Infections associated with reproductive disorders in cattle in Tanzania: occurrence, characterisation and impact

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Dedication

To my mother

Jane Fabian Shirima

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Coletha Mtenga Mathew

Summary

The livestock sector in Tanzania is huge in animal numbers but the production is disproportionally small. Most of the infections known to cause reproductive disorders are generally regarded endemic in Africa, but more specific information is lacking. The overall aim of the study was therefore to investigate the occurrence, characteristics, and impact of selected infections causing reproductive disorders in cattle in Tanzania.

A cross-sectional study was performed in two locations in Njombe and Mbeya regions in the southern highlands of Tanzania. In total, 202 cattle herds were visited once, and relevant information and biodata were collected through direct observation and interview of farmers. Biomaterials from 658 animals were collected for laboratory analysis.

The most common reproductive disorders encountered in the area were abortion (11.3%) and retained placenta (17.2%) (Paper 1). Antibodies specific to bovine viral diarrhoea virus (BVDV) were found in 15.2% of the animals in 17.9% of the herds, and antibodies to *Brucella* spp. in 5.4% of the animals in 7.4% of the herds. Herd level seroprevalence varied considerably between the two locations, from 6.5% to 66.7% for BVDV and from 0.6% to 36.1% for *Brucella spp*. A regression model identified large herds (odds ratio (OR): 14.5), location (OR: 23.1) and grazing (OR: 22.7) as risk factors for *Brucella* seropositivity and location (OR: 12.7) as risk factor for BVDV seropositivity at herd level. *Brucella* (OR: 15.5) and BVDV (OR: 5.0) seropositivity was associated with abortion at herd level. *N. caninum*-specific antibodies were found in 4.5% of animals and in 8.4% of the herds but no associations with reproductive disorders nor risk factors were found.

Brucellosis was further studied in one of the herds, which was experiencing abortions (Paper 2). The within-herd seroprevalence in cattle (n = 200) was 48% (95% CI 41-55), using an indirect antibody ELISA. Three *Brucella* isolates were cultured from an aborted foetus and associated foetal membranes. A multiplex PCR (Bruce-ladder), 16s rDNA gene sequencing and classical biotyping classified the isolates as *B. abortus* biovar 3. A multiple locus variable number of tandem repeats analysis (MLVA-16) revealed two different but closely related genotypes. High within-herd prevalence, isolation of the pathogen, and abortion confirm that *B. abortus* is circulating in this herd with cattle as reservoir hosts.

In Paper 3, sera from all cattle were analysed using an antibody ELISA kit for Schmallenberg virus (SBV), and 61% were positive. SBV virus neutralization test (VNT) was then performed

on 110 sera collected, of which 51 % were positive. Additional sera from the same area collected in 2008/2009were then included and 54.6% were positive in the antibody ELISA while 21% were positive in the VNT.45 ELISA positive sera were further analysed in VNTs for the related viruses Akabane, Douglas, Peaton, Sabo, SBV, Sathuperi, Shamonda, Aino, Tinaroo and Simbu viruses. Antibodies to all except Simbu virus were detected. For SBV, 29 sera (64.4%) were positive. These results indicate that SBV or a closely related virus was present in Tanzania before the European epidemic, however, cross-reactivity complicates the interpretation of serological studies.

Overall, the study shows that the selected infections represent both animal and human health risks in the study area. Two new genotypes of *B. abortus* that are not related to other African strains and antibodies to eight new Simbu serogroup viruses in Tanzania, of which four are also new to Africa, were found. The highly variable prevalence and impact of *Brucella* spp. and BVDV infections between geographically closely related locations require awareness. Animal contact represents a serious risk of introducing infections to infection-free sub-populations, and changes in management factors might alter conditions for spread and survival within the subgroups. Unpasteurised milk is commonly consumed in the area which underscores the need for strategic ‘One health’ control measures.

Sammendrag (Norwegian summary)

Antall storfe i Tanzania er høyt, men produksjonen hos hvert enkelt dyr er lav. Mange infeksjoner som gir reproduksjonsproblemer hos storferegnes å være endemisk i Afrika, men det mangler mer detaljert kunnskap. Målet med studien var derfor å identifisere og karakterisere slike smittestoff samt undersøkederes forekomst og betydning for reproduksjonsproblemer hos storfe i Tanzania.

En tverrsnittstudie ble gjennomført i to områder i regionene Njombe og Mbeya i høylandet i det sørlige Tanzania. Totalt 202 storfebesetninger ble besøkt en gang hver. Materiale fra 658 dyr ble samlet inn for videre laboratorieanalyse. Relevant informasjon og biodata ble samlet inn ved hjelp av direkte observasjoner og intervju av eierne.

Det vanligste reproduksjonsproblemet var tilbakeholdt etterbyrd (17.2%) og abort (11.3%) (artikkel 1). Antistoffer mot bovin virusdiarévirus (BVDV) ble påvist hos 15.2% av dyra i 17.9% av besetningene. Antistoffer mot *Brucella* spp. ble funnet hos 5.4% av dyra i 7.4% av besetningene. Prevalensen var svært ulik i de to områdene; I Njombe var 6.5% av besetningene positive for BVDV og 0.6% positive for *Brucella* spp., mens i Mbeya var 66.7% positive for BVDV og 36.1% positive for *Brucella* spp. Regresjonsanalyse viste at risikofaktorer for *Brucella* seropositivitet på besetningsnivå var: stor besetning (odds ratio (OR): 14.5), område (OR: 21.1) og beitebruk (OR: 22.7). For BVDV seropositivitet ble område (OR: 12.7) funnet å være risikofaktor. *Brucella* seropositivitet var assosiert med abort både på enkeltdyrnivå (OR: 4.6) og besetningsnivå (OR: 15.5). BVDV var signifikant assosiert med abort på besetningsnivå (OR 5.0). Antistoffer mot *N. caninum* ble påvist hos 4.5% av dyra i 8.4% av besetningene. Her var det ingen forskjell i prevalens mellom områdene, og det ble ikke påvist assosiasjon med reproduksjonsproblem eller identifisert risikofaktorer.

Forekomsten av brucellose ble videre studert i en stor besetning som hadde problemer med abort hos storfe (artikkel 2). Prevalensensen av positive dyr i besetningen (n=200) ble funnet å være 48% ved bruk av en indirekte antistoff-ELISA. Tre *Brucella*-isolater ble dyrket fra et abortert foster med fosterhinner. Mulitpleks-PCR ('Bruce-ladder'), 16s rDNA-sekvensering og klassisk biotyping viste at isolatene tilhørte *B. abortus* biovar 3. Såkalt 'multiple locus variable number of tandem repeats' (MLVA-16) viste at de tre isolatene besto av to ulike, men nært beslektede, genotyper. Disse var ikke beslektet med andre isolater funnet i Afrika. Høy

besetningsprevalens, forekomst av aborter og isolering av smittestoffet konfirmerer at *B. abortus* sirkulerte i denne besetningen, med storfe som sannsynlig hovedvert.

I artikkel 3 beskrives en serologisk undersøkelse for Schmallenberg virus (SBV) og nært beslektede Simbu serogruppe-virus. Sera fra alle storfe ble analysert med en ELISA for påvisning av antistoffer mot SBV, og 61% var positive. Virusnøytralisasjonstest (VNT) med SBV ble utført på 110 sera, og 51% var positive. Sera fra storfe fra området samlet tidligere år ble analysert, og 54.6% var positive i antistoff-ELISA og 21% var positive i VNT. 45 sera analysert videre i VNT for ti ulike Simbu serogruppe-virus. Antistoffer mot Akabane, Douglas, Peaton, Sabo, SBV, Sathuperi, Shamonda, Aino- og Tinaroovirus ble funnet. 29 sera (64.4%) var positive for SBV antistoffer, og ett av dyra hadde høyest titer for dette viruset. Resultatene indikerer at SBV eller nær beslektet virus sannsyligvis var tilstede i Tanzania før utbruddet i Europa. Kryssreagering kompliserer tolkningen av serologiske studier.

Totalt viser studien at de utvalgte infeksjonene representerer risiko både for dyrehelse og human helse i området. To nye genotyper av *B. abortus*, samt antistoffer mot åtte nye Simbu serogruppevirus i Tanzania ble funnet. Prevalensen og betydning av *Brucella* spp. og BVDV var svært ulik i de to geografisk nært plasserte områdene. Dyrekontakt representerer en alvorlig smitterisiko for de frie dyrepopulasjonene, og endringer i driftsopplegg kan endre forholdene for spredning og overlevelse av smittestoff i dyregrupper og områder. Resultatene representerer også alvorlig risiko for humanhelse siden det konsumeres upasteurisert melk i området. Det tydeliggjør behovet for 'en helse' - strategi for sykdomskontroll.

Abbreviations

AI	Artificial Insemination
BVDV	Bovine Viral Diarrhoea Virus
CNS	Central Nervous System
CI	Confidence Interval
cELISA	Competitive Enzyme Linked Immunosorbent Assay
DNA	Deoxyribonucleic Acid
EPINAV	Enhancing Pro-poor Innovations in Natural Resource and Agriculture Value-Chain
iELISA	Indirect Enzyme Linked Immunosorbent Assay
MRT	Milk Ring Test
MLVA	Multiple Locus Variable Number of Tandem Repeat
NAIC	National Artificial Insemination Center
NMBU	Norwegian University of Life Sciences
OR	Odds Ratio
OD	Optical Density
PI	Persistently Infected
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
RBT	Rose Bengal Test
RT-PCR	Real Time-PCR
ROC	Receiver Operating Curve
SUA	Sokoine University of Agriculture
SBV	Schmallenberg Virus
URT	United Republic of Tanzania
VNT	Virus Neutralisation Test
VIC	Veterinary Investigation Centers

List of papers

Paper 1:

C. **Mathew**; S. Klevar; T. Løken; R.H. Mdegela; G. Mwamengele; E. Skjerve J. Godfroid and M. Stokstad. Reproductive infections in cattle in Tanzania – lessons for control priorities (*SOJ Microbiol Infect Dis 2017 5(1):1-9*).

Paper 2:

C. Mathew; M. Stokstad; T. B. Johansen; S. Klevar; R. H. Mdegela; G. Mwamengele; P. Michel;·L. Escobar; D. Fretin and J. Godfroid. First isolation, identification, phenotypic and genotypic characterization of *Brucella abortus* biovar 3 from dairy cattle in Tanzania (*BMC Veterinary Research 2015* ***11****:156* ***DOI:*** *10.1186/s12917-015-0476-8).*

Paper 3:

C. Mathew; S. Klevar; A.R.W. Elbers; W. H. M. van der Pool; P. Kirkland; J. Godfroid; R.H. Mdegela; G. Mwamengele and M. Stokstad. Detection of serum neutralizing antibodies to *Simbu* sero-group viruses in cattle in Tanzania. (*BMC Vet Research 2015 Aug 15; 11:208. doi: 10.1186/s12917-015-0526-2*).

1.0 Introduction

1.1 Tanzania

Country profile and demography

Tanzania is located in east Africa and is bordered by Uganda and Kenya to the north; Burundi, Rwanda, and Congo to the west; Mozambique, Zambia, and Malawi to the south and the Indian Ocean to the east. It has massive wilderness areas, which include the plains of Serengeti National Park and Kilimanjaro National Park. Offshore are the tropical islands of Zanzibar and Mafia. The country covers a total area of 364,898 sq. mi. Eighty percent of the land in Tanzania is classified as semi-arid (with about 25–50cm annual rainfall) and the main source of livelihoods in these areas is agriculture and livestock keeping (Quinn et al., 2003). The climate of Tanzania is tropical; coastal areas are hot and humid while the northwestern and southern highlands are cool and temperate. The central plateau is arid/desert (very dry, with very little available water and extreme high temperatures) throughout the year. There are two rainy seasons, the short rains from October through December and the long rains from March to June.

Tanzania is a multiethnic country with more than 120 ethnic groups. It has an estimated population of nearly 50 million and a growth rate of 3.0% per year (URT, 2013). The proportion of the population under the age of 15 is 44.6%, while the proportion of the population 65 years and above is only 2.9%. More than 80% of the population live in the rural areas where agriculture and livestock keeping by smallholder producers who keep small numbers of cattle are the major economic activities (URT, 2009).

Agriculture

Agriculture is the backbone of Tanzania's economy and accounted for more than 29.0% of gross domestic product (GDP) in 2016. As in other African countries, more than two thirds of the population depends on smallholder agriculture for their livelihood. In Tanzania small holder agriculture constitute the main part of agriculture and provide more than three fourths of the food supply (Salami et al., 2010). However, its contribution to the national growth in general is limited due to several factors including access to land, poor farming skills and obstacles to trade (Dixon et al., 2003; Jayne et al., 2006). In 2006 the livestock subsector contributed about 5.9% of total GDP. Out of the subsector's contribution to GDP, about 40% originates from beef production, 30% from dairy production and another 30% from poultry and small ruminant production (MLFD., 2010). Furthermore, agriculture employs about 80% of the workforce (URT, 2014), although statistics show low productivity among farmers in rural areas (SNV,

2012). There is a growing demand for animal protein due to population growth and a rising middle class which stresses the importance of improved productivity of livestock.

Livestock production

Livestock production is one of the major agricultural activities in Tanzania. Tanzania is rich in indigenous cattle animal genetic resources. The livestock numbers have been increasing, and the cattle population ranks third in Africa after Ethiopia and Sudan, making the country important in terms of breed diversity (MLFD., 2012). The estimated population of livestock includes 22.8 million cattle, 15.6 million goats, 7.0 million sheep, 2.01 million pigs, 4.5 million improved poultry and 35.5 million indigenous poultry (MLFD., 2012). Most of the genetic resources in Tanzania are indigenous cattle of the East African Zebu breed. They account for about 75% of the total livestock population (Mlote et al., 2013) and provide a good source of animal protein as well as manure for crop production and are therefore vital to economic development. The sale of livestock and their products is the main source of cash income in rural areas and enables farmers to buy consumer goods and improved seeds, fertilisers, and other farm inputs needed to improve crop yield.

Pastoral and agro-pastoral production: Sub-Saharan Africa is home to more than 25 million pastoralists (people whose livelihoods are based on nomadic livestock keeping) and over 200 million agropastoralists (people combining livestock keeping with crop production). These two groups represent over a quarter of the total population in Africa and occupy 43% of the continent's total land mass (SNV, 2012). They depend on livestock for their livelihoods. The pastoral livestock production system in Tanzania uses indigenous cattle (Zebu) with relatively big herds (more than 10 cattle/herd) and is characterised by extensive grazing. Pastoralists tend to keep as many cattle as possible mainly for prestige and social purposes (Lupindu, 2007). Pastoralists are mainly concentrated in the arid and semi-arid areas of the country. The agropastoral system combines crop production and improved crossbred livestock production, which is characterised by semi-intensive grazing to zero grazing.

Dairy production: Dairy production in Tanzania plays a key role in people's livelihoods. It contributes to the socio-economic status of rural people as it increases monetary and social advantages and empowers marginalised women. Dairy production also plays a vital role by providing manure for sustainable agriculture and, to some extent, energy production. Smallholder dairy production dominates the urban and peri-urban areas where farmers keep small numbers of cattle indoors, usually one to six cattle per household, together with other

animal species like sheep, goats, pigs, ducks, or chickens. The main breeds are Friesian, Ayrshire, and Jersey cows crossed with East African Zebu. This system is characterised by zero grazing where cattle are supplemented with agricultural leftovers and industrial by-products. There are also a number of medium-scale privately owned dairy farms. In addition, there are a few state-owned dairy farms, including Kitulo, Ngerengere, Sao Hill, Nangaramo, and Mabuki, which are also used as livestock multiplication units for the production of replacement heifers. Most small-scale dairy farmers sell their milk locally on a retail basis to individuals in a neighbourhood. Some small-scale farmers sell their milk to processing plants. There are about 23 milk processing plants in the country which are all privately owned. Seven of them were state-owned under Tanzania Dairies Limited (TDL) but have been privatised since 1995 in line with the market liberalisation policy. The milk that is sold to dairy processing plants is collected in common collection centres, and the dairy products are sold all over the country.

Despite the large size of the livestock sector and the increased demand for dairy products, production is disproportionally low due to various challenges. Management problems/poor knowledge, inefficient production methods, poor feeding, scarcity of genetic resources, poor veterinary services, poor marketing infrastructure, culture, prevalence of endemic diseases and reproductive disorders are some of the obstacles to achieving optimal dairy production (Swai and Karimuribo, 2011; Swai et al., 2014).

1.2 Major livestock constraints

Animal genetic resources

Productive and reproductive potential are mainly determined by two factors: genetic potential and environment. The lack of a reliable supply of improved breeds, together with poor management, is a major barrier to increasing the production and sustainability of the dairy sector in many African countries. Mitigating this challenge, genetic improvement strategies for cattle in Tanzania have been implemented by modifying the breed composition of local populations either by artificial insemination (AI) or through direct importation of exotic cattle from other countries. The National Artificial Insemination Center (NAIC) was established in Arusha in 1978 to meet this increased genetic demand. The NAIC uses AI to provide crossbred cattle with improved exotic genetic material and to maintain the genetic resources in indigenous cattle breeds for disease resistance and drought tolerance, which are essential for survival in tropical climates. In the past, the NAIC was run under the Swedish International Development Agency, but it is now fully operated by the government of Tanzania (Lazaro, 2006). The centre

has a capacity of 68 bulls, but to date they are only 22 bulls that are used in semen production (personal communication). The NAIC has eight breeds of cattle: three dairy (Friesian, Jersey and Ayrshire), two beef (Boran and Bonsmara), and three dual-purpose (Simental, Sahiwal and Mpwapwa) breeds. Apart from selling semen and offering AI services, the NAIC also has a liquid nitrogen production machine, which is used for the preservation of semen, and offers training services to the public for better dairy production. Despite the efforts made to improve the genetic composition for animals used in dairy production, a high proportion of farmers use natural breeding with unimproved breeds due to unaffordable and unreliable AI services. The main limitations of AI in rural settings are the high cost, the difficulty of storing semen, and the lack of trained personnel. Therefore, natural breeding bulls are normally shared between farms and are likely to spread sexually transmitted infections such as campylobacteriosis and trichomonasis between herds. In addition, practical and logistical challenges in moving a bull to a cow in heat at appropriate time may result in along calving interval.

Feed and feeding

Inappropriate feed and feeding is one of the main constraints in dairy farming in East Africa (Gillah et al., 2012). Performance of animals depends not only on their genetic potential but also on their environment, of which nutrition is a critical part. The main feed resources can be grouped into natural grasslands, established pastures, cereals and root crops residues, and agricultural by-products (Lwoga and Urio, 1985). In traditional pastoral systems cattle depend on natural grasslands. The main limitations of natural grasslands are its seasonal characteristics, low dry matter, and low quality of herbage (Kavana et al., 2005). Smallholder farmers in Africa largely depend on grazing areas as the main source of fodder for their herds; however, land for agricultural activities is less available (Jayne et al., 2014; Lowder et al., 2016). In Tanzania, there are few established pastures and therefore their roles as a feed resource are limited. Cereal and root crops are produced mainly for human consumption. During the dry season these crops are scarce and therefore less accessible for animal feed. Agricultural by-products also contribute to animal feed resources; however, improvement in handling, processing, and transportation is necessary to meet the required standard since they are generally of low quality (Lwoga and Urio, 1985). Commercial feeds are less available and they are expensive, which make them unaffordable to farmers.

Studies show interaction between nutrition and reproductive performance (van Knegsel et al., 2005). Malnutrition may lead to a number of reproductive disorders including abortion and

retained placenta (Taylor and Njaa, 2011). Energy, proteins, and minerals are essential dietary components for optimum reproductive performances. These nutrients are seasonally scarce in most animal feeds in Tanzania (Mtengeti et al., 2008; Mtui et al., 2007; Pereka and Phiri, 1998). Poor feeding can result in negative energy balance, which in turn affects progesterone production and renders the uterine environment suboptimal for embryo development (Butler, 2003). The negative energy balance also causes delayed first ovulation as a result of reduced blood glucose level, luteinizing hormone (LH), insulin and insulin-like growth factors, which together inhibit estrogen production by dominant follicles (Butler, 2003).

Livestock diseases and veterinary services

Veterinary services in Tanzania are both public and private. The public veterinary service is organised hierarchically at the national, regional, and district levels. At the district level, routine field services are done in collaboration with zonal livestock veterinary investigation centers (VIC). At the regional level, both veterinary and public health services are delivered. At the national level, with the Ministry of Agriculture, Livestock and Fisheries Development animal health policy, strategy, regulations and guidelines are prepared. Occurrence of disease is confirmed at the Central Veterinary Laboratory at the national level. This laboratory, in collaboration with zonal VICs, plays a key role in disease investigation, prevention, and control activities. Its role is to safeguard human health, animal health and productivity as it relates to food security, safety and trade. Despite the well described responsibility of each institution in the public sector, none of the services are adequately delivered due to limited resources. Therefore, most of the disease control responsibilities are left to the private sector. The private veterinary service is involved mainly in the importation and selling of veterinary equipment and pharmaceuticals, therefore, concentrate on selling the veterinary inputs and very little attention is paid to clinical service delivery. The main challenge is that the private sector is business-oriented and therefore farmers with few resources cannot afford the services required.

Livestock production in the tropics is challenged by many endemic infectious diseases, some of which are also transmitted to humans. They include bacterial, viral, and parasitic diseases. Bacterial diseases include bovine tuberculosis which is an economically important and potentially zoonotic infection that is endemic in Tanzania (Katale et al., 2013). Mastitis, both clinical and subclinical, are prevalent in the country and may contribute to low productivity of dairy cattle (Mdegela et al., 2012). Several infectious agents including bacteria are involved in mastitis. Contagious bovine pleuropneumonia caused by *Mycoplasma mycoides* subspecies *mycoides* is endemic throughout most of semi-arid sub-Saharan Africa (Bölske et al., 1995;

Msami et al., 2001). Foot and mouth disease is endemic in Tanzania; its presence is a major obstacle to the development of the national livestock industry as it decreases production and affects cross border export markets of animals and animal products (Kivaria, 2003; Picado et al., 2011). Tick-borne diseases, namely anaplasmosis, babesiosis, cowdriosis, and theileriosis, rank high in reducing production efficiency, leading to considerable economic losses in the country from cattle mortality, loss of animal body weight, reduced milk production, and management costs (vaccination, chemotherapy, and tick control).The total annual national loss due to tick-borne diseases was estimated to be US $364 million (Kivaria, 2006). Bovine anaplasmosis is a tick-borne disease of cattle caused by *Anaplasma marginale* which is an intraerythrocytic parasite. The disease is prevalent in Tanzanian cattle (Swai et al., 2005) and is associated with significant economic losses (Kivaria, 2006). Bovine babesiosis is also prevalent in the country (Swai et al., 2004) and is caused by protozoan parasites of the genus *Babesia*. Principally *B. bovis*, *B. bigemina* and *B. divergens* are involved but so are other species including *B. major*, *B. ovata*, *B. occultans* and *B. jakimovi*. Cowdriosis, or heartwater, is also a tick-borne rickettsial disease caused by *Ehrlichia ruminantium* and is an important infectious disease both in domestic and wild ruminants in Tanzania (Swai et al., 2008). Theileriosis (East Cost fever) is a tick-borne protozoan disease of cattle, sheep, and goats caused by *Theileria* species. It is an economically important endemic disease in Tanzania (Kivaria et al., 2007) and has been implicated in neurological disorders (Ormilo disease) in cattle (Catalano et al., 2015).

Infections causing reproductive disorders are also a significant hindrance to productivity (Kanuya et al., 2000; Kanuya et al., 2006). However, they receive relatively little attention. One explanation could be that they cause trivial epidemics and minimal clinical ill-health in adult cattle, but the long-term effects and costs associated with the loss of calves/foetuses or long calving interval are substantial.

Reproductive disorders

Reproductive disorders are responsible for huge economic losses in dairy cattle production worldwide as a result of loss of replacement animals for the herd, prolonged inter-calving periods, more open days, reduced milk production, additional costs for veterinary inputs, and unnecessary culling (Escamilla et al., 2007; Grohn and Rajala-Schultz, 2000; Inchaisri et al., 2010; Yoo, 2010). Reproductive disorders in cattle includeearly embryonic death, abortion, stillbirth, birth of weak or malformed calves, dystocia, endometritis, retained placenta, repeat breeding, and increased calving intervaland are broadly classified as eitherinfectious or non-

infectious based on their aetiology (Anderson, 2012). Abortion and stillbirth are among the most common disorders in the dairy industry in many parts of the world (Asmare et al., 2013a; Serrano-Martinez et al., 2007).

Non-infectious causes of reproductive disorders include poor management, traumatic and metabolic causes, chemical toxins, mycotoxins, and genetic disorders (Sheldon and Dobson, 2004 239). Genetic disorders such as chromosomal defects, metabolic diseases such as ketosis, and toxic agents such as toxic plants can result in different forms of reproductive disorders. Poor management skills related to feeding, housing, breeding, and grazing affect the performance of the small-scale dairy industry in Tanzania (Chang'a et al., 2010).Most of the diagnosed cases of reproductive disorders worldwide are caused by infectious agents, of which some are zoonotic (Givens, 2006). A high percentage of the abortions are caused by organisms present in the environment of the cow and are not contagious (Anderson, 2007). In addition, many systemic illnesses can cause reproductive disorders and fever may induce abortion. The most important primary bacterial infections in cattle include *Brucella abortus*, *Leptospira* spp., *Campylobacter foetus*, *Coxiella burnetti, Listeria* spp., *Haemophylus somnus* and *Chlamydia* spp. Parasites include protozoans such as *Neospora caninum*, *Trichomonas foetus*, and *Sarcocystis neuroni*. Viruses include bovine viral diarrhoea (BVDV), bovine herpes virus-1 (BHV-1), Simbu serogroup viruses, and bluetongue virus (Biuk-Rudan et al., 1999; Givens, 2006; Yoo, 2010). In Tanzania, an abortion rate of 14.3% in cattle has been reported (Kanuya et al., 2006). However, the cause of abortion and other reproductive disorders is seldom diagnosed. Effective control strategies require the identification of specific causes, and therefore for the purpose of this thesis some selected infections were investigated.

1.3 Brucella

Aetiology

Brucella spp. are small (0.5 to 0.7 by 0.6 to 1.5µm), gram-negative, facultative intracellular parasite, non-motile, non-encapsulated, non-spore forming, rod shaped (coccobacilli) bacteria. They cause brucellosis, a disease of worldwide public health and economic significance (Schelling et al., 2003). Among domestic animals, ruminants are highly susceptible to brucellosis. Brucellosis in cattle is mainly caused by *B. abortus*, but other species of *Brucella,* such as *B. melitensis* and *B. suis,* can also infect cattle (Ledwaba et al., 2014; Muendo et al., 2012; Wareth et al., 2014). To date there are 12 recognised species of *Brucella,* which are genetically very similar although each has different host preferences (Godfroid et al., 2011; Hofer et al., 2016; Scholz and Vergnaud, 2013; Whatmore et al., 2014; Yu and Nielsen, 2010).

They are *B. abortus*, *B. melitensis*, *B. ovis*, *B. suis*, *B. canis*, *B. neotomae*, *B. microti*, *B. pinnipedialis*, *B. inopinata, B. ceti, B. papionis and B. vulpis.*

Three species are of great zoonotic and economic importance, namely *B. abortus*, *B. melitensis* and *B. suis* (Godfroid et al., 2011), which preferentially infect cattle, small ruminants, and swine, respectively, with the possibility for cross-transmission (Godfroid et al., 2013b). The zoonotic potential for the remaining species has yet to be established. *Brucella* spp. are further classified into biovars/biotypes. To date there are eight biotypes of *B. abortus* (biovars 1, 2, 3, 4, 5, 6, 7, and 9) that can be differentiated by classical biotyping methods (phage typing, monospecific antisera, biochemical reactions, and growth inhibition tests) (Alton et al., 1988) and molecular methods such as multiple locus variable number of tandem repeats (MLVA) (Le Flèche et al., 2006), which can provide genetic typing information for accurate epidemiological investigations.

Multilocus sequence typing (MLST) and whole genome sequencing have become available and should help in further understanding the evolution, host specificity and pathogenicity of the genus *Brucella* (Scholz and Vergnaud, 2013). *Brucella* spp. are divided into two groups based on their colony and cell morphology, namely smooth and rough (which are in accordance with cell surface and lipopolysaccharide (LPS) structure, immunological and biochemical reactions, and virulence) (Mancilla, 2015).The rough strains usually lack the O-polysaccharide antigen, the most external LPS moiety, which is the trait linked to their reduced virulence. LPS antigens are widely used for serodiagnosis of brucellosis. Structurally, the *Brucella* LPS consists of three parts that are anchored in the outer membrane (Figure 1). Lipid A, which is the hydrophobic part attached to the core, and the core, which is made of oligosaccharides that create a bridge to the O-polysaccharides (O-PS), which is the outermost surface. The O-PS confers resistance by interfering with innate immunity, hence preventing early host immune response and allowing successful intracellular infection (Gorvel and Moreno, 2002). The O-polysaccharide carries three antigenic sites (A, M and C) as determined with monoclonal antibodies (Douglas and Palmer, 1988) and are species- and biovar-specific. Of the twelve species so far known, *B. canis* and *B. ovis* are regarded as naturally rough strains while the rest are smooth strains.

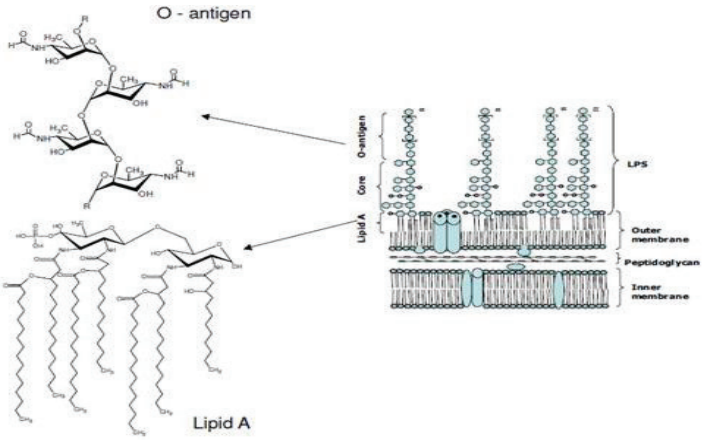


Figure 1:*Schematic presentation of Brucella spp. lipopolysaccharides (LPS) (Cardoso et al., 2006)*

Epidemiology and risk factors

Brucellosis is endemic in Africa, South America, Middle East, Latin America, and parts of Southern Europe (Corbel, 1997; Pappas et al., 2006). In most countries in the developed world, it has been successfully eradicated through routine screening of livestock, test-and-slaughter, and vaccination programmes (Al-Khalaf et al., 1992; B Lopes et al., 2010; Godfroid and Käsbohrer, 2002; Refai, 2002). In sub-Saharan Africa, seroprevalence of bovine brucellosis varies from country to country, but due to large variations in selection criteria, serological tests and validation of the serological methods, the different prevalence estimates should be interpreted carefully (McDermott and Arimi, 2002). In Kenya, seroprevalence in cattle kept in pastoral and agropastoral systems is reported to range from 9.9% to 15% (Njeru et al., 2016b). In Uganda, a national average seroprevalence of 10% is reported with a range of 10.2%–15.8% in pastoral systems and 5% in urban areas where the small-scale dairy system is dominant (Mugizi et al., 2015a). In Ethiopia, a range of 2.9% and 45.9% herd level seroprevalence is reported (Yilma et al., 2016). In Zambia, a seroprevalence of 20.7% was reported in traditionally managed cattle in which the majority were Zebu (Muma et al., 2013). In Tanzania, the first case of brucellosis was reported in an exotic breed of cattle in 1928 in the northern part of the country (Kitalyi, 1984) and later in humans (EVANS, 1936). Since then, a number of studies have been done and indicating a wide range of animal-level prevalence from 0.3%–60.8% (Alonso et al., 2016).

Several risk factors are responsible for the introduction, maintenance and spread of *Brucella* infection. The incidence of brucellosis varies with the level of contact between animals. Intensive management systems have been found to favour the spread of bovine brucellosis possibly because they facilitate contact with infected individuals (Omer et al., 2000). Indeed any factor that facilitates contact between animals, including herd size, movement, and congregation of animals in pasture, water, or points of sale, are potential risks (Ducrotoy et al., 2017; Megersa et al., 2011; Mekonnen et al., 2010).

Open herd systems also favour the introduction of *Brucella* spp. since it is possible to bring in infected cattle from other herds. Free movement of animals from one region to another contributes to the spread of infections. The purchase of infected cattle to upgrade or replace stock and lack of routine screening of new animals brought into herds contribute to the risk of introduction and spread of *Brucella* infection (B Lopes et al., 2010). Mixed farming, where cattle are kept together with small ruminants, increases the chance of infection transmission from small ruminants to cattle and vice versa as cross-transmission is possible (Godfroid et al., 2013b; Godfroid et al., 2011). The source of infections in a herd is abortion materials from infected cows and uterine discharge after normal but infectious calving. Therefore, improper handling of calving and abortions and absence of calving pens could predispose other susceptible cattle to the infections. Generally, a lack of proper biosecurity measures enhances the risk for transmission and spread of *Brucella* spp.

Transmission and pathogenesis

The main route of infection in cattle is through ingestion of contaminated feed or water. Licking of an infected placenta, calf or foetus or the genitalia of an infected cow soon after abortion or calving is also a potential source of infection. Calves can acquire the infection in utero or through the ingestion of contaminated colostrum or milk. Venereal transmission is not the main route, but artificial insemination with infected semen can also be a potential source of infection to naïve cows. In humans, brucellosis is mainly transmitted through ingestion of contaminated dairy products or as an occupational disease through direct contact with infected animals by precarious handling of abortions, dystocia and parturitions on farms or lack of biosecurity in slaughter houses. High-risk groups include farmers, veterinarians, slaughter house workers, and other animal workers (Godfroid et al., 2004).

Ingested bacteria penetrate the mucous membrane of the alimentary tract where they multiply in cells from the reticuloendothelial system (Neta et al., 2010). Bacteria are then phagocytosed

by neutrophils and macrophages and taken to the regional lymph nodes where they multiply and cause lymphadenitis with nasopharyngeal lymphadenitis as the initial lesion. Once bacteria have entered through digestive or respiratory tract, they are capable of surviving intracellularly in phagocytic or non-phagocytic host cells (Neta et al., 2010). From the lymph nodes, bacteria are released into the blood, causing bacteraemia.

During pregnancy, bacteraemia will reoccur and bacteria will be taken within neutrophils and macrophages to various organs including the uterus, udder, supra-mammary lymphnodes and spleen. The pregnant uterus is particularly susceptible to infections. *Brucella* replicate in the macrophage phagosome and non-phagocytic trophoblast of the pregnant uterus (Braude, 1951). In the uterus, bacteria have an affinity to erythritol, a four-carbon sugar preferentially utilised by *Brucella* spp., which is elevated around the fifth month of gestation and enhances massive growth and multiplication of bacteria (Petersen et al., 2013; Smith et al., 1962). Erythritol is present in the placenta of cows, goats, and pigs (Letesson et al., 2017; Petersen et al., 2013). From the uterus, bacteria are transported into chorionic epithelium of foetal membranes and foetal fluids which then can easily reach the foetus. This results in necrotic placentitis which leads to abortion or stillbirth in a naïve cow. In pregnant cows, bacteria move and localise in the placenta and during delivery/abortion they are massively secreted out through the uterine discharge and foetal membranes. In non-pregnant cows, the bacteria are usually localised in the supramammary lymph nodes and mammary glands and hence are secreted in milk (Harmon et al., 1988).

As an intracellular bacterium, *Brucella* incites both a specific antibody response and a typical cell-mediated immunity, mediated through T-cell cytokines (Gu et al., 2013). Humoral immunity is mediated through antibodies directed against outer membrane proteins (OMPs) or smooth lipopolysaccharides (S-LPS) A and M of *Brucella*. However, the protection conferred by anti-OMP is lower compared with anti S-LPS (Cloeckaert et al., 1991). Cellular immune response plays a role in the long-term protection of cattle against brucellosis.

Clinical disease

In cattle, brucellosis presents as abortion, stillbirth, birth of weak offspring, retained placenta, low fertility rate, fever, septicaemia, lymphadenopathy, weakness, weight loss, mastitis and reduced milk production (Emminger and Schalm, 1943). In males with chronic infection unilateral/bilateral hygroma of the carpal joints, orchitis, epididymitis, and arthritis are the main clinical signs (Bracewell and Corbel, 1980; Fensterbank, 1978). Infected animals can also be

asymptomatic (Megid et al., 2010). Abortion in cattle usually occurs at five to eight months of gestation (Neta et al., 2010), and in susceptible herds the abortion rate varies from 30–80% (Godfroid et al., 2004). Animals infected with *Brucella* usually abort only once, mainly in the first pregnancy, with subsequent normal calving although their placentas are heavily infected with the bacteria (Bang, 1906; Nicoletti, 1980). After the first abortion, animals are usually protected but still harbour the bacteria, and if introduced into an immunological naïve population, massive abortions in the herd will result within a short time (Ducrotoy et al., 2017; Godfroid et al., 2010). If infection is introduced in a cattle herd, up to 90% of the infected cows will proceed to chronic infection and the infection may persist for life, remaining confined to the udder and lymph nodes (Nicoletti, 1980). After the acute stage of the infection, the disease enters chronic state where the clinical signs become less distinct due to reduced bacterial challenge as a result of herd immunity. Stress related to poor management practices may cause fluctuation between acute and chronic states. Congenital transmission can result in initially asymptomatic and seronegative animals, which later become a source of infection for other cattle (Racloz et al., 2013).

Diagnosis

Diagnostics fall into two groups: i) indirect tests based on detection of *Brucella*-specific antibodies or allergy tests such as the Brucellin skin test and ii) direct microbiological analysis or detection of *Brucella* DNA (Geresu and Kassa, 2016). Serology is widely used to detect *Brucella* infection (Alton et al., 1988; Nielsen, 1990). During acute brucellosis, IgM isotypes are detected. Thereafter, IgG1 predominate and usually persist for as long as the animal remains infected, usually throughout its life. IgG2 and IgA isotypes of antibodies are produced at a later stage of infection (Nielsen and Yu, 2010). Most serological tests use antigens derived from smooth LPS which makes it impossible to detect antibodies from *Brucella* rough strains, and with the smooth strains detected, it is impossible to ascribe which *Brucella* spp. induced antibodies in the host (Godfroid et al., 2013a; Godfroid et al., 2011; John et al., 2010; Lucero et al., 2005). The absence of a perfect test (easy and robust, affordable, and with 100% sensitivity and 100% specificity) and complex biological, epidemiological, and socio-economic factors that affect applicability of diagnostic tests explain the use of different tests (Ducrotoy et al., 2016). The tests recommended by the OIE are Rose Bengal test (RBT), complement fixation test (CFT), Slow (tube) Agglutination Test (SAT), ELISAs, Milk Ring Test (MRT), and fluorescence polarisation assay. Table 1 shows the most common serological tests and their challenges.

A test used to demonstrate an allergic reaction to *B. abortus* is also available but less commonly used. It is based on a delayed type hypersensitivity reaction that detects cellular immune response induced by *Brucella* spp. measured by the increase in skin thickness at the site of inoculation (OIE, 2009; Saegerman et al., 2010). It is highly specific (99%) but less sensitive at animal level (Alton et al., 1988; Godfroid et al., 2010). It can discriminate false positive reactions due to other microorganisms (Nielsen and Yu, 2010) but cannot discriminate field from vaccine strains. This test, therefore, can be used to complement serological tests in the diagnosis of *Brucella* infection.

Table1: *Most common serological tests used for diagnosis of bovine brucellosis and their pros and cons as described in (Corrente et al., 2010; Gall and Nielsen, 2004; Godfroid et al., 2010; Nielsen, 1990; Nielsen, 2002; Nielsen and Yu, 2010; OIE, 2009)*

SN	CHALLENGES	TESTS					
		RBT	CFT	SAT	MRT	iELISA	cELISA
1	Non-specific reactions due to cross-reacting bacteria	Yes	Yes	Yes	Yes	Yes	No/Yes
2	Inability to differentiate vaccine from field strains	Yes	Yes	Yes	Yes	Yes	Yes
3	Inability to trace back to the source of infection	Yes	Yes	Yes	Yes	Yes	Yes
4	Inability to detect type of smooth *Brucella* spp.	Yes	Yes	Yes	Yes	Yes	Yes
5	Inability to detect *Brucella* rough strains	Yes	Yes	Yes	Yes	Yes	Yes
6	Inability to detect early exposed/infected animals	No	No	No	Yes	No	No
7	Used as a single test	Yes	Yes	Yes	No	Yes	Yes
8	Relative cost and time used	Low	High	Low	Low	Low	Low

Culture and isolation of *Brucella* spp. from foetal tissues and membranes, uterine discharge, and/or milk is the gold standard for the diagnosis of brucellosis (Nielsen and Yu, 2010). In clinical brucellosis, relevant samples include aborted foetal organs (stomach, spleen and lungs), foetal membranes, uterine and vaginal secretions, milk, colostrum and orchitis or hygroma fluid. At slaughter, mammary glands and associated lymph nodes, genital and oropharyngeal lymph nodes, and spleen are preferred and should be shipped to the laboratory immediately (Padilla Poester et al., 2010). The most preferred medium is the Farrell medium which contains antibiotics to inhibit the growth of other bacteria present in the sample. Some *Brucella* species like *B. aborus* biovars 1,2,3,4 require CO_2 for growth while others like other biovars of *B.*

abortus, *B. abortus* S19 vaccine strains, *B. melitensis* and *B. suis* do not require CO_2 for growth (Alton et al., 1988). Growth is expected after 2–4 days but will be considered negative after 2–3 weeks of incubation (Alton et al., 1988).

Biotyping provides epidemiological information that allows tracing back the source of infection especially where more than one biovar co-circulate. However, this method does not differentiate isolates belonging to the same biovar, hence less useful in areas where one biovar is overwhelmingly predominant (Godfroid et al., 2010) and biotyping of the strains always require bacterial isolation. Detection of *Brucella* spp. by culturing and isolation or detection of its DNA by PCR methods are the only methods that allow certainty of diagnosis (Godfroid et al., 2010). There are several PCR methods that have been developed for detection of *Brucella* DNA which are genus, species, and biovar-specific (Lopez-Goni et al., 2011; Ocampo-Sosa et al., 2005; Scholz and Vergnaud, 2013). The PCR methods are based on the detection of specific DNA sequence of *Brucella* spp. which can be genu- or species-specific. These sequences include 16S rDNA gene sequence, IS711 and *bcsp31,* which are genus specific; and BruAb2_0168 and B.MEII0466, which are species specific for *B. abortus* and *B. melitensis* respectively. Another potential molecular marker for genotyping based on the *rpoB* gene polymorphism has been proposed (Marianelli et al., 2006). This method can be used to identify almost all *Brucella* species and most of the biovars, hence offering an improvement over conventional typing methods.

For molecular typing, multiplex PCR techniques have been developed using a combination of different primers to identify and differentiate *Brucella* at genus, species and some at biovar levels. Several multiplex PCR (Bruce-ladder) are available including AMOS-PCR, named after its applicability to "*abortus*, *melitensis*, *ovis* and *suis*" species, which uses a primer of five oligonucleotides to differentiate four species of *Brucella* and their biovars: *B. abortus* (biovar 1, 2 and 4), all three biovars of *B. melitensis*, all biovars of *B. ovis* and *B. suis* biova 1(Bricker and Halling, 1994). Another AMOS multiplex PCR was later developed with additional primers to differentiate field strains from vaccine strains S19 and RB51 (Ewalt and Bricker, 2000). A novel multiplex Bruce-ladder PCR that is able to identify most of the present recognised *Brucella* species and accurately differentiate certain biovars of *B. abortus* and *B. suis* has also been developed (García-Yoldi et al., 2006; Huber et al., 2009). The limitation with PCR methods is that they do not differentiate all the biovars in a given *Brucella* species (Bricker et al., 2003; Bricker and Halling, 1994) and cannot discriminate strains of a given biovar within a given species. The sensitivity of PCR methods is influenced by the DNA extraction protocol

used and is usually lower than that of a culture, but its specificity is close to 100% (Leyla et al., 2003). Fingerprinting methods such as single nucleotide polymorphisms (SNPs), which detect single nucleotide differences in a DNA sequence, and MLVA, which assess the variability of a loci containing repeated sequences, are also available for typing of *Brucella* species and biovars and differentiating strains from the same biovars. MLVA is a useful tool for tracing back the source of infection (Al Dahouk et al., 2007) especially in cases where several biovars of a given *Brucella* species are cocirculating (Godfroid et al., 2010). MLVA-16 based on 16 loci has been developed (Le Flèche et al., 2006). The whole genome sequence (WGC) of *B. abortus* is available and was compared with that of *B. suis* and *B. melitensis* (Halling et al., 2005). This shows high similarity between *Brucella* spp. but provides an important resource for further investigation of *Brucella* spp. These novel methods help in further understanding the evolution, host specificity, and pathogenicity of the genus *Brucella*.

Treatment, prevention, and control

Infected animals are usually not treated because of the high cost associated with prolonged antibiotic treatment with no guarantee of clearing the infection. Furthermore, *Brucella* spp. may undergo L-transformation when exposed to certain antibiotics (e.g. penicillin, oxytetracyclines) resulting in cell wall deficiency. This will interfere with serological detection of the bacteria and create carrier animals (Hatten and Sulkin, 1966) and is thought to result in chronic disease.

Vaccination has been used in many countries to protect against brucellosis. In cattle, vaccines such as *B. abortus* strain 19 (S19), a live attenuated vaccine, strain 45/20, a rough killed vaccine, RB51, a rough live attenuated vaccine and BS2, a smooth strain of *B. suis* biovar 1 have been used. S19 has been widely used because of its safety, potency, practicality of production, and convenience of use in cattle (Al-Khalaf et al., 1992; Godfroid et al., 2013a). However, this strain induces production of immunoglobulins that are detected in serological tests (Godfroid et al., 2010), and in pregnant cows it can cause abortion (Nicoletti, 1978). In an outbreak, mass vaccination of adult cattle with S19 can be done if they have not been previously vaccinated. Strain 45/20 (rough strain derived from *B. abortus* smooth strain) does not interfere with serological reactions. However, it can revert to its smooth form and is not as protective as S19, hence it is no longer in use (Schurig et al., 2002). Strain RB51 does not induce post-vaccination antibody titres but is considered less efficient than S19 (Moriyon et al., 2004). A reduced dose can be used safely in pregnant animals. In China, the BS2 vaccine, has been widely used for control of brucellosis in cattle but its use is not recommended by the OIE (OIE,

2009). Bulls also contribute significantly in the transmission of disease. However, they are not usually involved in vaccination programmes because they can shed the vaccine strain in the semen and thus transmit the infection.

Other control measures are geared towards preventing the spread of the disease between animals and between herds, monitoring of brucellosis-free areas and elimination of infected animals through test-and-slaughter strategy. Vaccination combined with test and slaughter can be used effectively in the control and eradication of brucellosis. In most developed countries, a combination of vaccination, test-and-slaughter, and depopulation of herds has been successful for controlling and eradication of the disease (Caetano et al., 2016; Godfroid and Käsbohrer, 2002; Zamri-Saad and Kamarudin, 2016). The challenge with these methods in developing countries is the cost and logistics associated with these programmes. Systematic vaccination of young replacement stock and slaughter of adult animals may not be realistic in the extensive nomadic pastoralist system that predominates in most of sub-Saharan Africa. Culling of positive individuals and depopulation require that compensation be paid to farmers (making it expensive), who may otherwise refuse to cooperate. Repeated mass vaccination has been suggested as a method of choice in sub-Saharan African conditions (Ducrotoy et al., 2017). In many sub-Saharan countries where control strategies have been put in place, mainly ad-hoc vaccination using either S19 or RB 51 that has been implemented (Ducrotoy et al., 2017). Vaccination alone has been successful in reducing the prevalence of the disease (McDermott and Arimi, 2002) but not its eradication.

1.4 Bovine viral diarrhoea virus (BVDV)

Bovine viral diarrhoea virus belongs to genus Pestivirus within the family Flaviviridae. Three species of pestiviruses are important animal pathogens: BVDV, border disease virus, and classical swine fever virus. BVDV virions are spherical and approximately 50 nm in diameter with a tightly adherent envelope containing glycolipids. They have structural proteins that include the internal capsid protein and the virion surface envelop protein. Transmembrane glycoproteins E1 and E2 form a dimer on the virion surface where E2 is the main antigen and the target of virus-neutralising antibodies. The BVDV genome is a single stranded, positive sense ribonucleic acid (RNA) molecule consisting of about 12,500 base pairs (Qi et al., 1992).Two genotypes (1 and 2) have been identified (Qi et al., 1992). BVDV also exists as two biotypes according to cell culture properties: cytopathic and non-cytopathic. The non-cytopathic strains have the ability to produce persistently infected (PI) animals (Brownlie et al., 1984).

BVDV has a worldwide geographical distribution and causes infections and/or diseases in domestic ruminants, pigs, rabbits, and a wide range of wildlife (Potgieter, 2004). In East Africa, little is known about the occurrence of BVDV. Most studies concentrate on viral infections with clinical symptoms that affect international trade (Callaby et al., 2016). One study in Tanzania reported a 12% seroprevalence in cattle (Msolla et al., 1988). Internationally, the prevalence varies between regions partly depending on the presence or absence of control programmes. In endemic situations, the incidence risk varies from 0.08 to 0.48 while in areas with control programmes it varies from 0.02 to 0.03 (Lindberg, 2003). Within-herd prevalence varies, but is usually high in herds with PI animals (Lindberg, 2003). Although BVDV has been reported in many animal species and interspecies transmission is possible, the source of the virus is cattle (Løken, 1995). The exact role of other species in the epidemiology of BVDV is still unclear (Potgieter, 2004). Nevertheless, PI animals have also been reported to a lesser extent in sheep and goats. Management systems can favour or disfavour survival of the agent in the population. Purchase of untested cattle, lack of biosecurity measures, mixing cattle from different sources, large herds, high stocking densities and sharing of communal grazing land have been found to be risk factors (Houe, 1999; Lindberg, 2003; Lindberg and Houe, 2005; Solis-Calderon et al., 2005; Van Campen, 2010). In an endemic situation, the prevalence of PI animals usually lies between 1 and 2%. However, the prevalence is likely to be higher since most PI animals are culled or die before they are tested (Houe et al., 1995).

Transmission is by both vertical and horizontal direct and indirect contact, but the primary mechanism of transmission is direct contact between susceptible and PI animals (Lindberg and Houe, 2005). PI animals continuously shed high levels of the virus in their blood, nasal secretions, saliva, tears, semen, milk, urine, and faeces (Houe, 1995). Animals with acute postnatal infections are generally not considered infectious (Niskanen et al., 2000). Secretions and excretions of these animals contain the virus from four to ten days post-infection, but the viral load is usually too low to infect new animals. Cattle can also get the infection from sheep; however, strains passed from sheep to cattle have been proved to originate from cattle (Paton et al., 1995).

In pregnant animals, BVDV can cross the placenta to infect the embryo/foetus. The outcome of the infection for the pregnancy and foetus depends on the stage of gestation, the development of the foetus, and the strain of virus. Acute infection in very early pregnancy can lead to poor fertilisation and repeated breeding, abortions or mummification (McGowan et al., 1993). Infection in the first trimester often results in foetal death particularly between 40–120 days,

result in the birth of PI calves (Moennig and Liess, 1995). In the second trimester, the virus may induce a variety of congenital defects, particularly of the CNS and musculoskeletal system, with or without PI status. During the third trimester of gestation, foetuses usually mount relatively normal immune responses and are born healthy and antibody positive (Moennig and Liess, 1995; Potgieter, 2004).PI animals are infected before the immune system is developed enough to recognise the virus as non-self, and therefore do not mount any immune response to it. They are born virus positive and antibody negative, and remain so for life. If they have offspring, the offspring will also be PI (Meyling et al., 1990). Semen from PI bulls contain a high concentration of BVDV and may result in poor conception rates in susceptible heifers (Gard et al., 2007; Niskanen et al., 2002). These heifers may be transiently infected and may have reduced conception rate or produce PI calves (Potgieter, 2004).

Acutely infected animals typically develop lifelong protection, which is mediated through both cellular and humoural immunity (Bolin, 1993). The immune response results in protection of the foetuses in subsequent pregnancies (Fredriksen et al., 1999). Since strains of BVDV can cross-react, cross-protection between the strains is also possible. Calves can get passive immunity through colostrum that will last for about 4–6 months. Vaccination of cattle with live or inactivated virus stimulates antibodies to numerous viral proteins including the major pestivirus envelope glycoprotein E2 (Bolin and Ridpath, 1989, 1990; Bolin, 1993). Antibodies to other BVDV glycoproteins such as E^{rns} and E1 may be induced in calves after natural infection or vaccination with live virus; however, they may not be an important component of the humoural defence mechanism. Live modified vaccines induce long-lasting immune responses, while inactivated virus induce short-lived immunity with a narrow antigenic spectrum (Bolin, 1995; Kimman et al., 1993).

At herd level, BVDV usually affects reproductive performance and causes gastrointestinal and respiratory disease. Acute infection usually results in subclinical disease, and if there are signs these include inappetence, fever, and leucopenia. The virus also has an immunosuppressive effect. Some BVDV strains can cause severe symptoms including fever, diarrhoea, respiratory disease, and generalised haemorrhagic syndrome (Potgieter, 2004). Acute infection in early pregnancy can lead to poor fertilisation and repeated breeding/insemination (McGowan et al., 1993). Infection can also lead to abortions or mummification, intrauterine growth retardation, stillbirth and birth of weak foetuses, and foetal malformations (Moerman et al., 1994). In bulls, a transient impairment of semen quality is encountered (Niskanen et al., 2002).

PI or non-PI offspring born after intrauterine infection will often appear small due to intrauterine growth retardation; they might be stillborn or born weak. They may have malformations of the CNS or musculoskeletal system, which typically include cerebellar hypoplasia, hydrocephalus, microencephaly, microphthalmia, brachygnathism, and other skeletal defects, and they might have an abnormal shape (Moerman et al., 1994; Stokstad and Løken, 2002). PI animals can be clinically healthy but most often appear unthrifty, have a reduced growth rate, and have secondary infections. They are also at risk of developing mucosal disease. In the acute stage, they present with general depression, fever, anorexia, massive gastrointestinal mucosal erosions, and profuse and progressive diarrhoea, which results in wastage and death. In the chronic stage, similar clinical manifestations are evident but more protracted. In addition, erosive skin lesions and laminitis may develop (Baker, 1995).

Control of BVDV might be done at the individual, herd, regional, or national level, with or without the use of vaccines (Moennig et al., 2005). Systematic control programmes across entire regions have been implemented, particularly in northern Europe. The aim of any such control programme in a region is to determine herds' BVDV status to identify infected herds followed by elimination of the source of infection (PI animals) (Lindberg, 2003; Løken and Nyberg, 2013; Ståhl and Alenius, 2012). Biosecurity methods and control of direct animal contacts have proven to be the best methods of controlling BVDV (Lindberg, 2003). A systematic control measure without the use of vaccines that target persistently infected animals has proven to have substantial results (Moennig et al., 2005). Among the Scandinavian countries, Norway, has successfully eradicated BVDV without vaccination. The method used was based on initial identification of infected herds followed by implementation of systematic zoo-sanitary measures on a national scale to prevent introduction of BVDV in non-infected herds. For infected herds, the target was to reduce the prevalence by identification and elimination of PI animals (Løken and Nyberg, 2013).

In other parts of the world, vaccines against BVDV are widely used. The vaccines have been developed to prevent foetal infections and subsequent losses caused by the development of PI animals and further spread of the virus(Van Campen, 2010). Both live modified and inactivated vaccines are available. The live vaccine is quite effective but has significant safety issues, while inactivated vaccines are less effective. Vaccines against BVDV might reduce the negative consequences of the virus, but it is difficult to control BVDV by use of vaccines only (Ridpath, 2012). Control programmes can also be based on a combination of vaccination and systematic classification of herds and control of transmission(Grooms et al., 2007).

1.5 Neospora caninum

Neosporosis is a protozoan disease caused by *Neospora caninum*, an apicomplexan protozoan parasite (which closely resembles *Toxoplasma gondii*) that was first recognised in dogs in Norway in 1984 (Bjerkås et al., 1984). In cattle it was first identified in calves in the United States in 1987 (Dubey et al., 1989; Thilsted and Dubey, 1989). *N. caninum* is an obligate intracellular parasite that has three different infectious stages: the rapidly replicating tachyzoites, the slowly dividing bradyzoites within tissue cysts, and sporozoite*s* within oocysts (Dubey et al., 2007). The life cycle of this parasite involves an intermediate and a definitive host (Figure 2). Asexual development occurs in many intermediate hosts including cattle, sheep, goats, and horses whereas sexual reproduction occurs in the intestines of a canine definitive hosts producing unsporulated oocysts that are shed in the faeces. The oocysts sporulate and become infective to an intermediate host when it consumes feed or water contaminated with oocysts (Dubey et al., 2007).

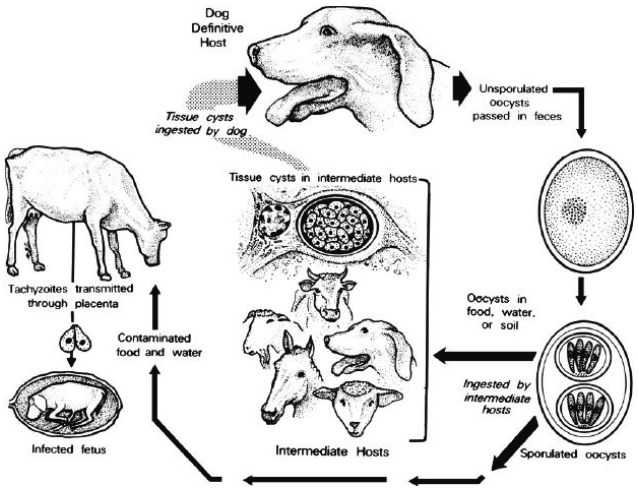


Figure 2: *Life cycle of Neospora caninum (Dubey et al., 2007)*

Neospora caninum is recognised as a major cause of infectious bovine abortions worldwide, with important economic losses to the cattle industry (Dubey and Schares, 2011; Reichel et al., 2014). Bovine *N. caninum*-associated abortions have been reported from all over the world including Canada, the United States, Argentina, Brazil, and several countries in Europe, Asia,

and Africa (Dubey et al., 2007; Klevar et al., 2010). Although information from African countries is scarcer, neosporosis has been reported in Sudan (Ibrahim et al., 2012), Senegal (Kamga-Waladjo et al., 2010), Egypt (Ibrahim et al., 2009), and Ethiopia (Asmare et al., 2013b). In Ethiopia it has been found to cause abortions and stillbirth (Asmare et al., 2013a; Asmare et al., 2013b; Vanleeuwen et al., 2010). In Tanzania, a prevalence study in the canid population showed that 22% of 49 dogs had antibodies to *N. caninum* (Barber et al., 1997). Furthermore, a prevalence of 8.1% and 2% was found in two different areas in cattle native to Tanzania; however, neosporosis' impact on reproductive performance has received little attention (Latham, 2003). The presence of infected dogs in herds increases the risk of infection in cattle (Corbellini et al., 2006; Pare et al., 1998; Wouda et al., 1999). Other risk factors include climate, age of cattle, herd size and density, movement of animals between herds, and presence of intermediate hosts other than cattle (Dubey et al., 2007).

In cattle, there are two transmission routes. The most important route is the vertical/endogenous transplacental route which occurs when congenitally infected dams transmit the parasite to their offspring after reactivation of bradyzoites in the tissue cysts during pregnancy (Anderson et al., 1997; Dubey et al., 2006). The horizontal/exogenous transplacental route is the minor route which occurs when pregnant cattle ingest food or water contaminated with sporulated oocysts (Dubey et al., 2006).

Infection is usually asymptomatic in healthy non-pregnant cows (Otter, 1997). In pregnant cows, tachyzoites infect maternal caruncles and cross the placenta to infect the developing foetus. In case of severe placental damage, abortion will occur due to either direct foetal damage or release of maternal prostaglandins that in turn cause luteolysis and abortion (Dubey et al., 2006).

Abortion is the only clinical sign observed in adult cows, and occurs from three months of gestation to term but mostly between five and six months (Wouda et al., 1998). Depending on the transmission route, abortion will either be sporadic or there will be an outbreak with an abortion rate of up to 20%-40%. There can also be repeated abortions from the same cow. Calves that are infected transplacentally will be persistently infected for the rest of their life. Neosporosis can increase the calving to conception interval and the number of inseminations to conception (Kamga-Waladjo et al., 2010). Cattle infected during pregnancy develop some degree of immunity; however, this immunity is not completely protective and abortion may occur also in subsequent pregnancies.

Unfortunately, there are neither approved treatments nor dependable vaccines on the market to prevent neosporosis in cattle. Since cellular immunity is important in protecting cattle against neosporosis, live vaccines are more likely to simulate the natural infections; however, the safety and potency of live vaccines is an issue of concern (Innes et al., 2002). Therefore, prevention and control strategies largely depend on the infection status of the herd and the associated management practices (Reichel et al., 2014). These include having a closed herd and purchasing replacement animals from a free herd. Cattle feed, water, and calving areas should be protected from definitive hosts (wild and domestic dogs) (Dubey et al., 2007). In infected herds, it is important to avoid vertical and horizontal transmission; therefore, it is important to test young and old stock and rear negative young stock separately (Reichel et al., 2014).

1.6 Simbu serogroup viruses

Simbu serogroup viruses belong to the family Orthobunyaviridae, genus Orthobunyavirus and are arthropod-borne (Calisher, 1996). Viruses in the genus Orthobunyavirus are divided into 18 serogroups. The largest group is the Simbu serogroup which consists of 25 antigenically closely related viruses (Saeed et al., 2001) including the Akabane, Shamonda, Aino, Tinaroo, Peaton, Simbu, Sabo, Sango, Yaba, Sathuperi, Shuni, and Schmallenberg viruses. They have spherical enveloped virions with a 90–100nm diameter and consist of three segments of single stranded negative sense RNA (Elliott et al., 1991).

Orthobunya viruses have a worldwide distribution but occur most frequently in Africa, Asia, Australia, and Israel (Della-Porta et al., 1977; Dubey, 2004; Kurogi et al., 1975; Zeller and Bouloy, 2000). The distribution of the virus is determined by climate, seasonal activity, and abundance and distribution of the vectors, which are culicoides (biting midges) (Geoghegan et al., 2014). The viruses have been isolated from both domestic and wild animals. Akabane, Shamonda, and Aino viruses have been the most recognised in this group (Hoffmann et al., 2012). Akabane virus is regarded as endemic in Africa, Asia, and Australia (Kirkland, 2004). Antibodies to Akabane virus in cattle and sheep have been reported in Asia, the Middle East, Australia, and Africa (Al-Busaidy et al., 1987; Hamblin et al., 1990; Jun et al., 2012; Kurogi et al., 1975; Leask et al., 2013). Simbu serogroup viruses originating from Africa has been recovered from cattle and *Culicoides* midges in Asia (Yanase et al., 2004; Yanase et al., 2005). In Africa, Sabo, Sango, Sathuperi, Shamonda, Shuni, Simbu and Yaba viruses have been isolated from domestic animals (Causey et al., 1972; Lee, 1979). Likewise, Sabo, Sango, Sathuperi, Shamonda and Shuni have been isolated from culicoides midges (Lee, 1979). Serological evidence of presence of Akabane virus has been reported in cattle in Nigeria and

Sudan (Elhassan et al., 2014; Oluwayelu et al., 2016). Antibodies to Akabane virus have also been identified in wild animals in Africa (Al-Busaidy et al., 1987; Hamblin et al., 1990).

Schmallenberg virus, another Simbu serogroup virus, was first detected in Germany in 2011 and has since been reported in many European countries (Doceul et al., 2013). It is not known if this virus is present in Africa. Serological indication of its presence has been reported in Nigeria (Oluwayelu et al., 2015). Diseases with similar clinical presentation without definitive diagnosis have been reported in South Africa (Kirkland, 2004; Leask et al., 2013).

Most of the studies on Simbu serogroup viruses in Africa were done to determine their presence, but little has been done on their impact in livestock. In Tanzania, apart from Akabane virus, little is known about other Simbu serogroup viruses and their impact on reproductive performance in cattle.

Since there is little information about most of the viruses in this group, Akabane virus is often used as a model. It is believed that their pathogenesis is relative similar. Viruses in this group are transmitted mainly by culicoides and mosquitos (*Aedes* and *Culex*) but also, phlebotoms, ticks, and trypanosomes (Calisher, 1996). Infection can cause significant outcomes if the animal is naïve and pregnant. A virus in the blood will cross the placenta to infect the placenta and the foetus. The virus will multiply in the trophoblastic cells of the placenta and in the rapidly dividing cells of the foetus, especially in the brain, spinal cord, and skeletal muscles, inducing necrotising encephalomyelitis and polymyositis (Kitano et al., 1996; Konno et al., 1988). This virus has high tropism to the neurons of the central nervous system resulting in cerebral malacia, vacuolation, porencephaly, and extensive tissue damage (Varela et al., 2013). Depending on the stage of gestation, foetal death and abortion or congenital malformation can occur. When foetuses are infected during the early gestational stage, they develop more severe lesions (Zeller and Bouloy, 2000). Surviving foetuses will develop several congenital CNS abnormalities. The duration of the infection in the foetus depends on immune-competence to develop antibodies which can neutralise the virus (St George and Kirkland, 2004). After natural infection with these viruses, animals develop some form of immunity. However, the duration of the immunity developed is not yet known.

In non-pregnant animals, these viruses cause clinically inapparent/subclinical infections. Some of these viruses cause fever, diarrhoea, loss of appetite, and reduced milk production in up to 50% of infected cows and small ruminants. Symptoms usually disappear within few days (Doceul et al., 2013; Garigliany et al., 2012). Infection in pregnant animals may result in late

abortion in the third trimester, premature birth, stillbirth, and congenital abnormalities, especially of the CNS in calves, lamb, and kids (Doceul et al., 2013; St George and Kirkland, 2004). The abnormalities include congenital arthrogryposis, porencephaly, hydrocephalus, cerebella hypoplasia and congenital hydrancephaly (Bilk et al., 2012; Doceul et al., 2013; George and Kirkland, 1994; Hoffmann et al., 2012; Muskens et al., 2012; van den Brom et al., 2012). Infection in early stages of gestation usually results in abortion; however, foetuses may survive and develop polio encephalomyelitis, and hence be born unable to stand and with flaccid paralysis or incoordination of the limbs (St George and Kirkland, 2004). Such abnormalities may cause dystocia. Depending on the extent of brain damage, some calves may be blind and may show other clinical signs associated with blindness while some can be reared to maturity and calve normally (Kirkland, 2004).

Control measures include vector (culicoides and mosquitos) control which is possible but difficult and may be impractical. Vaccination can be used to control diseases caused by these viruses. Anti-Akabane and Aino killed-virus vaccines are available, and the vaccine for Akabane virus has proven economically justified in Australia (Kim et al., 2011; Kirkland and Barry, 1986). For SBV, efforts have been made to develop an inactivated Schmallenberg virus vaccine to prevent viraemia in cattle and to reduce the level of viraemia in sheep. The vaccine is available and initial investigation of the vaccine candidate in cattle and sheep proved successful (Wernike et al., 2013). However, the cost of vaccinating livestock is not justified as the disease is regarded as a low impact disease.

1.7 Knowledge gaps

To summarise, both reproductive disorders (Kanuya et al., 2006) and infections capable of causing such disorders have been reported in Tanzanian cattle (Table 2).The occurrence of reproductive disorders and the seroprevalence status of infectious agents have not been studied in combination. Several infections are associated with reproductive disorders, but it is unclear which ones are of importance. It is also not known which risk factors are important for reproductive disorders and for each infection. Possible exclusion of infectious causes could facilitate diagnosis of non-infectious causes of reproductive disorders.

Brucella spp., BVDV, *Neospora caninum,* and Simbu serogroup viruses are generally known to be among the most important causes of infectious reproductive disorders. Table 2 summarises the studies conducted on the selected infections in different animal species in

Tanzania. As shown, the reports are few, fragmented, and old. Due to diversity in management systems and climatic conditions, it is also challenging to extrapolate information between areas.

While *Brucella* infection in cattle is relatively well-studied in the country, evidence on the types of strains circulating in different animal species and their association with reproductive disorders is scarce. Most of the studies are based on serology, which faces challenges including cross-reaction with bacteria and the impossibility of inferring which *Brucella* spp.-induced antibodies are in the host, which in turn makes it impossible to trace the source of the infection. *Brucella* spp. from cattle and small ruminants were isolated and identified more than 50 years ago (Mahlau, 1967), and there was no characterisation of the isolates. Recently, typing of strains from Tanzania was done, but because a method that can detect only *Brucella abortus* biovars 1, 2, 4 and 7 was used and the strains obtained could not be biotyped (Assenga et al., 2015). Integration of serology, bacteriology, and molecular typing would be necessary to understand the complex epidemiology and transmission dynamics of this complex multispecies pathogen. Identification of strains infecting animals would facilitate understanding of transmission patterns, as human infections always originate from animals.

Table 2: *Summary of studies conducted on Brucella spp., BVDV, Neospora caninum and Simbu serogroup viruses in different types of animals in Tanzania*

Infections	Animal Type	References
Brucella spp.	Cattle	Assenga et al., 2015; Jiwa et al., 1996; Karimuribo et al., 2007; Kiputa et al., 2008; Mahlau, 1967; Mellau et al., 2009; Roug et al., 2014; Shirima et al., 2007; Shirima et al., 2010; Shirima and Kunda, 2016; Swai and Schoonman, 2012; Weinhaupl et al., 2000.
	Small ruminants	Assenga et al., 2015; Mellau et al., 2009; Shirima et al., 2010.
	Humans	Assenga et al., 2015; Bouley et al., 2012; John et al., 2010; Kiputa et al., 2008; Shirima et al., 2007; Shirima et al., 2010; Swai and Schoonman, 2009.
	Wildlife	Assenga et al., 2015; Fyumagwa et al., 2009; Hamblin et al., 1990; Shirima et al., 2007.
BVDV	Wildlife	Hamblin et al., 1990.
	Cattle	Msolla et al., 1988.
Neospora caninum	Dogs	Barber et al., 1997.
	Cattle	Latham, 2003.
Simbu serogroup viruses	Cattle	Taylor et al., 2001.
	Wild life	Al-Busaidy et al., 1987; Hamblin et al., 1990.
	Small ruminants	(Levin, 2015)

There are only two studies on BVDV in cattle, and they are old. Occurrence of BVDV might have changed overtime, and there might be differences between different geographical areas and management systems. The virus has never been isolated or characterised in Tanzania. The effects of this virus on reproductive performance in cattle are unclear, but BVDV is recognised as one of the most important reproductive infections worldwide.

Climate change and recent global warming, and the subsequent insect vector expansion, are important factors in the distribution and impact of vector-borne viruses, including Simbu serogroup viruses. The only Simbu serogroup virus reported in Tanzania is Akabane virus, and information on all other related viruses is lacking. The study area is a hotspot for most vector-borne diseases with favourable climatic conditions for their survival and propagation. The area must be considered a high-risk area for vector-borne infections with high animal density and an outdoor grazing system. Despite this, it is unknownwhether or not other Simbu serogroup viruses are presentas is their impact on reproductive performance in cattle. Particularly interesting is the lack of investigation of SBV, since the origin of the SBV causing the outbreak in Europe, as well as the global geographic distribution, is unknown.

Neospora caninum can be termed a neglected abortifacient disease in cattle in Tanzania since it has received very little attention. Its prevalence in cattle is reported in only one study from the northern parts of the country (Latham, 2003) and there is no study of its impact on reproductive performance in cattle. Since studies in other countries have highlighted this infectious agent's high impact, it should be investigated whether this is also the case in Tanzania.

Altogether, the lack of systematic information on the occurrence of reproductive infections in cattle in Tanzania is an obstacle for designing targeted preventive strategies to improve productivity, human health, and the community's livelihood.

2.0 Aim of the study

The overall aim was to investigate the occurrence, impact, and characteristics of infections causing reproductive disorders in cattle in Tanzania.

Sub-aims:

1. To investigate the degree of reproductive disorders and exposure status of BVDV, *Brucella* spp. and *Neospora caninum* (Paper 1).
2. To investigate the risk factors for BVDV, *Brucella* spp., and *Neospora caninum* seropositivity and their association with reproductive disorders (Paper 1).
3. To isolate, identify, and characterise *Brucella* spp. causing abortion in cattle (Paper 2).
4. To investigate the presence of antibodies against SBV and closely related viruses and their possible association with reproductive disorders (Paper 3).

3.0 Materials and methods

3. 1 Area of the study and target population

This study was part of a large research and training programme in Tanzania entitled 'Enhancing Pro-poor Innovations in Natural Resources and Agricultural Value Chains' (EPINAV) https://www.nmbu.no/en/faculty/landsam/department/noragric/institutional_coop/epinav. EPINAV was a collaborative programme between SUA and the Department of International Environment and Development Studies (Noragric) at NMBU.

The study area was selected based on the requirements of the overall EPINAV programme and included Njombe and Mbeya regions in the southern highlands of Tanzania. In Mbeya region, Mbarali district was included, and in Njombe region, Wanging`ombe district, Njombe urban, and Njombe rural districts were included (Fig. 3). Njombe is located at 1600–1800m above sea level with an annual rainfall of about 1000–1600mm, temperatures ranging from 12 to 23ºC, and a rainy season from December to April. Mbarali district borders Njombe region on the east and is located in Ihefu valley, which is part of Usangu plain (basin). Ihefu, a catchment basin of Rufiji river in Mbeya city, is also known as the Usangu wetland. Mbarali sits at about 1252m above sea level with average temperatures between 25 and 30ºC and a mean annual rainfall of about 450–650 mm, which means it is slightly lower, warmer, and dryer than Njombe.

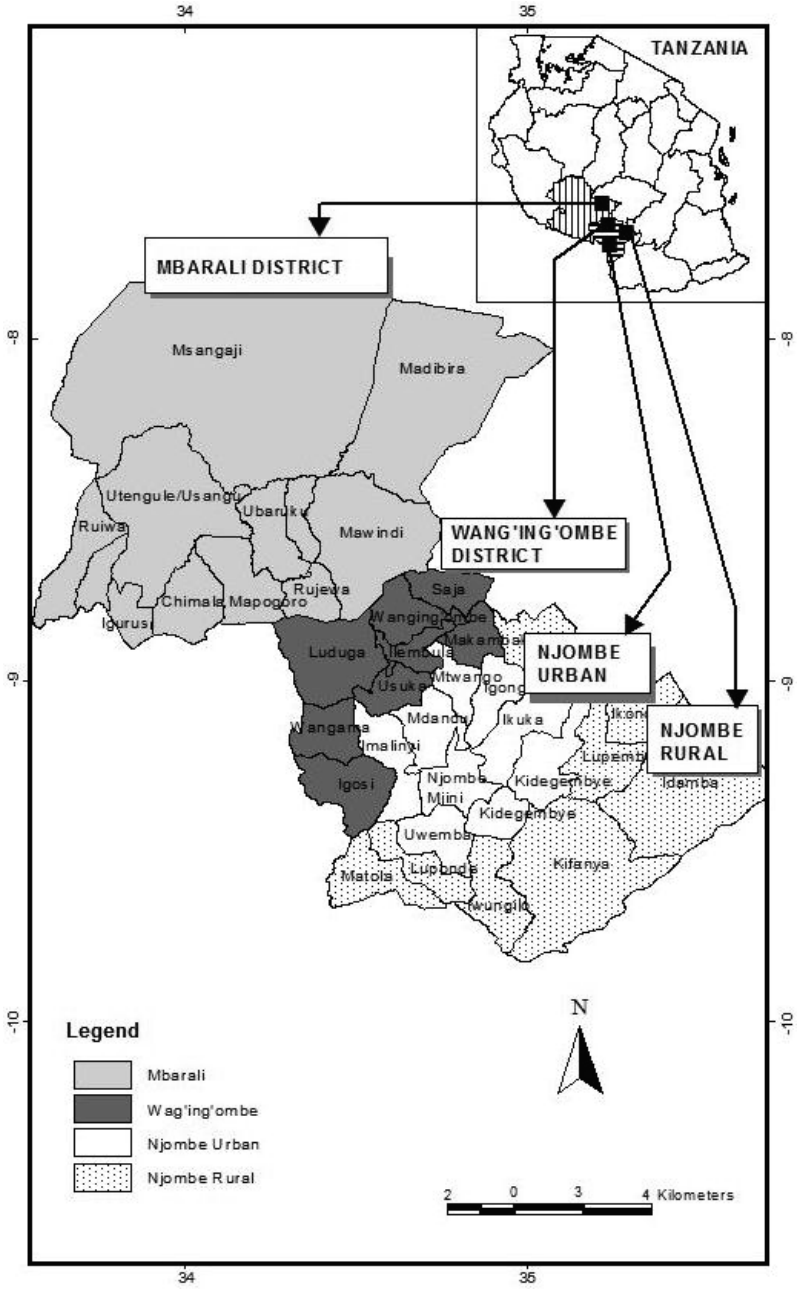


Figure 3: *A map of Tanzania showing the study areas*

The study area is known to be an area where small-scale dairy herds are most common, but larger dairy herds, which serve as a source of in-calf heifers and replacement stock for many parts of the country are also present (Fig 4). In addition, both pastoral and agropastoral herds exist. It therefore includes all management systems representative of the country. Dairy farmers generally keep European breeds and crosses with the local Zebu while pastoralists keep Zebu (Fig 5). Most farmers keep several animal species together with cattle such as goats, sheep, chickens, ducks, dogs and cats. Dairy cattle are usually housed in small barns made of either concrete walls or branches of trees with wooden, earthen, or concrete floors. For Zebu cattle, extensive grazing or combination with indoor keeping are common, and herds are moved according to where they can find feed and water. Artificial insemination is generally uncommon in the area; instead natural breeding using bulls from neighbourhood farms is practised.

Figure 4: *Herds with different management systems in the study area: small-scale with only one dairy cow which is crossbred (far left), medium-scale with mainly Zebu cattle (in the middle), and large-scale with crossbred dairy cattle (to the right) (Pictures: C. Mathew)*

Figure 5: *Zebu cattle (far left), crossbred dairy cattle (middle), and mixed (cattle and small ruminants) grazing (far right) in the study area (Pictures: C. Mathew)*

Calculation of sample size

The optimum sample size for a prevalence study was calculated using the following formula:

$$n = \frac{Z^2 P (1-P)}{d^2}$$

Where n = sample size, P = expected/assumed prevalence (that can be obtained from the same study or pilot study). P can also be obtained from previous studies in the same area, Z = statistics corresponding to level of confidence, usually 95%, and d = precision corresponding to effect size (Ausvet., 2011). For the present study, the minimum sample size was determined based on 50% individual prevalence, 95% level of confidence and 5% absolute precision. This provided a minimum sample size of 385 animals. Because of the diversity in management systems in the study area and the lack of previous studies, prevalence estimates from other studies could not be used as a benchmark. It was therefore decided to increase the number of animals as much as possible within the framework of the EPINAV programme in order to increase the accuracy of the results.

Herds, animals, and management

In total, 202 herds were visited and 658 animals included. 183 herds were small-scale dairy herds (1-6 cattle) and 18 medium-scale herds (7-100 cattle). In addition, a large dairy herd with about 350 cattle were included, and 200 of the animals were sampled. Table 3 shows the distribution of herds of different sizes in the two regions. Dairy cattle breeds were Friesian, Ayrshire or Jersey crossed with Zebu. Most of the crossbred dairy cattle from small or medium sized herds were housed in small barns kept indoors all the time and supplied with feed stuffs from the communal grazing areas. Zebu cattle were taken out for grazing during the day and housed at night.

Table 3: *Size and number of herds sampled from the two study locations (regions)*

Herd size	**Region** (Districts)	
	Mbeya (Mbarali)	**Njombe** (Njombe Rural, Njombe Urban, Wanging`ombe)
>100 cattle	1	0
7–100 cattle	13	5
1–6 cattle	28	155

3.2 Study design

The study districts and villages were selected through the EPINAV programme. Within the present study, selection was done at two stages: herd and animal level. Inclusion criteria for herds were to have at least one female above six months of age and willingness of the farmer to participate. For cattle, animal inclusion criteria were to be female above six months to avoid interference of maternal antibodies in the seroprevalence studies (Chase et al., 2008). A maximum of five cattle were selected using simple random techniques from herds with more than five cattle. In herds with fewer than five animals, all animals were included. In addition to females, 28 breeding bulls were also included. All material for the project was collected during field trips conducted from September 2012 to April 2014, where each herd was visited once. Information was gathered through direct observation at farms, interviews of farmers, and collection of biological material from animals.

A graphical overview of the study design, the materials collected, and the analysis performed is provided in figure 6. Papers 1 and 3 were based on serological investigations of sera from all included animals using cross-sectional study design. In addition, Paper 3 included serological investigations of archived sera from 130 cattle from the same area collected in another study during 2008/2009. Paper 2 was designed as a clinical case investigation based on only one herd, which was the largest investigated. Serum from cattle, small ruminants, and dogs; milk from cows; and aborted bovine foetuses and foetal membranes were used in this study. Detailed information on the study design is available in the materials and methods sections of the specific papers.

3.3 Laboratory analysis

Laboratory techniques used in this study include ELISAs (antigen & antibody), VNTs, RBT, MRT, bacterial culture, classical biotyping, PCR, and brucellosis MLVA-16. The analyses were conducted in different research and diagnostic laboratories. All serum samples collected were analysed for antibodies against *Brucella* spp., *N. caninum*, BVDV, and Schmallenberg virus using commercially available ELISA kits. In addition, for *Brucella* spp. antibodies, RBT and MRT were used. For BVDV antigen, ELISA was used and RT-PCR was used to detect the virus in the serum. For Schmallenberg virus, ELISA results were further confirmed using Schmallenberg VNT and VNT for other Simbu serogroup viruses. Investigation of *Brucella* started with gross and standard histopathological examination of tissues from an aborted foetus and culture of abortion materials and milk, classical biotyping, molecular identification and

characterisation using RT-PCR and genotyping using MLVA-16. These methods were used first to isolate and identify bacteria both at the genus and species level and then to characterise the isolates both phenotypically and genotypically and compare their characteristics with isolates from other countries. Details of each method are provided in the respective papers.

Some laboratory methods were used that were not included in any of the papers. These include *Brucella* spp. real-time multiplex PCR (Bruce-ladder), *B. abortus* and *B. melitensis* singleplex PCRs and 16S rDNA gene sequencing. Analysis of serum collected from herds other than the large-scale herd using RBT was done and results were thereafter compared with iELISA results.

Brucella genus specific RT-PCR (*bcsp31)* was performed as earlier described (Bounaadja et al., 2009). The isolates obtained that were classified as *Brucella* spp. based on IS *711* and *bcsp31* results were also subjected to Bruce-ladder and 16s rDNA gene sequencing (Saua et al., 1999) to further identify the *Brucella* species. Thereafter, *B. abortus* and *B. melitensis* simplex-specific real-time PCR (BruAb2_0168 and B.MEII0466 respectively) (Hinić et al., 2008) were performed to confirm the *Brucella* species.

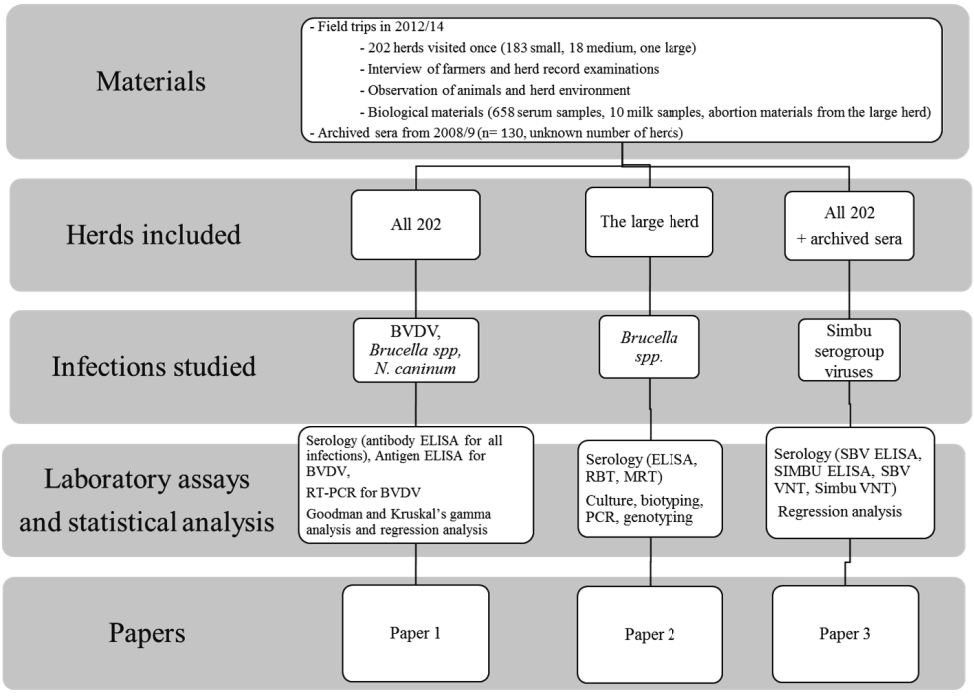


Figure 6: *Overview of the study; data and materials collected, infections studied, laboratory and statistical analysis performed and resulting publication*

3.4 Data management and statistical analysis

Statistical analysis of data was done using STATA version 12 for Windows (Stata Corp., College Station, TX, USA). Categorical variables were compared using chi-square tests at critical probability value of $P<0.05$ (95% confidence interval). Animal and herd level seroprevalences for *Brucella* spp., *N. caninum* and BVDV were obtained using survey command with cluster (herd) adjustment according to the herd sampling fraction. Within-herd prevalence was calculated using the following formula: $WHP= (n^+/N^+)*100$ where WHP= within-herd prevalence, n^+ =number of positive animals, N^+ =number of animals in positive herds.

Associations between animal- and herd-level serostatus for *Brucella* spp., *N. caninum*, and BVDV with covariates (reproductive disorders and risk factors) were determined using univariable logistics regression models adjusting for herd clustering effect for animals (Papers 1 and 3). Tabular analysis using Goodman and Kruskal's gamma was used to determine association between the infections. Predictors with P values ≤ 0.2 were further analysed for collinearity in cross-tabulation before being entered into a multivariable logistic regression model.

Taking into consideration the biological plausibility of the factors in addition to their statistical relevance, a final multivariable logistic regression model was formed using a backward elimination procedure to include significant variables in the model both at animal and herd levels (inclusion criteria $P\leq0.05$ of the likelihood ratio test). Predictive ability of the model and multicollinearity were then assessed using the variance inflation factor (VIF). The models constructed were further assessed using a Pearson chi-square test for goodness of fit, and by using the receiver operating characteristic (ROC) curve for its reliability and its predictive ability (Dohoo, 2010).

For the molecular study, the genotype profiles obtained by MLVA were compared with the sequence of *Brucella abortus* biovar 3 available in Genbank using the *Brucella* 2007 public database (MLVAbank2009) (http://mlva.u-sud.fr/mlvav4/genotyping/query.php). Cluster analysis of MLVA-16 loci data was performed with the software Bio Numerics 2.1 (Applied Maths, Sint-Martens-Latem, Belgium) following standard published methods (Le Flèche et al., 2006) (Paper 2).

4.0 Results

4.1 Main results of individual papers

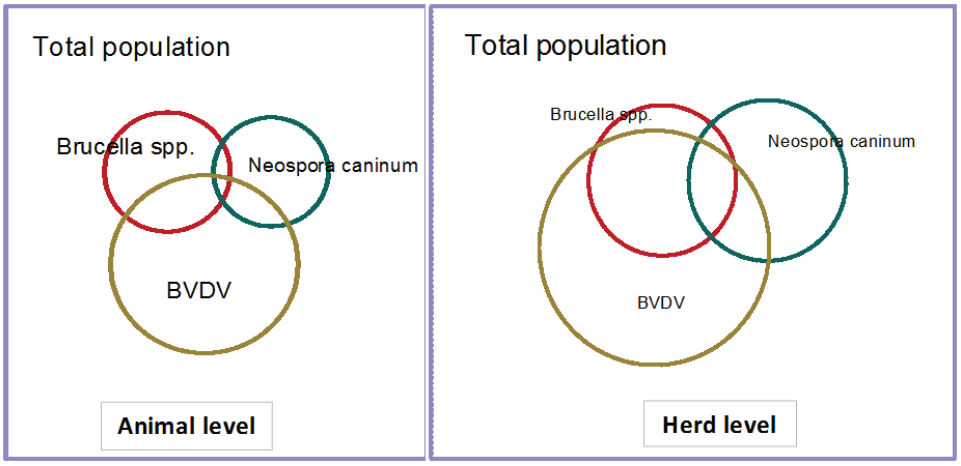
Paper 1: Reproductive infections in cattle in Tanzania – lessons for control priorities

Seroprevalences

The overall animal prevalence for BVDV, *Brucella* spp., and *N. caninum* antibodies were 15.2%, 5.4%, and 4.5% respectively. No serum was positive for BVDV antigens. Herd-level prevalence (at least one positive animal) for BVDV, *Brucella* spp., and *N. caninum* was 17.9%, 7.4%, and 8.4%, respectively. Interestingly, the seroprevalence for BVDV and *Brucella* spp. varied significantly between Njombe and Mbeya. In Mbeya region, the herd-level seroprevalence was 66.7% for BVDV and 36.1% for *Brucella* spp.; the animal-level seroprevalence was 38.3% and 17.8% respectively. In Njombe region, herd-level seroprevalence was 6.5% for BVDV and 0.6% for *Brucella* spp. and animal-level seroprevalence was 5.7% and 0.3%, respectively.*Brucella* spp. and BVDV seropositivity were significantly associated with each other both at animal ($\gamma = 0.64$) and herd level ($\gamma = 0.9$). BVDV and *N. caninum* were not associated with each other at animal level ($\gamma = 0.01$) but a weak association was observed at herd level ($\gamma = 0.38$). *Brucella* spp. and *N. caninum* were weakly associated at animal level ($\gamma = 0.04$) but more strongly associated at herd level ($\gamma = 0.58$) (Fig. 7). The large-scale herd had a seroprevalence of 73.1%, 47.8%, and 5.6%, for BVDV, *Brucella* spp. and *N. caninum* respectively. Out of the 28 breeding males, 32.1% were seropositive to BVDV, 14.3% to *Brucella* spp. and 10.7% to *N. caninum*.

Occurrence of reproductive disorders

Reproductive disorders were encountered in98 out of 658 animals over the last three years, giving an overall prevalence of 33% (95% CI: 28–39). These included abortion, stillbirth, retained placenta, dystocia, and calf malformation. Abortion (11.3%) and retained placenta (17.2%), shown in figure 8 were the most frequently encountered reproductive disorders. Animal-level prevalence of abortion was significantly higher in Mbarali (23.4%) than in Njombe (7.0%).



BVDV: bovine viral diarrhoea virus

Figure 7: *Venn diagram showing the relationship between prevalence of serum antibodies to Brucella spp., bovine viral diarrhoea virus, and Neospora caninum at animal and herd level in cattle in the southern highlands of Tanzania*

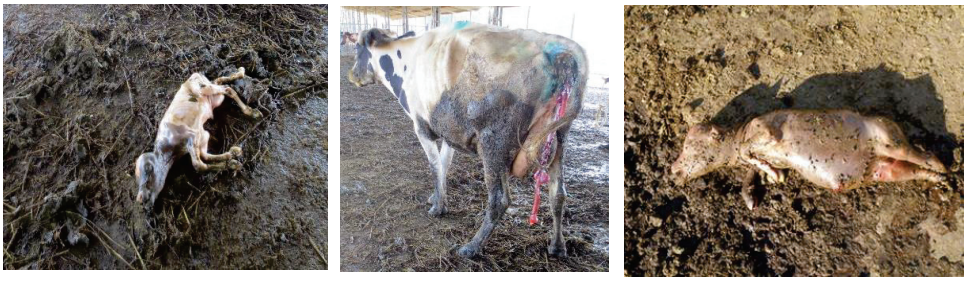


Figure 8: *Reproductive disorders (abortion and retained foetal membranes) encountered in the study area (Pictures: C. Mathew)*

Association between serostatus and reproductive disorders

At the animal level, *Brucella* spp. seropositivity was significantly associated with a history of abortion (OR: 4.6, 95% CI 1.5–14.2) while the other disorders were not associated with any of the infections. At herd level, abortion was also strongly associated with *Brucella* spp. (OR: 15.5, 95% CI 4.6–51.3) and BVDV (OR: 5.0, 95% CI 1.9–12.9) while *N. caninum* was not associated with any of the reproductive disorders. A combined *Brucella* spp. and BVDV seropositivity was associated with abortion both at animal (OR: 11.7, 95% CI 2.7-50.3) and herd level (OR: 10.1, 95% CI 2.9-35.5).

Association between serostatus and risk factors

At animal level, hypothesised risk factors for the three infectious agents were location, breed, and parity. *Brucella* spp. seropositivity was significantly associated with both location (OR: 21.5, 95% CI: 1.9–248) and breed (OR: 5.3, 95% CI: 1.2-23.5) while BVDV was associated only with breed (OR: 4.9, 95% CI: 1.8–13.6). Altogether, zebu cattle were more likely to be seropositive for *Brucella* spp. and BVDV than crossbred dairy cattle while breed did not affect the prevalence of *N. caninum*. There was no association between *N. caninum* seropositivity and presence of dogs on farms.

At herd level, location of the herd, size of the herd, and management system were hypothesised as potential risk factors for seropositivity to the infections. *Brucella* spp. seropositivity was significantly associated with all the risk factors including location (OR: 23.1, 95% CI: 1.9–292), herd size (OR: 14.5, 95% CI: 2.2–94.4) and management system (OR: 22.7, 95% CI: 3.5–150). BVDV seropositivity was significantly associated with location (OR: 12.7, 95% CI: 4.7–34.8) while *N. caninum* seropositivity was not associated with any of the risk factors.

Paper 2: First isolation, identification, and phenotypic and genotypic characterisation of *Brucella abortus* biovar 3 from dairy cattle in Tanzania

Serological findings

Ninety-six out of the 200 serum samples from cattle were positive in ELISA giving a within-herd prevalence of 48% (95% CI 41–55), while 43 of the 200 serum samples were positive with RBT resulting in a within-herd prevalence of 21.5% (95% CI 16–27). Thirty-six sera were positive in both tests, 60 were positive in ELISA but negative in RBT, and seven were negative in ELISA but positive in RBT. All 10 milk samples were positive in MRT. All sheep, goat, and dog sera were negative in RBT. However, two out of 35 sheep (prevalence: 5.7%; 95% CI 0-17) and one out of 50 goat (prevalence: 2%; 95% CI 0-7) sera were positive in ELISA.

Bacterial culture, isolation, identification, and biotyping

Three isolates of *Brucella* spp. were obtained, all from the same case: one from the aborted foetal liver and two from foetal membranes. No *Brucella* spp. was isolated from milk samples. Real time PCR (IS*711*) confirmed the three isolates as *Brucella* spp. Bruce-ladder PCR identified the isolates as *B. abortus* biovar 3 wild type as five fragments of 152, 450, 587, 774 and 1682 base pair in sizes were amplified. The isolates showed common phenotypic characteristics typical for the genus *Brucella*. Classical biotyping indicated that all the three isolates were *B. abortus* biovar 3 (Table 4), although they did not require CO_2 for growth.

Table 4: *Phenotypic characteristic profiles for three Brucella spp. strains isolated from aborted material from cattle in Tanzania compared to three different reference strains*

ID	CO_2 depend-ency	H_2S	Thionin		Fuchsin	Safranin	Anti A	Anti M	Urease		Tb	Tb	Wb	Iz	Species	Biovar
			10	20	20µg	100µg			30	60	RTD	RTD10^4	RTD	RTD		
Isolate 1	-	+	+	+	+	+	+	-	-	-	+	+	+	+	*abortus*	3
Isolate 2	-	+	+	+	+	+	+	-	-	-	+	+	+	+	*abortus*	3
Isolate 3	-	+	+	+	+	+	+	-	-	-	+	+	+	+	*abortus*	3
B.abortus biovar 2	+	+	-	-	-	-	+	-	-	-	+	+	+	+	*abortus*	2
B.abortus biovar 9	-	+	+	-	+	+	-	+	-	-	+	+	+	+	*abortus*	9
B. canis	-	-	+	+	-	-	-	-	+	+	-	-	-	-	*canis*	

RTD=Routine Test Diagnostic; IS*711*=Insertion Sequence *711;* Tb=Tbilisi; Wb=Weybridge; Iz=Izatnagar

MLVA-16 genotyping

The MLVA-16 loci identified three related *B. abortus* biovar 3 Tanzanian genotypes. Out of the three isolates, two were identical but different from the other one at one locus. The three isolates were identical using panel 1 loci but different at the locus Bruce 16 in panel 2. These genotypes were different from the reference strain's genotype and from the genotypes of strains isolated in Kenya and Uganda. Despite the Tanzanian genotypes being unique, they were more closely related to genotypes originating from Europe, Turkey and China than to genotypes from neighboring countries Uganda and Kenya.

Paper 3: Detection of Simbu serogroup virus serum-neutralising antibodies in cattle in the southern highlands of Tanzania

SBV antibody ELISA

Out of the 658 serum samples collected in 2012/2013, a total of 405 (61%) were positive in the SBV antibody ELISA, and out of 202 herds, 175(87%) had one or more seropositive animals. In Njombe, out of 160 herds, 133(83%) were positive, and out of 324 animals, 211 (65%) were positive. In Mbarali, however, all herds (n=42) were positive while out of 335 animals, 194 (58%) were positive. Seventy-one (55%) out of 130 sera collected in 2008/2009 were positive in the SBV ELISA. Antibodies were observed in sera collected from all herd size categories, locations (Njombe and Mbarali); and breeds (dairy and pastoral herds). The results of

univariable regression analysis indicated no significant differences between serostatus and location or herd size or animal breed.

SBV VNT

Fifty-six (51%) out of 110 serum samples collected in 2012/2013 were positive, with titers ranging from 1:16 to 1:512. Fifteen (21%) of 71 serum samples collected in 2008/2009 were also positive in this assay, with positive titers ranging from 1:16 to 1:768. Out of 71 samples from 2008/2009, 42 were toxic to the cell cultures, which made it impossible to interpret the test results. Four were positive but with no end point titers due to insufficient sample volume; therefore their titers could not be determined.

Other Simbu serogroup virus VNTs

Antibodies against nine out of the ten Simbu serogroup viruses (Aino, Akabane, Douglas, Peaton, Sabo, SBV, Sathuperi, Shamonda, Simbu, and Tinaroo) were detected in one or more of the 45 serum samples from 2012/2013. Their titers ranged from 80 to 1280. Most animals had antibodies against two or more viruses.

Twenty-nine (64.4%) sera were positive for SBV, out of which seven had high titers (≥160) to this virus. One serum sample had the highest titers for SBV, while two had the same titers for SBV and Douglas or Sathuperi virus. A high proportion (91.1%) of the sera had antibodies against Aino virus, and antibodies against Tinaroo, Douglas, Peaton, Shamonda, Sabo and Sathuperi viruses were detected in 75.6%, 73.3%, 71.1%, 55.6%, 46.7%, and 31.1% of the animals respectively. Twenty-four samples had high titers to either Aino or Peaton viruses and five sera had high titers only to Tinaroo virus. Eleven serum samples showed low antibody titers to Akabane virus while a single sample had a titer of 160. However, all of these sera had very high titers to at least one of the other viruses (Table 5). None of the samples had positive a titer to Simbu virus. Due to toxicity or bacterial contamination, sera collected in 2008/2009 were not suitable for this assay and were not included in the results.

Table 5: *Antibody titers to Simbu serogroup viruses detected in a virus neutralising test (VNT) performed on 45 cattle sera collected from Tanzania in 2012/2013 (Paper3). *Samples negative in the first SBV VNT, SBV=Schmallenberg virus, SATIV=Sathuperi virus, SHAV=Shamonda virus, NT=Not tested.*

ID	AINO	AKABANE	DOUGLAS	PEATON	SABO	SBV	SATIV	SHAV	SIMBU	TINAROO	Highest titer
1	10	-	20	-	-	20	-	-	-	-	SBV/Douglas
2	20	-	40	40	-	20	-	-	-	-	Douglas/Peaton
3*	20	-	-	20	-	-	-	80	-	80	Tinaroo/SHAV
4	640	10	-	640	640	-	-	160	-	160	Aino/Peaton/Sabo
5	40	-	80	-	80	320	320	80	-	-	SBV/SATIV
6	10	-	-	20	-	-	-	-	-	40	Tinaroo
7*	-	-	40	-	-	-	-	-	-	-	Douglas
8*	40	10	40	80	≥1280	40	-	160	-	≥1280	Sabo/Tinaroo
9	20	-	-	640	160	-	-	-	-	-	Peaton
10	640	-	10	40	NT	10	NT	NT	NT	160	Aino
11	160	-	-	-	160	-	-	-	-	-	Aino/Sabo
12	20	-	20	160	-	10	80	-	-	160	Peaton/ Tinaroo
13	80	-	-	20	80	-	-	-	-	80	Aino /Tinaroo/Sabo
14	-	-	-	-	80	-	-	160	-	160	Tinaroo/SHAV
15	320	-	-	-	-	-	-	-	-	80	Aino
16	-	-	-	-	80	80	160	80	-	-	SATIV
17	80	-	20	20	-	10	80	160	-	20	SHAV
18	160	-	20	40	-	40	80	320	-	80	SHAV
19	-	-	20	160	-	10	80	-	-	40	Peaton
20	160	-	-	-	-	-	80	160	-	10	Aino/SHAV
21	20	80	10	40	80	40	-	320	-	320	Tinaroo/SHAV
22	160	-	80	-	-	-	80	-	-	-	Aino
23	20	-	40	640	-	320	-	≥1280	-	40	SHAV
24	1280	-	20	160	160	10	80	160	-	20	Aino
25	20	-	20	80	80	20	160	320	-	80	SHAV
26	40	-	80	≥1280	-	160	160	-	-	-	Peaton
27	10	-	20	160	320	10	-	320	-	80	Sabo/SHAV
28	160	-	40	320	640	20	-	320	-	80	Sabo
29	40	-	80	-	-	160	-	-	-	-	SBV
30	80	10	-	320	160	-	-	320	-	160	Peaton/SHAV
31	40	-	20	160	-	20	-	80	-	160	Peaton/Tinaroo
32*	1280	-	40	1280	NT	40	NT	NT	NT	80	Peaton/Aino
33	10	-	160	-	≥1280	320	-	320	-	-	Sabo
34	1280	10	40	10	-	10	80	-	-	160	Aino
35*	20	20	20	160	320	40	-	320	-	≥1280	Tinaroo
36*	20	10	20	1280	80	40	-	640	-	320	Peaton
37	640	10	20	160	-	-	80	-	-	320	Aino
38	20	-	20	320	-	20	-	-	-	40	Peaton
39*	640	80	20	640	-	-	-	-	-	≥1280	Tinaroo
40*	80	-	80	-	-	160	-	640	-	160	SHAV
41	80	-	10	-	80	-	-	160	-	20	SHAV
42	≥1280	40	320	320	-	160	-	160	-	640	Aino
43*	80	-	-	80	320	-	-	160	-	80	Sabo
44*	≥1280	40	80	320	80	40	-	-	-	80	Aino
45	160	160	40	320	80	20	160	1280	-	≥1280	Tinaroo/SHAV

Association between reproductive disorders and serological results

There was a statistically significant association between the occurrence of one or more reproductive disorders and SBV ELISA seropositivity (OR= 1.9, 95% CI= 1.2–2.9) on an animal level. There was no association demonstrated between animal seropositivity and any reproductive disorder alone. There was also no association observed between herd seropositivity and any specific reproductive disorder. With the SBV VNT, there were no significant associations between any reproductive disorders and SBV seropositivity. Of the 45 sera subjected to Simbu serogroup VNT, eight originated from animals with a history of reproductive disorders. With Simbu serogroup VNT, there was only an association between Akabane virus antibodies and abortion, and this association was not statistically significant (OR=3.9, P=0.059). Antibodies to other viruses were not associated with any of the reproductive disorders.

4.2 Additional results

Results that are not presented in the three papers

The following results were obtained in the course of the present study, but are not included in any of the papers.

Within-herd seroprevalence for BVDV

Eight of the nine positive herds in Njombe had only one seropositive animal. In Mbarali, six out of 14 herds had one seropositive animal; the remaining had higher numbers, and the average within-herd prevalence was 59%. For the large herd, within-herd prevalence was 73%.

***Brucella* spp. iELISA and RBT**

The results presented in paper 2 included only animals from the big herd and in paper 1 RBT results were not presented (Fig. 4). RBT results obtained from serum originating from animals collected from other herds included in the present study are therefore presented here. The overall seroprevalence using RBT was 15% (95% CI: 12–18) while with iELISA, the overall prevalence was 18.2% (95% CI: 15–21). Forty sera were positive in both tests while 420 sera were negative in both test. Seventy-seven sera were negative in RBT but positive in iELISA and 47 sera were negative in iELISA but positive in RBT.

***Brucella* spp. PCRs and sequencing**

The 16S rDNA gene sequencing and *Brucella* spp. specific real-time PCR/Bruce-ladder gave positive results confirming *Brucella abortus* biovar 3 wild type. MLVA-16 gave a profile typical for *Brucella abortus* biovar 3. Both the *B. abortus* and *B. melitensis* simplex specific PCR targeting BruAb2_0168 and B.MEII0466 were negative.

Animal- and herd-level seroprevalences of selected infections based on herd size

In the present study, the majority of herds included were small-or medium-scale with female cattle as the main targeted animals. Nevertheless, results of samples from one large-scale herd and few male cattle are presented in Paper 1 based on these categories of herds and animals (Fig. 4). In this section, the overall results using all the animals sampled in the study (male and animals from the large-scale herd inclusive) are therefore presented. Seroprevalence based on ELISA results for overall, small-scale, and medium-scale herds is presented in Table 6. The large-scale herd with more than 100 cattle was positive for all the studied infections. The

difference in seroprevalence between small-scale and medium-scale herds was statistically significant ($P<0.01$).

Table 6: *Animal- and herd-level seroprevalences for BVDV, Brucella spp. and Neospora caninum in different herd size categories*

	Seroprevalence (%)						
	Animals				Herds		
Infection	Overall	Small-scale (1-6 animals)	Medium-scale (7-100 animals)	Large-scale(>100 animals)	Overall	Small-scale	Medium-scale
	n = 656	n = 336	n = 120	n= 200	n = 202	n = 202	n = 18
BVDV	32.6	10.4	26.7	73.1	18.8	13.7	66.7
Brucella spp.	18.2	2.7	12.5	47.8	8.4	2.7	61.1
Neospora caninum	5.0	5.4	3.3	5.6	9.9	8.7	16.7

The large herdwas positive for all infections so herd-level prevalence = 100%, overall indicates all animals/herds included in the study

5.0 Discussion

5.1 Methodological considerations

Study Design

Cross-sectional study design combined with serological investigations, as used here, is widely used in veterinary epidemiology (Dohoo et al., 2010). The advantage of cross-sectional design is that it is straightforward, inexpensive, and requires only one sampling occasion. It provides descriptive characteristics of a population at a particular point in time and includes both old and new cases (Dohoo et al., 2010). However, it is not suitable for determining when the disease occurred or for how long it has lasted. The finding of antibodies in a single serum sample only indicates that infection has occurred sometime in the past which make its diagnostic value as indicator of present active infection limited (Levin, 2006; Smith, 1995). It is impossible to determine the sequence of events, namely whether exposure occurred before, during, or after the onset of disease outcome (Levin, 2006). One disadvantage of cross-sectional design compared to, for example, longitudinal study design is therefore the weakness in determining cause-effect relationships (Dohoo et al., 2010). The association between seropositivity and reproductive disorders found in the present study is therefore not necessarily causal although it is statistically significant. Other study designs, such as longitudinal study or case-control study, would have been stronger. Few cases of reproductive disorders and small herds (1–3 cattle) made it difficult to choose a case-control study, and limited financial and practical resources for the project made cohort study design unfeasible, as it would have required several field visits.

In Tanzania, there is no farm registry. It is therefore not possible to get an accurate sampling frame, and the target population size of any study is actually not known. Furthermore, for financial and other practical reasons, the present study was limited to only villages that were already enrolled and participating in the EPINAV programme. The advantage of this was that it increased farmers' willingness to participate, as many had had positive experiences with other parts of EPINAV and earlier programmes. The field work had to be performed as a team where other people in the EPINAV programme were collecting other information. This saved resources but limited the number of villages that could be sampled and the limited time allocated for each activity. The overall implication was that being a part of an already existing programme was in some ways an advantage, but the frames and limitations of the programme

affected the freedom in choice of design, inclusion of herds, and type of information and material that could be collected.

Sampling technique and sample size

Ideally, to get a true prevalence estimate of a given infection in a population with good precision, all animals should be included (census). Due to scarcity of resources, only a fraction (sample) of the population is used to represent the whole population. This fraction needs to be optimal and representative to allow inferences to be made about the target population (Dohoo et al., 2010), which necessitates a random sampling strategy. Since random sampling of villages and herds was not possible because of the study's affiliation with the overall EPINAV project, the only level at which random sampling was possible was the individual animal level when a herd had more than five cattle. The calculation of sample size needed was complicated by several factors: unknown prevalence, heterogeneity in management systems, and different geographical location. Relevant literature on seroprevalence in Tanzania is scarce and concerns studies conducted in other parts of the country with different management systems, study design, and laboratory techniques, limiting its relevance. The minimum sample size was increased to take into account the mentioned challenges. It is therefore likely that the sample size in the present study allows inferences to be made about the target population; moreover, the herds included were generally typical and are very likely representative of other herds in the area.

Possible sources of bias

Selection and/or non-response bias

Since the villages were already chosen as part of the EPINAV programme, and only farmers willing to participate were included, selection bias in the current project is possible. Farmers with high interest and willingness to participate might be more informed, aware, and interested in their animals, and therefore more likely to have better management and healthy animals. It has been suggested that unwillingness to participate in research projects can be an indication of management difference (Dohoo et al., 2010) and possibly have an effect on the outcome of the study. In Paper 1, this might have led to an underestimation of the true magnitude and effects of the infections and the risk factors. On the other hand, it might be that farmers chose to participate because they were experiencing animal health problems. Willingness to participate might also indicate higher competence, and therefore farmers were more likely to

notice and report disease. It would be interesting to assess whether there were systematic differences between those who participated and those who did not.

Confounding factors

A confounding factor is associated with both the outcome and predictor variables and might therefore influence the association between them (Dohoo et al., 2010). In Paper 1, confounding factors were controlled for by introducing all known and available non-significant predictors, one by one, into the model, but no significant change in the model estimates were observed. The possible presence of confounding factors related to herd management that are difficult to measure was also controlled for by including a herd random effect. Although no confounding factors were detected, the presence of confounders not adjusted for cannot be excluded.

Selection of infectious agents

The infectious agents studied were selected based on their well-recognised impact as cattle reproductive pathogens. They all have global epidemiological distribution (Anderson, 2007), the ability to cause reproductive disorders in cattle, public health and economic importance, and likely local importance (Dubey et al., 2007; Ducrotoy et al., 2014). There are several other important infectious agents known to cause reproductive disorders in cattle such as *Coxiella burnetti*, *Leptospira* spp., *Campylobacter foetus*, *Listeria* spp., *Haemophylus somnus, Chlamydia* spp. *Trichomonus foetus*, *Sarcocystis neuroni* and Bovine Herpes Virus -1 (BHV-1) (Yoo, 2010). Practical and financial considerations made it necessary to focus on only a few. Generally, in Tanzania, there is a paucity of data on endemic zoonosis (Halliday et al., 2015); therefore, it would be interesting to include *Leptospira* spp., *Coxiella burnetti* and *Campylobacter foetus* in the study. Leptospirosis, like malaria and brucellosis, also causes febrile symptoms, which is likely to complicate diagnosis and management of all diseases, leading to systematic underreporting. Despite the lack of reliable data, it is prevalent in sub-Saharan Africa with more incidences originating from East Africa (Costa et al., 2015). In Tanzania, few studies have been done on humans and livestock (Allan, 2016; Maze et al., 2016; Muller et al., 2016), emphasising the need for more research on this infection. *Coxiella burnetti* is also a neglected zoonosis causing Q-fever in humans. It is prevalent in Africa, including in neighbouring countries Kenya (Browne et al., 2017; Njeru et al., 2016a), Sudan (Botros et al., 1995), and Malawi (personal communication). In Tanzania, few reports are available, but prevalence in humans, livestock, and game animals is indicated (Chipwaza et al., 2014; Crump et al., 2013; Hummel, 1976). Studies have indicated the prevalence of campylobacteriosis in

both humans and animals in Tanzania (Komba et al., 2013), but molecular epidemiological information is still lacking.

Diagnostic challenges

Brucella species

In the present study, RBT and iELISA were used because they are widely used and also recommended by OIE for screening of brucellosis (OIE, 2009). It is common to use RBT as a screening tool and ELISA as a confirmatory test in series. These tests do not discriminate between natural infection and vaccination with *B. abortus* S19, but, importantly, there is no history of vaccination against brucellosis in the region. Thus, the presence of antibodies due to vaccination can be excluded, which simplifies the interpretation of serological results.

In the present study, the iELISA had a much higher number of positive reactors than RBT (Paper 2 and additional results). Other studies have given similar results, both under experimental conditions (Godfroid et al., 2002) and in a field study (Shirima, 2005). The reasons for these differences are very difficult to identify; however, the dynamic of the infection must always be considered. Indeed, depending on the stage of the disease (acute/subacute/chronic), a different serological profile will be seen, which might explain discrepancies among studies (Godfroid et al., 2010). The vast majority of the brucellosis studies in the developing world are based almost exclusively on serological testing. If more than one serological test is used, the interpretation can be done either in parallel (high sensitivity) or in series (high specificity). Importantly, in serial testing, the sensitivity and specificity of the second test are modified when applied in a proportional population that has been selected by another test. Unfortunately, this is rarely taken into account and thus true prevalence estimates may be incorrectly evaluated. This is important since serial testing has been the practice for many serological studies done in Africa.

Furthermore, RBT is assumed to be more sensitive in the acute stage of the infection than iELISA, since RBT can detect both IgM and IgG (OIE, 2009). During infections in ruminants, the IgM/IgG shift occurs very rapidly, usually within 7 days, which illustrates that detection of IgG1 is essential in the serodiagnosis of cattle (Ducrotoy et al., 2016). The shifting of different IgG subtypes depends on the cytokine environment that develops during the interplay between the infectious agent and the immune response. RBT detects only IgG_1 while iELISA detects both IgG_1 and IgG_2, and the amount of IgG needed to induce agglutination in RBT is higher than the amount needed to induce a positive signal in iELISA (Saegerman et al., 2010). The

high seroprevalence result obtained with iELISA in the big herd (paper 2) could be explained by a high number of chronic infected individuals and a lower specificity of iELISA. False positive serological reactions could be caused by cross-reaction with other bacteria, in particular *Yersinia enterocolitica* 0:9, but also other gram-negative bacteria; this is a well-known phenomenon in brucellosis tests (Perry and Bundle, 1990).

Another explanation for the different tests results in the present study might be that the tests used were not properly validated, which could have led to inappropriate conclusions. In agglutination tests such as RBT, antibodies react with the O-polysaccharide on S *Brucella* cells in suspension and make the bacteria aggregate to clumps that become visible on a glossy surface. Even though the test is apparently straightforward, it has some challenges related to the antigen preparation and the testing conditions. Firstly, the preparation of stable antigens requires a total absence of mutant R *Brucella* cells in the antigen suspension because R cells will interfere with the agglutination and hence the sensitivity of the test. In addition, the antigen batch needs to be standardised with a reference serum, which does not necessarily mean optimal Se and Sp. Secondly, under standard conditions, brucellosis agglutination is affected by the existence of non-agglutination antibodies, prozones (blocking antibodies), and unspecific reactions. However, some of these three phenomena will almost disappear if low Ph agglutination tests like those recommended by OIE and used in the present study are used. Ideally, an appropriate test should be selected and standardised/validated in the specific population in which it is to be used. These tests and cut-off values have not been for regions where brucellosis is not enzootic and therefore they could have different sensitivity and specificity values than the published ones. However, it is likely that this would not modify the overall conclusions in the present study.

Simbu serogroup viruses

For Simbu serogroup viruses, SBV iELISA, Pan Simbu ELISA, and VNT were used (Paper 3).ELISA and VNT gave different results, which may indicate cross-reactivity in one or both tests. Cross-reactivity is possible since the samples originated from an area where little is known about the presence of viruses within this group, but it is assumed that several closely related viruses circulate in the area. Generally, any serological test depends on the quality of antigens, antibodies, and the cut-off points. Test validation needs to take into consideration the purpose and status of the agents in the country/region (OIE, 2013). For non-endemic regions, the aim is to maximise the test sensitivity, while for endemic regions a focus on specificity is important. Most commercially available serological tests are validated in non-endemic regions,

such as Europe or North American, where sensitivity and specificity are adjusted to suit the purposes of testing in those regions, and might not be ideal in endemic regions. Financial and technical challenges in resource-poor countries often make it impossible to re-test suspicious herds where the cut-off can be optimised. Although commercial ELISA kits are standardised using OIE reference sera, they require validation, and cut-offs must be assessed for specific epidemiological contexts.

Reproductive disorders and questionnaire study

Most of the information on reproductive disorders and risk factors was collected using an interview-based questionnaire, which is susceptible to communication and language challenges. To minimize the risk of information bias/misinformation, both a qualified veterinarian with proper understanding of the Swahili language together with a person who knew the local language conducted the interviews; this method was resource-demanding but advantageous compared to online or paper-based data collection. However, the information farmers provide depends largely on the their knowledge, record keeping, and capacity to remember what happened up to three years before the interview. Since written records are rare and farmers have a poor understanding of reproductive disorders, the frequency of reproductive disorders in the area might have been underestimated. For a dairy herd, the only record important to most farmers is milk yield. Information on reproductive performance indicators in general (age at first service, age at first pregnancy, conception rate, and calving interval) was sought but regarded unsuitable for analysis due to poor quality/lack of reliable information from most herds. Data on reproductive disorders were the only available data that could be used. Furthermore, the only reproductive disorders reported were those observed in the advanced stage of gestation. Early embryonic loss, such as fertilisation/conception failure and early embryonic mortality, was not possible to assess, which might cause further underestimation of the occurrence of reproductive disorders. BVDV mainly results in early embryonic losses, which are usually not observed by farmers and therefore this study again might have underestimated the impact of BVDV on reproductive performance/disorders. *Brucella* spp. typically results in abortion in late gestation that is relatively easily recognised by farmers.

Seropositivity and reproductive disorders

In Papers 1 and 3, an association between seropositivity and reproductive disorders was detected. However, seropositivity often lasts many years after infection. The link in time between the seroconversion, which is the actual risk period, and the reproductive disorder,

might be weak, and this will contribute to an overestimation of the association. Collecting the history of reproductive disorders for the last three years reduces the challenge of determining a link, as it is more likely that the three-year timeframe includes the actual time of infection. Seropositive animals may be protected against new infections and therefore not at risk of developing reproductive disorders due to the same infections. This will contribute to an underestimation of the association. However, the situation is different for the three infections. For intracellular parasites such as *Brucella* spp. and *N. caninum,* antibodies do not protect against infection, as the protection mechanisms rely mainly on cellular immune responses and incomplete immunity is conferred (Mackaness, 1964; Moore et al., 2011). For BVDV, seropositive animals normally are protected against new infection for the rest of their life (Bolin, 1995). Due to these biological differences in the infections studied, general interpretation is misleading. It is likely that the association between BVDV seropositivity and reproductive disorders was underestimated because seropositive animals are protected. On the other hand, the association between *N. caninim* seropositivity and reproductive disorders is likely to be real. For *Brucella* spp., interpretation of the association is challenging since even though seropositive animals are not protected, infected animals usually abort once (Godfroid et al., 2004) with a small proportion aborting in subsequent pregnancies. Therefore, the association between *Brucella* spp. seropositivity and abortion is likely to be underestimated.

Each individual immune system is different and responds differently to infectious agents, which poses a challenge in interpreting serological results, especially with cross-sectional study design. A cohort based on paired serum samples would enable detection of seroconversion. Nevertheless, detection of seropositive individuals in a herd provides valuable information about the herd's status in a general health monitoring programme. Moreover, *Brucella* spp. seropositive animals pose a great risk to susceptible animals in the herd, as they harbor the bacteria, and many BVDV seropositive animals in a herd indicates the presence of PI animals.

Although difficult to achieve, a study based on antigen detection would provide a stronger connection with reproductive disorders. For SBV and other Simbu serogroup viruses, virus isolation from cattle would have been ideal for the definitive diagnosis and for further molecular characterisation. However, isolation of virus in cattle is difficult due to shorter viremia (about 2–6 days). In the absence of an outbreak of congenital defects or clinical disease in mature animals, the chance of detecting the virus is very low. Alternatively, virus detection

in a vector population would be valuable to show which viruses are present in the area. The drawback of this is that it does not necessarily explain what is present in the cattle population.

5.2 General discussion

The focus of this study was to investigate the occurrence and characteristics of infections causing reproductive disorders in cattle in Tanzania. The study area included a relatively small number of villages, which had already been selected to participate in EPINAV project. In general, the chosen villages are typical of this region and therefore probably representative. However, since the livestock production system in the study area is quite heterogeneous, as is typical for Tanzanian conditions, external validity is always a concern. The selected herds, with different sizes, animal breeds, and management strategies included, area true representation of herds in Tanzania and, to some degree, other sub-Saharan African countries with similar climatic and management conditions. The epidemiology of the evaluated infections depends largely on the local management, weather, and ecological context.

Associations between seropositivity and reproductive disorders

Retained placenta and abortion are probably the two most easily recognised and remembered reproductive disorders, which might have contributed to them being the most frequently reported disorders. *Brucella* spp., BVDV and SBV seropositivity were associated with abortion (Papers 1 and 3). A strong association between *Brucella* spp. and abortion, together with isolation of *Brucella* spp. from aborted materials (Paper 1), suggests that *Brucella* spp. causes abortion in cattle in this area, as has also been reported in countries like Zambia, Tunisia, Sudan, and Indonesia (Barkallah et al., 2014; McDermott and Arimi, 2002; Muflihanah et al., 2013; Muma et al., 2007a). In Ethiopia, *N. caninum*, and not *Brucella* spp. or BVDV, was strongly associated with abortion (Asmare, 2014; Asmare et al., 2013a). This indicates a varying epidemiological pattern for these infections in African countries. The SBV ELISA used in Paper 3 is most likely not specific to SVB. The positive association with reproductive disorders therefore merely indicates that SBV, or one or more related viruses, is associated with reproductive disorders in the area. This is further supported by an association between Akabane virus seropositivity in Simbu serogroup viruses VNT and reproductive disorders (Paper 3). It can generally be concluded that there are viruses in the Simbu serogroup that are associated with reproductive disorders in cattle in Tanzania. This finding is supported by the presence of vectors that transmit these viruses in Tanzania and by the fact that they are known to cause

reproductive disorders and malformation in the young stock in other countries (Hoffmann et al., 2012; St George and Kirkland, 2004).

Brucella spp. and risk factors

Both in Paper 2 and additional results, discrepancies were observed between results based on iELISA and RBT assays. The discrepancies could be explained by the differences in specificity and sensitivity between the two tests. It has been shown that iELISA is more sensitive than RBT while their specificities are highly correlated (Sanogo et al., 2013b). The sensitivity of RBT is also highly influenced by the quality of the antigen batch. Taken together, this suggests that animals classified falsely by RBT have been picked up by iELISA. The difference in seroprevalence between the two tests are so high that RBT, if used alone, would have given a much lower prevalence estimate because of false negative test results. A previous study in Tanzania has also observed that RBT is less sensitive when compared to ELISA (Shirima, 2005), and similar observations have been reported in India (Mythili et al., 2011). Our findings show that screening with RBT and confirmation of positives with ELISA, which is commonly done, would have biased the prevalence estimate, namely making it too low. Caution must be taken when interpreting serological results, especially when RBT is being used with ELISA in series. Re-testing after some time might be necessary, as suggested by OIE (OIE, 2009).

A higher seroprevalence in older cows compared to younger ones has also been reported previously, which may indicate endemic stability, where the infectious agents are likely to produce a latent infection (Al-Majali et al., 2009; Matope et al., 2011). Another possible explanation could be lowered immunity during pregnancy, which facilitates new infection or activates latent infection. Another study has shown a similar trend of higher seropositivity in adult than in young cattle in Tanzania (Assenga et al., 2015). To the contrary, a study by Shirima et al. found that age was not associated with *Brucella* seropositivity (Shirima and Kunda, 2016).

Brucella spp. seropositivity was strongly associated with location at both animal and herd level. The high within-herd seroprevalence and the high overall animal seroprevalence in Mbarali indicate widespread infection in one of the studied regions where no control strategy is in place. In Njombe, where the prevalence was almost zero, a brucellosis testing strategy is in place emphasising the importance of the implementation of a sound control strategy. Furthermore, in Njombe, farmers have long experience in keeping dairy cattle (Urio et al., 2006) and had several trainings on proper animal keeping through NORAD projects. Farmers are therefore

highly motivated to properly manage their animals. In Mbarali, on the other hand, farmers have less experience in keeping cattle and invest less time and focus on cattle compared to other economic activities. A large-scale systematic control and prevention strategy for brucellosis in the country has not been in place since the 1980s when vaccination was practiced mainly in parastatal farms (Keekstra, 2009) and has never been done in the traditional and smallholder dairy sub-sectors.

A recent meta-analysis concluded that among other factors, region as a geographical area was a predictor of variability of *Brucella* spp. seroprevalence (Alonso et al., 2016), although another meta-analysis conducted earlier in sub-Saharan Africa contradicts this finding (Mangen et al., 2002). Differences between regions and countries, in addition to different risk factors, are probably also due to differences in management systems and ecoclimatic conditions. Variation between areas is also based on culture, herd size, and breed composition, as well as microclimatic features (Racloz et al., 2013), which all determine survival and hence transmission of infectious agents. Our study also showed that management system and herd size were risk factors for seropositivity to *Brucella* spp. Small herd size (1–3 cattle) in Njombe and zero grazing strategy with minimal contact between animals/herds which reduces the potential for herd-to-herd transmission further explains the low prevalence. Others have also found herd size to be associated with seropositivity to *Brucella* spp., (Makita et al., 2011; Muma et al., 2007b; Racloz et al., 2013). Grazing animals on communal land increases the likelihood of infections being transmitted between herds, due to an increase in both direct and indirect contact (B Lopes et al., 2010). However, it has also been found that the grazing strategy used in nomadic herding imposes a natural limit on the rate of *Brucella* spp. infection in cattle (Ducrotoy et al., 2014; Racloz et al., 2013). Large herds and a lack of biosecurity measures, such as testing animals before bringing them into the herd and having a separate calving pen, could explain the higher prevalence in Mbarali. Furthermore, there are usually more logistical limitations in maintaining hygienic standards in large herds to limit survival of most infections. In Tanzania, most small-scale herds source their replacement stock from large-scale herds. The high prevalence detected in the larger herds therefore represents a severe risk of transmission of these infections to the rest of the population.

The finding of few seropositive small ruminants tested with the iELISA (Paper 2) in an area without any control programme indicates limited infection due to *Brucella* spp. This finding is similar to the observation made in domestic animal-wildlife interfaces of the Katavi-Rukwa

ecosystem (Assenga et al., 2015) and in the Mikumi-Selous ecosystem (Temba, 2012). In addition, other studies have found a seronegative small ruminant population in areas with high seroprevalence in cattle (Shirima and Kunda, 2016). High seroprevalence in cattle (Papers 1 and 2) and few positive sheep and goats (Paper 2) is a strong indication that the seropositivity in small ruminants is likely due to *B. abortus,* that spilled over from cattle to small ruminants and the possible absence of *B. melitensis* in the studied regions. Findings of (Assenga et al., 2015 249) and (Chota et al., 2016; Shirima and Kunda, 2016) also support this conclusion. Usually, the presence of *B. melitensis* results in high seroprevalence in sheep and goats while *B. abortus* infection in sheep and goats results in low seroprevalence (Racloz et al., 2013). Only a few small ruminants from one herd were included in this study, and investigation of a higher number of small ruminants would have provided stronger epidemiological information. *B. melitensis* can also infect cattle but less frequently causes abortion compared to *B. abortus* (Kahler, 2000). Nevertheless, high numbers of *B. melitensis* abortions have been documented in cattle in regions free of *B. abortus* (Verger et al., 1989). A trend of high seroprevalence in cattle and low seroprevalence in small ruminants has also been reported in Ethiopia, Chad, Nigeria, and Sudan (Cadmus et al., 2006; Megersa et al., 2011; Mokhtar et al., 2007; Schelling et al., 2003). However, *B. melitensis* has been isolated from cattle in neighboring Kenya suggesting the presence of *B. melitensis* in the small ruminant population. In West Africa, only *B. abortus* has been isolated in cattle and small ruminants, suggesting that *B. melitensis* may be virtually absent in the region. Lastly, *B. aborus*, *B. melitensis,* and *B. suis* have been isolated from cattle in Egypt (Wareth et al., 2014). Altogether, these studies highlight that the situation in Africa is very diverse and therefore sound epidemiological studied have to be conducted to correctly assess the brucellosis situation on the continent.

Isolation and characterisation of Brucella abortus biovar 3

Three isolates of *Brucella abortus* biovar 3 were obtained, all from one of the aborted foetuses. The isolated strains did not require CO_2 for growth, which is the exception, not the rule, for *B. abortus*. Growth in the absence of CO_2 has already been reported in strains from other countries, including the reference strain Tulya isolated from a human patient in Uganda. Worldwide, the prevalence of *B. abortus* biovar 1 is dominant, followed by *B. abortus* biovar 3, which is reported to be dominant in Africa (Corbel, 1989; Sanogo et al., 2013a). *B. abortus* biovar 3 has been reported in Kenya, Gambia, and Togo (Bankole et al., 2010; Dean et al., 2014; Muendo et al., 2012). Other biovars have also been reported in Africa. In other regions of Tanzania, for instance, *B. abortus* biovar 1 has been reported (Assenga et al., 2015), which

demonstrates the diversity of circulating strains in the country. In Zimbabwe, *B. abortus* biovars 1 and 2 have been reported (Matope et al., 2009). In Uganda, *B. abortus* without a biovar designation (but possibly the former biovar 7) has been reported (Mugizi et al., 2015b), emphasising the diversity of the circulating *Brucella* strains in Africa.

The two closely related genotypes of *B. abortus* that were identified by MLVA-16 from the same animal differed on one marker in panel two (Paper 2). Neither genotype has been described before. Dual infection could be the possible but it is more likely that a mutation explains the presence of two very similar genotypes in the same animal. The genotypes clustered separately but with more similarity to European and Asian strains than to African strains. Different MLVA profiles for *B. abortus* have been documented in Africa (Bertu et al., 2015; Dean et al., 2014; Dean et al., 2013; Menshawy et al., 2014; Muendo et al., 2012). This shows the diversity of the *Brucella* spp. and strains in Africa. However, the similarity with Asian and European strains was only about 40% with differences in 11 markers. Nevertheless, this genetic similarity might explain the origin of the Tanzanian strains. It is likely that importation of infected cattle from Europe, China, and Turkey brought the strains into the country but the time point is unknown. This explanation is further supported by the history of importing exotic cattle breeds to Tanzania from Europe that dates back to colonial times, when colonial farmers introduced them. In males, *Brucella* are secreted in semen and artificial insemination using infected semen can be a potential source of infection to naïve cows (Díaz, 2013). DNA based methods for detection of *Brucella* in semen are available (Junqueira Junior et al., 2013). Importation of semen from Holland is also practiced in Tanzania to date. This could further explain the possible origin of *Brucella* spp. in the country. However, the Netherlands has had bovine brucellosis-free status since 1999 (Emmerzaal et al., 2002) and there is an ongoing surveillance programme, which makesexport of infected semen less likely.

From the time of their introduction in Africa, strains have evolved separately from European strains. Further investigation of our isolates would have been interesting. Indeed, a PCR method called AMOS-ery (*Abortus*, *Melitensis*, *Ovis*, *Suis* (with ery primers)) PCR as described by Ocampo-Sosa et al. (Ocampo-Sosa et al., 2005) would enable us to distinguish between *B. abortus* biovar 3a (strains of African origin) and *B. abortus* biovar 3b (strains of European origin). Whole genome sequencing would also better and properly describe the strains. Due to limited financial resources and unavailability of established laboratory methods, this was not possible but will be considered in the future.

Bruce-ladder, classical biotyping, and MLVA-16 identified *B. abortus* biovar 3 but the same could not be identified by *B. abortus* specific simplex real-time PCR (Bru Ab2_0168). The findings for *B. abortus* specific real-time PCR could be due to deletion of specific segment(s) in *Brucella* DNA, including areas where primers attach. Most likely, the deletion part of the DNA is BruAb2_0168, as has been described on *Brucella* strains isolated from Togo (Dean et al., 2014). This suggests that this target may not be a suitable for *B. abortus* specific PCRs and that *B. abortus* specific identification in the region requires multiple targets in more conserved regions (Dean et al., 2014).

Another possible explanation for negative results in the *B. abortus*-specific PCR is insertion or rearrangement. Insertion has already been reported to occur in *B. abortus* (Mancilla et al., 2011). Mutations in the DNA can cause modifications by adjustment of a single base unit, deletion, insertion or rearrangement, as has been recorded for other microbes like *Mycoplasma mycoides* and *Chlamydia trachomatis* (Cheng et al., 1995; Ripa and Nilsson, 2006). Full genome sequencing of the isolates would confirm if one or more of these changes has occurred to the isolated *Brucella*.

This indicates that some *Brucella* strains from Africa may not contain the targets for PCRs designed outside the region, emphasising again the need for validation of diagnostic tests for the specific region of interest. The lack of validated tests presents a challenge in the diagnosis and control of brucellosis from these endemic regions. The gold standard for diagnosis of brucellosis is isolation of the bacteria; however, this process requires a lot of time and resources and is likely to pose risks to laboratory personnel. For that reason, PCR and other DNA-based molecular techniques are becoming popular methods for identification of *Brucella* at species and biovar levels (Yu and Nielsen, 2010). It is therefore pragmatic to consider mutations when using PCR as diagnostic and typing tool for *Brucella* spp.

Isolation of *B. abortus* from an aborted foetus associated with seropositivity in the cow is a strong indication that *B. abortus* may be an important cause of abortion in cattle in this region. The lack of biosecurity measures increases the risk to susceptible animals. In this area, it is common to introduce new animals into a herd without testing; separate calving pens are usually unavailable, and calving often takes place on pasture which consequently contaminates the environment. Proper biosecurity measures should be implemented to prevent transmission to cattle and other livestock species. Since consumption of unpasteurised milk is still common in

some communities in Tanzania, raising awareness and knowledge among people is necessary to reduce human health risks.

Bovine viral diarrhoea virus and risk factors

The seroprevalence study (Paper 1) showed that BVDV was the most prevalent infection compared to *Brucella* spp. and *N. caninum*. Like *Brucella* spp., it was also associated with abortion. In a large seroprevalence survey done in Tanzania in 1988 involving 18 regions, 12% seroprevalence was observed (Msolla et al., 1988). In the northern parts of the country, 34% seroprevalence was observed in 1991 and BVDV was isolated (Hyera et al., 1991), which indicates the presence of PI animals and virus circulation in the region, as is expected when the seroprevalence is this high. Since animals acutely infected with BVDV will be seropositive and have protective immunity the rest of their life, seropositivity only tells that the animal or herd has been infected at some point in time, but the virus might not still be present unless there are PI animals present.

With the high seroprevalence detected in the area, the population most likely contains PI animals. PI animals are generally seronegative, but can have a low level of antibodiesresulting from colostral transfer of maternal immunity or from infection with heterologous strains (Fulton et al., 2003). When none of the investigated seronegative animals were positive for virus, it is likely because PI animals often are eliminated from the herd at a young age because they underperform or develop health problems and may therefore not have reached the age of six months required to fulfil the inclusion criteria in our study. During sample collection, clinically abnormalities typical of PI animals were not observed, but the growth curve is generally low and it might be more difficult to pick out suspected animals such as typical 'poor doers', unthrifty calves, and so forth, under these conditions. PI animals might also appear clinically normal. Detecting PI animals would be interesting since they shed high levels of the virus all the time in their blood, nasal secretions, saliva, tears, semen, milk, urine, and faeces (Houe, 1995). The virus could be identified and characterised, which would indicate the type of BVDV strains present, a potential epidemiological information necessary for implementing a control strategy.

The observed association with abortion indicates the potential impact of this infection. BVDV is one of the most important viruses that is globally known to be associated with abortion in cattle (Ali et al., 2012). The most typical clinical problems due to BVDV are repeated breeding due to early embryonic death and general calf health problems. The present study was not

particularly well designed to monitor either of these conditions, and the impact of BVDV was therefore likely underestimated. BVDV therefore probably has a higher negative impact in this population than was observed in this study and should receive more attention.

The finding that BVDV and *Brucella* spp. seropositivity were associated could be due to the fact that they share the same risk factors, such as location, breed, herd size, and grazing strategy. In addition, BVDV is known to be immunosuppressive, which increases chances of infection with other pathogens (Potgieter, 1995). In addition, BVDV can also induce pathological changes in the placenta and therefore facilitate transplacental infections with other microorganisms (Murray, 1990). The finding that seroprevalence for both infections increased with parity (Paper 1), although not statistically significant, is not surprising. The seroprevalence usually increases with age in endemic areas, especially for infections with lifelong seropositivity. For BVDV, markedly higher seroprevalence in older than in younger cattle may suggest old infection and absence of PI animals in the herds (Solis-Calderon et al., 2005), as typically seen at the end of eradication programmes. On the other hand, in areas where BVDV is newly introduced, there might be no age differences. The observed difference of seroprevalence among age groups strongly suggests limited circulation of BVDV in the area, and seropositivity observed could be from old infections.

Seroprevalence for BVDV was surprisingly different in the two locations. In Njombe, the seroprevalence of BVDV was very low, which might indicate no circulation of BVDV (Paper 1). The low within-herd prevalence in the positive herds in Njombe (additional results) is in line with no or low virus circulation in the area, which is supported by the absence of PI animals (Paper 3). It can be speculated that the positive individuals have old infections or were brought in from other regions. In Mbarali, on the other hand, the picture is different: within-herd seroprevalence for BVDV was relatively high, including from the big herd high (additional results). These results suggest virus circulation in this area. Weather conditions influence the ability of many infectious agents to survive in the environment (Aune et al., 2012). Mbarali is warmer and drier than Njombe, which disfavours survival of infectious agents outside the host. The fact that the prevalence of BVDV and *Brucella* spp. was higher in Mbarali than Njombe suggests that climate might not be the reason for the differences observed. Management factors are more likely to explain the findings. Others have found herd size to be a determinant of BVDV transmission and associated productivity losses (Damman et al., 2015). When stocking density is high, the degree of contact between animals increases and the number of susceptible animals infected also increases. Animals that are infected with or PI with BVDV will carry the

agents for life, which represents an increased risk of transmission to susceptible animals in the herd as long as they live. The small herd size (1–3 cattle) in Njombe disfavours survival of the agents in the herds. Zero grazing strategy, with minimal contact between animals/herds, also reduces the contact with other herds and therefore protects against introduction of agents. Grazing animals in communal pasture also increases the likelihood of infections' being transmitted between herds (Damman et al., 2015). Use of common pasture and herd-to-herd contact have already been identified as risk factors for BVDV infection in Norway (Valle et al., 1999).

Neospora caninum

Among the studied infections, the seroprevalence of *N. caninum* was the lowest. No risk factors, including the presence of dogs, were identified, and the impact on reproductive disorders was not significant (Paper 1). The lack of a significant difference in seroprevalence between the two locations, unlike for *Brucella* spp. and BVDV, suggests a different epidemiological pattern for this infection.

The 5% animal-level seroprevalence observed is far below other reports from African countries. In Ethiopia, the animal- and herd-level seroprevalence were13.3% and 39.6% respectively (Asmare et al., 2013b); in Sudan the animal- and herd-level seroprevalence were 10.7% and 44% respectively (Ibrahim et al., 2012); and in Senegal, the animal-level prevalence was found to be 17.9% (Kamga-Waladjo et al., 2010). In Nigeria, on the other hand, a lower prevalence of 2.8% was observed (Ayinmode and Akanbi, 2013).

The lack of association between *N. caninum* and reproductive disorders may suggest endemic stability in the area since in a naïve population, introduction of *N. caninum* usually causes an abortion storm (Dubey et al., 2007). In subsequent pregnancies, animals may abort but do so at a lower rate. *N. caninum* is known as an important cause of abortion in cattle worldwide especially in the United States and Europe (Dubey and Schares, 2011; Dubey et al., 2007). It can be concluded that *N. caninum* is not a significant cause of reproductive disorders in cattle in this area, and possibly other areas in the country too, contrary to other countries like Ethiopia where a higher prevalence and greater impact were observed (Asmare, 2014; Asmare et al., 2013a).

Domestic dogs and coyotes are crucial in the epidemiology of *N. caninum* as definitive hosts that shed infective oocysts in the environment (Dubey et al., 2007). Low seroprevalence in

cattle and lack of association between *N. caninum* seropositivity and the presence of dogs on the farm may suggest limited infection in the canine population.

Simbu serogroup viruses

Detection of seropositive animals represents the first indication of the presence of Simbu serogroup viruses in cattle in Tanzania. For Aino, Douglas, Peaton, and Tinaroo viruses there are no other publications indicating their presence in Africa. Viruses within the Simbu serogroup are generally regarded as endemic in Africa (George and Kirkland, 1994; Levin, 2015; P.J. Timoney, 2004) and our findings support that. The presence of suitable vectors and conducive weather for their survival and propagation make transmission of infection possible. Surprisingly, in the present study, there is little evidence of the presence of Akabane virus, despite the fact that antibodies to Akabane virus have been reported in wildlife (Al-Busaidy et al., 1987; Hamblin et al., 1990) and in cattle (Taylor et al., 2001) in Tanzania. Antibodies to Akabane virus have also been reported in domestic and wild animals in Kenya (Davies and Jessett, 1985) and Sudan (Elhassan et al., 2014) and in domestic animals in Nigeria (Oluwayelu et al., 2016) and South Africa (Theodoridis et al., 1979).

Despite the extended use of serological investigations to establish exposure to viruses in this group, the close genetic and antigenic relation between them (Kinney and Calisher, 1981; Lee, 1979; Saeed et al., 2001) makes it challenging to establish which virus(es) are present. Since animals had positive titers to more than one virus, cross-reactivity or exposure to more than one virus might be present. For animals with high antibody titers to a single virus, infection with homologous or very closely related viruses is suspected. Since several animals had high antibody titers to more than one virus (Paper 3), infection with one virus and cross-reactivity with others was suspected. Alternatively, these results may indicate dual or multiple infection. A screening study done in small ruminants in Tanzania indicated the presence of antibodies to SBV but with contradicting results between cELISA and iELISA (Levin, 2015). It is often assumed that the animal is infected with the virus that shows the highest titer. This might be wrong, for example in immune-compromised individuals or in latent infections.

The traditional way of confirming antibody ELISA results is to perform VNTs. The present study clearly shows that VNTs are also subject to unspecific results, cross-reactivity, and interlaboratory and interassay differences. They are also susceptible to the choice and availability of viruses to include, the cell lines etc. Interestingly, different VNT results were obtained from two laboratories that tested Simbu serogroup viruses using the same samples

and methodology (VNT) (Paper 3). This could be due to different strains of the viruses and cell type used in the two laboratories. Even for the same viral strains, when maintained in different laboratories after several repeated passages they may change as they are usually not antigenically stable (Edwards, 1990). The interpretation of the results by examination of the wells microscopically for cytopathic effects is subjective.

Other risk factors

The risk factors accounted for in the study are interlinked, and multicollinearity was suspected. However, testing before and after the models were built did not significantly alter the results and the models were also checked for reliability. Since the area under the ROC curve was 0.9824 and the curve extended reasonably well into the upper left-hand corner of the graph, indicating that the model has the best predictive ability.

Other risk factors that were not included in this study but were observed in the region and probably influence the seroprevalence include sharing of bulls, use of artificial insemination, and movement of animals between herds. For some infections that are sexually transmitted, bulls may be an important source of infection transmission. Bulls that are PI with BVDV can also transmit the virus via semen which may lead to the birth of PI calves if the semen is used in AI centres (Meyling et al., 1990; Meyling and Jensen, 1988). The virus can replicate within the seminal vesicles and prostate gland and is shed in semen (Meyling et al., 1990). In the study area, more than 98% of the farmers use natural breeding. For the studied infections, bulls play a minor role in transmission, but they can also facilitate indirect spread. It is possible that factors not considered for inclusion in the questionnaire can represent true risk factors that were not possible to detect in the present study. Further studies to evaluate their association with seroprevalence are recommended.

Public health implications

In addition to its economic importance (McDermott et al., 2013), the presence of *Brucella* spp. in cattle is of public health importance. Globally, about 500,000 new human cases of brucellosis are estimated to occur yearly (Pappas et al., 2006). Human brucellosis is a chronic and debilitating disease that can lead to permanent sequelae and requires long-term antibiotic treatment (Dean et al., 2012). Since there is no human vaccine and human-to-human transmission is rare, most human cases originate from animals. Therefore, controlling animal brucellosis is the only reliable option to reduce human incidence.

The socio-economic and human health impacts of brucellosis are higher in resource-poor countries (Halliday et al., 2015; Moreno, 2014) which cannot afford proper control strategies. A significant number of human and animal cases are found in sub-Saharan Africa (Racloz et al., 2013) where substantial number of cattle, sheep, and goats are found (FAO, 2013). In a study done in Tanzania and Kenya, an overall incidence of 22.7% in the human population was reported, and in Tanzania alone, the incidence was reported to be 28.2% (Chota et al., 2016). Another study done in Tanzania on children reported a 15.4% and 7% prevalence of *B. melitensis* and *B. abortus* respectively (Chipwaza et al., 2015). Although the relevance of ascribing the species of *Brucella* as the origin of the positive serological result is unclear, the study showed the potential human health concern. In addition, brucellosis and malaria have similar non-specific febrile symptoms and are both common in Tanzania, which further complicates diagnosis and might lead to under diagnosis of brucellosis (Halliday et al., 2015). The burden of brucellosis and its public health implications in developing countries, although not well documented, is undoubtedly high. Repeated mass vaccination in cattle has been suggested in sub-Saharan African conditions due to challenges associated with other control methods (Ducrotoy et al., 2017). An effective control strategy of brucellosis is indispensable and requires a "One Health" approach (Godfroid et al., 2013a; Godfroid et al., 2014; Halliday et al., 2015; Zinsstag et al., 2011). Cooperation between veterinarians, medical doctors, anthropologists, epidemiologists, economists and others will benefit the effort by helping us understand and tackle the root causes of the disease so that it can be controlled with eventual eradication. This is especially important in developing countries like Tanzania where cattle and small ruminants are kept together and majority of people in the rural areas live in close contact with their animals. Also, consumption of raw milk in some communities is culturally regarded as the best way to enjoy milk's benefits. Furthermore, *B. abortus* and *B. melitensis* are host-specific, but they can both infect cattle and small ruminants in mixed livestock keeping; at the domestic animal-wildlife interfaces, domestic animals, humans and wildlife can be infected (Godfroid et al., 2013b). It is certainly essential to document which *Brucella* species infects which host and which reservoir hosts are present in order to design the best control strategy.

The other studied infections are not zoonotic and therefore have mainly economic implications. There is some serological evidence of human exposure to *N. caninum,* which is not considered to be zoonotic (Donahoe et al., 2015). To date, there is no confirmed human infection with *N. caninum* but there is a high incidence of *N. caninum* antibodies in immune compromised patients, especially those with HIV, which suggests a potential role of *N. caninum* as an

opportunistic organism (Barratt et al., 2010). The studied Simbu serogroup viruses have never been associated with human illnesses, including the newly discovered Schmallenberg virus, which has proven less likely to have human health risks (Reusken et al., 2012). There is no evidence of zoonotic potential for BVDV, and in endemic regions, the impact of BVDV is lower due to herd immunity. However, cumulative losses over several years greatly surpass the losses occurring in non-endemic areas. Furthermore, the reduced productivity associated with BVDV in terms of impaired fertility, lost replacement stock, reduced food supply and lack of better nutrition, together with the loss of resources, which might cause economic and emotional stress for farmers, is of public health importance.

6.0 Main conclusions and recommendations

Infections by BVDV, *Brucella* spp. *N. caninum* and Simbu serogroup viruses were found to be prevalent in the study area. For BVDV and *Brucella* spp., the prevalence was highly variable in the two study locations. Njombe appeared to be almost free from *Brucella* spp. with low or no BVDV circulation. In Mbarali, the prevalence and impact of both infections were high. The difference is most likely due to different management systems' influencing the survival of the agents in the cattle populations. This shows that within areas traditionally regarded as endemic, infection free areas and high-prevalence areas might be located close to each other. Direct and/or indirect animal contact between the different areas represents a serious risk of introducing infections to infection-free sub-populations, and changes in management factors might easily contribute to altered conditions for spread and survival within the subgroups.

Brucella spp., BVDV and Simbu serogroup viruses were found to cause reproductive disorders in the area. *Brucella* showed the strongest association with abortion. The impact of *N. caninum* appeared to be low in the study area, with no association with reproductive disorders and no risk factors detected. This indicates that this infection is of less importance than in other countries.

The new *B. abortus* biovar 3 strains identified had unique MLVA profiles and were different from those isolated in neighbouring countries. Its origin is therefore unclear. This highlights that transmission patterns in the region are virtually unknown. The isolation of the pathogen and the high within-herd prevalence suggest a chronic infection in the herd.

This study is the first report of nine new viruses in cattle in Tanzania (Aino, Akabane, Douglas, Peaton, Sabo, SBV, Sathuperi, Shamonda and Tinaroo viruses), of which four are new to Africa. This shows that this area is a likely hotspot for the vector-borne Simbu serogroup viruses. SBV, or a closely related virus, was present in this area before the European SBV epidemic, which opens up for a possibly African origin of this new virus.

The presence of the infections highlights their negative impact on animal health and production and their public health implications. Appropriate control measures need to be instituted to reduce the burden of these infections on poor farmers and the population at large. Individual farmers should be aware of status, risk factors and biosecurity measures to protect themselves and their animals against infection. An interdisciplinary collaboration that aims at mitigating risks to human health arising from microorganisms present in non-human animal species ('One

Health') is necessary for the control of brucellosis in the area. Combined efforts from the animal and human health sectors and those working with ecosystem health would facilitate the control and possibly the eradication of zoonotic diseases such as brucellosis and other diseases with economic and public health implications at the wildlife-livestock-human interface.

7.0 Future perspectives

Reproductive performance and status with regard to infections in Tanzanian cattle is relatively little studied, and this study has revealed new knowledge in this field. It has also detected areas that should be followed up with further studies:

- To study the impact of infections on reproductive performance in general, studies with a design better suited to detect a wider range of negative consequences should be performed.
- The human-domestic animal-wildlife interfaces should be further studied to map *Brucella* spp. biotypes and genotypes in order to uncover potential reservoir and transmission patterns of brucellosis in Tanzania. Further molecular characterisation of the isolated and other *Brucella* strains circulating in Tanzania, including whole genome sequencing, would generate molecular epidemiological information. The prevalence of brucellosis in small ruminants should be more extensively studied since they can be spill-over hosts of *B. abortus* from cattle, or reservoir hosts for *B. melitensis*. Since this was only studied in one herd, further studies would further establish epidemiological role of small ruminants in the area.
- Screening for BVDV PI animals including relevant age group is recommended. This will enable identification and molecular characterisation of the BVDV strains present in the country. To further increase the impact, studies on reproductive performance in general and on calf health, should be performed.
- Efforts should be geared towards isolating, identifying and characterising Simbu serogroup viruses in Tanzania from both cattle and vector populations and should be based on molecular methods. This will be essential for final documentation of which viruses are present and to understanding the epidemiology and evolution of SBV and related viruses.
- Dogs should be screened for *N. caninum* antibodies to confirm if the low prevalence in cattle is associated with a low prevalence in dogs.

8.0 Errata

Page 1: Coletha Mathew, changed to Coletha Mtenga Mathew; Name of the faculty (Faculty of Veterinary Medicine) has been added

Page 6: Coletha Mathew, changed to Coletha Mtenga Mathew

Page 13: Tanzania, font size changed from 13 to 14

Page 15: Major livestock constrains, font size changed from 13 to 14

Page 19: *Brucella*, font size changed from 13 to 14

Page 28: Bovine viral diarrhea virus, font size changed from 13 to 14

Page 32: *Neospora caninum,* font size changed from 13 to 14

Page 34, Simbu serogroup virus, font size changed from 13 to 14

Page 36: Knowledge gap, font size changed from 13 to 14

Page 40: Area of the study and target population, font size changed from 13 to 14

Page 44: Study design and Laboratory analysis, font size changed from 13 to 14,

Page 46: Data management and statistical analysis, font size changed from 13 to 14

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10.0 Appendix I: Questionnaire used in the study

SECTION A: IDENTIFICATION

Farm/Herd Number ----------

Village----------Hamlet----------

Ward----------Division----------

Farm classification [] Urban, [] Peri-urban [] Rural

Name of Enumerator----------Date of interview----------

Name of Field Supervisor----------Date of editing----------

SECTION B: FARMER'S DATA

1.1 Name of the farmer (Number) ----------

1.2 Age of the farmer----------

1.3 Gender of the farmer 1. Male 2. Female

1.4 Marital status of household head
1. Single 2. Divorced 3. Widow 4. Widower 5. Monogamous married 6. Polygamous married (No of wives)

1.5 Role of interviewee **1. Household head 2. Other household member (state) 3. Attendant 4. Relative 5. Other (state)**

1.6 Gender of interviewee **1. Male 2. Female**

1.7 Family size----------

1.8 Owner's level of education
1. Standard seven 2. Form four 3. Form six 4. College 5. University 6. Others

1.9 Other occupation **1. Yes** **2. No**

1.10 Average income per month---------- (Tsh)

1.11 Experience (duration of keeping dairy cattle)
1. Less than one year 2. One year 3. More than one year

SECTION C: LIVESTOCK/CATTLE DATA

2.1 Number of cattle in the farm ----------

2.2 Is this all the dairy cattle you have? **1. Yes 2. No**

2.3 If no, please give details of cattle on other premises ----------

2.4 What is the total number of dairy cattle on your farm?

	Cows		**Heifers (>1 year)**		**Bulls (>1 year)**		**Calves**	
	Milking	*Dry*	*Pregnant*	*Empty*	*Breeding*	*Fatten*	*Male*	*Female*
Total								

2.5 How many local breed cattle do you own? ----------

2.6 What type of production system do you practise?
1. Intensive 2. Semi-intensive
3. Extensive 4. Backyard
5. Tethering 6. Others (specify) ----------
2.7 If the animals graze outside have you seen them in contact with other animals from other farms?
1. Yes **2. No**

2.8 Have you seen them in contact with wild animals? **1. Yes** **2. No**

2.9 If Yes mention where……

2.10 Which animal species……

2.11 Have you seen them in contact with pastoral cattle? **1. Yes** **2. No**

2.12 If Yes mention where……

2.13 Who takes care of the animals? (Source of labour)
1. Children 2. Own work 3. Hire someone

3.1 How are your dairy animals housed?
1. Cowshed with roof 2. Cowshed without roof
3. Temporary boma 4. Others (state)

3.2 Where do the milking cows sleep?
1. Same place as feeding area 2. Area separated from feeding place

3.3 Are the animals tethered while in the house? **1. Yes** **2.No**
If yes how often?

1. Throughout the day 2. During the day only 3.At night only
3.4 On what type of floor do the milking cows sleep?
1. Concrete 2. Eartherned 3. Other (state) ----------

Do you provide them with bedding? **1. Yes 2. No**
3.5 What type of bedding is used on floor?
1. Leftover forage 2. Dried grass 3. Wood shavings 4. Others (state) ----------

2.14 How often do you change the beddings?
1. Every week 2. Every month 3. Occasionally 4. Others (Specify)

2.15 Do you take your animals for dipping/spraying? **1. Yes** **2. No**

2.16 If yes mention where ----------

2.17 What type of breeding system do you practice?1. Natural 2. Artificial Insemination

2.18 If you are using natural breeding what is the source of the bull?
1. From own farm 2. Neighbourhood 3. Other farms

2.19 What is the source of drinking water for your animals?
1. Surface 2. Underground 3. Others (specify) ----------

2.20 What type of feed do you give to the animals? ---------

2.21 Where is the source of the feeds for your animals?
1. Own farm 2. From grazing land 3. Others (Specify) ----------

2.22 Do you provide your animal with supplements? **1. Yes** **2. No**

2.23 a) If yes, what types of supplements feeds?
1. Maize bran 2. Sunflower seed cake 3. Cotton seed cake
4. Minerals 5.Lime 6. Bone meals7.MPTs 8. Others

b) How do you feed? **1. Mix the above** **2. Feed the above separately**

c) If you mix, at what ratio? ----------

2.24 Do you access veterinary services? **1. Yes** **2. No**

2.25 Do you keep animal records? **1. Yes** **2. No**

2.26 Rate the production efficiency/performance of your animals
1. Very good 2. Good 3. Poor

2.27 Do you have other animal spp. in your farm? **1. Yes** **2. No**

2.28 How many of the following animals do you have in your farm today?

Animal	Adult (breeding age +)	Young stock (<breeding age)	Total	Purpose: 1.Cash 2.Food 3.Both 4. Others (state)
Pigs				
Goats				
Sheep				
Donkeys				
Dogs				
Cats				
Chicken				

Others e.g. rabbits, ducks, guinea pigs				

2.29 When did you get your first?

Dairy cattle ---------- *(Give month and year)*

Local cattle ---------- *(Give month and year)*

2.30 How did you get your first dairy cattle?

1. Bought using own money 2. Bank Loan 3. Bought at subsidized price 4. Gift from relative/friend 5. Others (state)

2.31 Do you employ labourers on your farm at any time of the year? **1. Yes** **2. No**

2.32. If yes, what type of labour do you employ? **1. Full-time/Permanent (>6 months) 2.Seasonal/Temporary (1–6 months) 3.Casual (on daily basis) 4. Other (state)**

2.33 How many labourers are engaged in dairy farm activities currently? ----------

2.34 What kind of labourers do you employ? **1. Boys 2. Girls 3. Men 4. Women 5. Other (state)**

2.35 Where do the labourers stay? **1. Employer's house 2. Own place 3. Other (state)**

2.36 How much are the labourers paid?

Per day (Tshs) ----------

Per month (Tshs) ----------

Per year (Tshs) ----------

Other (state) ----------

2.37 Who is responsible for following dairy farm activities?

Activity	**<u>Person</u>:** **1. Husband 2.Wife 3.Son 4. Daughter** **5. Attendant 6. Relative 7. Other (state)**
Tick control (acaricide application)	
Cleaning cowshed/Sanitation	
Cut and carry forage	
Feed dairy cattle	
Milking	
Selling milk	
Who makes decisions to buy animal feeds, drugs etc.?	
Who provides money to purchase animal feeds, drugs, etc.?	
Who receives money from milk and dairy product sales?	
Who receives money from animal sales?	

2.38 Did any of the household members attend any dairy husbandry training course in the last five years? **1. Yes** **2. No**

If yes, give details in the table below:

Organiser & Venue	**Financial contribution to the course:** *1.Free 2.Partial contribution 3.Full contribution*	**Participant:** *1. Husband 2. Wife 3.Son 4.Daughter 5.Relative (M/F) 6.Animal attendant 7. Other specify*	**Year**	**Course aspect:** *1.General animal husbandry 2.Dairy farming 3.Disease control specify 4.Other specify*

2.39 How often does the extension officer visit your farm?
1. Daily 2. Weekly 3. Bi-monthly 4. Monthly 5. Not at all 6. Other (state)
Reasons for visit

1. For extension advice 2. For treatment/clinical consultation 3. Both

2.41 What constraints do you face in your dairy farming?
List down and rank them in ascending order using Arabic numerals i.e. 1, 2, 3, etc.

Constraint	**Rank**

Hint**: Important constraints may include **1. Availability of animal feed/water 2. Lack of market for milk 3. Animal diseases 4. Lack of money to buy inputs 5. Lack of enough/good land 6. Lack of breeding bulls/AI service etc 7. Others (state)

2.42 Do you sell milk ***1.Yes*** ***2.No***

2.43 If yes, what is the;
a) Total milk yield in your herd at present (litres/day) ----------
b) Amount of milk sold (litres/day) ----------
c) Amount of milk left for home consumption or processing (litres/day) ----------
d) Amount of milk fed to calves ----------

2.44 To whom do you sell your milk?
1. Neighbours 2.Milk vendor 3.Primary co-operative milk collection centre4. Private milk collection centre 5.Restaurant/hotel 6.Processing factory 7. Milk kiosk 8.Others (state)

3.14 How would you assess the availability of water in your farm?
1. Readily available 2. Available 3.Not readily available

3.15 How would you assess the availability of water for udder washing?
1. Readily available 2. Available 3.Not readily available

3.16 When do you water your animals?

SN	Time	Quality
1	Morning	
2	Noon	
3	Evening	
4	Liberal (all the time)	

3.17 Is the water sufficient for both animal and human consumption?

	Wet season	Dry season
Yes		
No		

4.21 Which are the most important diseases affecting your dairy animals? ----------

4.22 Can you rank the above diseases in order of importance in terms of causing deaths, milk loss, costs of treatment (***rank them in ascending order using Arabic numbers i.e. 1, 2, 3 etc.)***

Disease	*Deaths*	*Milk loss*	*Treatment costs*	*Other (state)*

4.23 Are you aware of diseases which are communicable between animals and humans? **1. Yes** **2.No**

If yes, please mention them; ----------

5.0: <u>REPRODUCTIVE PERFPRMANCE</u>

5.20 What is the status of the calves born by this cow in your farm?
1. Normal 2. Weak 3. Others. Please specify----------

5.23 Do the calves born in your farm survive to maturity? **1. Yes** **2. No**

5.24 If No what causes them not to survive? ----------
5.25 At what age did they die?
1. Immediate after birth **2.** After few months **3.** After one year

5.26 When did it happened
1. This year **2.** Last year **3.** Two years ago **4.** Others

5.30 How do you dispose the after-birth?
1. Buried 2. In the pit 3. Burn 4. Others (Please specify)

5.31 Have the animals been vaccinated **1. Yes** **2. No**

5.32 If yes mention the diseases

1. Brucellosis 2. Leptospirosis 3. TB 4. BVDV 5. Others (Specify)

5.33 Did you experience any disease in your farm? **1. Yes** **2. No**

5.34 If yes mention the disease(s) ----------

5.35 How did you manage the disease (s)
1. Own attempt 2. Call a Veterinarian 3. Ask assistance from a neighbour

5.36 Are there cats at home or from the neighbourhoods? **1. Yes** **2. No**

5.37 Are there dogs at home or from the neighbourhoods? **1. Yes** **2. No**

5.38 Have you seen any rodents around your farm/ home? **1. Yes** **2. No**

5.39 Can you rate reproduction performance of this cow?
1. Very good 2. Good 3. Fair 4. Poor

5.40 If poor what do you think are the reasons for this performance? ----------

5.41 Do you have any views regarding reproduction of your animals? ----------

DETAILS OF ANIMALS REPRODUCTIVE HISTORY /EVENTS																			
Cow Name/ID	Age	Source	Parity	Number of calves	*Age at first calving*	Date of 1st calving	Date of 2nd calving	Date of 3rd calving	Calving interval	Milk prod/day	Lactation lenght	Pregnancy status+ stage	No of services before conceptiom	Calving to conception	History of abortion+NO+when+stage	Dystocia	Still birth	Calf malformation?type	Retained placenta

Any additional information: ----------

--

For cattle that have been tested for *Brucella* spp. and or TB

SN	Cow's name	Breed	Sex	BCS	*Brucella*/TB test

11.0 Appendix II: Paper 1-3

Paper 1

Symbiosis

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Reproductive Infections in Cattle in Tanzania – Lessons for Control Priorities

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Abstract

Reproductive disorders have negative impact on performance in cattle worldwide. Studies on infections causing reproductive disorders in Tanzania are few and fragmented, which complicates targeted disease prevention. To investigate the prevalence of selected infections and their associations with reproductive disorders and risk factors in cattle under different management systems, a cross-sectional study was conducted in two bordering regions in the southern highlands in Tanzania. Herd and individual animal level data were collected by direct observation and a semi-structured questionnaire interview of the farmer. Sera from 658 cattle from 202 herds were analyzed using a commercial ELISA kits for antibodies to Bovine Viral Diarrhea Virus (BVDV), *Brucella* spp. and *Neospora caninum*. The logistic regression model identified herd size (odds ratio (OR): 14.5), location (OR: 23.1) and management system (grazing strategy) (OR: 22.7) as risk factors for *Brucella* spp. The same risk factors were also identified for BVDV herd size (OR: 2.8), location (OR: 12.7) and management system (OR: 2.9). History of abortion was associated with seropositivity for *Brucella* spp. (OR: 4.6). No risk factors, including location and presence of dogs, nor any association with reproductive disorders were identified for *N. caninum.* In one region the herd level sero-prevalence was 66.7% for BVDV and 36.1% for *Brucella* spp., while in the other it was 6.5% for BVDV and 0.6% for *Brucella* spp. In total, BVDV specific antibodies were found in 15.2% of the animals in 17.9% of the herds, and *Brucella* spp. specific antibodies were detected in 5.4% of the animals in 7.4% of the herds. Anti- *N. caninum* antibodies were found in 4.5% of animals in 8.4% of the herds. In conclusion, prevalence and impact of BVDV and *Brucella* spp. differed significantly between geographically closely related areas, most probably due to differences in management system that affects the potential for survival of the agents in the population. This shows that all control measures must be based on accurate epidemiological knowledge of the occurrence of the infection. Low-prevalence areas are highly susceptible for introduction of infection, while in the high-prevalence areas control measures must be implemented to reduce the impact and the risk of transferring *Brucella* spp. from livestock to humans.

Keywords: Abortion; Antibody-ELISA; Bovine; *Brucella* spp; BVDV; *N caninum;* Pestivirus; Reproductive-Disorders; Serology

Introduction

Livestock keeping is a major agricultural activity in Tanzania. Although the cattle population is large, the production output is disproportionately low and management systems are diverse. Smallholder dairy production dominates the urban and peri-urban areas, while pastoralism dominates the rural areas. All types of management systems, from large industrialized dairy herds to traditional pastoralism, where big herds are pastured more freely, may be present in the same area. The herd size, management system and degree of contact between cattle herds, as well as contact with other livestock and wild animals, are highly variable.

Reproductive disorders contribute significantly to suboptimal performance and production in cattle. Studies on reproductive performance including estimation of the frequency of abortion and stillbirth have been reported in different parts of Tanzania but little is known of different risk factors associated with reproductive disorders [1-3]. Causes of reproductive disorders are broadly categorized as infectious and non-infectious. *Brucella* spp., Bovine Viral Diarrhea Virus (BVDV) and *Neospora caninum* are known to be among the most common infections associated with reproductive disorders in many parts of the world, but the information about which ones are implicated in reproductive disorders in cattle in Tanzania is scarce [4]. These infections may cause different reproductive disorders including early

embryonic death, abortion, stillbirth and fetal malformations [5-7]. In addition, *Brucella* is an important zoonotic agent, and its seroprevalence in cattle varies between regions in Tanzania [8-11]. In Tanzania, the prevalence of antibodies against BVDV has been found to be 12% and 17% in cattle and wildlife populations respectively [12,13]. Neosporosis caused by the protozoan parasite *N. caninum*, has emerged as one of the most frequently diagnosed causes of abortion in cattle in many parts of the world [14]. In Tanzania, only a few reports exist on *N. caninum* in cattle and canid populations [15,16].

All three infections are generally considered endemic in the cattle populations in Tanzania, as in the rest of Africa. Climatic factors and the diverse management systems of the cattle industry are likely to influence the epidemiology of these infections, but the impact of these infections on reproductive disorders has received little attention.

The aim of the present study was to investigate the occurrence of selected infections and their impact on reproductive disorders in cattle under different management systems in the southern highlands of Tanzania. Specifically, the study was carried out to establish the i) animal and herd level prevalence of serum antibodies to *Brucella* spp., BVDV and *N. caninum*, ii) the association between serostatus and reproductive disorders, and iii) management and other risk factors associated with serostatus and reproductive disorders.

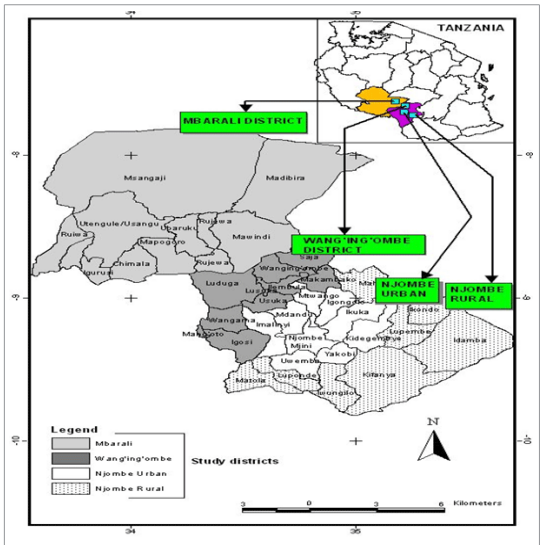
Materials and Methods

Study design

The study was a cross-sectional including selected dairy and pastoral herd in four districts in two regions. Epidemiological information regarding the selected animals and herds were collected by interviews and direct observation.

Study area

The study was part of a larger research and education program (EPINAV) taking place in the same area the study was conducted in Mbeya and Njombe regions in the southern highlands of Tanzania (Figure 1). Njombe is located in the altitude between 1600-1800m above sea level with annual rainfall of about 1000-1600mm and temperature ranges from 12-23oC. Mbarali is in altitude of about 1252m above sea level with average temperature between 25-30oC and mean annual rainfall of about 450-650 mm. In the Mbeya region, the Mbarali district was included, and in the Njombe region, the Wanging`ombe district, Njombe urban and Njombe rural districts were included. For practical reasons, the herds identified were in a limited number of villages; fifteen villages in the Njombe region and nine in the Mbeya region all of which participated in EPINAV program. Contact between villages, farmers and researchers were already established and well-functioning due to the EPINAV program. The herds selected were thus a mix of randomly selected herds in villages selected more by convenience.



	Region (Districts)	
Herd size	**Mbeya** (Mbarali)	**Njombe** (Rural, Urban,Wanging`ombe)
> 100 cattle	1	0
7–100 cattle	13	5
1–6 cattle	28	155

Figure 1: A map of Tanzania showing the study areas and the associated table indicating size of herds from the study regions.

Sampling strategy and sample size

The sample size was determined based on an 50% individual prevalence, 95% level of confidence and 5% absolute precision [17]. This provided a minimum sample size of 385 cattle. Due to the diversity of the production and management systems in the area, the total sample size was increased to 658. Inclusion criteria for herds were: presence of at least one female aged six months or above and that the farmer was willing to participate. A total of 201 herds were selected. Simple random sampling technique was used to select cattle in medium and large herds. In addition, serum from 200 cattle in a large herd with about 350 cattle and 28 breeding bulls in the primary selected herds were sampled as subgroups.

Blood sampling

About 5 ml of whole blood was aseptically collected from each animal. The blood samples were left at room temperature for a maximum of 12 hours for serum separation. Serum samples were then pipette into sterile tubes, transported on ice to a local laboratory and immediately frozen at approximately -20ºC. The material was shipped on ice, then kept frozen at -20ºC until analysis.

Collection of epidemiological information

The farmers were interviewed by enumerators with good knowledge of the local language using a structured questionnaire including questions on relevant biodata, past or present occurrence of reproductive disorders, management and possible risk factors for the past three years. The animal level biodata included age, sex, breed, source, parity and Body Condition Score (BCS) while the herd level data included location, herd size and management strategy. Reproductive disorders included abortions, stillbirth, and delivery of weak/malformed calves, dystocia and retention of fetal membranes.

Serological examination

All sera were analyzed at the Norwegian Veterinary Institute in Norway. Positive and negative control sera provided by the kits were included in all tests. The presences of antibodies to *Brucella* spp. were analyzed using indirect ELISA commercial kits following the manufacturer's instructions (SVANOVA® Brucella-Ab I-ELISA Svanova Biotech AB-Uppsala). The sensitivity and specificity provided by the manufacturer were 95.1% and 97.6%. Serum samples with ≥ 15 % positivity (PP) values were considered positive and PP value < 15 were considered negative. Anti-BVDV antibodies were analyzed using indirect ELISA commercial kit following the manufacturer's instructions (SVANOVA® BVDV -Ab I-ELISA Svanova Biotech AB-Uppsala), with a sensitivity of 99% and a specificity of 96% according to the manufacturer. Serum samples with PP values ≥ 10 were considered positive and PP value < 10 as negative. About 200 BVDV antibody negative samples were subjected to BVDV antigen test using commercial ELISA kit following the manufacturer's instructions (IDEXX BVDV Antigen Test kit/ serum plus Idexx Switzerland AG/ Switzerland). *N. caninum* specific antibodies were analyzed using an indirect ELISA commercial kit following manufacturer's instructions (SVANOVA® Neospora -Ab I-ELISA Svanova Biotech AB-Uppsala). The sensitivity and specificity provided by the manufacturer were 99% and 96%. Serum samples with PP values ≥ 20 were considered positive and PP value < 20 as negative.

Data analysis

Analysis of data was done using STATA version 12 for Windows (Stata Corp., Collage station, TX, USA) with herd as primary sampling unit. Most of the independent variables were categorical. Continuous variables were converted to categorical variables. Associations between dependent variables (infection status and reproductive history) and independent variables were estimated using univariable logistics regression adjusted for herd clustering effect at individual animal level. With consideration to biological plausibility of the factors in addition to their statistical relevance a final multivariable logistic regression model was formed using backward elimination procedure (inclusion criteria $P \leq 0.05$ of the likelihood ratio test). Tabular analysis using Goodman and Kruskal's gamma was used to determine association between the infections. Prevalence estimation for the males and the big herd subpopulations was done separate from other animals in the general study population. Some animals were not included in the analysis due to lack of reliable information.

Results

Herds, animals and management

Out of the 201 primary sampled herds, 183 had one to six cattle (small-scale herds) and 18 had seven to 100 (medium-scale herds). In addition one large-scale herd with about 350 cattle was included as a subpopulation group. From Njombe region, 155 herds were small-scale and five medium-scale. In Mbeya region (Mbarali district referred to Mbarali in Tables and Figures), 28 herds were small-scale 13 herds were medium-scale and one was large scale. In Njombe, all herds kept cross-bred dairy cattle, while in Mbeya both dairy and zebu cattle herds were present. In total, there were 392 female cross-bred dairy cattle (Holstein Friesian and Ayrshire crossed with Zebu) from 186 herds and 66 female zebu cattle from 15 herds sampled. Female cattle included in the final analysis were 65 heifers without calves (nulliparous), 94 with one calving (primiparous) and 229 with two or more calvings (multiparous) while 70 of them we did not get their information on parity. From one large-scale herd of cross-bred dairy cattle, 200 sera were collected and the 28 breeding bulls were from 12 herds; nine bulls from Njombe and 19 from Mbeya.

None of the sampled cattle were vaccinated against the studied infections. Most of the cattle in Mbeya region were kept on pasture during the day and indoors at night, while the majority of cattle in Njombe were confined in open barns with concrete walls or branches of trees with earthened, wooden or concrete floor. For zero grazed animals, roughage was obtained from communal grazing land with little supplementation from agricultural leftovers and industrial by-products. Grazing was on communal land except for a few herds that grazed on the farm.

Seroprevalence of BVDV, *Brucella* spp. and *N. caninum* and association between the infections

The overall, animal prevalence for BVDV, *Brucella* spp. and *N. caninum* antibodies were 15.2%, 5.4%, and 4.5% respectively. No serum was positive for BVDV antigens. Herd level prevalence (at least one positive animal) for BVDV, *Brucella* spp. and *N. caninum*, were at 17.9%, 7.4%, and 8.4%, respectively. In Mbeya region the herd level sero-prevalence was 66.7% for BVDV and 36.1% for *Brucella* spp. (animal level was 38.3% and 17.8% respectively) while in Njombe region it was 6.5% for BVDV and 0.6% for *Brucella* spp. (animal level was 5.7% and 0.3% respectively). The sero-prevalence for all the three infections in Njombe and Mbarali is shown in Figure 2. *Brucella* spp. and BVDV sero-positivity were associated with each other both at animal ($\gamma = 0.64$) and herd level ($\gamma = 0.9$). BVDV and *N. caninum* was not associated with each other at animal level ($\gamma = 0.01$) but a weak association was observed on herd level ($\gamma = 0.38$). *Brucella* spp. and *N. caninum* was weakly associated at animal level ($\gamma = 0.04$) but much more at herd level ($\gamma = 0.58$). The large-scale herd, from which 200 sera were collected, had a sero-prevalence of 73.1%, 47.8%, and 5.6%, for BVDV, *Brucella* spp. and *N. caninum* respectively. Out of the 28 breeding males, 32.1% were seropositive to BVDV, 14.3% to *Brucella* spp. and 10.7% to *N. caninum.*

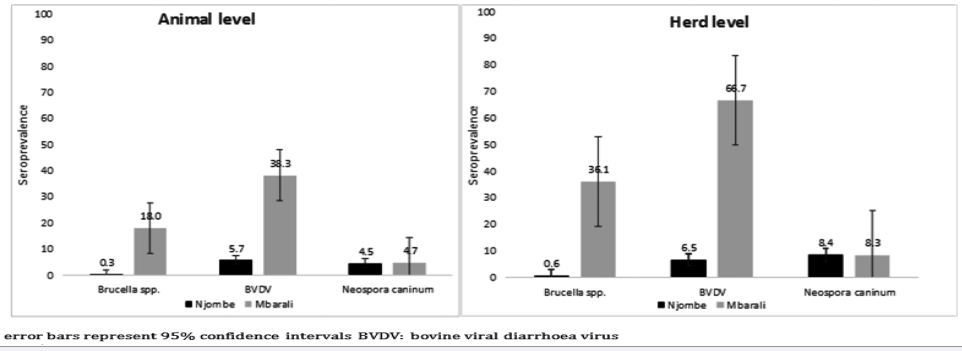


Figure 2: Prevalence of serum antibodies to *Brucella* spp., bovine viral diarrhea virus and *Neospora caninum* in cattle in the southern highlands in Tanzania

Prevalence of reproductive disorders

Reproductive disorders were observed in 98 animals with an overall prevalence of 33% (95% CI: 28-39). Table 1 indicates proportions for each disorder. Retained placenta and abortion were the most frequent encountered reproductive disorders. Dystocia was encountered in 29 animals, but due to missing information in many herds this parameter was not included in statistical analysis. Mbeya had higher proportions of abortion on animal and herd level than Njombe (Table 1).

Table 1: Prevalence of reproductive disorders in cattle in the southern highlands of Tanzania

Disorders (n)	P=Animal level prevalence (%)					P=Herd level prevalence (%)			
	P	95% CI	Location	p	95% CI	P	95% CI	p	95% CI
Abortion (38)	11.3	8-16	Njombe	7.0	4-11	11.6	7.7-17	7.8	4-13
			Mbarali	23.4	14-35			27.8	16-45
Retained placenta (51)	17.2	13-20	Njombe	18.2	12-25	22.6	17-29	23.4	17-31
			Mbarali	14.3	7-27			19.4	9-36
Stillbirth (5)	1.7	0.7-4	Njombe	1.4	0.4-4	2.6	1-6	1.9	0-6
			Mbarali	2.6	0.6-9			5.6	1-19
Malformations (4)	1.4	0.5-4	Njombe	1.4	0.4-4	1.6	0.5-5	1.9	0-5
			Mbarali	1.3	0.1-8			0	-

CI: Confidence Interval, n: number of cases

Association between sero-status and risk factors

At animal level, hypothesized risk factors for the three infectious agents were location, breed and parity. *Brucella* spp. sero-positivity was significantly associated with both location and breed while BVDV was associated with only breed. Altogether, zebu cattle were more likely to be seropositive for *Brucella* spp. and BVDV than crossbred dairy cattle while the prevalence of *N. caninum* was not affected by breed (Table 2). There was no association between *N. caninum* sero-positivity and presence of dogs on the farm (Table 2).

Table 2: Association between animal (n=292) level sero-status for *Brucella* spp., BVDV and *Neospora caninum* and hypothesized risk factors in a multivariable logistic regression model in cattle in the southern highlands of Tanzania

Risk factors	Level	Prevalence (95% CI)	OR	95% CI	*p*
		***Brucella* spp.**			
Breed	Dairy Cross	1.6 (0.5-5.0)	1.00	-	-
	Local	35 (22-51)	5.34	1.22-23.5	0.03
Location	Njombe	0.46 (0.07-3.1)	1.00	-	-
	Mbarali	22.1 (13.4-34.1)	21.5	1.9–248	0.01

Parity	Primiparous	2.3 (0.6-8.3)	1.00	-	-
	Multiparous	7.8 (4.0-15.0)	3.72	0.65–21.3	0.14
BVDV					
Breed	Dairy Cross	7.9 (4.6-13.6)	1.00	-	-
	Local	50 (37.6-62.3)	4.9	1.76–13.6	0.002
Location	Njombe	6.5 (3.1-13.4)	1.00	-	-
	Mbarali	33.8 (23.2-46.3)	2.89	0.92–9.1	0.09
Parity	Primiparous	10.5 (5.6-18.7)	1.00	-	-
	Multiparous	15.1 (10.4-21.5)	1.44	0.69-3.1	0.33
Neospora caninum					
Breed	Dairy Cross	5.2 (2.7-9.6)	1.00	-	-
	Local	5.0 (0.7-28)	0.37	0.04-3.53	0.38
Location	Njombe	4.2 (2.0-8.7)	1.00	-	-
	Mbarali	7.8 (2.9-19.4)	3.12	0.74–13.2	0.12
Parity	Primiparous	2.3 (0.6-9.0)	1.00	-	-
	Multiparous	6.3 (3-3-11.7)	3.18	0.77–13.2	0.11

CI: Confidence Interval, BVDV: Bovine Viral Diarrhoea Virus, OR: Odds Ratio, p: associated p values from multivariable logistic regression

At herd level, location of the herd, size of the herd and management system were hypothesized as potential risk factors for sero-positivity to the infections. *Brucella* spp. sero-positivity was significantly associated with all the risk factors. BVDV sero-positivity was significantly associated with location while *N. caninum* sero-positivity was not associated with any of the risk factors (Table 3).

Table 3: Association between herd (n=201) level sero-status for *Brucella* spp., BVDV and *Neospora caninum* and hypothesized risk factors in a multivariable logistic regression model in cattle in the southern highlands of Tanzania.

Risk factors	Level	Prevalence (%) (95%CI)	OR	95% CI	*p*
***Brucella* spp.**					
Location	Njombe	0.63 (0.09-4.3)	1.00	-	-
	Mbarali	36.5 (23.3-52.2)	23.1	1.96-292	0.013
Herd size	Small-scale (≤ 6)	2.7 (1.1-6.4)	1.00	-	-
	Medium-scale (6-100)	61.1 (37.7-80.3)	14.5	2.2–94.4	0.005
Management system	Indoor	1.1 (0.3-4.4)	1.00	-	-
	Outdoor	63.6 (42.1-80.8)	22.7	3.45-150	0.15
BVDV					
Location	Njombe	6.9 (3.8-12.0)	1.00	-	-
	Mbarali	63.4 (47.7-76.7)	12.7	4.7-34.8	< 0.001
Herd size	Small-scale (≤ 6)	13.7 (9.4-19.4)	1.00	-	-
	Medium-scale (6-100)	66.7 (42.7-84.3)	2.8	0.65–11.8	0.17
Management system	Indoor	11.7 (7.7-17.4)	1.00	-	-
	Outdoor	72.7 (50.9-87.3)	2.9	0.75-11.3	0.12
Neospora caninum					
Location	Njombe	9.4 (5.7-15.0)	1.00	-	-
	Mbarali	9.8 (3.7-23.4)	0.69	0.15-3.1	0.62
Herd size	Small-scale (≤ 6)	8.7 (5.4-13.8)	1.00	-	-
	Medium-scale (6-100)	16.7 (5.4-41.1)	2.1	0.36-12.2	0.40
Management system	Indoor	8.9 (5.5-14.1)	1.00	-	-
	Outdoor	13.6 (4.4-35.0)	1.4	0.19–9.9	0.74
Presence of dogs	Yes	9.7(5.2-17.1)	1.05	0.4-2.7	0.8
	No	9.3(4.9-16.9)	1.0	-	-

CI: Confidence Interval, BVDV: Bovine Viral Diarrhoea Virus, OR: Odds Ratio, p: associated p values from multivariable logistic regression

Association between reproductive disorders and risk factors

Factors associated with reproductive disorders were breed, parity of the animal, location, herd size and management system. Both at animal and herd level only abortion gave a model with explanatory power. At animal level, abortion was associated with herd size (OR: 4.4 CI 1.7-11.2). At herd level, abortion was mainly associated with size of the herd (OR: 5.7, CI 1.6-20.6). Other reproductive disorders did not show any significant association with any of the risk factors.

Association between reproductive disorders and sero-status

At the animal level, *Brucella* spp. sero-positivity were significantly associated with history of abortion (OR: 4.6, 95% CI 1.5-14.2), while other disorders were not associated with any of the infections. At herd level, abortion was also strongly associated with *Brucella* spp. (OR: 15.5, 95% CI 4.6-51.3) and BVDV (OR: 5, 95% CI 1.9-12.9) while *N. caninum* was not associated with any of the reproductive disorders. A combined *Brucella* spp. and BVDV sero-positivity was associated with abortion both on animal (OR: 11.7, 95% CI 2.7-50.3) and herd level (OR: 10.1, 95% CI 2.9-35.5).

Discussion

These results indicated that BVDV, *Brucella* spp. and *N. caninum* antibodies are present in the study area. BVDV sero-prevalence was the highest, followed by *Brucella* spp. and *N. caninum.* Furthermore, important differences in the seroprevalence of *Brucella* spp. and BVDV and the frequency of abortions were revealed between the two bordering regions in the study area. This indicates high diversity in the epidemiological pattern of these agents within a geographically closely related areas. These differences are most likely influenced by many factors. Since the heterogeneous livestock production system in the study area is typical for African conditions, a complex epidemiology is probably a general pattern.

The observed sero-prevalence for *Brucella* spp. calls for attention, as human brucellosis originates from animals [18]. Veterinary public health measures need to be in place as this is a zoonotic infection and consumption of unpasteurized dairy products is still a practice in some communities in Tanzania. *Brucella abortus* ,biovar 3 has earlier been isolated from an aborted cattle fetus from the large-scale herd included, illustrating the risk for transmission. *Brucella abortus* biovar 1 has been detected in the Katavi-Rukwa ecosystem in Tanzania [8,19]. Previous studies have reported the prevalence of brucellosis in cattle to range from 2.2-12.3 % in different regions and management systems in Tanzania [10,11,20,21]. Similarly the present study indicates a difference in sero-prevalence in two geographically very closely areas. Interestingly, Njombe, with a total of 160 herds investigated, had only one seropositive animal, which could be a false positive, and therefore, it is possible to regard the area as *Brucella* free. This is further supported by information from a local milk factory. They require that farmers test their animals for brucellosis before milk is accepted, and no positive animal has been detected for the past five years (personal communication). The prevalence of brucellosis in Mbarali could be explained by management strategies which allow for more direct or indirect contacts between infected and susceptible animals, as has been observed elsewhere [22]. High prevalence of abortion, strong association with *Brucella* spp. on both animal and herd level, and isolation of the agent in the same area suggest that *Brucella abortus* causes abortion in this area.

The prevalence of BVDV was found to be higher than that detected in 18 regions about 25 years ago, but more similar to that observed in the northern parts of the country [13,23]. With this relatively high sero-prevalence, the cattle population most likely also includes Persistently Infected (PI) animals, but such animals are frequently weak-born, unthrifty and underperform and are often eliminated from the herd early in life under these management conditions [24]. Since only animals over six months of age were included in the present study, this might explain why no PI animals were detected. It is not unlikely that BVDV could also has been introduced directly or indirectly from outside as most herds were open, but the general trend of very small herds and little contact probably limits the survival of BVDV in Njombe.

The higher prevalence of both BVDV and *Brucella* spp. in the two subgroups investigated is interesting, since both subgroups represent particular risk of inter-herd transmission. Breeding males represent a risk because they are commonly moved from herd to herd for natural breeding, and the large-scale herd as it represents typical procedures of replacement heifers for smaller herds.

The low sero-prevalence for *N. caninum* indicates a different epidemiological pattern from BVDV and *Brucella* spp. Contrary to our findings, in Ethiopia, a higher sero-prevalence for *N. caninum* than BVDV and *Brucella* spp. has been reported, and is regarded as more important for reproductive performance [25,26]. This highlights the difference in epidemiological patterns for these infections in African countries. Presence of infected dogs, which shed infective oocysts in the environment is crucial to dissemination into the cattle population. Investigation of the dog population in the area would have been valuable to explain if a low prevalence in the dogs may be the main reason for the observed low prevalence in cattle. The lack of association between presence of dogs and *N. caninum* sero-prevalence could be because exposure is more evenly distributed as stray dogs move easily between farms. This lack of association between presence of dogs at farm and *N. caninum* seropositivity has also been reported in Ireland [27].

The observed association between larger herds and *Brucella* spp. sero-positivity is in accordance with other observations [28,29]. Evidence suggests that when *Brucella* spp. is introduced into herds, a large proportion of animals will be infected and the infection will persist for a longer time [30-32]. Sharing of pasture and drinking water facilitate transmission of most infectious diseases, which is in line with the present findings of grazing as risk factor for *Brucella* and BVDV infections [33]. This might be caused by a higher degree of contact with

animals from other herds [34]. In addition the pasture may have been contaminated with infectious agents from animal secretion particularly with *Brucella* spp. since it is common for cattle to give birth outdoor which contaminate the surrounding environment.

The trend in this study that *Brucella* spp. and BVDV prevalence is linked to breed, has also been observed earlier for *Brucella* spp. [35]. However, all the zebu cattle herds in the present study were located in the Mbeya region with the higher seroprevalence of *Brucella* spp. and BVDV so the finding that Zebu cattle was more likely to be seropositiv for these infections should be carefully interpreted. Since breed, location, grazing strategy and management are often interlinked, confounding effects are possible.

The quality of the information gained from the interviews is a concern, as written recordings by farmers are uncommon. The data on reproductive disorders were the only reproductive performance information that was regarded suitable for analysis. Since *Brucella* spp. typically gives abortion where it is easily observed, the consequences on reproductive performance is most likely better estimated than for BVDV, which often leads to early embryonic death and repeated breeding/prolonged calving interval [36]. The impact of BVDV on reproduction is therefore probably underestimated in the present study.

The present findings indicate that co-infections with BVDV and *Brucella* spp. may have a greater influence on occurrence of reproductive disorders than mono-infection. Immunosuppressive properties of BVDV is known, and although the mechanisms of abortion caused by BVDV is unclear, it has been speculated that pathological changes induced in the placenta may allow other pathogens to cross the fetal membrane barriers [37,38]. The most likely explanation for the co-infections in this study is, however, that they share the same risk factors.

Serological investigations and cross sectional design has both advantages and disadvantages as methods to establish the prevalence of infection. In the absence of vaccination, sero-positivity can be regarded as an earlier infection. For all three infections, animals are generally sero-positive for several years after the infection [39-41]. The risk period for reproductive disorders caused by the agent is only when during pregnancy, and when the agent is actually present. Later, the animal will be fully or partly protected, which leads to underestimation of the association between infection and reproductive performance. Collection of reproductive history for the past three years, as in this study, reduces this challenge.

Conclusion

Antibodies to all the three studied infections were detected in cattle in the area, but the impact of the infections seems to be highly variable. For BVDV and *Brucella* spp., the prevalence was high but variable, with some areas almost free from *Brucella* spp. and very low BVDB. Location, herd size and grazing strategy influence the sero-prevalence. The high-prevalence area represent a risk to the low-prevalence area especially because purchase of replacement stock is common. In the high prevalent areas, the infections have a significantly impact both on cattle reproduction and possibly human health consequences.

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Ethical approval

The protocol for field studies and collection of animal material was approved by the University Ethics committee using guidelines from the Code of Conduct for Research Ethics of Sokoine University Agriculture SUA/VET/012/04. Farmer's verbal consent was sought before embarking on data and biological material collection.

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Paper 2

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RESEARCH ARTICLE Open Access

First isolation, identification, phenotypic and genotypic characterization of *Brucella abortus* biovar 3 from dairy cattle in Tanzania

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Abstract

Background: Brucellosis is a disease of worldwide public health and economic importance. Successful control is based on knowledge of epidemiology and strains present in an area. In developing countries, most investigations are based on serological assays. This study aimed at investigating a dairy herd experiencing abortions in order to establish within-herd seroprevalence to *Brucella* spp., identify, characterize *Brucella* strains by Multiple Loci Variable Number of Tandem Repeats Analysis (MLVA-VNTR) and investigate possible spillover to other species.

Results: The within-herd seroprevalence in cattle ($n = 200$) was 48 % (95 % CI 41–55), using an indirect ELISA, while the Rose Bengal Test (RBT) yielded lower prevalence (21.5 %; 95 % CI 16–27). Two sheep ($n = 35$) and one goat ($n = 50$) were seropositive using ELISA while none of the dogs ($n = 6$) was positive with the RBT. Three *Brucella* were isolated from an aborted fetus and associated membranes. Real time PCR (IS*711*), Bruce-ladder and classical biotyping classified the isolates as *B. abortus* biovar 3. MLVA-VNTR revealed two different but closely related genotypes. The isolates showed unique profiles, providing the first genotypic data from Tanzania. These genotypes were not related to *B. abortus* biovar 3 reference strain Tulya originally isolated from a human patient in Uganda in 1958, unlike the genotypes isolated and characterized recently in Kenya. High within-herd prevalence, isolation of the pathogen and abortion confirm that *B. abortus* is circulating in this herd with cattle as reservoir hosts. A low seroprevalence in sheep and goats suggests a spillover of *B. abortus* from cattle to small ruminants in the herd.

Conclusions: This is the first isolation and characterization of *B. abortus* biovar 3 from a dairy cow with abortion in Tanzania. The origin of the Tanzanian genotypes remain elusive, although they seem to be related to genotypes found in Europe, Turkey and China but not related to *B. abortus* biovar 3 reference strain or genotypes from Kenya. Importantly, replacement heifers are commonly sourced from large farms like this to smallholder farmers, which poses risk of spread of bacteria to other herds. *B. abortus* is a significant zoonotic risk and animal health problem in this production system, therefore further studies on humans is recommended.

Keywords: Abortion, Bovine, Biotyping, *Brucella*, Dog, Genotyping, MLVA, Prevalence, Small ruminants, Zoonosis

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Background

Brucellosis is a zoonotic disease of high economic and public health importance worldwide [1–3]. It is caused by *Brucella* spp. and manifests itself as abortion and infertility in domestic and wild animal species and reduced milk production in cattle. In cattle the disease is mainly caused by *B. abortus*. However, other species of *Brucella* can also be isolated [4–8]. Brucellosis in humans is almost always associated with infected domestic and wild animals or their products and poses more risk to farmers, animal handlers, abattoir workers and veterinarians [9]. It causes a debilitating disease with unspecific symptoms comparable to other febrile conditions such as malaria, which may be chronically disabling. Treatment of human brucellosis is long and costly.

Brucella are small (0.5 to 0.7 by 0.6 to 1.5 μm), gram negative, non-motile, non- encapsulated, non-spore forming, rod shaped (coccobacilli) bacteria which are facultative intracellular parasites. The genus shows little variation genetically. To date there are 11 recognized species of *Brucella* which are genetically very similar although each has different host preferences [6]. Six are regarded as classical *Brucella* spp. Four members have recently been classified as additional species [10, 11] and recently the eleventh *Brucella* spp. has been described [12]. Three species are of great zoonotic and economic importance; these are *B. abortus*, *B. melitensis*, and *B. suis* which preferentially infect cattle, small ruminants and swine respectively. Some *Brucella* spp. are further divided into several biovars. So far, *B. abortus* has been subdivided into biovars 1, 2, 3, 4, 5, 6, 7 and 9 [13]. Several biovars of *B. melitensis* (biovar 1, 2, 3) and *B. suis* (biovar 1, 2, 3, 4, 5) are also recognized [14]. *Brucella abortus* biovar 1 accounts for more than 80 % of the total number of isolates worldwide whereas in Africa *B. abortus* biovar 3 has been reported in most of the few published studies [2, 4].

Screening of brucellosis can be performed by serological methods detecting antibodies directed against epitopes associated with the smooth lipopolysaccharide (S-LPS) [5]. Confirmation of the infection is done by culture and isolation of the bacteria. However, this bacterium is difficult to grow and the procedure is time consuming. Furthermore, the procedure poses a risk to laboratory personnel and should be performed in biosafety level 3 laboratories. Nevertheless this method remains the "Gold standard" for diagnosis of brucellosis and *Brucella* infections. Biotyping of *Brucella* spp. provides additional information. Polymerase Chain Reaction (PCR) and other molecular techniques have been developed and have found diagnostic application [1]. Detection of *Brucella* spp. or its DNA provide the only certain diagnosis [5]. Genotyping of *Brucella* spp. can be achieved by Multiple Loci Variable Number of Tandem Repeats Analysis (MLVA-VNT) which shows a very good discriminatory power [14]. Such data can provide molecular epidemiological information for elucidating transmission pattern.

Brucellosis is widely spread in African countries [2, 3, 15, 16]. Serological studies done in different parts of Tanzania indicate that the infection is widely spread in domestic animals, wildlife and human beings [17, 18]. In Tanzania the problem is bigger in pastoral systems and wildlife than in the dairy farming system [18]. Data on isolation of *Brucella* spp. both in humans and animals, with further characterization is scarce. Isolation of *B. abortus* and *B. melitensis* from cattle and small ruminants in Tanzania was reported more than 50 years ago. However characterization of the isolates was not performed [19]. *Brucella melitensis* and *B. abortus* have been isolated and characterized from cattle in Uganda and Kenya. In Tanzania, similar studies need to be performed to trace back the reservoir host species [7, 20]. It is not known whether cattle in Tanzania are infected with *B. abortus* or *B. melitensis* or both. Successful control of brucellosis requires knowledge of its epidemiology in different animal species and the circulating strains in the region have to be assessed. This information is scarce in Tanzania.

Therefore the aims of the present study were to investigate a dairy herd experiencing abortion in order to:

- Establish within-herd prevalence of *Brucella* seropositive animals.
- Isolate, identify and characterize *Brucella* spp. from milk and abortion materials.
- Compare molecular characteristics of the obtained isolates with other strains in the region and outside.
- Investigate a possible spillover to small ruminants and dogs as they are a potential source of infection to cattle and can as well acquire infection from cattle.

Methods

The present study is part of an extensive project on infectious cause of reproductive disorders in dairy cattle. During sampling on the present study farm, abortions were encountered and became available for the current investigation.

Study farm

The farm is located in Mbarali district in Mbeya region in the southern highlands of Tanzania. At the time of sampling the farm had a total of 350 cattle which were crossbreeds of Friesian and Ayrshire with Boran and Zebu, 130 goats, 90 sheep and six dogs. The animals mingled with close interactions among them. All cattle and small ruminants grazed in controlled areas. The study herd had minimal contacts with pastoral herds

and other dairy herds around, most of which took place during the dry season. There was no history of vaccination against brucellosis on the study farm.

Animal material

Samples included serum, milk and one aborted fetus including fetal membranes collected in 2012–2013. Cattle were purposively selected to include only those above 6 months of age, while sheep and goats were randomly selected. Blood samples were collected from 200 cattle aged above 6 months, 50 goats, 35 sheep and six dogs. All female cattle above 6 months of age ($n = 187$) and all breeding bulls were included ($n = 13$). About 5 ml of whole blood was collected aseptically into plain vacutainer tubes. Blood samples were left at room temperature for about 12 h to allow serum separation. Serum was then pipetted into sterile tubes. Individual milk samples were collected from 63 cows, altogether from both Rose Bengal Test (RBT) positive and negative cows in sterile containers and properly sealed. Both serum and milk samples were transported to the laboratory on ice and stored at −20 °C until analysis. The aborted fetus and fetal membranes were examined on the farm. The fetus was examined externally for gross lesions and then aseptically dissected for examination of its internal organs. Examination revealed a relatively fresh fetus and its gestation stage was estimated to be 6 months.

Samples from all visceral organs (liver, lungs, kidneys, spleen, heart and brain) including foetal membranes were collected in a sterile plastic bag and were tight sealed and thereafter preserved at −20 °C for bacterial culture and isolation.

Ethical statement

The protocol for field studies and collection of animal materials was approved by Njombe and Mbarali districts veterinary and agricultural authorities. Farmers were informed of the study and their verbal consent was sought before commencement of data collection.

Serological examination

Rose Bengal test

All sera from cattle, goats, sheep and dogs were tested for presence of *Brucella* antibodies using RBT antigen following the manufacturer's instructions (Standardized *B. abortus* Rose Bengal Test Antigen Central Veterinary laboratory New Haw Addlestone, Surrey, UK), in accordance with the OIE manual [1]. *Brucella* positive control serum was always included in the test.

Indirect ELISA

Serum samples from cattle, sheep and goats were analyzed for the presence of *Brucella* spp. specific antibodies using indirect ELISA commercial kits following manufacturer's instructions (SVANOVA® *Brucella*-Ab I-ELISA Svanova Biotech AB-Uppsala). To monitor interassay variations, *Brucella* positive control serum was always included.

Milk ring test

Individual milk samples from RBT positive cows were tested on farm using Milk Ring Test (MRT) antigen (Atlas Medical William James House, Cambridge, UK) following the manufacturer's instructions and in accordance with the OIE manual [1]. Due to shortage of reagents in the field, only ten milk samples were tested.

Bacterial culture, isolation and identification

Bacteriological analysis was performed in a safety level-3 bio-containment facility at the Norwegian Veterinary Institute. Nineteen individual milk samples and aborted fetal organs as well as fetal membranes from one aborted fetus were subjected to bacterial culture. Primary isolation of *Brucella* spp. was done by inoculating the samples on a *Brucella* selective media (Selective Serum Dextrose Agar (SSDA)) (Oxoid) and Farrell's medium. Two plates per sample, (one per medium) were used. From milk samples, 100 μL of milk were inoculated per plate. All plates were incubated both aerobically, and in 5 % CO_2 atmosphere, at 37 °C and examined regularly after two, and up to 14 days, for *Brucella* like colonies. Such colonies were examined further with Gram staining. The plates were discarded if no growth was evident after 14 days of inoculation. Colonies typical of *Brucella* spp. were sub-cultured from which subsequent bacterial isolates were examined under phase contrast microscope and by Gram staining for organism morphology and size. Typical colonies revealing small Gram-negative coccobacilli, were further analyzed to obtain full identification and biotype.

Classical biotyping

Classical biotyping was done as described by [21] at The National Reference Center for Brucellosis, Veterinary and Agrochemical Research Centre (CODA-CERVA) in Belgium. *Brucella* monospecific antisera A and M and *Brucella* phages Tb, Wb and Iz obtained from FAO/WHO Collaborating Center for Brucellosis Reference and Research at the Veterinary Laboratory Agency, Weybridge, UK were used. A panel of biotyping tests were performed and interpretation of the results was performed according to the OIE manual [1].

DNA preparation and PCR

Suspected *Brucella* spp. isolates were subjected to genomic DNA extraction by heat treating a loopful of bacterial material dissolved in MQ water at 99 °C for 15 min [22]. After centrifugation, the supernatant was used as DNA template.

Molecular identification

The extracted DNA was subjected to real time PCR for the *Brucella* spp. specific targeting IS*711* [13]. Primers and probe were developed at the Swedish Institute for Communicable Disease Control (unpublished protocol). Positive results were obtained for the three extracted DNA (results not shown).

Bruce-ladder analysis

Species-level molecular identification was undertaken by multiplex PCR (Bruce-ladder) which was performed as described [23, 24] with the following conditions: Step 1: 95 °C 15 min, Step 2: 94 °C 30 s, Steps 3: 58 °C 90 s, Step 4: 72 °C 3 min, Step 5: 72 °C 10 min. Step 2, 3 and 4 was repeated in 25 cycles. The size of the PCR products was analyzed by capillary electrophoresis with Bioanalyzer®, Agilent Technologies, Santa Clara, CA, USA.

MLVA-VNTR genotyping

The isolates identified as *B. abortus* biovar 3 were analyzed using MLVA-VNTR 16 loci as described before [14]. Primers used were those described by Le Fleche et al. [14]. A PCR master mix was prepared using the following reactives: buffer (10×), bethain, dNTP 2.5 mM, Taq DNA polymerase rec (5U/ul Invitrogen), MgCl2 and H_2O. The following PCR program with iCycler BioRad was used: Step 1: 96 °C 5 min, Step 2: 96 °C 30 s, Step 3: 60 °C 30 s, Step 4: 70 °C 1 min, Step 5: 70 °C 5 min, Step 6: 8 °C. Step 2, 3 and 4 was repeated in 30 cycles. For the markers bruce 06, bruce 11, bruce 42, bruce 55 with repeat unit size 134 bp, 63 bp, 125 bp and 40 bp respectively, the PCR fragment size was analyzed by 2 % agarose gel electrophoresis. For the markers bruce 08, bruce 12, bruce 43, bruce 45, bruce 18, bruce 19, bruce 21, bruce 04, bruce 07, bruce 09, bruce16 and bruce 30, the size of the PCR products were analyzed by capillary electrophoresis with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Indianapolis, IN, USA). The size of the PCR products were then converted to a corresponding tandem repeat number for each locus as described by Le Fleche et al. [14] to get the genotype.

To classify the Tanzanian *Brucella* strains, a polyphasic strategy that included phenotypic (classical biotyping) as well as genomic criteria (presence of IS*711*, Bruce-ladder and MLVA) was used. Accordingly, MLVA analysis within *B. abortus* biovar 3 was performed. The profile of the Tanzanian strains were compared to *B. abortus* biovar 3 genotypes deposited in the *Brucella* aggregated database on MLVAnet (http://mlva.u-psud.fr/) hosted by the Université Paris-Sud. Four *B. abortus* biovar 3 genotypes from Belgian strains were also included in the analysis.

Cluster analysis of MLVA data was performed with the software BioNumerics 2.1 (Applied Maths, Sint-Martens-Latem, Belgium) following previous methods by Le Fleche et al. [14]. Cluster analysis was done with Euclidean distance which gives the quantitative difference. Only isolates of 100 % similarity with the same number of tandem repeats in each locus were assigned to the same cluster. The most similar strains clustered closely together with short and thick edges, while the strains with high genomic variations had thin and longer edges. The dendrogram was generated using a distance matrix calculated with the categorical coefficient and the unweighted-pair group method using average linkages as previously described [14]. An identical weight was given to each marker. The MLVA profile of the isolates was also subjected to a minimum spanning tree (MST) analysis in BioNumerics (MLVA plugin 2.1), illustrating the relationship and possible mutation pathways within the clusters based on single locus variations (SLV). Only the units (and not the sizes) from each marker were considered for the analysis. The nodes (circles) consist of identical genotypes and the edges (lines) of weight based on number of mutations (steps) taken from the loci were used. Long weight (steps) indicates multiple mutations while short weight indicates few mutations.

Results

Serological findings

Ninety six out of 200 serum samples from cattle were positive in ELISA giving a within-herd prevalence of 48 % (95 % CI 41–55), while 43 of the 200 serum samples were positive with RBT resulting into within-herd prevalence of 21.5 % (95 % CI 16–27). Thirty six sera were positive in both tests, 60 were positive in ELISA but negative in RBT, seven were negative in ELISA but positive in RBT. All 10 milk samples were positive in MRT. All sheep goat and dog sera were negative in RBT. However, two out of 35 sheep (prevalence: 5.7 %; 95 % CI 0–17) and one out of 50 goat (prevalence: 2 %; 95 % CI 0–7) sera were positive in the ELISA.

Bacterial culture, isolation, identification and biotyping characteristics

Three isolates of *Brucella* spp. were obtained, one from the aborted fetal liver and two from fetal membranes (all from the same animal). No *Brucella* spp. was isolated from milk samples. Real time PCR (IS*711*) confirmed the three isolates as *Brucella* spp. Bruce-ladder identified the isolates as *B. abortus* wild type as five fragments of 152, 450, 587, 774 and 1682 bp in sizes were amplified. The isolates showed common phenotypic characteristics typical for the genus *Brucella*. They grew anaerobically, in a 5 % CO_2 atmosphere, and aerobically after 3–14 days incubation at 37 °C. Bacterial colonies were small, convex, and regular with smooth surface, honey colored, shiny and translucent. The organisms were gram negative, small

(0.5–1 μm wide) single coccobacilli. The isolates were catalase and oxidase positive, also producing urease but not before 24 h of incubation. They were all H_2S positive, and were agglutination with the monospecific anti-A serum but not the monospecific anti-M serum. The isolates grew in the presence of Thionine, Fuchsin and Safranin dyes. They were lysed by Tb both in RTD and RTD10[4], Wb and Iz phages. The Tanzanian strains were characterized as *B. abortus* biovar 3, although they did not require CO_2 for growth.

MLVA genotyping

The MLVA 16 loci identified three closely related *B. abortus* biovar 3 Tanzanian genotypes (C64, C65 and C66). Out of the three isolates, C65 and C66 were identical while C64 was different at one locus (Table 1). The three isolates were identical using panel 1 loci but different at the locus Bruce 16 in panel 2. There was no amplification at locus Bruce 19 for strain C64. The genotypes were different from the reference strain's genotype and from genotypes from Kenya. Despite the Tanzanian genotypes being unique, they were more closely related to genotypes originating from Europe, Turkey and China than to genotypes from Uganda and Kenya (Figs. 1 and 2).

Table 1 Sixteen loci variable number of tandem repeat for the three Tanzanian *Brucella abortus* genotypes (C64, C65 and C66)

		B. abortus biovar 3			
	Locus	C64	C65	C66	Reference Tulya
Panel 1	Bruce 06	2	2	2	3
	Bruce 08	4	4	4	5
	Bruce 11	2	2	2	4
	Bruce 12	12	12	12	11
	Bruce 42	3	3	3	2
	Bruce 43	2	2	2	2
	Bruce 45	3	3	3	3
	Bruce 55	3	3	3	3
	Bruce 19	[a]	42–44	42–44	40
Panel 2	Bruce 04	7–8	7–8	7–8	6
	Bruce 07	2	2	2	5
	Bruce 09	6	6	6	3
	Bruce 16	7	8	8	11
	Bruce 18	5	5	5	8
	Bruce 21	8	8	8	8
	Bruce 30	4	4	4	5

B. abortus biovar 3 strain Tulya was used as reference strain
[a]indicates no amplification

Discussion

High within-herd seroprevalence in affected cattle herds has also been reported in Nigeria and Uganda [20, 25]. In Uganda within-herd prevalence of seropositive animals varied from 1 to 90 % [26]. Management systems such as common grazing increase the contact between cattle. Extensive movement and sharing of pasture enhances contact of cattle from different areas and facilitates transmission of most infectious diseases, including brucellosis. However, other studies have shown that management systems, where cattle are in constant movement puts a natural limit on the rate of *Brucella* infection accumulation or transmission and that the prevalence decreases [27, 28].

Positive MRT for individual animals in the herd suggests infection, but mastitis and colostrum might have caused false positivity [1] as 65 % of animals from this herd gave positive results in a California mastitis test (results not shown).

Only few of the small ruminants had antibodies against *Brucella* spp. The presence of seropositive small ruminants from mixed farming systems has been reported by others in Tanzania [19], Uganda [29] and Ethiopia [30], while in Togo, no seropositive small ruminants in mixed farming systems with seropositive cattle were found [31]. The presence of *B. abortus* in cattle, high within-herd seroprevalence, and the small number of seropositive small ruminants suggests a spillover of *B. abortus* from cattle to small ruminants, although the presence of *B. melitensis* or its DNA was not investigated in the later species [32]. In case of *B. melitensis* infection in small ruminants, given the *B. melitensis* basic reproductive number (R_0) of 1.2 (if greater than 1, the number of infected animals increases) as calculated in Mongolia [28], a higher seroprevalence should have been seen in small ruminants in our study. Such an epidemiological situation has recently been described in the Sudan were *B. abortus* biovar 6 had spilled over from cattle to sheep [32]. Naturally acquired *B. abortus* infection in dogs associated with infected cattle has been reported [33]. Although dogs may be valuable indicators (sentinels) of brucellosis in cattle, this study suggests that dogs did not play any significant role in the epidemiology of bovine brucellosis in this farm.

Both RBT and ELISA are OIE prescribed screening tests for brucellosis [1]. In the present study, both tests were used in parallel in cattle. Discrepancies between the two tests in the present study could be due to several reasons, including differences in sensitivity and specificity as indirect ELISA (iELISA) was reported to be more sensitive than RBT [5] and RBT more specific than the iELISA [34]. In some studies, iELISA has been shown to detect more cattle chronically infected with *Brucella* than the RBT [9]. It is worth noting that cut-off points

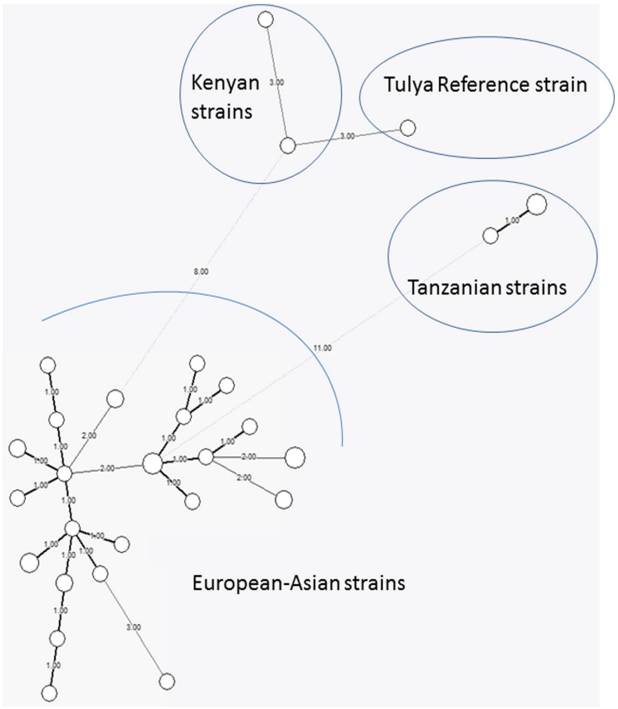

Fig. 1 Minimum Spanning Tree (MST) analysis of the MLVA-16 profiles of three *Brucella abortus* biovar three genotypes isolated from cattle in Tanzania, compared with *Brucella abortus* biovar three strains isolated worldwide. MLVA profiles were determined for a selection of 28 *B. abortus* biovar 3 genotypes including the Tulya reference strain (MLVA profiles derived from publicly available data (http://mlva.u-psud.fr/), four recent Belgian isolates) and the three Tanzanian isolates. Clustering by minimum spanning tree was performed with Bionumerics. Circles outline the genetic profiles of strains. Numbers on the connecting lines refer to the number of markers differing between samples. The size of the circles is proportional to the number of strains (1 or 2) bearing the same genetic profile

for iELISA have been defined for use in *Brucella*-free regions to optimize sensitivity [34]. The performance of such tests in endemic regions such as sub-Saharan Africa is unknown. Commercial ELISA kits need thus to be evaluated and cut-offs need to be established for specific epidemiological regions. The present iELISA results may therefore overestimate the actual within-herd seroprevalence. With this word of caution, the results suggest that there is a high within-herd prevalence and indicate that cattle in this farm were chronically infected.

This is the first report on the isolation, identification and characterization of *B. abortus* biovar 3 from cattle in Tanzania. It is important to isolate and characterize *Brucella*, as with serological methods it is not possible to infer which smooth *Brucella* spp. induced antibodies in the host [6, 9]. Some serological tests lack sensitivity and it is impossible to differentiate antibodies produced after vaccination from those produced after infection [5].

Biotyping profiles of the isolated strains indicated characteristics typical for *B. abortus* biovar 3, except CO_2 requirement for growth [21]. However *B. abortus* biovar 3 reference strain Tulya, which was isolated from a human patient in the neighboring country Uganda, is reported to be CO_2 independent [35]. Growth in the absence of CO_2 has been observed to occur within the same biovars [36, 37].

In the present study, two different genotypes were obtained from the same animal. To the best of the author's knowledge, both genotypes have never previously been described. The genetic polymorphism observed is incongruent with that observed in Uganda [38] and Kenya [7]. The genetic polymorphism shown at the panel two at one locus that is usually polymorphic might explain the difference between the two genotypes. Both genotypes are more related to European and Asian genotypes than to African genotypes. This suggests that the Tanzanian genotypes were introduced from Europe, possibly through importation of infected animals, although the time frame when this occurred remains elusive.

Brucella abortus biovar 3 has also been isolated from other African countries including Kenya, Gambia and

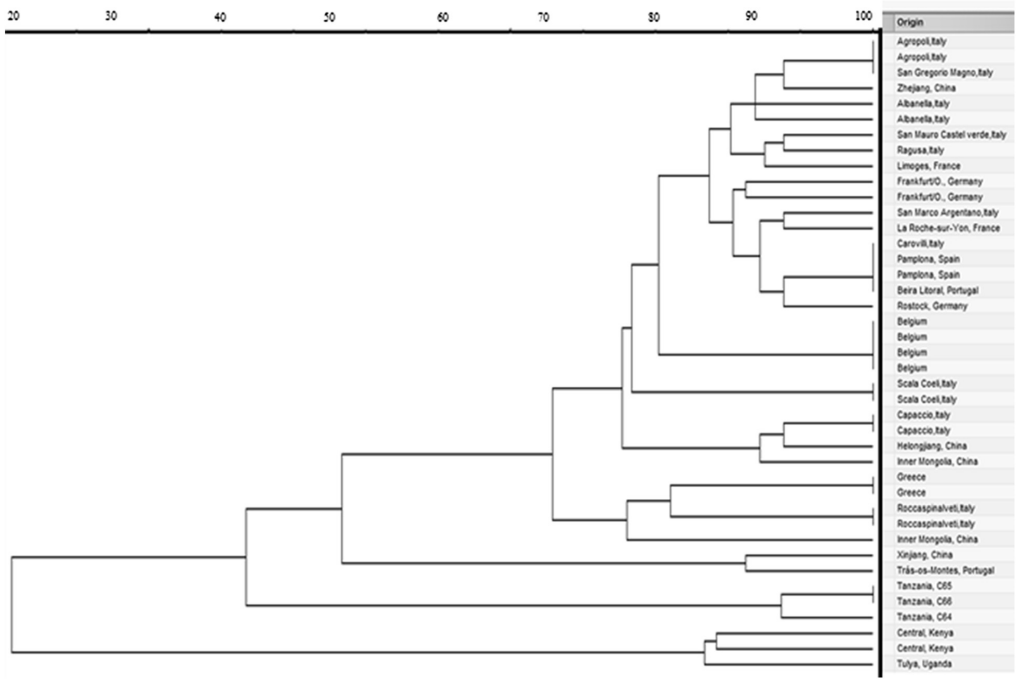

Fig. 2 Clustering analysis of 40 *B. abortus* field strains and Tulya *B. abortus* biovar 3 reference strain with the two panels of markers (MLVA-16). Scale (%) shows the MLVA genetic similarity. The geographic origin is given in the column. All strains were isolated from cattle, except one of the Belgian strain that was isolated from a dog in the farm where *B. abortus* biovar 3 was isolated from cattle and the Tulya reference strain originally isolated from a human patient in Uganda in 1958

recently Togo [4, 7, 36]. In Zimbabwe and Nigeria, *B. abortus* biovar 1 and 2 are common while *B. abortus* biovar 3 is rare [3, 37]. Specific biovars of *Brucella* are said to predominate certain geographical regions with *B. abortus* biovar 3 being commonly encountered in cattle in Africa [39]. In Egypt, infection of cattle by *B. melitensis* [40] and recently also *B. abortus*, and *B. suis* [15] have been reported. Some authors have proposed using AMOS-ery PCR to divide biovar 3 into two groups: one group 3a that will contain strain Tulya and field strains isolated from Africa while group 3b will contain strains from Europe [13]. This was not performed in the present study but the MLVA results suggest that the Tanzanian strains are not related to strain Tulya. Hence classifying *B. abortus* biovar 3 strains according to their geographical origin should be carefully considered.

The absence of culture positive milk samples could be due to the low number of samples tested, too few bacteria in the sample, or due to a low volume of milk inoculated. The excretion of organisms in milk is intermittent [21]. Freezing of milk might also have been a negative factor since the bacteria are easier to culture from fresh samples or samples stored at refrigeration temperature [41]. Consumption of raw milk is practiced in some communities in Tanzania. Under such conditions brucellosis is a public health issue.

This report further highlights the role of *Brucella* spp. as cause of reproductive problems on this farm, as the bacteria were cultured from the aborted fetus and associated membranes. In addition, large and medium scale dairy farms represents a risk for spread of the bacteria to other herds as they are sources of replacement heifers to small-scale dairy herds.

Conclusion

This is the first isolation, identification and characterization of *B. abortus* biovar 3 from a cow in a dairy herd in Tanzania. In the absence of any control program, the isolation of the pathogen and the high within-herd prevalence suggest chronic infection in this herd. Importantly, big herds like this serves as potential sources of replacement heifers to smallholder farmers, posing risk of infection transmission to other herds. Since *B. abortus* is a zoonotic agent, there is a risk of transmission to humans hence further studies on human brucellosis in the region are recommended. Information

on the prevalence and the circulating *Brucella* strains in different livestock species and possibly wildlife is important to understanding transmission patterns and risk factors. It is also a necessary first step in designing appropriate control policies and strategies. The results suggest that the Tanzanian strains are not related to other *B. abortus* biovar 3 strains isolated in the neighboring countries, Uganda and Kenya. This highlights that transmission patterns in the region are virtually unknown. In order to decipher such transmission patterns in the region, more strains should be isolated and characterized.

Abbreviations

NMBU: Norwegian University of Life Sciences; PCR: Polymerase chain reaction; AMOS-ery: Abortus, Melitensis, Ovis, Suis (with ery primers); MLVA-VNTR: Multiple locus variable number of tandem repeat; MST: Minimum spanning tree; SLV: Single locus variation; iELISA: indirect Enzyme Linked Immunosorbent Assay; RBT: Rose bengal test; MRT: Milk ring test; DNA: Deoxyribonucleic acid; RTD: Routine test diagnostic; MQ: Milli-Q; SSDA: Selective serum dextrose agar; IS*711*: Insertion sequence 711; Tb: Tbilisi; Wb: Weybridge; Iz: Izatnagar; Bp: Base pair.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CM performed the study, analyzed the data and drafted the manuscript. MS, GM, RHM, SK and JG conceived and designed the study, critically revised the paper and acted as the first author's study supervisors. PM and LE performed some of the analysis. TBJ, DF and JG analyzed the data and critically revised the paper. All authors read and approved the final manuscript.

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Detection of serum neutralizing antibodies to Simbu sero-group viruses in cattle in Tanzania

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Abstract

Background: Orthobunyaviruses belonging to the Simbu sero-group occur worldwide, including the newly recognized Schmallenberg virus (SBV) in Europe. These viruses cause congenital malformations and reproductive losses in ruminants. Information on the presence of these viruses in Africa is scarce and the origin of SBV is unknown. The aim of this study was to investigate the presence of antibodies against SBV and closely related viruses in cattle in Tanzania, and their possible association with reproductive disorders.

Results: In a cross-sectional study, serum from 659 cattle from 202 herds collected in 2012/2013 were analyzed using a commercial kit for SBV ELISA, and 61 % were positive. Univariable logistic regression revealed significant association between ELISA seropositivity and reproductive disorders (OR = 1.9). Sera from the same area collected in 2008/2009, before the SBV epidemic in Europe, were also tested and 71 (54.6 %) of 130 were positive. To interpret the ELISA results, SBV virus neutralization test (VNT) was performed on 110 sera collected in 2012/2013, of which 51 % were positive. Of 71 sera from 2008/2009, 21 % were positive. To investigate potential cross reactivity with related viruses, 45 sera from 2012/2013 that were positive in SBV ELISA were analyzed in VNTs for Aino, Akabane, Douglas, Peaton, Sabo, SBV, Sathuperi, Shamonda, Simbu and Tinaroo viruses. All 45 sera were positive for one or more of these viruses. Twenty-nine sera (64.4 %) were positive for SBV, and one had the highest titer for this virus.

Conclusions: This is the first indication that Aino, Akabane, Douglas, Peaton, Sabo, SBV, Sathuperi, Shamonda and Tinaroo viruses circulate and cause negative effect on reproductive performance in cattle in Tanzania. SBV or a closely related virus was present before the European epidemic. However, potential cross reactivity complicates the interpretation of serological studies in areas where several related viruses may circulate. Virus isolation and molecular characterization in cattle and/or vectors is recommended to further identify the viruses circulating in this region. However, isolation in cattle is difficult due to short viremic period of 2 to 6 days, and isolation in vectors does not necessarily reflect the situation in cattle.

Keywords: Antibody ELISA, *Orthobunyavirus*, Serology, Schmallenberg virus, Virus neutralizing test

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Introduction

Simbu sero-group viruses belong to the genus *Orthobunyavirus*, in the family *Bunyaviridae* and contain three RNA segments. These viruses are naturally capable of genetic reassortment, which can lead to development of new viral strains with altered biological properties. They are transmitted by arthropods, mainly biting midges from the genus Culicoides and mosquitoes [1]. Most cause sub-clinical infections in non-pregnant animals. In pregnant animals, some of these viruses readily cross the placenta causing fetal infections that are associated with abortion, premature birth, still birth and congenital abnormalities in calves, lamb, and kids. The abnormalities include arthrogryposis, porencephaly, hydrocephalus, cerebella hypoplasia and congenital hydranencephaly [2].

The Simbu sero-group includes Schmallenberg virus (SBV), a newly emerged livestock virus first identified in Germany in 2011 [3, 4]. Its genome has been found to be closely related to Douglas, Sathuperi and Shamonda viruses [5]. Full genome investigation has indicated that SBV belongs to the species *Sathuperi virus* and is a possible ancestor of the reassortant Shamonda virus [6]. The origin of SBV is unclear [5, 7].

A number of Simbu sero-group viruses have been found to be present in different parts of the world, including Africa, Asia, Australia and Israel [8–10]. They have been isolated from domestic and wild animals as well as from vectors. Akabane virus has been the most recognized virus in this group together with Shamonda and Aino virus [4]. Antibodies to Akabane virus have been found in cattle and sheep in Asia, the Middle East, Australia and Africa [11, 12]. Diseases associated with some Simbu sero-group viruses have been reported to occasionally cause significant economic losses in the Australian and Japanese livestock industries [12], while in Africa both viruses and their consequences are poorly reported. A disease with characteristic signs of SBV has been observed in cattle and sheep in South Africa and Zimbabwe [1].

In Africa, members of the Simbu sero-group have been isolated from *Culicoides* midges and domestic animals. These include Sabo, Sango, Sathuperi, Shamonda, Shuni, Simbu and Yaba viruses [13, 14]. Neutralizing antibodies to Akabane virus have been found in wild animals in different African countries south of the Sahara including Tanzania [15–17]. In Asia, Simbu sero-group viruses originally recognized in Africa have been recovered from cattle and *Culicoides* midges in 2004 [18, 19].

Diagnosis of infections caused by Simbu sero-group viruses has traditionally been accomplished by detection of specific antibodies using virus neutralization assays but more recently enzyme-linked immunosorbent assays (ELISA) have been used and some are available as commercially prepared kits. However, as these viruses were originally clustered on the basis of serological assays, extensive cross reactivity is often observed [20, 21]. Virus isolation can be achieved in cell cultures and direct virus detection and identification is possible using RT- PCR and other molecular detection methods. Subsequent sequencing of the genome gives more detailed information of the type of the virus infecting animals.

In Tanzania, little is known about the presence of Simbu sero-group viruses and their impact on reproduction in cattle. In addition, the origin and global geographic distribution of SBV is not known. Mixed farming and extensive management systems and the presence of wildlife protected areas may facilitate transmission of these viruses between wild and domestic animals. This environment is conducive for vector activity. The present study was therefore undertaken with the following aims:

1. To investigate the prevalence of SBV-antibodies in cattle in the southern highlands of Tanzania, using a commercially available indirect ELISA kits.
   - o Investigate the SBV antibody prevalence before and after the epidemic in Europe.
   - o Investigate the association between seropositivity and reproductive disorders.
2. To further analyze ELISA results using a series of VNTs for SBV and other Simbu sero-group viruses closely related to SBV.

Materials and methods

Study area

The present study was part of a larger project on infectious agents causing reproductive disorders in dairy cattle in Tanzania. It was conducted in four districts that included Mbarali district in Mbeya Region and Wanging`ombe, Njombe rural and Njombe urban districts in Njombe Region in the southern highlands of Tanzania between July 2012 and March 2013. The selected farms from Njombe and Mbeya regions practice two different grazing systems. Most of the cattle in Mbeya graze on pastures during the day and are kept indoors at night, while the majority of herds in Njombe are confined in cattle houses. However, cattle houses or barns in both locations are not sealed to vectors. Both dairy cattle, which are mixed breed (Holstein Friesian and Ayrshire with Tanzania short horn zebu), and pastoral local breed of cattle (zebu), were included.

Study design and sampling

A cross-sectional study design was chosen. A total of 202 herds were included in the present study. Of these, 181 dairy herds were small scale with an average size of three cows per herd, two were medium scale dairy herds with about 20 cows each, one was a large scale dairy herd with about 350 cows and 18 were pastoral herds with an average of 15 cows per herd. Inclusion criteria for herds were to have one female above 6 months of age and that the farmer

was willing to participate. All animals below 6 months of age were excluded. From herds with less than five cattle above 6 months, all were included in the study. From herds with more than five cattle, five were randomly selected. This made a total of 659 animals. In addition, serum samples from 130 cattle from the same area, collected in connection with another study during 2008–2009, were also included in the present study. Blood samples were collected once from each animal.

All herds were visited by qualified personnel and farmers were interviewed using a structured questionnaire. The questionnaire aimed to gather information about reproductive disorders in the herd and individual animals over the last 3 years. The questionnaire was designed to determine the presence or absence of abortions, delivery of weak/malformed calves, stillbirth, dystocia and retained fetal membranes. Abortion was defined as a cow giving birth to a calf at any stage of gestation before term. Stillbirth was defined as giving birth to a dead calf, dystocia was defined as any assisted birth and retained placenta when fetal membranes fail to expel 24 h after delivery. Farmers were informed on the study before commencement of data and sample collection and willingly agreed to participate.

Sample treatment and antibody analysis

Blood samples (about 5 ml) were collected aseptically from the jugular vein in plain evacuated tubes. The samples were left at room temperature for about 12 h to allow serum separation, and then serum was decanted into sterile tubes and transported on ice to the local laboratory and immediately frozen at approximately −20 °C. Between laboratories samples were shipped to the destination laboratories on ice and then kept frozen at −20 °C until analysis.

Indirect enzyme linked immunosorbent assay

All serum samples were analyzed at the Norwegian Veterinary Institute using a commercial ELISA kit (ID Screen® Schmallenberg Virus Indirect multi-species screening test ID.Vet Innovative Diagnostics) according to the manufacturer's instructions. For each sample the S/P percentage was calculated: S/P = (OD sample-OD negative control)/OD positive control-OD negative control ×100. Samples presenting S/P values less than or equal to 50 % were considered negative, S/P values between 50 and 60 % were considered doubtful and S/P values greater than 60 % were considered positive.

SBV Virus Neutralization Test (VNT)

The SBV VNT was performed on selected serum samples that tested positive in the SBV ELISA. This included 110 of 405 serum samples obtained in 2012/2013 (Fig. 1) and 71 of 130 sera collected in 2008/2009. Samples from 2012/2013 were selected to represent the whole study area. The test was performed at the Central Veterinary Institute, Lelystad, The Netherlands. In this test an SBV isolate from brain tissue of a lamb, fourth passage on Vero (African green monkey kidney) cells, was used. The VNT was performed on serum samples according to the method published in Loeffen et al. [22] with some small modifications: dilutions tested started at 1:4 and ended at 1:512. All samples were tested in duplicate. Titers were determined using the Reed-Muench method [23].

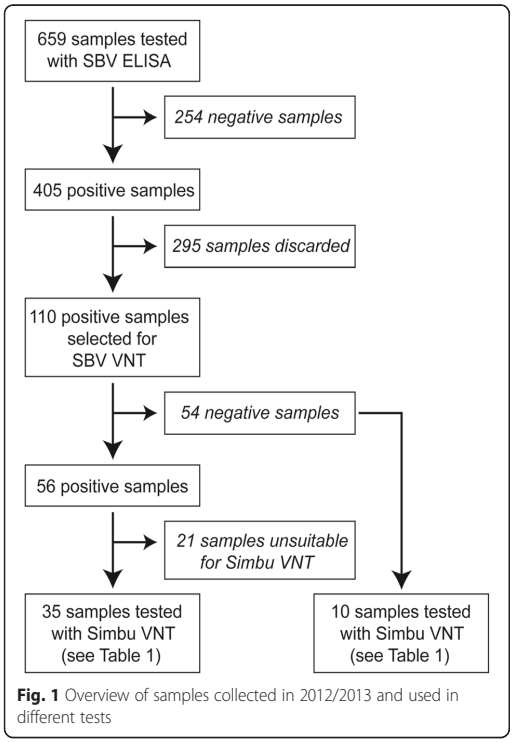

Fig. 1 Overview of samples collected in 2012/2013 and used in different tests

VNT for other simbu sero-group viruses

SBV VNT positive ($n = 35$) and negative ($n = 10$) serum samples from 2012/2013 (Fig. 1) and 10 SBV ELISA positive (which were also positive in SBV VNT) and 10 negative serum samples from 2008/2009 were subjected to a VNT for Simbu sero-group viruses including Aino, Akabane, Douglas, Peaton, Sabo, SBV, Sathuperi, Shamonda, Simbu and Tinaroo at the Elizabeth MacArthur Agriculture Institute, Virology Laboratory, Menangle NSW Australia. Australian prototype strains of Aino, Akabane, Douglas, Peaton and Tinaroo viruses and prototype of Sabo, Sathuperi, Simbu and Shamonda viruses [20, 21] were used for these VNTs as described by Kirkland et al. [24] with the inclusion of the relevant virus in the test. For SBV the prototype strain originally isolated from Germany [4] was used. Selection of samples from 2008/

2009 for this assay was based on availability of samples with enough volume.

Data analysis

Data were analyzed using STATA version 12 for Windows (Stata Corp., College station, TX, USA) software. Positively infected animals were reported in proportions. The association between presence or absence of reproductive disorders and herd or individual animal serostatus (using SBV ELISA, SBV VNT and Simbu sero-group virus VNT) were analyzed using univariable logistic regression and reported as OR at 95 % CI, and level of significance at $P < 0.05$.

Ethical statement

The protocol for field studies and collection of animal material was in accordance with ethical approval by the University Ethics Committee using guidelines from the Code of Conduct for Research Ethics of Sokoine University of Agriculture SUA/VET/012/04. Farmer's verbal consent was sought before embarking on data and biological material collection.

Results

SBV antibody ELISA

Out of the 659 serum samples collected in 2012/2013, a total of 405 (61 %) were positive in the SBV antibody ELISA and out of 202 herds, 175 (87 %) had one or more seropositive animals. In Njombe out of 160 herds, 133 (83 %) were positive and out of 324 individual animals, 211 (65 %) were positive. In Mbarali however, all herds ($n = 42$) were positive while out of 335 individual animals, 194 (58 %) were positive. Seventy one (55 %) out of 130 sera collected in 2008/2009 were positive in the SBV ELISA. Antibodies were observed in sera collected from small, medium and large scale herds, Njombe and Mbarali, dairy and pastoral herds. The results of univariable regression analysis indicated no significant differences between serostatus and location or herd size or animal breed.

SBV VNT

Fifty six (51 %) out of 110 serum samples collected in 2012/2013 were positive with titers ranging from 1:16 to 1:512 (Fig. 1). Fifteen (21 %) of 71 serum samples collected in 2008/2009 were positive in this assay with positive titers ranging from 1:16 to 1:768. Out of 71 samples from 2008/2009, 42 were toxic to the cell cultures which made it impossible to interpret the test results, while four were positive but with no end titers due to insufficient sample volume therefore their titers could not be determined.

Other simbu sero-group virus VNTs

Antibodies against nine out of the ten Simbu sero-group viruses (Aino, Akabane, Douglas, Peaton, Sabo, SBV, Sathuperi, Shamonda and Tinaroo) were detected in one or more of the 45 serum samples from 2012/2013. Their titers ranged from 80 to 1280 (Table 1). Most animals had antibodies directed against two or more viruses. No antibody titers were detected against the species Simbu virus.

Twenty nine (64.4 %) sera were positive for SBV, out of which seven had high titers (≥160) to this virus. One serum sample had highest titers for SBV, while two had the same titer to SBV and Douglas or Sathuperi virus. A high proportion (91.1 %) of the sera had antibodies against Aino virus and antibodies against Tinaroo, Douglas, Peaton, Shamonda, Sabo and Sathuperi were detected in 75.6, 73.3, 71.1, 55.6, 46.7 and 31.1 % of the animals respectively. There were 24 samples that had high titers to either Aino or Peaton viruses and five sera that had high titers only to Tinaroo virus. Eleven serum samples showed low antibody titers to Akabane virus while a single sample had a titer of 160. However all of these sera had very high titers to at least one of the other viruses (Table 1). None of the samples had positive titer to Simbu virus. Due to toxicity or bacterial contamination, sera collected in 2008/2009 were not suitable for this assay and were not included in the results.

Association between reproductive disorders and serological results

Reproductive disorders were encountered in 104 out of 659 individual animals and they included either abortions, still birth, retained placenta, dystocia or calf malformations. There was a statistically significant association between the occurrence of one or more reproductive disorders and SBV ELISA seropositivity (OR = 1.9, 95 % CI = 1.2–2.9) on an individual animal level. There was no association demonstrated between individual animal seropositivity and any reproductive disorder alone. There was also no association observed between herd seropositivity and any specific reproductive disorder.

With the SBV VNT, there were no significant associations between any reproductive disorders and SBV seropositivity. Of the 45 sera subjected to Simbu sero-group VNT, eight originated from animals with a history of reproductive disorders. With Simbu sero-group VNT, there was only association between Akabane virus antibodies and abortion, and this association was not statistically significant (OR = 3.9, $P = 0.059$). Antibodies to none of the other viruses were associated with any of the reproductive disorders.

Discussion

This is the first report of the presence of antibodies against viruses from the Simbu sero-group in cattle in Tanzania.

All animals tested with Simbu sero-group virus VNT had antibodies against one or more of the viruses. This is in line

Table 1 Antibody titers to Simbu sero-group viruses detected in virus neutralizing test (VNT) performed on 45 cattle sera collected from Tanzania in 2012/2013

ID	AINO	AKABANE	DOUGLAS	PEATON	SABO	SBV	SATIV	SHAV	SIMBU	TINAROO	Highest titer
1	10	-	20	-	-	20	-	-	-	-	SBV/Douglas
2	20	-	40	40	-	20	-	-	-	-	Douglas/Peaton
3[a]	20	-	-	20	-	-	-	80	-	80	Tinaroo/SHAV
4	640	10	-	640	640	-	-	160	-	160	Aino/Peaton/Sabo
5	40	-	80	-	80	320	320	80	-	-	SBV/SATIV
6	10	-	-	20	-	-	-	-	-	40	Tinaroo
7[a]	-	-	40	-	-	-	-	-	-	-	Douglas
8[a]	40	10	40	80	≥1280	40	-	160	-	≥1280	Sabo/Tinaroo
9	20	-	-	640	160	-	-	-	-	-	Peaton
10	640	-	10	40	NT	10	NT	NT	NT	160	Aino
11	160	-	-	-	160	-	-	-	-	-	Aino/Sabo
12	20	-	20	160	-	10	80	-	-	160	Peaton/Tinaroo
13	80	-	-	20	80	-	-	-	-	80	Aino/Tinaroo/Sabo
14	-	-	-	-	80	-	-	160	-	160	Tinaroo/SHAV
15	320	-	-	-	-	-	-	-	-	80	Aino
16	-	-	-	-	80	80	160	80	-	-	SATIV
17	80	-	20	20	-	10	80	160	-	20	SHAV
18	160	-	20	40	-	40	80	320	-	80	SHAV
19	-	-	20	160	-	10	80	-	-	40	Peaton
20	160	-	-	-	-	-	80	160	-	10	Aino/SHAV
21	20	80	10	40	80	40	-	320	-	320	Tinaroo/SHAV
22	160	-	80	-	-	-	80	-	-	-	Aino
23	20	-	40	640	-	320	-	≥1280	-	40	SHAV
24	1280	-	20	160	160	10	80	160	-	20	Aino
25	20	-	20	80	80	20	160	320	-	80	SHAV
26	40	-	80	≥1280	-	160	160	-	-	-	Peaton
27	10	-	20	160	320	10	-	320	-	80	Sabo/SHAV
28	160	-	40	320	640	20	-	320	-	80	Sabo
29	40	-	80	-	-	160	-	-	-	-	SBV
30	80	10	-	320	160	-	-	320	-	160	Peaton/SHAV
31	40	-	20	160	-	20	-	80	-	160	Peaton/Tinaroo
32[a]	1280	-	40	1280	NT	40	NT	NT	NT	80	Peaton/Aino
33	10	-	160	-	≥1280	320	-	320	-	-	Sabo
34	1280	10	40	10	-	10	80	-	-	160	Aino
35[a]	20	20	20	160	320	40	-	320	-	≥1280	Tinaroo
36[a]	20	10	20	1280	80	40	-	640	-	320	Peaton
37	640	10	20	160	-	-	80	-	-	320	Aino
38	20	-	20	320	-	20	-	-	-	40	Peaton
39[a]	640	80	20	640	-	-	-	-	-	≥1280	Tinaroo
40[a]	80	-	80	-	-	160	-	640	-	160	SHAV
41	80	-	10	-	80	-	-	160	-	20	SHAV
42	≥1280	40	320	320	-	160	-	160	-	640	Aino

Table 1 Antibody titers to Simbu sero-group viruses detected in virus neutralizing test (VNT) performed on 45 cattle sera collected from Tanzania in 2012/2013 *(Continued)*

43[a]	80	-	-	80	320	-	-	160	-	80	Sabo
44[a]	≥1280	40	80	320	80	40	-	-	-	80	Aino
45	160	160	40	320	80	20	160	1280	-	≥1280	Tinaroo/SHAV

SBV schmallenberg virus, *SATIV* sathuperi virus, *SHAV* shamonda virus, *NT* not tested
[a]Samples negative in the first SBV VNT

with the general view that Simbu sero-group viruses are endemic in Africa. There are reports of detection of antibodies to viruses like Shamonda, Sabo, Sango, Shuni, Igwavuma, Sathuperi and Akabane in the 1970s in Nigeria, South Africa and Kenya [13, 14, 25, 26]. However, due to the extensive cross reactivity that can occur between these viruses that are both genetically and antigenically closely related [14, 20, 27], it can be difficult if not impossible in some instances to determine with which virus(es) an animal has been infected.

Some animals had antibodies to only one virus, suggesting infection with the homologous or a very closely related virus. In other cases, there were very high titers to at least one virus and low titers to the others. Such patterns of reactivity could be interpreted as antibodies resulting from infection with one virus, with cross reactivity to other viruses. Alternatively, particularly for older animals, the low titers could indicate successive infections with different viruses in the Simbu sero-group. Dual or multiple infections in individual animals is also possible which would also complicate serological investigations.

It is possible that other viruses in the same group that were not included in the test are present. The occurrence of reassortants from two or more parental viruses can complicate the situation even further. It is known that some of these Simbu sero-group viruses are themselves reassortants. Phylogenetic studies have shown that Aino and Peaton viruses, Akabane and Tinaroo viruses and Shamonda and Sathuperi viruses are reassortants [7]. Phylogenetic analyses have also indicated that SBV is a reassortant with Sathuperi and Shamonda viruses, hence the possibility that an ancestor of SBV was created by co-infection with both viruses in the past [7]. However, in the context of this study, for interpretation of VNT results, only sharing or mobility of the M RNA segment is of relevance as it encodes the glycoproteins, against which the neutralising antibody response is developed. The ELISA kit used in the present study uses an entire recombinant N protein. There is extensive cross reactivity against N proteins as these are the group reactive antigens and also found in some of the reassortants [7].

Nevertheless, recognising these limitations in the interpretation of serological results, some broad trends can be deduced from the data. There is no evidence of infections with Simbu virus. There is little evidence of current infections with Akabane virus. Only a small proportion of animals were positive for Akabane virus and they had low antibody titers. Low titers may be due to declining antibody levels or could be caused by cross reactivity with other related viruses. Akabane has been shown to have cross reactivity with Shamonda, Sabo, Tinaroo and Yaba-7 viruses [20]. Our results support this finding since most of the samples with positive titers to Akabane also showed higher titers to Shamonda, Sabo or Tinaroo viruses. Therefore seropositivity to Akabane in our samples might be due to cross reactivity with the above viruses. As Simbu sero-group viruses are transmitted intermittently, it is possible that Akabane virus may not have been active in the 15-year period that the study animals represent (age data not shown). Akabane virus has been reported in wildlife in Tanzania in the past [16] and from neighboring Kenya [28]. Akabane virus has also been reported in cattle in many other African countries [26, 28–30] which shows that Akabane virus is endemic in Sub Saharan Africa that share ecological characteristics with parts of Tanzania.

Of the tested viruses, a large proportion of animals were seropositive to Aino, Peaton and Tinaroo viruses. Many of the positive animals also had very high antibody titers. Aino virus has only been reported previously in Japan and Australia [31, 32] not in Africa. This is also the first report of antibodies to Douglas virus, Peaton virus and Tinaroo virus in Africa and the first report of Sathuperi, Shamonda and Sabo viruses in Tanzania. Sathuperi, Shamonda and Sabo viruses have already been reported in Nigeria in 1970s [14] It is likely that these or very closely related viruses are each present as there were animals with very high antibody titers to a single virus and very low or no antibodies to the other viruses.

Antigenic relationships between Simbu sero-group viruses isolated in Africa and Australia have been documented. It is of particular interest to see how closely related these viruses are since both isolates have been used in the present study. Cross reactivity between Douglas and Sathuperi virus, Peaton and Sango virus and Tinaroo and Sabo viruses have been reported before [20]. However, in the present study there was no clear pattern of antibody levels to these virus pairs (Douglas/Sathuperi and Tinaroo/Sabo) being more associated with each other than to the other viruses. The close relationships between some of these viruses is reflected in their current taxonomic classification where the species Sathuperi virus includes Sathuperi, Douglas and Schmallenberg viruses; species Shamonda virus includes Shamonda, Peaton and Sango viruses;

species Akabane virus includes Akabane, Tinaroo and Sabo viruses; species Shuni virus includes Aino and Shuni viruses and species Simbu virus includes only Simbu virus [6]. In the present study, the prevalence of positive animals to the traditionally Australian viruses Peaton, Tinaroo and Douglas viruses, was higher than to the Sabo, Sango and Sathuperi viruses that are traditionally African viruses. This suggests that cross reactivity is not the reason for the seropositivity to Australian strains in Tanzania, but rather that the animals are infected with these or other closely related unknown viruses. Among the positive animals, there was also no trend of higher titers to the African strains than the Australian strains.

Although virus neutralizing antibodies to SBV were detected in the 2012/2013 sera, there is no conclusive evidence that animals were infected with this virus. Most of the animals that were positive for SBV also had similar or higher titers in the VNT for one of the other Simbu serogroup viruses. Based on the interpretation that an animal is infected with the virus that gives the highest titre in a VNT, the results indicate the presence of SBV in Tanzania but only one animal had the highest titer to this virus. There are also some other possible explanations. Firstly, it might be that the animals have even higher titers for other viruses that were not included in the test assay, hence cross reactivity as discussed earlier. Secondly, the animals may not always produce the highest titers towards the infecting agent. In multiple infections especially with closely related agents, interpretation of the antibody titer may be a challenge [33]. Another study from Africa has reported antibodies against SBV in cattle and small ruminants in Mozambique [34]. However, the report is based only on results from tests with a commercial iELISA, which in the current study has been shown to lack specificity for SBV, and without confirmation with VNT or virus isolation. Disease with clinical presentation similar to SBV has also been reported in South Africa [17] but the clinical signs associated with SBV infections are not specific and no further confirmation of the diagnosis was undertaken.

Antibodies against SBV were also observed in the samples collected in 2008/2009, which if these are really SBV-specific antibodies, shows that it was probably already present in the area before the European epidemic. Further studies to confirm the presence of SBV in different African countries and compare its molecular characteristics with the European strains would be valuable. To definitively confirm if SBV is present or not, virus detection in the host will be necessary. The viremia induced by SBV is short lived, lasting for 2 to 6 days in cattle [5]. In the absence of an outbreak of congenital defects or clinical disease in mature animals, the chance of detecting the virus is very low. In the present study, 100 serum samples were tested in SBV RT-PCR, but all yielded negative results (results not shown). Surveillance in vectors could also be considered for virus detection and isolation which would allow further characterization using molecular methods. However, such studies will not prove that the virus is present in the cattle population. Nevertheless, this approach is most likely to unequivocally demonstrate the circulation of SBV in Africa.

The antibody ELISA indicated that many of the animals were positive for antibodies against SBV. However, a large proportion of ELISA positive samples were negative in the VNTs. As shown in table one, no SBV-specific antibody was detected in 16 out of 45 sera tested in multiple VNTs. A single sample that was negative in the SBV VNT had a low titre only to the closely related Douglas virus while the other 15 sera had neutralizing antibodies either to one other Simbu sero-group virus (two samples) or to multiple viruses (13 samples). The fact that several samples were negative to SBV and at the same time strongly positive for closely related viruses indicates that cross reactivity may not be the main reason for the seropositivity to SBV in the VNTs.

In general, commercially available ELISA assays are sensitive, specific and robust, but cross reactivity with other members of Simbu sero-group has been reported for the assay used in this study previously [35]. The initial validation of the assay was undertaken in Europe where other Simbu sero-group viruses have not been detected in cattle or sheep. As there is a high degree of similarity between the N protein antigens of members of the Simbu sero-group (the basis upon which these viruses were initially grouped), it is not surprising that cross reactivity is observed in the ELISA when animals are infected with one or more other Simbu sero-group viruses. Consequently, when interpreting the results of this and similar ELISAs, care must be exercised because a positive result may not indicate infection with SBV but could be due to infection with another Simbu sero-group virus. While VNTs are considered to be the 'gold standard' for the assessment of other assays, it is well recognised that even they are prone to cross reactivity for viruses belonging to the Simbu sero-group. As the ELISA is relatively easy to perform, requires minimal laboratory equipment, and laboratories do not need to have all reference viruses, it will be preferred in many of the regions where multiple Simbu sero-group viruses may be present. There is a need to validate ELISA kits for use in these endemic areas but this will be challenging due to the complex cross reactivity. Limited cross reactivity is even observed in monoclonal antibody based ELISAs run in either blocking or competitive formats (D.S. Finlaison and P.D. Kirkland, unpublished data). A goal of demonstrating specificity for the detection of antibodies to the Simbu sero-group should be the primary objective for an assay that is to be used for the confirmation of the cause of congenital defects in ruminants. Other virus specific assays such as real time

PCR can then be used to undertake further testing of fetal specimens.

The present study showed an association between SBV ELISA positivity and reproductive disorders. As the ELISA is not SBV-specific, this reactivity may also be due to other Simbu sero-group viruses which are known to cause late abortion, premature birth, still birth and congenital malformations [3, 36, 37]. A clinical outbreak has not been reported in the study area. The lack of clinical outbreak could be due to endemic stability as it has been postulated for Akabane virus in Australia and for epizootic hemorrhage disease in white-tailed deer in the US [38]. However, the present study detected abortions but without any obvious fetal abnormalities. The reasonable interpretation of this result is that one or more of the Simbu sero-group viruses that are prevalent in the area result in a negative effect on reproductive performance. The syndrome of congenital defects associated with infection with Simbu sero-group viruses that has been reported in Japan, Australia and Israel has not been observed in Africa despite the presence and wide spread occurrence of antibodies to Simbu sero-group viruses in different animals [28].

Antibodies against Akabane virus were associated with abortion, however the association was not statistically significant and the serological evidence of the presence of Akabane virus is inconclusive. It is well known that Akabane virus causes abortion in bovine animals [2, 28]. The small sample size limits the power of the analysis of association between the different viruses included in VNTs and reproductive disorders.

Conclusion

This is the first serological indication of Simbu sero-group viruses including SBV and their possible association with reproductive disorders in cattle in Tanzania. It is possible that viruses from the Simbu sero-group, other than the ones included in the test, are also present. The origin of the virus that caused the recent SBV epidemic in Europe is still a mystery but this study demonstrates the possibility that the virus may have been present in Tanzania already and other parts of Africa also where the same vectors are abundant. Isolation and further genetic characterization of the viruses, including isolates from different geographical origins, will be essential for understanding the molecular epidemiology and evolution of SBV related viruses.

Abbreviations

SBV: Schmallenberg virus; SATIV: Sathuperi virus; SHAV: Shamonda virus; ELISA: Enzyme linked immunosorbent assay; VNT: Virus neutralization test; OR: Odds ratio.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CM performed the study, collected the data and sample, analysed and interpreted the data and drafted the manuscript. GM, RHM, SK, JG and MS conceived and designed the study, participated in data and sample collection, critically revised the paper and acted as the first author's study supervisors. ARWE, WHMP and PDK advised on the study design, analyzed the data and critically revised the paper. All authors read and approved the final manuscript.

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