Flows going into the compartment of seropositive cattle I_c are infected cattle as aforementioned. Flows out of compartment I_c are mortality of seropositive cattle. Mortality of seropositive cattle I_c= - $\mu_c I_c$ is alike to the mortality of susceptible cattle but for compartment I_c. The differential equation for camels is constructed with an equivalent schematic (see equations 3 & 4). Flows into the camel compartment are with an equivalent for cattle.

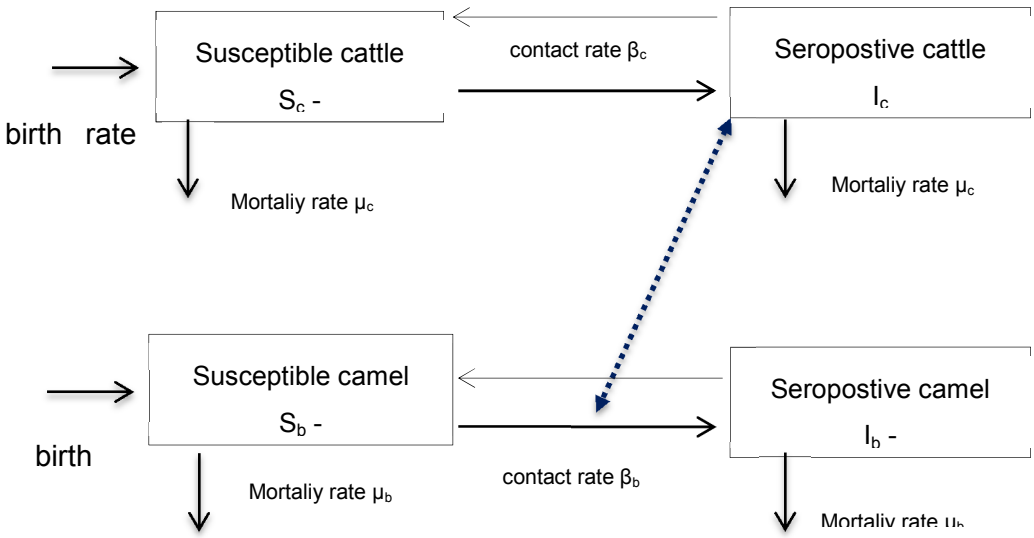

Figure 7. 1 The model framework

The model framework which is composed of compartments for susceptible camel and cattle (serologically negative by the Rose Bengal test). Susceptible camels and cattle become infected and move to the compartments of seropositive camel and cattle (Rose Bengal test).

The following equations have been used to describe the flows in and out of compartments

For susceptible cattle

$\frac{dS_c}{dt} = b_c(S_c + I_c) - \mu_c S_c - \beta_{cc} S_c I_c - \beta_{bc} S_c I_b$ (1) cattle to cattle

For seropositive cattle

$\frac{dI_c}{dt} = \beta_{cc} S_c I_c + \beta_{bc} S_c I_b - \mu_c I_c$ (2) cattle to cattle

Birth rate; mortality rates for cattle:

$b = S_c I_c$

$mortality\ rate = -\mu_c S_c$ for susceptible cattle

$mortality\ rate = -\mu_c I_c$ for seropositive cattle

For susceptible camel

$\frac{dS_b}{dt} = b_b(S_b + I_b) - \mu_b S_b - \beta_{bb} S_b I_b - \beta_{cb} S_b I_c$ (3) cattle to camel

For seropositive camel

$\frac{dI_b}{dt} = ((\beta_{bb} S_b I_b) + (\beta_{cb} S_b I_c)) - (\mu_b I_b)$ (4) cattle to camel

Birth rate; mortality rates for camel:

$b = S_b I_b$

$mortality\ rate = -\mu_b S_b$ for susceptible camel

$mortality\ rate = -\mu_b I_b$ for seropositive camel

7.2.2.2. Fitting the transmission model

The fitting of the model to data was done with Vensim system analysis software (Ventana System Inc., 60 Jacob Gates Road, Harvard, MA, USA; www.vensim.com) using the Powell nonlinear maximum-likelihood optimization algorithm (Zinsstag *et al.*, 2005). Parameters were optimized on the basis of the goodness-of-fit, which is called "payoff" in Vensim software. The payoff compares the log likelihood of the current model with the log likelihood of a perfect model (having as many parameters as data points). The best model is the one with the payoff value closest to zero. In a first step, mortality and birth rates were optimized for the susceptible cattle (S_c) and camel (S_b) (Figure 1). Birth rates were expressed proportionally to the total populations; mortality parameters in livestock included natural mortality. In the second step, the transmissions within cattle and within camels were fitted by fixing demographic parameters. To fit the transmission process, the proportions of infected I_c and I_b were expressed as uniform probability distributions and their boundaries were varied to identify the best fit (in terms of the deviance) of contact rates for the transmission between

cattle and between camel. The transmission to camel is expressed as additive contributions of transmission from cattle to camel (Figure 1).

7.2.2.3. Sensitivity analysis

Sensitivity analyses were carried out for the fitting of the model without interventions. For this we used multivariate Monte Carlo sensitivity simulation (MVSS) in Vensim with 200 simulations over the range of parameters specified in Table 1. Monte Carlo multivariate sensitivity works by sampling a set of numbers from within bounded domains. To perform one multivariate test, the distribution for each parameter specified is sampled, and the resulting values used in s simulation. All simulations then were summarized by calculating the mean values and 95% confidence limits.

7.3. Results

The cattle population grew steadily, while the camel numbers decreased between 2011 and 2014. Seropositivity of brucellosis increased in both cattle and camel populations in the two provinces. The model fitted very well for both the cattle and camel populations (Figures 2 and 3). The fits to the seropositive cattle and camel for brucellosis are presented in Figures 2 and 3. For cattle and camels, proportions infectious were estimated by variation of their boundaries in a uniform distribution. The boundaries with the best payoff were then used in model.

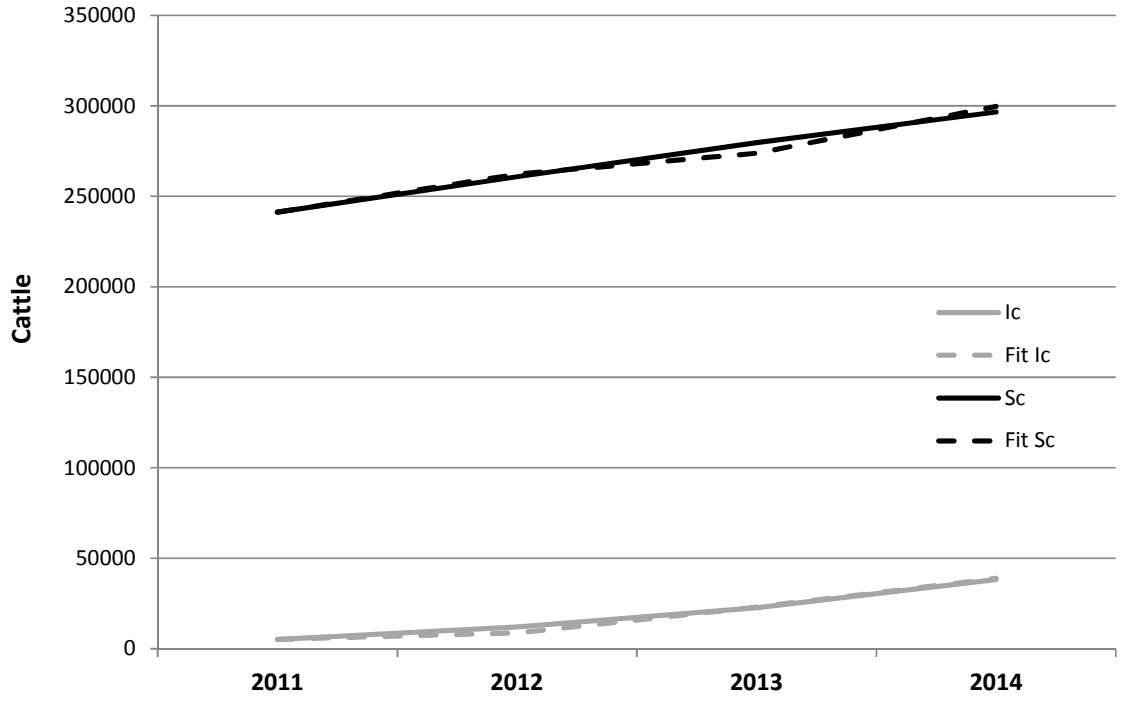

Figure 7. 2 Fit of the model to the susceptible and seropositive cattle populations between 2011 and 2014.

The two upper (black) lines show the reported (full line) and fitted (dotted line) cattle populations; the two lower lines the estimated and fitted population of seropositive cattle in the two Eastern provinces.

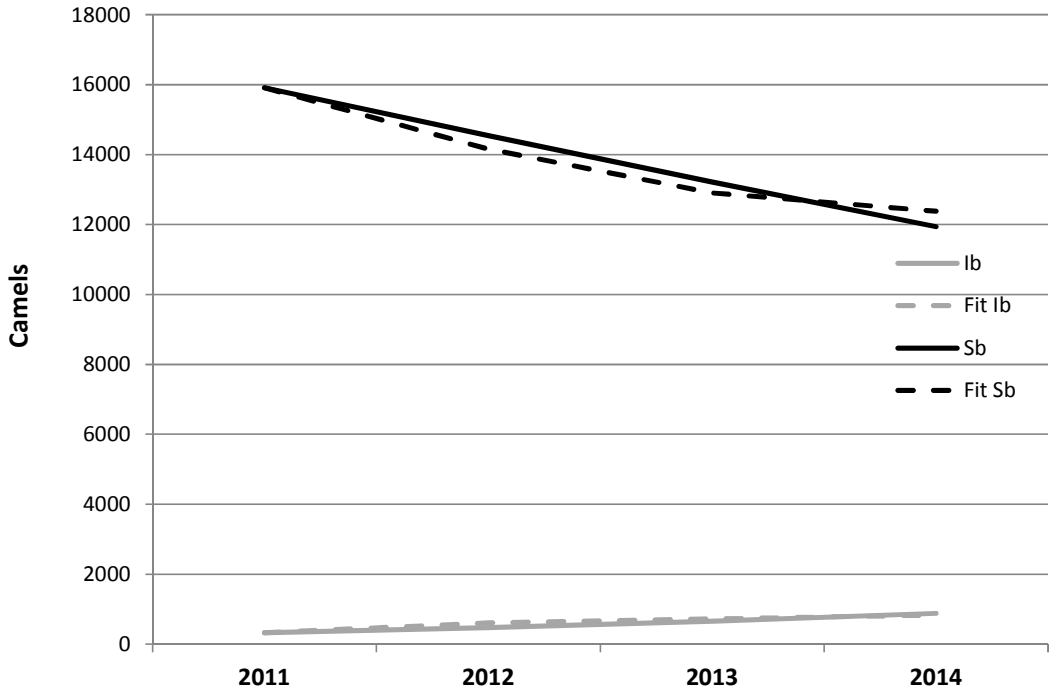

Figure 7. 3 Fit of the model to the susceptible and seropositive camel populations between 2011 and 2014.

The two upper (black) lines show the reported (full line) and fitted (dotted) cattle populations; the two lower (grey) lines the estimated and fitted population of seropositive cattle in the two Eastern provinces.

The following scenarios of transmission (based on the demographic model) were modeled i) within cattle-cattle and camel-camel transmission (with no linkages between the two); ii) cattle-cattle and cattle-camel; iii) camel-camel and camel-cattle; iv) cattle-cattle, camel-camel and cattle-camel; v) cattle-cattle, camel-camel and camel-cattle; and finally vi) cattle-cattle, camel-camel, cattle-camel and camel-camel. The full model vi had the best pay-off. Which is also seems reasonable. Interestingly, catte-camel transmission was then near 0.

7.4. Discussion

The demographic model on cattle and camel demography performed a 4-years period in Eastern provinces (Sukhbaatar and Dornod) performed very well. The model using steps of

one year was validated with livestock demographic and disease data from 2011 to 2014 (before introduction of the vaccination campaign).

The reproductive efficiency of Camelidae, particularly of the Bactrian camels and dromedaries, is generally considered low. In camels, birthing rates rarely exceed 40% in nomadic herds and 70% in more intensive herds (i.e. a calf every 2 and a half and 1 and a half years, respectively). In addition to low birthing rates, camel herds suffer from high neonatal losses; sometimes reaching epizootic proportions (Ali *et al.*, 2009; Tibary *et al.*, 2006). Actually, in our fitted model, birth rates of camels were even smaller.

Shabb *et al.* (2013) modeled the demographics of cattle, sheep, goats and horses. Camels were not included in this demographic model, partially due to lack of data. Therefore, we first had to establish a demographic model for camels including the compartments of susceptible and seropositive animals. We have foreseen an infectious contact rate between cattle and camels.

Zinsstag *et al*. (2005) have modeled brucellosis in cattle, sheep and humans using three compartments, susceptible (X), seropositive (Y) and immunized animals (Z) in Mongolia. The validation of the vaccination intervention used data from the first three years (2000 – 2012) of the past brucellosis mass vaccination campaign in Mongolia. This will be a next step: to consider the introduction of vaccination in cattle. We will use the data from seromonitoring of the achieved vaccination coverage in cattle. A survey in five randomly selected districts of Dornod province after the first vaccination campaign showed that 50.5% of surveyed cattle herds had a within herd seropositivity of lower than 60% and almost 25% lower than 10% meaning that they were not reached by a vaccination team at all. The overall coverage was critically at the 40%, the minimally needed cut-off. Veterinarians were asked to start vaccination with the furthest away herds from the district centres. Indeed, they have done this and coverage was higher the further away. However, once they were to vaccinate the nearest herds, several veterinarians no longer had sufficient vaccine doses. This data, together with more recent vaccination and monitoring, will be collated to be able to model a compartment of ‘recovered’ for cattle and see the implications for the camel seropositivity. However, acknowledging that the best fit of the transmission model allowed cattle-camel transmission and camel-cattle transmission (with rather strong camel-cattle transmission and not cattle-camel transmission) – it remains exciting to follow-up on the theories if there is no epidemiological linkages between brucellosis in cattle and in camels – or even more interesting (and contradicting literature) if eventually rather camels transmit brucellosis to cattle than vice versa.

8. General Discussion

8.1. Mongolia's efforts to eliminate brucellosis

Brucellosis is still one of the most important endemic zoonotic disease in Mongolia. Human incidences are dropping after introduction of mass livestock vaccination, however, there still are many human cases and the disease continues to cause economic losses in the livestock sector (notably the second most important sector in Mongolia) due to abortions, decreased animal products (milk, meat, wool), death of weak new-borns, and infertility. Export bans of livestock due to brucellosis have been imposed in the past by important import countries such as Russia. Finally, the zoonotic potential of the disease in camels and livestock production in this so far neglected species should not be over-looked (M. Gwida *et al.*, 2012).

Over the past five decades, different control strategies have been implemented in an attempt to control brucellosis. They have achieved temporal reduction of its burden in animals and humans. After the change of the government in the 1990s from socialist to private economy, brucellosis rapidly spread again once vaccination stopped and due to lack of governmental funding, less surveillance and uncontrolled new private veterinarians. A new national ruminant vaccination campaign is ongoing since 2010 in Mongolia. Animal vaccines, such as *B. melitensis* Rev.1 and *B. abortus* S19 play a key role to reduce brucellosis transmission, particularly in the mobile livestock keeping system where tracing of individual animals is not possible. Several countries, including Mongolia and Kyrgyzstan, have recently adopted conjunctival vaccination that is now recommended by the OIE (Racloz *et al.*, 2013). Monitoring of vaccination campaigns showed that coverage was sufficient for small ruminants, but rather critical for cattle due to difficulties of veterinarians to restrain the animals. Therefore, the decision makers have decided to carry out again vaccination campaigns using injection (intramuscular vaccination). The latter is, however, more prone to cause vaccine-induced abortions if campaigns are carried out not early enough before mating season, because vaccinal bacteria circulate longer in the animals compared to conjunctival inoculation.

The minimally needed vaccination coverage in cattle and small ruminants to interrupt further transmission must be monitored and are 40% for small ruminants and 60% for cattle (Zinsstag *et al.*, 2005). Such a monitoring in cattle and small ruminants after vaccination campaigns is now ongoing. But in none of the provinces camel herds (nor horses) have been covered during vaccination campaigns and monitoring. Umnogobi is the only of the 22 Mongolian provinces with no livestock brucellosis vaccination at all. The rational for this

governmental decision was based on the fact of its large proportion of camels and the vastness of the province.

The camel husbandry in Mongolia - after its steep decease during the change from socialism to private economy and slight increase again in past years – remains largely in the hands of pastoralists of the Gobi Desert. Camels are herded in close contact with other livestock. It is not known if they are primary hosts who can maintain the disease (and thus potentially re-infect other livestock after vaccination campaigns), or if they are solely spill-over hosts.

The current institutional set-up for brucellosis research and diagnostic in Mongolia is that there are three institutes working apart: i) The strain and sera bank of brucellosis and a National Reference Laboratory for Brucellosis have been established at the State Central Laboratory since 2013, ii) the Veterinary Research Institute has actually most experience on culture and research on brucellosis; and iii) this study of camel brucellosis was carried out at the School of Veterinary Medicine that has competencies in epidemiology and laboratory work on animal diseases, but so far not on brucellosis. In fact, all three institutes lack sound bacteriological experience, phenotypic characterization and genotyping methods. They are limitated in funding and there is only poor cooperation and exchange of information between the institutes. Also, the Mongolian brucellosis sera-bank (gold standard positive and negative serum) is not yet fully established and can yet hardly validate ruminant serological tests for their use in Mongolia. Only one *B. abortus* isolate form a camel is in the bank – and this isolate was collected by this study.

8.2. Overall methodology

Previous surveys determined sero-prevalences of brucellosis in different regions of Mongolia. Despite having included camels, no risk factors have camel brucellosis have been assessed.

Veterinary laboratories have reported that cases of brucellosis in camels in some localities of Mongolia are increasing. A study found a notable 3% seroprevalence in a population-based survey in Sukhbaatar province in 2010 (Baljinnyam *et al.*, 2011). A large screening survey for brucellosis of livestock diseases in 2011 showed rather high seropositivity in camels in Dornod and Sukhbaatar provinces (37% of 260 tested camels in Dornod and 1% of 469 camels in Sukhbaatar (Unpublished results, 2011).

This is the first epidemiological study that was aimed at assessing risk factors on camel seropositivity using repeated multi-stage cross-sectional study, and in a complementary study, also serological test characteristics for use in camels in Mongolia. This multi-stage cross-sectional study was conducted in five provinces (Eastern provinces: Dornod and

Sukhbaatar; and South-Western: Dornogobi, Umnogobi and Khovd provinces. Each 6 districts were selected proportional to size of their camel populations in the provinces for 2 consecutive years between 2013 and 2015.

The surveys were planned from April to end of July, during and just after parturition in order to take samples for bacteriology. Herders with major income from goat cashmere kept often during the sampling period their camel herd far away because they were occupied with combing of the cashmere goats. Also, herders were moving continuously. Lactation of female animals depended on the availability of good pasture during our sampling between mid-spring and summer, which has complicated sampling. But also, at the beginning of the warm season in Southern and Eastern provinces, camel herds were kept closer to the households during the wool shearing period and to protect new born animals from wild animals or to avoid that female camels ran away during the calving period. For several reasons, in initially selected camel herd (belonging to a hot ail) could not be enrolled in the study and a replacement hot ail had to be contacted. This was the case in less than 6% in Eastern provinces, while it went up to 30% of cases in Southern and Western provinces and we could not re-visit all herds that were sampled the year before. Essentially, if it had not been for the sampling of vaginal swabs and milk for bacteriology (where harvest is best in the calving season), a sero-survey in camel herds are easier during summer and autumn in these mobile herds. Serological monitoring of camels should be coupled with monitoring of vaccination coverage of other livestock in October (one month after vaccination), or, specific surveys in camels can be done after the summer break.

The overall seroprevalence by RBT in camels was 2.3% - and the whole provincial range was between 0.3% and 6.1%. Eastern provinces had significantly higher seroprevalences than Southern & Western provinces. Indeed, being in an Eastern province was the most important risk factor of camel brucellosis seropositivity with an OR of 13.2 when compared to the Southern & Western provinces. The camel densities in the Eastern provinces are lower than in the Southern & Eastern provinces. However, before introduction of vaccination, the Eastern provinces had the highest seroprevalences in livestock reported for Mongolia. This is an indication that camels were exposed here more frequently by close contact to cattle or small ruminants.

Camel seroprevalences were stable for the two survey years, despite introduction of ruminant vaccination: 5.7% (95% CI 3.1-10.2%) and 5.8% (95% CI 3.3-10.1%) in Eastern provinces and much lower at 0.4% (0.2-1.2%) and 0.5% (0.1-2.0%) in Southern & Western provinces. We have isolated *Brucella abortus* from camel. Indeed, camel seropositiviy was associated to herding together with cattle and was closest correlated to cattle herd

seropositivity. Note that - where there was ruminant vaccination - sampling was more than 5-6 months after vaccination campaigns and therefore ruminants would have lost their seropositivity due to vaccination.

The results of the present study indicate that brucellosis exists up to important seroprevalences in camel herds in Mongolia and is likely endemic in Eastern provinces. Further monitoring is needed to assess if camel seroprevalances decrease with ongoing ruminant vaccination. This should be coupled with more confirmation about *Brucella* spp. isolates from Mongolian camels (up to date only one *B. abortus* was isolated and it is not known if *B. melitensis* does not infect Mongolian camels).

Camels might not only act as reservoir for *Brucella* spp. after vaccination campaigns and re-infect other livestock as they are kept together and spread the disease through uncontrolled animal movements, brucellosis may also be transmitted from camels to humans, especially through milk, traditional practices of livestock product consumption and lack of an effective control program. Consumers of camel products say that raw and fermented camel milk has a curative effect on health. Since pastoralism involves a lack of stable diagnostic facilities and access to veterinary and public health professionals, the disease is likely to remain untreated in many nomadic settings, with both humans and livestock being infected (Racloz *et al.*, 2013).

Effective control of camel brucellosis could be achieved by establishing an effective diagnostic and surveillance system coupled with rigorous monitoring. Cost-effectiveness of control measures in cattle and small ruminants have been shown for Mongolia. The cost-effectiveness of such a surveillance programme in camels could also be established. The main purpose of the system would be to assess if seroprevalences in camels drop in parallel to ongoing vaccination of other livestock and to obtain more *Brucella* spp. isolates from camels. Without this knowledge, vaccination of camels cannot be recommended. In any case, the vaccine dose for camels and the vaccine strain to be used remain for the time being unassessed.

The levels of disease reported in literature appear to depend on the diagnostic test used. Initial testing with the Rose Bengal test (RBT) is usually conducted as a sensitive rapid screening test, yet, due to *Brucella*'s cross-reactivity with other bacteria, further serial testing with another test, the complement fixation test (CFT), serum agglutination test (SAT), competitive enzyme-linked immunosorbent assay (C-ELISA), and, most recently, the fluorescence polarization assay (FPA) is recommended.

The most appropriate serology test for Mongolian camels was so far not been assessed. In a brucellosis endemic country such as Mongolia professionals should use standardized

diagnostic tests and reagents according to internationally accepted standards. Brucellosis diagnostic tests are commonly validated and cut-offs set with gold standard reference sera from national sera bank. Therefore, it is of paramount importance to further establish the national collection of a sera bank from culture positive and negative animals in Mongolia. Up to date it is incomplete for cattle and small ruminants and camel samples are missing all together. Bacteriology capacity at the State Central Laboratory hosting the reference bank must be maintained and improved. Rigorous biosafety measures must also be maintained: *Brucella* spp. are considered as one of the most hazardous laboratory pathogens given their high infectivity. This would be enable the implementation of initial quality control of laboratories, validation of different serological tests and improvement of overall quality of the national diagnostic system.

We have attempted to assess test characteristics of use of serological tests in camels. Fortunately, there were few other camel *Brucella* spp. culture positive sera available from the Veterinary Research Institute that has follow-up on a report of mass abortion in a camel herd to be used as true seropositive sera. We have also used pairwise test comparison using Kappa statisitics and ROC curve analysis as well as a comparison to serological results obtained in other species. Due to lower sensitivity of the RBT in camels when compared to other livestock species, we do not recommend it as a screening test for brucellosis monitoring in camels, despite that it is the cheapest and most easily to handle test among the five tests assessed. We recommend either the I-ELISA or FPA for screening. Another confirmatory test such as the CFT can added – or both tests combined, however, the former two tests also have good specificity. The combination of these serological tests, although more expensive, time consuming, and require more specialized laboratories, will reduce false positive and particularly false negative results.

Previous studies have genotyped *B. melitensis* using Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA) in 2013 as dominant strain in ruminant (Baljinnyam, 2016). In this study, four *Brucella* cultures were identified as *Brucella abortus* by Bruce-ladder multiplex PCR.

Further, by using qPCR the absence of *B. melitensis* detection was shown in our samples. However, the fact of *B. ovis* and mixed infections detection in our field sera were detected (and was assessed with cross-checking with different positive and negative controls) should be followed up. The confirmation of *B. ovis* and the absence of *B. meilitensis* in more recent samples would have important implications for samples needed in the new brucellosis sera and culture reference bank in Mongolia, detection methods to be used and on the assessment of serological tests.

This study was conducted during 2 years in each selected areas. Longer time sequences would have helped to more clearly understand the epidemiological picture for camel brucellosis. We have assumed a measurable drop of seroprevalences before and after introduction of vaccination campaigns as has been seen in people during monitoring survey. A first transmission model for brucellosis in camel of Eastern province was established. The compartmental model considered transmission within and between camel and cattle populations. The fits to the seropositive camel and cattle data were used for four years by Rose Bengal Test. The model including both cattle to camel as well as camel to cattle transmission had the best pay off. Actually, the model set inexpectantly cattle to camel transmission to zero (but still was having the potential flow included in the model, improved the model), whereas there was important infectious contact rates of camel to cattle. In conclusion, the model did not exclude transmission between camels and exchange between camels and cattle.

9. Recommendations

The results of this survey confirm the presence of *Brucella* spp. in camel herds in Mongolia. Camel seropositivity was significantly higher in Eastern than in Southern & Western provinces. It was closest associated to the infection in cattle. We did not observe a decrease of camel brucellosis seropositivity with ongoing ruminant vaccination. Repeated studies are needed to see if seroprevalenes in camels are dropping over years with ongoing vaccination in other livestock species. Spill-over from cattle is possible, but also own maintenance of brucellosis in the camel population could not be excluded. Therefore, much attention should be given to achieve sufficient vaccination coverage in cattle in Mongolia to interrupt this assumed spill-over. Future and continued monitoring of camels is highly recommended. Also, more isolates are needed to confirm that seropositivity in camels is due to *B. abortus* alone.

Due to lower sensitivity of the RBT in camels when compared to other livestock species, we do not recommend RBT as a screening test for brucellosis monitoring in camels, despite that it is the cheapest and most easily to handle test among the five tests assessed. We recommend either the I-ELISA or FPA for screening. Another confirmatory test such as the CFT can be added – or both tests combined, however, the former two tests also have good specificity. The combination of these serological tests, although more expensive, time consuming, and require more specialized laboratories, will reduce false positive and particularly false negative results, which is needed as brucellosis elimination efforts with mass vaccination continue in Mongolia and the situation in camels (that are not vaccinated) needs to be closely monitored. In parallel, the brucellosis reference strain and sera bank in Mongolia finally needs also true positive and true negative samples from camels.

We recommend to further evaluate the threshold of detection of the qPCR, but primarily to follow-up on further detection of *B. ovis* with direct qPCR detection in samples (milk and swabs) that have been classified as *B. suis, B. melitensis* or Rev-1 by Bruce-ladder. The confirmation of *B. ovis* and the absence of *B. meilitensis* in more recent samples would have important implications for samples needed in the new brucellosis sera and culture reference bank in Mongolia, detection methods to be used and on the assessment of serological tests.

This study on camel brucellosis and past epidemiological and monitoring studies in Mongolian livestock and people have been funded by external agencies. Research on brucellosis was done in parallel by the Veterinary Research Institute. The Mongolian government invests important money in the vaccination of livestock to work towards elimination of brucellosis in Mongolia that causes important burden of disease. Monitoring of

achieved vaccination coverage is now ongoing by the Veterinary services. However, better exchange between the three veterinary institutes with shared interest in brucellosis diagnostics and research must be improved. Without sharing of facilities, results and experiences, there is risk of duplication of known, but the unknowns of progress towards elimination will remain unrecognized until they become an urgent problem that needs a fast but uninformed fix. We therefore recommend that an inter-institutional brucellosis diagnostic and research working group is set-up. This inter-institutional group should also include the human health sector in view that has several brucellosis programs. Good progress and needed actions for correction towards elimination can only be achieved if all interest groups share information and approaches also to jointly apply for grants to maintain research and monitoring.

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Appendix 1 Additional files for Chapter 4

The interview included Hot ail questions on herd risk factors (including buying/selling of animals, sharing of pastures and watering places), herd and human health management (including disposal of aborted fetuses/placentas), vaccination of cattle and small ruminants.

Hot ail (nomadic camp) questionnaire

Used for the survey on camel brucellosis in selected aimags (provinces) between 2013 and 2015. SDC Animal Health Project

Identification code:

|__|__| |__¦__| |__¦__|

Aimag Soum Hot ail/hh

1. Date of interview |__|__|__|__| |__|__| |__|__|
Year Month Day

2. Name of interviewer: ……

3. Coordinates of the hot ail:
North I__I__I I__I__I__I__I__I__I, East I__I__I I__I__I__I__I__I__I

4. Surname of the hot ail's head..... Name ……..

5. Number of the households in hot ail I___I___I

N	Name of household head	Number of livestock					
		Sheep	Goat	Horse	Cattle	Camel	Other
1.							
2.							
3.							
4.							
5.							

6. Has brucellosis been diagnosed in your livestock?

	Species	Answer		If yes, when (year)	Result	
		Yes	No		Positive, how many?	Negative, how many?
1.	Cattle			I__I__I__I__I		
2.	Yak			I__I__I__I__I		
3.	Sheep			I__I__I__I__I		
4.	Goat			I__I__I__I__I		
5.	Camel			I__I__I__I__I		
6.	Horse			I__I__I__I__I		
	Total					

7. Has your livestock been vaccinated during the past autumn?

N	Species	Answer		If yes, when (year, month)	Number of vaccinated livestock
		Yes	No		

1.	Cattle			I__I__I__I__I. I__I__I	
2.	Yak			I__I__I__I__I. I__I__I	
3.	Sheep			I__I__I__I__I. I__I__I	
4.	Goat			I__I__I__I__I. I__I__I	

8. Did you buy any animals the past 12 months? Yes I__I No I__I

If yes (please fill in a table)

1.	From where	
2.	When (within last months)	a). 1-3 b). 3-6 c). 6-12 d). .....
3.	Was that animal tested for brucellosis?	
4.	Was it noted in your herdbook?	

9. How many families shared the pasture for their herd? I__I__I

10. How many families shared the watering points for their livestock? I__I__I

11. How do you handled aborted material?
(please write) ……..

12. During the past calving season, did you have abortions in cattle Yes I__I No I__I
If yes, which period of the pregnancy? I__I__I

13. Did you observe swollen front knees and creaky noises of the joint in a camel?
Yes I__I No I__I

Thank you very much for your participation

Additional file 2. Herder questionnaire

The interview included questions on knowledge on epidemiology of brucellosis and history of brucellosis in the household.

Individual (herder) questionnaire

Used for the survey on camel brucellosis in selected aimags (provinces) between 2013 and 2015. SDC Animal Health Project

Identification code:

|__|__| |__|__| |__¦__| |__| |__¦__|

Aimag ① Soum ② Hot ail/hh ③ Species ④ Numerator ⑤

Name of interviewer ……..

A. Information on the person who is a livestock owner (herder)

1. **Date of the interview and blood sampling:** |__¦__¦__¦__| |__¦__| |__¦__|
   Year Day Month
2. **Surname**…… **Name**……
3. **Date of birth** |__¦__¦__¦__| |__¦__| |__¦__|
4. **Sex** Male |__| Female |__|
5. **Occupation:** A: Herder |__| B: Other |__|
6. **Phone numbers**: |__|__|__|__|__|__|__|__| or: |__|__|__|__|__|__|__|__|
7. **Have you ever given blood to test for human brucellosis?** Yes |__| No |__|
   If yes, when? |__|__|__|__| |__|__|
   Year Month
   Was the result positive? Yes |__| No |__|

B: Asking about Brucellosis knowledge

8. **Which symptoms can brucellosis patients have?**

A. Skin rash	yes |__| no |__|	G. Weakness	yes |__| no |__|
B. Fever	yes |__| no |__|	H. Night sweat	yes |__| no |__|
C. Arm and leg pain	yes |__| no |__|	I. Depression	yes |__| no |__|
D. Back pain	yes |__| no |__|	K. Abortion	yes |__| no |__|
E. Muscle pain	yes |__| no |__|	L. Tsticle pain	yes |__| no |__|
F. Exhaustion	yes |__| no |__|	M. Headache	yes |__| no |__|

9. **Which animals transmit brucellosis infection to human?**

	Yes	No		Yes	No
Cattle	I__I	I__I	Horse	I__I	I__I
Wolf	I__I	I__I	Dog	I__I	I__I
Goat	I__I	I__I	Sheep	I__I	I__I
Cat	I__I	I__I	Deer	I__I	I__I
Camel	I__I	I__I	Gazelle	I__I	I__I

10. Which of the following symptoms can animals infected with brucellosis show?

A. Abortion	Yes I__I	No I__I
B. Delivery with difficulty	Yes I__I	No I__I
C. Weight loss	Yes I__I	No I__I
D. Lack of milk	Yes I__I	No I__I
E. Swollen leg joints	Yes I__I	No I__I
F. Limping for a long time	Yes I__I	No I__I
G. Animal tongue becomes blue	Yes I__I	No I__I

11. How can a herd become infected with brucellosis?

A. By mixing with a brucellosis infected herd	I__I
B. By sharing the same pasture with a brucellosis infected herd	I__I
C. By sharing watering places (well, river) with a brucellosis infected herd	I__I
D. By introducing a single brucellosis infected animal into a herd	I__I

12. How can people become infected with brucellosis?

A. By consuming raw milk	I__I
B. By consuming raw milk products	I__I
C. By consuming half-done meat	I__I
D. By milking animals	I__I
E. By contact with animal wool and skin	I__I
F. By combing cashmere	I__I
G. By shearing wool	I__I
H. By contact with animal placenta	I__I
I. By assisting in obstetric work	I__I

13. Do you use personal protective clothes during contact with animals?

Yes I__I No I__I

If yes, what kind of personal protective clothes do you wear? (Please write)

.....

……..……..……..……..

14. From where do you get information on brucellosis?

A. From soum physicians and health care workers	I__I
B. From veterinarian	I__I
C. From promotion materials	I__I
D. From radio and TV	I__I
E. From newspapers and magazines	I__I
F. From friends and relatives	I__I

15. Do you have a traditional way of raw livestock produces?

Yes I__I No I__I

If yes, which production and how does it use? (please write)

……..

16. Do you use personal protective clothes during the lambing season?

Yes I__I No I__I

If yes, what kind of personal protective clothes do you wear? (please write)

……..

17. Do you disinfect the livestock pen? What do you do?

(please write)……..….

Thank you very much for your participation

STable 1Analysis of risk factors for camel seropositivity, univariate analysis, adjusted ORs

						Univariate			Adjusted	
		n neg	% neg	n pos	% pos	OR	95% CI OR	p-value*	Adjusted OR	95% CI of AOR
Province	Dornogobi	385	99.2	3	0.8	1				
	Dornod	228	94.6	13	5.4	7.4	2.1-26.4	0.002**	7.9*	2.1-30.1
	Khovd	368	99.7	1	0.3	0.4	0.04-3.5	0.4	0.4	0.05-3.2
	Sukhbaatar	280	94.0	18	6.0	8.4	2.4-29.7	0.001***	10.2*	2.7-38.6
	Umnogobi	524	99.6	2	0.4	0.5	0.1-2.7	0.4	0.5	0.1-2.4
Age class	≤ 4years	248	98.2	5	1.8	1				
	> 4years	1537	98.0	32	2.0	1.1	0.4-2.6	0.9	1.2	0.4-3.2
Sex	Female	1403	99.2	26	1.8	1				
	Male	322	97.0	10	3.0	1.4	0.6-3.1	0.4	0.8	0.3-1.8
Year	2013	224	94.5	13	5.5	1				
	2014	1177	98.2	22	1.8	0.4	0.15-0.8	0.017*	1.0	0.4-2.4
	2015	384	99.5	2	0.5	0.09	0.02-0.4	0.002**	1.0	0.2-5.6
Cattle present	no	238	100	0	0.00	1				
	yes	1547	97.7	37	2.3	8.1	1.5 +∞	0.01[ł]	-	-
Sheep present	no	126	100	0	0.00	1				
	yes	1659	97.8	37	2.2	4.0	0.7 +∞	0.1	-	-
Goats present	no	102	100	0	0.00	1				
	yes	1683	97.8	37	2.2	3.2	0.6+∞	0.2	-	-
Ruminant vaccination in province	No	748	98.0	15	2.0	1				
	Yes	1037	97.9	22	2.1	0.9	0.4-2.1	0.8	1.0	0.2-5.6
Distance to district centre	< district median	853	98.6	12	1.4	1				
	≥ district median	906	97.5	23	2.5	1.7	0.8-3.7	0.2	2.1	0.9-4.9

Prior brucellosis testing of household members	No		901	98.2	17	1.9	1				
	Yes		501	96.5	18	3.5	1.9	0.8-4.3	0.1	1.3	0.5-3.2
Positive human cases	No		467	96.1	19	3.9	1				
	Yes		100	98	2	2.0	0.4	0.1-1.9	0.3	0.7	0.1-3.6
Knowledge on transmission between herds	< scores	50%	584	98.5	9	1.5	1				
	≥ scores	50%	1201	97.7	28	2.3	1.5	0.6-3.6	0.4	1.4	0.5-4.1
Knowledge on transmission from animals to humans	< scores	50%	632	98.4	10	1.6	1				
	≥ scores	50%	1153	97.7	27	2.3	1.4	0.5-3.8	0.5	1.1	0.4-3.1
Knowledge on clinical signs of animal brucellosis	≤ scores	50%	1097	98.2	20	1.8	1				
	> scores	50%	688	97.6	17	2.4	1.2	0.6	0.6-2.8	0.9	0.2-3.0
Number of information sources	No source		580	99.2	5	0.8	1				
	1 source		36	97.3	1	2.7	3.4	0.4-29.5	0.3	2.2	0.2-19.7
	≥ 2 sources		307	96.2	12	3.8	4.7	1.4-15.9	0.01**	2.5	0.7-8.6
Veterinarian provides information	No		81	91.0	8	9.0	1				
	Yes		134	96.4	5	3.6	0.4	0.1-1.3	0.1	0.3*	0.1-1.0
Disinfection within the fence	No		109	96.5	4	3.5	1				
	Yes		103	91.9	9	8.0	2.4	0.65-8.8	0.2	2.6	0.7-9.4
Buying	No		979	97.9	21	2.10	1				

animals	Yes	139	99.3	1	0.7	0.4	0.05-3.2	0.4	0.7	0.1-5.5
Destroy abortion material	No	300	99.3	2	0.66	1				
	Yes	887	97.8	20	2.2	2.7	0.4-21.7	0.3	3.7	0.3-43.6

Analysis of risk factors for camel seropositivity, univariate analysis showing odds ratios while using a gee model considering a random effect at herd level. We also present adjusted ORs, adjusted to province, year, sex and age classes. [ł] -exact logistic regression, * $p \leq 0.05$

Appendix 2 Supplementary data for Chapter 5

STable 2 Sample of cattle, sheep and goat sera from the five provinces collected

Species	Total no. of sera (all tested with RBT)	CFT	I-ELISA	C-ELISA	FPA
Cattle	1155	117	177	124	114
Sheep	1492	149	199	184	132
Goats	1531	156	220	170	150

Sample of cattle, sheep and goat sera from the five provinces collected during the epidemiological survey on brucellosis in Mongolia for 2013-2015. All sera were tested with the RBT and sub-samples with other diagnostic tests

STable 3 The cross-table values and Kappa statistic

		CFT		I-ELISA		C-ELISA		FPA	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
RBT	Pos	34	24	45	13	35	23	45	13
	Neg	5	31	11	25	4	32	7	29
K value		0.41 (0.24-0.58)		0.47 (0.28-0.65)		0.45 (0.29-0.61)		0.56 (0.40-0.73)	
CFT	Pos			34	5	27	12	33	6
	Neg			22	33	12	43	19	36
K value				0.44 (0.28-0.61)		0.47 (0.29-0.66)		0.48 (0.31-0.65)	
I-ELISA	Pos					34	22	43	13
	Neg					5	33	9	29
K value						0.44 (0.28-0.61)		0.52 (0.35-0.70)	
C-ELISA	Pos							31	8
	Neg							21	34
K value								0.40 (0.22-0.57)	

The cross-table values and Kappa statistic (K value) of pairwise test result comparisons of cattle sera.

STable 4 Test comparison of cattle sera between results

Tests		Combination 1		Combination 2		Combination 3	
		Pos	Neg	Pos	Neg	Pos	Neg
I-ELISA	Pos	22	3	54	17	22	3
	Neg	34	35	2	21	2	21
K value		0.30 (0.13-0.42)		0.55 (0.38-0.72)		0.55 (0.38-0.72)	
FPA	Pos	22	1	49	21	22	1
	Neg	30	41	3	21	3	21
K value		0.37 (0.23-0.52)		0.46 (0.30-0.63)		0.83 (0.67-0.99)	

Test comparison of cattle sera between results obtained with the I-ELISA and FPA and three combinations of classification: Combination 1 considering as positive those that were all positive in other 4 tests, Combination 2 considering as positive if positive in any other test, but as negative if negative in all others, and Combination 3 all positive and all negative.

STable 5 The % S/P ratio of the I-ELISA outcomes of cattle sera

Serological test	I-ELISA													
	Cut-off	Se %	Sp %	LR+	LR-	AUC				Cut-off	Se %	Sp %	LR+	LR-
						Area	95% CI		P value					
RBT	≥85.5	81.0	69.4	2.6	0.3	0.80	0.70	0.90	0.05	≥96.2	72.4	86.1	5.2	0.3
CFT	≥85.5	92.3	60.0	2.3	0.1	0.82	0.74	0.91	0.04	≥142.7	18.0	98.2	9.9	0.8
C-ELISA	≥85.5	92.3	60.0	2.3	0.1	0.83	0.75	0.91	0.04	≥176.8	10.3	98.2	5.6	0.9
FPA	≥85.5	86.5	69.1	3.0	0.2	0.84	0.75	0.93	0.04	≥108.7	55.8	92.9	7.8	0.5
Test combination 1	≥85.5	96.0	52.7	2.0	0.1	0.83	0.75	0.91	0.04	≥118	52.0	88.4	4.5	0.5
Test combination 2	≥85.5	78.9	91.3	9.1	0.2	0.89	0.83	0.96	0.03	≥ 91.8	71.8	95.7	16.5	0.3
Test combination 3	≥ 89.4	96.0	91.3	11.0	0.04	0.99	0.98	1.0	0.007	≥91.9	92.0	95.7	21.2	0.1

The % S/P ratio of the I-ELISA outcomes of cattle sera analysed with ROC curve statistics while taking other test outcomes and combinations hereof as references. The manufacturer's recommended cut-off is at ≥ 80%.

STable 6 The millipolarisation level (mP) of the FPA outcomes of camel sera

Serological test	FPA													
	Cut-off	Se %	Sp %	LR+	LR-	AUC				Cut-off	Se %	Sp %	LR+	LR-
						Area	95% CI		P value					
RBT	≥20.9	72.4	83.3	4.3	0.3	0.84	0.76	0.9	0.04	≥61.2	60.3	97.2	21.7	0.4
CFT	≥20.9	82.1	70.9	2.8	0.3	0.80	0.71	0.90	0.05	≥69.6	69.2	85.5	4.8	0.4
C-ELISA	≥20.9	76.9	67.3	2.4	0.3	0.78	0.69	0.88	0.05	≥88.4	61.5	89.1	5.6	0.4
I-ELISA	≥20.9	71.4	80.0	3.4	0.4	0.81	0.71	0.90	0.05	≥79.5	51.8	92.1	6.6	0.5
Test combination 1	≥20.9	91.3	62.0	2.4	0.1	0.83	0.74	0.93	0.05	≥139.1	13.0	98.6	9.3	0.9
Test combination 2	≥20.9	65.7	91.7	7.9	0.4	0.84	0.74	0.93	0.05	≥45.1	54.3	95.8	13.0	0.5
Test combination 3	≥20.9	91.3	91.7	11.0	0.1	0.93	0.83	1.0	0.05	≥69.6	87.0	95.8	20.9	0.1

The millipolarisation level (mP) of the FPA outcomes of camel sera analysed with ROC curve statistics while taking other test outcomes and combinations hereof as references. The manufacturer's recommended cut-off is at ≥ 20 mP.

Appendix 3

1. Photos from the field


SFigure 1 Mongolian Bactrian camel husbandry


SFigure 2 Mobile livestock husbandry: sharing water points


SFigure 3 Mobile livestock husbandry: sharing pastures

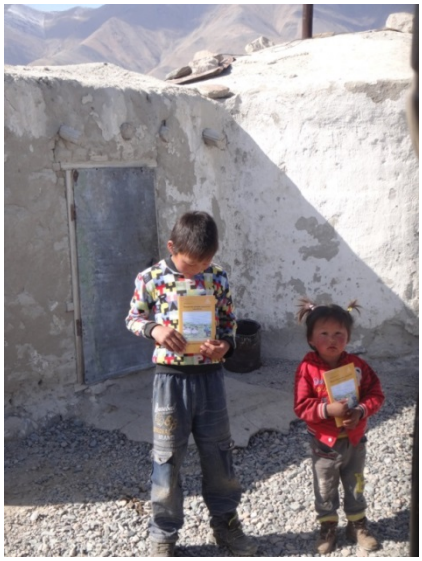
SFigure 4 Mobile livestock husbandry: camel transportation; They helped to catch camels in the field.

SFigure 5 mobile livestock husbandry: camel milking

SFigure 6 long journey to walk for sample collection

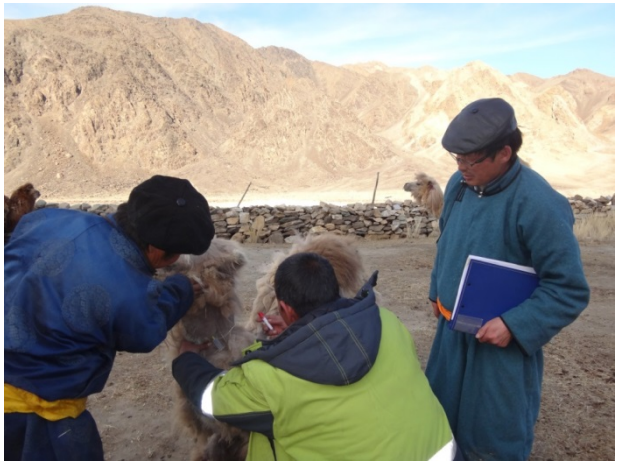
SFigure 7 Difficult local road conditions

SFigure 8 Interview with herder



SFigure 9 A handbook about brucellosis prevention was distributed to children

SFigure 10 Handbook about brucellosis was distributed to children



SFigure 11 Samples were taken from yaks instead of cattle in Western Provinces

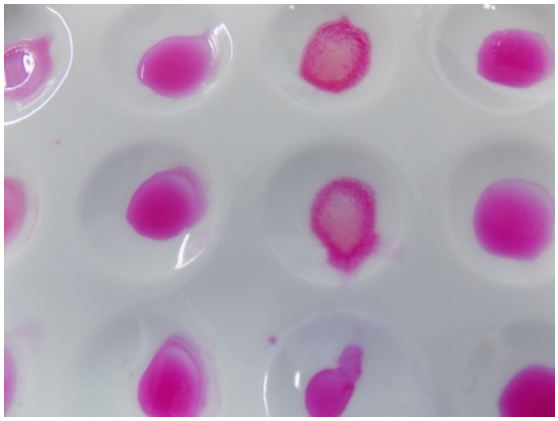

SFigure 12 Milk samples were collected from camels

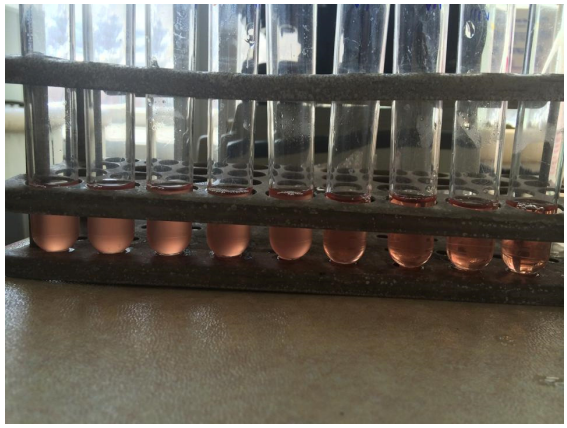

SFigure 13 Tricky handling of camels for sampling

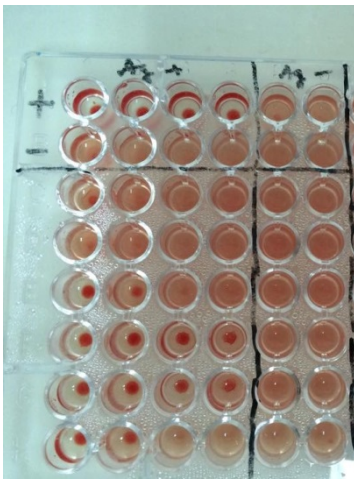

SFigure 14 Uterine swabs were collected from camels

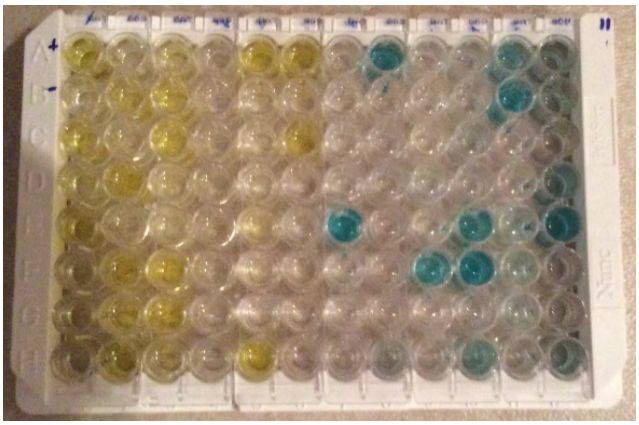
SFigure 15 some animals were treated with the team during field trip

2. Photos from the laboratory

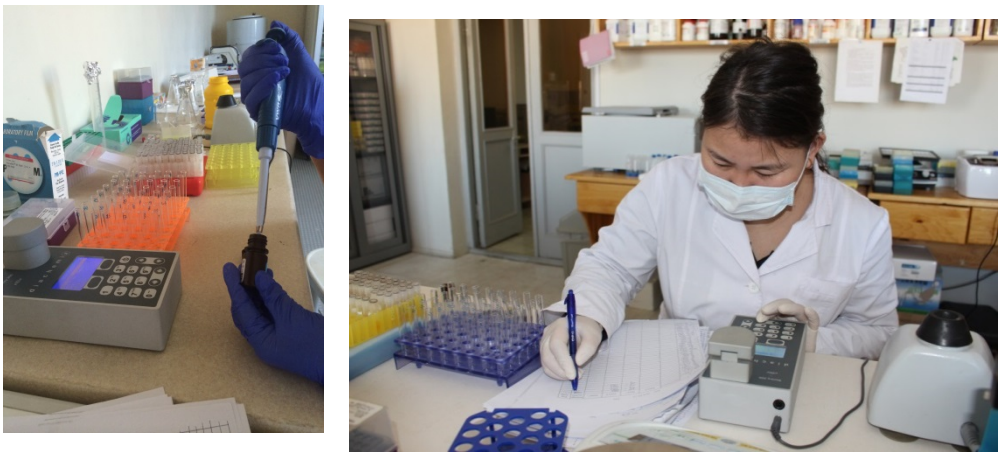




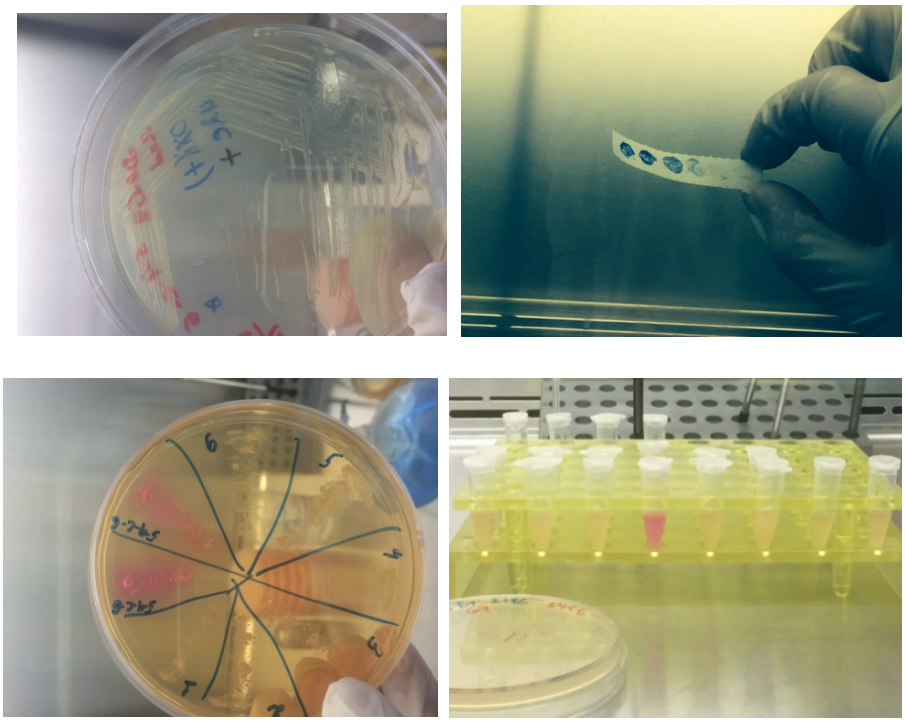




SFigure 16 Serological tests: Rose Bengal test; Titration for hemolysis and complement; Complement Fixation Test and indirect ELISA



SFigure 17 Serological tests: Fluorescence polarization assay



SFigure 18 Biochemical tests on Brucella cultures: Incubation and oxidase test; Pure culture of Brucella; Urea agar and broth tests

SFigure 19 Bruce-ladder PCR

CHIMEDTSEREN BAYASGALAN

Zaisan Khoroo 11, Ulaanbaatar, Ulaanbaatar 17024
97688016567 - chimee_vet@muls.edu.mn

SKILLS

- Self-motivated
- Skilled in all Microsoft, Stata
- Data management
- Process implementation

WORK HISTORY

03/2009 to Current **Senior Lecturer**
Mongolian University Of Life Sciences – Ulaanbaatar, Mongolia

EDUCATION

2017 **PhD**: **Epidemiology**
Swiss Tropical And Public Health Institute, Basel University - Basel Switzerland
The PhD entitled 'Epidemiology and Diagnosis of Brucellosis in Mongolian Bactrian Camels' supervised by PD Esther Schelling, University of Basel, Switzerland, and Dr. Tungalag Chultemdorj of the School of Veterinary Medicine, Mongolian University of Life Sciences, Mongolia.

2006 **Master Degree**: **Veterinary Medicine**
Mongolian State University Of Agriculture - Ulaanbaatar Mongolia
Master degree thesis entitled "The epidemiology on Bluetongue disease in Eastern province in Mongolia" Mongolian State University of Agriculture

2006 **Bachelor** : **Veterinary Medicine**
Mongolian University Of Life Sciences - Ulaanbaatar, Ulaanbaatar

2002 **Certificate**: **Secondary Education**
57th Secondary School - Ulaanbaatar Mongolia

RELEVANT PROFESSIONAL EXPERIENCE

- Part of a team to establish the prevalence, diagnosis and surveillance of Bluetongue disease in livestock in Mongolia2007 to 2012
- Worked in the FAO project "Strengthening Early Warning for Transboundary animal disease diagnosis" 2006-2011, Mongolia
- Participation to writing of the project report on "Strengthening Early Warning for Transboundary Animal Disease Diagnosis" project, 2010
- Participated in designing and developing diagnosis of Bluetongue by C-ELISA 2007-2009
- Have been trained in epidemiology and use of ELISA diagnostics organized by FAO (TCP MON 3101) and the Department of Microbiology and Infectious Disease of the School of Veterinary Medicine, Mongolian State University of Agriculture 2008
- I have organized several local and international workshops between 2007 and 2017

TRAINING EXPERIENCE

- Advanced Security in the Field (English) UNDSS in 2018
- Basic Security in the Field II (English) UNDSS in 2018
- Biosafety and Biosecurity train the trainer training by EU at Bishkek, Kyrgyzstan in 2017
- **At the University of Basel I have successfully taken 14 ECTS** with the following courses: Interdisiplinary Research in Epidemiology and Infection Biology; Ecology of Infectious disease at the Human-Anima Interfeace; Chronic Disease and Molecular Epidemiology; Modelling of infectious diseases; Advanced One Health; Cultural epidemiology and Epidemiological Methods
- English course stage 10 "Talk Talk English" in Mongolia 2012-2013
- Graduated from an intermediate level of English at the AEC college in Singapore, 2011
- International veterinary epidemiology course at Colorado University, USA, 2009

PUBLICATIONS AND PRESENTATIONS

Peer-reviewed Publications in English

- Risk factors of brucellosis seropositivity in Bactrian camels of Mongolia BMC Vet Res 2018, 14(1):342 Bayasgalan, C. Chultemdorj, T. Roth, F. Zinsstag, J. Hattendorf, J. Badmaa, B. Argamjav, B. Schelling, E.
- Serological tests for brucellosis in Mongolian Bactrian camels, forthcoming, Bayasgalan, C., Chultemdorj, T., Vanabaatar,.B., Jantsandorj, M., Felix, R., Argamjav, B., Badmaa, B., Zinsstag, J.,& Schelling, E.
- Evidence for camels (Camelus bactrianus) as the main intermediate host of Echinococcus canadensis G6/G7 in Mongolia", for possible publication in Parasitology ResearchGérald UMHANG; Bolor Bold; Franck Boué; Christian

Schindler; Battsetseg Badmaa; Belgutei Batbekh; Bayanzul Argamjav; Chimedtseren Bayasgalan; Akira Ito; Uranshagai Narankhuu; Agiimaa Shagj; Jakob Zinsstag

Peer-reviewed Publication and Proceedings in Mongolian Journals

- On the problem of animal infectious diseases. Mongolian journal of infectious disease research, №6 (25) p29-31, 2008, Ch. Tungalag, B. Chimedtseren, S. Ganzorig, J. Dabaadorj
- Proceedings Current advances in Microbiology, Immunology and Allergology, Second International Conference "Surveillance of Antibodies to Bluetongue Virus in Livestock in Mongolia using C-ELISA: Preliminary Results", Ulaanbaatar, Mongolia, 2010, Ch. Tungalag, Ts. Altansukh,A. Bayanzul,B. Chimedtseren,S. Ganzorig,J. Dabaadorj
- Emerging and Remerging infectious diseases and Climate change, Second Conference on urgent issues on the world warming and ecological disorder, Ulaanbaatar, Mongolia, 2010, Ch. Tungalag, A. Bayanzul, B. Chimedtseren.
- Parvovirus in the Ulaanbaatar city, Mongolian journal of infection disease research, p27, №6 (37), 2010, Ch. Tungalag, Ts. Ariunaa, B. Chimedtseren.
- Emerging and known infectious diseases and climate change challenge Ch. Tungalag, Ts. Ariunaa, B. Chimedtseren, 2013

Proceedings in Internationol Symposia and Workshop

- Surveillance of Antibodies to Bluetongue Virus in Livestock in Mongolia using C-ELISA: Preliminary Results. FAO/IAEA International Symposium on Sustainable Improvement of Animal Production and Health Vienna, Austria 2009, Tungalag. Ch, Altansukh. Ts, Bayanzul. A, Chimedtseren. B, Ganzorig. S & Dabaadorj. J
- Poster presentation on the "Chemical for creating the Future – Fourth Hope-Meeting" for young scientists by JSPS in Tsukuba, Japan, 2012
- Seroprevalence of bluetongue virus in Mongolia, 2013, Chimedtseren. B, Baynzul. A, Tungalag. Ch Joint Seminar ChonbukNational University and MSUA,
- Predominance of canine parvovirus type 2b in dogs in Ulaanbaatar city, Tumenjargal.Sh, Chimedtseren.B, Ariunaa.Ts, Tungalag.Ch, Агарная Наука-Сельсхозяйственному производству Сибири, Казакстана, Монголии, Беларуси и Болгари, Сборник научных докладов, 2015
- Poster presentation "The surveillance on Brucellosis in Sukhbaatar province in Mongolia", Battsetseg. B, Chimedtseren. B, Tungalag. Ch, JST Sakura Science Plan, Azabu University, Japan, 2016
- Poster presentation "The surveillance on Brucellosis in Sukhbaatar province in Mongolia", Battsetseg. B, Chimedtseren. B, Tungalag. Ch, JST Sakura Science Plan, Azabu University, Japan, 2016

- Study of Camel brucellosis in Mongolia, 2017, Bayasgalan. C, Chultemdorj, T., Badmaa, B., Argamjav, B., Zinsstag, J., Hattendorf, J. & Schelling, E. Joint Symposium Zoonotic Infectious Diseases, Ulaanbaatar, Mongolia
- Epidemiology of Brucellosis in Bactrian Camels in Mongolia, 2017, Bayasgalan, C., Chultemdorj, T., Badmaa, B., Argamjav, B., Zinsstag, J., Hattendorf, J. & Schelling, E. New generation towards agricultural sustainable development scientific conference
- A review of study on animal Brucellosis in Mongolia, 2017, Bayasgalan. C Scientific conference for 75th Aniversary of School of Veterinary Medicine Mongolian University of Life Sciences