Seroprevalence of brucellosis and Q-fever among cattle in high risk herds in the Bethlehem area, Free State, South Africa

By

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Submitted in partial fulfilment of the requirements for the degree of

Master of Science (Tropical Animal Health)

In the

Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

University of Pretoria

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August 2019

Declaration

I, Johannes Christoffel Du Plessis, hereby declare that this dissertation, which I hereby submit for the Master of Science degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, to be my own work and has not been previously submitted by me for degree purposes at another tertiary institution.

8 August 2019

Acknowledgements

Belgian Directorate-General for Development Co-operation Framework Agreement (FA4 DGD-ITM 2017-2021) awarded to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria and the Free State Department of Agriculture and Rural Development for funding the study.

Thanks are to Michael Khosa for assistance with *Coxiella* ELISA in the Department of Tropical diseases serological laboratory.

Marlize Nel, Animal Health Technician Bethlehem for assisting in the sample taking as well as the distribution of the samples to be tested for bovine brucellosis.

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Foetal loss can be devastating to a cattle farmer. In the Free State province many commercial cattle farms contend with foetal loss due to abortion. The causes of most of these abortions are never diagnosed because of inappropriate samples submitted; diagnostics being too expensive or non-submission of samples due to ignorance. The aim of this study was to investigate the apparent seroprevalence of *Brucella* species and *Coxiella burnetii* in commercial cattle of five epidemiological units. The overall apparent seroprevalence in this study was 22% and 11% respectively for *Brucella* species and *Coxiella burnetii* antibodies. Although the presence of antibodies does not lead to a definitive diagnosis, it is now known that there are bacterial challenges in these commercial cattle, and this could form the basis of future studies. Improved education of the public as well as communication with the human health sector is necessary to effectively control brucellosis and Q-fever.

Chapter 1: Literature review

1.1. Introduction

In Bethlehem in the Free State province, many cases of foetal loss are reported to the private and state veterinarians. These herds have cases of more than 5% in most of the herds; these herds are seen as "high risk cattle herds" with regards to the two diseases in this study. An aetiological diagnosis is not always made on specific cases due to various factors as described below in the study. The major role of the state veterinarian is controlling diseases, particularly *Brucella abortus*. In the field the common assumption is that these abortions are due to *Brucella* spp. infection, however in some cases Brucella is ruled out and causative agent is not identified. The aim of this study was to look at herds that are reporting foetal loss and to determine the seroprevalence of antibodies against *Brucella* spp. and *Coxiella burnetii* in these herds as both organisms can cause late term abortions.

Abortions and embryonic deaths are part of a disease complex in the field of veterinary science where it is very difficult to find a correct definitive diagnosis (Borel, *et al* 2014). Yet, abortions can have massive financial repercussions due to direct loss of calves (Gwida *et al* 2015; De Vries, 2006). Late abortions are the most economically important losses in ruminants due to the time lost before the cow reconceived (Diskin & Morris, 2008). A late abortion is defined as the expulsion of a calf that does not have the ability to survive outside the dam as opposed to early embryonic death (losses earlier than 45 days of pregnancy, before organogenesis is complete) (Holler, 2012; Miller, 1977). A stillbirth is defined as the premature expulsion of a foetus that has the ability to survive outside the dam (Holler, 2012; Miller, 1977). Ante-partum deaths are distinguished from peri-partum deaths by a higher occurrence of autolysis in the organs of the expelled foetus (Holler, 2012; Miller, 1977).

In addition, certain infections such as *Brucella* species can lead to major economic losses as well as trade restrictions but above all this disease is zoonotic (Gwida *et al*., 2015; De Vries, 2006). In Florida in America in the 1990's it was confirmed that more that 14% of the herds were infected with bovine brucellosis and the calculated effect that the disease had in monetary value was more than 300 million rand per annum (De Vries, 2006).

Various factors can cause loss of foetuses or embryos. Causes of foetal loss may be classified into three broad categories namely genetic, environmental and infectious (Miller, 1977). Infectious causes of abortion can be subdivided further as: bacterial, viral and protozoal causes (Diskin & Morris, 2008; Givens & Marley, 2008). According to Holler (2012), a rate of abortion greater than 5-8% is seen as significant. Intervention is advisable in these cases (Jonker, 2004). The expensive nature of the different tests available for abortion investigation limits their use, which in turn reduces the chances of a definitive diagnosis being made (Holler, 2012). The results of a study in America conducted over more than 10 years by the personnel at the Veterinary Science Department of South Dakota State University indicated that bacterial agents were implicated in 14.49% of cases, viruses in 10.57% of cases and fungi in 5.31% of cases. In 67.17% of abortions no diagnosis could be made (Kirkbride, 1992). In a study in Argentina showing similar results of 354 cases of abortion were assigned to different aetiologies. Only 45.5% of the cases could be assigned aetiology. Most of these cases (34.4%) had a bacterial cause of which the highest number was *Brucella abortus* (Contagious abortion), *Campylobacter fetus* and *Escherichia coli*. *Neospora* was implicated in 7.3% of the cases (Campero *et al*, 2003).

In California a similar study revealed that bacterial causes of abortion were more frequently encountered than viral and protozoal causes. Out of the 468 abortions that were analysed only 29.5% of these abortions had a specific cause of which 16% were bacterial, 5.6% viral and 3.2% protozoal (Anderson *et al*, 1990). In South Africa a study by Schutte *et al*, 1976 implicated *Coxiella burnetii* in cases of cattle abortion by examination of stained placental impression smears, culture and serology. Out of a total of 80 cattle tested for presence of antibodies, 65 tested positive. This was a study done on animals that aborted as well. No reports of subsequent investigations were found. These studies all have the following in common, the majority of diagnoses point to bacterial aetiologies, it is difficult to find an aetiological diagnosis and many abortions go undiagnosed.

The most important part of an abortion investigation is the compilation of a proper clinical history, the description of the calf size as well as the macroscopic description of lesions seen at necropsy (Holler, 2012). A definitive diagnosis of a specific abortion can only be achieved by a combination of histopathology and culture of the agent (Anderson, 2007; OIE, 2016).

These methods are dependent on correct sampling methods and good quality samples from the foetus and foetal membranes were possible, because abortions are usually seen by the farmers a day later and scavenger animals could have removed some of the above-mentioned tissue. If the dam is slaughtered, direct samples for bacterial culture can also be collected from her, samples include the mammary lymph nodes, uterine tissue, sub iliac lymph nodes as well as udder tissue (Holler, 2012). However, a history of abortion is not always available, and the foetus and placenta may be eaten or removed by scavenger animals before samples can be taken, so often the only samples available to collect are serum from the dam.

Serology can detect antibodies produced after exposure to bacteria (Godfroid *et al.*, 2005). Serum is easy to collect from the coccygeal vein in the tail into a sterile vacutainer tube without anticoagulant. These samples may include serum from the dam as well as other members of the herd if a herd problem is suspected. If possible, a paired serum sample should be taken from a cow that aborted. If the latter is not possible, a sample at the time of abortion will contain most antibodies to the specific causative agent (Holler, 2012). According to Anderson & Barr (1993) serology can be useful in the investigation of abortion however; a single sample might be of limited or no diagnostic value. Often a result is only seen as significant if a four-fold change in titres can be demonstrated from paired serum samples collected two weeks apart. Comparison of serology results of animals that abort to normal controls is often recommended (Holler, 2012). In addition, serology results must be evaluated together with the vaccination history of the animal of interest as some vaccines can lead to false positive titres, for example when S19 *Brucella abortus* vaccine is used in cattle that are older than seven months (Stevens *et al*, 1994; Godfroid *et al*, 2005).

1.2. *Brucella* spp. in cattle

There are several *Brucella* species: *Brucella abortus* infects mostly cattle, *Brucella melitensis* mostly sheep and goats, *Brucella ovis* sheep, *Brucella suis* pigs and *Brucella canis* dogs. Most of these *Brucella* species can infect humans as well as wildlife, and the wildlife can act as reservoirs (Godfroid *et al*, 2013). This study will focus on *Brucella* spp. antibodies of cattle.

Brucella abortus are facultative, intracellular non-motile bacteria (Kubuafor *et al*., 2000). The clinical signs most commonly seen in cattle are late abortions, stillbirths, retained placentas and weak calves (Bosilkovski, 2015; OIE, 2016A).

After the first abortion, normal live calves are born, but the causative bacteria are shed in foetal fluids as well as in milk. Survival of these bacteria in the environment depends on moisture and exposure to sunlight this makes the control of disease difficult. In wet soil the bacteria can survive up to three months; whereas it only survives for about a month or two in drier soil (Godfroid *et al* 2004). There is a high risk of spreading the disease to uninfected animals if the infected animals are not removed from the herd (OIE, 2016A). If a heifer calf is born to a positive cow, between 2.5-9% of these animals will be infected and will only seroconvert later in their first pregnancy (Godfroid *et al*., 2004).

Infection can be transmitted via ingestion, inhalation as well as via the mucous membranes such as the conjunctiva. The pathogenesis of the bacteria is dependent on the number of bacteria that the animal is exposed to, which is referred to as the infective dose (Stevens *et al.,* 1994). The vaccination status and sex of the animals also can play a role in susceptibility since heifers, as well as pregnant cows are more susceptible to infection than bulls. *Brucella abortus* can cause infertility in bulls since the bacteria cause inflammation of the testis (orchitis) (Godfroid *et al*., 2004). The incubation period is very long, it can vary from one week up to several months depending on the sex as well as the pregnancy status of the animal that the bacteria infects (Seleem *et al.*, 2010; Lopes *et al.*,2010).

Once the bacteria pass through the mucous membranes it is exposed to cells of the immune system. From there the bacteria are moved to the lymph nodes where it causes local infection and replicates. A bacteraemia results and the bacteria spreads to the pregnant uterus or to the lymph nodes around the udder (Godfroid *et al*., 2004). The bacteria are attracted to the pregnant uterus, where the carbohydrate erythritol, present in high concentrations in the last half of gestation, enhances their growth (Godfroid *et al*, 2004).

The bacteria cause a placentitis, the outcome of which may be abortion or the birth of a weak calf depending on the degree of inflammation (Jonker, 2004). The process of abortion is brought on by the placentitis (Miller, 1977), which leads to a decrease in supply of nutrition

as well as oxygen to the calf resulting in stress. This leads to increased production of cortisol by the calf combined with lower progesterone levels produced by the infected placenta (Miller, 1977). This is the hormonal combination that will start normal parturition but at the wrong gestational period (Godfroid *et al*., 2004).

Antibody production is a consequence of antigenic exposure to the immune system. This antigen in most cases of *Brucella* is the lipopolysaccharide (LPS) molecule. It comes into contact with the immune system when the bacteria cross the mucous membranes (Godfroid *et al.*, 2004). The LPS molecule is not specific to only *Brucella* species, it is also found in *Escherichia hermanni*, *Escherichia coli* serovar O:157, *Yersinia enterocolitica* serovar O:9, *Vibrio cholera* as well as *Salmonella* serovars of group N that can lead to false positive results in serological tests (Godfroid *et al*., 2005).

When *B. abortus* as well as other species of *Brucella* are exposed to the immune system antibodies are produced against these bacteria especially the O-polysaccharide (Neta *et al.*, 2010). Multiple cells that interplay with each other to present the bacteria to the antibody producing cells and they in turn create antibodies against the bacteria (Corbel, 1997). The innate immunity is the main protection against a bacterial challenge; most of these processes occur on the mucous membranes. The dendritic cells, macrophages and neutrophils are the first cells that encounter the bacteria. The phagocytes present parts of the bacteria through Toll-like receptors to the rest of the immune cells called CD4 cells that stimulate the immune system (Neta *et al*., 2010). The antibodies that are produced are IgM first and thereafter the IgG1, with IgG2 being produced last. These antibodies are the targeted antibodies when the disease is tested for, especially the IgG1 (Neta *et al*., 2010). In chronic infections IgG is more prominent. Vaccination stimulates more IgM antibodies (Allan *et al.*, 1976).

The Rose Bengal plate agglutination test (RBT) and the Compliment Fixation Test (CFT) are commonly used tests to detect antibodies to *Brucella* species in ruminants. Rose Bengal reagent is a killed *Brucella abortus* strain S99 or S1119-3 stained with a rose-Bengal stain (Nielsen, 2002). The RBT is performed by mixing test serum and Rose-Bengal reagent. These tests are done at a low pH to discourage false positive/irregular reactions. A positive result will be indicated as a red ring at the surface of the serum, which specifies that there are

antibodies, which bind to the antigen to form an agglutinate. Results are recorded as positive or negative. The test is very sensitive (Nielsen, 2002; OIE, 2016A).

The CFT is performed by diluting the sample serum and adding antigen as well as complement. Indicator red blood cells are added to indicate binding of antibody in the serum to the antigen. If an antibody-antigen complex forms, it will activate the complement system and the indicator red blood cells will not be destroyed. The cells sink to the bottom of the well to form a button; this is read as a positive result. If there are no antibodies, the complement will be available to lyses the indicator red blood cells leading to a reddish colour change in the wells. This can be visualized mechanically or automatically using a spectrophotometer (Nielsen, 2002; OIE, 2016A).

Routine test only the RBT test is done once it is positive the CFT is done. A CFT titre of 20 IU/ml is considered positive in unvaccinated animals. In animals that have been vaccinated with either *B. abortus* S19 or *B. melitensis* Rev-1 vaccines at the prescribed ages, a titre greater than 30 IU/ml is considered positive. These values and cut-offs are also adapted to the country and the legislation of the country (OIE, 2016A).

The Serum Agglutination Test is a simple agglutination test that can be used in the detection of antibodies against *Brucella* spp. The test targets the IgM antibody that is the most likely to agglutinate and this can lead to false positives, as the major amount of antibodies made by S19 vaccine is also IgM and can give false positive results (Nielsen, 2002).

The most common way of controlling the disease is through testing and slaughter protocol with calf vaccination (S19) as well as adult vaccination (RB51). In South Africa *Brucella abortus* is controlled on a test and slaughter protocol. Farms where a suspicion of the disease is present are placed under quarantine and animals can only be moved to a registered abattoir. The herds are re-tested until three complete negative tests are produced of all the female animals on the farm. All of these movements must take place with proper documentation from the state veterinarian office. S19 is implemented to be vaccinated by law to all heifers 4 to 7 months of age.

If heifers are vaccinated at an age older than recommended by the manufacturer for the vaccine S19, the vaccine bacterial LPS antigen can stimulate the production of antibodies, especially IgM. This can lead to difficulty interpreting the results as they will also test positive on the Rose Bengal test (Stevens *et al*, 1994).

Although culture and typing of *Brucella* remains the gold standard for confirmation of diagnosis, serological screening for *Brucella* by means of immune response to its antigens is effectively used in eradication programmes (Rahman *et al*, 2006). The United States of America (USA) implemented an eradication program for *Brucella abortus* in 1934. In their case the complicating factor is the presence of carriers or hosts that are difficult to control such as bison (*Bison bison*). In 2010 a review reported a seroprevalence of 50% in bison in the Yellowstone Park (Lopes *et al*, 2010)

In the review by Lopez *et al.* (2010), *Brucella* seroprevalence was reported as 10.5% in South America when the ELISA method was used. In Los Lagos in Chile in 1991 seroprevalence was reported as between 23 and 38%. A milk ring test study in the same country found prevalence to be 19.7%. This serves as indication of the lower sensitivity of this test when compared to the ELISA method. By 1996 there was a reduction by half of the seroprevalence due to control strategies implemented. The authors attributed this change in prevalence to the usage of RB51 vaccine instead of S19 vaccine (Lopes *et al*., 2010).

In the European Union the prevalence of *Brucella* is very low. There are multiple countries that are officially *Brucella* free (OBF). These countries include Austria, Denmark, Finland and Germany. In the non-official *Brucella* free countries prevalence is reported to be between 0.12% in Ireland and 1.31% in Italy. Overall prevalence is much lower than reported from American and African countries (Lopes *et al*., 2010). A study in Turkey from 2001 to 2006 reported an apparent prevalence of brucellosis of 35.30% by the Rose Bengal test, 32.92% by the serum agglutination test (SAT) and 39.45% by the ELISA. This was the highest prevalence reported in European countries (Lopes *et al*, 2010). In this study the SAT was the least sensitive method. For this reason it was not considered for this study.

In a study in Chad by Schelling *et al* (2003) *Brucella* seroprevalence was found to be higher by 7% in cattle than other livestock. In addition, the study indicated that positive brucellosis

titres were a significant factor for abortion in cattle (OR = 2.8). A seroprevalence study in Cameroon from samples collected from an abattoir reported a seroprevalence of 8.4% was found among this study (Lopes *et al*., 2010; Bayemi, *et al*, 2009). In Ghana in a study in 2000 seroprevalence was found to be 17.2% at the highest in one of the provinces (Kubuafor *et al*., 2000).

In South Africa there is no true prevalence determined for Bovine brucellosis (Godfroid *et al*., 2004). However, two studies in South Africa, one by Bishop in 1984 and one by Bosman in 1980 found that *Brucella* seroprevalence was 3.7% and 6% respectively at animal level as these animals were tested when they were slaughtered. A study on communal cattle in KwaZulu-Natal reported a serological prevalence of 1.45% (Hesterberg *et al*, 2008). World Health Organization (WHO) data on reported disease outbreaks in South Africa as well as the number of cases of *Brucella* reported is only available for 2005, in these reports the number of outbreaks recorded are 343 and the number of cases in these outbreaks are 6599 (OIE, 2016A).

1.3. *Coxiella burnetii* in cattle

Coxiella burnetii is a strict intracellular gram-negative bacterium (OIE, 2016B). The bacteria can survive for long times in the environment as a spore-like structure. It is commonly found and therefore the presence of antibody titres in serum is not unusual in healthy animals (Agerholm, 2013). The main mode of transmission is inhalation of moisture droplets contaminated by products of abortion such as placenta and foetal fluids. Milk and unpasteurized cheese might also be implicated. This bacterial disease is also thought to be transmitted by ticks (Knobel *et al*, 2013). Infection does not result in clinical signs in 50-60% of the cattle, sheep and goats (Akbarian *et al*, 2015). Clinical signs in ruminants are primarily abortions, stillbirths and weak calves. International studies indicate that the highest rate of infection with *C. burnetii* may be found in goats (Guatteo *et al*, 2012; Roest *et al 2011*).

New Zealand is the only country that is believed to be free of *C. burnetii* (Guatteo *et al*, 2011). *Coxiella burnetii* in domestic animals in Afghanistan was reported a seroprevalance at 41.3 % overall in this study, when the seroprevalance prevalence was split into different species, goats had the highest prevalence at 52.7%, sheep at 43.4% and lastly cattle at 5.2% (Akbarian

et al., 2015). A literature review by Knobel *et al*. (2013) reported the prevalence of *C. burnetii* in cattle in Kenya to be 7-57%; and in goats 33-34%. In a cross-sectional study done by Knobel *et al*. in 2013 the prevalence in the different species affected in Kenya was found to be 28.3% in cattle; 32% in goats and 18.2% in sheep that was much lower than the literature review done above. A literature review by Guatteo *et al,* in Zimbabwe in 1993 reported the apparent prevalence to be between 39-41%. A literature review in Egypt found an overall prevalence below 8% with the highest prevalence reported between 10 and 32%. A close correlation between animals that lost a foetus and antibodies was reported (Anderson *et al*, 1990).

Several serological tests targeting IgG antibodies have been developed (OIE, 2016B). In this study an ELISA was used due to availability and ease of use. In South Africa, few prior research articles could be found, therefore this investigation of serological prevalence of agent is a very valuable study in its own.

According to the OIE website only two outbreaks were reported in the past and both these cases were found in goats. A study in South Africa including serology as well as placentas from cattle and sheep that aborted found that 58% (7/12) of herds tested were infected with *Coxiella burnetii* (Schutte *et al*., 1976).

The presence of antibodies will be regarded as potential exposure to *Brucella species* and *Coxiella burnetii*. The apparent prevalence can be calculated (Czaplicki *et al*, 2012).

1.4. *Brucella* and *Coxiella burnetii* in humans

Both *Brucella* spp. and *C. burnetii* are zoonotic, meaning that the bacteria can spread to humans and cause disease (Agerholm, 2013; McDermott *et al*, 2013). *Brucella abortus* can infect humans and is referred to as 'Undulating fever' or 'Malta Fever', as it manifests as a chronic, recurring infection. Humans are exposed because of their work. The main modes of transmission to humans and other animals are ingestion of unpasteurized milk and contact with birth products from infected animals (Chang *et al*, 2010). The routes of infection can be via the respiratory system, mucous membranes or alimentary tract or by the ingestion of unpasteurized milk from an infected cow (OIE, 2016A; Kubuafor*et al*, 2000). Shedding of the bacteria is very high in the colostrum of a cow that has aborted a calf, but it is not constantly shed trough the milk during lactation (Godfroid *et al*., 2004). Accidental human vaccination

with Strain 19 can cause disease that might resolve on its own, but the disease can also manifest in a chronic form (Godfroid *et al*., 2004). Clinical signs of *Brucella abortus* infection in humans include fever, pain in muscle and joints and in some cases night sweat (Godfroid, *et al*, 2013). The infection can become chronic if not treated early resulting in recurrent fever (undulant fever). A blood culture in the febrile stage of the disease or PCR is available for diagnosis of the infection in humans (Mantur *et al*., 2007). The occurrence of human brucellosis is very high with over five hundred thousand human cases reported worldwide annually (Godfroid *et al,* 2013). However, the overall prevalence worldwide is most likely underappreciated as the clinical signs might be misdiagnosed (Mantur *et al*., 2007). There is no vaccine for humans yet (Mantur *et al*, 2007).

Coxiella burnetii can affect humans, particularly people that come into direct contact with animals due to their occupation, particularly veterinarians, farmers and their workers and abattoir workers are at greatest risk. The main modes of transmission are ingestion of unpasteurized milk and contact with birth products from infected animals (Chang *et al*, 2010). In the acute form clinical signs are: fever, encephalitis, hepatitis and pneumonia. The chronic form can manifest as infection of the heart valves presenting as endocarditis (Akbarian *et al,* 2015; OIE, 2016B; Vanderburg *et al* 2014).

In a study in western Kenya that included humans as well as on domestic animals, 30.9% of people had antibodies to *C. burnetii* (Knobel *et al,* 2013). During an outbreak of *C. burnetii* infection in the Netherlands between 2007 and 2009 reported by Roest *et al*. (2011), 3523 human cases were reported. The clinical sign that was seen in most of these cases was respiratory involvement. *Coxiella burnetii* infections in humans in South Africa were first reported in 1950 by Gear, Wolstenholme & Cort as well as Saner & Fehler (Schutte *et al*., 1976; Vanderburg *et al*, 2014). A human serological survey conducted by the South African institute for Medical Research and published in 1953 indicated that antibodies to *C. burnetii* was common in humans in South Africa (Schutte *et al*., 1976).

1.5. Aim

This study will investigate the prevalence of antibodies to *Coxiella burnetii* and/or *Brucella* spp. in cattle on selected high-risk cattle farms in the Bethlehem area of the Free State province in South Africa.

1.6. Hypotheses

H0: No antibodies to *Brucella* spp. and/or *Coxiella burnetti* are present in cattle on the farms in question.

H1: Antibodies will be found to *Brucella* spp. and/or *Coxiella burnetti* in cattle on the farms in question.

Chapter 2: Materials and methods

2.1. Sample size

This is a cross sectional descriptive study. When farmers report abortions to their state veterinarian, the whole herd is sampled to screen for *Brucella* spp., which is the controlled disease. Eighteen herds that experienced abortions were referred by a private veterinarian to the state veterinary office during a particular month. Five of these herds, four beef and one dairy, were selected at random to test for both *Brucella* spp. and *C. burnetii*. The inclusion criteria were a history of abortions amongst the cattle, reported by the farmers as a problem. None of these herds were sampled for these two diseases previous to the sampling done for this study.

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

Z (Z statistic for 95%) = 1.96
P = Prevalence = 50%
d = Precision = 0.06

Using the formula above, the number of cattle required was calculated as 267 with a confidence interval (CI) of 95%; with a precision (d) of 0.06 for a prevalence (p) of brucellosis at 50%. This was also included as an over estimation as most of the herds tested were herds that have a problem with abortions. The prevalence of *C. burnetii* is unknown in this study population and in South Africa, therefore the prevalence was estimated at 50% with a precision of 0.06 and a 95% confidence 267 samples were necessary. Three hundred samples were tested as the C*oxiella* ELISA plates had space for 300 samples so the maximum amount of samples were taken.

A sample was calculated out of the total number of herds presented to the office that month via cluster sampling. Due to the many unknown variables the equation for sample size was done using a simple sampling size equation to determine the amount of clusters needed (Kodinariya & Makwana, 2013).

$$k = \frac{\sqrt{n}}{2}$$

k = the number of clusters to select
n= average number of cattle per cluster

The eighteen herds referred to the State Veterinarian's office, comprised of a total of 1856 cattle. Thus the average number (n) of animals in the herds was 103. The number of clusters needed was 5. Five clusters were selected at random.

2.2. Consent form

Informed consent was obtained from owners of animals as follows. A consent form (Annexure A) were designed and approved by the Animal Ethics Committee. Appointments were made with the selected farmers prior to the sampling date and on arrival on each farm everyone was introduced by stating their name, qualifications and what their job will be on the day of the sample collection. The forms were explained to the farmer and each farmer had to sign the documentation before the commencement of any physical work.

The following parameters were used to describe the study population for interest sake but also for clinical significance: closed herd, pregnancy diagnosis, calving percentage, set breeding period, time of abortion, vaccination status towards *Brucella* spp. and whether the nutrition was extensive or intensive. The question regarding time of abortion was stated using comparison of foetal size to the size of dogs. The dog used for comparison in the questionnaire was a full-grown Jack Russel terrier weighing about 10kg. Respondents were asked if the foetuses seen were larger (mid stage abortion) or smaller than this dog (early foetal loss). A set measurement would usually be included, but to get more information from the staff working on the farm the use of a comparison was more appropriate. Respondents were also asked if the calf was fully developed with sufficient hair covering the body (late stage abortion or stillbirth). This helped to narrow down the specific aetiologies of the different abortions.

Confidentiality and protection of the identities of these farmers is very important, therefore the farms will be referred to as epidemiological units and allocated numbers in the form of abbreviations derived from the sequence of farms sampled, and the researcher determined the numbers and is the only party that has this information. See attached an example of the indemnity form in Annexure B.

The sampling personnel or researcher were equipped with correct protective clothing, these included overalls, gumboots, latex gloves and protective glasses, which is important to decrease the risk of infection to the person collecting the samples. For biosecurity reasons it is important when working at more than one site per day, that a new set of clean protective clothing and equipment was used or that the protective gear, like the glasses were disinfected between visits to prevent spread of diseases between farms.

The welfare of animals was very important in this study and no harm was done to any animals, the animals were handled in a respectful manner to prevent any additional stress. The environment was kept as safe as possible for the people working with the animals and for the animals themselves to prevent injury. Handling the cattle in a calm manner was of utmost importance, this was done using techniques such as taking small groups of cattle at a time into the crush, ensuring that there are no visual distractions at the front of the crush. Combined with that, no electric prodders were used, animals were not rushed into crushes and no loud noises were permitted around the crush to keep the animals as calm as possible (Pas *et al*, 1998).

2.3. Sample collection

Cattle were restrained one by one in the crush just tight enough to prevent the animal from excessive movement and to cause as little discomfort or injury as possible. The tail was elevated trying to keep it as straight as possible. A clean, sterilised needle was used on each animal with a clean cuff holding the needle. The needle was inserted perpendicular to the skin between two coccygeal vertebrae.

The serum tube was inserted into the cuff and kept in place until a minimum of 4 ml of blood was obtained; after which the tube was removed. The process was repeated to fill a second tube. These two tubes were individually tested for *Brucella* and *Coxiella.* Tubes were marked

with the number of the cow. The needle was removed, the cuff was cleaned, and a sterile needle was inserted.

If the serum samples could not reach the lab within 24 hours, they were stored upside-down. The blood clots adhering to the lid was then removed to prevent haemolysis which would render the sample unsuitable for testing.

2.4. Transport and *Brucella* analysis

One of the two serum samples collected from each animal was packaged in serum sample boxes together with the sample submission forms. This set of samples was couriered to Allerton Provincial Laboratory, Pietermaritzburg, KwaZulu-Natal were appropriately skilled staff performed the RBT and CFT tests for Brucellosis. The second serum sample collected from each animal was transported to the Department of Veterinary Tropical Diseases.

2.5. *Coxiella burnetii* analysis

At the Serology Laboratory of the Department of Veterinary Tropical Diseases the serum samples were centrifuged for five minutes to separate the serum from any remaining red blood cells. Then 500 µl of the serum was aliquoted into Eppendorf tubes and stored in the fridge at 4°C. The remainder of the samples were refrigerated and only removed before use in the ELISA.

Serological tests were performed to detect antibodies to *C. burnetii* as follows: The serum was tested by means of an ELISA kit (IDEXX Q Fever Ab Test Q Fever/*C. burnetii*), according to the manufacturer's instructions. Two kits were used: serial nr: M401 (Plate SN: 0120-145, exp: 30.04.2020) and K231 (Plate SN 0107-141, exp: 30.06.2019).

The IDEXX Q FEVER (*Coxiella burnetii*) Antibody Test Kit contains antigen-coated plates, positive and negative controls, conjugate, TMB substrate (3,3',5,5'-tetramethylbenzidine) (ThermoFisher Scientific, 2018), Stop solution and wash buffer at 10-fold concentration.

The ELISA kits were left out at room temperature to warm up. Test sera and duplicate controls were added at a 1/400 dilution in wash buffer to appropriate wells. Plates were incubated for 60 min at 37 °C in a humid chamber. After incubation, plates were washed three times,

using a BioRad pw40 microplate washer. Plates were dried by lightly tapping on a paper towel. One hundred micro litre conjugate was added to all the wells followed by incubation as above. Plates were washed three times and 100 µl of the TMB Substrate was added to each well. The plate was incubated in the dark at room temperature for 15 minutes. Last step was to add 100 µl Stop solution to all the wells to stop the colour change, thereafter the microplate was read in the BioTek ELx808 microtiter reader at a wavelength of 450nm.

The assay was considered valid if the mean OD for the negative control was ≤ 0.500 and the mean OD for the positive control ≤ 2.500 and ≥ 0.300. Test results are reported as a percentage of the positive control (PP) using the following equation.

$$\mathrm{PP} = \frac{OD\ SAMPLE_{405} - Mean\ OD\ NEG\ CONTROL_{405}}{Mean\ OD\ POS\ CONTROL_{405} - Mean\ OD\ NEG\ CONTROL_{405}} \times 100$$

Negative: Sample PP < 30%. Suspect: Sample PP > 30% and < 40%. Positive: Sample pp > 40%

In the study there was only one opportunity to test the sera so, the positive samples were taken as PP > 40% (Astobiza *et al*, 2012).

2.6. Records

The CA5 form is the official form for Brucellosis monitoring in South Africa. It is used by any official that is responsible for taking serum samples be it a veterinarian or an animal health technician. These forms were used to record animal numbers, the farm of origin, contact details of the specific farmers and number of samples collected as prescribed by the Bovine Brucellosis SOP of the Department of Agriculture and Rural Development, Free State. Results of Brucellosis serological tests were recorded on the CA5 forms by the testing laboratory and sent to the researcher. The hard copy data is stored in a file with all the raw data as well as all the questionnaires and consent forms. The different epidemiological units as well as the test results were recorded electronically in an Excel spreadsheet.

Chapter 3: Results

The results in Table 3.1 reveal an overall apparent prevalence at animal level of 22% (66/300) for *Brucella* and 11% (33/300) for *C. burnetii*. These findings correlate with a presentation by Mbizeni in 2015 where the seroprevalence of brucellosis-infected herds is estimated at above 25% with some variation across provinces. Only cattle from unit 1 and 2 had *Brucella* antibodies. Unit 2 had the highest apparent prevalence at animal level for *Brucella* at 12.3% (37/300) of the total 37% (66/300). In contrast to the original estimate of 50% Brucella seropositivity, three of the herds (Units 3, 4 &5) tested completely negative (0%) for *Brucella* antibodies.

Table 3.1 The numbers of animals tested on each of the farms; and the results and apparent prevalence for the *Brucella* RBT and CFT as well as *Coxiella* ELISA

Epidemiological unit	Number of samples	*Brucella* Positive	*Coxiella burnetii* Positive	*Coxiella burnetii* Suspect
Unit 1	81	37 (45.7%)	5 (6.17%)	3
Unit 2	61	29 (47.5%)	2 (3.27%)	
Unit 3	61	0	8(13.11%)	1
Unit 4	68	0	17 (25%)	2
Unit 5	29	0	1 (3.44%)	
Total	300	66 (22%)	33 (11%)	

Positive Brucella: RBT positive and CFT titre above 60.

Positive Coxiella: pp > 40%; Suspect: Sample PP > 30% and < 40%; Negative: Sample PP < 30%.

Chapter 4: Discussion

It was interesting to note that the first two units, which were not managed as closed herds and where the presence of late abortions were reported, returned the highest apparent prevalence at herd level of *Brucella* antibodies at 12.3% (37/300)and 9.6% (29/300) respectively (Table 3.1). These were two of the three units that reported not keeping a closed herd. The value of having a closed herd is the reduction of the possibility of introduction of a new disease. If new animals are acquired, they should be isolated and tested before introducing them to the primary herd. This is particularly relevant in the case of brucellosis. In addition, no pregnancy diagnosis was performed. These observations are in agreement with Brennan & Christley (2012) who found that inadequate biosecurity measures such as buying in animals from herds with an unknown disease history was a risk for introducing diseases into a herd.

Vaccination strategies on the farms are also a concern as only 32% (97/300) of the animals tested were vaccinated against *B. abortus* (Strain 19 or RB51). This is one of the most important ways to control the disease this is a concern.

All herds tested positive for *C. burnetii* antibodies. Unit 4 returned the highest apparent prevalence at 5.6% (17/300). There were suspected positive results as well, as indicated in Table 3.1, these animals should ideally be re-tested to see if there is an increase or decrease in titres: this will give an indication of active infection. An interesting finding was that two of the *Brucella* negative units that maintained closed herds under extensive conditions (Unit 3 & 4) returned the highest apparent seroprevalence of *C burnetii* at 13.11% and 25% respectively. On the other hand, one of the *Brucella* positive units (Unit 2) and the intensively fed unit (Unit 5) returned the lowest apparent seroprevalence for *C. burnetii* at 3.27% and 3.44% respectively. The reasons for this phenomenon are not clear and should be investigated.

Unit 3 reported a high calving percentage of 86%. No antibodies to *Brucella* were detected on this unit, but there were antibodies to *Coxiella.* Unit 4 had the highest number of animals with *Coxiella* antibodies at 25% and reported a lower calving percentage of 75%. Further

investigation by molecular methods will be necessary to determine whether this infection plays a role in the abortions.

An interesting finding was that at one of the units unit 4, two distinct breeds were farmed and tested, the Angus (*Bos taurus*) as well as Thuli (*Bos indicus*) cattle. The Angus cattle had higher antibody titres to *C. burnetii* than the Thuli cattle. The possibility of inter-breed differences in cattle towards infections such as *Coxiella* needs to be researched on its own.

In future, seroprevalence of *B. abortus* and *C. burnetii* should be investigated in the human population in this area. Particularly in the case of *Brucella* there are antibodies in cattle, which raise concern for human exposure to the bacteria. This has significant public health implications and can be an incentive for the control of the disease, especially when looking at the risk the infection poses to workers on the farm, workers in the abattoir slaughtering the animals or the veterinarians that assist with dystocia on the farm. A study in Afghanistan demonstrated a correlation between animal and human seroprevalence for both *Brucella* and *C. burnetii* (Akbarian *et al*. 2015).

The best way to predict seroprevalence would be to follow all the herds tested in the area over time, or to include every herd in the Bethlehem area. Further investigations of aborted foetuses combining necropsy, histopathology and molecular methods will be necessary to determine whether *C. burnetii* could be the cause of some of the abortions. If the *Coxiella* ELISA could be validated for South African conditions more samples could be tested annually to look at the presence of antibodies.

The most important means of control of these infections will be to take the 'One Health' approach. Covering all the different areas were disease control can be implemented (Godfroid & Dahouk, 2013). This would include the wildlife side (as we are not completely aware which animals might be carriers of the diseases and what role they play), domestic animal health, human health as well as the environment. Mitigation steps may include the proper disposal of foetuses to prevent further spread. Government funded investigations and mitigations will allow a country like South Africa to control the disease and decrease the risk of zoonotic infections.

While compulsory testing of all animals susceptible to *B. abortus* is still not a prerequisite by law, the disease will not be controlled. This leads to a high risk of spreading this disease as the infection might not result in great abortion storms immediately and once it spread to multiple cows, abortions are noticed. Other factors that contribute to the successful control of this disease include man power to test animals for this disease, enough laboratories to test and send out results promptly. When results are available positive animals need to be removed from herds and slaughtered as soon as possible to prevent the spread of the disease. Animals that are not removed as soon as possible, pose a risk to spread the disease further within and between herds.

The overall findings in this study indicate that there are antibodies circulating in the population and that a more in depth search needs to be done to find out if the presence of antibodies can be linked to the causative agent in cases of abortions or foetal loss.

The study was difficult in the sense of sample selection. The samples were only selected from farmers that presented to the Department of Agriculture and Rural Development and not a true representation of the total number of farms in the area. It is compulsory for these farmers to report any foetal loss, but this is not the case at all. This makes sample selection difficult. Sampling for these diseases was done at a time when the recourses were available. This is also not always possible as the resources such as travel allowance, bleeding tubes and laboratories for testing for *Brucella* spp. are not always available due to various reasons. There was no laboratory to do serological tests for *C. burnetii* when the samples were collected and the test has not been validated under South African conditions. The ELISA kit was procured and the testing was done by the researcher at the Department of Veterinary Tropical Diseases.

The results are significant in the sense that foetal loss is recoded and there is serological evidence that Brucella species and C. burnetii is circulating in the area. If not controlled, these diseases will continue to be a problem and might become a bigger problem in the future.

Chapter 5: Conclusion

The conclusion is that there is a seroprevalence of 22.5% for *Brucella* spp. and 11% for Coxiella thus rejecting the null hypothesis. There are *Brucella* as well as *C. burnetii* antibodies circulating in cattle in the Bethlehem area in the Free Sate, although the validity of the ELISA for Q fever needs to be considered.

The next step would be to investigate the occurrence of these bacteria in aborted foetuses by conventional bacterial culture and/or molecular methods the bacterial culture for *Brucella abortus* requires a Biosafety Level 3 laboratory.

Better education of the public is necessary to raise awareness of brucellosis and how it presents as well as the clinical signs in humans. Improved communication between the animal and human health sectors is necessary as seroprevalence of *Brucella* spp. can be an early warning sign to human health practitioners to include brucellosis as a differential diagnosis for people working on farms where positive animals have been identified.

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ANNEX A: Animal ethics consent form

INFORMED CONSENT FORM

We, the undersigned, hereby agree that the animal(s), as specified below, may be used by the researcher(s), as specified below, in the procedures as explained below:

To be completed by the researcher(s)

- **NAME OF THE RESEARCHER(S):**
  Dr JC Du Plessis

- **NAME OF RESEARCH PROJECT:**
  Seroprevalence of *Brucella* sp. and *Coxiella burnetti* among cattle in the Bethlehem area, Free State, South Africa

- **PURPOSE OF RESEARCH PROJECT:**
  Determine if animals in Bethlehem area in the Free State Province of South Africa have been exposed to the bacteria described above.

- **DETAILED PROCEDURE(S) TO BE PERFORMED:**
  Collection of Serum samples from cattle on the farm

- **RISK(S) INVOLVED IN SPECIFIED PROCEDURE:**
  Animals can become injured in the process of restraining in the crush during collection

- **IDENTIFICATION OF ANIMAL TO BE USED:**
  Ear tag number will be used

Title of project: Seroprevalence of *Brucella* spp. and *Coxiella burnetti* among cattle in the Bethlehem area, Free State, South Africa

Details of the study

Researcher: Dr JC Du Plessis

Serum samples will be collected from cattle on the farm, depending on the amount of farmers that participate that will determine the amount of samples that will be collected.

Samples can be small volumes, but it must be good quality serum. The Cow identification number, date collected and place of origin will be recorded on the sample submission form and samples should be accompanied by a veterinary movement permit.

Serum from biobanks can be returned to the owner subject to and in accordance with an agreement.

Procedures regarding confidentiality

Unless recognition of contributions is requested (please refer to sections eight and nine of the consent form), data retained for this project is limited to species (essential), date and place of collection (subject to availability).

The use of the samples, data in research, publications, sharing and archiving.

Serum samples remaining after use in this project, not returned to the owner/supplier, and not destroyed in terms of the Animal Diseases Act, Act 35 of 1984, will be stored in a DAFF approved biobank of the University of Pretoria for use in subsequent studies and validation of assays resulting from these studies.

Data from this project and subsequent studies will be used for academic purposes and publication. Sharing of owner specific data according to an agreement is permitted. Data will be stored and archived in accordance with the processes and principles of the University of Pretoria.

If so requested, owners of serum samples will be acknowledged in publications.

1. To be completed by the animal's owner or person duly authorized to sign on his/her behalf:

   - **NAME OF OWNER: _____**
   - **HAVE YOU RECEIVED DETAILED INFORMATION REGARDING THE PROPOSED STUDY?**

     ☐ **YES**

     ☐ **NO**
   - **HAVE ALL THE RISKS INVOLVED IN THE PROCEDURE BEEN EXPLAINED TO YOU AND DO YOU FULLY UNDERSTAND THESE RISKS?**

     ☐ **YES**

     ☐ **NO**
   - **DO YOU GRANT FULL CONSENT FOR THE PROCEDURE TO BE PERFORMED?**

     ☐ **YES**

     ☐ **NO**

2. The undersigned parties further agree that no compensation will be payable to the animal's owner or anybody else and that all research associated costs will be covered by the researcher(s).
3. The undersigned parties further agree that this form would serve to fully indemnify the University of Pretoria and the undersigned researcher(s) against any future claims resulting from the specified procedure by or on behalf of the animal's owner.

4. The undersigned parties further agree that no material of any kind, including data and research findings, obtained or resulting from the procedure, would be passed on to any third party or used for any purpose other than that specified in this form, except with the written consent of the undersigned owner of the animal.
5. No personal information will be disclosed but may be used unanimous in publications. As owner it is my right to withdraw my animal(s) from the trial

_____	_____
SIGNATURE RESEARCHER(S)	SIGNATURE OWNER
_____	_____
SIGNATURE WITNESS	DATE

ANNEX B: Animal ethics approval certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Investigation of seroprevalence of Brucella sp. and Coxiella burnetii among cattle in the Bethlehem area of the Free State province of South Africa**
PROJECT NUMBER	**V044-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. JC du Plessis**

STUDENT NUMBER (where applicable)	**U_28106700**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPESIES/SAMPLES	**Cattle**
NUMBER OF ANIMALS	**300**
Approval period to use animals for research/testing purposes	**June 2018 - June 2019**
SUPERVISOR	**Dr. A Jonker**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 27 July 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15