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Bacterial and fungal causes of abortion in domestic ruminants in South Africa

by

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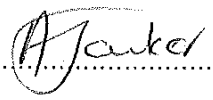
Declaration

I Annelize Jonker, declare that the thesis which I hereby submit for the degree DOCTOR OF PHILOSOPHY, in the Faculty of Veterinary Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.



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Ethics statement

The author, whose name appears on the title page of this thesis, has obtained the required ethics approval/exemption for the research described in this work.

The author declares that they have observed the ethical standards required in terms of the University's Code of ethics for scholarly activities.

ABSTRACT

Abortions in cattle, sheep and goats represent important economic losses in the agricultural industry. Determining causes of abortions is important for control efforts, but can be challenging.

This study investigated detection methods for and significance of bacteria and fungi as agents of abortion in domestic ruminants in South Africa. Retrospective data was collected by searches of case reports (2006-2016) of participating pathology and bacteriology laboratories. During the prospective study (2017-2019), samples were analysed by a combination of conventional bacteriology methods. Quantitative real-time PCR assays for detection of the *Chlamydiales*, *Chlamydia abortus*, *Chlamydia pecorum*, *Parachlamydia acanthamoeba* and *Waddlia chondrophila* were created by combining primers and probes selected from literature. These assays were optimized and employed to analyse samples from 25 cases, with placentitis and/or pneumonia lesions, selected from the prospective study.

The retrospective study reported 288 cases from six provinces. Diagnostic rate was 35.1%. The prospective study reported 135 cases from six provinces. Diagnostic rate was 42.2%. *Brucella* species were most commonly isolated in both the retrospective and prospective studies at 7.3% and 7.4% of cases, respectively. The qPCR assays detected *Chlamydiales* in 60% of cases with placentitis and/or pneumonia. *Chlamydia abortus*, *P. acanthamoeba* and *W. chondrophila* were detected in bovine; and *C. pecorum* and *W. chondrophila* in ovine and caprine cases. *Chlamydiales* were detected in three previously inconclusive cases. Identification was improved from genus to species level (*C. pecorum*).

In conclusion, retrospective laboratory records yielded valuable passive surveillance data. Submission of placenta was an important factor in successful diagnosis. The most effective combination of conventional culture methods was aerobic culture together with selective *Brucella*, *Campylobacter* and fungal culture. This combination lead to improvement of the diagnostic rate in comparison with the retrospective study. *Brucella abortus* was the most common cause of bovine abortion over 12 years in the retrospective and prospective studies. *Trueperella pyogenes* was the second most common. Real-Time qPCR

assays improved detection of *Chlamydiales* and differentiation to species level. The first detection of *P. acanthamoeba* and *W. chondrophila* in abortion cases in South Africa was reported indicating a potential role in abortions in this country.

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SUMMARY

Investigation into bacterial and fungal causes of abortion in domestic ruminants in South Africa

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Infectious abortions in domestic ruminants represent an important cause of economic losses in the agricultural industry worldwide. Bacterial and fungal agents of abortion may be contagious or opportunistic. Many are zoonotic posing an occupational risk to humans in close contact with animals. Retrospective data from veterinary diagnostic laboratories could be a valuable, cost-effective source of passive surveillance. Studies worldwide highlight significant abortion agents and percentage of successful diagnoses. Molecular methods can improve detection of bacteria such as the *Chlamydiales*.

This study investigated detection methods for and significance of bacterial and fungal agents of abortion in cattle, sheep and goats in South Africa. In Objective I a retrospective survey was conducted including data from 2006 to 2016 in South Africa. Objective II was a prospective study of bacterial and fungal agents of abortion by means of conventional bacteriological methods. In Objective III PCR

assays were employed to detect bacterial agents of abortion to extend diagnostic ability beyond the limits of conventional culture.

In this study a retrospective survey of cases from participating laboratories revealed a diagnostic rate (proportion of cases in which a diagnosis was made) of 35%. Availability of placenta samples were often associated with successful diagnoses. Furthermore, *Brucella* emerged as the most common abortifacient in the provinces covered and *Trueperella pyogenes* the second most common.

During the prospective study, routine clinical samples from aborted foetus cases were analysed. Analyses employed for conventional bacteriology were aerobic, anaerobic, *Campylobacter*, *Leptospira*, *Mycoplasma* and fungal culture. This removed test selection bias in the bacteriology laboratory, but not overall. The diagnostic rate improved to 42.2%. Bacteria and fungi such as *Campylobacter jejuni* and *Rhizopus* species were isolated as result of the selective methods employed. *Brucella* and *T. pyogenes* were the most common isolates.

Real-Time qPCR assays were optimised and applied for detection of the order *Chlamydiales*, *Chlamydia abortus*, *Chlamydia pecorum*, *Parachlamydia acanthamoeba* and *Waddlia chondrophila.* Primers and probes were taken from literature to create one singleplex and two duplex assays. Twenty-five clinical cases from the prospective study, with pathological lesions of placentitis or pneumonia, were investigated by application of the Real-Time qPCR assays in this study. *Chlamydia abortus*, *P. acanthamoeba* and *W. chondrophila* were detected in bovine and *C. pecorum* and *W. chondrophila* in ovine and caprine cases. *Chlamydiales* were detected in three previously inconclusive cases. Identification was improved from genus to species level (*C. pecorum*). Four cases remained inconclusive.

In conclusion, valuable passive surveillance data was retrieved from retrospective laboratory records. Submission of placenta was an important factor in successful diagnosis. The most effective combination of conventional culture methods was aerobic, selective *Brucella*, *Campylobacter* and fungal analyses. This combination lead to improvement of the diagnostic rate in comparison with the retrospective study. *Brucella abortus* was the most common cause of bovine abortion over 12 years in the retrospective and prospective studies. *Trueperella pyogenes* was the second most common isolate in both studies.

Real-Time qPCR assays improved detection of *Chlamydiales* and differentiation to species level. The first detection of *P. acanthamoeba* and *W. chondrophila* in abortion cases in South Africa was reported indicating a potential role in abortions in this country.

CHAPTER 1

Introduction: Literature Study

Bacterial and fungal causes of abortion

Abstract

Infectious abortion is a worldwide phenomenon that leads to production losses. Bacterial and fungal agents of abortion may be contagious or opportunistic. In addition, many are zoonotic, posing a risk to humans in close contact with animals giving birth, aborting or products of abortion. Studies across the world have served to highlight significant agents of abortion as well as the number of successful diagnoses. Improving diagnostic methods could lead to increased successful diagnoses leading to increased submission of foetuses. This in turn would lead to increased opportunities for implementing control measures. Data from veterinary diagnostic laboratories could serve as a valuable form of passive surveillance. Conventional and/or molecular investigation by means of a set panel of tests targeting agents of abortion will contribute to eliminate test selection bias. Molecular methods will improve detection of difficult to culture bacteria such as the *Chlamydiales*.

Introduction

Pregnancy wastage (the combination of embryonal, foetal and neonatal deaths) (Baumgartner 2014) is a worldwide problem that leads to production losses in ruminants, translating to economic losses in the industries dependent on them (Holler 2012; Wheelhouse & Dagleish 2014; Borel *et al*. 2014). The impact on production is easy to underestimate (Baumgartner, 2015). In a study conducted in California in 2016, Clothier & Anderson indicated that the cost of a lost bovine

foetus may amount to US$1900 per foetus. The total cost of bovine foetal loss per year was estimated at 22 to 45 million Swiss Francs in Switzerland and 165 million US$ in Argentina (Campero *et al*. 2003; Borel *et al* 2006).

The scope of this study includes foetal death and abortion. Embryonal death, stillbirth and neonatal death are excluded. Abortion forms part of pregnancy wastage and is defined as the expulsion of the foetus from the uterus before it is full term and viable (Holler 2012, Baumgartner 2014). The foetus is the part of the conceptus that develops to a live neonate from the end of organogenesis to the end of second stage parturition (Givens & Marley 2008). The gestational age of a foetus may be estimated by comparing the crown-rump length, weight and external characteristics to a standardised table (Kirkbride 1986, Njaa 2012). Foetal death is the death *in utero* of the foetus before it is full term, whereas stillbirth is the delivery of a dead full-term foetus (Holler 2012). Foetuses that die *in utero* may present with immature, oedematous red tissues, clear red fluids in body cavities, pale livers and friable kidneys (Baumgartner 2016). Death during birth may result in lesions such as fractured ribs, partial aeration of the lungs or a ruptured liver with haemorrhages (Baumgartner 2016). Neonatal death refers to mortalities during the first four weeks after birth (Dennis 1970). Neonatal deaths shortly after birth are characterised by aeration of the lungs, milk in the stomachs, umbilical artery thrombosis (Baumgartner 2016). Advanced autolysis may mask distinguishing features which can complicate age estimation.

Abortion rates less than 2% usually do not indicate that an investigation is necessary (Menzies 2011). Most abortions are sporadic with less than 5% of pregnant animals aborting (Givens & Marley 2008). Abortion rates higher than 5 to 8% are routinely seen as an indication that investigation is necessary. However, an abortion rate of between 2% and 5% should also be investigated as it may be an indication that an endemic infectious agent is present (Menzies

2011). Abortion storms, when abortion rates may be 10% to 40% or greater, can be disastrous to the producer (Holler 2012).

Causes of abortion may be categorised as infectious or non-infectious (Givens & Marley 2008). Infectious causes of abortion are microorganisms, such as bacteria, fungi, viruses (Bovine viral diarrhoea virus, Bovine herpesvirus-1, Bluetongue virus, Rift Valley Fever virus), protozoa (*Tritrichomonas foetus*, *Neospora caninum*, *Toxoplasma gondii*), and/or their toxins (Givens & Marley 2008; Baumgartner 2014). The pregnant uterus is not a sterile environment. Low numbers of bacteria are often present without any associated inflammation (Karstrup *et al*. 2017). Infectious agents may have a direct effect on the foetus by infection or toxins, or an indirect effect by infection of the placenta (Givens & Marley 2008). Non-infectious causes are plant toxins, heat shock, stress, severe trauma, hormonal imbalances, and malnutrition (Menzies 2011; Baumgartner 2014). Studies from both developed and developing countries report more infectious than non-infectious aetiologies. Selection of topics for studies and/or better characterisation and understanding of infectious causes may contribute to this phenomenon (Baumgartner 2014). Most of the infectious causes of foetal loss reported are bacterial and several of these are zoonoses presenting a potential occupational risk to humans that encounter products of abortion (da Silva *et al*. 2009; Barkallah *et al*. 2014; Borel *et al.* 2014, Clune *et al*. 2021a). Bacterial and fungal causes of foetal death and abortion will be the focus of this study. Viral and protozoal causes of abortion are excluded.

Bacterial causes of abortion

Bacteria implicated in abortion may be grouped as contagious or opportunistic. Contagious agents of abortion are bacteria such as *Brucella*, *Chlamydia*, *Campylobacter fetus* subspecies venerealis and *Leptospira interrogans* (Anderson *et al*. 1990). *Brucella* species are members of the family

Brucellaceae. They are Gram-negative, partially acid fast, facultative intracellular bacteria that infect a range of mammals including ruminants, humans, pigs, cervids, lagomorphs, rodents, canids, and marine mammals (Songer & Post 2005). *Brucella abortus* and *Brucella melitensis* are primarily found in ruminants. Cattle are most susceptible to *B. abortus*, however they may also on occasion become infected by *B. melitensis*. Sheep and goats are most susceptible to *B. melitensis*, although they can become infected by *B. abortus* (WOAH 2016).

In domestic ruminants, infection occurs mainly via ingestion (Anderson 2007). Sources of infection are placenta, foetuses, uterine discharges, milk, and urine from infected animals (WOAH 2022). *Brucella* spp. multiply in macrophages in local lymph nodes and then spread via the blood circulation to other organs such as the uterus, mammary gland and supramammary lymph nodes. Growth is stimulated by erythritol produced by the gravid uterus. *Brucella* spp. invade the placental trophoblasts leading to placentitis and infection of the foetus. Late term abortions as result of placental damage and endotoxaemia usually follow initial infection of a pregnant animal (Anderson 2007; Markey *et al*. 2013).

Bacteria in four families in the order *Chlamydiales* are of significance in contagious abortions, namely *Chlamydiaceae*, *Waddliaceae*, *Parachlamydiaceae* and *Rhabdochlamydiaceae*. Members of these families fall in the difficult to culture category (Vidal *et al*. 2017). They are Gram-negative, partially acid fast, obligate intracellular bacteria that do not stain with the Gram stain method and do not grow on conventional growth media (Songer & Post 2005; Borel *et al.* 2014).

The most important family is the *Chlamydiaceae* to which *Chlamydia* species belong (Markey *et al.* 2013). Infection by *Chlamydiaceae* occurs via ingestion or inhalation (Borel *et al.* 2018). Faeces, urine, placenta or discharges from the nose, eyes or vulva of an infected animal may serve as the source of infection. A

study by Berri *et al*. (2004) suggests that the pathway of infection may lead via the blood circulation to the placenta where trophoblasts are infected. *Chlamydia abortus (C. abortus)*, the agent of ovine enzootic abortion, can also infect cattle and goats and become endemic in herds (Markey *et al*. 2013; Borel *et al.* 2014). Infection with *C. abortus* may result in late term abortion or weak full-term calves, lambs or kids (Givens & Marley 2008; Borel *et al.* 2014; Giannitti *et al*. 2016). *Chlamydia pecorum* (*C. pecorum)* infects cattle, sheep and goats leading to an intestinal carrier state (Gianitti *et al.* 2016; Clune *et al*. 2021b; Westermann *et al*. 2021). It has been implicated in cases of small ruminant abortion in the USA (Gianitti *et al*. 2016), France, Morocco, Algeria, Tunisia and Australia (Berri *et al*. 2009; Clune *et al*. 2021b; Westermann *et al*. 2021). The role of *C. pecorum* as an agent of abortion in ruminants and as a zoonotic agent needs to be investigated further (Berri *et al*. 2008, Gianitti *et al*. 2016, Clune *et al*. 2021b).

Waddlia chondrophila (*W. chondrophilia*) belongs to the family *Waddliaceae* and can cause abortion in cattle (Markey *et al*. 2013). Since 1986, cases of bovine abortion where *W. chondrophilia* was isolated were reported from the northern hemisphere in the United States, Germany and Switzerland (Borel *et al.* 2018). The presence of *W. chondrophilia* was also detected by polymerase chain reaction (PCR) in African cattle in Tunisia, but no comparison was made to histopathological lesions (Barkallah *et al.* 2014). *W. chondrophilia* is often found in combination with other agents of abortion such as *Listeria monocytogenes* and *Parachlamydia acanthamoebae* (Barkallah *et al* 2016). No literature could be found on *W. chondrophilia* in South Africa or other countries in the southern hemisphere. Reasons for this may be that these bacteria require isolation in cell culture which is not routinely offered in diagnostic bacteriology laboratories. In addition, molecular abortion investigations do not commonly include testing for *W. chondrophilia* (Borel *et al.* 2018) hence it could be missed, contributing to the

fraction of abortion cases without aetiological diagnosis. The role *W. chondrophilia* plays in abortion in South Africa and elsewhere therefore needs to be investigated further (Barkallah *et al* 2016; Borel *et al.* 2018).

Parachlamydia acanthamoebae (*P. acanthamoebae*) of the family *Parachlamydiaceae* was first reported in 1997 by Amann *et al*. An amended description of the order *Chlamydiales* providing for the family *Parachlamydiaceae* was proposed in 1999 by Everett *et al*. Since then, *P. acanthamoebae* has been identified as an emerging agent of bovine, ovine and caprine abortion in several northern hemisphere countries, e.g., Switzerland, United Kingdom, Scotland, Ireland and Hungary (Borel *et al*. 2007, Ruhl *et al*. 2009, Wheelhouse *et al*. 2010, Deuchande *et al*. 2010, Wheelhouse *et al.* 2014, Borel *et al* 2018). A study conducted in Tunisia reported *P. acanthamoebae* in post abortion vaginal samples from cattle (Barkallah *et al*. 2014). As is the case with *W. chondrophilia*, mixed infections with other agents of abortion are reported (Blumer *et al*. 2011, Borel *et al* 2018). More work is necessary to define the role of *P. acanthamoeba* in abortion, particularly isolation from abortion material (Borel *et al*. 2018). As for *W. chondrophilia*, the role of this agent in abortions in South Africa must be determined.

Campylobacter and *Arcobacter* species are members of the *Campylobacteraceae* family. These bacteria are microaerophilic, facultative intracellular, motile, thin, spiral or curved Gram-negative rods. They are mucosal commensals of the intestine or genital tract of mammals and birds (Campero *et al*. 2005; Markey *et al* 2013). Species most associated with abortion in ruminants are *Campylobacter fetus* subspecies *venerealis*, *Campylobacter fetus* subspecies *venerealis* biovar intermedius, *Campylobacter fetus* subspecies *fetus (C. fetus* subsp. *fetus)*, *Campylobacter jejuni* (*C. jejuni*) (Moeller 2001, Anderson 2007, Van der Graaf-van Bloois *et al.* 2014, Hameed *et al*. 2019), *Arcobacter*

skirrowii (A. skirrowii), *Arcobacter cryaerophilus* (*A*. *cryaerophilus*) and *Arcobacter butzleri* (*A. butzleri*) (Songer & Post 2003).

Campylobacter fetus subspecies *venerealis (C. fetus* subsp. *venerealis)* occurs worldwide, most commonly in cattle (Sprenger *et al*. 2012). It is the only member of the family that becomes endemic in herds. It is a resident of the genital tract and is transmitted venereally. From the vagina the bacteria migrate through the cervix into the uterus where they attach to and invade epithelial cells to cause a chronic endometritis which results in abortion and infertility (Campero *et al*. 2005; Sprenger *et al*. 2012). In South Africa, *C. fetus* subsp. *venerealis* is reported to be more common than *C. fetus* subsp*. fetus* (Coetzer & Oberem 2018). *C. fetus* subsp. *venerealis* biovar intermedius has been isolated from bovine samples in South Africa and presents challenges when it comes to phenotypic identification (Van der Graaf-van Bloois *et al.* 2014).

Campylobacter fetus subsp. *fetus*, *C. lari*, *C. jejuni*, *A. skirrowii*, *A cryaerophilus* and *A butzleri* have been associated with abortion in sheep (Campero *et al*. 2005; Bath *et al*. 2013). Transmission occurs orally after which the mucosa of the intestine is colonised. If a bacteraemia develops, bacteria are carried to the pregnant uterus where an infection is established in the placenta. Abortions are sporadic (Campero *et al*. 2005).

Leptospira belongs to the family *Leptospiraceae*. They are Gram-negative, motile spiral bacteria (spirochaetes) with hooked ends (Coetzer & Oberem 2018, Vidal *et al* 2017). The pathogenic leptospirae are serovars of *Leptospira interrogans (L. interrogans)*. There are 250 known serovars worldwide which are divided in 25 serogroups according to their serological phenotype. (Vidal *et al*. 2017). Since the advent of molecular characterisation pathogenic leptospirae were also divided into 13 genomospecies. There is no correlation between serovars and genomospecies (Jobbins *et al*. 2014).

Infection occurs via contact with a moist environment contaminated by urine containing *L. interrogans*. *Leptospira interrogans* can penetrate intact mucous membranes or skin wounds (Anderson 2007). They find their way to the bloodstream and are transported to the liver where they localise. Primary replication takes place and leptospirae are released into the blood again. During the second bacteraemic phase they localise in the lung, brain, kidneys, eyes or the pregnant uterus. *Leptospira interrogans* may cross the placenta and infect the foetus (Coetzer & Oberem 2018).

It is likely that leptospirosis is an underdiagnosed cause of ruminant abortion over the world as it is difficult to culture (Vidal *et al.* 2017). Internationally, *L. interrogans* serovars implicated in cattle abortion are *Leptospira* Hardjo (*L.* Hardjo) and *Leptospira* Pomona (*L.* Pomona) (Songer & Post 2005). Abortion rates are usually below 10% in cattle infected with *L.* Hardjo. *L.* Pomona infection leads to a higher abortion rate (Anderson 2007). In sheep, *L* Hebdomadis, *L* Australis, *L* Pomona, and *L*. Sejroe have been implicated in cases of abortion. *L.* Pomona, *L*. Sejroe, *L*. Icterohaemorrhagiae and *L* Grippotyphosa have been implicated in cases of abortion in goats (Coetzer & Oberem 2018). Abortions or stillbirths may occur or weak young may be born (Coetzer & Oberem 2018).

There are many opportunistic agents of abortion. Opportunistic bacteria may be part of the animal's normal flora (*Trueperella pyogenes* and *Histophilus somni*) or may be common in the environment (*Bacillus* species and *Escherichia coli*) (Holler 2012; Baumgartner 2014). These bacteria usually cause sporadic abortions and are implicated in cases of abortion most often (Holler 2012).

Coxiella burnetii belongs to the family *Coxiellaceae*. It is a Gram-negative, partially acid fast, obligate intracellular bacterium (Coetzer & Oberem 2018; Markey *et al* 2013; Borel *et al* 2014). *C. burnetii* may infect a variety of mammals such as cattle, water buffalo, sheep, goats, and humans (Khademi *et al*. 2019,

Robaj *et al*. 2021). It also infects ticks (Coetzer & Oberem 2018). Seropositivity is common among ruminants, but clinical manifestation of disease such as abortion is rare in comparison. Goats appear to be most likely to abort due to *C*. *burnetii* infection (Agerholm 2013).

The main route of infection is respiratory when dust particles with adherent environmentally resistant bacteria in the form of small dense cells are inhaled. However, *C. burnetii* can also be transmitted by tick bite (Agerholm 2013, Coetzer & Oberem 2018). After entering the body small dense cells are taken up by phagocytes and distributed via the blood circulation. Small dense cells are shed in large numbers in products of abortion or normal birth, milk and faeces (Songer & Post 2003, Van den Brom 2015, eds. Coetzer & Oberem 2018).

Listeria species are small, motile Gram-positive rods belonging to the *Listeriaceae* family (Markey *et al*. 2013). Most are saprophytes that live on decomposing plant material (Songer & Post 2005). *Listeria monocytogenes,* the most well-known abortigenic member of the family, is a facultative intracellular bacterium (Dreyer *et al*. 2015). Another member of the family, *Listeria ivanovii,* was implicated in abortion in cattle in the USA (Anderson 2007). Abortions caused by *Listeria* spp. are usually sporadic and late-term, but abortion storms have been reported (Songer & Post 2005, Anderson 2007). Subsequent pregnancies may be affected (Baumgartner 2014). Infection often occurs via ingestion of silage that was not fermented properly (Anderson 2007). Pathogenic *Listeria* spp. invade epithelial cells of the gastrointestinal tract by inducing phagocytosis. Inside the cell, they escape the phagosome and multiply in the cytoplasm. From the infected cell, they will spread laterally to neighbouring cells or they will leave the cell via exocytosis leading to bacteraemia. In the pregnant animal, the cells of the placentomes may be invaded and the placental barrier

damaged. Subsequently the bacteria may also infect the foetus (Songer & Post 2005).

Mycoplasma and *Ureaplasma* species belong to the family *Mycoplasmataceae*. These bacteria are of Gram-positive lineage, but they do not have cell walls so they do not stain by the Gram stain method. Very limited metabolic functions oblige them to exist in close association with host cells (Songer & Post 2005). Many *Mycoplasma* and *Ureaplasma* species are commensals of the upper respiratory and lower reproductive tract (Trichard *et al* 1981, Songer & Post 2005). The most pathogenic is *Mycoplasma bovis*, an extremely virulent mycoplasma of cattle that has been implicated in cases of abortion. *Mycoplasma canadense* has also been implicated in abortions in cattle (Songer & Post 2005). *Ureaplasma diversum* has been associated with opportunistic abortions in cattle. *Ureaplasma diversum* was often reported in Canada, but not in the United States (Anderson 2007). The significance of *Mycoplasma* species and *U. diversum* as agents of abortion in South Africa is not clear (Coetzer & Oberem 2018). Transmission of both *Mycoplasma* species and *U. diversum* occurs by direct contact by means of droplets of body secretions that are inhaled or venereally transmitted. Virulent *Mycoplasma* such as *M. bovis* can enter the bloodstream and reach many organs including the pregnant uterus (Songer & Post 2005).

Salmonella species belong to the family *Enterobacteriaceae* (Markey *et al.* 2013). These Gram-negative, facultative intracellular bacteria form part of the normal flora of the intestine. Carrier animals commonly contribute to the introduction and maintenance of *Salmonella* in a herd. Internationally, the *Salmonella* serovars most associated with abortions are *Salmonella* Dublin and *Salmonella* Munster in cattle and *Salmonella* Abortusovis in sheep (Songer & Post 2005). Salmonellae survive well in the environment. Water, soil and feed may be sources of infection. After ingestion, *Salmonella* that survived the acid

environment of the stomach will adhere to and enter enterocytes in the small intestine. When they exit the enterocytes, Salmonellae may be phagocytised by macrophages where they survive by preventing the fusion of the phagosome and lysosome. The infected macrophages are carried in the bloodstream to the lymph nodes and organs including the pregnant uterus (Songer & Post 2005). The aborting animal is often clinically ill (Borel *et al* 2014).

Anaerobic bacteria are fastidious in their growth requirements. They cannot tolerate oxygen; therefore, they require an anaerobic environment for growth (Songer & Post 2005). They are not commonly reported as causes of abortion; this phenomenon may be ascribed to the fact that anaerobic culture is not routinely included in abortion investigations (Borel *et al* 2014). Anaerobic bacteria such as the Gram-negative rods *Fusobacterium necrophorum*, *F nucleatum* (family: *Fusobacteriaceae*) and *Bacteroides fragilis* (family *Bacteroidaceae*) are implicated in cases of ruminant abortion on rare occasions. They are common in the environment, on mucosal surfaces and in the intestines of animals. As is the case with many other bacterial and fungal causes of abortion, these bacteria are opportunists (Songer & Post 2005).

Several other opportunistic bacteria may cause infections of the placenta and foetus. Examples are *Pasteurella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Helcococcus ovis, Propionibacterium* spp*., Vibrio metschnikovii* and *Yersinia pseudotuberculosis* (Agerholm *et al* 2006; Anderson 2007; Givens & Marley 2008; Holler 2012; Borel *et al*. 2014; Vidal *et al.* 2017). Multiple abortions in a herd caused by one of these agents often serve as an indication of another underlying health problem such as bovine viral diarrhoea virus infection (Anderson 2007; Borel *et al.* 2014).

Mycotic or fungal abortion in cattle, sheep and goats may be caused by several opportunistic fungi that are normally present as saprophytes in the environment

of the animal. These fungi may be hyphal or yeast forms (Songer & Post 2005; Anderson 2007). Fungi most associated with abortion are *Aspergillus* species (especially *Aspergillus fumigatus*) and *Candida* species (Anderson 2007; Holler 2012). Other species implicated are *Lichtheimia* (*Absidia*) *corymbifera*, *Mortierella wolfii*, *Mucor* species and *Rhizopus* species (Anderson 2007; Borel *et al* 2014). Abortion is sporadic and subsequent pregnancies are not affected (Pal 2014). Mycotic abortions are usually reported after feeding of large quantities of mouldy feed. Spores that are inhaled or ingested may lead to a primary infection in the lungs or intestines from where fungal elements may enter the bloodstream and disseminate to the placenta where an infection is established. From here the fungus can grow into the amniotic fluid to reach the foetus and colonize the skin to form fungal plaques (Holler 2012, Pal 2014).

The most common macro- and microscopic lesions in foetal tissues associated with bacterial and fungal infection are summarised in Table 1.1.

Table 1.1 The most common clinical signs, placental and foetal necropsy and/or histopathological lesions associated with selected bacterial and fungal agents of abortion in ruminants. (Adapted from 'Table of infectious causes of abortion in cattle', http//www.partners-in-reproduction.com/reproduction-cattle/table-infectious-abortions.asp Accessed: 2016/04/05)

INFECTIOUS AGENT	**ANIMAL SPECIES AFFECTED**	**NECROPSY/ HISTOPATHOLOGY LESIONS**	**REFERENCES**
BACTERIA *Arcobacter skirrowii*	Sheep	Placenta: purulent placentitis Foetus: mummified, autolytic, or alveolar immaturity	Bath *et al*. 2013
Bacillus spp. *B cereus* *B licheniformis*	Cattle, sheep, goats	Placenta: necrotic, haemorrhagic or suppurative placentitis	Logan 1988 Baumgartner 2014

		Foetus: suppurative pneumonia, meningoencephalitis, fibrinous pericarditis Histology: disseminated vascular emboli, lymphoid hyperplasia of spleen and lymph nodes	
Brucella abortus *Brucella melitensis*	Cattle, sheep, goats	Clinical signs: retained placenta Placenta: cotyledons necrotic, red-yellow thickened intercotyledonary area with yellow exudate, placentitis with oedema. Foetus: normal or autolytic with bronchopneumonia.	Anderson 2007 Givens & Marley 2008 Borel *et al.* 2014
Campylobacter fetus subsp. *venerealis* *Campylobacter fetus* subsp. *fetus*	Cattle, sheep	Placenta: mild placentitis, haemorrhagic cotyledons and an oedematous intercotyledonary area. Foetus: fresh or autolysed; mild fibrinous pleuritic, peritonitis, bronchopneumonia.	Moeller 2001 Anderson 2007
Campylobacter jejuni	Sheep	Placenta: necro-purulent placentitis (occasionally with vasculitis). Foetus: fresh or autolysed; bronchopneumonia, hepatic necrosis.	Hedstrom *et al* 1987
Chlamydia abortus	Cattle, Sheep, Goats	Placenta: Purulent or necrotic placentitis with vasculitis Foetus: necrotic pneumonia or hepatitis	Buxton *et al*. 2002
Chlamydia pecorum	Sheep, Goats	Placenta: Purulent or necrotic placentitis with vasculitis. Foetus: necrotic pneumonia or hepatitis, necrotic enteritis, necrotic nephritis, meningoencephalitis.	Buxton *et al*. 2002 Gianitti *et al*. 2016 Westermann *et al*. 2021
Coxiella burnetii	Cattle, Sheep, Goats	Placenta: suppurative and necrotizing placentitis mainly of	Borel *et al*. 2014

		the intercotyledonary chorioallantois without vasculitis.	
Enterobacteriaceae-*Escherichia coli*	Cattle, Sheep, Goats	Clinical signs: Aborting animal clinically ill. Placenta: Congestion. Suppurative placentitis. Membranes thickened. Yellow/brown exudate. Foetus: Petechial haemorrhages on pleura, pericardium and kidney. Fibrinous foetal fluids. Bronchopneumonia	Sargison *et al*. 2001 Holler 2012 Baumgartner 2014
Enterobacteriaceae-*Salmonella* spp.	Cattle, sheep	Clinical signs: Aborting animal clinically ill. Placenta: Autolysed and emphysematous. Membranes thickened. Yellow exudate and caruncular fragments on cotyledons. Foetus: Autolysed and emphysematous. Foetal fluids fibrinous. Necrotic foci in the liver.	Baumgartner 2014
Leptospira interrogans	Cattle, sheep, goats	Placenta: light tan cotyledons and oedematous, yellowish intercotyledonary areas (necrotic placentitis). Foetus: autolysed, icterus may be present. Histology: interstitial nephritis and renal tubular necrosis.	Borel *et al*. 2014 Vidal *et al*. 2017
Listeria monocytogenes *Listeria ivanovii*	Cattle, sheep, goats	Clinical signs: Fever, anorexia Placenta: retained, small, pale foci in cotyledons (suppurative placentitis) Foetus: autolysed, no lesions or white necrotic foci in liver (hepatitis), fibrinous	Baumgartner 2014

		polyserositis (fibrin in body cavities), abomasal erosions	
Mycoplasma bovis	Cattle	Placenta: fibrinonecrotizing placentitis Foetus: bronchopneumonia, fibrinonecrotic epicarditis, myocarditis	Baumgartner 2014
Nocardia spp.	Cattle	Placenta: Suppurative, necrotic placentitis. Foetus: Miliary lung abscesses.	Bawa *et al*. 2010
Pajaroellobacter abortibovis (Foothill abortion/ Epizootic bovine abortion)	Cattle	Placenta: non-specific findings Foetus: hepatomegaly (nodular liver), splenomegaly, generalised lymphadenopathy. Histopathology: lymphoid hyperplasia in spleen and lymph nodes. Granulomatous inflammation in most organs.	Givens & Marley 2008
Parachlamydia acanthamoebae	Cattle	Placenta: purulent to necrotizing placentitis sometimes with vasculitis Foetus: Pneumonia	Deuchande *et al.* 2010 Ruhl *et al*. 2008
Trueperella pyogenes	Cattle	Placenta: Diffuse placentitis, Reddish brown to brown exudate. Thickened intercotyledonary tissues. Foetus: Autolysed. Fibrinous pericarditis, pleuritis or peritonitis	Baumgartner 2014
Ureaplasma diversum	Cattle	Placenta: Intercotyledonary areas thickened. Non-suppurative necrotic placentitis. Foetus: not autolysed. No macroscopic lesions. Non-suppurative pneumonia.	Anderson 2007 Baumgartner 2014
Waddlia chondrophilia	Cattle	Placenta: purulent, suppurative to necrotic placentitis with vasculitis Foetus: Acute suppurative bronchopneumonia	Blumer *et al* 2011 Henning *et al* 2002

Vibrio metschnikovii	Cattle	Placenta: necrotic placentitis with vasculitis	Vidal *et al.* 2007
Yersinia enterocolitica	Sheep	Placenta: Suppurative and necrotic placentitis. Thickened cotyledons. Foetus: Fluid accumulation. Serosanguinous, haemorrhagic organs.	Menzies 2007
Yersinia pseudotuberculosis	Cattle, Sheep	Placenta: Cotyledons thickened and red/tan. Little exudate. Intercotyledonary fibrosis. Foetus: Little autolysis. Fibrinous fluid in body cavities. Bronchopneumonia. Histopathology: Necrosis, vasculitis, bacterial colonies	Baumgartner 2014
FUNGI *Aspergillus* spp. *Mortierella wolfii* *Mucor* spp. *Lichtheimia* (*Absidia*) *corymbifera* *Rhizopus* spp. *Candida* spp.	Cattle	Placenta: retained placenta, thickened, leathery intercotyledonary areas, necrotic, haemorrhagic infarcts of cotyledons Foetus: Little autolysis. No lesions or raised plaques on the skin. Microscopic: Suppurative placentitis with vasculitis and associated fungal hyphae, bronchopneumonia, inflammation of the GIT	Baumgartner 2014 Borel *et al*. 2014

Foetal infections by viruses such as Bovine alphaherpesvirus-1 and Bovine viral diarrhoea virus can cause interstitial pneumonia and/or hepatic necrosis which can be difficult to distinguish from lesions caused by certain bacterial infections for example *Mycoplasma* and *Campylobacter* spp. without further analysis (De Oliveira *et al.* 2022; Anderson 2007).

Distribution of agents of abortion

In a 1990 report Anderson *et al*. stated that different agents are dominant in different areas for various reasons such as farming systems, climate and type of animals. When the most common agents of abortion found in various countries by retrospective and prospective studies are summarised as in Table 1.2, this variation is evident. In developed countries in the northern hemisphere, a variety of agents of abortion are reported in cattle, but *T. pyogenes* appears to be most common. In ovine and caprine abortions *Chlamydia* spp., *Campylobacter* spp. and *C. burnetii* were most reported. In developed countries in the southern hemisphere, the most common agents of abortion in sheep in New Zealand were reported to be *Campylobacter* spp. and *Salmonella* species (West 2002). *Campylobacter* spp. and *Listeria* spp. were most reported in sheep in an Australian study by Clune *et al*. (2021a). In developing countries (Argentina, Brazil and Iran), *Brucella* spp. is reported as common causes of abortion (Campero *et al* 2003, Moshkelani *et al*. 2011, Antoniassi *et al* 2016). Similar studies have not been published in South Africa since the 1970's.

Table 1.2 Most commonly reported bacterial and fungal agents of abortion from the northern and southern hemispheres

COUNTRY	**MOST COMMON BACTERIA AND FUNGI BY ANIMAL SPECIES**		
Northern Hemisphere	**Animal species**	**Bacteria/ Fungi**	**Reference**
Canada	Bovine	*Trueperella pyogenes* *Listeria monocytogenes*	Khodakaram-Tafti & Ikede 2005
Denmark	Ovine	*Yersinia pseudotuberculosis,* *Fusobacterium necrophorum*	Agerholm 2006
Finland	Bovine	*Ureaplasma* species *Trueperella pyogenes* *Bacillus licheniformus*	Syrjälä *et al.* 2007

Iran	Bovine	*Leptospira interrogans* *Brucella* species	Moshkelani *et al*. 2011
Netherlands	Ovine Caprine	*Campylobacter* species *Listeria monocytogenes*	van Engelen *et al* 2014
Switzerland	Ovine Caprine	*Chlamydia* species *Coxiella burnetii*	Schnydrig *et al*. 2017
United Kingdom	Ovine	*Chlamydia abortus* *Campylobacter* species	Pritchard *et al*. 2011 Carson *et al* 2017
United States of America	Bovine	*Campylobacter fetus* subspecies *fetus* *Salmonella* species	Jerrett *et al* 1984
		Trueperella (*Actinomyces*) *pyogenes*	Anderson *et al* 1990
		Pajaroellobacter abortibovis	Clothier & Anderson 2016
	Ovine	*Campylobacter* species	Kirkbride 1993
	Caprine	*Chlamydia psittaci* (later *C. abortus*) *Coxiella burnetii*	Moeller 2001
Southern Hemisphere	**Animal species**	**Bacteria/ Fungi**	**Reference**
Argentina	Bovine	*Brucella abortus* *Campylobacter fetus* subspecies *Leptospira interrogans*	Campero *et al* 2003 Morrell *et al* 2019
Australia	Ovine	*Campylobacter* species *Chlamydia pecorum* *Leptospira interrogans* *Listeria* species *Trueperella pyogenes* *Aspergillus fumigatus* *Mortierella wolfii*	Clune *et al* 2021a Clune *et al* 2021b Reichel *et al*. 2018
Brazil	Bovine	*Brucella abortus*	Antoniassi *et al* 2016
New Zealand	Ovine	*Campylobacter* species *Leptospira interrogans* *Salmonella* species *Trueperella pyogenes*	West 2002 Reichel *et al*. 2018

		Aspergillus fumigatus *Mortierella wolfii*	
Uruguay	Cattle	*Coxiella burnetii* *Campylobacter fetus* subspecies *venerealis*	Macías-Rioseco *et al*. 2019

Bacterial agents of abortion in South Africa

During the early 1970's a study was launched by the Veterinary Research Institute, Onderstepoort to investigate bacterial causes of abortion (Schutte *et al*. 1976). *Brucella* species emerged as the most significant cause of abortion in cattle (Coetzer & Schutte 1978). Other publications investigated the role of *Coxiella burnetii*, *Chlamydia* spp., *Leptospira interrogans* serovar Pomona and *Vibrio fetus* subspecies, *Mycoplasma* spp. and *Ureaplasma* spp. as causes of ruminant abortion in South Africa (Schutte *et al*. 1971, Ehret *et al*.1975, Schutte *et al*. 1976, Coetzer & Schutte 1978, Herr *et al*. 1982, Trichard & Jacobsz 1985).

Since then, limited information was published on the prevalence, significance, and zoonotic risk of specific bacterial agents of abortion (Njiro *et al*. 2011; Bath *et al*. 2013). Serological studies indicate the continued presence of *C. abortus* in South Africa (Musuka *et al*. 2001). However, the presence of antibodies does not necessarily indicate ongoing infection and cannot indicate a cause of abortion (Schnydrig *et al.* 2017). *Brucella abortus* is known to be common in South Africa (Mbizeni 2015), but little is known about the occurrence of *B. melitensis* in domestic ruminants. Only three outbreaks in domestic ruminants are documented. The first was an outbreak in sheep in the Transvaal reported by van Drimmelen in 1965, the second was in goats reported in 1990 by Ribeiro *et al*. and the third in goats in northern KwaZulu Natal reported by Reichel *et al* in 1996. More recently, Kolo *et al*. (2019) reported isolation of *B*. *melitensis* from cattle routinely slaughtered at abattoirs in Gauteng. *Brucella melitensis* was also isolated from sable antelope (Glover *et al*. 2020). According to Coetzer &

Oberem (2018), serological evidence indicates that *L.* Hardjo is the most significant cause of bovine leptospirosis. *Leptospira* Pomona is reported, but less commonly and more often as an agent of sporadic abortions in cattle. *Leptospira* Mini has also been implicated in cattle abortion. In a serological study *L.* Pomona was implicated in sheep abortions, but it has not been reported in goats. In Southern Africa abortions as result of *Listeria* species infection has been reported in sheep, goats, and cattle, but it is not considered to be economically important (Vorster & Mapham 2011). *Salmonella* Dublin is a known cause of abortions in cattle (Coetzer & Oberem 2018). The significance of these bacteria as agents of abortion must be investigated further in South Africa (Trichard *et al* 1981, Coetzer & Oberem 2018).

Surveillance and control of bacterial causes of abortion

Passive (event driven) surveillance systems can provide useful early warning of notifiable zoonotic agents that cause abortions. Such surveillance systems rely on the reporting of a defined clinical sign, e.g., abortion (Bronner *et al*. 2015). Countries such as Ireland, France and Switzerland implemented compulsory notification of aborted foetuses as a method of passive monitoring (Schnydrig *et al.* 2017). Unfortunately cost cutting measures often dictate that only the minimum analyses are done, targeting major zoonotic abortive agents such as *C. burnetii* and *Brucella* spp. and limiting the diagnostic value of the effort for the veterinarian and farmer (Bronner *et al*. 2015, Schnydrig *et al.* 2017). A study in France found that lower sensitivity due to non-compliance by veterinarians and farmers was a significant barrier to optimal functioning of the system (Bronner *et al*. 2015). The study reported that finding the cause of abortion was a greater incentive to notify than merely taking part in a detection programme for a specific agent (Bronner *et al*. 2015). South Africa does not have a compulsory notification system, and foetuses are submitted to veterinary laboratories only when

veterinarians and/or farmers need a diagnosis. Improving diagnostic methods could increase the number of successful diagnoses which in turn could encourage increased submission of foetuses. Collated data from veterinary diagnostic laboratories could serve as a valuable form of passive surveillance.

Data from passive surveillance could guide control efforts. Overall knowledge of the role and presence of various agents of abortion could inform improved vaccine development and formulation. Knowledge of abortive agents present in a province may aid veterinary clinicians in following a more targeted approach when considering immunization schedules and biosecurity measures (Givens & Marley 2008), because the approaches to control of different agents of abortion may vary significantly (Bath *et al*. 2013). In some cases, vaccination is an option (e.g., *Brucella* spp or *C. fetus* subsp. venerealis), in others antibiotics (e.g., *Chlamydia pecorum*) or improved management of feed (*Listeria monocytogenes* and fungi) is necessary.

Many bacterial agents of ruminant abortion are known to be zoonotic, therefore aborted material should be handled with appropriate hygiene and safety precautions to prevent infection of the handler (Clune *et al*. 2021a). The list includes *B. abortus*, *B. melitensis*, *C. abortus*, *C. burnetii*, *L. interrogans* serovars, *W. chondrophilia*, *P*. *acanthamoeba* and many more (Sprenger *et al*. 2012, Baumgartner 2015). *Brucella* species cause acute to chronic, debilitating infection with recurrent episodes of fever in humans (WOAH 2016). Cases of human brucellosis are reported in South Africa, for example in 1995 Hendricks *et al.* investigated the occurrence of brucellosis in children in the Western Cape province. Recently a case of human *Brucella melitensis* infection (also in the Western Cape) was reported (Wojno *et al*. 2016). *Coxiella burnetii* infection (Q fever) in humans can lead to flu-like symptoms, pneumonia, hepatitis, chronic fatigue syndrome, spontaneous abortion, premature birth, or foetal death (WOAH

2018). The first human clinical Q fever cases in South Africa were reported in 1950 by Gear *et al*. as well as Saner & Fehler. More recently, Simpson *et al*. (2018) reported serological evidence of human exposure to *C. burnetii*. No South African reports of human *C. abortus*, *W. chondrophilia* or *P*. *acanthamoeba* could be found, but these bacteria have been implicated in human pregnancy failure and miscarriage in European studies (Givens & Marley 2008; Borel *et al.* 2018). Leptospirosis in humans can be sub-clinical or manifest as influenza-like symptoms in mild cases and icterus in severe cases (Feresu 1990, Naidoo *et al*. 2015). In 1964 Rademan *et al.* reported the first isolations of *Leptospira* spp. in Cape Town in the Western Cape. More recently an outbreak of leptospirosis in a correctional facility was reported by Naidoo *et al*. (2015).

Diagnosis of infectious causes of abortion

Determining the cause of abortion may prove to be a challenge to both the clinician and the laboratory diagnostician (Clothier & Anderson 2016). The rate of successful diagnoses (diagnostic rate) is the number of confirmed diagnoses out of the total number of submissions (Reichel *et al*. 2018). Across the world studies report varying diagnostic rates (22.5% to 50%) when conventional methods of analysis are employed (Campero 2003, Anderson 2007, Wheelhouse & Dagleish 2014, Wolf-Jäckel *et al*. 2020). Cases of bovine, ovine and caprine abortion where the aetiology remains unknown, but the presence of an infectious agent is indicated by pathological lesions such as placentitis or pneumonia, are a problem encountered worldwide (Blumer *et al.* 2011). Major stumbling blocks tend to be lack of clinical history as well as the quality and range of samples received at the laboratory (Holler 2012, Schnydrig *et al*. 2017, Clune *et al*. 2021a). Characteristic macroscopic and microscopic lesions are rare which means that the pathological examination cannot stand alone (Kirkbride 1986, Schnydrig *et al* 2017). So, a combination of tools is best to investigate a case of

abortion (Schnydrig *et al.* 2017) and all available diagnostic procedures should be used in every abortion case (Kirkbride 1986; Borel *et al*. 2014). Unfortunately, due to high costs of abortion investigation and the large number of possible causes in ruminants this often does not happen, instead a low-cost option (necropsy and examination of smears stained by the Stamp's modification of the Ziehl Neelsen stain method (MZN) is offered and the causative agent is not determined (Vidal *et al*. 2017).

Histopathology

Histological investigation is essential to implicate a specific microorganism in a disease process since many bacteria and fungi are found as commensals or in the environment, (Vidal *et al* 2017). Histopathology methods allow microscopic visualisation of pathological changes and sometimes bacteria at cellular level in tissue samples (Johnson 2022). However, these methods can usually not identify bacteria or fungi to genus and species level (Gupta *et al*. 2009). Histopathology is combined with other methods such as immunohistochemistry, culture and/ or PCR to identify bacteria or fungi.

Immunohistochemistry (IHC)

Immunohistochemistry methods use monoclonal and polyclonal antibodies. These methods are used to detect specific antigens in tissue sections to allow visualisation and identification of bacteria in pathological lesions (Goswami *et al*. 2012, Vidal *et al*. 2017). Detection is often genus specific and sensitivity is more limited than that of PCR methods (Sachse *et al*. 2009, Borel *et al*. 2014, Vidal et al. 2014). Immunohistochemistry is combined with other methods such as culture or PCR to improve sensitivity and/or to identify bacteria to genus level.

Serology

Serum from the aborting female as well as body cavity fluid and heart blood from the foetus can be used for serological assays in combination with necropsy of the foetus. Presence of specific foetal antibodies is evidence of an infection (Njaa 2012, Borel *et al*. 2018). Maternal serology can be useful in combination with a foetal examination, however antibody titres as result of vaccination, natural immunity or cross reactions can interfere with interpretation (Kirkbride 1986). Seropositivity to some bacteria such as those in the order *Chlamydiales* is common and does not necessarily indicate an active infection, therefore serology cannot be used to diagnose abortion in individual animals (Schnydrig *et al.* 2017). In addition, antibodies to *Chlamydiales* are only genus specific (Rhodolakis & Laroucau 2015). Detection of antibodies to *C. burnetii* is of little use in abortion diagnostics in individual animals as seroconversion does not necessarily occur (Schnydrig *et al.* 2017). Confirmation of *Leptospira* abortion by serology in individual animals, particularly in the maintenance host, is difficult as maternal antibody production usually takes place before foetal death or not at all (Schnydrig *et al*. 2017; Vidal *et al* 2017). Serological examination of paired samples of 10% of the herd or comparison of serological results from animals that aborted to animals that did not abort, is often more useful (Holler 2012).

Conventional microbiological analyses

Over the years smear examination and conventional bacterial culture were tools commonly used in abortion investigation. However, there are limitations to these methods. Foetuses that died 24 to 48 hours before abortion may already be autolytic by the time they are aborted (Givens & Marley 2008). Autolysis masks lesions and the agent of abortion may be outcompeted by other organisms on conventional growth media (Kirkbride 1986, Clune *et al*. 2021a).

Abomasal smears stained by routine methods (Gram stain or Giemsa) may be useful if a pathogen is present in large numbers (Kirkbride 1986). Some bacteria, such as *Chlamydiales*, *C. burnetii* and *Leptospira* spp. cannot be stained by the Gram stain method. *Brucella* spp., *Coxiella burnetii*, and *Chlamydiales* are partially acid fast and can be stained by the MZN stain method (Pritchard *et al*. 2011; Markey *et al* 2013). Impression smears stained by the MZN method are often used to screen for *C. burnetii* and *Chlamydia* spp., although Pritchard *et al.* (2011) and Schnydrig *et al.* (2017) demonstrated that this method has low sensitivity. Other staining methods such as Giemsa, Gimenez and dark ground Methylene Blue, can also be used to detect *Chlamydiales* (Dagnall & Wilsmore 1990, Songer & Post 2005; Markey *et al*. 2013). Although *Leptospira* spp. are Gram-negative, they do not stain with the Gram stain method. Dark field microscopy of wet preparations is the routine method of visualisation for these bacteria (Markey *et al* 2013; Jobbins *et al*. 2014).

Most bacteria can be cultured by aerobic methods with addition of 5 to 10% CO_2 (Borel *et al*. 2014). Studies reviewed reported aerobic culture in 4 - 7% CO_2 as well as microaerophilic culture (Anderson *et al.* 1990, Kirkbride 1993, Moeller 2001, Campero *et al*. 2003, Agerholm *et al*. 2006, Syrjälä *et al*. 2007). Blood and MacConkey agar was commonly used (Anderson *et al.* 1990, Kirkbride 1993, Moeller 2001, Agerholm *et al*. 2006) and some studies included chocolate agar (Anderson *et al.* 1990, Moeller 2001).

Recovery of slower growing and more fastidious bacteria such as anaerobes, *Brucella* and *Campylobacter* spp. is enhanced by addition of selective growth media and an environment conducive to growth (Markey *et al*. 2013). Few studies included anaerobic culture (Agerholm *et al*. 2006, Syrjälä *et al*. 2007). Genus-specific selective culture methods for bacteria such as *Brucella* (Moeller 2001, Syrjälä *et al*. 2007) and *Mycoplasma* (Syrjälä *et al*. 2007) were mentioned

by only a few researchers. Fungal culture was mostly done when indicated (Anderson *et al.* 1990, Campero *et al*. 2003, Khodakaram-Tafti & Ikede 2005) except for Syrjälä *et al*. (2007) who reported it as part of a routine conventional culture panel. Optimising a panel of tests for detecting pathogens in abortion samples may contribute to a more effective approach to abortion diagnostics by removing test selection bias (Borel *et al*. 2014).

Molecular microbiological analyses

Molecular analysis offers the possibility of increasing diagnostic rate since nucleic acid of bacteria may still be harboured in autolytic and fixed tissues. Nucleic acid detection methods are increasingly used to detect agents of abortion. Polymerase chain reaction (PCR) methods can be gel-based or real-time and may be developed to detect pathogens in unpreserved clinical samples or paraffin embedded tissues. A gel-based PCR protocol uses an agarose gel to demonstrate the products of a reaction, while real-time quantitative PCR (qPCR) employs fluorescence to demonstrate the presence of a target. The drawback of these methods is their limitations where multiplexing is concerned (Jiang *et al*. 2017). A PCR assay may be simplex, when a target deoxyribonucleic acid (DNA) sequence of only one organism is detected (Jiang *et al.* 2017), or multiplex when target DNA sequences of multiple organisms are detected in a single reaction (Richtzenhain *et al* 2002).

Liquid reaction kinetics (qPCR and Bead Based Molecular Assays) are faster with more reproducible results compared to solid arrays (Christopher-Hennings *et al*. 2013). However, qPCR can only distinguish up to five analytes in a single reaction depending on the ability of equipment to detect fluorescent dyes (Christopher-Hennings *et al*. 2013). Despite limitations where multiplexing is concerned, several duplex and multiplex conventional and qPCR protocols were developed for various sample types and organisms. In 2006, Pelletier *et al*.

reported the development of a duplex PCR for *C. abortus* and *C. burnetii* in cattle abortion products. In 2009, Berri *et al.* reported the development of a multiplex PCR to detect *C. abortus*, *C. pecorum* and *C. burnetii*. A multiplex real-time PCR was developed at the Iowa state University to analyse samples from sheep and goat abortion cases for *Chlamydia* spp., *Campylobacter* spp. and *C. burnetii* (Azeem *et al.* 2015). Multiplex qPCR kits for ruminant abortion are available commercially, for example the VetMAX™ Ruminant Abortion Screening Kit (Thermo Fisher Scientific) that can detect *Anaplasma phagocytophilum*, Bovine herpesvirus 4, *Coxiella burnetii*, *Campylobacter fetus*. *Chlamydia* species, *Listeria monocytogenes*, *Salmonella* and pathogenic *Leptospira*.

Culture, PCR and serology results cannot stand alone as overestimation of the significance of an organism is always a possibility since many bacterial and fungal agents of abortion form part of the normal flora of mucosal surfaces in ruminants; or are common in the environment and can contaminate products of abortion. If no correlation with a pathological lesion can be demonstrated, results are inconclusive (Wheelhouse & Dagleish 2014; Clothier & Anderson 2016). Involvement of intracellular bacteria, such as *C. abortus*, *P. acanthamoeba* and *W. chondrophilia*, that are difficult and time consuming to culture may lead to inconclusive results if poorly sensitive methods are employed (Barkallah *et al.* 2016; Vidal *et al*. 2017).

Conclusion

Abortion, as part of pregnancy wastage, is a world-wide phenomenon with often underestimated economic losses for farmers. In South Africa some literature is available on detection of bacterial and fungal agents of abortion, but most date back to the early 1990's. More recent data are necessary to update knowledge on the occurrence and significance of these agents.

Abortion rates higher than 2% are an indication that investigation is necessary. Many bacteria and fungi are implicated in cases of abortion and a number of these are zoonotic. Several of these agents of abortion are common in the environment or are commensals of mucous membranes mainly leading to sporadic, opportunistic abortions. Others become endemic in herds and cause either abortion storms or sporadic abortions. Regional differences in the occurrence of agents of abortion become evident when many reports are available for comparison via passive surveillance. Such data are essential to guide control programmes. In South Africa there are differences in farming systems and climatic zones which could influence occurrence of agents of abortion, but passive surveillance data are lacking.

Due to a lack of history and clinical signs to guide investigations, laboratory investigation of cases of abortion should always include a combination of as many diagnostic tools as possible. Macroscopic pathology, histopathology, IHC, serology, bacterial and fungal culture, and PCR are methods commonly used in abortion investigations. None of these diagnostic methods can stand alone when determining an aetiological cause of abortion. Limited characteristic pathological lesions, incomplete sample sets, test selection for economic reasons, as well as fastidious growth requirements and limited viability of certain bacteria are stumbling blocks that can lead to an inconclusive result. A routinely applied set of methods for conventional culture and molecular analyses would remove test selection bias and potentially lead to identification of more agents of abortion.

Nucleic acid detection methods are increasingly used in abortion investigations. Multiplex PCR assays can detect several agents in one reaction saving time and cost. Limitations in multiplexing can be addressed by bead-based multiplex assays such as the Luminex xTAG® platform that offer increased multiplexing ability.

Aims and objectives

The aim of the study was to investigate detection methods for, and the significance of, bacteria and fungi as agents of abortion in cattle, sheep and goats in South Africa.

The objectives were:

To conduct a retrospective survey including data from 2006 to 2016 in South Africa on bacterial and fungal agents of abortion in cattle, sheep, and goats to determine which methods were used for detection of these agents and what the resulting diagnostic rate was.

To conduct a prospective survey of bacterial and fungal agents of abortion by means of a set panel of methods for conventional culture designed for the study to improve the range of previously applied methods (aerobic and *Brucella* selective culture) and determine if the improvement leads to increased isolation of significant bacteria and fungi.

To develop and optimise real-time qPCR assays to detect and identify *Chlamydiales* to extend diagnostic ability beyond the limits of conventional culture.

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CHAPTER 2

Retrospective study of bacterial and fungal causes of abortion in domestic ruminants in northern regions of South Africa (2006-2016)

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Abstract

This initial retrospective study presents information on bacterial and fungal causes of abortion in domestic ruminants in South Africa over 10 years (2006-2016). A complete set of data was collected at the Faculty of Veterinary Science by a keyword search of pathology and bacteriology laboratory registers. Additional electronic data was received from an external laboratory. A total of 288 cases were recorded from six provinces. Overall diagnostic rate was 35.1%. In 14.6% of cases histological evidence of an infectious process was found, but no aetiological agent was detected. Several cases did not include aetiological diagnoses because applicable diagnostic techniques were not available or not applied when necessary. Increased submission of placenta as well as additional conventional and molecular diagnostic methods can contribute to improved diagnostic rate. In addition, the study highlights the superior significance of *Brucella abortus* as a major bovine pathogen in South Africa.

Introduction

A retrospective or historical study is defined as a study that collects and records information after the outcome of an exposure is known (Klebanoff & Snowden 2018). Retrospective studies of data on ruminant abortion over a period at selected veterinary laboratories covering all major farming areas can provide useful data for the development of herd health programmes and state veterinary disease control programmes to reduce future losses due to abortion (Anderson *et al*. 1990). Data on number of cases, diagnostic rate, and regional incidence of causes of abortion and farming systems can be collected. In comparison with prospective field studies that are expensive, retrospective studies can produce valuable information at low cost (Khodakaram-Tafti & Ikede 2005).

A significant number of retrospective studies covering causes of abortion have been published internationally (Khodakaram-Tafti & Ikede 2005; Kirkbride 1992; Clothier & Anderson 2016; Reichel *et al*. 2018). Diagnostic rate (Reichel *et al*. 2018) emerged as an important parameter for the evaluation of the efficiency of laboratory investigation. Several studies from different laboratories in the United States of America (USA) and Canada reported data on bovine, ovine or caprine abortion cases (Anderson *et al*. 1990; Kirkbride 1992, 1993; Moeller 2001; Khodakaram-Tafti & Ikede 2005; Clothier & Anderson 2016; Reichel *et al*. 2018). Diagnostic rate in bovine cases ranged from 29 to 56% (Anderson *et al*. 1990; Kirkbride 1992; Khodakaram-Tafti & Ikede 2005; Clothier & Anderson 2016). Diagnostic rates of 44% and 47% were recorded for ovine and caprine abortions respectively (Kirkbride 1993; Moeller 2001). In European studies, diagnostic rate was reported as 22 to 26% in bovine cases, 56 to 74% in ovine cases and 67% in caprine cases (Syrjälä *et al*. 2007; Van Engelen *et al*. 2014; Wheelhouse & Dagleish 2014). Kirkbride (1993) observed that ovine abortions were more likely

to present with recognizable lesions and abortions caused by opportunistic agents were less frequent than in cattle in that study.

Bacteria and fungi were often implicated as major causes of abortion. The detection of pathogens responsible for abortion will provide an indication of the mitigation or control method to address the problem such as test and slaughter, vaccination, or improved feed management. In cases of bovine and ovine abortion, bacteria have been implicated in 14 to 67%; and fungi in 4 to 28% of all cases (Anderson *et al*. 1990; Kirkbride 1992, 1993; Wheelhouse & Dagleish 2014; Clothier & Anderson 2016). Studies of caprine abortion implicated bacteria in 30.5% and fungi in 0.5% of cases (Moeller 2001).

Results of a retrospective study can indicate the most common infectious agents in a country as well as regional differences (Anderson *et al*. 1990). These differences occur for various reasons such as farming systems, climate, and type of animals (Anderson *et al*. 1009). This information can prove useful in focussing control efforts. In selected developed countries in the northern hemisphere certain organisms have emerged as the most significant agents of abortion in cattle, sheep and goats including *Bacillus licheniformus* (Finland), *Campylobacter* species (Netherlands, United Kingdom and USA), *Chlamydia abortus* (UK, Netherlands, USA), *Coxiella burnetii* (USA)*, Listeria monocytogenes* (Canada, Netherlands), *Trueperella* (*Actinomyces*) *pyogenes* (Canada, Finland, USA) and *Ureaplasma diversum* (Finland) (Anderson *et al*. 1990; Khodakaram-Tafti & Ikede 2005; Syrjälä *et al*. 2007; Pritchard *et al*. 2011; Van Engelen *et al*. 2014; Wheelhouse & Dagleish 2014). In the southern hemisphere, the most common agents of abortion in sheep in New Zealand were reported to be *Campylobacter* and *Salmonella* species (West 2002). In addition, Reichel *et al*. (2018) have reported that most mycotic abortions in cattle in these countries were due to *Aspergillus* species and *Mortierella wolfii*.

Placentitis and other pathological lesions without detection of a causative agent are commonly associated with cases of abortion. Such reports range from 4% to 40.6% (Anderson *et al*. 1990; Kirkbride 1992; Khodakaram-Tafti & Ikede 2005; Syrjälä *et al*. 2007; Van Engelen *et al*. 2014). These reports serve to highlight the fact that there are still cases where an aetiological diagnosis cannot be reached, hence the need for improvement in laboratory techniques.

Conventional bacterial and fungal culture methods are commonly employed to analyse products of abortion. Internationally, the composition of diagnostic abortion panels varies. Most studies reported at least aerobic culture in 4 to 7% CO_2 as well as microaerophilic culture on blood and/or MacConkey agar (Anderson *et al*. 1990; Kirkbride 1993; Moeller 2001; Syrjälä *et al*. 2007). Some studies included chocolate agar (Anderson *et al*. 1990; Moeller 2001). Few studies included anaerobic culture (Syrjälä *et al*. 2007). Genus specific cultures such as *Brucella* (Moeller 2001; Syrjälä *et al*. 2007) and *Mycoplasma* (Syrjälä *et al*. 2007) were only mentioned by a few researchers. Fungal culture was mostly done when indicated (Anderson *et al*. 1990; Khodakaram-Tafti & Ikede 2005) except for Syrjälä *et al*. (2007) who reported it as part of a routine conventional culture panel.

Additional history such as farming systems, abortion rate and feeding practices are not often mentioned although it can play an important part in reaching a diagnosis (Wheelhouse & Dagleish 2014). Anderson *et al*. (1990) reported cases from intensive, zero-grazing dairy cattle farms. Another study by Clothier & Anderson (2016) had information associating cases with dairy or beef cattle. Syrjälä *et al*. (2007) received additional data in 242 (89.3%) cases indicating that 55% of submissions were from dairy and 45% of submissions from beef cattle.

The advantage of the retrospective studies mentioned above was that it gave animal health agencies, policy makers, researchers, and diagnosticians an

indication of the causative agents involved, relative prevalence and geographical diversities of these agents, as well as risk factors for abortion. Information generated is useful for planning purposes in several areas, for example at government level it can advise animal disease surveillance and control efforts. At farm level specific vaccines can be included in vaccine programmes to improve prophylaxis or treatment can be applied. At laboratory level the data can be used for financial planning since agents known to occur in the area can be targeted by inclusion of suitable assays in diagnostic panels.

The aims of the study were to exploit available retrospective laboratory data on cases of confirmed or suspected bacterial or fungal abortions in cattle, sheep, and goats for the purpose of determining the diagnostic rate including factors that could influence it as well as to identify the most significant bacterial and fungal causative pathogens per animal species and province.

Materials and methods

The observational retrospective study attempted to use cases from multiple diagnostic laboratory systems. A case was defined as samples from one or more foetuses or products of abortion from a single herd submitted at the same time for bacterial and fungal culture. Case results were used in the count of infectious agents. If two foetuses registered as a single case yielded different agents these were counted separately. Criteria for selection was foetus (not stillborn or neonate). Indications that the calf/ lamb/ kid was born alive such as aerated lungs, colostrum in the abomasum or worn golden slippers were criteria for exclusion. A full necropsy was recorded on all foetuses, including macroscopic and microscopic pathology. Samples were submitted for bacterial and fungal analysis at the discretion of the pathologist. A diagnosis was made on the

combined findings of necropsy, histopathology, microbiology, and other analyses such as immunohistochemical staining and polymerase chain reaction (PCR).

South African public and private veterinary diagnostic laboratories and their management (where appropriate) were approached for permission to collect data on cases received between 2006 and 2016. Data were recoded on a spreadsheet under the following headings: farming systems, feeding practices, geographical area of origin of foetuses/samples, condition of samples received, routine test methods used for foetuses/ abortion samples by the various laboratories, the number of abortion cases received (infectious and non-infectious), the number of confirmed diagnoses of infectious causes, the agents of abortion identified, the number of suspected diagnoses of infectious causes that could not be confirmed and the number of cases were there were macroscopic or microscopic indications of an infectious cause but no diagnosis.

At the Bacteriology laboratory, Department Veterinary Tropical Diseases and Histopathology laboratory, Section Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria (DVTD & Path) a search was done manually and electronically. Registers were searched for the keywords: bovine, ovine, caprine, foetus, calf, lamb, kid, abomasal content and foetal fluid. Lists of possible abortion cases were compiled at the histopathology and bacteriology laboratories. These were used as reference to search files of pathology reports. Pathology reports were opened individually, and data recorded. Electronic pathology reports received from a private laboratory, Vetdiagnostix Pathology Laboratory Services (Vetdiagnostix), were also opened individually and data recorded.

The overall diagnostic rate (percentage) as well as the diagnostic rate per species was calculated by means of the following formula: (number of cases with confirmed diagnoses/ number of cases submitted) x 100. Diagnostic rate was

also calculated for samples in good condition (no/ slight autolysis), samples in poor condition (advanced autolysis), cases where a placenta was submitted, and the different combinations of tests offered for conventional culture. Different combinations of conventional bacterial analyses were allocated a number each: 1 = laboratories offered only aerobic culture, 2 = laboratories offered aerobic culture as well as other cultures on request. 3 = laboratories offered a routine abortion panel including *Brucella*, 4 = laboratories included a routine abortion panel including aerobic culture, anaerobic culture, microaerophilic culture and fungal culture (Annex 1).

The percentage of cases that recorded additional data was calculated as well as abortion rate. The formula for abortion rate was: number of abortions/ number of animals pregnant in the herd at the farm of origin of the samples (Menzies 2011). Percentages with 95% confidence intervals (CI) were calculated. Association between diagnosis and completeness of submission, where inclusion of placenta was the predictor, was analysed by logistic regression using Epi info 7™ 7.2.3.1, (Centers for Disease Control and Prevention, Atlanta, Georgia).

Results

The country-wide 10-year retrospective study from 2006 to 2016 that was initially planned was not achievable. In total, 288 cases were recorded from six provinces. Two hundred and thirty-five (235) cases were recorded over 10 years at the Pathology and DVTD Bacteriology laboratories of the Veterinary Faculty of University of Pretoria. In addition, Vetdiagnostix Pathology Laboratory Services contributed data from a 2013 CEVA Animal Health sponsored study on causes of abortion in sheep and goats which yielded 18 cases; as well as data on 35 cases recorded in 2016 (Annex 1).

Overall, 193 cases (67%) were bovine, 57 (20%) ovine and 38 (13%) caprine. DVTD & Path recorded 159 bovine, 43 ovine and 33 caprine cases. Most of these cases (bovine: n=101, ovine: n=43, caprine: n=21) were submitted by farms in Gauteng province. In the CEVA Animal Health project 18 cases (ovine: n=13; caprine: n=5) were recorded. Ovine cases were mainly from farms in Mpumalanga and KwaZulu-Natal while caprine cases originated mainly from Mpumalanga and North-West. In 2016 Vetdiagnostix recorded 34 bovine and one ovine case, mostly from KwaZulu-Natal (Annex 1).

The total number of cases (n=288) consisted of 58 cases that had foetuses and placenta (complete sets) and 224 cases that comprised of foetuses only. Six cases had placenta only. At DVTD & P 43 complete sets were recoded (bovine: n=25, ovine: n=12, caprine: n=6). In 186 cases only foetuses were recorded (bovine: n=128, ovine: n=31, caprine: 27). Only DVTD & P recorded cases where only placenta was submitted (bovine: n=6). The CEVA Animal Health project included nine complete sets were recorded (ovine: n=7, caprine: n=2) Nine cases had a fetus only (ovine: 6, caprine:3). Vetdiagnostix 2016 cases included six complete sets which were all bovine and 29 cases where only a foetus was submitted (bovine: n=28, ovine: n=1) (Annex 1).

A diagnosis was made in 101 cases. The overall diagnostic rate was 35.1% (95% CI [26, 45]); 35.2% (95% CI [26,45]) in bovine, 33.3% (95% CI [24,43]) in ovine and 35.9% (95% CI [26,45]) in caprine cases.

Table 2.1 Microorganisms isolated arranged per animal species per province

Microorganisms	**Province**					
Bacteria	**FS**	**GT**	**KZN**	**LP**	**MP**	**NW**
Actinomyces israelii		B(1)				
Anaplasma marginale			B(2)			
Bacillus cereus					B(1)	
Bacillus licheniformus			O(1)			

Brucella abortus biovar 1	B(1)	B(3)			B(1)	B(1)
Brucella abortus biovar 2				B(1)		
Brucella spp.		B(5)	B(1)	B(1)	B(4)	B(3)
Burkholderia cepacia					B(1)	
Campylobacter spp.				O(1)		B(1)
Chlamydia abortus		C(1)				
Chlamydia pecorum		O(1)				
Chlamydia spp.		C(1)				
Corynebacterium spp.		B(1)				
Coxiella burnetii	O(1)					
Enterobacter cloacae		C(1)			B(1)	
Enterococcus faecalis				C(1)		
Escherichia coli		B(2);O(3);C(1)				B(1)
Fusobacterium spp.		B(1)				
Leptospira spp.		B(1)				
Listeria monocytogenes			O(1)	B(2)		
Nocardia asteroides		B(1)				
Salmonella spp.		B(1);C(1)				
Streptococcus canis				B(1)		
Trueperella pyogenes		B(1)	B(1)	B(3)	O(1)	
Fungi						
Aspergillus fumigatus		B(1)				
Penicillium spp.			O(1)			
Rhizopus spp.					C(1)	
Unidentified fungus		B(1)				

FS: Free State, GT: Gauteng, KZN: KwaZulu Natal, LP: Limpopo, MP: Mpumalanga, NW: North West, B: Bovine, O: Ovine, C: Caprine, (): Number of isolates

In 63 cases (21.9%, 95% CI [13,30]) a bacterial or fungal isolate was implicated considering presence of bacteria in the lesion as well as the type of lesion (Annex 1). A bacterial aetiology was identified in 59 cases (20.5%, 95% CI [13,29]) and a fungal aetiology in four cases (1.38%, 95% CI [0,5]). *Brucella* species constituted the most common isolates in all provinces. It was detected in 21 cases (7.3%, 95% CI [3,14]) and only in bovine foetuses. *Trueperella pyogenes* was isolated from five bovine (1.73% 95% CI [0,5]) and one ovine

foetus (0.34%, 95% CI [0,4]). Members of the *Enterobacteriaceae* were commonly isolated. Most common was *E. coli* (seven cases) (2.4%, 95% CI [0,7]) followed by *Salmonella* spp. (two cases) (0.69% 95% CI [0,4]) (Table 2.1).

Table 2.2 Number of case submissions with and without placenta.

	Number of cases	**Percentage (%) of cases**
Foetuses w/o placenta	224	77.8
Foetuses with placenta	58	20.1
Placenta only	6	2
Total placenta submitted	64	22.2

Only 58 (20.1%) cases were classified as complete (foetus with placenta) and six (2%) had only placenta (Table 2.2). In most cases (n=224) foetuses were submitted without placenta. Placenta submissions totalled 64 (22.2%). Samples in good condition returned 80 (79.2%, 95% CI [70,87]) diagnoses while samples in poor condition (autolytic) returned 21 (20.8%, 95% CI [13,29]) diagnoses.

Thirty-two (50%) placentas had lesions indicative of an infectious cause. Necrotic placentitis was recorded in 21 (32.8%) and necrotic placentitis with vasculitis in five (7.8%) cases. In 14 of these cases no pathogen was detected. Diagnostic rate for cases where placenta was included was 42.2% (95% CI [32,52]). There was no statistically significant association between diagnostic rate and completeness of the submission (inlusion of placenta) (Table 2.3).

Table 2.3 Logistic regression analysis investigating association between diagnosis and completeness of submission (inclusion of placenta).

Term	**Odds Ratio**	**Lower 95% C.L.**	**Upper 95% C.I.**	**P-Value**
Placenta (Yes/No)	1.46	0.83	2.58	0.193

Necropsy and/ or histopathological lesions indicative of an infectious process was recorded in 42 cases (14.6%) where no infectious agent was identified (Table 2.4). In these cases, the most common lesions recorded were placentitis 14 cases (21.8%) and pneumonia 10 cases (23.8%).

Table 2.4 Cases with pathological lesions indicative of infection, but no aetiological agent identified.

Macro- or microscopic lesion reported	**Number of cases**	**Percentage of cases (%)**
Abomasitis	1	2.4
Hepatitis/ Liver necrosis	5	11.9
Hepatosis	5	11.9
Kidney necrosis	3	7.1
Meningitis/ Meningoencephalitis	4	9.5
Nephritis	1	2.4
Placentitis	14	21.8
Pneumonia	10	23.8
Total	42	14.6

Abortion rate was available in 22.9% of case submissions. *Brucella* spp., *T. pyogenes*, *L. monocytogenes*, *C. burnetii*, *B. licheniformus* and *Rhizopus* spp. were implicated in cases where abortion rates higher than 5 to 8% were reported. Six cases with abortion rates below 4% were associated with agents that become endemic in herds such as *B. abortus*, *Leptospira spp.*, *Chlamydia pecorum* and *Campylobacter*. Farmers and veterinarians were more likely to report abortions over time than abortion rate. Four abortions in four months were associated with a *Brucella* sp. infection.

One hundred and twenty-nine cases (44.8%) in this study included information on production systems. Information received revealed that 17 cases were from

beef production and 11 from dairy systems. Sheep and goat production systems were mostly extensive (11 cases) or semi-intensive (seven cases). Feeding practices were reported in 54 cases revealing that most herds were pasture fed with supplementary feeding such as lick (30 cases). Feed quality is often incriminated in cases of mycotic, *Listeria* sp. and *Bacillus* sp. abortions. In this study, only one case involving these agents included a history of feeding practices.

Discussion

The aim of the study was to exploit available retrospective laboratory data on cases of confirmed or suspected bacterial or fungal abortions in cattle, sheep, and goats for the purpose of identifying the most significant bacterial and fungal causative pathogens per animal species and province, as well as the diagnostic rate and factors that could influence it.

The relatively small number of cases recorded (n=288) was expected as there were several limitations to this study. Submission of abortion cases is not compulsory in South Africa. Farmers and field diagnosticians will often only opt for an abortion investigation if the number of abortions in a herd is seen as a financial threat. Most cases originate from farms closer to the participating laboratories in the northern parts of South Africa, an indication that distance affects submission rate. In the few laboratories that offered to share their data, filing methods and manpower proved to be a challenge. Electronic filing systems were created to search accounts not reports. Non-electronic filing systems had to be searched manually. Both required manpower most laboratories could not assist with. The result was that data was collected from only two laboratories. In contrast to declining submission rates reported by van Engelen *et al.* (2014), submission rates in this study appeared to increase from 2014 onward. Future

investigation including data from veterinary laboratories in the southern parts of South Africa will be useful to identify similarities and differences between different parts of the country.

Common agents of bacterial or fungal abortion could be identified per species and per country, but not per province. This is most probably due to the low number of laboratories involved and cases recorded. The most detected bacterial species in all provinces was *Brucella* spp. in cattle. This was expected as South Africa has a high incidence of brucellosis (Mbizeni 2015). What was surprising was the low rate of detection, only two to three positive cases per year. No *B. melitensis* isolates were recorded. However, isolation of *B. melitensis* from cattle samples collected at Gauteng abattoirs in 2016 and 2017 was reported by Kolo *et al*. (2019). An increase in complete submissions, increased laboratory capacity, routine inclusion of *Brucella* isolation in conventional bacteriology panels as well as more sensitive methods of detection, such as Real-Time PCR, should lead to improved detection of these bacteria.

Abortions due to sporadic infections were common. *Trueperella pyogenes* was the second most common isolate at 10 (14.2%) bovine cases and one ovine case. It was implicated as the agent of abortion if histopathological findings indicated a suppurative placentitis and/ or if it was isolated in a pure or almost pure culture from the abomasal fluid and foetal tissues (Kirkbride 1992). *Trueperella pyogenes* is also a common isolate from bovine abortion cases in the USA and Canada (Anderson *et al*. 1990; Khodakaram-Tafti & Ikede 2005).

Salmonella was rarely detected. When detected, isolates were rarely identified to serovar level during the study period, most likely due to limited availability of serotyping and financial reasons. However, serotyping is important to determine whether vaccination is an option for control as vaccines are available in South

Africa against the two most common *Salmonella* serovars found in cattle, e.g., *S*. Dublin and *S*. Typhimurium.

An important finding of this study was the bacteria that were not detected, or detected in low numbers, such as the anaerobes, *Chlamydiales*, *Campylobacteriales*, *Leptospira*, *Listeria* sp. and *Mycoplasmatales*, as well as their identification to genus level only. Culture methods for fastidious bacteria are not routinely included in conventional abortion panels due to cost. Some of these bacteria fall in the difficult to culture category (Vidal *et al*. 2017) due to an intracellular lifestyle, e.g., the *Chlamydiales* and *C. burnetii*; or due to their fastidious nature, e.g., *Campylobacteriales*, *Leptospira*, *Mycoplasma* and *Ureaplasma*. Others such as *Listeria* spp. are quickly overgrown by contaminating bacteria. Inclusion of conventional and molecular detection methods is necessary to determine whether these bacteria are truly uncommon in South Africa. Identification to species level is preferable for bacteria such as *Campylobacter* and *Chlamydia* as vaccines are only available for some species, e.g., *Campylobacter fetus* subspecies *venerealis* and *Chlamydia abortus*.

In this study only four mycotic abortions were recorded. Pathological lesions reported in these cases were necrotic placentitis, dermatosis and bronchopneumonia. Successful diagnosis of mycotic abortion is dependent on the availability of placenta as foetal tissues are often not involved (Kirkbride 1992). Increased submission of placenta as well as inclusion of fungal isolation in conventional abortion panels should improve detection of fungi. Internationally, mycotic abortion appears to be rare in more recent years. In cases of bovine abortion, reports ranged from 0.5 to 6.8% (Anderson *et al*. 1990; Khodakaram-Tafti & Ikede 2005; Syrjälä *et al*. 2007). The reason for this phenomenon is not clear, but improved feed management or drier climatic conditions due to climate change may play a role.

Placentas were received in only 22.2% of cases. Diagnostic rate for cases including placenta was 42.2%, but there was no statistically significant association between presence of placenta and diagnosis. The reason for this phenomenon may be that although pathological lesions indicative of an infectious cause was reported for 32 placenta samples, 14 of these cases remained inconclusive because no pathogen was detected. Placenta associated bacteria and fungi detected were *B. licheniformis*, *Chlamydia*, *C. burnetii*, *Penicillium* and *Rhizopus*. These agents may have been missed if the placenta had not been included. Placenta was positively associated with diagnosis of agents of abortion in an American study by Moeller (2001) and is considered the most useful sample for abortion investigation. However, the rate of submission is low worldwide at 12.5% to 26%, for various reasons (Syrjälä *et al*. 2007; Clothier & Anderson 2016). In addition, placenta samples are often of poor quality due to contamination and autolysis reducing chances for isolation in the laboratory (Clothier & Anderson 2016; Vidal *et al*. 2017).

Necrotic placentitis was often associated with detection of *Brucella*, *T. pyogenes*, *L. monocytogenes*, *E. coli*, *Streptococcus*, *Campylobacter* and some fungi. Pneumonia was associated with detection of *Brucella*, *T. pyogenes*, *L. monocytogenes*, *Streptococcus* and *Enterobacteriaceae*. Hepatitis or hepatic necrosis was associated with detection of *L. monocytogenes*, *Brucella*, *Leptospira* and *Campylobacter*. Cholestasis and cholangiohepatitis were associated with detection of *Anaplasma marginale*. Nephritis or nephrosis was associated with detection of *Leptospira* and *E. coli*. *Brucella* (two cases) and T. pyogenes (two cases) were isolated in the absence of lesions. In these cases the isolates were most likely incidental.

Histological evidence of an infectious process was reported in 42 cases (14.6%) in which no cause could be identified. Reasons could be that the bacteria were

not culturable by conventional methods or overgrown by fast growing non-pathogenic organisms (Van Engelen *et al*. 2014). This serves as an indication that improved diagnostic methods that are not reliant on viable bacteria are necessary to determine causes of abortion. Similar results were reported in studies in other countries where placentitis or other signs of inflammation and no aetiological agent was reported in 7.3% to 11.7% of bovine cases (Khodakaram-Tafti & Ikede 2005; Clothier & Anderson 2016), 4 % of caprine cases and 11 to 15.4% of ovine cases (Kirkbride 1992; Van Engelen *et al*. 2014).

Additional clinical history could serve as an important component of a successful diagnosis. Half of the abortion rates in this study fell in the less than 5% category. Abortions in this category tend to be sporadic (Givens & Marley 2008). The reason for this may be that clients who can provide details on abortions in relation to herd size are also the ones who will submit abortions sooner. Abortion rates less than 2% are usually not seen as an indication for investigation (Menzies 2011). However, an abortion rate of between 2% and 5% may be an indication of an endemic infectious agent (Menzies 2011). The latter was evident in this study where such cases were associated with bacterial agents that become endemic in herds such as *B. abortus*, *Leptospira*, *Chlamydia pecorum* and *Campylobacter*. Abortion rates higher than 5 to 8% are usually seen as more than sporadic and an indication that investigation is necessary. In this study *Brucella* spp., *T. pyogenes*, *L. monocytogenes*, *C. burnetii*, *B. licheniformus* and *Rhizopus* sp. infections were implicated in such cases. In this study a cluster of four abortions in four months was associated with a *Brucella* spp. infection indicating that reports of abortions over time can also be useful. A similar finding was reported by Menzies (2011).

Conclusion

The results of this study emphasise the value of analysis of data collected over a long time. Increased submission of placenta and application of additional conventional and molecular diagnostic techniques may lead to increased successful diagnoses. *Brucella* species were the dominant isolates in all provinces included in this study. It would be prudent for all laboratories in these provinces to include *Brucella* detection in their routine abortion panels. Fungal isolates were rare. Different isolates were cultured form different animal species and provinces. The importance of submitting placenta, as well as recording abortion rate and other clinical information can be communicated to veterinarians and farmers at educational events.

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CHAPTER 3

Approaches to increase recovery of bacterial and fungal abortion agents in domestic ruminants

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Abstract

Abortions in domestic ruminants cause significant economic losses to farmers. Determining the cause of an abortion is important for control efforts, but it can be challenging. All available diagnostic methods in the bacteriology laboratory should be employed in every case due to the many limiting factors (autolysis, lack of history, range of samples) that complicate the investigation process. The purpose of this study was to determine whether the recovery of diagnostically significant isolates from domestic ruminant abortion cases could be increased by a combination of the existing aerobic culture and *Brucella* selective method with methods that are commonly recommended in literature reporting abortion investigations. These methods are examination of wet preparations and impression smears stained by the Stamp's modification of the Ziehl Neelsen method (MZN), anaerobic, microaerophilic, *Leptospira*, *Mycoplasma* and fungal culture. Samples of placenta and aborted foetuses from 135 routine clinical abortion cases of cattle (n=88), sheep (n=25) and goats (n=22) were analysed by the new combination of methods. In 46 cases bacteria were identified as aetiological agents and in one case a fungus. Isolation of *Brucella* species increased to 7.4% over 2 years compared to the previous 10 years (7.3%), as

well as *Campylobacter jejuni* (n=2) and *Rhizopus* species (n=1). *Salmonella* species (5.9%) and *Trueperella pyogenes* (4.4%) were also isolated more often. In conclusion, the approach was effective in removing test selection bias in the bacteriology laboratory. The importance of performing an in-depth study on the products of abortion by means of an extensive, combination of conventional culture methods was emphasized by increased isolation of *Brucella abortus* and isolation of *Campylobacter jejuni*. The combination of methods that yielded most clinically relevant isolates was aerobic, microaerophilic, *Brucella* and fungal cultures.

Introduction

Abortions in domestic ruminants cause significant economic losses to farmers worldwide (Borel *et al*. 2014). Abortion is defined as the expulsion of the foetus from the uterus before it is full term and viable (Baumgartner 2015). Diagnosis of the cause of an abortion plays an important role in the subsequent mitigation and control efforts (Borel *et al*. 2014). A diagnosis depends not only on detection of a bacterial/ fungal agent, but also on finding macro- and/ or microscopic lesions indicative of bacterial/fungal infection in foetal tissues which can be challenging (Wheelhouse & Dagleish 2014; Clothier & Anderson 2016). Autolysis is one of the most important factors the laboratory diagnostician must contend with, because it masks lesions and the agent of abortion may be outcompeted by contaminating bacteria (Kirkbride 1986). Foetuses may already be autolytic by the time they are aborted due to an infection process or maceration (Givens & Marley 2008; Clune *et al*. 2021). Other major stumbling blocks tend to be lack of history and incomplete range of samples received at the laboratory (Holler 2012; Wheelhouse & Dagleish 2014). Limited test selection, a cost reducing measure often aimed at detecting the most common agents or those that can be zoonotic,

can drastically reduce chances of reaching a diagnosis (Borel *et al*. 2014; Schnydrig *et al*. 2017).

Placenta and abomasal fluid samples are most likely to yield the organism of interest (Holler 2012; Clothier & Anderson 2016). Agents of abortion that only affect the placenta such as *Chlamydia* species, *Coxiella burnetii*, as well as *Bacillus licheniformis* and some fungi, may not be detected if the placenta is not included (Wheelhouse & Dagleish 2014; Clothier & Anderson 2016). Fungal infections may result in macroscopic lesions in the placenta, but not in the foetus (Borel *et al*.2014). Kidney and liver samples are useful for detection of *Leptospira interrogans* (Markey *et al*. 2013; Mgode *et al*. 2015).

The use of dark field microscopy of wet mounts as well as impression smears were reported by several studies. Kirkbride (1992) as well as Macías-Rioseco *et al*. (2020) used dark field microscopy to screen for motile bacteria, such as *Campylobacter*, in abomasal fluid. Impression smears stained by the Stamp's modification of the Ziehl Neelsen method (MZN) were included in routine abortion investigations although the method was shown to have limited sensitivity and specificity (Borel *et al*. 2014; Schnydrig *et al*. 2017). In a South African report of chlamydiosis in a beef herd, Ehret *et al*. (1975) mentioned the use of the MZN method.

Conventional culture methods vary between laboratories, different species of animals and countries. Bacterial isolation employing aerobic, as well as microaerophilic culture was commonly reported (Campero *et al*. 2003; Agerholm *et al*. 2006; Clothier & Anderson 2016; Schnydrig *et al*. 2017). Aerobic culture on agar plates with 5% blood in a 5% CO_2 atmosphere is sufficient to isolate most bacteria (Borel *et al*. 2014). Addition of microaerophilic culture increases sensitivity for bacteria that are sensitive to oxygen (Borel *et al*. 2014). Anaerobic bacteria such as *Fusobacterium necrophorum, Fusobacterium nucleatum* and

Bacteroides fragilis have been reported in cases of ruminant abortion on rare occasions (Agerholm *et al*. 2006; Syrjälä *et al*. 2007) which may be ascribed to the fact that anaerobic culture is not commonly included in abortion panels (Borel *et al*. 2014). Schnydrig et al. (2017) included selective media for *Brucella*, *Campylobacter*, *Listeria,* and fungi to increase chances of isolating these microorganisms by suppressing growth of contaminating bacteria.

Several bacterial agents of abortion are contagious and/ or become endemic in herds. *Brucella abortus* (*B. abortus*) is common and *Brucella melitensis* (*B. melitensis*) as well as *Brucella ovis* (*B. ovis*) are isolated in South Africa (Mbizeni 2015; Kolo *et al*. 2019; Matle *et al*. 2021). *Campylobacter fetus* subsp. *venerealis*, *Campylobacter fetus* subsp *fetus*, *Campylobacter jejuni* and *Arcobacter* spp. are occasionally implicated in cases of abortion in South Africa (Bath *et al*. 2013; Coetzer *et al*. 2018), while *Campylobacter* species were more commonly reported as causes of abortion in Argentina and New Zealand (West 2002; Campero *et al*. 2003). The families *Chlamydiaceae*, *Waddliaceae*, *Parachlamydiaceae* and *Rhabdochlamydiaceae* are of significance in contagious abortions (Borel et al. 2014). However, members of these families fall in the difficult to culture category, because they do not grow on conventional growth media (Vidal 2017).

Serological studies indicate that *Chlamydia abortus* plays a role in ruminant abortions in South Africa (Sri Jeyakumar 2001; Ndou & Dlamini 2012). *Leptospira interrogans* serovars have been implicated in bovine abortion in South Africa and other countries such as the USA (Herr *et al*. 1982; Clothier & Anderson 2016). Several *Salmonella* subspecies and serovars are associated with abortion in ruminants, for example *Salmonella enterica* subspecies *diarizonae*, *Salmonella enterica* subspecies *enterica* serovar Dublin and *Salmonella enterica* subspecies *enterica* serovar Abortusovis (Markey *et al*.

2013). *Salmonella enterica* subspecies *enterica* serovar Dublin is a known cause of abortions in cattle in South Africa (Coetzer *et al*. 2018).

Opportunistic bacterial agents of abortion may be commensals of mucous membranes or may be common in the environment, for example *Bacillus* spp., *Escherichia coli* (*E. coli*), *Histophilus somni*, *Pasteurella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Trueperella pyogenes* (*T. pyogenes*) and *Mycoplasma* spp. Abortions caused by these bacteria are sporadic but their occurrence is widespread (Clothier & Anderson 2016).

Mycotic or fungal abortion is caused by opportunistic fungal pathogens that are normally present as saprophytes in the environment of the animal. Fungi most associated with abortion are *Aspergillus* spp. and *Candida* spp. (Knudson & Kirkbride 1992; Anderson 2007; Holler 2012; Mee 2020).

Additional herd information and history such as the number of abortions, the number of pregnant animals, clinical signs in dams and changes in husbandry are useful (Borel 2014). Number of abortions per number of pregnant animals (abortion rate) is a useful guideline to determine whether an investigation is indicated or not. Abortion rates less than 2% is usually not seen as an indication for investigation (Menzies 2011). Most abortions are sporadic with less than 5% of pregnant animals aborting (Givens & Marley 2008). However, it must be borne in mind that an abortion rate of between 2% and 5% may be an indication that an endemic infectious agent is present (Menzies 2011). Additional information must be interpreted with caution as it can be misleading. For example, etiological agents should not be ruled out simply because animals were vaccinated as vaccination does not result in complete immunity (Kirkbride 1986). In addition, unvaccinated, infected animals could have been introduced into a herd prior to an abortion event.

The aim of this study was to analyse samples of placenta and aborted foetuses from cattle, sheep and goats by means of a set combination of conventional culture and identification methods for bacteria and fungi. The objective was to determine if more diagnostically significant isolates could be cultured if anaerobic, *Campylobacter*, *Leptospira*, *Mycoplasma* and fungal culture were added to the existing aerobic and *Brucella* selective culture; and if applying the new combination of methods to every abortion case could remove test selection bias.

Materials and methods

Routine diagnostic samples from products of abortion from cattle, sheep and goats submitted to the Bacteriology laboratory of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria over a two-year period (September 2017 to September 2019) were included in the study. Samples were accepted from the Pathology section at the Faculty of Veterinary Science, regional government laboratories, private laboratories, private veterinarians, or State veterinarians. Criteria for selection was abortion meaning a foetus expelled from the uterus before it was full term and viable (Baumgartner 2015). Indications that the calf/ lamb/ kid was born alive such as aerated lungs, colostrum in the abomasum or worn golden slippers were criteria for exclusion. One or more foetuses submitted as a single case was recorded as a single case, except when different agents were recovered from the different foetuses. Samples accepted were any of the following: placenta, stomach content, lung, liver, spleen, and kidney from aborted foetuses. Samples were accepted whether they were in good (no or limited autolysis) or poor (advanced autolysis) condition.

Sample size was calculated as 235 foetuses for this descriptive study (Survey toolbox-www.epitools.ausvet.com.au) (Fosgate 2009), therefore the aim was to analyse samples from between 200-300 foetuses.

Conventional microbiological analyses

The methods that were previously applied to placenta and foetus samples in the bacteriology laboratory at the DVTD were impression smears stained by the Gram and MZN methods as well as aerobic culture, *Brucella* selective culture and fungal culture. *Brucella* selective culture and fungal culture were performed on request. For the purpose of this study wet mounts, microaerophilic culture, anaerobic culture, *Mycoplasma* selective culture and *Leptospira* selective culture were added.

Direct smears

Wet mounts were made of stomach content samples, if included in abortion cases, and examined by dark field microscopy for *Campylobacter* spp., *Leptospira* spp. and other motile bacteria. Impression smears were made of all placenta and foetus samples, heat fixed and stained by Gram and MZN methods (Markey *et al*. 2013).

Aerobic culture

All samples were inoculated on Columbia blood agar with 5% horse blood (CBA) (Selecta-media, Thermo Fischer, Gauteng, South Africa), MacConkey agar (McC) (without crystal violet) (Selecta-media, Thermo Fischer, Gauteng, South Africa) and Xylose-Lactose-Deoxycholate medium (XLD) (Oxoid CM0469B, Thermo Fischer, Gauteng, South Africa). Inoculated CBA plates were incubated in a 5% carbon dioxide (CO_2) atmosphere at 37 ºC ± 1 ºC and McC and XLD plates were incubated in normal air at 37 ºC± 1 ºC. CBA plates were examined daily for 10 days. McC plates were examined after 24 hours. Primary identification consisted of Gram stain as well as catalase and oxidase tests. The

outcome of these tests determined carbohydrate combinations for secondary identification (Markey *et al*. 2013).

Anaerobic culture

Only stomach content was inoculated on pre-reduced CBA and incubated anaerobically at 37 ºC ± 1ºC for five days in an anaerobic workstation (Bactron anaerobic chamber, United Scientific, Gauteng, South Africa). If stomach content was not received, this culture method was not performed. An anaerobic detector strip (Oxoid, Thermoscientific, Gauteng, South Africa) was used to confirm anaerobiosis. Primary identification consisted of Gram stain, catalase and oxidase tests. Depending on the outcome of these tests, isolates were identified by Mastring (Mast ID, Davies Diagnostics, Gauteng, South Africa) or Rapid ID 32A (Biomerieux, Gauteng, South Africa) (Markey *et al*. 2013).

Brucella selective culture

In addition to aerobic culture, placenta, stomach content and/ or lung were inoculated on Farrell medium (Oxoid CM0169 with supplement (SR0083A), Thermoscientific, Gauteng, South Africa). If these samples were not received, this culture method was not performed. One plate of Farrell medium was inoculated with an in-house *Brucella abortus* control culture for quality control purposes. Plates were incubated in CO_2 at 37 ºC ± 1 ºC for 10 days and examined daily. Cultures were regarded as negative when no characteristic growth was obtained by day 10. Suspect colonies were subcultured onto two CBA plates and one McC plate. One CBA plate was incubated in CO_2, the other CBA plate was incubated in normal air together with the McC plate. Primary identification included a Gram stain, MZN stain, oxidase test and a Urease slant (Selecta-media, Thermoscientific, Gauteng, South Africa) (WOAH 2016). Presumptive *Brucella* spp. identified were submitted to the Agricultural Research

Council Onderstepoort Veterinary Research (ARC-OVR) for confirmation and phenotyping.

Salmonella selective culture

In addition to direct culture for *Salmonella*; placenta, stomach content and organ samples were inoculated on Rappaport-Vassiliadis Broth (RV) (Oxoid CM 0866B, Thermoscientific, Gauteng, South Africa) and incubated at 42 ºC ± 1 ºC in normal air. After 24 hours, the broth culture was inoculated on XLD medium (Oxoid CM0469B, Thermoscientific, Gauteng, South Africa) and incubated at 37 ºC ± 1 ºC in normal air. Primary identification included a Gram stain, catalase, oxidase, and spot indole tests. API® 10S (Biomerieux, Gauteng, South Africa) was used for secondary identification (Markey *et al*. 2013). If no characteristic colonies were seen the culture was regarded as negative. *Salmonella* isolates were serotyped according to the White-Kauffman-Le Minor scheme (Grimont & Weil 2007).

Mycoplasma selective culture

In addition to aerobic culture, stomach content, placenta and lung were inoculated directly on a plate of *Mycoplasma* medium (Oxoid CM0401 & SR0059, Thermoscientific, Gauteng, South Africa) and in *Mycoplasma* broth (Oxoid CM0403 & SR0059, Thermoscientific, Gauteng, South Africa). If these samples were not received, this culture method was not performed. The broth was incubated in normal air at 37 ºC ± 1 ºC and plated onto *Mycoplasma* medium after 24 hours. Plates were incubated in CO_2 at 37 ºC ± 1 ºC; examined at 48 and 96 hours after inoculation and thereafter once a week. If no characteristic growth was obtained after 14 days' incubation, the culture was regarded as negative. Suspect cultures were identified to genus level as described by Markey *et al*. (2013).

Campylobacter culture

Placenta, stomach content and liver were inoculated on CBA. If none of these samples were received, this culture method was not performed. Inoculated plates were incubated in a microaerophilic atmosphere (6% O_2, 10% CO_2 and 84% N) (CampyGen, Oxoid Ltd, Thermoscientific, Gauteng, South Africa) in anaerobic jars (Oxoid Ltd, Thermoscientific, Gauteng, South Africa) at 37 ºC ± 1 ºC for five days. The plates were examined after 72 hours and again at the end of five days. If no suspect cultures were noted by day five, plates were discarded as negative. Primary identification included Gram stain, catalase, and oxidase tests. Secondary identification included growth in a microaerophilic atmosphere at 42 ˚C, growth at 37 °C in normal air, growth at 22 °C, growth in the presence of 1% glycine, production of H_2S in triple sugar iron medium (TSI) (Selecta-media, Thermoscientific, Gauteng, South Africa) and hippurate hydrolysis (Remel, Thermoscientific, Gauteng South Africa) (Markey *et al*. 2013).

Charcoal-Cefoperazone-Dextrose-Agar (CCDA) (Selecta media, Thermoscientific, Gauteng, South Africa) was inoculated with placenta, stomach content and liver samples from cases that yielded *Campylobacter* isolates on CBA. The *Campylobacter* isolates from these cases and a control strain, *Campylobacter jejuni* (ATCC 33560) were also inoculated on CCDA. The inoculated medium was incubated in a microaerophilic atmosphere at 37 °C ± 1 ºC for five days. The plates were examined for suspect colonies after 72 hours and again after five days. If no suspect colonies were observed by day five, plates were discarded as negative. Primary and secondary identification of suspect colonies was performed as described above.

Leptospira culture

One tube of *Leptospira* Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco 279410 & supplement 279510, Becton Dickinson, Gauteng,

South Africa) was inoculated with 1 g kidney or liver. If none of these samples were received, this culture method was not performed. Cultures were incubated at 30 ºC ± 1 ºC in normal air and examined weekly for 13 weeks. Contaminated cultures were subcultured in to a new tube of EMJH medium with 5'-fluorouracil (Sigma, Gauteng, South Africa). Turbidity and/or the formation of Dinger's ring as well as characteristic morphology on dark-field microscopy was regarded as presumptive positive. If no turbidity is evident by week 13, cultures were regarded as negative (Markey *et al*. 2013). Presumptive positive cultures were submitted to the Agricultural Research Council Onderstepoort Veterinary Research (ARC-OVR) for PCR confirmation.

Fungal culture

Stomach content and placenta (as well as skin lesions, if submitted) were inoculated on Potato Dextrose agar (Selecta-media, Thermoscientific, Gauteng, South Africa). If none of these samples were received, this culture method was not performed. The plates were incubated at 37 ºC for 5 days. Plates were examined twice a week. A sticky tape colony smear was made of suspect colonies and stained by Lactophenol Cotton Blue stain (Merk, Gauteng, South Africa). Fungi were identified by colony morphology. Yeasts were identified by Gram stain and API® 20C AUX (Biomerieux, Gauteng, South Africa) (Markey *et al.* 2013).

Supplementary information

Data such as pathology lesions, immunohistochemical staining results and PCR results were extracted from diagnostic reports for supplementary information. The methodologies for these procedures were, however, not available.

Herd history

Additional production data such as farming system, feeding system, number of pregnant animals, number of abortions and vaccination status were extracted from pathology reports.

Data capture and analysis

Data was captured in an Excel spreadsheet from which summary tables were created. The aim was to investigate patterns with respect to diagnostic rate, completeness of submission, condition of samples, methods used and season. Diagnostic rate was calculated by dividing the number of confirmed diagnoses by the total number of submissions and multiplying by 100. The result was expressed as a percentage. A confirmed diagnosis was defined as a diagnosis made by the pathologist considering the pathological lesion and the bacterial or fungal isolate. Abortion rate was calculated by dividing the number of abortions by the number of pregnant animals in a herd (if available) and multiplying by 100. The result was expressed as a percentage. Abortion rate is used to determine the significance of the number of abortions in a herd. Data was exported into a statistical program (Epi info 7™ 7.2.3.1, Centers for Disease Control and Prevention). Percentages with 95% confidence intervals (CI) were calculated; and multivariable logistic regression was used to investigate associations between completeness of submission, condition of samples, confirmed diagnosis and season.

Results

Number of cases

The calculated number of 235 abortion cases was not reached due to lower-than-expected submission rates during 2018 and 2019. Samples from 135 abortion cases were received for bacterial and fungal culture. Eighty-eight were

bovine, 25 were ovine and 22 were caprine cases (Annex 2). Forty-six complete submissions (foetus and placenta) were received; 26 bovine, 11 ovine and 9 caprine cases. Eighty-nine submissions were incomplete; 85 cases of foetus only and 4 cases of placenta only. Most samples (n=100) were considered good quality and 35 were poor quality (Table 3.1).

Table 3.1 Diagnosis and diagnostic rate (with 95% confidence intervals) compared to the completeness of submissions as well as quality of samples.

Submission	**Number of submissions**	**Confirmed diagnosis (Diagnostic rate)**	**Quality of samples**	**Number**	**Confirmed diagnosis (Diagnostic rate)**
Foetus and placenta	46	20 (43.5%, 95% CI 33, 53)	Good	33	15 (45.5%, 95% CI [35,55])
			Poor	13	5 (38.5%, 95% CI [28,48])
Foetus only	85	36 (42.4%, 95% CI [32,52])	Good	63	31 (49.2%, 95% CI [39,59])
			Poor	22	5 (22.7%, 95% CI [14,31])
Placenta only	4	1 (25%, 95% CI [17,35])	Good	4	1 (25%, 95% CI [17,35])
Total	135	57 (42.2%, 95% CI [32, 52])	Good	100	47 (47%, 95% CI [37,57])
			Poor	35	10 (28.6%, 95% CI [19,38])

Diagnostic rate

Overall diagnostic rate for cases submitted to the bacteriology laboratory was 42.2% (95% CI [32, 52]). Diagnostic rate per animal species was 36 of 88 bovine cases (32.9%, 95% CI [23,42]), 14 of 25 ovine cases (48%, 95% CI [38,58]) and 7 of 22 caprine cases (27.3%,95% CI [19,37]). Bovine cases were most often received (88/135). Complete submissions (foetus and placenta) had the highest

diagnostic rate overall at 43.5% (95% CI [33, 53]) although this was not the case for all species. Diagnostic rate for complete bovine cases was 10 of 26 (38.5%, 95% CI [28,48]) and for incomplete cases 24 of 62 (38.7%,95% CI [28,48]). Diagnostic rate for complete ovine cases was five of 11 (45.5%, 95% CI [35,55]) and for incomplete cases seven of 14 (50%, 95% CI [40,60]). Diagnostic rate for complete caprine cases was three of nine (33.3%, 95% CI [24,43]) and for incomplete cases, three of 13 (23%, 95%CI [15,32]). Submissions where the placenta was included (n=50) had a diagnostic rate of 42% (95% CI [32,52]). The diagnostic rate was 47%, 95% CI [37,57] for submissions where samples were of good quality (no or limited autolysis) and 28.6%, 95% CI [19,38] in cases with poor quality samples (moderate autolysis) (Table 3.1). In 47 of 57 submissions where a diagnosis was made, bacteria or fungi were implicated. In the remaining 10 cases where a diagnosis was made, viral (Bovine herpesvirus-1, BVDV) or non-infectious aetiologies were implicated.

Most cases were submitted in winter (n=59) followed by spring (n=34). Most *Brucella abortus* positive cases (n=8) and *Trueperella pyogenes* positive cases (n=6) were submitted in winter and spring. Multivariate logistic regression analysis was performed to investigate the association between diagnosis and degree of autolysis, completeness of submission as well as season (Table 3.2). There were no significant associations between diagnosis complete submission (inclusion of placenta) or season. Although there appeared to be a significant difference in diagnostic rate between good and poor quality samples (Table 3.1), there was no significant association between degree of autolysis and diagnosis (P>0.05) (Table 3.2)

Table 3.2 Multivariate regression analysis investigating association between diagnosis and degree of autolysis, completeness of submission and season.

Term	Odds Ratio	Lower 95% C.L.	Upper 95% C.L.	P-Value

Autolysis (Yes/No)	11 215	0.454	27 704	0.804
Complete submission (Yes/No)	13 444	0.6393	28 272	0.435
Season (Spring/Autumn)	18 074	0.4681	69 785	0.391
Season (Summer/Autumn)	19 606	0.4737	81 152	0.353
Season (Winter/Autumn)	16 928	0.4787	5 986	0.414

No wet mounts were positive for motile bacteria. Modified Ziehl-Neelsen stained impression smears were positive for small, partially acid-fast coccobacilli in six of 10 cases (60%, 95% CI [3,9]) that yielded *Brucella abortus* positive cultures. In addition, three cases that were positive for partially acid-fast rods on stained impression smears were PCR positive for *Coxiella burnetii*. No *Chlamydiales* positive cases were positive for partially acid-fast bacteria on impression smears.

Eighty-five potentially abortifacient isolates were cultured from 135 submissions. Bacteria were most common (73/85). Twelve isolates were endemic/contagious bacteria and 73 were environmental or commensal bacteria that cause sporadic abortions. Twelve isolates were fungi. In 46 cases bacteria were identified as aetiological agents and in one case a fungus (Table 3.1). *Brucella abortus* was the most common of the endemic/contagious bacteria at 10/12 isolates. It was also one of the most common bacterial isolates overall (10/71).

Submissions were received from six of nine provinces of South Africa. Farms of origin were mainly in Gauteng province (n=45) followed by North West province (n=30) and Mpumalanga (n=20) (Annex 2). *Brucella abortus* was isolated from bovine submissions from North West, Free State, Mpumalanga, and Northern

Cape provinces. *Campylobacter jejuni* was isolated from ovine and caprine submissions from Gauteng province. *Campylobacter fetus* was isolated from submissions from the Free State and North West provinces. *Trueperella pyogenes* was isolated from bovine cases from Gauteng, North West, Eastern Cape, and Free State provinces.

Several co-infections (n=12) were identified, notably *Bacillus licheniformis* and *Rhizopus* spp., *Brucella abortus* biovar I and *Salmonella* sp., *Chlamydia abortus* and *Chlamydia pecorum* as well as *Chlamydia* spp. and *Enterobacteriaceae*.

Eighteen cases were recorded where there was pathological evidence of an infectious process, but no potential pathogen was isolated. Most cases had placental lesions (n=12); necrotic placentitis (n=6), necrotic placentitis with vasculitis (n=2) or placentitis (n=4) (Table 3.3).

Table 3.3 Significant bacterial and fungal isolates from products of abortion detected by impression smears stained by the Modified Ziehl Neelsen method (MZN), conventional culture, immunohistochemistry (IHC) and PCR. (A=Aerobic culture, B=Brucella selective culture, C=microaerophilic culture, F=Fungal culture, M=Mycoplasma selective culture; X=positive, N=Negative, ND=Not done)

Case no	**Pathology lesions**	**Bacterial/fungal isolate**	**Detection method**			
	Bovine abortions		**MZN**	**Culture**	**IHC**	**PCR**
1	Purulent bronchointerstitial pneumonia	*Trueperella pyogenes*	X	A	ND	ND
2	No specific lesions (No placenta)	*Trueperella pyogenes*	N	A	ND	ND
3	Meningitis	*Brucella abortus* biovar 1	X	A, B	ND	ND
4	Meningitis, bronchointerstitial pneumonia	*Brucella abortus* biovar 1	X	A, B	ND	ND

5	Non-specific lesions (No placenta)	*Campylobacter fetus* subsp. *venerealis* biovar intermedius	N	A, M	ND	ND
6	Purulent bronchopneumonia	*Trueperella pyogenes*	N	A	ND	ND
7	Non-specific lesions (No placenta)	*Salmonella* sp.	N	A	ND	ND
8	Necropurulent placentitis	*Trueperella pyogenes*	N	A	ND	ND
9	Purulent bronchopneumonia	*Brucella abortus* biovar 1	X	A, B	ND	ND
10	Necrotic placentitis, purulent pneumonia	*Chlamydia* sp.	N	ND	X	ND
		Salmonella sp.	N	A	ND	ND
11	Purulent bronchopneumonia	*Trueperella pyogenes*	N	A	ND	ND
12	Bronchointerstitial pneumonia	*Brucella abortus* biovar I	X	A, B	ND	ND
13	No specific lesions (No placenta)	*Salmonella* sp.	N	A	ND	ND
14	Necrotic placentitis with fungal hyphae, purulent bronchopneumonia	*Bacillus licheniformis*	N	A	ND	ND
		Rhizopus sp.	N	F	ND	ND
15	Necrotic placentitis	*Streptococcus dysgalactiae*	N	A	ND	ND
		Acholeplasma sp.	N	M	ND	ND
16	Pulmonary & hepatic congestion	*Brucella abortus* biovar I	N	A, B	ND	ND
		Acholeplasma sp.	N	M	ND	ND
17	Generalised congestion, hepatomegaly, bronchopneumonia	*Brucella abortus* biovar 1	N	A, B	ND	ND
		Salmonella sp.	N	A	ND	ND
18	Yellow liver	*Salmonella* sp.	N	A	ND	ND
19	Neutrophilic interstitial pneumonia	*Pseudomonas luteola*	N	A	ND	ND

20	Necrotic placentitis	*Campylobacter fetus*	N	A, C	ND	ND
		Mannheimia varigena, Streptococcus pluranimalium	N	A	ND	ND
		Mycoplasma sp.	N	M	ND	ND
21	Necrotic placentitis and vasculitis	*Mycoplasma* sp.	N	C	ND	ND
22	S/C oedema, hydrothorax, ascites,	*Trueperella pyogenes*	N	A	ND	ND
23	Necrotic placentitis with vasculitis	*Coxiella burnetii*	X	ND	X	ND
24	Interstitial pneumonia	*Brucella abortus* biovar 1	N	A, B	ND	ND
25	Purulent bronchopneumonia	*Aeromonas hydrophila*	N	A	ND	ND
26	Hepatitis, Vasculitis, bacteria in organs	*Staphylococcus delphini*	N	A	ND	ND
27	Bronchopneumonia, hepatic necrosis	*Brucella abortus* (Field strain)	N	A, B	ND	ND
28	Purulent bronchopneumonia	*Brucella abortus* biovar 2	X	A, B	ND	ND
29	No lesions (No placenta)	*Brucella abortus* biovar 1	X	A, B	ND	ND
	Caprine abortions					
30	Meningoencephalitis	*Campylobacter jejuni* subsp. *jejuni*	N	C	ND	ND
31	Lung haemorrhages, Hepatic necrosis	*Salmonella enterica* subsp. *arizonae*	N	A	ND	ND
32	Necrotic placentitis	*Coxiella burnetii*	X	ND	X	ND
		Salmonella Typhimurium	N	A	ND	ND
33	No lesions (No placenta)	*Chlamydia abortus*	N	ND	ND	X
		Chlamydia pecorum				
34	Brain congestion, Severe interstitial pneumonia	*Escherichia coli*	N	A	ND	ND
35	Bacteraemia	*Escherichia coli*	N	A	ND	ND

	Ovine abortions					
36	Necrotic, purulent placentitis Bronchopneumonia	*Chlamydia abortus*	N	ND	ND	X
		Escherichia coli	N	A	ND	ND
37	Purulent bacterial placentitis	*Escherichia coli*	N	A	ND	ND
38	Congestion of placenta, brain, liver; Lymphoplasmacytic infiltration of myocardium	*Salmonella* D	N	A	ND	ND
39	Non-specific lesions (No placenta)	*Chlamydia* sp.	N	ND	X	ND
		Salmonella sp.	N	A	ND	ND
40	Pleuropneumonia, Bacterial & fungal placentitis	*Chlamydia abortus*	N	ND	ND	X
41	Bacterial placentitis	*Arcobacter* sp.	N	A	ND	ND
42	Necrotic placentitis	*Salmonella* Budapest	N	A	ND	ND
		Mycoplasma sp.	N	M	ND	ND
43	Necrotic placentitis, purulent pneumonia	*Chlamydia* sp.	N	ND	X	ND
44	Necrotic placentitis	*Stenotrophomonas maltophilia*	N	A	ND	ND
45	Hydrothorax, bacteria in blood vessels of brain and liver	*Escherichia coli*	N	A	ND	ND
46	Necrotic placentitis	*Coxiella burnetii*	X	ND	X	ND
47	Non-specific lesions	*Campylobacter jejuni*	N	C	ND	ND
		Staphylococcus sp.	N	A	ND	ND

Additional production related history (farming system, livestock species, vaccinations, feeding system, herd size, number of abortions) was reported for 50 cases (37%). In 38 of these cases farming systems were recorded. Farming systems were mostly meat production (n=30) and dairy (n=9). Cattle (n=19) and goats (n=9) were the most common livestock species. In 12 cases vaccinations

were reported. Five of these cases included *Brucella* vaccination. Two cases included *Chlamydia abortus* vaccination. Feeding systems were mostly pasture based with supplementary feeding (n=6). One owner reported feeding silage the quality of which was sometimes poor. Nine owners reported number of abortions and number of pregnant animals or abortion percentage. Abortion percentage ranged from 1% to 75%. Two owners reported abortions over time: nine abortions in five days and four abortions in two days.

Vaccination history was recorded in 12 cases. A wide range of vaccines were used many of which do not have prevention of abortion as their primary application. Vaccines for the prevention of abortion were *Brucella* S19 and RB51 recorded in five cases; as well as Enzootic abortion vaccine (*C. abortus*) recorded in two cases. Abortions in these seven cases were due to agents other than those vaccinated against indicating that the vaccines were effective or that animals were not exposed to the agents the vaccines are protective against.

Discussion

In this study, the standard diagnostic approach for abortion investigations in this laboratory namely impression smears stained by the Gram and MZN methods, aerobic culture with the addition of *Brucella* culture if requested, was challenged. The effect of adding anaerobic, *Brucella*, *Campylobacter*, *Mycoplasma* and *Leptospira* culture methods and applying the combination of methods to every submission of foetus and placenta samples, was investigated. The objectives were to determine if the extended combination of conventional culture methods could increase recovery of bacterial and fungal causes of abortions. In addition, pathological lesions as well as results of other methods of detection employed by pathologists in case investigations such as PCR and IHC were recorded.

The number of submissions per year in this study; 83 (2018) and 72 (2019) increased markedly compared to submissions recorded by a retrospective study (2006-2016) by Jonker & Michel (2021) where submissions varied between 11 and 55 per year. The increased number of submissions was probably due to this research project where the conventional bacteriology fees were subsidised. Despite this, submissions were less than the statistically calculated sample size for the study and still biased toward a bacterial or fungal aetiology for the following reasons. Notification of abortion is not compulsory in South Africa. Products of abortion are only submitted for investigation when the field diagnostician and the farmer need a diagnosis; and the farmer can afford the fees. Fees for abortion investigation can be considerable, so cost cutting measures are commonly employed leading to test selection bias. Only samples that are highly suspect for a bacterial or fungal infection will be submitted to a bacteriology laboratory. This approach is contrary to recommendations by Kirkbride (1992) and Borel *et al*. (2014) that all diagnostic methods available should be employed in every abortion case to ensure the best possible chance of a diagnosis.

Number of submissions decreased as distance increased. Most submissions were received from farms in Gauteng province which is closest to the Veterinary Faculty at Onderstepoort, followed by neighbouring North West and Mpumalanga provinces. This is probably due to increased costs related to transport of products of abortion and other veterinary laboratories in closer proximity.

Inclusion of additional methods resulted in two *Campylobacter jejuni* isolates that grew only in a microaerophilic atmosphere and would not have been isolated by the previous options for culture. Detection of *Brucella* improved from 7.3% over 10 years (Jonker & Michel 2021) to 7.4% over two years. Anaerobic culture did

not yield isolates that could be implicated as causes of abortion. *Leptospira* cultures did not yield isolates. Anaerobes and *Leptospira* are labile and could die before samples reach the laboratory leading to false negative results when culture is attempted. Molecular methods should be investigated as an alternative for detection of these bacteria since genetic material can still be detected after the death of an organism. Agerholm *et al*. (2006) reported *Fusobacterium necrophorum* as the second most common aetiology in their ovine study. In their study *F necrophorum* was never cultured, but was detected by 16S rRNA gene PCR after bacteria associated with inflammation was noted during histological examination.

The overall diagnostic rate of 42.2% in this study was significantly higher than the 35.1% found in the previous retrospective study (2006-2016) (Jonker & Michel 2021). Of the cases where a diagnosis was made 82.5% had a bacterial/fungal cause. This was expected, because products of abortion are only submitted when a bacterial or fungal infection is suspected. Among the remaining 17.5% cases where diagnoses were made, three cases with hepatic necrosis had a viral aetiology. In two cases Bovine herpesvirus-1 was implicated and in a third case Bovine viral diarrhoea virus. The rest of the cases had non-infectious causes. Pathological lesions caused by viral or protozoal infections can sometimes be macroscopically indistinguishable from that caused by bacteria so samples are submitted to the bacteriology laboratory to rule out bacterial infection.

The diagnostic rate for ovine cases (48%) was highest followed by bovine cases (32.9%). This phenomenon is also noted by another study (Kirkbride 1993) where it was remarked that infectious agents that cause ovine abortions tended to cause more recognisable lesions. In this case the difference in diagnostic rate may also be related to number of submissions for the different species.

As previously reported, availability of placenta appeared to positively influence diagnostic rate. Cases where a placenta was included had a diagnostic rate of 42%. In this study placentas were received in 37% cases, an improvement over 22.2% placentas recorded in the retrospective study (Jonker & Michel 2021). This is probably due to continued efforts to emphasize the importance of submission of placenta in reports to clients. Good quality samples appeared to have a more positive influence on diagnostic rate (47%) than inclusion of placenta. However, no statistically significant association ($p=<0.05$) was found between overall diagnostic rate and completeness of submission or quality of samples. This is probably due to the small number of cases in these two studies.

The significance of a bacterial isolate is indicated by compatible history, isolation in large numbers in pure or almost pure culture from one or more than one sample, as well as histopathological findings that correlate with the isolate (Anderson *et al*. 1990; Kirkbride 1993; Borel *et al*. 2014). Determination of the significance of a fungal isolate is similar with the additional requirement that fungal hyphae must be visualised microscopically in tissues (Campero *et al*. 2003; Borel *et al*. 2014). Contamination by bacteria or fungi result in a mixed culture without any dominant isolates (Kirkbride 1986).

In this study *Brucella abortus* was the most common isolate recovered by conventional bacteriological methods reported overall, as well as from three provinces; North West, Free State and Mpumalanga. In this study 7.4% cases were positive for *B. abortus* which is not significantly more than 7.3% reported previously in South Africa (Jonker & Michel 2021). Modified Ziehl Neelsen-stained impression smears were negative in 40% of *Brucella* culture positive cases confirming insufficient sensitivity of MZN-stained smears as reported by Schnydrig *et al*. (2017). *Brucella melitensis* was not isolated. Successful *B. melitensis* isolation was reported in a retrospective study by Matle *et al*

2021.This could be due to a difference in sample quality, numbers of bacteria in samples and/or processing. Matle *et al* 2021 reported homogenizing samples and plating it on 5% sheep blood agar in addition to Farrell's medium.

In this study *Campylobacter* species was only isolated in two cases of bovine, one case of ovine and one case of caprine abortion. Only *Campylobacter jejuni* could be cultured on CCDA. Although *Campylobacter* spp. did not appear to play a significant role in abortion the findings clearly highlight the importance of including a non-selective culture medium in *Campylobacter* culture. In contrast with this study, *Campylobacter* spp. was reported as a significant cause of abortion in countries in the northern hemisphere (the Netherlands and United Kingdom) as well as Argentina in the southern hemisphere where it was the second most common aetiology in bovine cases (Campero *et al*. 2003; Van Engelen *et al*. 2014; Carson 2017). Vaccine can be used to control *C. fetus* subsp. *venerealis* infection. Therefore, it is important to differentiate *Campylobacter* spp. from one another although phenotypic differentiation of species in this family does present challenges (Van der Graaff-Van Bloois *et al*. 2014).

Salmonella species (5.9%) and *T. pyogenes* (4.4%) were implicated more commonly as agents of abortion compared to the retrospective study by Jonker & Michel (2021). The reason for the increased isolation of *Salmonella* spp. could be reduction of test selection bias in this study.

In this study, like several other studies, several co-infections were recorded, such as *Bacillus licheniformis* and *Rhizopus* spp., *Brucella abortus* biovar I and *Salmonella* spp. as well as *Chlamydia abortus* and *E. coli*. A South African study by Schutte *et al*. (1976) reported co-infection of *C. burnetii* and *C. psittaci*. Schnydrig *et al*. (2017) reported 31.2% cases with co-infections. Campero *et al*. (2003) reported a case of *B. abortus* and *Campylobacter* sp. co-infection.

Agerholm *et al*. (2006) reported a dual infection of *F. necrophorum* and *L. monocytogenes* in an ovine foetus. In cases where dual infections occur complete investigation by histopathology, IHC as well as detection of the pathogen is required to determine the causative agent.

In 12 cases (8.8%) evidence of an infectious process was seen macro- and/or microscopically, but no agent of abortion was isolated. This is a reduction of cases from 14.6% previously recorded previously by Jonker & Michel (2021). Application of an extended combination of diagnostic methods to all cases probably contributed to this improvement. More *Brucella* sp were detected by adding *Brucella* selective culture. *Campylobacter jejuni* was detected by microaerophilic culture. *Mycoplasma* species was detected by *Mycoplasma* selective culture. *Salmonella* was detected by aerobic culture in cases where the client only requested *Chlamydia* detection. Immunohistochemistry and PCR methods were rarely used, increased application of these methods may lead to increased detection of bacteria such as *Brucella* sp, *Chlamydia* sp and *Coxiella burnetii*.

Conclusions

The new combination of conventional culture methods applied to all abortion cases only served to remove test selection bias in the bacteriology laboratory, but not sample selection bias. Nonetheless, the importance of performing an in-depth study on the products of abortion by means of an extensive, combination of conventional culture methods was emphasized by an increase in the number of *B*. *abortus* and *Campylobacter jejuni* isolations. Some of these isolates would have been missed if culture in a microaerophilic atmosphere and *Brucella* selective culture were not included. The combination of methods that yielded the

highest number of significant isolates was aerobic, microaerophilic, *Brucella* and fungal culture.

Recommendations

Submission of good quality samples is important to ensure the best chance of recovering bacterial or fungal abortion agents.

Further investigation is recommended to extend the use of molecular methods to detect and identify difficult to culture agents of abortion to species level.

Brucella abortus was the most common agent of abortion isolated in this laboratory over a period of two years. Due to the common occurrence of brucellosis, the recommendation is that all laboratories in South Africa employ *Brucella* species detection.

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Chapter 4

Optimization and application of quantitative Real-Time PCR assays in detection and identification of *Chlamydiales* in products of domestic ruminant abortion

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Abstract

Domestic ruminant abortions due to infectious agents represent an important cause of economic losses in the agricultural industry. Placentitis and pneumonia are pathological lesions associated with *Chlamydiales* infection. In a previous study twenty-five clinical cases had pathological lesions of placentitis or pneumonia. This study aimed to optimise and apply qPCR assays for detection of *Chlamydiales* in domestic ruminant abortion cases. Primers and probes for detection of the order *Chlamydiales*, *Chlamydia abortus*, *Chlamydia pecorum*, *Parachlamydia acanthamoeba* and *Waddlia chondrophila* were taken from literature to create one singleplex and two duplex assays and the assays were optimised. These cases were investigated further by application of the qPCR assays in this study. *Chlamydiales* were detected in 16 cases. *C. abortus*, *P. acanthamoeba* and *W. chondrophila* were detected in bovine; and *C. pecorum* and *W. chondrophila* in ovine and caprine cases. *Chlamydiales* were detected in three previously inconclusive cases. Identification was improved from genus to species level (*C*. *pecorum*). Four cases remained inconclusive. In conclusion, detection of *Chlamydiales* and differentiation to species level was improved

compared to the previous study (Chapter 3). This study reports the first detection of *P. acanthamoeba* and *W. chondrophila* in abortion cases in South Africa indicating a potentially significant role in abortions in this country.

Introduction

Infectious agents are the most common causes of domestic ruminant abortions (Sebastiani *et al*. 2018) representing important economic losses in the agricultural industry (Clothier & Anderson 2016). Abortion is defined as the premature end of a pregnancy with the expulsion of a non-viable foetus (Baumgartner 2015). Three families in the order Chlamydiales are of significance as agents of abortion, namely the *Chlamydiaceae*, *Waddliaceae* and *Parachlamydiaceae* (Borel *et al*. 2018). Chlamydiales are adapted to a very broad spectrum of hosts and are agents of a variety of diseases in animals and humans (Borel *et al*. 2018). Several members of the order are of zoonotic significance. *Chlamydia abortus* (*C. abortus*), *Waddlia chondrophila* (*W. chondrophila*) and *Parachlamydia acanthamoebae* (*P*. *acanthamoebae*) have been detected in human cases of miscarriage and pneumonia (Greub *et al*. 2002; Ammerdorffer *et al*. 2017; Pichon *et al*. 2020).

Members of the order Chlamydiales are small, gram negative, obligate intracellular bacteria with a biphasic developmental cycle (Borel *et al*. 2018). Faeces, urine, placenta and/or discharges from the nose, eyes or vulva of an infected animal may serve as sources of infection (Borel *et al*. 2014; Essig *et al*. 2015; Walker *et al*. 2015). A primary infection is established in the tonsils following which the bacteria may disseminate via blood or lymph to the placenta (Borel *et al*. 2018).

The most important family is the *Chlamydiaceae* to which *Chlamydia* species belong (Markey *et al*. 2013). *Chlamydia abortus*, the cause of ovine enzootic abortion (OEA), can also infect cattle and goats and is among the most common bacterial causes of ruminant abortion worldwide (Borel *et al*. 2018). It can become endemic in herds (Markey *et al.* 2013) and result in late term abortion or weak full-term calves, lambs, or kids (Givens & Marley 2008; Borel *et al*. 2014; Gianitti *et al*. 2015, Di Paolo *et al*. 2019). Studies suggest that *Chlamydia pecorum* (*C. pecorum*) is endemic in cattle, sheep, and goats worldwide (Sachse *et al*. 2009; Walker *et al*. 2015). It is often carried in ruminant gastrointestinal tracts, but only causes sporadic abortions (Sachse *et al*. 2009; Walker *et al*. 2015). An Australian study by Clune *et al.* (2021) indicated that it could be an important cause of abortion in that country. *Chlamydia pecorum* was also reported in a study in Chile where it was detected by real-time PCR in an aborted caprine foetus (Saldías *et al*. 2014). Development of detection methods and research into the significance of *C. pecorum* in domestic ruminants has increased in recent years in other countries (Walker *et al*. 2015). However, little is reported on these bacteria in South Africa.

Other *Chlamydiales* of interest are *Waddlia chondrophila* (family *Waddliaceae*) (Markey *et al*. 2013) and *Parachlamydia acanthamoebae* (family *Parachlamydiaceae*). Both species were implicated in bovine abortions in several studies in the northern hemisphere (Henning *et al*. 2002; Ruhl *et al*. 2009; Borel *et al*. 2014; Barkallah *et al*. 2014). *P. acanthamoeba* was detected in material form ovine abortion cases in a Tunisian study by Barkallah *et al*. (2018). No literature on *P. acanthamoebae* or *W. chondrophila* detection in the southern hemisphere was found. The reason may be that routine abortion investigation do not commonly include detection of these bacteria (Borel *et al*. 2014; Borel *et al*. 2018). The role *P. acanthamoebae* and *W. chondrophila* plays in abortion

worldwide needs to be investigated further (Borel *et al*. 2014; Barkallah *et al*. 2016).

Chlamydiales infections often lead to placentitis, and bronchopneumonia in the foetus (Borel *et al*. 2014). *Chlamydia abortus*, *C. pecorum* and *W. chondrophila* infections lead to a purulent and/or necrotizing placentitis often including vasculitis (Henning *et al*. 2002; Blumer *et al*. 2011; Hazlett *et al*. 2013; Gianitti *et al*. 2015; Borel *et al*. 2018). *Parachlamydia acanthamoeba* infection leads to a purulent and/or necrotizing placentitis most commonly without vasculitis (Borel *et al*. 2007; Blumer *et al*. 2011). Both *P. acanthamoeba* and *W. chondrophila* infection have been associated with pneumonia in the foetus (Henning *et al*. 2002; Ruhl *et al*. 2008).

Members of the *Chlamydiales* fall in the difficult to culture category (Vidal *et al*. 2017). Historically, isolation in cell culture or chicken embryos is the 'gold standard' (Rodolakis *et al*. 2015). Due to the low sensitivity conventional cell culture methods are seldom used and molecular based methods are investigated for detection of these bacteria (Goy *et al*. 2009). Cases of bovine, ovine and caprine abortion where the aetiology remains unknown, but the presence of an infectious agent is indicated by a purulent or necrotising placentitis, or septicaemia are a problem encountered worldwide (Clothier & Anderson 2016; Barkallah *et al*. 2016). Foetuses that died 24 to 48 hours before abortion may already be autolytic by the time they are aborted (Givens & Marley 2008). This presents an additional challenge as autolysis masks lesions and the agent of abortion may be outcompeted by other organisms (Kirkbride 1986). These challenges prompted the application of methods that are less affected by autolysis.

Immunohistochemical staining methods (IHC) uses antibodies directed against chlamydial lipopolysaccharides or other surface antigens to demonstrate these

bacteria in cells. However, these methods are often not specific for a bacterial species (Sachse *et al*. 2009; Borel *et al*. 2014).

Molecular analysis offers more sensitive and specific methods in the form of conventional or quantitative real-time PCR (qPCR). These methods offer the opportunity to improve the diagnostic rate of traditional methods, such as smear examination and culture (Jiang *et al*. 2017). However, sensitivity and specificity differ between different PCR protocols. Real-time PCR methods provide increased sensitivity compared to conventional PCR, and qPCR methods provide quantification of target DNA (Iraola *et al*. 2016). A study in Switzerland reported an overall diagnostic rate of 51.9% when qPCR was employed in abortion investigation in addition to conventional culture (Schnydrig *et al*. 2017). Duplexing is the simultaneous amplification and quantification of two target sequences in a single qPCR assay (Barkallah *et al*. 2016). This approach increases sample throughput, reduces labour, minimizes the impact of pipetting errors, and saves on reagent costs (Barkallah *et al*. 2016).

A screening PCR for the order *Chlamydiales* followed by species specific PCRs is valuable for detection of *Chlamydia* and *Chlamydia*-like bacteria in products of abortion and swabs of the genital tract (Lienard *et al*. 2011; Barkallah *et al*. 2014). A *Chlamydiales* specific real-time qPCR (pan-Chlamydiales qPCR) with locked nucleic acids targeting the conserved 16S rRNA gene was published by Lienard *et al* (2011). This PCR was successfully applied to screen human and cattle samples for *Chlamydiales* (Lienard *et al*. 2011; Barkallah *et al*. 2014; Wheelhouse *et al*. 2014). In the study by Wheelhouse *et al*. (2014), amplicons from PCR positive cattle abortion samples were sequenced and revealed *C. abortus*, *P. acanthamoebae* and *Neochlamydiae hartmannellae*. In the Tunisian study by Barkallah *et al*. (2014) further analysis of amplicons from PCR positive cattle vaginal swab samples by PCR together with sequencing lead to the

identification of *C. abortus*, *C. pecorum*, *P. acanthamoeba* and *W. chondrophila*. As vaginal swab samples were used the bacteria detected could not be correlated with placental or foetal lesions to determine their role in abortions.

Species-specific singleplex qPCR assays were developed to detect the ompA gene of *C. abortus* (Pantchev *et al*. 2009) and *C. pecorum* (Pantchev *et al*. 2010), a fragment of the 16S rRNA gene of *W. chondrophila* (Goy *et al*. 2009) and of *P. acanthamoeba* (Casson *et al*. 2008). These qPCR assays were successfully used to detect DNA of *C. abortus*, *C. pecorum, P. acanthamoeba* and *W. chondrophila* in animal samples (Pantchev *et al*. 2009; Pantchev *et al*. 2010; Blumer *et al*. 2011; Gutierrez *et al*. 2012; Barkallah *et al*. 2014).

Different multiplex PCR assays for detection of Chlamydiales and other bacteria in products of abortion are reported in literature (Berri *et al*. 2009; Tramutra *et al*. 2011; Azeem *et al*. 2015; Barkallah *et al*. 2016). In addition, multiple potential agents of abortion are often reported (Barkallah *et al*. 2014; Barkallah *et al*. 2016; Schnydrig *et al*. 2017). This raises the question of the role mixed infections play in abortion. In order to differentiate between infection and colonisation by *C. abortus*, a study by Gutierrez *et al.* (2012) established a diagnostic cut-off point (DNA copies: $\geq 10^6$) for the duplex qPCR they used.

The aim of this study was to optimise and apply singleplex screening and duplex species-specific qPCR assays for detection of *Chlamydiales* DNA in products of domestic ruminant abortion, where placentitis and/or pneumonia was previously reported (Chapter 3) as pathological lesion regardless of previous detection of an aetiological agent. The use of a diagnostic cut-off point to determine the clinical significance of *C. abortus* results was investigated.

Materials and methods

In this descriptive study synthetic controls as well as DNA from bacterial cultures and ruminant abortion cases were used to optimise three qPCR assays for the detection of Chlamydiales DNA in products of ruminant abortion cases. The qPCR assays were optimised by means of a two-phase test validation process. During the first phase analytical test validation comprised the following steps: a) testing synthetic DNA positive controls of the organisms of interest (sensitivity), b) testing pure cultures of non-target organisms that are commonly found in products of abortion (specificity), c) testing extracted DNA of foetal stomach content, lung and placenta spiked with synthetic DNA controls of the pathogens of interest to determine limit of detection. During the second phase diagnostic test validation comprised: a) parallel testing of field abortion samples where the causative pathogen was detected previously by histopathology, immunohistochemistry, or PCR to demonstrate diagnostic sensitivity and b) testing of foetal tissue/placenta of known uninfected animals to demonstrate diagnostic specificity.

Synthetic controls

Synthetic oligonucleotides (Annex 3) were used for determination of specificity, sensitivity, and limit of detection of the qPCR assays. Synthetic oligonucleotides (gBlocks® Gene Fragments) were obtained from Integrated DNA Technologies, Coralville, Iowa, USA (www.idtdna.com) (IDT). The synthetic oligonucleotides were designed to contain three regions, the forward and reverse primers, and the probe of each target. The design was based on genomic reference sequences obtained from Genbank (http://www.ncbi.nlm.nih.gov/GenBank/index.html): Nr 036834.1 *Chlamydia abortus* strain Ov/B577 AY601755.1 *Chlamydophila abortus* clone 5 OmpA gene, KX388207.1 *Chlamydia pecorum* isolate M17/Ocular, Nr 074886.1 *Waddlia chondrophila* WSU86.1044 and Nr 026357.1

Parachlamydia acanthamoeba Strain B179. The synthetic controls were reconstituted according to manufacturer's instructions.

Microorganisms

Specificity of the PCR assays was tested using DNA extracted from different non-target bacteria often associated with products of abortion. Live intracellular control strains *C. abortus* (ATCC VR-165), *P. acanthamoeba* (ATCC VR-1476) and *W. chondrophila* (ATCC VR-1470) were procured from the American Type Culture Collection, Manassas, Virginia, USA. These bacteria were cultivated in five millilitres Serum-Casein-Glucose-Yeast extract medium (SCGYEM) at 30°C for 6 days with *Acanthamoeba castellani* (ATCC 50739) as host according to ATCC instructions. Cultures were then harvested and centrifuged at 5000g for 5 minutes. The supernatant was discarded and the pellet was resuspended in one millilitre of sterile PCR grade water.

Live bacterial control strains that grow on acellular media (Table 4.1) were streaked on 5% blood agar plates and incubated in 5% CO_2 at 37°C until growth was observed. Bacterial suspensions were made in 5% saline to a turbidity equal to a 0.5 McFarland standard. Fifty microlitre of *Acholeplasma laidlawlii* (NCTC 10116) suspension was inoculated in *Mycoplasma* broth (Oxoid CM0403 & SR0059, Thermoscientific, Gauteng, South Africa) and incubated in normal air at 37°C until the broth was turbid. The bacterial suspensions and *Acholeplasma laidlawlii* broth culture were then diluted 1:100 in sterile PCR grade water.

Table 4.1 Bacteria and amoeba species used as negative controls to test specificity

Bacteria	**Source or strain**
Acholeplasma laidlawlii	NCTC 10116
Brucella abortus	Clinical sample

Chlamydia abortus	ATCC VR-165
Escherichia coli	ATCC 25922
Ochrobactrum anthropi	ATCC 49687
Parachlamydia acanthamoeba	ATCC VR-1476
Pasteurella multocida	ATCC 12945
Salmonella Typhimurium	ATCC 13311
Staphylococcus aureus	ATCC 25923
Streptococcus agalactiae	ATCC 27956
Trueperella pyogenes	Clinical sample
Waddlia chondrophila	ATCC VR-1470
Amoeba	
Acanthamoeba castellani	ATCC 50739

Clinical samples

All 25 clinical bovine, ovine and caprine abortion cases where necrotic placentitis and/or pneumonia was reported, were selected from the 135 clinical abortion cases in the same study (Jonker *et al*. 2023; Chapter 3). Fifty samples (17 placentas, 19 stomach contents, 14 lungs) from these 25 cases were analysed in this study.

DNA from forty-nine samples (14 placenta, 17 stomach content, 18 lung) from clinical ruminant abortion cases (13 bovine, 9 ovine, 10 caprine) which were available from a previous study (Jonker *et al*. 2023; Chapter 3), were used in

specificity and limit of detection analyses. Only samples that had pathological lesions other than placentitis or pneumonia or no lesions, were selected.

DNA extraction

DNA was extracted according to manufacturer's instructions from all culture suspensions and the *Acholeplasma* broth culture (500 µl) and clinical samples (25mg) using the NucleoSpin®Tissue kit (Macherey-Nagel GMBH & Co., Düren, Germany).

Primers and probes for detection of Chlamydiales

Primers and probes were selected from published studies according to base pair size and annealing temperature (Casson *et al*. 2008; Goy *et al*. 2009; Pantchev *et al*. 2009; Pantchev *et al*. 2010; Lienard *et al*. 2011). The probes for Pan-Chlamydiales, *W. chondrophila* and *P. acanthamoeba* were slightly modified by replacing locked nucleic acids by a Minor Groove Binder (MGB) probe. Primers were synthesised by Integrated DNA Technologies, Coralville, Iowa, USA (www.idtdna.com) and probes by Thermo Fisher Scientific, Waltham, Massachusetts, USA (www.thermofischer.com). Sequences and targets for primers and probes are as in Table 4.2.

Table 4.2 Sequences, target genes, base pair sizes and concentration of primers and probes (For: Forward; Rev: Reverse; P: Probe; FAM 6-carboxy-fluorescein; TAMRA 6-carboxy-tetramethylrhodamin; VIC 2'chloro-7phenyl-1,4-dichloro-6-carboxy-fluorescein; MGB Minor Groove Binder).

Chlamydiales	**Target gene**	**Primer and probe sequences (5' to 3')**	**Base pair sizes**	**Concentration (µM)**	**References**
		Singleplex qPCR assay			
Pan-Chlamydiales	16S rRNA	For CCGCCAACACTGGGACT	207 to 215	0.1	Lienard *et al*. 2011
		Rev GGAGTTAGCCGGTGCTTCTTTAC		0.1	

		P VIC-CTACGGGAGGCTGCAGTCGAGAATC-MGB		0.1	
		Multiplex qPCR assays			
Assay 1 *Chlamydia abortus*	ompA	For GCAACTGACACTAAGTCGGCTACA	82	0.9	Pantchev et *al*. 2009
		Rev ACAAGCATGTTCAATCGATAAGAGA		0.9	
		P FAM-TAAATACCACGAATGGCAAGTTGGTTTAGCG-TAMRA		0.2	
Chlamydia pecorum	ompA	For CCATGTGATCCTTGCGCTACT	76	0.9	Pantchev *et al*. 2010
		Rev TGTCGAAAACATAATCTCCGTAAAAT		0.9	
		P VIC-TGCGACGCGATTAGCTTACGCGTAG-TAMRA		0.2	
Assay 2 *Waddlia chondrophila*	16SrRNA	For GGCCCTTGGGTCGTAAAGTTCT	101	0.5 (This study)	Goy *et al*. 2009
		Rev CGGAGTTAGCCGGTGCTTCT		0.5	
		P VIC-CATGGGAACAAGAGAAGGATG-MGB		0.2	
Parachlamydia acanthamoebae	16SrRNA	For CTCAACTCCAGAACAGCATTT	103	0.5 (This study)	Casson *et al*. 2008
		Rev CTCAGCGTCAGGAATAAGC		0.5	
		P FAM-TTCCACATGTAGCGGTGAAATGCGTAGATATG-MGB		0.2	

The Pan-*Chlamydiales* qPCR was used as a singleplex assay. The *C. abortus* and *C. pecorum* qPCRs were combined in a duplex qPCR assay; while the *P. acanthamoeba* and *W. chondrophila* qPCRs were combined in a second duplex qPCR assay.

Quantitative Real-time PCR (qPCR)

Amplification and detection of PCR products were performed in clear PCR strip tubes with caps (Applied Biosystems, Carlsbad, CA, USA) using a StepOnePlus® Real Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The final volume of reaction mixtures was 20 µl. Pan-*Chlamydiales* qPCR primer and probe concentrations were as published by Lienard *et al*. (2011). The *C. abortus and C. pecorum* qPCR primer and probe concentrations were used as published (Pantchev *et al*. 2009; Pantchev *et al*. 2010). In this study, *P. acanthamoeba* and *W. chondrophila* qPCR primer and probe concentrations were increased, to 0.5 and 0.2 µM respectively. The reaction mixture was completed by addition of 4 µl 5x Hot FirePol Probe Universal qPCR mix (Solis BioDyne, Tartu, Estonia), 10 µl ultrapure water and 2.5 µl DNA sample. Cycling conditions were: 95°C for 10 min (initial activation), and 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 1 min (annealing/elongation).

Analytical sensitivity

PCR amplification efficiency was established by standard curves where the amplification efficiency is calculated from the slope of the log-linear portion of the standard curve (Bustin *et al*.2009; Kralik *et al*. 2017). Reconstituted synthetic controls were diluted to 10^8 DNA copies/µl. Then 1:10 serial dilutions were prepared to 10 DNA copies/ µl. Analytical sensitivity was determined in triplicate.

Analytical specificity

Analytical specificity for each qPCR assay was evaluated using three *Chlamydiales* and nine non-*Chlamydiales* bacteria that are likely to occur as

contaminants in products of abortion. The *Acanthamoeba castellani* ATCC control was included since it was used as a host for cultivating the live *Chlamydiales* controls.

Limit of detection of the qPCR assays

Samples of stomach content, placenta, and lung from cases where no placentitis or pneumonia was reported, were analysed with the qPCRs in this study. DNA from samples where there was no amplification of target DNA were spiked with 10, 5, 2 and 1 DNA copies (*C. abortus*/ *C. pecorum* & *P. acanthamoeba*/ *W. chondrophila*) or 1000, 500, 250 and 62 DNA copies (Pan-*Chlamydiales*) of the synthetic controls (Integrated DNA Technologies, Coralville, Iowa, USA). Six repetitions of the spiked samples were analysed to determine detection limits of the singleplex and duplex qPCR assays.

Diagnostic sensitivity

Diagnostic sensitivity is the ability of a test to detect samples identified by a reference method as positive. It is calculated as follows: TP/(TP+FN), where TP=True positive and FN=False negative (Kralik *et al*. 2017). Samples of placenta, stomach content and lung from cases where placentitis and/or pneumonia was observed previously by histopathology were analysed by the qPCR assays in this study and the results were used to calculate diagnostic sensitivity.

Diagnostic specificity

Diagnostic specificity is the ability of a test to identify samples as negative that were found to be negative by a reference method. The formula is: TN/(TN+FP), where TN=True negative and FP=False positive (Kralik *et al*. 2017). Samples of placenta, stomach content and lung from cases where no placentitis or pneumonia was observed previously by histopathology were analysed by the qPCR assays in this study.

Quality assurance

A β-actin internal amplification control (Taqman® Gene Expression Assays, Applied Biosystems, Carlsbad, CA, USA) was included to assess the efficiency of the DNA extraction process and presence/absence of DNA polymerase inhibitors. Nuclease free water was included as a negative control.

Data analysis

Data analysis was performed with Applied Biosystems software in the StepOnePlus® Real Time PCR system as well as Microsoft Excel.

Results

Sensitivity, specificity, efficiency, and limit of detection of qPCR assays for detection of Chlamydiales

Analytical sensitivity

Standard curves for the Pan-Chlamydiales*, C abortus*/*C pecorum* and *P acanthamoeba*/*W chondrophila* are represented in Figures 1 to 3. The data for the singleplex Pan-*Chlamydiales* qPCR assay was not linear which had a negative influence on correlation coefficient (R^2) and efficiency calculations. An R^2 of 0.9585 and an efficiency of 104.432% was calculated. The R^2 and efficiency were lower than the *C. abortus/C. pecorum* duplex assay, but higher than the *P. acanthamoeba/ W. chondrophila* duplex assay. The R^2 values in the *C. abortus*/ *C. pecorum* duplex assay were slightly better than the R^2 of the singleplex qPCRs. Efficiency in the duplex assay was 99.5% (*C. abortus*) and 99% (*C. pecorum*). Multiplexing of *P. acanthamoeba* and *W. chondrophila* qPCR assays resulted in data that was not linear also with a negative influence on R^2 and efficiency calculations. Efficiency of the *W. chondrophila* qPCR decreased from 97.98% (singleplex) to 77.85% (duplex), R^2 decreased from 0.97

(singleplex) to 0.94 (duplex). The Ct value was 21 when a sample contained 10^5 DNA copies. The *P. acanthamoeba* qPCR increased slightly in efficiency from 74.51% (singleplex) to 78.11% (duplex), R^2 decreased from 0.97 (singleplex) to 0.93 (duplex). The Ct value was 19 when a sample contained 10^5 DNA copies.

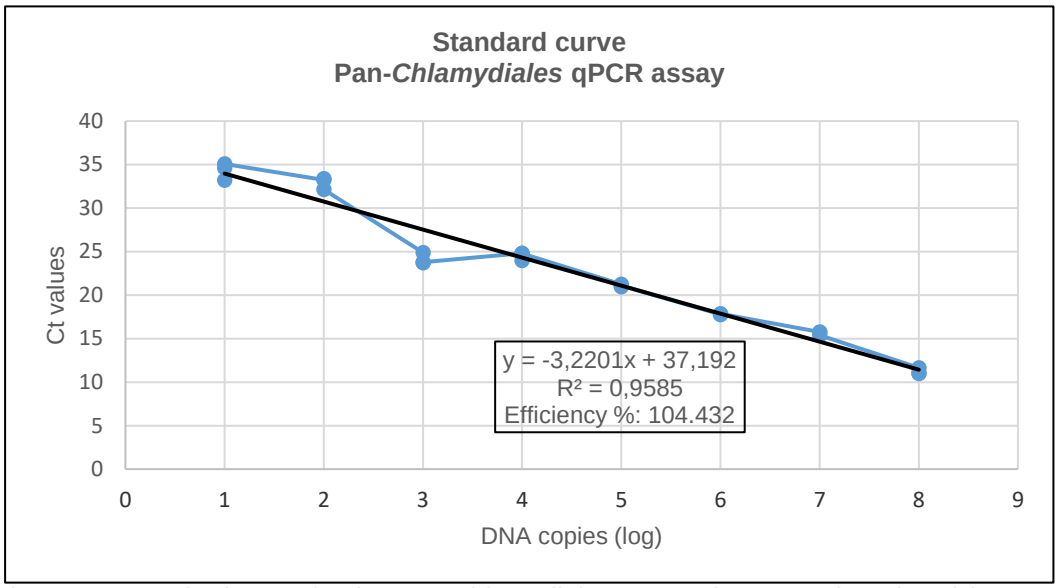

Figure 4.1 Standard curve for the Pan-*Chlamydiales* assay where Ct values (y-axis) are plotted against the log of the concentration of DNA copies (x-axis).

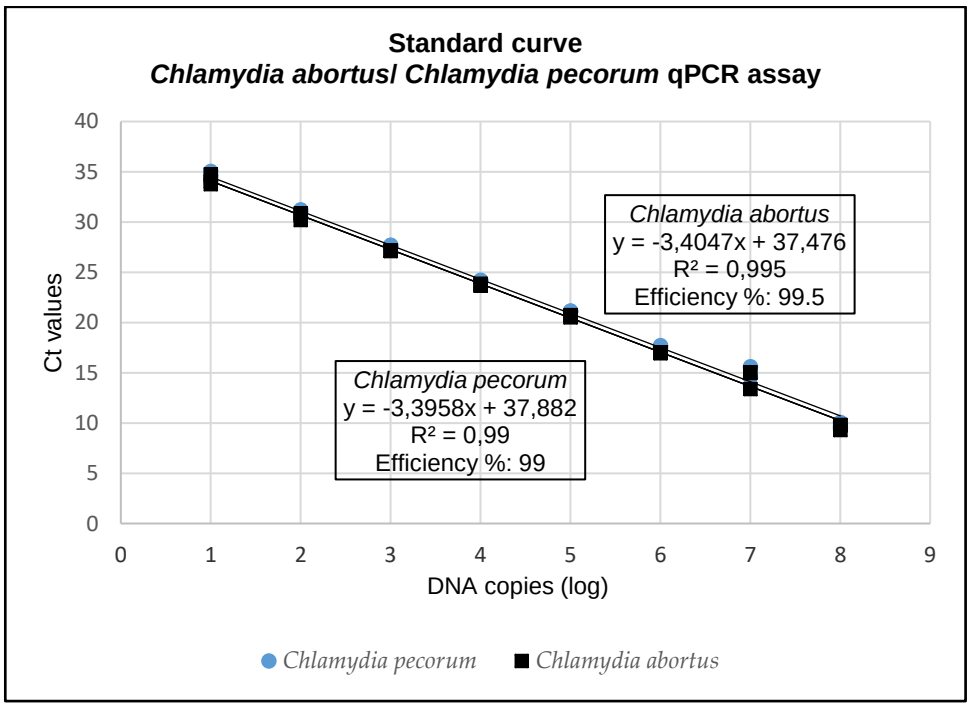

Figure 4.2 Standard curve for the *Chlamydia abortus*/ *Chlamydia pecorum* assay where Ct values (y-axis) are plotted against the log of the concentration of DNA copies (x-axis).

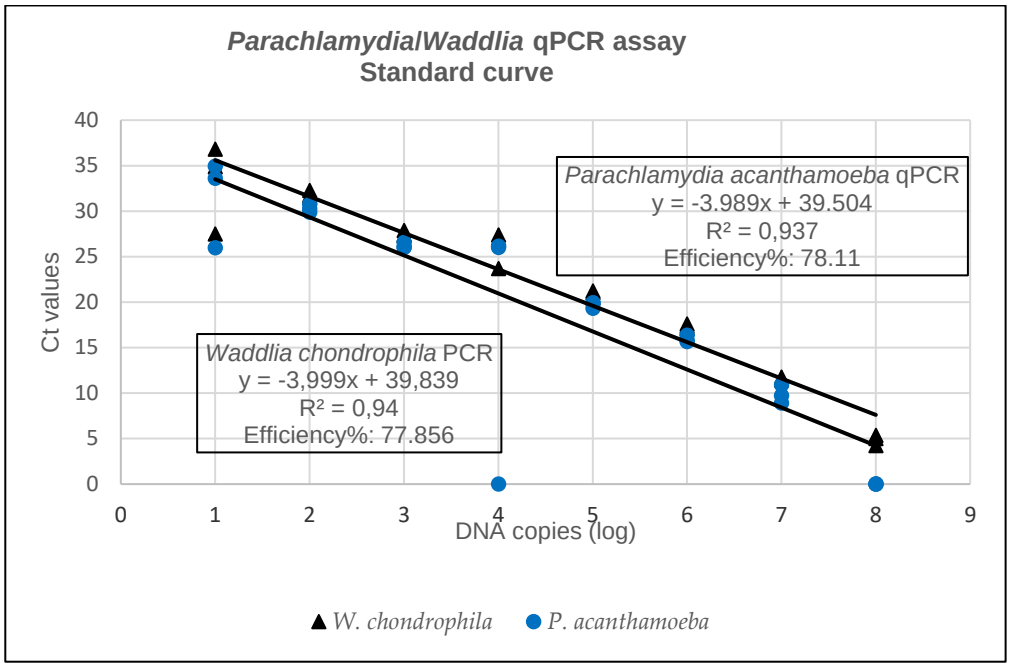

Figure 4.3 Standard curve for the *Parachlamydia acanthamoeba*/*Waddlia acanthamoeba* duplex qPCR where Ct values (y-axis) are plotted against the log of the concentration of DNA copies (x-axis).

Limit of detection

Synthetic controls were added to the extracted DNA of placenta, stomach content and lung samples. Spiked samples were analysed to determine the limit of detection of the qPCR assays. The limit of detection was defined as the lowest amount of target DNA that could be detected in 95% of replicates (Bustin *et al*. 2009; Kralik *et al*. 2017). Pan-Chlamydiales qPCR limits of detection were <62.5 DNA copies in placenta, stomach content and <125 DNA copies in lung DNA. Limits of detection of the *Chlamydia abortus*/ *Chlamydia pecorum* qPCR assay were 5 DNA copies in placenta and stomach content; and 2 DNA copies in lung DNA. Limits of detection for the *P. acanthamoeba*/ *W. chondrophila* qPCR assay were <1 DNA copy (*P. acanthamoeba*) and 1 DNA copy (*W. chondrophila*) in

placenta, < 1 DNA copy in stomach content and < 1 DNA copy in lung. (Annex 4 tables A4-A12).

Analytical specificity

None of the three qPCR assays detected any of the non-target organisms.

Clinical samples

Fifty clinical samples with necrotic placentitis and/or pneumonia lesions from 25 abortion cases (17 placentas, 19 stomach contents and 14 lungs) were analysed. Animal species involved were bovine (n=17), ovine (n=6) and caprine (n=2) (Table 4.3). In 16 cases *Chlamydiales* were detected in at least one sample. In four of these cases *Chlamydiales* were detected in two samples each. In two cases *Chlamydia* had been detected previously by immunohistochemistry (Jonker *et al*. 2023) and in this study *Chlamydiales* were again detected using qPCR. In eight cases no *Chlamydiales* were detected in this study or the previous study by Jonker *et al.* (2023). A cut-off point (10^6 DNA copies) experimentally determined by Gutierrez *et al*. (2012) was applied to *C. abortus* results to determine diagnostic significance of results.

Table 4.3 *Chlamydiales* DNA detected in bovine, ovine and caprine cases in this study together with the samples where they were detected, necropsy lesions, bacteria detected previously and cycle thresholds. (ND =Not detected, Ct=Cycle threshold, *PanChl*=Pan-*Chlamydiales*, *Chlab=Chlamydia abortus*, *Chlpec=Chlamydia pecorum*, *Pacanth=Parachlamydia acanthamoeba*, *Wchon=Waddlia chondrophila*)

Case no.	**Pathology**	**Bacteria/ Fungi (Previous study Jonker *et al*. 2023)**	**Samples**	**DNA detected (Mean Ct values)**
1	**Bovine** Necrotic placentitis Purulent bronchopneumonia	*Bacillus licheniformis* *Rhizopus* sp.	Stomach content	ND
			Lung	ND
2	Necrotic placentitis and vasculitis	*Mycoplasma* sp. *Aspergillus fumigatus*	Placenta	*PanChl* (36.05)

			Stomach content	*PanChl* (33.38) *Chlab* (32.33) *Chlpec* (36.90) *Wchon* (31.85)
			Lung	ND
3	Necrotic placentitis	ND	Stomach content	ND
4	Necrotic placentitis	*Salmonella* sp.	Placenta	*PanChl* (32.90) *Wchon* (26.02)
5	Necrotic placentitis with vasculitis	*Coxiella burnetii*	Stomach content	*PanChl* (32.25) *Chlab* (32.18) *Chlpec* (35.86)
6	Necrotic placentitis	*Streptococcus dysgalactiae* *Acholeplasma* sp.	Placenta	*PanChl* (28.63)
			Stomach content	*Wchon* (34.36)
			Lung	ND
7	Purulent placentitis	ND	Placenta	ND
8	Necrotic placentitis	*Lichtheimia corymbifera*	Placenta	*PanChl* (31.21) *Wchon* (23.97)
			Stomach content	ND
			Lung	ND
9	Meningitis Bronchointerstitial pneumonia	*Brucella abortus* biovar 1	Stomach content	ND
10	Meningoencephalitis Necrotic placentitis Hepatic necrosis	ND	Stomach content	ND
11	Necrotic placentitis with vasculitis	ND	Placenta	ND
		ND	Lung	ND
12	Necrotic placentitis	*Campylobacter fetus* *Mannheimia varigena* *Streptococcus pluranimalium* *Mycoplasma* sp.	Placenta	*PanChl* (30.02) *Pacanth* (36.88) *Wchon* (32.47)
			Stomach content	ND
			Lung	ND
13	Necrotic placentitis Purulent pneumonia	*Chlamydia* sp. *Salmonella* sp.	Placenta	*PanChl* (31.63)
			Stomach content	*PanChl* (33.94)

			Lung	ND
14	Necrotic placentitis	ND	Placenta	*PanChl* (5.03) *Chlab* (5.65)
			Stomach content	ND
15	Viral placentitis	*Pasteurella multocida*	Placenta	*Chlab* (30.79)
16	Interstitial pneumonia	*Brucella abortus* biovar 1	Stomach content	ND
17	Necropurulent placentitis	*Trueperella pyogenes*	Placenta	*PanChl* (34.68) *Pacanth* (33.48)
18	**Ovine** Necrotic placentitis Pneumonia	*Chlamydia* sp.	Placenta	*PanChl* (23.13) *Chlpec* (18.96)
			Stomach content	*PanChl* (31.69) *Chlpec* (29.83)
19	Bacterial & fungal placentitis Pleuropneumonia	*Chlamydia abortus*	Stomach content	ND
			Lung	ND
20	Bronchopneumonia	ND	Stomach content	*PanChl* (27.42) *Chlpec* (22.19) *Wchon* (35.17)
			Lung	*PanChl* (25.74) *Chlpec* (23.42) *Wchon* (35.39)
21	Bacterial placentitis	*Arcobacter* sp.	Placenta	ND
			Lung	ND
22	Necrotic placentitis	*Salmonella* Budapest *Mycoplasma* sp.	Placenta	*PanChl* (35.03) *Wchon* (33.35)
			Stomach content	ND
23	Fibrinopurulent bacterial pneumonia	ND	Placenta	*PanChl* (29.49) *Chlpec* (34.48) *Wchon* (32.52)
			Lung	ND
24	**Caprine** Necrotic placentitis	*Coxiella burnetii* *Salmonella* Typhimurium	Placenta	ND
			Stomach content Foetus A	ND
			Lung Foetus A	PanChl (32.41)

				Chlpec (35.84) *Wchon* (31.57)
			Stomach content Foetus B	ND
			Lung Foetus B	ND
25	Necrotizing placentitis Subcutaneous oedema Hydrocephalus	*Escherichia coli* *Lichtheimia corymbifera*	Placenta	*PanChl* (10.27)
			Stomach content	ND
			Lung	ND

Chlamydiales were detected in ten bovine, four ovine and two caprine cases and most often in placenta (n=12). The number of DNA copies was highest in placenta. Where *Chlamydiales* DNA was detected in more than one sample in a case, DNA copies were lower in stomach content and lung than in placenta. *C. abortus* was detected in three bovine cases only. *C. pecorum* was only detected in ovine and caprine cases. *P. acanthamoeba* was detected in three bovine cases. *W. chondrophila* was detected in bovine, ovine and caprine cases. *Chlamydia pecorum* (n=2) and *W*. *chondrophila* (n=1) were detected in cases where pneumonia lesions had been reported previously.

One case which was previously positive for *C abortus* by PCR was not positive in this study. In one bovine and one caprine case *Chlamydiales* could not be identified further by the assays in this study.

Seven cases had pathological lesions (necrotic placentitis (n=5); pneumonia (n=2) that pointed to bacterial infection, but previously no agent could be detected (Jonker *et al*. 2023). In this study *Chlamydiales* were detected in samples from three of these cases. *Chlamydia pecorum* was detected in this study in one case where a *Chlamydia* sp. was detected previously by IHC. In

four cases no agent of abortion could be detected in this study and the previous study by Jonker *et al*. (2023).

β-actin was detected in all samples indicating efficient extraction and absence of inhibition factors that could lead to false negative results.

Diagnostic sensitivity

Diagnostic sensitivity was calculated using the data in Table 4.3. The results were as follows: Pan-*Chlamydiales* assay: 0.38 (38%); *C. abortus* / *C. pecorum* assay: 0.2 (20%); *P. acanthamoeba* / *W. chondrophila* assay: 0.22 (22%).

Diagnostic specificity

Forty-nine samples of placenta, stomach content and lung, from bovine (n=19), ovine (n=9) and caprine (n=10) cases where no placentitis or pneumonia was observed, were analysed using the qPCR assays in this study (Annex 5). In 45 samples either no target DNA was detected or Ct values higher than 32 were returned. *Chlamydiales* were detected at Ct values lower than 32 in five samples. Results of diagnostic specificity calculations were as follows: Pan-*Chlamydiales* assay: 0.67 (67.3%); *C. abortus* / *C. pecorum* assay: *C. abortus*: 0.94 (94%) and *C. pecorum*: 1 (100%); *P. acanthamoeba* / *W. chondrophila* assay: *P. acanthamoeba*: 0.18 (18.4%) and *W. chondrophila*: 1 (100%).

Discussion

The aim of this study was to optimise and apply qPCR assays for detection of members of the order *Chlamydiales* and differentiation of *C. abortus*, *C. pecorum*, *P. acanthamoeba* and *W. chondrophila* in products of abortion, with necrotic placentitis and/or pneumonia lesions, from domestic ruminants in order to improve detection of intracellular bacteria that cannot be cultured on acellular media. Primers and probes for detection of DNA of the order *Chlamydiales*, and

of the species *C. abortus*, *C. pecorum*, *P. acanthamoeba* and *W. chondrophila* were selected from literature. One singleplex and two duplex qPCR assays were optimised. These assays were applied to 50 samples from 25 clinical abortion cases, where necrotic placentitis and/ or pneumonia was described histopathologically. An additional 49 samples where no pathological lesions were reported or where lesions other than placentitis and pneumonia was reported, were also analysed using the three qPCR assays.

Slight modification of the Pan-*Chlamydiales* qPCR using a MGB probe instead of locked nucleic acids resulted in a correlation coefficient (R^2) of 0.959 and an efficiency of 104.4%. Although efficiency lies between 90 and 105% (Kralik *et al*. 2017), R^2 is only 0.959 indicating that the serial dilution of the control was not accurately done (Kralik *et al*. 2017). The efficiency of this screening assay is much lower than the *C. abortus*/ *C. pecorum* assay. This could be due the use of a fragment of 16S RNA for the design of the primers and probe. Consequently, the Pan-*Chlamydiales* assay could miss positive cases which would be detected by the *C. abortus*/ *C. pecorum* assay. Improvement of this assay should be attempted by amplifying a fragment from a different gene such as *ompA*. The *C. abortus*/ *C. pecorum* assay had a high efficiency percentage at 99.5% (*C. abortus*) and 99% (*C. pecorum*) and R^2 of 0.995 and 0.99 for *C. abortus* and *C. pecorum* respectively. These results were slightly better than results when the assays were run in singleplex fashion. Efficiency of the *W*. *chondrophila* qPCR which was 97.98% as a singleplex assay decreased in the duplex assay while efficiency of the *P. acanthamoeba* qPCR improved slightly (*P. acanthamoeba*: 78.11% and *W. chondrophila*: 77.86%). The use of an MGB probe improved the efficiency of this assay slightly, however, detection of targets is still impaired (Bustin *et al*. 2009). Improvement of the efficiency of the *P. acanthamoeba*/ *W. chondrophila* qPCR assays must be attempted in future studies. Similar to the

Pan-*Chlamydiales* assay amplifying a fragment from a different gene such as *ompA* must be investigated to improve the assay. Improvement is essential if this assay is to be used for quantification studies in future since PCR efficiency plays an important role in quantification of target DNA (Kralik *et al*. 2017).

Direct diagnosis of *Chlamydiales* consist of demonstration of microorganisms in tissues by methods such as the Stamp's modification of the Ziehl Neelsen staining method (MZN) and IHC (Rodolakis *et al*. 2015). *Chlamydiales* were detected in 16 of the 25 clinical cases with histopathological lesions of necrotic placentitis or pneumonia. These results provide a preliminary indication of diagnostic sensitivity of the assays in this study, because *Chlamydiales* were detected in DNA from samples where histopathological lesions, that are associated with infection by these bacteria, were reported (Borel *et al.* 2018). The calculated diagnostic sensitivity was quite low for all three assays, indicating that either the inclusivity of the PCR is low or that the limit of detection of the reference method is higher than the tested qPCR. Another reason for low sensitivity can be low amounts of DNA due to inefficient DNA extraction. Unfortunately results of previous IHC or PCR analyses for *Chlamydiales* were only available in three cases. In two of these cases *Chlamydiales* were also detected in this study. Further analysis of diagnostic sensitivity of the qPCR assays in this study by comparison with culture, IHC and other PCR methods is necessary. The most sensitive combination of methods in a diagnostic setting needs to be determined.

Analysis of samples from cases without lesions resulted in several Ct values higher than 32. Since there were no histopathological lesions, these results are more likely to be an indication of contamination. Diagnostic specificity results of the *C. abortus*/ *C. pecorum* assay was highest indicating good ability to identify samples found negative by the reference method. The results for the Pan-

Chlamydiales and *P. acanthamoeba*/ *W. chondrophila* assays were lower indicating poorer exclusivity of the assays (Kralik *et al*. 2017). Another explanation is that the sensitivity of the reference method (histopathology) is low and the assays can detect more positive samples (Kralik *et al*. 2017). In addition to the reproductive tract, *Chlamydiales* can also colonise the intestinal tract (Borel *et al*. 2007). Elementary bodies, the environmentally stable, infectious phase of the *Chlamydiales* developmental cycle (Borel *et al*. 2014), are shed into the environment together with products of abortion or faeces. Environmental contamination of placentae and foetuses can also lead to positive PCR results (Borel *et al*. 2007; Sachse *et al*. 2009). Therefore, the interpretation of PCR results by the laboratory diagnostician is of major importance. qPCR includes the option to quantify the amount of an agent present in a sample (Sachse *et al*. 2009) which can be helpful in the discrimination between etiologically significant pathogens and contaminants to prevent overestimation of the significance of a result.

A singleplex qPCR only detects one pathogen at a time. Several singleplex qPCR assays may be performed to detect different pathogens, but this can become quite costly and time consuming (Selim *et al*. 2014). The minimum number of tests is often selected for economic reasons. Partly for this reason, results of abortion investigations are often inconclusive (Schnydrig *et al*. 2017). A multiplex qPCR on the other hand, can screen samples for several different pathogens at the same time, allowing the development of a more time and cost-effective analysis (Selim *et al*. 2014). In this study detection of potential agents of abortion improved from 19 to 21 out of 25 cases when multiplex qPCR was used and sample bias was removed by analysing all samples with necrotic placentitis and/ or pneumonia lesions.

The assays in this study enabled the detection of *C. pecorum* in two ovine cases. In both cases pneumonia was reported, which is not the case in reports by other authors (Gianitti *et al*. 2015; Clune *et al*. 2021). The role of *C. pecorum* in ovine abortion was investigated by an Australian study (Clune *et al*. 2021) and it was found to be significant in some herds. In South Africa the significance of this *Chlamydia* sp. is currently unknown and its investigation is warranted.

Waddlia chondrophila was detected in five bovine, three ovine and one caprine case and *P. acanthamoeba* in two bovine cases in this study. *P. acanthamoeba* and *W*. *chondrophila* was detected before in samples from bovine, ovine and caprine abortion cases in countries in the Northern Hemisphere (Henning *et al*. 2002; Ruhl *et al*. 2008; Deuchande *et al*. 2010; Wheelhouse *et al*. 2014) To the best of the authors' knowledge this is the first report of detection of *P. acanthamoeba* and *W. chondrophila* in South Africa. The role of these members of the *Chlamydiales* as agents of domestic ruminant abortion in South Africa is currently unknown.

In this study, like many others, several co-infections were detected. In an effort to differentiate between infection and colonization or contamination a diagnostic cut-off point (10^6 DNA copies) experimentally determined for *C. abortus* by Gutierrez *et al*. (2012) was applied. In this study, this cut-off point excluded all cases where *C. abortus* was detected except for one bovine case. However, there may be less than 10^6 DNA copies of a causative agent in a sample due to sampling or processing. So, although a diagnostic cut-off point can be a useful tool it can not be used in isolation to determine the significance of a qPCR result. Since *C. abortus* is not commonly associated with bovine abortion it may not be selected from a list of singleplex PCR options. In this study the *C. abortus* was detected, because the analysis was included in a multiplex assay targeting bacteria that cause necrotic placentitis or pneumonia and test selection was not

possible. This illustrates the advantage of a multiplex assay that can detect several agents associated with a particular pathological lesion in one reaction.

Chlamydiales were detected in samples from two ovine cases, *C. pecorum* in one case and *W. chondrophila* in the other, where only pathological lesions pointing to an infectious cause were reported previously by Jonker *et al.* (2023). Lack of any other possible agents of abortion increases their significance in these cases. However, the efficiency of the *P. acanthamoeba*/*W. chondrophila* qPCR used in this study were considered too low to aid in the diagnosis, indicating the need for the design of new assays.

In one case *C. abortus* was detected previously, but not in this study. The reason may be that a different sample from the same case was used and that there was not enough DNA in the sample. *Chlamydiales* detected in three bovine and one caprine case could not be identified further by the assays in this study. Internationally, more than one author has reported *Chlamydiales* they could not identify (Schnydrig *et al*. 2017; Merdja *et al*. 2015). This serves as an indication that other *Chlamydiales* may be agents of abortion. Further investigation by PCR and sequencing is necessary to identify these agents and determine their role in abortions. The findings in this study indicate that further investigation of the role of *Chlamydiales* in ruminant abortion in South Africa is warranted.

Conclusions

Application of the three qPCR assays in this study improved detection of members of the order *Chlamydiales* in samples from cases with necrotic placentitis and/or pneumonia. In addition, differentiation of the *Chlamydiales* to species level was improved. Application of a diagnostic cut-off point was useful to identify clinically significant Ct values for *C. abortus*. Detection of *C pecorum,*

P acanthamoebae and *W chondrophila* in ruminant abortion cases in South Africa indicate that these bacteria potentially play a role in abortions. The prevalence of the different *Chlamydiales* in South Africa needs to be evaluated in an epidemiological study.

It is recommended that in future, these qPCR assays form part of a comprehensive diagnostic approach to ruminant abortions. These results serve as a reminder that ruminant abortion material should be handled with appropriate safety precautions since *C. abortus*, *P. acanthamoebae* and *W. chondrophila* are potentially zoonotic agents.

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Chapter 5

Final discussion and conclusions

Infectious abortion is a worldwide phenomenon that affects animal welfare and leads to production losses. The 423 cases reported in the studies in Chapters 2 to 4 represent at least 423 cattle, sheep and goats that were lost to the agricultural production system often due to bacterial or fungal infection and subsequent abortion. The one or two aborted foetuses presented for investigation often represent a much larger number of abortions on the farm of origin since suspicion is only raised when the number of abortions in a herd reach 5% (Menzies 2011). In these studies that was often the case as well.

Brucella abortus and *Chlamydiales* were the most commonly detected bacteria in this study. *Brucella abortus* (*B. abortus*) and members of the order *Chlamydiales* are intracellular Gram-negative bacteria (Borel *et al*. 2014, WOAH 2022). *Brucella abortus* is an agent of abortions in domestic ruminants, particularly cattle (WOAH 2022). Several members of the order *Chlamydiales* are agents of cattle, sheep and goat abortion (Borel *et al*. 2018). The most well-known is *Chlamydia abortus*, the cause of Ovine Enzootic Abortion, which also causes abortion in cattle and goats (Borel *et al*. 2014). Both *B. abortus* and *Chlamydiales* are shed in large numbers by infected animals in products of abortion or normal birth and uterine discharges (Borel *et al*. 2018, WOAH 2022). These bacteria survive in products of abortion and contaminate pastures where they are ingested or inhaled by new hosts (Borel *et al*. 2018, WOAH 2022).

Fungi were rarely implicated as agents of abortion in this study. *Rhizopus* species were the most common. These fungi are saprophytes that are normally found in the environment. They exist in hyphal form and can grow at 37°C. The latter is a characteristic that is important for survival in a mammalian body

(Songer & Post 2005). The latter characteristic enables them to cause opportunistic, systemic infections in animals. These fungi are not implicated in cases of abortion as often as *Aspergillus* species or *Candida* species (Borel *et al*. 2014). The source of infection is often mouldy feed (Pal 2014). Spores produced by fungi are inhaled or ingested and reach the placenta via the blood circulation (Holler 2012, Pal 2014).

The aim of this study was to investigate detection methods for and significance of bacteria and fungi as agents of abortion in cattle, sheep, and goats in South Africa. Objective I (Chapter 2), the retrospective survey of data from 2006 to 2016 in South Africa, investigated detection methods used and the bacterial and fungal agents of abortion detected. Objective II (Chapter 3), the prospective study, attempted to find the most efficient combination of conventional bacteriology methods for analysis of samples from abortion cases. Bacterial and fungal agents of abortion detected during the study were reported. Objective III (Chapter 4) attempted to improve existing real-time qPCR assays for detection of *Chlamydiales* by creating multiplex assays.

The retrospective and prospective studies indicated that data from veterinary diagnostic laboratories can serve as a valuable form of passive surveillance. These studies contributed more recent information on bacterial and fungal causes of abortion in domestic ruminants in South Africa compared to the available literature of which most dated back to the 1990's and earlier. Subsidising laboratory fees served as an incentive to clients to submit samples for abortion investigation.

The prospective study found that an abortion panel including several culture methods removes test selection bias in the bacteriology laboratory, but not sample selection bias in the field. In addition, it offers increased opportunity to detect potential bacterial and fungal agents of abortion. In the retrospective

study, reduced range of analyses due to economic constraints often hampered a final diagnosis. This phenomenon was also reported by other authors (Kirkbride 1992, Clothier & Anderson 2016). The new combination of conventional culture and identification methods applied in the prospective study lead to an improved diagnostic rate (42.2%) in comparison with the retrospective study (35.1%). However, the difference was not statistically significant. In the prospective study, the most effective combination of methods was aerobic, microaerophilic, *Brucella* and fungal culture. The value of applying this extensive combination of conventional culture methods was emphasized by isolation of *B. abortus* from an increased number of cases in comparison with the period 2006 to 2016, and isolation of *Campylobacter jejuni*.

Submission of placenta improves chances of detecting agents of abortion since these bacteria and fungi can often only be detected in cotyledons and intercotyledonary areas (Kirkbride 1992, Clothier & Anderson 2016). In this study there were a few cases where agents of abortion were only detected in the placenta. Submission of placenta increased over the study period from an initial 22.2% in the retrospective study to 37% in the prospective study. Sample quality was more important to the diagnostic rate in the prospective study than the presence of placenta.

Regional similarities and differences in the occurrence of agents of abortion became evident when data were analysed. No specific pattern or regional occurrence of abortion agents was detected. *Brucella abortus* emerged as a major cause of bovine abortions in these studies. It was detected in cases from all six provinces in the retrospective and four of the six provinces from which samples were received in the prospective study. *Brucella abortus* was not detected from samples from Gauteng during the prospective study. *Brucella abortus* is also reported in other countries, for example Argentina, Brazil, Iran

and USA (Campero *et al*. 2003, Anderson 2007, Moshkelani *et al*. 2011, Antoniassi *et al*. 2016).

A difference in diagnostic rate between cattle and sheep abortion was found where an aetiological diagnosis was made more often in cases of sheep abortion. Kirkbride (1993) observed that sporadic abortions by different agents, that are not commonly isolated, appear to be less common in sheep, but that could not be concluded in this study.

Sporadic infections by opportunistic bacteria were common in both the retrospective and prospective studies. *Trueperella pyogenes* was the second most common isolate in both studies. It was most often detected in bovine cases from four of the six provinces in the retrospective study and four of the nine provinces in the prospective study. *Trueperella pyogenes* isolates from bovine abortions are also reported from countries such as the USA, Canada, Finland, Australia and New Zealand (Anderson *et al*. 1990, Khodakaram-Tafti *et al*. 2001, Syrjälä *et al*. 2007, Reichel *et al*. 2018). Chlamydiales and *Coxiella burnetii* (*C. burnetii*) were reported as causes of bovine, ovine and caprine abortion in both studies. Abortions due to Chlamydiales and *C. burnetii* infection are reported from many countries (Moeller 2001, Borel *et al*. 2014, Schnydrig *et al*. 2017, Clune *et al*. 2021). Detection of *Salmonella* increased during the prospective study to include almost all the provinces in the study. This was most likely due to removal of test selection bias. *Salmonella* is only reported as a common isolate by the USA and New Zealand (Jerret *et al*.1984, West 2002). *Campylobacter* species was a rare isolate in both studies. *Campylobacter jejuni* could be isolated in the prospective study due to addition of microaerophilic culture. *Campylobacter* species are more commonly reported by several other countries, including Australia, New Zealand, Argentina, USA, United Kingdom and

Netherlands (Campero *et al*. 2003, Clune *et al*. 2021, West 2002, Jerret *et al*.1984, Kirkbride 1993, Carson *et al*. 2017, van Engelen *et al*. 2014).

Abortions due to fungal agents were rare in these studies. *Aspergillus fumigatus* and *Rhizopus* species were isolated occasionally from cases from Gauteng and Mpumalanga. Mycotic abortions are also rarely reported internationally (Pal 2014).

Several co-infections were detected in the prospective study for example *Chlamydia* species and *Escherichia coli* as well as *Bacillus licheniformis* and *Rhizopus* species. Co-infections were reported previously in South Africa (Schutte *et al*. 1976). Studies in other parts of the world also report this phenomenon (Campero *et al*. 2003, Schnydrig et al. 2017). It is not clear what the roles of different microorganisms in co-infections are. This is a field for future investigation.

During the prospective study three cases were submitted for bacterial culture where samples with lesions of necrotic hepatitis were recorded. Bovine alphaherpasvirus type 1 was detected by IHC in two of the samples, while Bovine Viral Diarrhoea virus was detected by IHC in the third case. This serves to emphasize that certain viral infections can cause similar lesions in foetuses to bacterial infections. This must be kept in mind when multiplex real-time qPCR assays are designed.

To improve and explore the value of non-culture methods, previously published primers and probes for detection of the order *Chlamydiales*, *Chlamydia abortus*, *Chlamydia pecorum*, *Parachlamydia acanthamoeba* and *Waddlia chondrophila* were used to create one singleplex and two duplex assays. These qPCR assays were optimised and applied to detect *Chlamydiales* DNA in products of domestic ruminant abortion from twenty-five of the clinical cases in the prospective study

where pathological lesions of placentitis or pneumonia was observed. These cases were analysed further since placentitis and pneumonia are pathological lesions often associated with *Chlamydiales* infection (Borel *et al*. 2014). The result was detection of *Chlamydiales* in 16 of 25 cases. Real-Time qPCR analysis aimed at specific pathological lesions and bacteria, reduced test selection bias and offered an increased opportunity to detect potential agents of abortion. In agreement with the findings of Schnydrig *et al.* 2017, it was found that it is important that the laboratory diagnostician evaluate any positive results together with histopathology and IHC results to avoid over- or underestimating their significance. Real-time PCR can detect very low levels of DNA which could also be due to environmental contamination.

The new assays improved detection and identification of *Chlamydiales* overall. The singleplex screening assay proved valuable as it enabled detection of *Chlamydiales* that could not be identified further by the species-specific duplex assays in this study. This indicates that other members of the order may also play a role in domestic ruminant abortions in the provinces included in this study. Inclusion of species-specific assays led to a first detection of *P. acanthamoeba* and *W. chondrophila* in abortion cases in South Africa. In some cases which were inconclusive before, *Chlamydiales* were detected and in others identification was improved from genus to species level. *Chlamydia abortus* was detected in bovine cases indicating that it should not be disregarded as a cause of abortion in cattle.

Limitations of the study

Limited sample numbers and coverage of the country made it impossible to estimate prevalence or incidence of specific aetiological agents in different

animal species and geographical areas. Quality of samples from products of abortion is often a limiting factor since these samples are usually autolysed to a certain degree since abortion often takes place several days after foetal death (Holler 2012). Autolysis can lead to overgrowth of the aetiological agent by environmental bacteria and degrading of DNA leading to a false negative result. Samples discarded accidentally reduced the sample numbers and prevented further investigation of lesions. Limited multiplexing ability of equipment prevented development of an assay where all five PCR assays could be performed at once. Low efficiency of some of the assays may lead to false negative results. Lack of data from previous PCR analysis necessitated the use of histopathology data for optimization. Exclusion of other infectious causes of abortion such as protozoa and viruses from the study may have lowered the diagnostic rate.

Conclusions

The study demonstrated that passive surveillance can be employed to track agents of ruminant abortion in South Africa by collating and publishing data extracted from laboratory reports. An important finding was that *Brucella* remains the most common bacterial agent of ruminant abortion in South Africa. *Brucella melitensis* was not isolated which is a concern.

Increased submission of complete cases (foetus and placenta) and good quality samples together with the use of a set combination of methods for conventional culture, including *Campylobacter*, *Brucella* and fungal selective culture, provide the best chance of isolating culturable bacterial and fungal agents of abortion leading to improved diagnostic rate.

The simplex and duplex real-time qPCR methods improved detection of *Chlamydiales* and differentiation to species level. This study reports the first detection of *P. acanthamoeba* and *W. chondrophila* in abortion cases in South Africa. In future the detection of *C. pecorum*, *P. acanthamoeba* and *W. chondrophila* can inform decisions on vaccine development.

Recommendations

Further investigation is recommended to extend the use of non-culture methods to detect and identify difficult to culture agents of abortion to species level. Veterinary laboratories in all provinces should be equipped with appropriate facilities and accredited methods to detect *Brucella* species in clinical samples. Increasing submission of complete sample sets from small ruminant abortions should receive particular attention to increase the possibility of detecting *B melitensis*. When multiplex real-time qPCR assays are designed to include microorganisms that cause specific pathological lesions, all the relevant agents of abortion (bacteria, fungi, viruses and protozoa) in the country should be included, in addition to the agents specifically monitored, to provide additional value to the veterinarian and the farmer.

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Annex 1

Retrospective study data

Table A1: Retrospective study data including laboratories, species, provinces, condition of samples, sample sets, bacteria and fungi reported, pathology and culture methods. Condition of samples: 1=Good, 2=Autolysed; Samples: Foetus=1, Foetus and placenta = 2, Placenta = 3; Culture methods: 0=No bacterial culture, 1=Aerobic culture, 2=Aerobic culture + Brucella, 3=Aerobic culture + other tests on request, 4=Routine abortion panel (Aerobic, Brucella, Campylobacter, Fungi), 5=Routine abortion panel (more extensive than above)

Year	No.	Laboratory	Species	Province	Condition of sample	Sample set	Bacteria reported	Fungi reported	Pathology	Culture methods
2006	1	DVTD & Pathology	Bovine	North West	1	1	*Listeria monocytogenes*	-	Abomasitis	2
	2	DVTD & Pathology	Bovine	North West	1	1	*Listeria monocytogenes*	-	Pneumonia, Hepatitis, Abomasitis	2
	3	DVTD & Pathology	Bovine	Limpopo	1	1	*Streptococcus canis*	-	Pneumonia, Abomasitis	2
	4	DVTD & Pathology	Bovine	North West	2	1	-	-	-	2
	5	DVTD & Pathology	Bovine	Limpopo	1	1	*Brucella abortus biovar 2*	-	Bronchopneumonia	2
	6	DVTD & Pathology	Bovine	Limpopo	1	2	*Streptococcus canis*	-	Placentitis, Bronchopneumonia	2
	7	DVTD & Pathology	Bovine	Limpopo	2	1	*Escherichia coli, Streptococcus dysgalactiae* subsp. *equisimilis*	-	Necropurulent placentitis	2
	8	DVTD & Pathology	Bovine	North West	2	1	-	-	-	2
	9	DVTD & Pathology	Bovine	North West	2	1	-	-	-	2
	10	DVTD & Pathology	Bovine	Limpopo	1	2	-	-	Abomasitis, Kidney necrosis	2

	11	DVTD & Pathology	Bovine	Limpopo	1	3	*Trueperella pyogenes*	-	Necrotic placentitis	2
	12	DVTD & Pathology	Bovine	Limpopo	1	1	*Klebsiella ozaneae*	-	Bronchopneumonia	2
	13	DVTD & Pathology	Ovine	Gauteng	2	1	*Escherichia coli*	-	Ascites, Subcutaneous oedema	2
	14	DVTD & Pathology	Bovine	Gauteng	2	2	-	-	Autolysis	2
	15	DVTD & Pathology	Bovine	North West	1	1	-	-	Petechiae	2
	16	DVTD & Pathology	Bovine	Mpumalanga	1	1	-	-	-	2
	17	DVTD & Pathology	Ovine	Gauteng	2	1	*Escherichia coli*	-	Autolysis	2
	18	DVTD & Pathology	Ovine	Gauteng	1	1	*Pantoea agglomerans*	-	Retained placenta	2
	19	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	20	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	Autolysis	2
	21	DVTD & Pathology	Bovine	Gauteng	1	3	-	-	Necrotic placentitis & vasculitis	2
	22	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	2
	23	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	Autolysis	2
	24	DVTD & Pathology	Bovine	Limpopo	1	2	*Trueperella pyogenes Streptococcus canis*	-	Necropurulent placentitis fibrinopurulent pneumonia	2
	25	DVTD & Pathology	Bovine	Limpopo	1	1	*Escherichia coli*	-	Congestion	2
	26	DVTD & Pathology	Bovine	North West	1	1	-	-	Hydrocephalus Cerebellar hypoplasia	2
	27	DVTD & Pathology	Bovine	Gauteng	2	1	*Actinomyces israelii*	-	Oedema	2
2007	28	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	Autolysis	3
	29	DVTD & Pathology	Bovine	North West	1	1	-	-	-	2
	30	DVTD & Pathology	Bovine	Gauteng	1	1	*Brucella abortus biovar 1*	-	Pleuropneumonia Peritonitis Ascites Hydrothorax	2
	31	DVTD & Pathology	Bovine	North West	1	1	*Escherichia coli*	-	Bacterial colonies in lung & liver	2
	32	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	33	DVTD & Pathology	Bovine	Gauteng	1	2	-	-	Placentitis	3
	34	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	2

	35	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	36	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	37	DVTD & Pathology	Bovine	Gauteng	2	3	-	-	Protozoa on cotyledons	2
	38	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	3
2008	39	DVTD & Pathology	Caprine	Gauteng	2	1	*Escherichia coli*	-	-	3
	40	DVTD & Pathology	Bovine	Gauteng	1	1	*Leptospira sp.*	-	Interstitial nephritis Hepatic necrosis Myocarditis	3
	41	DVTD & Pathology	Bovine	Gauteng	1	1	*Brucella abortus biovar 1*	-	Bronchopneumonia Bacteria in abomasum Pyelonephritis Hepatitis	2
	42	DVTD & Pathology	Bovine	Gauteng	2	1	*Escherichia coli (smooth)*	-	Subcutaneous oedema	2
	43	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	44	DVTD & Pathology	Bovine	Gauteng	2	1	-	*Aspergillus fumigatus*	Pus floccules in abomasum Bronchopneumonia	2
	45	DVTD & Pathology	Bovine	Gauteng	1	1	-	*Aspergillus fumigatus*	Plaque-like dermatosis Bronchopneumonia	3
	46	DVTD & Pathology	Bovine	Gauteng	1	1	-	*Aspergillus fumigatus*	-	3
	47	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Interstitial pneumonia Hepatitis	2
	48	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Arthrogryposis anencephaly	2
	49	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	3
	50	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	2
	51	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Foetal teratology	2
	52	DVTD & Pathology	Bovine	North West	2	1	-	-	Interstitial nephritis Meningitis	2
	53	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	54	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	55	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	Intestinal atresia	2

	56	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	Congenital malformation Arthrogryposis Hydrocephalus	2
2009	57	DVTD & Pathology	Bovine	Gauteng	1	1	*Escherichia coli*	-	Bronchopneumonia, Nephrosis	2
	58	DVTD & Pathology	Bovine	Gauteng	1	1	-	Fungus	Mycotic dermatitis	2
	59	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Purulent pneumonia	2
	60	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	Yellow liver	2
	61	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	-	2
	62	DVTD & Pathology	Bovine	Free State	2	1	*Brucella abortus* biovar *1*	-	-	2
	63	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	64	DVTD & Pathology	Caprine	Gauteng	2	1	-	-	Dystocia	2
	65	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	-	2
	66	DVTD & Pathology	Ovine	Gauteng	2	2	-	-	Necropurulent placentitis	2
	67	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Teratology	2
	68	DVTD & Pathology	Bovine	Gauteng	2	2	*Escherichia coli*	-	Placentitis	2
	69	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	3
	70	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	Macerated	2
	71	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	Hepatosis	2
	72	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	Arthrogryposis	2
	73	DVTD & Pathology	Bovine	Gauteng	1	1	*Nocardia asteroides*	-	White foci on skin & lung. Filamentous bacteria	2
	74	DVTD & Pathology	Bovine	North West	1	1	-	-	-	2
	75	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	76	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Splenomegaly	2
	77	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Lymphocytic infiltrate in organs	2
	78	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	79	DVTD & Pathology	Bovine	Gauteng	1	2	-	-	Mummified	2
	80	DVTD & Pathology	Bovine	Limpopo	1	1	-	-	-	2

	81	DVTD & Pathology	Bovine	North West	2	1	-	-	-	2
2010	82	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	-	-
	83	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	Dystocia	-
	84	DVTD & Pathology	Ovine	Gauteng	2	2	-	-	-	-
	85	DVTD & Pathology	Ovine	Gauteng	1	2	-	-	Petechiae, Lung necrosis, necrotic placentitis	-
	86	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	Hepatosis	2
	87	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	-	-
	88	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	89	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	-	-
	90	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	-	-
	91	DVTD & Pathology	Ovine	North West	1	2	-	-	-	-
	92	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	-
	93	DVTD & Pathology	Bovine	Mpumalanga	1	2	-	-	Meningoencephalitis	-
	94	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	-
	95	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	Autolysis	-
	96	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	-
	97	DVTD & Pathology	Bovine	Mpumalanga	1	1	*Brucella abortus* biovar 1	-	Splenomegaly	3
	98	DVTD & Pathology	Caprine	Mpumalanga	1	1	-	-	-	-
	99	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	-
	100	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Splenomegaly	-
	101	DVTD & Pathology	Bovine	North West	2	1	-	-	Anasarca	-
	102	DVTD & Pathology	Caprine	Mpumalanga	1	1	-	-		2
	103	DVTD & Pathology	Bovine	Gauteng	2	2	-	-	Autolysis	2
	104	DVTD & Pathology	Bovine	Gauteng	1	2	*Aeromonas hydrophila* *Escherichia coli*	-	Placentitis, Necropurulent bronchopneumonia	2
	105	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	-
	106	DVTD & Pathology	Bovine	Mpumalanga	1	1	*Brucella* sp.	-	Brain congestion	2

	107	DVTD & Pathology	Ovine	Gauteng	2	2	-	-	Lymphoplasmacytic placentitis with intracellular organisms	3
	108	DVTD & Pathology	Bovine	North West	1	1	*Brucella* sp.	-	Splenomegaly	2
	109	DVTD & Pathology	Bovine	Noth West	1	1	-	-	Congested brain & meninges	-
	110	DVTD & Pathology	Ovine	Limpopo	2	1	-	-	Congestion of brain & lung	-
	111	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Kidney & Liver necrosis, Petechiae	-
	112	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Petechiae	-
	113	DVTD & Pathology	Bovine	Gauteng	1	1			Petechiae	-
2011	114	DVTD & Pathology	Bovine	Gauteng	1	1	-	-		2
	115	DVTD & Pathology	Caprine	Gauteng	2	2	*Escherichia coli*	-	Purulent placentitis, Hydroperitoneum	3
	116	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	-	-
	117	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	1
	118	DVTD & Pathology	Bovine	Limpopo	1	1	-	-	Orange liver, Epicardial haemorrhages	3
	119	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	-
	120	DVTD & Pathology	Ovine	North West	2	2	-	-	Anasarca, Hydroperitoneum, Hydrothorax, Petechiae	-
	121	DVTD & Pathology	Caprine	North West	2	1	-	-	Anasarca, Hydrothorax	-
	122	DVTD & Pathology	Bovine	Mpumalanga	1	2	-	-	Necrotic placentitis	-
	123	DVTD & Pathology	Bovine	Gauteng	1	2	-	-	-	2
	124	DVTD & Pathology	Bovine	Gauteng	2	2	-	-	Oedema	2
	125	DVTD & Pathology	Bovine	Gauteng	2	2	-	-	Ascites, Hydrothorax	2
	126	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	127	DVTD & Pathology	Caprine	Gauteng	2	1	-	-	-	-
	128	DVTD & Pathology	Caprine	North West	2	1	-	-	-	-

	129	DVTD & Pathology	Ovine	North West	2	1	-	-	Anencephaly, Limb agenesis	-
	130	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Hydrothorax	2
	131	DVTD & Pathology	Bovine	Limpopo	1	1	-	-	-	-
	132	DVTD & Pathology	Caprine	North West	1	1	-	-	Ascites, Lymphadenopathy	2
	133	DVTD & Pathology	Ovine	Gauteng	1	2	-	-	Cerebral congestion & haemorrhage	
	134	DVTD & Pathology	Bovine	Gauteng	1	2	-	-	Placentitis & vasculitis, Pneumonia	2
	135	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Liver & Kidney necrosis, Gallbladder oedema, Petechiation & ecchymoses	2
	136	DVTD & Pathology	Caprine	Limpopo	1	1	*Enterococcus faecalis*	-	-	2
	137	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Interstitial pneumonia	2
	138	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	Micropthalmia, Brachygnathia	-
	139	DVTD & Pathology	Bovine	Gauteng	1	1	*Escherichia coli*	-	Ascites, Hydrothorax	2
	140	DVTD & Pathology	Caprine	North West	1	1	-	-	Meningeal haemorrhages	1
	141	DVTD & Pathology	Bovine	Gauteng	1	1	*Brucella* sp.	-	Hydropericardium, Splenomegaly, Hepatomegaly	2
	142	DVTD & Pathology	Ovine	Gauteng	1	2	*Escherichia coli*	-	Placentitis, Hydrothorax, Ascites, Patent foramen ovale	1
	143	DVTD & Pathology	Bovine	North West	1	1	*Brucella* sp.	-	Bronchopneumonia	2
	144	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	1
	145	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	Ascites, Hydrothorax, Hydropericardium, Hydrocephalus, Hepatosis	1
	146	DVTD & Pathology	Caprine	North West	1	1	-	-	Ascites, Hydrothorax	2

	147	DVTD & Pathology	Caprine	North West	1	1	-	-	-	2
2012	148	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Hydrothorax	3
	149	DVTD & Pathology	Ovine	Mpumalanga	2	2	*Trueperella pyogenes*	-	Placental congestion	1
	150	DVTD & Pathology	Bovine	North West	1	1	*Brucella abortus biovar 1*	-	Bronchopneumonia	2
	151	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	Autolysis	1
	152	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Bronchopneumonia	3
	153	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	Ascites	1
	154	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	155	DVTD & Pathology	Bovine	Gauteng	1	1	*Brucella abortus biovar 1*	-	Anasarca, Ascites, Hydrothorax, Bronchopneumonia	2
	156	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	2
	157	DVTD & Pathology	Bovine	Gauteng	1	3	*Trueperella pyogenes*	-	-	1
	158	DVTD & Pathology	Bovine	North West	1	1	-	-	-	2
	159	DVTD & Pathology	Caprine	Gauteng	1	2	-	-	-	1
	160	DVTD & Pathology	Bovine	Limpopo	1	1	*Brucella sp.*	-	Pneumonia	3
	161	DVTD & Pathology	Caprine	North West	1	1	-	-	-	1
	162	DVTD & Pathology	Bovine	Gauteng	1	2	*Corynebacterium* sp.	-	Hydrothorax	2
	163	DVTD & Pathology	Caprine	Gauteng	1	1	*Chlamydia abortus*	-	Hydrothorax, Hydropericardium, Pulmonary oedema	-
	164	DVTD & Pathology	Ovine	Gauteng	2	1	-	-	-	2
	165	DVTD & Pathology	Caprine	Gauteng	1	2	*Salmonella* sp.	-	Hydrothorax	2
	166	DVTD & Pathology	Caprine	North West	1	2	-	-	-	1
	167	DVTD & Pathology	Caprine	North West	1	1	-	-	-	2
	168	DVTD & Pathology	Bovine	Mpumalanga	1	1	-	-	Anasarca	2
	169	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	-	2

	170	DVTD & Pathology	Ovine	Limpopo	1	1	-	-	-	2
2013	171	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Multifocal hepatic necrosis	2
	172	DVTD & Pathology	Bovine	Mpumalanga	1	2	-	-	Mycotic placentitis	2
	173	DVTD & Pathology	Bovine	Mpumalanga	1	2	*Burkholderia cepacia*	-	Placentitis with vasculitis	2
	174	DVTD & Pathology	Bovine	Mpumalanga	2	1	-	-	Autolysis	2
	175	DVTD & Pathology	Bovine	Mpumalanga	1	3	*Brucella* sp.	-	Placentitis	2
	176	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	177	DVTD & Pathology	Bovine	Limpopo	2	1	*Trueperella pyogenes*	-	Purulent bronchopneumonia	2
2014	178	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	2
	179	DVTD & Pathology	Bovine	Mpumalanga	2	1	-	-	Yellow liver, splenomegaly	2
	180	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	181	DVTD & Pathology	Bovine	Limpopo	2	1	-	-	-	2
	182	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	183	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	2
	184	DVTD & Pathology	Bovine	Gauteng	1	2	*Brucella* sp.	-	Necropurulent placentitis	2
	185	DVTD & Pathology	Bovine	Mpumalanga	2	1	-	-	Mineralization	2
	186	DVTD & Pathology	Bovine	Free State	1	2	-	-	Necrotic placentitis, pneumonia, necrotic hepatitis	2
	187	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	188	DVTD & Pathology	Ovine	Gauteng	1	1	*Chlamydia pecorum*	-	-	3 AnO2 Brucella
	189	DVTD & Pathology	Ovine	Gauteng	2	2	-	-	Mineralization	2
	190	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	2
	191	DVTD & Pathology	Caprine	Gauteng	1	2	-	-	-	2
	192	DVTD & Pathology	Ovine	Gauteng	1	2	-	-	-	2
	193	DVTD & Pathology	Bovine	Gauteng	2	1	*Fusobacterium* sp.	-	Autolytic	3 AnO2 Brucella
	194	DVTD & Pathology	Bovine	Mpumalanga	1	3	-	-	-	2
	195	DVTD & Pathology	Bovine	Gauteng	1	1	*Brucella* sp.	-	Broncho-alveolar pneumonia	2

	196	DVTD & Pathology	Bovine	North West	1	1	-	-	-	2
2015	197	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	3 O2 ANO2 Brucella
	198	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	2
	199	DVTD & Pathology	Bovine	Mpumalanga	1	1	*Brucella* sp.	-	-	3 O2 ANO2 Brucella
	200	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	Hydranencephaly	2
	201	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	2
	202	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Anasarca, Hydropericardium	2
	203	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	-	2
	204	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	2
	205	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	206	DVTD & Pathology	Bovine	Mpumalanga	2	1	-	-	Hepatic necrosis	2
	207	DVTD & Pathology	Bovine	Gauteng	1	1	*Brucella* sp.	-	Bacterial necrofibrinous splenitis	2
	208	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	2
	209	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Trophoblast mineralization	2
	210	DVTD & Pathology	Bovine	KwaZulu Natal	1	2	-	-	Necropurulent placentitis Pneumonia	-
	211	DVTD & Pathology	Bovine	North West	1	2	-	-	-	2
	212	DVTD & Pathology	Caprine	Gauteng	1	1	*Enterobacter cloacae*	-	Bronchopneumonia Meningitis	3 O2 ANO2 Brucella
	213	DVTD & Pathology	Ovine	Limpopo	1	1	*Campylobacter* sp.		Purulent broncho-alveolar pneumonia Multifocal hepatic necrosis	2
	214	DVTD & Pathology	Ovine	Northern Cape	1	1	Clostridial enterotoxaemia	-	Nephrosis	2
	215	DVTD & Pathology	Bovine	Gauteng	2	1	-	-		2
2016	216	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	217	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2

	218	DVTD & Pathology	Bovine	Mpumalanga	1	2	*Bacillus cereus*	-	Necrotic placentitis	2
	219	DVTD & Pathology	Ovine	Gauteng	2	2	*Escherichia coli*	-	Autolysis	2
	220	DVTD & Pathology	Bovine	Gauteng	2	1	-	-	-	2
	221	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	222	DVTD & Pathology	Bovine	Gauteng	2	1	*Salmonella* sp.	-	Oedema, Hydrothorax Ascites Suggilations	2
	223	DVTD & Pathology	Bovine	Gauteng	1	2	-	-	Oedema Necropurulent placentitis	2
	224	DVTD & Pathology	Caprine	Gauteng	1	2	*Chlamydia* sp.	-	Necrotic placentitis	2
	225	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	226	DVTD & Pathology	Bovine	Mpumalanga	1	1	*Enterobacter cloacae*	-	Pneumonia	2
	227	DVTD & Pathology	Bovine	Mpumalanga	2	1	-	-	Hydrothorax Hydropercardium Hydroperitoneum	2
	228	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	229	DVTD & Pathology	Ovine	Gauteng	1	1	-	-	-	2
	230	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	-	2
	231	DVTD & Pathology	Bovine	Mpumalanga	2	1	*Brucella* sp.	-	Bronchopneumonia	2
	232	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	2
	233	DVTD & Pathology	Bovine	Free State	1	2	-	-	-	2
	234	DVTD & Pathology	Caprine	Gauteng	1	1	-	-	-	2
	235	DVTD & Pathology	Bovine	Gauteng	1	1	-	-	Oedema, anasarca	2
2013	236	CEVA - Vetdiagnostix	Ovine	Mpumalanga	1	1	-	-	-	4
	237	CEVA - Vetdiagnostix	Ovine	Mpumalanga	2	2	-	-	Brain & cotyledonary necrosis	4
	238	CEVA - Vetdiagnostix	Ovine	Mpumalanga	2	2	-	-	Cotyledonary necrosis	4
	239	CEVA - Vetdiagnostix	Ovine	KwaZulu-Natal	2	2	*Listeria monocytogenes*	-	Necrotic placentitis	4
	240	CEVA - Vetdiagnostix	Ovine	Free State	1	1	-	-		4
	241	CEVA - Vetdiagnostix	Ovine	KwaZulu-Natal	1	2	-	*Penicillium* sp.	Cotyledonary necrosis	4
	242	CEVA - Vetdiagnostix	Caprine	Mpumalanga	1	2	-	-	Necrotic placentitis	4

	243	CEVA - Vetdiagnostix	Ovine	Mpumalanga	1	1	-	-	-	4
	244	CEVA - Vetdiagnostix	Ovine	Mpumalanga	2	1	-	-	-	4
	245	CEVA - Vetdiagnostix	Caprine	Mpumalanga	1	2	-	*Rhizopus* sp	Necrotic placentitis	4
	246	CEVA - Vetdiagnostix	Ovine	KwaZulu-Natal	1	1	-	-	-	4
	247	CEVA - Vetdiagnostix	Ovine	Free State	1	2	*Coxiella burnetii*	-	Autolysed cotyledon, yellow plaques on intercotyledonary areas. No vasculitis. Intracellular bacteria.	4
	248	CEVA - Vetdiagnostix	Ovine	KwaZulu-Natal	2	1	-	-	-	4
	249	CEVA - Vetdiagnostix	Caprine	North-West	2	1	-	-	Bronchopneumonia	4
	250	CEVA - Vetdiagnostix	Ovine	KwaZulu-Natal	1	2	*Bacillus licheniformis*	-	Necrotic cotyledons	4
	251	CEVA - Vetdiagnostix	Caprine	North-West	1	1	-	-	-	4
	252	CEVA - Vetdiagnostix	Caprine	Eastern Cape	1	1	-	-	-	4
	253	CEVA - Vetdiagnostix	Ovine	Eastern Cape	2	2	-	-	Necrotic placentitis	4
2016	254	Vetdiagnostix	Bovine	KwaZulu-Natal	1	1	-	-	-	4
	255	Vetdiagnostix	Bovine	Mpumalanga	1	1	-	-	Non specific	
	256	Vetdiagnostix	Bovine	North West	1	2	*Campylobacter* sp.	-	Necropurulent placentitis with vasculitis Bronchopneumonia	4
	257	Vetdiagnostix	Bovine	North West	1	1	*Brucella* sp.	-	Bronchopneumonia	4
	258	Vetdiagnostix	Bovine	Mpumalanga	1	1	-	-	-	4
	259	Vetdiagnostix	Ovine	Free State	1	1	-	-	No lesions	4
	260	Vetdiagnostix	Bovine	Free State	1	1	-	-	-	4
	261	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	-	4

	262	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	*Trueperella pyogenes*	-	-	4
	263	Vetdiagnostix	Bovine	Free State	1	1	-	-	-	4
	264	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	*Anaplasma marginale*	-	Cholestasis, Anaemia	4
	265	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	Lung congestion, Myocardial petechiation	4
	266	Vetdiagnostix	Bovine	Free State	1	1	-	-		4
	267	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-		4
	268	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	Anasarca	4
	269	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	-	4
	270	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	-	4
	271	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	Thyroid goitre, Degenerative hepatopathy	4
	272	Vetdiagnostix	Bovine	Free State	1	1	-	-	-	4
	273	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	*Brucella* sp.	-	Hypercellular alveoli	4
	274	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	-	4
	275	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	Hydrothorax Hydropericardium Ascites	4
	276	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	*Anaplasma marginale*	-	Cholestasis, Colangiohepatitis Bacterial emboli Purulent adrenolitis	4
	277	Vetdiagnostix	Bovine	KwaZulu Natal	1	2	-	-	-	4
	278	Vetdiagnostix	Bovine	KwaZulu Natal	1	2	-	-	Bronchopneumonia	4
	279	Vetdiagnostix	Bovine	KwaZulu Natal	1	2	-	-	-	4
	280	Vetdiagnostix	Bovine	KwaZulu Natal	1	2	-	-	Necrotizing placentitis with vasculitis	4
	281	Vetdiagnostix	Bovine	KwaZulu Natal	1	2	-	-	-	4
	282	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-		4
	283	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	Pneumonia	4
	284	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	Necrotic hepatitis	4
	285	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	-	4
	286	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	Pleuropneumonia	4
	287	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	-	-	Hepatic necrosis	4

	288	Vetdiagnostix	Bovine	KwaZulu Natal	1	1	*Brucella abortus*	-	Hypercellular alveoli	4

Annex 2

Prospective study data

Table A2 Cases per province and species as well as total number of bovine, ovine and caprine cases

Province	Species	Number of cases
Gauteng	Bovine	29
	Ovine	8
	Caprine	8
Total cases		45
North West	Bovine	22
	Ovine	3
	Caprine	5
Total cases		30
Mpumalanga	Bovine	16
	Ovine	2
	Caprine	2
Total cases		20
Free State	Bovine	7
	Ovine	6
	Caprine	2
Total cases		15
Eastern Cape	Bovine	1
	Ovine	2
	Caprine	1
Total cases		4
Limpopo	Bovine	11
	Ovine	2
	Caprine	4
Total cases		17
Northern Cape	Bovine	2
	Ovine	1
Total cases		3
Western Cape	Ovine	1
Total cases		1
Total cases per species	Bovine	88
	Ovine	25
	Caprine	22
Total cases overall		135

Annex 3

Sequences of synthetic controls

Table A3: Sequences (5' to 3') of synthetic controls used for the optimisation of the Pan-Chlamydiales assay and the *C. abortus*/*C. pecorum* and *P. acanthamoeba*/*W. chondrophila* assays.

Synthetic controls	Sequence (5' to 3')
Pan-Chlamydiales assay	CCG CCA ACA CTG GGA CTG AGA CAC TGC CCA GAC TTC TAC GGA AGG CTG CAG TCG AGA ATC TTT CGC AAT GGA CGA AAG TCT GAC GAA GCG ACG CCG CGT GTG TGA TGA AGG CTC TAG GGT TGT AAA GCA CTT TCG CTT GGG AAT AAG AGA GAT TGG CTA ATA TCC AAT CGA TTT GAG CGT ACC AGG TAA AGA AGC ACC GGC TAA CTC C
***C. abortus/C. pecorum* assay** ***P. acanthamoeba/W. chondrophila* assay**	CTC AAC TCC AGA ACA GCA TTT GAA ACT ATA TTT CTT GAG GGT AGG CGG AGA AAA CGG AAT TCC ACA TGT AGC GGT GAA ATG CGT AGA TAT GTG GAA GAA CAT CGG TGG CGA AGG CGG TTT TCT AGC TTA TTC CTG ACG CTG AGA CTA GGC CCT TGG GTC GTA AAG TTC TTT CGC ATG GGA ACA AGA GAA GGA TGC TAA TAT CAT CTG GAT TTG AGC GTA CCT TGT AAA GAA GCA CCG GCT AAC TCC GAC TAG CAA CTG ACA CTA AGT CGG CTA CAA TTA AAT ACC ACG AAT GGC AAG TTG GTT TAG CGC TCT CTT ATC GAT TGA ACA TGC TTG TAC TAC CAT GTG ATC CTT GCG CTA CTT GGT GCG ACG CGA TTA GCT TAC GCG TAG GAT TTT ACG GAG ATT ATG TTT TCG ACA

Annex 4

Supplementary tables – Limit of detection

Sensitivity: Synthetic controls: Limit of detection in placenta, abomasal fluid and lung. Testing foetal tissues and placenta spiked with the synthetic DNA fragment of interest.

Pan-Chlamydiales qPCR

Table A4 Results of limit of detection analysis of spiked placenta DNA

Tissue DNA: Placenta	
DNA copies	**Cт (Target: Pan-Chlamydiales)**
500	34,74
500	34,79
500	34,52
500	34,32
500	34,79
500	36,89
250	31,08
250	30,86
250	30,72
250	30,81
250	30,83
250	30,90
125	30,46
125	30,67
125	30,21
125	30,60
125	30,72
125	32,04
62,5	30,27
62,5	30,13
62,5	29,99
62,5	30,27

62,5	30,51
62,5	30,52

Table A5 Results of limit of detection analysis of spiked stomach content DNA

Tissue DNA: Stomach content	
DNA copies	**C_T (Target: Pan-Chlamydiales)**
500	35,15
500	35,96
500	34,35
500	35,26
500	35,27
500	Undetermined
250	36,47
250	36,06
250	35,38
250	Undetermined
250	35,59
250	35,44
125	36,08
125	35,39
125	35,23
125	35,94
125	35,15
125	35,25
62,5	35,51
62,5	35,91
62,5	35,58
62,5	35,60
62,5	33,99
62,5	36,47

Table A6: Results of limit of detection analysis of spiked lung DNA

Tissue DNA: Lung

DNA copies	CT (Target: Pan-Chlamydiales)
1000	32,12
1000	32,38
1000	32,17
1000	32,61
1000	32,36
1000	32,47
500	32,49
500	32,61
500	33,17
500	33,58
500	33,81
500	33,64
250	34,14
250	35,43
250	35,19
250	33,94
250	34,99
250	34,98
125	36,00
125	35,96
125	35,27
125	36,01
125	36,02
125	35,92

Limit of detection: *C abortus*/*C pecorum* assay

Table A7: Results of limit of detection analysis of spiked placenta DNA

Tissue DNA: Placenta		
DNA copies	**CT (Target: *C. abortus*)**	**CT (Target: *C pecorum*)**
10	33,31	32,94
10	33,73	33,10
10	32,70	32,28
10	32,79	32,34

10	33,52	32,87
10	33,76	33,18
5	34,22	33,51
5	35,28	34,53
5	33,62	32,74
5	34,43	33,38
5	37,05	35,08
5	Undetermined	Undetermined
2	34,51	34,04
2	35,28	34,42
2	33,84	33,39
2	35,69	34,98
2	Undetermined	Undetermined
2	Undetermined	Undetermined
1	37,51	36,94
1	35,18	34,69
1	36,79	34,79
1	35,95	33,95
1	37,27	36,56
1	35,82	33,90

Table A8: Results of limit of detection analysis of spiked stomach content DNA

Tissue DNA: Stomach content		
DNA copies	**CT (Target: *C. abortus*)**	**CT (Target: *C. pecorum*)**
10	36,49	35,58
10	33,57	32,99
10	34,39	34,55
10	35,60	36,60
10	34,75	34,63
10	35,49	34,96
5	34,04	34,07
5	34,07	33,21
5	34,72	33,68
5	37,10	36,25
5	Undetermined	37,14
5	34,97	33,93
2	34,74	34,75

2	Undetermined	Undetermined
2	Undetermined	Undetermined
2	35,87	35,23
2	35,63	34,33
2	34,85	33,80
1	35,92	35,53
1	34,67	34,02
1	36,39	35,94
1	36,99	36,29
1	35,63	36,64
1	36,30	35,17

Table A9: Results of limit of detection analysis of spiked lung DNA

Tissue DNA: Lung		
DNA copies	**CT (Target: *C abortus*)**	**CT (Target: *C. pecorum*)**
10	32,79	32,53
10	32,28	31,86
10	32,96	32,28
10	34,91	34,82
10	34,36	34,09
10	33,36	33,73
5	34,61	33,89
5	34,27	34,41
5	34,58	34,73
5	33,99	34,10
5	34,68	34,43
5	34,51	33,68
2	34,65	34,35
2	35,61	34,54
2	17,11	32,34
2	35,21	35,68
2	34,65	34,08
2	37,74	37,16
1	Undetermined	35,56
1	35,85	34,60
1	Undetermined	Undetermined
1	34,56	35,63

1	Undetermined	35,82
1	35,17	37,492

Parachlamydia/Waddlia qPCR assay

Table A10 Results of limit of detection analysis of spiked placenta DNA

Tissue DNA: Placenta		
DNA copies (log)	**Cт (Target: Parachlamydia)**	**Cт (Target Waddlia)**
10	35,18	33,01
10	33,96	35,15
10	Undetermined	34,00
10	34,30	33,72
10	36,23	33,67
10	36,74	33,72
5	33,96	34,50
5	34,58	34,81
5	34,83	34,04
5	34,66	34,84
5	33,78	34,31
5	33,72	34,41
2	34,87	36,34
2	35,80	35,61
2	35,34	36,31
2	34,37	35,64
2	34,91	34,33
2	34,72	34,67
1	34,75	36,26
1	34,44	35,41
1	35,44	35,65
1	36,41	36,17
1	34,35	36,87
1	35,66	Undetermined

Table A11 Results of limit of detection analysis of spiked stomach content DNA

Tissue DNA: Stomach content		
DNA copies (log)	**Ct (Target Parachlamydia)**	**Ct (Target: *Waddlia*)**
10	30,75	31,76

10	30,75	31,85
10	30,83	31,72
10	30,76	32,26
10	30,27	31,79
10	30,34	31,58
5	34,96	35,52
5	31,65	32,77
5	31,62	32,76
5	30,78	32,22
5	31,43	32,89
5	31,55	32,61
2	33,23	34,52
2	32,64	34,29
2	32,77	34,09
2	34,91	36,99
2	32,38	33,51
2	32,98	34,33
1	34,58	34,59
1	33,48	35,18
1	32,88	34,35
1	33,26	34,80
1	33,32	35,20
1	33,61	35,34

Table A12: Results of limit of detection analysis of spiked lung DNA

Tissue DNA: Lung		
DNA copies (log)	**Cт (Target: *Parachlamydia*)**	**Ct (Target: *Waddlia*)**
10	27,90	28,75
10	27,87	28,76
10	27,59	28,60
10	27,67	28,75
10	27,79	28,59
10	27,90	28,49
5	30,85	29,62
5	31,17	29,16
5	30,57	29,16
5	30,04	28,69

5	30,73	29,48
5	30,17	29,83
2	31,51	30,02
2	30,96	30,78
2	31,19	31,11
2	30,28	30,73
2	30,33	30,68
2	32,14	30,85
1	33,32	31,78
1	31,68	31,44
1	31,62	30,52
1	38.00	31,70
1	32,18	32,24
1	32,74	32,40

Annex 5

Results testing of placenta foetal tissue of known uninfected animals

Table A13 Testing of foetal tissue/placenta of known uninfected animals to demonstrate specificity. (Samples from uninfected animals was defined as samples from abortion cases where no typical pathological lesions were noted.)

No.	Primer/Probe and tissue					
	Target: Pan-Chlamydiales	Lab. number	Animal	Pathological lesion	Ct	Ct
1	Placenta	B01488-18	Ovine	None	34.57	35.23
2	Placenta	B01800-18	Ovine	None	34.24	33.08
3	Placenta	B00920-19	Ovine	None	30.78	29.28
4	Placenta	B01010-19	Ovine	Generalised congestion	37.00	34.94
5	Placenta	B02033-19	Ovine	None	33.55	32.43
6	Stomach content	B01800-18	Ovine	None	37.12	ND
7	Stomach content	B02302-19	Ovine	Non-specific	ND	ND
8	Lung	B0215-18	Ovine	None	ND	ND
9	Lung	B01800-18	Ovine	None	ND	ND
10	Lung	B00902-19	Ovine	None	ND	ND
11	Lung	B01010-19	Ovine	Generalised congestion	12.40	ND
12	Lung	B02033-19	Ovine	None	ND	ND
13	Placenta	B01765-18	Bovine	None	8.35	ND
14	Placenta	B02311-18	Bovine	None	33.95	ND
15	Placenta	B01167-19	Bovine	None	34.51	32.62
16	Placenta	B01309-19	Bovine	None	35.56	36.71
17	Stomach content	B01259-18	Bovine	None	ND	ND
18	Stomach content	B01355-18	Bovine	None	36.26	ND
19	Stomach content	B01457-18	Bovine	None	ND	ND
20	Stomach content	B01498-18	Bovine	None	ND	ND

21	Stomach content	B01499-18	Bovine	None	21.92	ND
22	Stomach content	B01631-18	Bovine	Splenic necrosis	ND	ND
23	Stomach content	B01011-19	Bovine	None	32.93	33.75
24	Stomach content	B01431-19	Bovine	None	36.67	ND
25	Stomach content	B01482-19	Bovine	Epicardial ecchimoses, subcut haemorrhages, anasarca	ND	ND
26	Lung	B02311-18	Bovine	None	ND	21.12
27	Lung	B01457-18	Bovine	None	11.87	ND
28	Lung	B01167-19	Bovine	None	32.82	31.71
29	Lung	B01860-19	Bovine	None	3.54	35.49
30	Lung	B02063-19	Bovine	Nodular liver	18.18	12.44
31	Lung	B00490-18	Bovine	Pulmonary leukostasis	ND	ND
32	Lung	B00883-19	Bovine	Yellow liver	ND	ND
33	Placenta	B00771-18	Caprine	None	36.92	36.89
34	Placenta	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	ND
35	Placenta	B01582-18	Caprine	None	ND	ND
36	Placenta	B01756-19	Caprine	None	17.84	ND
37	Placenta	B01980-19	Caprine	Brain congestion, pericardial effusion	33.08	31.57
38	Stomach content	B01638-18	Caprine	Abomasitis, Vascular leucostasis	34.13	34.60
39	Stomach content	B0876-19	Caprine	None	ND	ND
40	Stomach content	B1456-19	Caprine	None	ND	12.82
41	Stomach content	B01756-19	Caprine	None	ND	ND
42	Stomach content	B02378-19	Caprine	Pleuritis, Hydropericardium	ND	ND
43	Stomach content	B01756019	Caprine	None	ND	ND
44	Lung	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	ND

45	Lung	B01582-18	Caprine	None	39.05	ND
46	Lung	B01638-18	Caprine	Abomasitis, Vascular leucostasis	ND	ND
47	Lung	B01456-19	Caprine	None	ND	ND
48	Lung	B02245-19	Caprine	Bacteraemia	36.63	5.87
49	Lung	B02378-19	Caprine	Pleuritis, Hydropericardium	ND	ND
	Target: *Chlamydia abortus*	**Lab. number**	**Animal**	**Pathological lesion**	**Ct**	**Ct**
1	Placenta	B01488-18	Ovine	None	ND	ND
2	Placenta	B01800-18	Ovine	None	ND	32.23
3	Placenta	B00920-19	Ovine	None	ND	ND
4	Placenta	B01010-19	Ovine	Generalised congestion	ND	ND
5	Placenta	B02033-19	Ovine	None	9.26	7.17
6	Stomach content	B01800-18	Ovine	None	ND	ND
7	Stomach content	B02302-19	Ovine	Non-specific	ND	ND
8	Lung	B0215-18	Ovine	None	ND	ND
9	Lung	B01800-18	Ovine	None	ND	ND
10	Lung	B00902-19	Ovine	None	19.40	ND
11	Lung	B01010-19	Ovine	Generalised congestion	13.14	ND
12	Lung	B02033-19	Ovine	None	ND	ND
13	Placenta	B01765-18	Bovine	None	ND	ND
14	Placenta	B02311-18	Bovine	None	ND	ND
15	Placenta	B01167-19	Bovine	None	ND	ND
16	Placenta	B01309-19	Bovine	None	ND	14.07
17	Stomach content	B01259-18	Bovine	None	29.90	ND
18	Stomach content	B01355-18	Bovine	None	ND	ND
19	Stomach content	B01380-18	Bovine	None	33	ND
20	Stomach content	B01457-18	Bovine	None	ND	ND

21	Stomach content	B01498-18	Bovine	None	ND	ND
22	Stomach content	B01499-18	Bovine	None	ND	5.54
23	Stomach content	B01631-18	Bovine	Splenic necrosis	ND	ND
24	Stomach content	B01011-19	Bovine	None	ND	ND
25	Stomach content	B01431-19	Bovine	None	ND	4.49
26	Stomach content	B01482-19	Bovine	Epicardial ecchimoses, subcut haemorrhages, anasarca	ND	ND
27	Lung	B00470-18	Bovine	Pulmonary leukostasis	ND	ND
28	Lung	B02311-18	Bovine	None	10.21	ND
29	Lung	B01167-19	Bovine	None	3.68	ND
30	Lung	B01457-19	Bovine	None	ND	ND
31	Lung	B01498-19	Bovine	None	ND	14.52
32	Lung	B01860-19	Bovine	None	15	ND
33	Lung	B02063-19	Bovine	Nodular liver	35.79	ND
34	Placenta	B00771-18	Caprine	None	ND	ND
35	Placenta	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	ND
36	Placenta	B01582-18	Caprine	None	ND	5.13
37	Placenta	B01756-19	Caprine	None	4.91	8.72
38	Placenta	B02593-19	Caprine	Brain congestion, pericardial effusion	ND	ND
39	Stomach content	B01638-18	Caprine	Abomasitis, Vascular leucostasis	35.10	ND
40	Stomach content	B0876-19	Caprine	None	13.02	ND
41	Stomach content	B1456-19	Caprine	None	ND	ND
42	Stomach content	B02378-19	Caprine	Pleuritis, Hydropericardium	ND	5.64
43	Stomach content	B01756-19	Caprine	None	ND	ND
44	Lung	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	ND

45	Lung	B01582-18	Caprine	None	3.14	ND
46	Lung	B01638-18	Caprine	Abomasitis, Vascular leucostasis	16.72	ND
47	Lung	B01456-19	Caprine	None	ND	ND
48	Lung	B02245-19	Caprine	Bacteraemia	ND	ND
49	Lung	B02378-19	Caprine	Pleuritis, Hydropericardium	ND	ND
	Target*: Chlamydia pecorum*	**Lab. number**	**Animal**	**Pathological lesion**	**Ct**	**Ct**
1	Placenta	B01488-18	Ovine	None	ND	ND
2	Placenta	B01800-18	Ovine	None	ND	ND
3	Placenta	B00920-19	Ovine	None	ND	ND
4	Placenta	B01010-19	Ovine	Generalised congestion	ND	ND
5	Placenta	B02033-19	Ovine	None	ND	ND
6	Stomach content	B01800-18	Ovine	None	ND	ND
7	Stomach content	B02302-19	Ovine	None	ND	ND
8	Lung	B0215-18	Ovine	None	ND	ND
9	Lung	B01800-18	Ovine	None	ND	ND
10	Lung	B00902-19	Ovine	None	ND	ND
11	Lung	B01010-19	Ovine	Generalised congestion	ND	ND
12	Lung	B02033-19	Ovine	None	ND	ND
13	Placenta	B01765-18	Bovine	None	ND	ND
14	Placenta	B02311-18	Bovine	None	ND	ND
15	Placenta	B01167-19	Bovine	None	ND	ND
16	Placenta	B01309-19	Bovine	None	ND	ND
17	Stomach content	B01259-18	Bovine	None	ND	ND
18	Stomach content	B01355-18	Bovine	None	ND	ND
19	Stomach content	B01380-18	Bovine	None	ND	ND
20	Stomach content	B01457-18	Bovine	None	ND	ND

21	Stomach content	B01498-18	Bovine	None	ND	ND
22	Stomach content	B01499-18	Bovine	None	ND	ND
23	Stomach content	B01631-18	Bovine	Splenic necrosis	ND	ND
24	Stomach content	B1011-19	Bovine	None	ND	ND
25	Stomach content	B01431-19	Bovine	None	ND	ND
26	Stomach content	B01482-19	Bovine	Epicardial ecchimoses, subcut haemorrhages, anasarca	ND	ND
27	Lung	B0490-18	Bovine	Pulmonary leukostasis	ND	ND
28	Lung	B00883-19	Bovine	Yellow lung	ND	ND
29	Lung	B01167-19	Bovine	None	ND	ND
30	Lung	B01457-18	Bovine	None	ND	ND
31	Lung	B01860-19	Bovine	None	ND	ND
32	Lung	B02063-19	Bovine	Nodular liver	ND	ND
33	Lung	B02311-18	Bovine	None	ND	ND
34	Placenta	B00771-18	Caprine	None	ND	ND
35	Placenta	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	ND
36	Placenta	B01582-18	Caprine	None	ND	ND
37	Placenta	B01756-19	Caprine	None	ND	ND
38	Placenta	B01980-19	Caprine	Brain congestion, pericardial effusion	ND	ND
39	Stomach content	B01638-18	Caprine	Abomasitis, Vascular leucostasis	ND	ND
40	Stomach content	B0876-19	Caprine	None	ND	ND
41	Stomach content	B1456-19	Caprine	None	ND	ND
42	Stomach content	B02378-19	Caprine	Pleuritis, Hydropericardium	ND	ND
43	Stomach content	B01756-19	Caprine	None	ND	ND
44	Lung	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	ND

45	Lung	B01582-18	Caprine	None	ND	ND
46	Lung	B01638-18	Caprine	Abomasitis, Vascular leucostasis	ND	ND
47	Lung	B01456-19	Caprine	None	ND	ND
48	Lung	B02245-19	Caprine	Bacteraemia	ND	ND
49	Lung	B02378-19	Caprine	Pleuritis, Hydropericardium	ND	ND
	Target: *Parachlamydia acanthamoeba*	**Lab. number**	**Animal**	**Pathological lesion**	**Ct**	**Ct**
1	Placenta	B01488-18	Ovine	None	36.49	36.67
2	Placenta	B01800-18	Ovine	None	36.60	35.44
3	Placenta	B00920-19	Ovine	None	35.53	ND
4	Placenta	B01010-19	Ovine	Generalised congestion	35.65	37.02
5	Placenta	B02033-19	Ovine	None	36.89	34.98
6	Stomach content	B01800-18	Ovine	None	35.75	36.95
7	Stomach content	B02302-19	Ovine	Non-specific	ND	ND
8	Lung	B0215-18	Ovine	None	36.78	36.07
9	Lung	B01800-18	Ovine	None	35.50	35.88
10	Lung	B00902-19	Ovine	None	34.93	34.10
11	Lung	B01010-19	Ovine	Generalised congestion	36.69	37.02
12	Lung	B02033-19	Ovine	None	35.92	35.61
13	Placenta	B01765-18	Bovine	None	34.36	35.84
14	Placenta	B02311-18	Bovine	None	34.54	35.22
15	Placenta	B01167-19	Bovine	None	35.79	ND
16	Placenta	B01309-19	Bovine	None	35.99	37.05
17	Stomach content	B01259-18	Bovine	None	33.78	30.99
18	Stomach content	B01355-18	Bovine	None	35.49	33.56
19	Stomach content	B01457-18	Bovine	None	35.32	35.85
20	Stomach content	B01498-18	Bovine	None	35.04	33.07

21	Stomach content	B01499-18	Bovine	None	36.33	36.94
22	Stomach content	B01631-18	Bovine	Splenic necrosis	ND	35.51
23	Stomach content	B1011-19	Bovine	None	35.86	36.12
24	Stomach content	B01431-19	Bovine	None	36.39	ND
25	Stomach content	B01482-19	Bovine	Epicardial ecchimoses, subcut haemorrhages, anasarca	36.53	35.92
26	Lung	B00490-18	Bovine	Pulmonary leukostasis	36.78	36.07
27	Lung	B00883018	Bovine	Yellow liver	34.93	34.10
28	Lung	B02311-18	Bovine	None	35.88	35.33
29	Lung	B01167-19	Bovine	None	36.83	36.11
30	Lung	B01457-18	Bovine	None	35.32	35.85
31	Lung	B01496-19	Bovine	Viral placentitis	34.94	36.05
32	Lung	B01860-19	Bovine	None	35.42	36.45
33	Lung	B02063-19	Bovine	Nodular liver	34.98	34.88
34	Placenta	B00771-18	Caprine	None	35.82	36.07
35	Placenta	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	35.57
36	Placenta	B01582-18	Caprine	None	ND	ND
37	Placenta	B01756-19	Caprine	None	33.27	36.05
38	Placenta	B02593-19	Caprine	Brain congestion, pericardial effusion	35.98	36.13
39	Stomach content	B01638-18	Caprine	Abomasitis, Vascular leucostasis	36.43	36.31
40	Stomach content	B0876-19	Caprine	None	36.79	36.12
41	Stomach content	B1456-19	Caprine	None	36.07	35.35
42	Stomach content	B02378-19	Caprine	Pleuritis, Hydropericardium	35.89	35.97
43	Stomach content	B01756-19	Caprine	None	32.75	35.57
44	Lung	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	35.92	ND
45	Lung	B01582-18	Caprine	None	ND	35.58

46	Lung	B01638-18	Caprine	Abomasitis, Vascular leucostasis	35.36	35.46
47	Lung	B01456-19	Caprine	None	36.10	35.96
48	Lung	B02245-19	Caprine	Bacteraemia	35.83	36.03
49	Lung	B02378-19	Caprine	Pleuritis, Hydropericardium	34.78	36.92
	Target: *Waddlia chondrophila*	**Lab. number**	**Animal**	**Pathological lesion**	**Ct**	**Ct**
1	Placenta	B01488-18	Ovine	None	ND	ND
2	Placenta	B01800-18	Ovine	None	ND	ND
3	Placenta	B00920-19	Ovine	None	ND	ND
4	Placenta	B01010-19	Ovine	Generalised congestion	ND	ND
5	Placenta	B02033-19	Ovine	None	ND	ND
6	Stomach content	B01800-18	Ovine	None	ND	ND
7	Stomach content	B02302-19	Ovine	Non-specific	ND	ND
8	Lung	B0215-18	Ovine	None	ND	ND
9	Lung	B01800-18	Ovine	None	ND	ND
10	Lung	B00902-19	Ovine	None	ND	ND
11	Lung	B01010-19	Ovine	Generalised congestion	ND	ND
12	Lung	B02033-19	Ovine	None	ND	ND
13	Placenta	B01765-18	Bovine	None	ND	ND
14	Placenta	B02311-18	Bovine	None	ND	ND
15	Placenta	B01167-19	Bovine	None	ND	ND
16	Placenta	B01309-19	Bovine	None	ND	ND
17	Stomach content	B01259-18	Bovine	None	ND	ND
18	Stomach content	B01355-18	Bovine	None	ND	ND
19	Stomach content	B01457-18	Bovine	None	34.29	ND
20	Stomach content	B01498-18	Bovine	None	ND	ND
21	Stomach content	B01499-18	Bovine	None	ND	ND

22	Stomach content	B01631-18	Bovine	Splenic necrosis	ND	ND
23	Stomach content	B01011-19	Bovine	None	ND	ND
24	Stomach content	B01431-19	Bovine	None	ND	ND
25	Stomach content	B01482-19	Bovine	Epicardial ecchimoses, subcut haemorrhages, anasarca	ND	ND
26	Lung	B00490-18	Bovine	Pulmonary leukostasis	ND	ND
27	Lung	B00883018	Bovine	Yellow liver	ND	ND
28	Lung	B02311-18	Bovine	None	ND	ND
29	Lung	B01167-19	Bovine	None	ND	ND
30	Lung	B01457-18	Bovine	None	ND	ND
31	Lung	B01498-19	Bovine	None	ND	ND
32	Lung	B01860-19	Bovine	None	ND	ND
33	Lung	B02063-19	Bovine	Nodular liver	ND	ND
34	Placenta	B00771-18	Caprine	None	ND	ND
35	Placenta	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	ND
36	Placenta	B01582-18	Caprine	None	ND	ND
37	Placenta	B01756-19	Caprine	None	ND	ND
38	Placenta	B01980-19	Caprine	Brain congestion, pericardial effusion	ND	ND
39	Stomach content	B01638-18	Caprine	Abomasitis, Vascular leucostasis	ND	ND
40	Stomach content	B0876-19	Caprine	None	ND	ND
41	Stomach content	B1456-19	Caprine	None	ND	ND
42	Stomach content	B02378-19	Caprine	Pleuritis, Hydropericardium	ND	ND
43	Stomach content	B01756-19	Caprine	None	ND	ND
44	Lung	B01567-18	Caprine	Lung haemorrhages, Hepatic necrosis	ND	ND
45	Lung	B01582-18	Caprine	None	ND	ND
46	Lung	B01638-18	Caprine	Abomasitis, Vascular leucostasis	ND	ND

47	Lung	B01456-19	Caprine	None	ND	ND
48	Lung	B02245-19	Caprine	Bacteraemia	ND	ND
49	Lung	B02378-19	Caprine	Pleuritis, Hydropericardium	ND	ND

Annex 6

Permissions

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

06 December 2021

LETTER OF APPROVAL

Ethics Reference No	**REC070-21**
Protocol Title	**Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa**
Principal Investigator	**Dr A Jonker**
Supervisors	**Prof AL Michel**

Dear Dr A Jonker,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC070-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

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Faculty of Veterinary Science
Fakulteit Veeartsenykunde
Lefapha la Disaense tša Bongakadiruiwa

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Faculty of Veterinary Science
Animal Ethics Committee

7 March 2024

Study completed

Dr A Jonker
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

Dear Dr A Jonker,

RE: Acknowledgement Study Completed for AEC

Protocol No	REC070-21 Line 3
Title	Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa
Investigator	Dr A Jonker
Supervisor	Prof AL Michel
Sponsor	Andani Sengani Mphephu - AgriSeta

We hereby acknowledge receipt of the documents received on 11 February 2024 and note the content thereof.

Please note that the AEC may ask further questions and/or seek additional information.

Yours sincerely

Prof V Naidoo

CHAIRMAN: UP-Animal Ethics Committee

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Faculty of Veterinary Science
Animal Ethics Committee

09 February 2023

Approval Certificate
Annual Renewal
(EXT2)

AEC Reference No.:	REC070-21 Line 2
Title:	Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa
Researcher:	Dr A Jonker
Student's Supervisor:	Prof AL Michel

Dear Dr A Jonker,

The **Annual Renewal** as supported by documents received between 2023-01-13 and 2023-01-30 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-01-30.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Approved
Domestic ruminants	
Samples	Approved
Cattle, Sheep, Goats - Foetus & Placenta - RSA – (Stored/historic V029-17)	235

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-02-09.
3. Please remember to use your protocol number (REC070-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Faculty of Veterinary Science
Animal Ethics Committee

24 October 2022

Approval Certificate with Conditions
Annual Renewal (EXT1)

AEC Reference No.:	REC070-21 Line 1
Title:	Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa
Researcher:	Dr A Jonker
Student's Supervisor:	Prof AL Michel

Dear Dr A Jonker,

The **Annual Renewal** as supported by documents received between 2022-07-18 and 2022-09-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-09-26.

Please note the following about your Conditional ethics approval: (section 20 permit expires the 1th of Jan 2023) AEC approval period granted only until **1 January 2023.**

1. The use of species is approved:

Species	Approved
Domestic ruminants	
Samples	Approved
Foetus & Placenta (Stored/retrospective-historic) **V029-17**	235

2. Please remember to use your protocol number (REC070-21) on any documents or correspondence with the AEC regarding your research.
3. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
4. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
5. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

- **Ethics approval is subject to the following:**

The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRPERSON: UP-Animal Ethics Committee

UNIVERSITEIT VAN PRETORIA
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Faculty of Veterinary Science
Animal Ethics Committee

30 July 2021

Approval Certificate
New Application

AEC Reference No.:	REC070-21
Title:	Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa
Researcher:	Dr A Jonker
Student's Supervisor:	Prof AL Michel

Dear Dr A Jonker,

The **New Application** as supported by documents received between 2021-05-24 and 2021-07-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-07-26.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Domestic ruminants	
Samples Foetus & placenta	235 Stored- Historic/Retrospective (V092-17)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-07-30.
3. Please remember to use your protocol number (REC070-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Faculty of Veterinary Science
Animal Ethics Committee

12 May 2020

Approval Certificate
Annual Renewal (EXT3)

AEC Reference No.:	V092-17
Title:	Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa
Researcher:	Dr A Jonker
Student's Supervisor:	Prof AL Michel

Dear Dr A Jonker,
The **Annual Renewal** as supported by documents received between 2020-04-14 and 2020-05-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-05-04.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number Available
Cattle, Sheep, Goats	235

2. Ethics Approval is valid for 1 year. Please submit a NEW APPLICATION next year, this is the final extension that will be granted.

3. Please remember to use your protocol number (V092-17) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Animal Ethics Committee

Extension No. 2

PROJECT TITLE	**Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa**
PROJECT NUMBER	**V092-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. A Jonker**

STUDENT NUMBER (where applicable)	**U_89080875**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SAMPLES	**Cattle, Sheep, Goats**
NUMBER OF ANIMALS	**235 (Used 95)**
Approval period to use animals for research/testing purposes	**March 2019 – March 2020**
SUPERVISOR	**Prof. A Michel**

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 2 March 2019
CHAIRMAN: UP Animal Ethics Committee	Signature

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Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa**
PROJECT NUMBER	**V092-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. A Jonker**

STUDENT NUMBER (where applicable)	**U_89080875**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SAMPLES	**Cattle, Sheep, Goats**	
NUMBER OF SAMPLES	**235**	
Approval period to use animals for research/testing purposes		**March 2018 – March 2019**
SUPERVISOR	**Prof. A Michel**	

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 4 April 2018
CHAIRMAN: UP Animal Ethics Committee	Signature 

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Animal Ethics Committee

PROJECT TITLE	**Investigation of bacterial and mycotic causes of abortion in domestic ruminants in South Africa**
PROJECT NUMBER	**V092-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. A Jonker**

STUDENT NUMBER (where applicable)	**U_89080875**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SAMPLES	**Cattle, Sheep, Goats**	
NUMBER OF ANIMALS	**235**	
Approval period to use animals for research/testing purposes		**September 2017 – September 2018**
SUPERVISOR	**Prof. A Michel**	

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 26 September 2017
CHAIRMAN: UP Animal Ethics Committee	Signature