"*Coxiella burnetii* as the causative agent of abortions in sheep and goats in Cyprus"

A project submitted to Middlesex University in partial fulfilment of the requirements for the degree of Master in Professional Studies

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Title of Project : "*Coxiella burnetii* as the causative agent of abortions in sheep and goats in Cyprus"

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Summary

One of the most serious problems that we deal with in veterinary services is the issue of abortions in sheep and goats. It is an issue that includes many aspects, among which is the annual cause of negative effects in animal population, in the economy, the loss of animals, the pregnancy time wasted the loss in milk production, etc.

Abortions are caused by a number of factors, such as infections by bacteria and viruses, nutritional causes etc.

The project is focused on the implementation of molecular methods as a diagnostic tool in cases of abortions in sheep and goats, where *C. burnetii* could be one of the causative agents

The survey took place in Cyprus, and samples from abortions such as placentas and embryos were collected and transferred to the State Official Veterinary laboratories that deal with animal health, for the detection of *C. burnetti*, using molecular diagnostic methods. At the same time samples were examined in order to exclude other agents that may cause abortions, such as *Brucella melitensis, Campylobacter fetus*, *Chlamydophila psittaci* and *Anaplasma spp.*

The results of the research indicated a significant involvement of *C. burnetti* in abortions in sheep and goats observed in Cyprus. Similar involvement in those abortions seems to have also the pathogen *Chlamydophila psittaci*. The findings further indicate that the introduction of PCR in the laboratories of the Veterinary Services of Cyprus can be a great diagnostic tool for detection of *C. burnetii* in abortion materials in combination with the routine method Giemsa stain.

Suggestions and recommendations have been made emphasizing the importance of the freshly-implemented technique (PCR) to be adopted in the routine diagnosis and prevention of the pathogen

Chapter 1

Introduction

Several bacterial pathogens, which infect small ruminants, can cause abortion or fertility reduction, some of which, such may also infect humans (zoonotic diseases).

The present study is focused on the implementation of molecular methods as a diagnostic tool in cases of abortions in sheep and goats, where *C. burnetii* could be one of the causative agents.

C. burnetii is an intracellular bacterium and can cause the zoonotic disease Q fever.The main reservoir for the pathogen is domestic animals such as cattle, sheep and goats. A large variety of hosts, such as mammals (humans, ruminants, small rodents, dogs, and cats) as well as birds, fish, reptiles, and arthropods can also be infected by the pathogen (Marmion B.P and Stoker M.G.P,1950),(Davoli R. and Signorini L F, 1951), (Ioannou I, Chochlakis D et al,2009),(Ioannou I, Sandalakis V et al ,2011)Ticks can serve as a reservoir, and also spread the disease (Angelakis, E., Raoult, D.,Q ,2010)

The most common clinical signs of the disease.in animals are pneumonia, abortion, still birth and delivery of weak offspring (Arricau-Bouvery, Annie Rodolakis, 2005)

The main shedding route of *C. burnetii* is via birth products (birth fluids, placenta), but it can also shed via vaginal mucus, milk and faeces, **(**Berri, M.et al, 2002), urine (Heinzen, R.A.et al 1999), semen (Kruszewska, et al, 1997).

The diagnosis of *C. burnetii* in Cyprus Veterinary laboratories depends only on staining techniques using materials from abortions, such as placentas and embryos. However this method is characterized by its low sensitivity.

The inadequate diagnostic methods used in Cyprus Veterinary Services laboratories for the detection of *C. burnetti,* restrict our knowledge relating to the degree of involvement of the pathogen in abortions.The only evidence, regarding the circulation of *C. burnetii* in Cyprus, is based mainly on two serological surveys that took place in the past years. (Psaroulaki et al., 2006), (Ioannou I. et al., unpublished data)

In Cyprus, the officially reported number of sheep and goats is 332,597 and 279,717 respectively. Although abortions in Cyprus are included in the list of mandatory notifiable animal diseases, they are still underreported and the real number of abortion in sheep and goats remains unknown.

In consequence, the true involvement of *C. burnetii* in abortions in sheep and goats in Cyprus remains unknown. In fact according to the Veterinary Services of Cyprus there is no report, recording *C. burnetii* as the causative agent for the observed abortions. Seroprevalence data indicate that in Cyprus Q fever had been present in cows, sheep and goats for several decades and in fact is an endemic disease. Nevertheless, the degree of participation of the pathogen as an etiological factor of abortions has never been quantitatively determined.

For the completion of the study, a combination of laboratory and field-based work took place. A number of appropriate books, journals and publications were used for the completion of the literature review.

The survey took place in the five largest districts of Cyprus, Pafos, Limassol, Larnaca, Nicosia and Ammochosto, and samples from abortions such as placentas and embryos were collected and transferred to the State Official Veterinary laboratories that deal with animal health, for the detection of *C. burnetii*, using molecular diagnostic methods. For completion of the proposed research, 50 samples fetuses and/or placentas (47 fetuses and 19 placentas) were collected randomly from sheep and goats during the breeding season from October 2012 until March 2013.These samples were analyzed alongside with the 130 samples (73 fetuses and 71 placentas) were kept in the tissue bank of the laboratory services of Cyprus and were collected the last 7 years from goats and sheep that had abortions.

The total number of samples that was finally included in the research was 180 placentas and/or embryos.

At the same time samples were examined for *Brucella melitensis, Campylobacter fetus*, *Chlamydophila psittaci* and *Anaplasma spp* in order to exclude other agents that might have caused the abortions.

Following collection of the necessary epidemiological information by interviewing farmers, appropriate epidemiological bulletins were completed accompanying the samples to the Cypriot Veterinary laboratory.

The fast and accurate diagnosis of coxiellosis in animals is very important within farming industry throughout the world. Loss of embryos that are still in their primary stage of life may not have any productive value at the moment when they are aborted, but they would certainly have a sheer value when becoming adults. Pregnancy is also a time consuming and stressful period for each female individual that could, with the absence of the particular bacterial infection, deliver a healthy young offspring that would eventually join the chain of the productive animals in specific farms.

Furthermore *C. burnetii* can be potentially dangerous to humans as well. Thus, veterinary personnel, farmers, their employees and even their families and all people involved in general in farming industry are facing the potential danger of infection, since no precautions are currently undertaken regarding the particular pathogen.Finally as *C. burnetii* can be transmitted in large distances via the wind, general population without any prior contact with animals, can also be infected. The most supportive example of this statement is the Netherlands Q fever outbreak.

The findings of the research could change the current policy and strategy followed so far, regarding the diagnosis, treatment and prevention of *C. burnetii* in sheep and goats.

Chapter 2

2. Terms of Reference/Objectives and Literature Review

2.1 Project aim

The aim of the project is to determine whether *C. burnetii* is responsible and to what extent for the recorded abortions in sheep and goats in Cyprus as suggested by the serological studies that have been performed in 1996 and 2006.(Psaroulaki A. et al., 2006),(Ioannou I. et al., unpublished data)

2.2 Project Objectives

1. Retrospective seroprevalence data indicated that coxiellosis (Q fever) had been present in sheep and goats for several decades in Cyprus.Although *C. burnetii* is endemic in Cyprus, its degree of participation as an etiological factor of abortions in animals has not been adequately studied. The project's primary objective thus was to investigate if and to what extent *C. burnetii* is responsible for abortions in sheep and goats in Cyprus.

2. To introduce PCR in the laboratories of Veterinary Services of Cyprus with the aim of detecting *C. burnetii* with greater speed, reliability and sensitivity on samples taken from abortions occurring in sheep and goats in Cyprus. For comparison and evaluation result purposes, examination of the samples was also attempted with current most commonly used methods in Cyprus veterinary laboratories aiming at the detection of the pathogen, *C. burnetti* (staining techniques).

3. To identify the high risk areas of Cyprus where the number of abortions in sheep and goats due *to C. burnetii* infection is increased and recorded and identify risk factors that may have caused infection by the pathogen.

4. To attempt to correlate incidents of abortions in sheep and goats due to *C. burnetii* infection with the risk factors which were recorded in epidemiological questionnaires.

5. To exclude other frequent causative agents of abortions, such as *Brucella melitensis*, *Campylobacter fetus*, *Chlamydophila psittaci* and *Anaplasma spp.*

6. To correlate PCR findings with the currently used routine methods of Cyprus veterinary services of *C. burnetii* detection.

7. To correlate the results of the present research with the results of the two past serological investigations performed in Cyprus in 1996 and 2006.

2.3 Project Boundaries

Ethical Issues

Ethical issues were not expected to arise during the execution of the present research project.

The field work was involved with taking samples from animals that have already aborted, such as placentas and dead fetuses, which already existed in the external environment and no human interference was made on animals that could cause pain, fear or suffering.

Proper handling of animals in general was complied in accordance with the provisions of the Protection and Welfare of animals Law of 1994.

Throughout the completion of the project, anonymity and confidentiality were respected in accordance with the provisions of the Laws of the Data Protection Authority

Research Activities

Although abortions in Cyprus are obligatory notifiable, they are still underreported and the real number of abortion in sheep and goats remains unknown.

By examining the records of the Cypriot Veterinary Services laboratories since 2006, we realized that there was a significant variation in the number of samples from abortions presented to the lab. Therefore in order to draw valid conclusions from the research, we decided alongside with the samples that had collected within the period from October 2012 to March 2013 to include more samples of abortions (fetuses and placentas from sheep and goats) that were collected the last 7 years and were kept at the tissue bank of the Veterinary Service laboratory. This in turn, was an alternative solution for the smooth running of the project within the specified timetable, since the number of abortions was unexpectedly small due to farmer's failure to declare abortions to veterinary services.

Recourses

The Literature review was an important learning tool that helped me tremendously with the project. Collection of the information was done through sound scientific articles published in internationally recognized books, journals or websites. Access to these was gained through the author's University library and the library of the Medical School, University of Crete under the guidance and supervision of my Academic Consultant Dr Anna Psaroulaki, Head of the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine, University of Crete.

Time: The project demanded a lot of time in the laboratory performing the necessary PCR analyses. It was thus important to make an effective use of my time and prepare a flexible but strict timetable, to ensure that the project would finish in the particular deadline.

2.4 Research Statement

In Cyprus Veterinary Services the use of staining techniques in order to diagnose *C. burnetti* from abortion materials has low reliability, and does not reinforce solid results.

Laboratories of Cyprus Veterinary Services using inadequate methods for diagnosis, limit our knowledge as to the findings of the agent of *C. burnetti* in abortions.

This study centers in recording *C. burnetii* as the etiological basis of abortions in sheep and goats in Cyprus. Within this study, samples from animals with recorded abortions will be examined by PCR method, whilst they will be also be examined with the same molecular diagnostic method, in an effort to exclude other frequent abortion causative agents such as *Brucella melitensis, Campylobacgter fetus, Chlamydophila psittaci and Anaplasma spp.*

2.5 Research Questions

In order to deal with the existing problem the following questions need to be answered:

1) Is *C. burnetii* responsible for any abortions in sheep and goats in Cyprus?

2) Is it essential for the laboratories of Cyprus Veterinary Services to remove the old diagnostic methods for *C. burnetii* and replace them with a more sensitive accurate and reliable diagnostic method, such as the molecular method PCR?

3) What is the prevalence of *C. burnetii* in goats and sheep in Cyprus and what are the risk factors?

4) Is there a direct correlation of *C. burnetii* infection to outbreaks of abortions in sheep and goat units in Cyprus?

5) Can abortions of sheep and goats recorded in Cyprus be due to bacterial infections other than *C. burnetii*?

6) How do the PCR findings of the present project correlate with the currently used routine methods of Cyprus veterinary services of *C. burnetii* detection?

7) Can the results of the present project be correlated to the results of the past 2 serological surveys performed in Cyprus which indicated that *C. burnetii* is endemic to the island?

2.6 Literature Review

2.6.1 Abortions in sheep and goats:

According to several studies, abortions in animals constitute a key problem not only in Cyprus but also in other European countries which have developed the livestock sector.Each year thousands of abortions are recorded worldwide in animals of agricultural importance due to various causes, nutritional, metabolic, bacterial (Al-Ani FK et al, 2004), viral and parasitic infections. (Buxton D, 1998)

There are numerous studies from different countries in search of the causative agent of abortion in sheep and goats:

In 2003-2005 in Sardinia, samples of placenta and embryos from goats and sheep that aborted were examined by PCR for detection of DNA from *C, burnetii, Chlamydophila abortus, Salmonella abortus ovis, Toxoplasma gondii* and *Neospora caninum.*

Toxoplasma gondii was found in both animal species in high numbers **(**Masala G. et al, 2007)

In two other studies conducted in Switzerland in 1996-1998 (Chanton-Greutmann H et al, 2002) and in Hungary in 1998-2005 (Szeredi L et al, 2006), it was found that the most frequent cause of abortion in sheep and goats was the infection by *Chlamydophila abortus.*

In 2003-2004 in Croatia there was an epidemic of abortions in sheep due to *Salmonella abortus ovis*. During examination of five sheep farms, where the abortion rate presented to be

22% -38%, *S. abortus ovis* was isolated from 13 vaginal samples and two fetuses. (Habrun B, et al, 2006)

In investigating cases of abortion in sheep and goats in Spain it was found that 1.7% of 973 abortions in sheep and of 2.6% of the 262 abortions in goats was caused by *Leptospiras. Sp* (Leon-VizcainoL et al, 1987)

Abortions in livestock cause various problems such as reduced production of livestock and milk. Some of the abortions are due to pathogens that can cause zoonoses, threatening the public health other than the negative financial effects relating to animal production, animal products and animal health.

Towards finding a solution, each country has moved to create a network that aims to record the elimination of causative factors, record the measures taken to this direction, and whether they have any effect

C. burnetii and *C. abortus* are two of the most important causes of abortions in ruminants.**(**Berri M. et al , 2009) Apart from the losses caused to animal production they are zoonoses transmitted to humans with great impact on public health .(Rodolakis A.et al ,1998)

Furthermore, *C. burnetii* is considered of being one of the most common causes of abortion in goats in several countries (Hatchette, T.F. et al, 2001), (Moeller, R.B., 2001)

2.6.2 ***Coxiella burnetii***

C. burnetii is an intracellular, polymorphic bacillus (0.2-0.4 mm width, 0.4 to 1.0 mm length) **(Figure I),** which has a cell membrane, similar to gram-negative bacteria (Maurin M, Raoult D.,1999),(Kovacova E, Kazar J, 2002). However, it stains poorly with pigmented Gram stain. Gimenez staining (Gimenez DF., 1964) is traditionally used to stain the pathogen from pathological materials and crops.

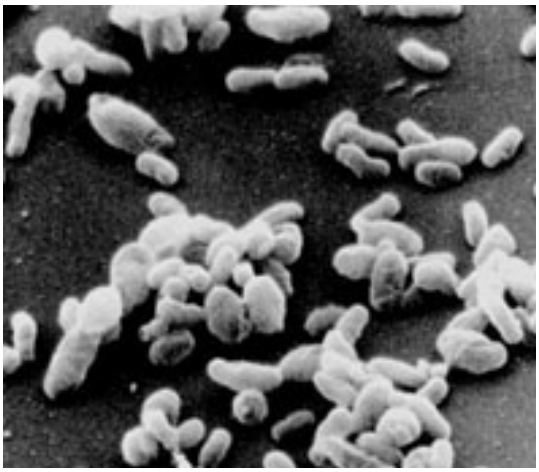


Figure I: *Coxiella. burnetii* as shown in electron microscope. Q fever- still a query and underestimated infectious disease (Kovacova E, Kazar J, 2002)

The bacterium is highly resistant to heat, pressure, and chemical stress (Heinzen RA.et al, 1999,) and thus it can survive for months in the environment. It's resistance to environmental conditions combined with its high infectivity and its aerosol transmission, places it in the "Category B" of biological warfare agents (Rotz LD. et al, 2002

As an obligatory intracellular bacterium, it can be multiplied only inside living cells and in this case, within fagolysosomata under harsh acidic ph(Hackstad T. and Williams Jc.1981).*C. burnetii* has a complex intracellular cycle (Williams JC, Peacock MG.,et al, 1981), giving rise to forms like spores **(Figure II).**

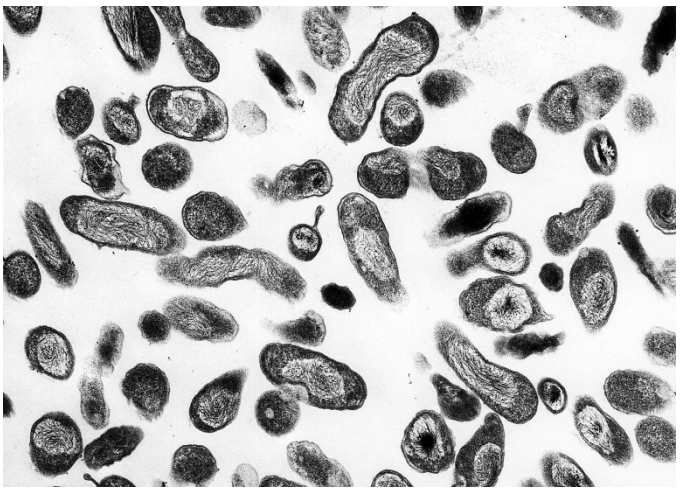


Figure II: Photograph from electron microscope showing the two structural forms of *C. burnetii*, namely the large cell variant and the small cell variant.

The stages of intracellular growth of *C. burnetii* include the "small-cell variant" (SCV), which correspond to the metabolically inactivate extracellular forms of *C. burnetii*, and "large-cell variants" (LCV), which correspond to the metabolically active intracellular forms of the bacterium (Maurin M, Raoult D., 1999)After entering host cells by phagocytosis, *C. burnetii* delays the fusion of lysosomes with the phagosome, using the delay likely to change from SCV to LCV form (Heinzen RA,et al,1999),(Howe D, Mallavia LP.,2000).**(Figure III**). The *C. burnetii* utilizes the acidic pH (4, 8) of the fused fagolysosomatos and survives (Vogel JP.2004)

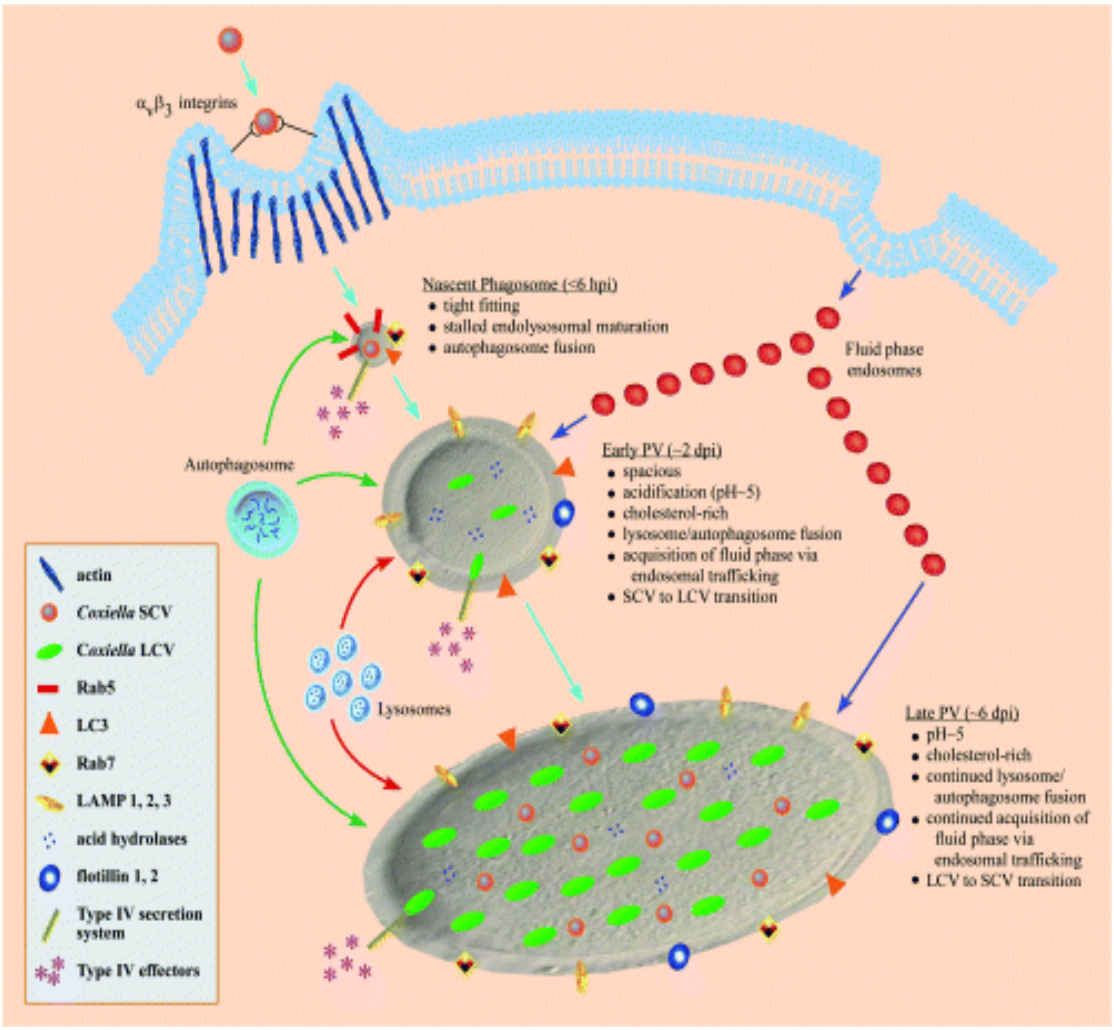


Figure III: Lounging in a lysosome: the intracellular lifestyle of *Coxiella burnetii* (Voth DE. and Heinzen RA., 2007)

C. burnetii displays two antigenic phases, phase I and phase II, that are liable to the lipopolysaccharide (LPS) of the membrane. Phase I bacteria corresponds to the smooth phase (Smooth) of Gram negative bacteria and are more highly infectious and phase II , to the granular phase (Rough) which has a lower virulence. Bacteria obtained directly from patients and animals are in phase I and those obtained after repeated passages in embryonated hen eggs are in phase II. (Fournier PE, et al, 1998)

Coxiella was originally considered to be a *Rickettsia*, then during various gene-sequence analyses *Coxiella* genus was classified in the order of Legionellale, family Coxiellaceae with *Rickettsiella* and *Aquicella* **(**Seshadri R et al, 2003). There is a high resemblance between the parasitic strategies and the lifestyle of *Rickettsiae* and *Chlamydiae* and *C. burnetii.* However ,their genome architectures are considerably different, in terms of mobile elements being present, extent of genome reduction, metabolic capabilities, and transporter profiles.(Seshadri R et al,2003)According to a 16S rRNA gene sequence analysis (Weisburg.et al, 1989).and genome analysis (which is based on shared proteins across genomes and phylogenetic analysis of a set of 20 highly conserved proteins) indications showed that since it is a γ-proteobacteria (order Legionellales) it therefore cannot belong to the a-proteobacterial Rickettsia group.

2.6.3 Q fever

In 1935 in Queensland, Australia, an outbreak of a febrile illness of unknown origin (Query fever) was observed among abattoir workers and that is when Q fever was first acknowledged.(Derrick E.H., 1937)

Q fever is a zoonosis caused by *C. burnetii*, with significant economic losses in the breeding of small ruminants and also unpleasant health complications for infected people that sometimes can result in death. (Porter Sara Rebecca et al, 2011

Q fever in humans

Q fever in humans can be observed as an acute disease or as a chronic disease. Acute disease can cause self-limited febrile illness, pneumonia, or hepatitis and chronic disease can cause endocarditis in patients immune compromised or suffering from valvulopathy, or abortions and stillbirth in pregnant women. (Angelakis, E., Raoult, D., 2010)

Inhalation of contaminated aerosols or dusts containing *C. burnetii* shed by infected animals are the main source of human infection.(Tissot-Dupont et al ,2004)

Epizootics of Coxiellosis in goats are related to Q fever outbreaks in humans as numerous studies have suggested. (Rousset E. et al, 2009

Q fever epidemiology

The usual sources of human infection with *Coxiella burnetii* are domestic ruminants such as cattle, goats and sheep, as the animals shed the bacteria in feces, urine, milk and products of labor / birth.The pathogen is excreted from the various secretions of infected animals in the ground, is particularly resistant to drying and remain viable in the environment even for years. Transmission to humans is via inhalation of contaminated aerosols (aerosols) which result directly from the infected animal secretions, or may contaminate the pups, placenta or wool.(Maurin M. and Raoult D., 1999). Because of long-term survival of *C. burnetii* surroundings, Q fever may be regarded as airborne infection from spreading to areas farms, in urban areas and in many unexpected places [Tissot-Dupont H. and others, 2004]. The infectious dust is a source of infection for humans and animals. (Parker NR. and others, 2006)

C. burnetii was classified as a category B potentional biological weapon due to the easy way that can be transmitted through air and inhalation, plus to its resistance and the ability to produce large quantities of C. *burnetii* in the placentas of aborted sheep or goats. (Kagawa F.T.et al, 2003)

The epidemiological and clinical features of Q fever may vary from one area of south Europe to another, as reports from France, Spain and Italy indicate. Q fever is less frequently reported in north Europe, therefore is not included in the nationally notifiable diseases list. **(**Pape M. et al, 2009)

Although Germany and Denmark, among other European countries, have also reported a change in epidemiology and an increase in cases of Q fever in 2008, they were not reported to be at the same extent as in The Netherlands.(Villumsen S, Nielsen HI.,2008)

In 1993 in Bulgaria, more than 2000 cases reported in a 6-month episode were probably due to Q fever. (Roest H.I.J et al, 2011

During 1983 in Switzerland *C. burnetii* infected animals descended from the alpine pastures to the valley passing through villages. As a result, a Q fever outbreak occurred, involving 415 serologically confirmed persons. (Dupuis, G.et al, 1987)

Epidemiological, clinical and therapeutic date of Q fever was also reported in Crete and northern Greece. (Tselentis Y.et al, 1995)

2.6.4 Coxiellosis in animals

The main reservoir for *C. burnetii* is domestic animals such as cattle, sheep and goats. A large variety of hosts, such as mammals (humans, ruminants, small rodents, dogs, and cats) as well as birds, fish, reptiles, and arthropods can be infected by the pathogen.(Marmion B.P and Stoker M.G.P,1950),(Davoli R. and Signorini L F, 1951),(Ioannou I, Chochlakis D et al,2009),(Ioannou I, Sandalakis V et al ,2011).Ticks can serve as a reservoir, and also spread the disease (Angelakis, E., Raoult, D.,Q ,2010).

The main shedding route of *C. burnetii* is via birth products (birth fluids, placenta), but it can also shed via vaginal mucus milk and faeces, **(**Berri, M.et al, 2002), urine (Heinzen, R.A.et al 1999), and semen (Kruszewska, et al, 1997)

C. burnetii is basically transmitted by air.The inhalation of aerosols that originate from infected placentas, body fluids or contaminated dust as a result of contaminated manure and desiccation of infected placenta and body fluids can cause the infection**.**(Arricau-Bouvery N., Annie Rodolakis,2005)

Many scientists consider inhalation as the most common way of *C. burnetii* transmission, both in animals and humans. (Tissot-Dupont, H.et al, 1999)

During experiments it was proven that humans can be infected and can show clinical signs of the disease by the inhalation of a single *C. burnetii* organism. (Tigertt, W.D et al, 1961)

The most common clinical signs of the disease.in mammals are pneumonia, abortion, still birth and delivery of weak offspring, are the most common clinical signs of the disease.(Arricau-Bouvery,Annie Rodolakis,2005)

Coxiellosis can also be asymptomatic. By the end of gestation abortions occur without specific clinical signs until abortion is imminent. *Coxiella* is then shed into birth products, urine, faeces and milk of infected animals. (Rodolakis, A., 2006)

Abortions in goats due to Coxiellosis occur more frequently than in sheep at a percentage up to 90% of females being affected **(**Berri M.et al, 2007)

2.6.5 Seroprevalence of *C. burnetii* in animals

Coxiellosis in animals is detected in all the 5 continents, (Africa, America, Asia, Europe, Oceania), whilst the only country with a reported apparent prevalence of zero is New Zealand. (Guatteo Raphael et al, 2011)

There is a plethora of *C. burnetii* seroprevalence studies throughout the world.

According to reports from the USA, sheep have been shown to have a lower seroprevalence of *C. burnetii* (16.5%) than goats, (41.6%) (McQuinston JH, Childs JE. 2002)

In Southern Croatia, antibodies for *C. burnetii* were detected in 16.4% of domestic animals (Punda-Polic V.et al, 1995)

In a serological study that took place in Egypt, 10.4% of sheep and 6.5% were found seropositive (Mazyad SA, Hafez AO.2007)

Q fever outbreaks that occurred to humans in Bosnia and Hergegovina were due to infected sheep and goats (Sukrija Z, Hamzic S, Cengic D et al. 2006)

In Northern Greece, 10.4% of sheep and 6.5% of goats were found positive for *C. burneti***i** **(**Pape M. et al, 2009)

C. burnetii distribution in sheep and goats in Italy is considered of being very high (38% and 47% respectively) according to a sero prevalence analysis. (Masala G. et al, 2004)

In a sero-epidemiological study done in 1996-1999 in the island of Cyprus in order to determine the prevalence of IgG antibodies to *C. burnetii,* 29.3% of goats, 17% of sheep and9.3% of cattle was found seropositive (Psaroulaki A. et al, 2006). A large Sero-prevalence was observed in the farming areas (60.8%) compared to the Suburban (48.4%) and urban (33.9%).

Ten years after this study, sero positivity rates in animals and the human population increased, confirming and reinforcing earlier studies concerning the significance of this pathogen in Cyprus. In a total number of 679 sheep and goats tested, 561 (82%) of them were positive in IgG antibodies C. burnetii. Of these, 292 goats (84%) and 269 sheep (81%) were seropositive. (Ioannou I. et al., unpublished data)

In 2012, in a recent seroepidemiological study in Crete by examining 493 serum samples from human populations with diagnostic methods IFA, 48% of them tested positive for IgG antibodies, while 34% were found positive in IgM antibodies against *C. burnetii.* The analysis of 225 serum samples from human populations at high risk showed the prevalence of *C. burnetii* at a rate of 62.2%. Their findings showed that *C. burnetii* is highly endemic in Crete, showing a large population exposure to the pathogen regardless of occupation or place of residence. . (Vranakis I., et al., 2012)

2.6.6 Q fever outbreak in Netherlands

The magnitude and duration of Q fever epidemic between 2007 and 2009 in Netherlands was such that attracted attention. Several decades before the disease was diagnosed in dairy goats and dairy sheep, it seems that Q fever was endemic in the Netherlands, as seroprevalence studies of 1981 and 1987 suggest**.(** Anon., 1981),(Houwers DJ,et al.,1987). In 2007 the increase in human infections started. (Roest H.I.J et al, 2011).

During the Q fever epidemic in Netherland, 1000 human Q fever cases were notified in 2008. A total number of 2355 human cases was the sharp increase observed in April 2009, whilst between January and May 2010 a decrease in the number of notified human cases was registered, with 208 human cases (Roest H.I.J et al, 2011)

The source of human infection, based on epidemiological research reports, was likely to be the inhabitancy of people near to dairy goat flocks where repeated abortions occurred. (Van der Hoek, et al, 2010)

According to Karagiannis et al study (Karagiannis,I et al,2009), in the Netherlands 2007 outbreak, factors that were found to be associated with the recent infection was smoking and contact with agriculture products, and living east where positive goat farm, cattle, or small ruminants were situated.

As genomic studies reported, multiple genotypes were involved. However, both in a dairy goat herd suffering from multiple abortions as well as in several human patients, an identical subtype were identified. A survival and propagation advantage seemed to be the status of this particular subtype when compared to other bacterial subtypes**.(**Klaassen C.H.W et al ,2009).

Within October 2008 the Ministry of Agriculture in Netherlands, in its effort to restrict the epidemic, ordered a voluntary vaccination of goats with phase I Q fever vaccine for ruminants. [Coxevac*1*, CevaSante´Animale, France**;]** in the high risk Q fever area in the Noord-Brabant (Roest H.I.J et al, 2011

A stringent hygiene protocol was presented in February 2009, which was made mandatory for all professional dairy goat and dairy sheep farms in the Netherlands, regardless of their Q fever status. General farm hygiene was improved by measures such as vermin control, compulsory rendering of aborted fetuses and placentas and measures for handling manure (Roest H.I.J et al, 2011)

Vaccination of all dairy sheep and goat farms with a population over 50 animals became mandatory within 2009, enlarging the area of vaccination, whilst it was exclusively performed in areas of the human outbreak. (Porter Sarah Rebecca et al, 2011)

Whilst The Netherlands had a major problem with Q fever, it is yet not clear why no other country had a similar problem. The epidemiology change in goats could happen due to two factors: The goat density increase in specific areas of The Netherlands, and secondly the extension of the farms through the years. In-herd and between-herd dynamics of Q fever could have been affected by these two factors. Nevertheless, there are pathogen-related factors that can't be ignored, involving the circulation of a highly virulent *C. burnetii* strain. (Roest H.I.J et al, 2011)

2.6.7 Diagnosis of Coxiellosis

The routine diagnosis of Coxiellosis in aborted ruminants is to detect the pathogen using staining techniques, such as Stamp, Gimenez, Machiavello, followed by a serological analysis of at least ten sera samples by the complement fixation (CF) test, or better by ELISA.(Kovacova E., Kazar J., Spanelova D. 1998). However, staining techniques cannot be specific and they have reduced sensitivity, especially with vaginal swabs, milk and fecal sample (Berri, M., et al., 2000)

Although CF test is prescribed by OIE as a diagnostic method for *C. burnetii*, its sensitivity is weak. Antibodies of *C. burnetti* in sheep and goats cannot be detected frequently by the antigen of the specific test (Kovacova E., Kazar J., Spanelova D. 1998). This test, on the contrary to ELISA, cannot detect all IgG subclasses. In ruminants, CFT can detect only IgG1 that fixes the complement. Moreover, IgG2, IgM and anticomplement substances potentially present in sera are capable of interfering with fixation of IgG1 to the complement lowering the titer of IgG1 detected by CFT. (Rousset E., et al., 2009)

Even though, the ELISA test, allows the testing of a greater number of animals and flocks, and despite the fact that it is a more sensitive test than the CF test and IFA, it does not also allow individual identification of animals that shed *C. burnetii* in faeces or milk, since there is no true relationship between the serological response and excretion. Although most animals that excrete *C. burnetii* in vaginal mucus, faeces or milk can be seropositive, still there are some animals that can be seropositive without shedding *C. burnetii* and a few ones can excrete and remain sero negative **(**Berri, M. et al, 2002).

The species- specific indirect immunofluorescence assay, IFA, does not have the ability to screen in a large scale, and it also cannot be automated and can be subjective. This is the reason why the diagnostic method IFA, which is considered as the gold standard in Q fever diagnosis in humans, is not preferred for a routine diagnosis of *C. burnetii* in animals. There is not yet any commercial kit using IFA for veterinary investigation.**(**Arricau-Bouvery N., Annie Rodolakis, 2005).

Srerological tests including enzyme-linked innunosorbent assay (ELISA), complement fixation test (CF) and immunofluorence (IFA) have the potential disadvantage to indicate exposure rather than to detect the organism. Moreover they have low sensitivity in the early stages of an infection and a cross reactivity between *C. burnetii* and *Chlamydia* strains in ELISA and immunoblot analysis can also be observed. (Lukacova M et al., 1999)

Due to the zoonotic nature of the agent, isolation of *C. burnetii* is not performed for routine diagnosis in veterinary medicine. The main reasons are the high level of expertise required, the time consumed and the requirement of BSL3 laboratories confinement**. (**Berri M.et al, 2000)

A simplified shell vial culture system traditionally used for virus isolation was proposed as an alternative technique for the isolation of *C. burnetii*, with increased sensitivity. (Raoult, D., Vestris, G., Enea, M., 1990

The diagnosis of *C. burnetii* in Cyprus veterinary laboratories is depended solely on staining techniques by using materials from abortions, such as placentas and embryos. For other pathogens that can also cause abortions in sheep and goats in Cyprus and which are target in this survey, such as *Brucella melitensis* and *Campylobacter fetus,* diagnosis is performed by culture and for *Chlamydia psittaci* with Immunochromatographic assay (Rapid test).

The diagnosis of Coxiellosis in veterinary medicine was aided by the introduction of polymerase chain reaction (PCR).Shedders could be identified whilst PCR kits are becoming available providing a specific, sensitive and rapid tool for the detection of *C. burnetii* in various clinical samples.(Berri, M., Arricau-Bouvery, N., Rodolakis, A., 2003).

PCR is considered to be highly specific sensitive and rapid diagnostic tool for *C. burnetii,* compared to other laboratory techniques, since it can detect the smallest concentration of bacterial DNA in biological samples (Willems et al., 1994).

2.6.8 Control measures

Biosecurity measures:

In the case of Coxiellosis epidemic, hygiene measures and precautions shall be applied to livestock herds and human level, to reduce the transmission of the pathogen.

Since birth is critical for transmission of *C. burnetii* in infected herds, animal births should take place only in a specified location, which should be disinfected as well as every tool used during labour. Since products of labour are considered to be high risk materials for the transmission of the pathogen, placentas and embryos must be destroyed as soon as possible to avoid their ingestion by domestic or wild carnivores (Arricau-Bouvery N., Annie Rodolakis, 2005)

Destruction of every high risk material, including contaminated bedding with incineration or burial with lime is recommended. (Porter Sarah Rebecca et al, 2011). Manure should be treated with lime or calcium cyanide prior to spreading, and spreading must be performed on a day of apnea.

Antibiotics

The quantities of *C. burnetii* shed during parturition, and the reduction of abortions can be minimized with antibiotic treatments such as tetracyclines. (Berri M., Souriau A., Crosby M., Rodolakis A., 2002)

Vaccines

Inactivated whole *C. burnetii* phase I vaccines are considered to be the most effective ,since during the experimental infection or in natural Q fever infection, vaccinated sheep and goats showed less bacterial shedding in placentas and milk (Sadecky E., Brezina R.,1977)

The excretion of *C. burnetii* in pregnant goats was studied by A. Bouvery , in order to investigate the effectiveness of phase I and phase II vaccines ,(Coxevac, phase I,CEVA Sant´e Animale and Chlamyvax FQ, phase II, MERIAL).Phase I vaccine proved to be effective and dramatically reduced both abortion and bacteria excretion in the milk, vaginal mucus and faeces, whilst the Phase II vaccine, failed to change the course of the disease or the excretion.(Arricau-Bouvery*N. et al, 2005)*

Nevertheless, shedding in milk in naturally infected cows prior to vaccination, according to several authors, phase I vaccines failed to be preventive.(Schmeer N. et al , 1987), underlining that a vaccine can only protect uninfected animals but is not able to treat an infected one.

Livestock vaccinated with phase II vaccine, cannot be protected against a *C. burnetii* infection and thus reduce the shedding of bacteria by the vaginal route and in milk. (Fishbein D.B., Raoult D., 1992)

As a summary, Q fever is a disease of interest for public policy makers and food industries (especially dairy production) based on public and animal health issues. A better risk assessment based on the knowledge of the true prevalence of the infection, can therefore lead to the decision to implement or not a control program. Veterinarians and policy makers could also be helped by this information to determine (i) the level (i.e. herd, local, regional level) on which this control program have to be implemented and (ii) control actions that must be taken against the infection (i.e. vaccination, trade restriction, etc.).(Guatteo Raphael et al, 2011

Chapter 3

3. Methodology

Research methodology is very important for the accomplishment of every research project. The approach that the researcher will adopt and the method of data collection techniques will depend on the type of information required. Therefore, the approaches and techniques will be the researcher's tool, which will serve him/her to answer the project's aims and objectives.

3.1 Research families/ approach

After addressing the objectives of the project, there was a need to consider a strategy that would provide answers to the problems under investigations. All the data collected from the analysis results and the information acquired from the participants were analysed in a quantitative way using statistics in order to answer the research questions. The aim was to investigate whether the microbiological factor *C. burnetti* is responsible for the abortions in sheep and goats recorded in Cyprus, as well as its percentage contribution. In the framework of this study samples from sheep and goats that had aborted in Cyprus were examined by PCR. At the same time the same samples were examined using the same diagnostic method, PCR in order to exclude other frequent causative agents of abortions, such as *Brucella melitensis*, *Campylobacter fetus*, C*hlamydophila psittaci* and *Anaplasma spp*

The majority of the research was carried out in the field. The research involved processes of collecting abortion materials from sheep and goats from farms distributed in the four largest districts of Cyprus. In addition, a part of the research was conducted in the controlled environment of a laboratory, where abortion samples from sheep and goats were examined by PCR method.

The practical source of information was combined with the necessary deskwork. As more findings were obtained from practise the appropriate through a continuing review of the literature was performed.The collection of information was performed through sound scientific articles published in internationally recognized books, journals or websites. Access to these was gained through the author's university library and the library of the Medical School, University of Crete under the guidance and supervision of Academic Consultant Dr Anna Psaroulaki, Head of the Unit of Zoonoses (Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine) Medical School, University of Crete.

Having chosen research families it was necessary to link them to the most appropriated research approach. Experimental Survey approach underpinned the work I produced for this project.

The experimental option would give the ideal opportunity to investigate the contribution of C. *burnetii* in the recorded abortions in sheep and goats in Cyprus, by performing experiments, such as laboratory analysis of abortion materials using the diagnostic method PCR.

The adoption of a survey, through the on-site farm inspections and the personal interviews with farmers allowed the author to obtain all the useful epidemiological information in order to extract the appropriate conclusions upon completion of the project.

3.2 Research Methodology

The methodology used for the project was a combination of an epidemiological investigation on farm level and laboratory work.

Epidemiological Research

The epidemiological research included the following steps:

-Recording of abortions observed in goats and sheep in Cyprus through an existing recording abortion system governed by legislation.

-Development of an epidemiological bulletin (questionnaire) which was completed during the sample collection and which helped to a more thorough collection of epidemiological information of the abortions.

The questionnaires were completed by the author and the findings were confirmed through inspections on each farm.

Within the daily contact with farmers and our collaboration in tackling various problems related to animal diseases or animal production problems they faced the author developed her personal social skills. The ability to create an atmosphere of trust during dealing with farmers helped acquiring the correct information via personal interviews and the completion of epidemiology questionnaire.

-Development of the sampling protocol.

Through practice over the last ten years as a veterinary officer in the Veterinary Services of Cyprus, the author had dealt with various veterinary issues. During this period, among others, implementation of several various surveillance and control programmes were performed, contingency plans for epizootic diseases were executed and epidemiological investigations were undertaken. The author's extensive working experience provided the necessary knowledge and skills for the proper design of the epidemiological questionnaire, as well as the sampling protocol

-Sample collection from abortions, placentas and embryos from sheep and goat farms which were distributed in the 5 districts of Cyprus.

In Cyprus, the officially reported number of sheep and goats is 332,597 and 279,717 respectively. Although abortions in Cyprus are obligatory notifiable, they are still underreported and the real number of abortion in sheep and goats remains largely unknown. This is also proven by the records of the laboratories of veterinary services in Cyprus, which show that the last 7 years there was a significant variation in the number of samples submitted for examination in the laboratory. This occurred because breeders randomly or deliberately neglected to declare abortions to their animals in veterinary services for fear of sanctions or any restrictive measures in their animals or animal products. Bearing this in mind and in order to extract valid results and complete the project within the set timeframe, it was decided alongside with the 50 sample that had collected within the period from October 2012 to March 2013, to include 130 more samples of abortions (fetuses and placentas from sheep and goats) that were collected the last 7 years and were kept at the tissue bank of the Veterinary Service laboratory.

The total number of samples that was finally included in the research was 180 placentas and/or embryos.

All samples were coded and stored in the laboratory of Veterinary Services until further analysis. Coded samples were recorded in a database created for this purpose.

Both types of samples were stored at -80 ° C until they were tested with PCR.

-Recording and statistical processing of the results. Mapping of results using a Geographic Information System (GIS)

A statistical analysis of data was performed and the corresponding parameters were selected based on the collected data.

A map of Cyprus was designed showing the sampling areas, regions and cases of positive samples and pathogens which were detected in the area. This resulted in the recording of specific areas where the number of abortions due to *C. burnetii* is increased and in my proposal to my service for any necessary actions

It was also an attempt to record and identify risk factors that may cause infection of the pathogen under study.

Laboratory Research

The laboratory research included the following steps:

-The study and process of all laboratory protocols

With the daily contact with Veterinary Services laboratories for sending of the samples and epidemiological information, intimacy and close collaboration with the laboratory personnel were developed. The author also developed various specific skills and knowledge for diagnostic methods used for each disease. At the same time the proper theoretical training, regarding the laboratory protocols, was gained through international bibliography. The successfully completion of the laboratory research and processing of laboratory protocols of the project was guaranteed by the author's training by the scientific staff of the Laboratory of Clinical Bacteriology, Parasitological, Zoonoses and Geographical medicine, University of Crete in Greece.

In this project internationally recognized protocols on diagnostic methods of *C. burnetii* such as PCR and Giemsa were used.

-*C. burnetii* detection by PCR

Up to date, the diagnosis of *C. burnetti* in Cyprus veterinary laboratories depends solely on staining techniques using materials from abortions, such as placentas and embryos. This

method suffers from low sensitivity, leading to observation of large number of falsely negative samples.

In this study alongside with the diagnostic method Giemsa, PCR was used for the detection of *C. burnetii* in abortion samples, which is based on identifying the bacterial DNA and therefore is a much more sensitive and specific method.

PCR has the ability to detect the smallest concentration of bacterial DNA and tends to establish itself as the reference method for the diagnosis of pathological organisms due to increased specificity, sensitivity, and immediacy of result

Regarding methods such as serology test and culture currently used for detection of *C. burnetii* the disadvantages are either reduced sensitivity in the early stages of infection (e.g. serology tests are based on the detection of antibodies to the pathogen) or the long time required to export a response (e.g. culture, requires up to 21 days).

The sample preparation protocols (DNA extraction) and PCR are specific and internationally accredited.

Samples of placenta and embryos that were collected and stored under appropriate conditions were examined, using PCR, to detect *C. burnetii* DNA.

At the same time, samples were examined using the same diagnostic method PCR, in order to exclude other frequent causative agents of abortions, such as *B. melitensis, C. fetus, C. psittaci and Anaplasma spp*

- Laboratory test of placentas and fetuses for the detection of *C. burnetii* using staining techniques

For comparison and evaluation result purposes, examination of the samples was attempted with the most commonly used methods in Cyprus veterinary laboratories aiming at the detection of the pathogen, *C. burnetti* (staining techniques).Fixed impressions or smears prepared from the placenta and from the fetus stomach content stained by Giemsa were examined in the microscope in order to detect the pathogen.

The diagnosis of Coxiellosis in aborted ruminants is usually established by bacterioscopic examination of stained smears are taken from the placenta of aborted ruminants, from the

fetus stomach content, or from other body tissues. Smears are usually stained by Stamp, Gimenez, Machiavello, or Giemsa stain.(Angelakis, E., Raoult, D., 2010), (Gimenez DF. 1964). The presence of large masses of red-coloured coccobacilli will indicate a strong presumptive diagnosis of *C. burnetii*. However these diagnostic methods are poorly sensitive and not specific due to possible confusion with other pathogens such as *Brucella spp*., *Chlamydophila spp.,* or *Chlamydia spp*. (Berri M. et al., 2009), (Guatteo R.,et al., 2006), (Woldehiwet . Zerai, 2004)

3.3 Confidential and ethical issues

The project was completed with consistency and professionalism and the required responsibility to colleagues and farmers without creating any moral issues involving human beings or animals.

The research was completed in collaboration with the Cyprus Veterinary laboratories, using the buildings, laboratory equipment and samples from the tissue bank of the institute. We had access to the laboratory database and were able to use the data obtained from previous surveys or reports of illnesses in Cyprus.The project was carried out with the approval of Cyprus Veterinary laboratories. All actions and access to databases were made known beforehand to the appropriate department and were constantly available for official inspection. With the completion of the research project the results were forwarded to the appropriate Cyprus Veterinary services department for assessment and further actions

In accordance with the relevant veterinary legislation owners of sheep and goats in Cyprus are obliged to declare abortion outbreaks presented in their animals to the Veterinary Services of Cyprus.

The author's working experience in the veterinary services of Cyprus as an official veterinarian, was not by any means manipulated to exercise any pressure, intimidation or penalties to farmers that either through negligence or intentionally did not declare to the competent authority any abortions of their animals.

On the contrary, efforts were made to inform farmers correctly and appropriately for the beneficial objectives and purposes of the particular project.

As such, their collaboration was presented as a prerequisite not only for the completion of the project but also for possible infections or epidemics in humans and animals improving the quality of animal products and animal health with a view to protecting public health and the health of livestock.

The field work was involved with taking samples from animals that have already aborted, such as placentas and dead fetuses, which already existed in the external environment and no human interference was made on animals that could cause pain, fear or suffering.

Proper handling of animals in general was complied in accordance with the provisions of the Protection and Welfare of animals Law of 1994.

Throughout the completion of the project, anonymity and confidentiality were respected in accordance with the provisions of the Laws of the Data Protection Authority

3.4 My role as a researcher

Having two roles as an insider researcher and as just a researcher, it is logical one may think that the combination of these two could have led to ethical issues during the conduct of my research programme.

Authority and power inevitably given to an insider researcher through his work, might have led to erroneous actions of abuse of power resulting in ethical issues such as the immoral guidance of participants based on the information they were giving, manufacturing information and data, using the practice of falsifying results or even coercion of farmers to participate in the research program.

All the years that I have worked into the Cyprus Veterinary Services, I learned to work with professionalism and sense of responsibility towards farmers and the Service which I have served for so long. It's clear in my mind that my role as a veterinary officer into the Veterinary Services of Cyprus should be independent from that of the researcher. As an insider researcher I focused my actions as if I was an independent researcher who at the end of the project after exporting the appropriate conclusions united the two roles again to help achieve the objectives of the research.

According to the relevant veterinary legislation all abortion outbreaks of sheep and goats in Cyprus are to be declared to the Veterinary Services of Cyprus from sheep and goats units owners.

I managed to keep the integrity of my personal and professional status, by not taking any advantage of any power given to me through my position in Cyprus Veterinary Services as an official veterinarian. I strictly avoided to exercise any kind of pressure, intimidation or penalties to farmers that either through negligence or intentionally did not declare as they were obliged to, to the competent authority any abortions of their animals.

I explained to them that the main target of my study is the recording of *C. burnetti* as an etiological cause of abortions in sheep and goats in Cyprus, locating particular farms and regions concerning abortions occurring by *C. burnetti* and a proposal to Cyprus Veterinary Services to take specific measures to prevent the disease.

I also tried to convince farmers that their prompt participation in my research project by stating directly to the Veterinary Services the cases of abortions in their livestock would be beneficial at individual level (microeconomics of their husbandry) since a modern new prompt diagnosis would be established having as the ultimate long-term goal the prevention of abortions and reduction of economic losses resulting there from and thereby contributing to the improvement of the economy at the national level

3.5 Strengths and weaknesses of the research

Nowadays, the diagnosis of *C. burnetti* in Cyprus veterinary laboratories depends only on staining techniques using materials from abortions, such as placentas and embryos. For this purpose samples from placentas and fetuses are taken from sheep and goats aborted and then are stained with Giemsa stain. During the bacterioscopic examination of stained smears the presence of large masses of red-coloured coccobacilli is a great indication for the presence of *C. burnetii*. However these diagnostic methods are poorly sensitive and not specific due to possible confusion with other pathogens such as *Brucella spp*., *Chlamydophila spp.,* or *Chlamydia spp*. (Berri M. et al., 2009), (Guatteo R.,et al., 2006), (Woldehiwet , Zerai, 2004).

The survey intended to identify the *C burnetii* in the placenta or fetus from sheep and goats which have aborted, using a new diagnostic method called PCR which until now was not

used by Cyprus Veterinary laboratories. The use of this method resulted to show that PCR technique supplements the conventional Giemsa stain technique already used and improves the reliability of the results in order to export right and valid conclusions. PCR is used by all internationally recognized and approved agencies and institutions, both for diagnosis and for epidemiological studies.Its sensitivity and reliability is based on the detection of pathogen DNA in a manner such that the probability and practical false-positive results due to cross-reactions are minimal to nil, which is not the case with other methods.That is also why PCR tends nowadays to develop, despite the higher cost compared with other methods, into a first-line technique.

For the completion of the laboratory part of the project, the buildings and the equipment of the veterinary laboratories were used in order to save and examine the samples.

Notification of abortions cases from farmers was crucial to the completion of the proposed project to the specified schedule and to extract accurate results. Although farmers were advised correctly and appropriately for the beneficial purposes of the whole research with the purpose to motivate them regarding their participation in the project, their response was not as expected. This resulted in the disclosure of abortions to Veterinary Services Cyprus during the breeding season from October 2012 to March 2013. The number of abortions reported by farmers was reduced either through negligence or intentionally out of fear of restrictions to be imposed to their animals or animal products. The economic crisis in Cyprus at that time was another important factor that led breeders to avoid declares abortions and pay examination fees of the samples as they should, according to the relevant veterinary legislation.As a result, instead of collecting 100 samples of placentas or / and embryos during the period of breeding season between October 2012 to March 2013, only 50 samples were collected, in which case in order to export correct and valid results for research purposes, another 130 samples that were saved since 2006 into the bank tissue of the veterinary services laboratory were used for laboratory examination.

Chapter 4

4. Project Activity

4.1 Project Journal

During the project a more detailed version of a laboratory book was always kept where all thoughts and activities were written. The data collection had started in a very early stage, so it was essential to write and record all activities, meetings, problems that were encountered and all the critical incidents that occurred over the data collection period. The journal was an important part of the project activity as it linked the various tasks the author was engaged in and provided an element of continuity.

4.2 Phases of the Project Activity

The study was designed in six phases and the estimated time of completion for all phases was calculated to be 9 months. Completion of the study lasted from September 2012 until May 2013.

Phase 1: Literature Review:

Literature review was a collection of summary of references from various sources such as books, reports, articles relevant on the project subject. All the information from the different kind of documents was integrated into a coherent study about *C. burnetii*, its pathology, epidemiology, diagnosis and prophylaxis. Access to internet resources such as PubMed bibliography was useful, since journal articles regarding diagnosis of *C. burnetii* were found.

References were found about several diagnostic methods used to detect *C. burnetii* in aborted goats and sheep.

Direct diagnosis of Coxiellosis in ruminants usually consists of modified Ziehl-Neelsen staining of placental or fetal sample smears (i.e., Stamp or Gimenez)**(**Rousset et al.,2004).

Serology tests such as ELISA, CF test (compliment fixation test) (Kovacova E., Kazar J., Spanelova D. 1998) and the indirect immunofluorescence assay (IFA) .(Arricau-Bouvery N.,Annie Rodolakis,2005) show either reduced sensitivity in the early stages of infection (since are based on the detection of antibodies to the pathogen) or cross-reactivity between other pathogens (Lukacova M,et al., 1994)

A culture can be used for the isolation of C. *burnetii,* using the simplified shell vial system. (Raoult, D., Vestris, G., Enea, M., 1990.The disadvantages of this method are either the long time required to export a response (requires up to 21 days) or the requirement of BSL3 laboratory.

Recently polymerase chain reaction (PCR) has become a useful tool for the detection of *C. burnetii* in biological samples. This technique has the ability to detect the smallest concentration of bacterial DNA and tends to establish itself as the reference method for the diagnosis of pathological organisms due to increased specificity, sensitivity, and immediacy of result**.(**Frasier et al 1990; Willems et al.,1994; Muramatsu et al ,1996; Yuasa rt al.,1996; Lorenz et al.,1998)

Phase 2: Analytical Process of Protocols:

a. Development of a questionnaire (epidemiological bulletin) that was filled in during sample collection

The questionnaire aimed to collect information on: a) the area of breeding, b) the total number of animals on farm c) the animal species which aborted d) the number of animals aborted, e) the relative age of animal, f) the month the abortion took place, g) the type of samples collected, h) biosecurity measures on the farm and any other information deemed useful and necessary to record. **(Appendix No 1)**

The questionnaire was completed in the presence of the farmer who had received knowledge for the purpose of this study and obtained the consent. The questionnaire was given a code that was unique to the animal that aborted and the region. The same code was given in the collected samples.

b. Development of a sample collection protocol for sheep and goats (placentas and embryos) that had aborted

During sampling all necessary measures were taken to avoid contamination of the sampler but also sample contamination. **(Appendix No 2)**

Phase 3: Collection of samples from abortions, placentas and embryos from sheep and goat farms which were distributed in the 5 districts of Cyprus.

This stage included sampling embryos and placentas from goats and sheep that exhibited abortions. Finally 50 samples (47 fetuses and 19 placentas) were collected randomly from sheep and goats during the breeding season from October 2012 until March 2013.These samples were analyzed alongside with the 130 samples (73 fetuses and 71 placentas) were kept in the tissue bank of the laboratory services of Cyprus and were collected the last 7 years from goats and sheep that had abortions.

Sampling was made by a person trained in sampling procedures and in this case it was made by me. During sampling all necessary measures were taken to avoid contamination of the sampler but also sample contamination.

After taking all necessary protection measures, samples were affixed in sterile plastic bags and isothermal container with ice packs (4 ° C) and were transferred to the laboratory of the Veterinary Service of Cyprus.

Samples were carried to the laboratory accompanied by the questionnaire (epidemiological form) which included useful epidemiological data for analyzing the results and was completed in each case during sampling.

Upon arrival of samples in the laboratory each sample was recorded, and coded.

Embryos after undergoing sterilization of the abdomen were opened and stomach content was taken with a sterile 10ml syringe to be stored in Cryon tubes at-80oC.

The placentas were subjected to successive washes with sterile normal saline to remove any impurities and contamination and with sterile instruments a share of the points of adhesion of the embryo (cotyledons in placentas of ruminants) was received to be kept in Cryon tubes at-80th.

Both types of samples were stored at -80 ° C until been tested with PCR.

Phase 4: Laboratory Procedures

Samples of placenta and embryos that were collected and stored under appropriate conditions, after the DNA extraction procedure, were examined with the molecular PCR method in order to detect DNA of *C. burnetii.*

At the same time, the same samples were examined using the same diagnostic PCR method, in order to exclude other frequent causative agents of abortions, such as *B. melitensis, C. fetus, C. psittaci* and Anaplasma *spp*

DNA Extraction

A small quantity of tissue was cropped from placentas and a small quantity of embryonic fluid from embryos was taken. These samples underwent a particular procedure (containing the sample boiling step and incubation with specific enzymes) needed before the isolation of bacterial DNA from tissues and fluids. In the final stage of this process the extracted DNA was stored in a sterile tube at-20c.The extraction of DNA from these samples was based on protocols used internationally. **(Appendix No 3**) (QIAGEN D Neasy Blood & Tissue Handbook,2006). In the final stage of the process the extracted DNA was stored in a sterile tube at - 20.

PCR

It included the process of PCR in involving an attempt of bacterial DNA propagation. Samples used in this stage were exported DNA of the previous stage. For this reason, specific primers (primers) in each of the pathogens under study were used. **(Appendix No 4)** (QIAGEN Taq PCR Handbook,2011).

For the detection of DNA of *C. burnetii* the primers used are:

5'-GTATCTTGAGTATGGTAGAGGGA-3'

5'-TCGGTTCCCGAAGGCACCAA-3'

Which encode the sequence of the gene of peroxide dismutase (superoxide dismutase gene) (Spyridaki I, Gikas A, Kofteridis D, Psaroulaki A, Tselentis Y,1998)

For the detection of DNA of *Brucella melitensis* the primers used are: (Tramuta C, Lacerenza D,et al 2011)

5'-TGGCTCGGTTGCCAATATCAA-3'

5-'CGCGCTTGCCTTTCAGGTCTG-3'

For the detection of DNA of *Campylobacter fetus*, the primers used are: (Tramuta C, Lacerenza D,et al 2011)

5'-TAACAGCTTAACTGTTAAACTGC-3'

5'-TCTCTTATCTCTAAGAGATTAGTT-3'

For the detection of DNA of *Chlamydophila psittaci* the primers used are: (Tramuta C, Lacerenza D,et al 2011)

5'-ACTTGGGAATAACGGTTGGAAAC-3'

5'-ACAYCTAACTTTCCTTTCCGCC-3'

For the detection of DNA of *Anaplasma spp* the primers used are: (Brouqui P. et al., 2001)

5'-GGT-ACC-YAC-AGA-AGA-AGT-CC-3'

5'-TAG-CAC-TCA-TCG-TTT-ACA-GC-3'

Upon completion of the protocols, an electrophoresis of a small amount of PCR products had taken place on agarose gels of specific in density and for specific time.

The gels were then placed in a solution that contained a specific dye and was incubated for a specified period. The "reading" of the gel was made in machine that emits ultraviolet radiation (UV light) capable of a specific wavelength so that the DNA would become visible.

Giemsa staining

For comparison and evaluation result purposes, examination of the samples was attempted with current most commonly used methods in Cyprus veterinary laboratories aiming at the detection of the pathogen, *C. burnetii* (staining techniques).

Pieces of the placenta and embryo samples were taken and permanently put over slides. Then the slides were treated with specific stains for the detection of the same pathogen. (Giemsa). **(Appendix No 5)**(Clinical Veterinary Microbiology)

Phase 5: Statistical analysis of results

All data were entered, clean and validated using a Microsoft excel package, data were then tranfered to STATA 11.0 (StataCorp LP) and analysed. Description analysis was performed, chi2 and ttest were calculated, giving a statistical signifigance of $p<0.05$ and risk factors were identified (OR with 95% confidence intervals) with a univariate analysis.

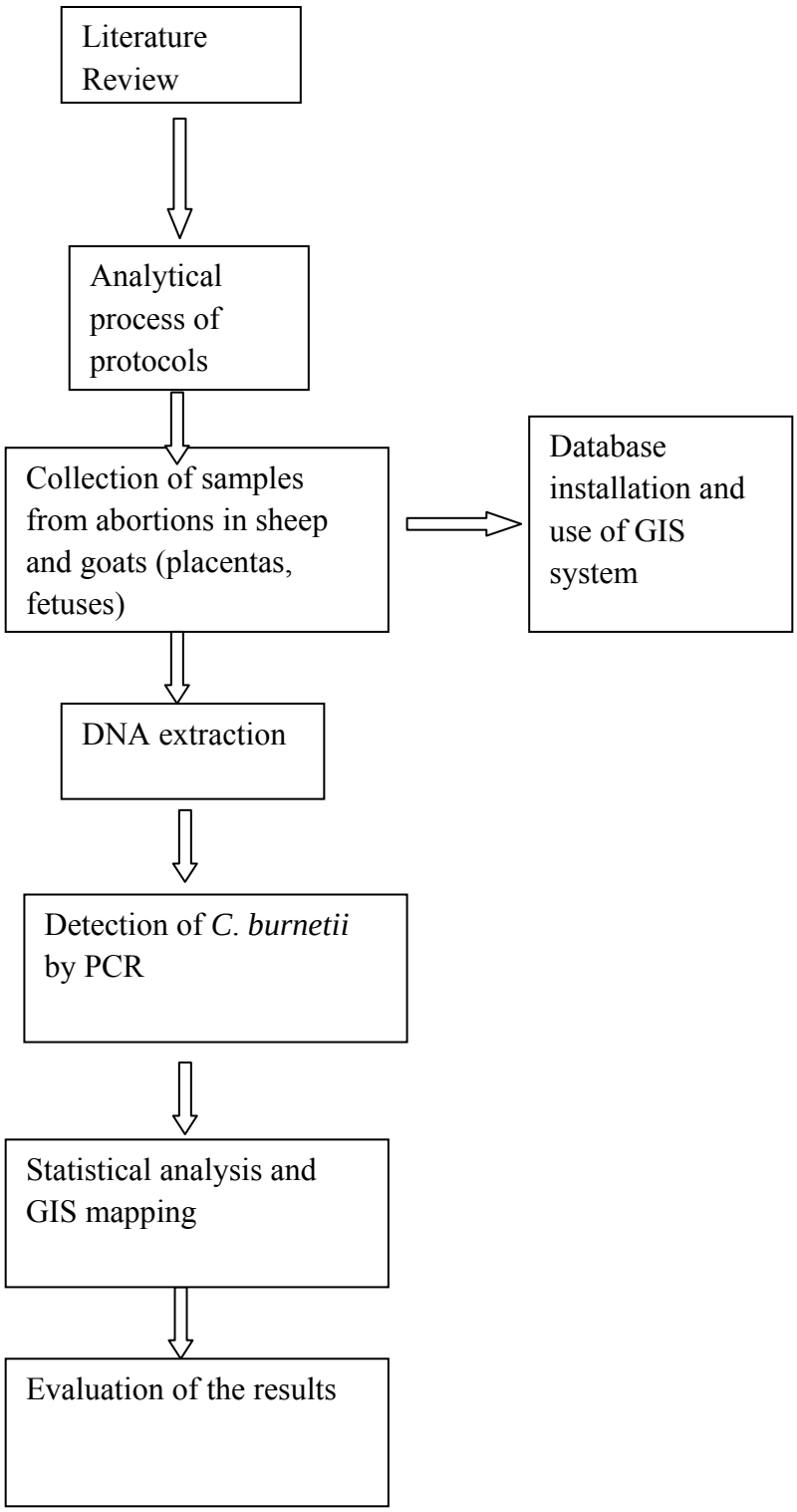
Through statistical analysis, tables and graphs were prepared to represent all the results obtained.

Phase 6: GIS mapping

A map of Cyprus was designed showing the sampling areas, regions and cases of positive samples and pathogens which were detected in the area. This resulted in the indication of specific areas where the number of abortions due to *C. burnetii* infection was increased.

An attempt was also made to identify risk factors that might have contributed to transmission of the pathogen under study.

Figure 1: Schematic representation of the Methodology



Chapter 5

Project Findings

Sampling Procedure

All samples were obtained from flocks in farms, where abortions had occurred. A total of 50 biological samples were collected prospectively from October 2012 till March 2013, while 130 samples were obtained retrospectively from the tissue banks of the vet lab services of Cyprus. In total 180 samples were included in this study from aborted sheep and goats, occurred in 2006 till 2013.

Particular samples concerned abortions that occurred between 2006 and March 2013 as shown in Table 1. These samples were obtained through the mandatory abortion disclosure system that fuctions in Cyprus by farmers themselves

Table 1

Year	No of samples
2006	6
2007	6
2008	6
2009	11
2010	18
2011	54
2012-September	29
October 2012-March 2013	50
Total	**180**

Description of the Sample:

Table 2: Number of animals tested

Type of animal	No of Animals tested	Percentage(%)
Sheep	98	54.44
Goat	82	45.6
Total	**180**	**100.0**

The sampling was conducted in 95 sheep and goat farms from all the 5 districts of free Cyprus based on abortions declared by farmers themselves. From each breeding farm a sample was taken from an animal that experienced abortion. Specifically samples were taken from 98 sheep and 82 goats, summing up to a total of 180 samples

Table 3 Number of samples tested

Type of animal	No of samples	Stomach content	Placentas
Sheep	98	64	50
Goats	82	56	40
Total	**180**	**120**	**90**

A total of 180 samples, 120 stomach content and 90 placentas were collected from 95 farms (flocks) corresponding to 180 animals.

These samples came from 82 goats and 98 sheep (double samples, placents and stomach content were taken from 14 goats and 16 sheep).

Table 4: Number of samples tested by district area

District area	No of samples tested	Percentage(%)
Pafos	15	8.33
Limassol	15	8.33
Larnaca	49	27.22
Ammochostos	19	10.56
Nicosia	82	45.56
Total	**180**	**100.00**

The samples were obtained from all five provinces of Cyprus. Analytically the following samples were received: 15 samples from Paphos (8.3%), 15 from Limassol (8.3%), 49 from Larnaca (27.3%), 19 from Ammochostos (10.56%) and 82 from Nicosia (45.6%).

Epidemiological Information

Abortions

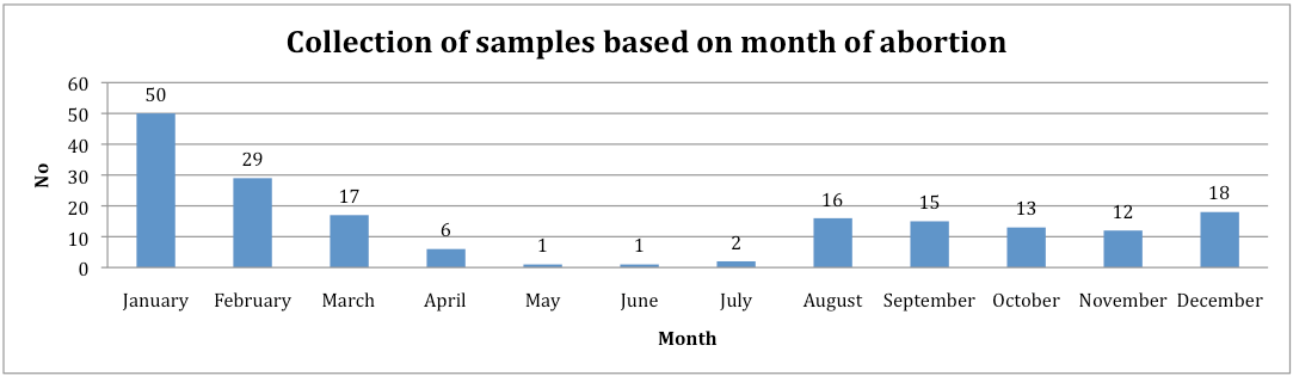
From each farm in which abortions occurred, immediately after sampling, an epidemiological questionnaire was completed with the following information concerning the farm, history of abortions in the herd, whether abortions concerned goats or sheep, month and year of abortions, area-location of the farm, number of animals in the herd, and whether the farm is adjacent to other farms. In addition, information was collected on which farms maintained dedicated birth place, which farms had isolation ward for sick animals, whether hygiene measures were properly applied by regular removal of manure and systematic disinfection of premises and materials and finally if the farm had satisfactory biosecurity measures

Table 5: Monthly distribution of the notified abortions (2006-2013)

Month	No of abortions	Percentage(%)
April	6	3.33
August	16	8.89
December	18	10.00
February	29	16.11
January	50	27,78
July	2	1.11
June	1	0.56
March	17	9.44
May	1	0.56
November	12	6.67
October	13	7.22
September	15	8.33
Total	**180**	**100.00**

The seasonal correlation of the number of abortions within the last 7 years is reflected in Table 5. It appears that most abortions were observed in January and February.

Graphic 1: Collection of samples based on month of abortion



Animals

Table 6: Number of animal aborted by age

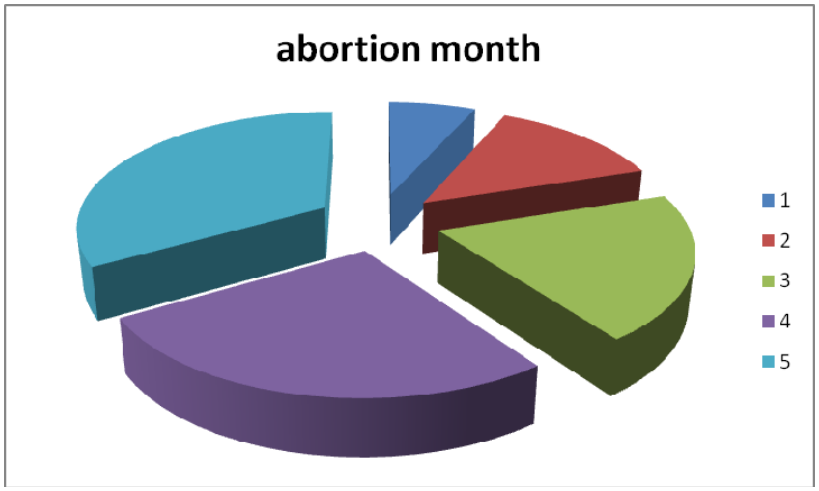
Animal age	No of animals	Percentage (%)
1	39	21.67
2	73	40.56
3	47	26.11
4	16	8.89
5	3	1.67
6	2	1.11
Total	**180**	**100.00**

The epidemiological investigation showed that animals of young age presented the highest abortion rate. Specifically 39 animals (21.67%) had aborted at one year of age, 73 animals (40.56%) at 2 years of age, 47 animals (26.11% 0 to 3 years of age, 16 animals (8.89%) at the age of 4, 3 animals (1.67%) and 2 animals (1.11%) at the age of 6.

Table 7: Number of animals by abortion month

Abortion month	No of animals	Percentage (%)
1	1	0.56
2	5	2.78
3	34	18.89
4	84	46.67
5	56	31.11
Total	**180**	**100.00**

Figure 2: Frequency of abortions by abortion month



On the contrary, an increase of abortion rate was noted in animals in advanced pregnancy stage. Specifically, one animal (0.56%) had abortion in the first month of pregnancy, 5 animals (2.78%) had an abortion in the second month, 34 animals (18.89%) aborted in the third month, 84 animals (46.67%) aborted at 4 month and 56 animals (31.11%) in the fifth month of pregnancy

Farms

Table 8: Number of farms tested by farm size

Farm size	No of farms tested	Percentage (%)
1-50	8	8.4
51-199	21	22.1
>200	66	69.5
Total	**95**	**100.00**

Sampling was conducted from farms with various capacities. Specifically, samples were taken from 8 farms (8.4%) of small capacity with animals of 1-50 from 21 farms (22.1%), of moderate capacity (51 to 199 animals) and from 66 farms (69.5%) of large capacity> 200 animals

Table 9: Number of farms with dedicated birth area

Birth area	No of farms	Percentage (%)
No	82	86.3
Yes	13	13.7
Total	**95**	**100**

The number of farms that had separate birth area was 13 (13.7%) while those that did not have this facility were 82 (86.3%)

Table 10: Number of farms with isolation ward

Iso ward	No of farms	Percentage (%)
No	21	22.1
Yes	74	77.9
Total	**95**	**100**

The number of farms that had an isolation ward was 74 (77.9%) while those that did not have were 21(22.1%)

Table 11: Number of farms applied disinfections

Disinfections	No of farms	Percentage (%)
No	25	26.3
Yes	70	73.7
Total	**95**	**100**

The farms which were conducting disinfection of both spaces and equipment were more than those which did not use any disinfection methods. In total 70 farms (73.7%) were conducting systematic disinfection while 25 farms (26.3%) did not

Table 12: Number of farms with biosecurity measures

Biosecurity	No of farms	Percentage (%)
Unsatisfactory	11	11.57
Satisfactory	84	88.43
Total	**95**	**100**

Regarding biosecurity measures out of the 95 farms on which sampling was performed, 84 of them implemented satisfactory biosecurity measures, while in 11 farms the biosecurity level was not satisfactory.

Sample Preparation and analysis

Embryos after undergoing sterilization of the abdomen were opened and stomach content was taken with a sterile 10ml syringe to be stored in Cryon tubes at-80oC.

The placentas were subjected to successive washes with sterile normal saline to remove any impurities and contamination and with sterile instruments a share of the points of adhesion of the embryo (cotyledons in placentas of ruminants) was received to be kept in Cryon tubes at -80oC

Both types of samples were stored at -80 ° C until been tested with PCR.

Laboratory analysis

Conventional Methods

All samples were first tested by methods used in the routine laboratory of the Veterinary services. Specifically for Coxiella burnetii diagnosis was performed by staining teghnique Giemsa(Clinical Veterinary Microbiology), *Brucella melitensis* and *Campylobacter fetus,* by culture and for *Chlamydophila psittaci* with immunochromatographic assay.
Subsequently all samples were tested by PCR method for genotyped detection of the above pathogens.

Detection of specific DNA

DNA extraction from samples

DNA was isolated using the DNeasy Blood &Tissue Kit (QIAGEN DNeasy Blood &Tissue Handbook,2006), according to the manufacturer's instructions. Organ samples were cut into 25 mg sections mechanically disrupted and digested over night in 180 μL lysis buffer (Buffer ATL) with 20 μL proteinase K at 56°C.

Polymerase chain reaction (PCR)

A PCR assay for the detection of specific DNA for Coxiella burnetii, Chlamydia psittaci , Campylobacter fetus and Brucella melitensis was conducted in thermal cycling protocol included an initial denaturation of 95°C for 15 min, followed by 40 cycles of 94°C for 1 min, annealing temperature for 1 min and extension of 72°C for 1 min. This was followed by a final extension step at 72°C for 10 min.(QIAGEN TaqPCR Handbook,2011). For Anaplasma spp, the thermal cycling protocol included an initial denaturation of 95°C for 15 min, followed by 45 cycles of 94°C for 30 sec, annealing temperature for 1 min and extension of 53°C for 45 sec. This was followed by a final extension step at 72°C for 1min

Results

Achievement of the Objectives

Objective one: To investigate if and to what extent *C. burnetii* is responsible for abortions in sheep and goats in Cyprus.

Laboratory testing of the samples was done by PCR tecnique. The samples were obtained from 98 sheep and 82 goats that had abortions and were obtained from farms of the districts of Paphos, Limassol, Larnaca, Ammochosto and Nicosia. Overall 180 samples were checked, of which 90 involved fetuses and 120 placentas. A flock (or a farm) was considered positive if at least one animal was tested positive.

Tables 13 to 14 show the results of control over the above samples by using PCR technique, by animal species and by type of sample.

Findings

Table 13: Prevalence of *C. burnetii* by animal aborted

Animal Species Aborted	Total Number Of Animal /SamplesTested	*Coxiella burnetii* With PCR	%
Sheep	98	22	22.4
Goat	82	24	29.3
Total	**180**	**46**	**25.5**

Using the PCR method *C. burnetii* was found in 46 out of 180 samples tested.More specifically positive were 22 out of 98 sheep samples and 24 out of 82 goat samples tested.The 46 positive samples were found in 30 out of 95 farms tested. The total percentage of C. burnetii positive in goat samples (29.3%) was slightly higher than the positivity rates of the samples in sheep (22.4%).

Table 14: Prevalence of *C. burnetii* by sample

Type of Sample	Total number of Sample	*Coxiella burnetii* with PCR	%
Stomach	120	18	15.0
Placentas	90	30	33.3
Total	**210**	**48**	**22.9**

Using PCR method, *Coxiella burnetii* was found in 48 from 210 biological samples (18 stomach content and 30 placentas) corresponding to 46 animals. Two animals were found positive for more than one biological sample.

Geographical distribution of C.burnetii

Table 15

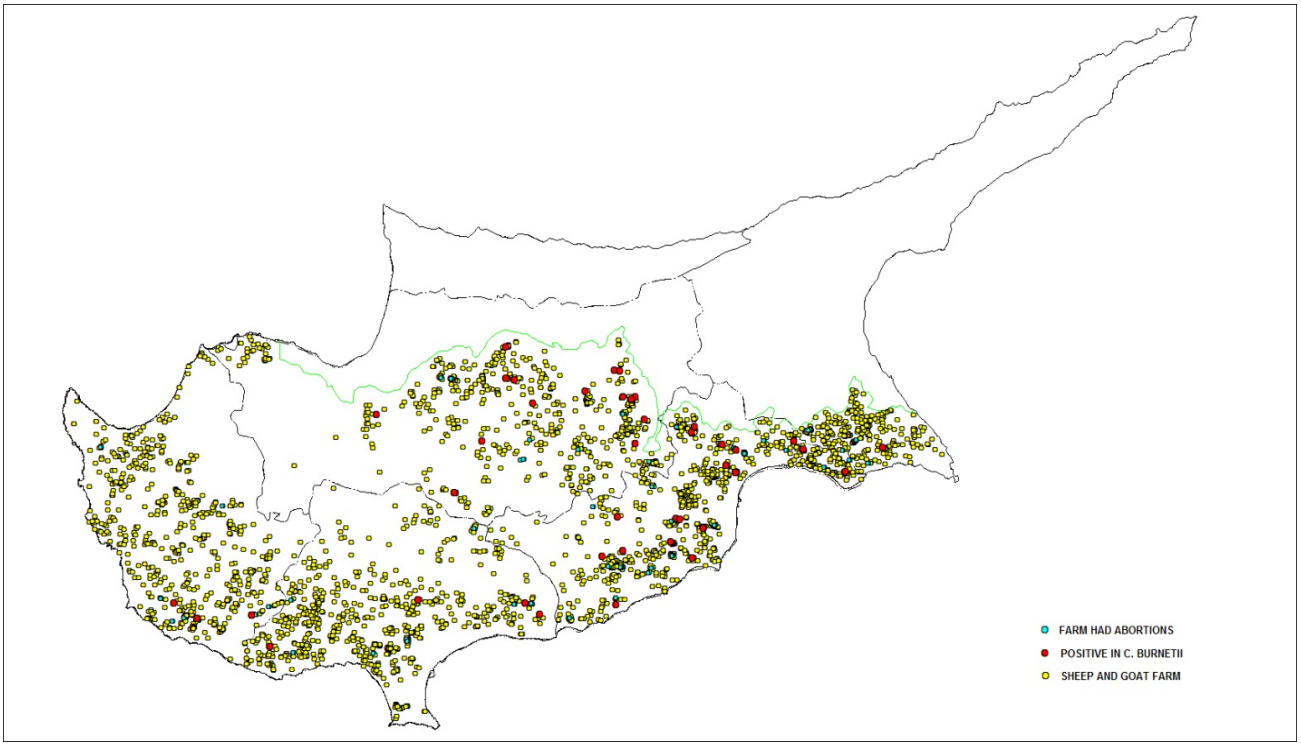
Area	Sheep	(+)	Goat	(+)	Total	Positive	(%)
Pafos	5	3	10	1	**15**	4	**26.6**
Limassol	5	0	10	3	**15**	4	**26.6**
Larnaca	31	10	18	4	**49**	14	**28.6**
Ammohostos	12	2	7	3	**19**	5	**26.3**
Nicosia	45	7	37	13	**82**	20	**24.4**
Total	98	22	82	24	**180**	46	**35.6**

Table 16: Prevalence of *C. burnetii* by farm and area

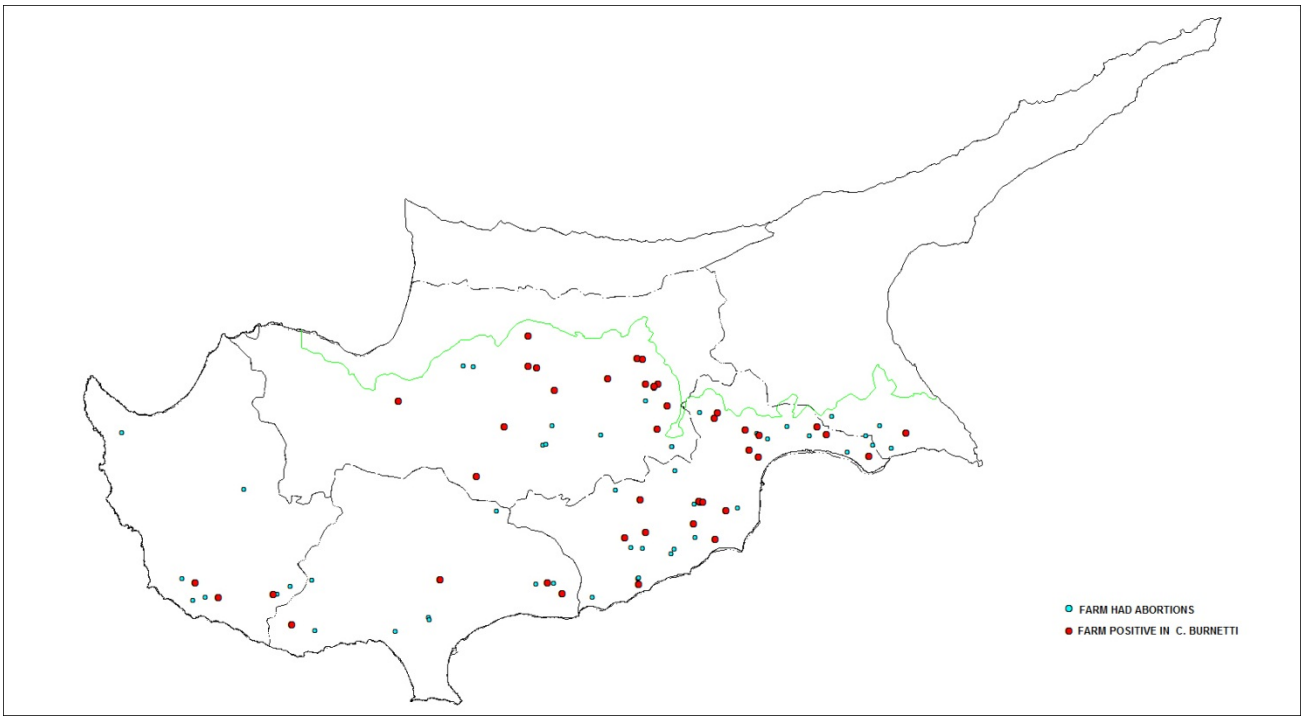
District Area	Farm Code
Pafos	Cys 6106008
Pafos	Cys 6107019
Pafos	Cys6200012
Limassol	Cys5126013
Limassol	Cys5101009
Limassol	Cys5227020
Larnaca	Cys4214009
Larnaca	Cys4110015
Larnaca	Cys4012088
Larnaca	Cys4126026
Larnaca	Cys4101027
Larnaca	Cys4309044
Larnaca	Cys4309085
Larnaca	Cys4100025
Larnaca	Cys4125006
Larnaca	Cys4215006
Larnaca	Cys4202018
Ammochostos	Cys3103015
Ammochostos	Cys41060807
Ammochostos	Cys4107011
Ammochostos	Cys4105018
Nicosia	Cys1303008
Nicosia	Cys1241011
Nicosia	Cys1013010
Nicosia	Cys1243047
Nicosia	Cys1120013
Nicosia	Cys1013010
Nicosia	Cys1213010
Nicosia	Cys1241016
Nicosia	Cys1024029
Nicosia	Cys1230004
Total	**30 Farms**

The geographical distribution but also the incidence of *C. burnetii* in sheep and goats per farm province is presented in **Maps 1 and 2**

<u>MAP 1</u>: Geographical distribution of *C. burnetii*



MAP 2: Geographical distribution of *C. burnetii*



Objective 2: To introduce PCR in the laboratories of Veterinary Services of Cyprus with the aim of detecting C. *burnetii* with greater speed and reliability, on samples taken from abortions occurring in sheep and goats in Cyprus

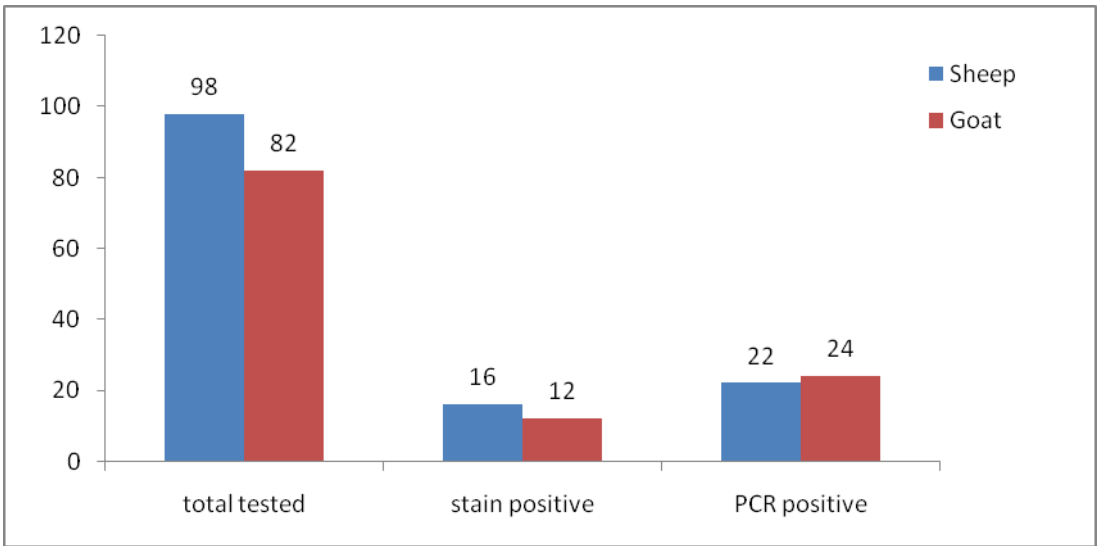
For comparison and evaluation result purposes, examination of the same 180 samples examined by PCR was also attempted with current most commonly used methods in Cyprus veterinary laboratories aiming at the detection of the pathogen, *C. burnetti* (staining techniques).

Findings

Table 17: Detection of *C.burnetii* in animals using PCR and Staining Giemsa

Animal species aborted	Total number of animal/samples tested	*Coxiella burnetii* with PCR	%	*Coxiella burnetii* with Staining technique	%
Sheep	98	22	22.4	16	16.3
Goat	82	24	29.3	12	14.6
Total	**180**	**46**	**25.5**	**28**	**15.6**

Figure 2: Detection of *C.burnetii* in animals using PCR and Staining Giemsa



Using the staining Giemsa method, *C. burnetii* was found in 28 out of 180 samples tested.More specifically positive were 16 out of 98 sheep samples and 12 out of 82 goat samples tested.The 28 positive samples were found in 41 out of 95 farms tested. The total percentage of *C. burnetii* positive in sheep samples (16.3%) was higher than the positivity rates of the samples in goats (14.6%).

Table 18: Prevalence of *C. burnetii* by sample using Staining Giemsa

Type of sample	**Total number of sample**	***Coxiella burnetii* with staining technique**	**%**
Stomach	90	9	10.0
Placentas	90	19	21.1
Total	**180**	**28**	**15.6**

Using the staining method, *Coxiella burnetii* was found in 28 from 180 biological samples (9 stomach content and 19 placentas) corresponding to 28 animals .In this case only 180 biological samples were tested instead of 210, because out of the 30 double samples (stomach contents and placenta) only placentas were tested

Table 19: Prevalence of *C. burnetii* by animal type, sample type tested and diagnostic methd used

Type of animal	Total animal tested	PCR Stomach	(+)	PCR placenta	(+)	Stain stomach	(+)	Stain placenta	(+)	Cox postive [1]
Sheep	98	64	11	50	12	48	6	50	9	33
Goat	82	55	7	40	18	42	3	40	10	30
Total	**180**	**119**	**18**	**90**	**30**	**90**	**9**	**90**	**19**	**66**

Specificity and Sensitivity of staining technique

The negative presence of *C. burnetii* in abortion samples, corroborated by both methods by 65% .Specifically 117 of the 180 tested negative in *C.burnetii* by both methods. A percentage of 6.1% (11 of 180 samples) were found positive by both methods and at a rate of 28.9% (52 out of the 180 samples) there was no identification of results between the two methods. From these results it is concluded that the two methods converge to the detection of the negative samples.

In the absence of existence reference method which would indicate the actual positive and actual negative samples; we consider PCR method as the reference method to help determine the specificity and sensitivity of the staining technique.

These results are further analyzed in table 20

Table: 20

	No of stain positive samples	**No of stain negative samples**	**N**
No of PCR positive samples	11	35	46
No of PCR negative samples	17	117	134
N	**28**	**152**	**180**

Method: staining GIEMSA (compared with PCR)	
sensitivity	23.9%
specificity	87.3%

From these it is concluded that the staining Giemsa method has relatively low sensitivity and high specificity always considering PCR as a reference method. As a gold standard method Real time PCR could be used in a future stage. At this stage due to lack of laboratory results from the Real time PCR, we suggest the simultaneous use of both methods in Cyprus veterinary laboratories for export accurate laboratory results. At a later stage it the strengthening of this study is proposed along with laboratory results using Real time PCR method

Objective 3: To identify the high risk areas of Cyprus where the number of abortions in sheep and goats due *to C. burnetii* infection is increased and recorded and identify risk factors that may have caused infection by the pathogen.

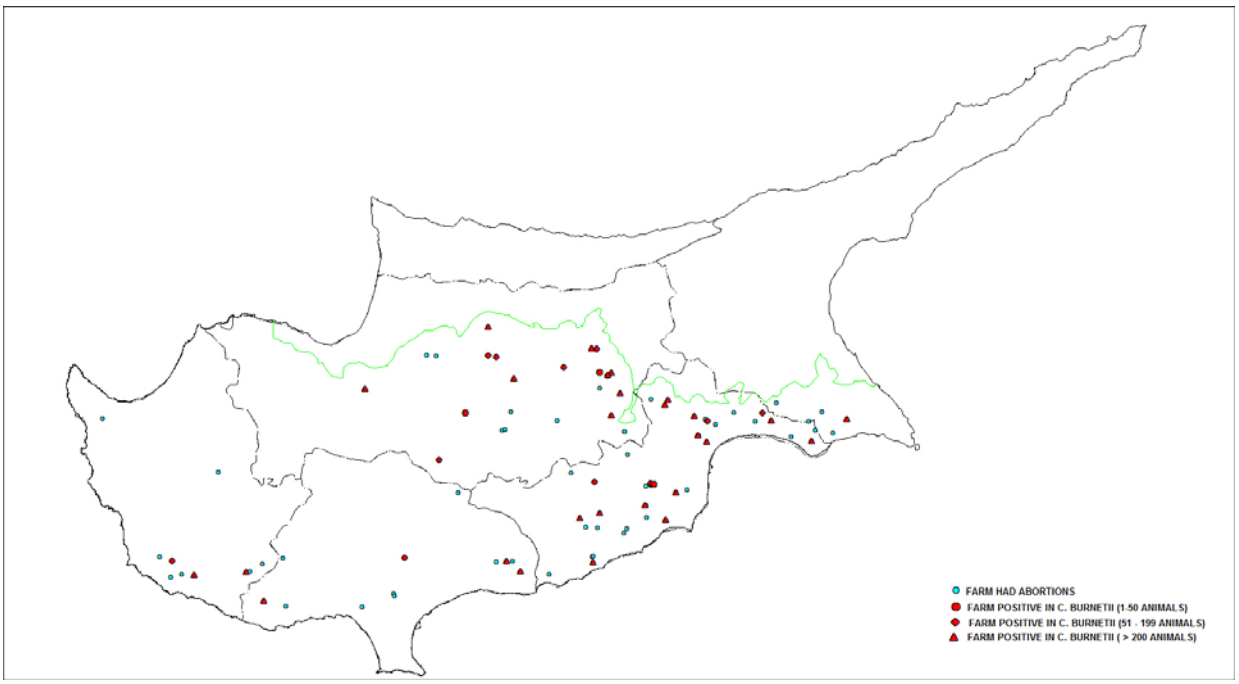
Findings

Table 21: Distribution of positive farms in *C.burnetii* by district area and by capacity

Area	Farm Size			
	1-50	51-199	>200	Total
Pafos	0	1	2	**3**
Limassol	0	1	2	**3**
Larnaca	1	1	9	**11**
Ammochostos	0	1	3	**4**
Nicosia	2	3	4	**9**
Total	**3**	**7**	**20**	**30**

Table 21 shows that from a a total of 95 farms that samples were examined for *C. burnetii,* three positive Farms were detected in Paphos, 3 in Limassol, 11 in Larnaca, 4 and 9 in Famagusta in Nicosia, 30 farms in total. Most positive Farms in *C. burnetii* are located in Larnaca and then in Nicosia. Concerning the farms positivity in pathogen in relation to the capacity, the above 20 positive farms, were of high capacity> 200 animals.

MAP 3: Positive farms in *C.burnetii* by district area and by capacity



Objective 4: To attempt to correlate incidents of abortions in sheep and goats due to *C. burnetii* infection with the risk factors which were recorded in epidemiological questionnaires

Findings

Table 22 and 23 show factors that have been recorded during the sampling of the farms. The occurrence of abortions in a farm was correlated (without the analysis to be statistical important) with the following factors: a) existence of a dedicated place for animal birth (separate birth place within the farms) b) existence of isolation ward c) disinfections have been carried out in the farm d) the existence of a nearby farm (close proximity concentration in specified livestock areas) e) the size (capacity) of the farm and f) satisfactory levels of biosecurity in the farm.

As showed by the analysis, an important risk factor was the level of biosecurity in a farm: farms with unsatisfactory biosecurity levels, were almost two times more likely (RR=1,91, CI1,01-3,61) to have abortions due to *C. brunetii* compared to those farms being recorded with satisfactory levels of biosecurity.

Table 22			Epidemiological data						Laboratory data	
A/A	Prefecture	Neighbor farms	No of animals in farm	Species aborted	Separate ward for delivery	Isolation ward	Disinfection control	Farm condition	Stomach positive by PCR /Collected	Placenta positive by PCR /Collected
C1	Pafos	Yes	>200	Goat	Yes	Yes	No	Unsatisf.	1/3	0
C2	Pafos	No	>200	Sheep	Yes	Yes	Yes	Satisf.	0	1/2
C3	Pafos	Yes	51-200	Sheep	No	No	No	Unsatisf.	1/1	1/1
C4	Limassol	Yes	>200	0	No	Yes	Yes	Satisf.	0/1	1/1
C5	Limassol	Yes	51-200	Goat	No	Yes	Yes	Unsatisf.	1/1	0
C6	Limassol	No	>200	Goat	No	Yes	Yes	Unsatisf.	1/2	0
C7	Larnaca	Yes	>200	Sheep	No	Yes	Yes	Unsatisf.	0	1/1
C8	Larnaca	No	>200	Sheep	Yes	Yes	Yes	Satisf.	1/2	1/1
C9	Larnaca	Yes	>200	Goat	No	Yes	Yes	Unsatisf.	0/1	1/3
C10	Larnaca	Yes	>200	Sheep/ goat	No	Yes	Yes	Unsatisf.	0	1/1
C11	Larnaca	Yes	>200	Sheep	No	Yes	Yes	Unsatisf.	4/4	0/1
C12	Larnaca	Yes	<50	Goat	No	Yes	Yes	Unsatisf.	0	1/1
C13	Larnaca	No	>200	Goat	No	Yes	Yes	Unsatisf.	0	1/2
C14	Larnaca	No	51-200	Sheep	No	No	Yes	Unsatisf.	1/1	0
C15	Larnaca	Yes	>200	Goat	No	Yes	Yes	Unsatisf.	0	1/1
C16	Larnaca	Yes	>200	Sheep	No	Yes	Yes	Unsatisf.	0/3	1/1
C17	Larnaca	Yes	>200	Goat	No	Yes	Yes	Unsatisf.	0	1/1
C18	Ammoch.	Yes	51-200	Sheep	No	Yes	Yes	Unsatisf.	0	1/1
C19	Ammoch.	No	>200	Sheep	No	Yes	Yes	Unsatisf.	0/3	1/4
C20	Ammoch.	Yes	>200	Goat	No	Yes	Yes	Unsatisf.	0	2/3
C21	Ammoch.	Yes	>200	Goat	No	Yes	Yes	Unsatisf.	0/2	1/1
C22	Nicosia	Yes	>200	Goat	Yes	Yes	Yes	Satisf.	0/2	1/1
C23	Nicosia	Yes	<50	Goat	No	No	No	Unsatisf.	0	1/1
C24	Nicosia	Yes	>200	Sheep/ goat	Yes	Yes	Yes	Satisf.	6/41	5/9
C25	Nicosia	No	51-200	Goat	No	No	No	Unsatisf.	1/1	0
C26	Nicosia	No	<50	Goat	No	No	No	Unsatisf.	0/1	1/1
C27	Nicosia	No	>200	Goat	No	No	No	Unsatisf.	0/1	1/1
C28	Nicosia	No	>200	Goat	Yes	Yes	Yes	Satisf.	0	2/4
C29	Nicosia	Yes	51-200	Goat	No	No	No	Unsatisf.	1/1	1/1
C30	Nicosia	Yes	51-200	Goat	No	No	No	Unsatisf.	0	1/1

Table 23: Risk of *coxiella burnetii* infection in comparison with recorded exposures.

Variable		N	%	RR	95% CI	P value
Dedicated birth place	Yes	6	46,1	1,57	0,80-3,10	0,223
	No	24	29,2	-		
Isolation ward	Yes	22	29,7	0,78	0,41-1,49	0,467
	No	8	38,1	-		
Disinfections	Yes	22	31,4	0,98	0,51-1,91	0,957
	No	8	32,0	-		
Biosecurity	Satisfactory	6	54,5	1,91	1,02-3,61	0,079
	Unsatisfactory	24	28,6	-		
Other farms in the same area	Yes	20	30,7	0,92	0,49-1,74	0,802
	No	10	33,3	-		
Farm size	<50			-		
	51-199			0,88	0,30-2,61	
	>200			0,80	0,31-2,12	
Area	Pafos			-		
	Limassol			0,69	0,19-2,48	
	Larnaca			0,91	0,35-2,34	
	Ammohostos			1	0,25-3,39	
	Nicosia			1,08	0,41-2,80	

Objective 5: To exclude other frequent causative agents of abortions, such as *Brucella melitensis, Campylobacter fetus, Chlamydia psittachi* and *Anaplasma spp*

To achieve this objective, the 180 abortion samples that were collected from aborted sheep and goats, during 2006 and Marh 2013 and were examined by PCR method for detecting *C.burnetii*, were also tested with the same method for other pathogens that are often considered a cause of abortion in sheep. Specifically, the samples were tested for *Brucella melitensis, Campylobacter fetus, Chlamydophila psittachi and Anaplasma spp*

Findings

The results of laboratory testing of the samples against the under study pathogens for each animal species but also separately for each type of sample are shown in Tables 24 and 25

Table 24: Detection of Brucella *melitensis, Campylobacter fetus, Chlamydophila psittachi* and *Anaplasma spp* in animals using PCR

Animal species aborted	Total number of animal/ sample tested	*Chlamydophila psittaci* with PCR	%	*Campylobacter fetus* with PCR	%	*Anaplasma Spp* with PCR	%	*Brucella melitensis* with PCR	%
Sheep	98	17	17.4	3	3.1	2	2.1	0	0
Goat	82	30	36.6	2	2.4	3	3.7	0	0
Total	**180**	**47**	**26.1**	**5**	**2.7**	**5**	**2.7**	**0**	**0**

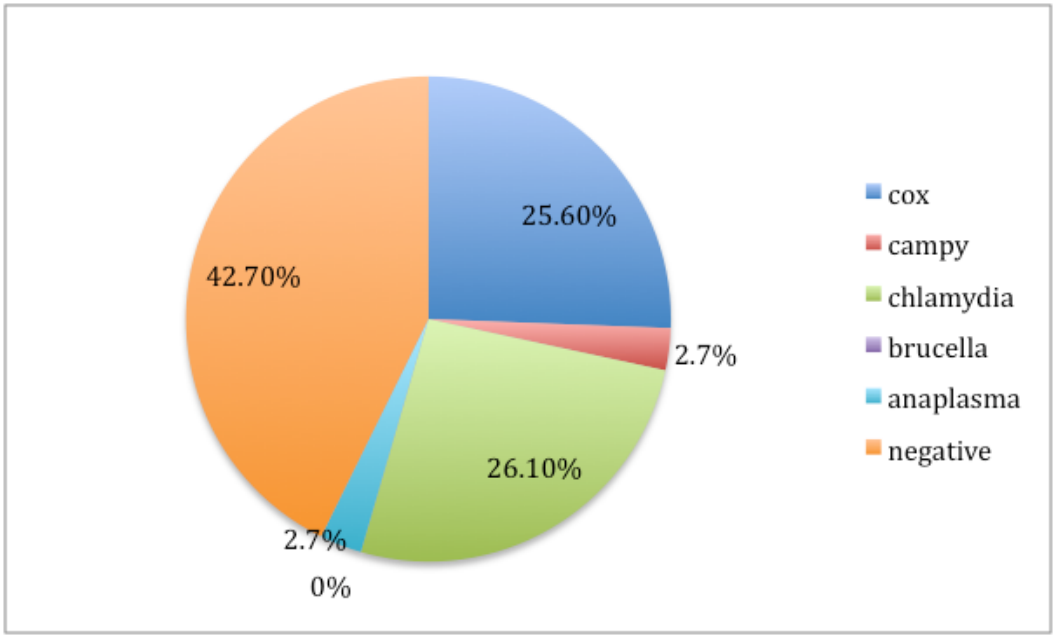
Using PCR, *C. psittachi* was found in 47 out of 180 samples tested.More specifically positive samples were 17 out of 98 sheep samples tested and 30 out of 82 goat samples tested.The 47 positive samples were found in 39 out of 95 farms tested. The total percentage of *C. psittachi* positive in goats samples (36.6%) was higher than the positivity rates of the samples in sheep (17.4 %).

Using the same diagnostic method, *Campylobacter fetus* was found in 5 out of 180 samples tested.More specifically positive samples were 3 out of 98 sheep samples tested and 2 out of 82 goat samples tested.The 5 positive samples were found in 4 out of 95 farms tested.

Concerning *Anaplasma spp*, it was found in 5 out of 180 samples tested.More specifically positive samples were 2 out of 98 sheep samples tested and 3 out of 82 goat samples tested.The 5 positive samples were found in 4 out of 95 farms tested.

The total prevalence of *Chlamydophila psittachi* in the samples is significantly higher than the other pathogens and indeed the same as that of *C. burnetii*. Prevalence rates in samples of pathogenic *Campylobacter fetus, and Anaplasma spp* are low.

Figure 3: Prevalence of 5 pathogens in among the 180 aborted animals in five districts in Cyprus



Especially impressive is the zero positivity rate of *Brucella melitensis* observed during the examination of samples. This result confirms the effectiveness of National brucellosis control programme that had been applied in 2002 in Cyprus. That year, there was a nationwide epidemic of Brucellosis and a National brucellosis control programme had been applied. In low level contamination cases, we followed the method "test and slaughter", conducting blood sampling from all the animals of the affected unit every month in order to eliminate the positive animals with seizure and slaughter until the unit was freed from the disease. The control programme of brucellosis was completed in 2006 and Cyprus was considered to free from brucellosis since today

Table 25: PCR positive results obtained from stomach of fetus and placenta of animal aborted by pathogents

Type of sample tested	Total number of sample tested	*Chlamydophila Psittaci* with PCR	%	*Campylobacter fetus* with PCR	%	*Anaplasma Spp* with PCR	%	*Brucella melitensis* with PCR	%
Stomach	120	19	15.6	3	2.5	2	1.6	0	0
Placenta	90	34	37.7	3	3.3	3	3.3	0	0
Total	**210**	**53**	**25.3**	**6**	**2.9**	**5**	**2.4**	**0**	**0**

Using PCR method, *Chlamydophila psittachi* was found in 53 out of 210 biological samples tested (19 stomach content and 34 placentas) corresponding to 47 animals. Six animals were found positive for more than one biological sample. The frequency of detected specific *Coxiella burnetii* DNA**,** both in placenta and stomach content samples was 12.7 % of the tested animals

Campylobacter fetus was found in 6 out of 210 biological samples tested (3 stomach content and 3 placentas) corresponding to 5 animals. One animal was found positive for more than one biological sample. The frequency of detected specific *Coxiella burnetii* DNA**,** both in placenta and stomach content samples was 20% of the tested animals

Anaplasma spp was found in 5 out of 210 biological samples tested (2 stomach content and 3 placentas) corresponding to 5 animals.

Simultaneous Infections

Out of the 46 samples that tested positive in *C. burnetii* using PCR method, 18 of them, corresponding to 16 flocks were found simultaneously positive to other pathogens. Specifically, 17 of them were positive for *Chlamydophila psittaci*, while one of them was positive for *Anaplasma spp.* None of these samples were found simultaneously positive at all three pathogens. In addition, no sample that was positive in *C.burnetii* was simultaneously positive in *Campylobacter fetus*.

From the above 18 samples, which were positive in more than one pathogen, 4 were stomach content (corresponding to 4 farms) and 15 were placentas (corresponding to 14 farms).

<u>Objective 6:</u> To correlate and compare the PCR results with the results of the diagnostic methods that are used since today for *Brucella melitensis, Campylobacter fetus* and *Chlamydophila psittachi* in the Cyprus Veterinary laboratory

These 180 samples of the specific project were examined by Veterinary Officers in charge of the labs in the frames of the relevant veterinary legislation and various animal diseases surveillance program for pathogenic *Brucella melitensis, Campylobacter fetus* and *Chlamydophila psittachi* using other diagnostic methods as provided by the Protocol of the laboratory of my services. Specifically pathogens *Brucella melitensis, Campylobacter fetus,* were examined by culture and *Chlamydophila psittachi* using immunochromatographic assay. These results were compared with results from the examination of those specific pathogens by PCR method for comparison purposes of the methods.

<u>Findings</u>

Table 27:

Animal species aborted	Total number of samples tested	*Chlamydophila Psittaci* Method: Immunochromatographic Assay	%	*Campylobacter fetus* Method:Culture	%	*Brucella melitensis* Method: Culture	%
Sheep	98	17	17.4	1	1.0	0	0
Goat	82	32	39.0		0	0	0
Total	**180**	**49**	**27.2**	**1**	**1.0**	**0**	**0**

Using Immunochromatographic assay(Rapid Test), *C. psittachi* was found in 49 out of 180 samples tested.More specifically positive samples were 17 out of 98 sheep samples tested and 32 out of 82 goat samples tested. The 49 positive samples were found in 36 out of 95 farms tested. The total percentage of *C. psittachi* positive in goats samples (39.0%) was higher than the positivity rates of the samples in sheep (17.4%). Using culture, *Campylobacter fetus* was found in one sheep sample (originated from one sheep) out of 180 samples tested.

Discussion

This study was conducted within the framework of mandatory abortion disclosure system that functions in Cyprus by farmers themselves and covered 95 farms distributed relatively smoothly on 5 provinces of Cyprus. 180 animals, which presented abortions were, checked (98 sheep and 82 goats). Abortion biological samples were obtained from the above animals as follows: 90 samples of stomach contents, 90 samples of placenta. From 30 animals duplicate samples of stomach contents and placenta were obtained.

Epidemiological data regarding the distribution of abortions due to coxiellosis in Cyprus are scarce and are based solely on materials submitted for routine laboratory examination in the Cyprus Veterinary laboratories.Results obtained from such studies are frequently biased, since the materials are not randomly sampled.
These samples were analyzed both by conventional methods (Giemsa stain) used routinely by the Veterinary Services across Cyprus and by direct identification of the pathogen by PCR, applied as a diagnotic tool for the first time to the laboratories services
Conventional methods commonly used in routine do not seem to have high sensitivity or specificity, thus they create false positive or negative results.
In addition to the above weaknesses, results showed that the use of the new PCR technique to detect the *C.burnetii* supplements the conventional Giemsa stain technique already used and improves the reliability of the results. In this first stage of my research I do not recommend the exclusive use of PCR as the only reliable method of detecting the *C.burnetii.* Further research is needed to demonstrate this. Furthermore, the future research can be extended to the use of Real time PCR method.
Similar contribution seems to have the PCR technique over conventional methods for detection of the rest of pathogens discussed in this study.

The present study, aimed at estimating the prevalence of *C. burnetii* in sheep and goat flocks with a history of abortions during 2006-2013 in Cyprus.
We found that 46 (25,5%) out of 180 samples were positive in *C.burnetii*.These correspond to 30 (31.6%) positive flocks out of 95 farms tested. These results indicated a significant involvement of *C. burnetti* in abortions in sheep and goats observed in Cyprus

Similar studies were done in several countries in order to detect *C.burnetii* DNA in abortion materials in sheep and goats that had previously aborted. In France PCR analysis of vaginal mucus of goats aborted, showed that 70% of the aborting goats were positive in *C.burnetii*.The prevalence rate of the pathogen into vaginal mucus of the aborted goats was 44%. (Rousset E., M. Berri, et al.2009). In Sardinia PCR analysis was performed in 399 abortion samples from sheep and goats. Out of a total of 366 ovine aborted samples, *C.burnetii* was detected in 10.9% of fetuses and 13.1% of placentae.Out of a total of 31 caprine aborted samples *C. burnetii* was found in 12.5% of placentae.(Masala G. ,Rosaura P.et al.2007) . In another study (Mustapha B. et al.2009), PCR was performed in 67 abortion samples taken from ruminant's flocks that had abortions. From those, 33 vaginal samples and one placenta were found positive in *C.burnetii.* However, due to numerous parameters such as differences in study design and inclusion criteria (e.g. high abortion rates), flock size and management, prevalence of other abortifacient agents (e.g. *Brucellae, Salmonellae, Toxoplasma, Chlamydia, Campylobacter*) it is virtually impossible to compare the present study's prevalence findings with the afore mentioned studies.

Through the Data Information of the laboratories I found out that in 2011 a *C.burnetii* epidemic was diagnosed in sheep and goats in a particular farm in Nicosia district. In this specific farm we observed positive animals at various sampling times, indicating Coxiella circulation within the herd.. In 2011 the specific farm, experienced an increased number of abortions and out of the 24 samples taken from 24 animals that experienced abortions and were examined by PCR method, 8 of them were found positive in *C.burnetii.* At that time taken heightened hygiene measures were taken in the specific farm, through systematic disinfection of premises and materials and administration of tetracyclines in animals that had abortions. Within 2012 abortions continued with a downward tendency, until March 2013. Out of the 14 samples taken in 2012 only one was positive and from the 6 samples taken in 2013, two were positive in *C.burnetii.*

Chapter 6

Conclusions and Recommendations

The geographical spread of *C. burnetii* indicates that the pathogen is present throughout the island. This makes sense, since previous serological studies that have been performed in 1996 and 2006, had shown that the pathogen is endemic in the island. The highest percentage of positive samples was found in Larnaca (28.6%), followed by Nicosia (24.4%) .

The frequency of detecting *C. burnetii* in a biological differed significantly between stomach content or placenta samples, placentas were more reliable. Of the ten duplicate samples (stomach content and placenta) positive in *C. burnetii*, 8 placentas were positive and stomach fluid negative while no sample showed a negative placenta and a positive stomach liquid.

It seems that abortions in sheep and goats due to *C. burnetii* had an increased frequency in goats even in young animals.

From the analysis of all possible risk factors for the transmission of the disease four major risk factors had occurred: Unsatisfactory biosecurity measures, the high capacity of the farm, the close proximity of farms and their concentration in specified livestock areas and the separate birth place within the farms. Most abortions due to the *C. burnetii* occurred in high capacity farms, with more than 200 animals and farms that are placed in developed ranching regions where the distance between the farmsis very small and often there is immediate vicinity of farms. Bearing into account the spread of the pathogen through birth fluids, placentas and embryos, the very small percentage of farms which feature separate birth places, seem to favor the transmission of *C. burnetii* in Cyprus

Through a detailed report I will inform my services for the results and via my personal professional suggestions an efficient mechanism for effective prevention and control of coxiellosis in sheep and goats will be activated from the Veterinary Services of Cyprus in order to protect human and animal health respectively. Hygiene measures and precautions shall be applied to livestock herds and human level, to reduce the transmission of the pathogen.

Veterinary Services of Cyprus will organise educational seminars in order το inform and educate farmers and subordinate personnel of the veterinary services about the management of the infected with *C.burnetti* animals and the abortion materials.

Written guidelines for hygiene measures and precautions shall be applied to farmers to reduce the transmission of disease in animals and humans. Since birth is critical for transmission of *C. burnetii* in infected herds, animal births should take place only in a specified location, which should be disinfected as well as every tool used during labour. Antibiotics, such as tetracycline can be used in the infected animal in order to minimize the shedding of *C.burnetii* and the incidents of abortions.

The findings of my research will help my service to change its policy and strategy followed so far, regarding the diagnosis, treatment and prevention of *C.burnetii*

The knowledge of the true prevalence of the infection can lead to a better risk assessment, which could impact on to the decision to implement or not a control program. This information could also help policy makers and veterinarians to determine (I) at which level (i.e. herd, local, regional level) this control program has to be implemented and (II) the nature of the control actions.

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Appendix No 1

REPUBLIC OF CYPRUS

MINISTRY OF AGRICULTURE

NATURAL RESOURCES AND ENVIRONMENT

ABORTIONS EPIDEMIOLOGICAL FORM

1. Name of breeder: ..... Tel: .....

Full Postal Address: .....

2. Location of the farm:

	Pafos	Limassol	Larnaca	Ammoxwstos	Nicosia
Location	1	2	3	4	5

3. Other sheep and goats farms located in the same area:

Yes	No
1	2

4. Total number of animals in the farm:

1	Small capacity	1-50
2	Medium capacity	51-199
3	High capacity	> 200

5. Number of animals aborted:

	Species	Number
1.	Sheep	
2.	Goat	

6. Age of animal aborted:

1 yr	2 yrs	3 yrs	4 yrs	5 yrs	6 yrs	7 yrs	8 yrs	9 yrs	10 yrs

7. Abortion Month:

1st	2nd	3rd	4th	5th

8. Number and Type of Sample (s):

	Type of abortion material	Number
1	Placenta	
2	Fetus	

9. Nutrition and diet changes

……

……

……

……

10. Does the farm have specific area for animal births?

Yes	No
1	2

11. Does the farm have an isolation ward for diseased animals?

Yes	No
1	2

12. Disinfections are carried out at the farm?

Yes	No
1	2

13. Biosecurity of Farm

Satisfactory	Unsatisfactory
1	2

Comments:

……..…

……..……

……

…….

Appendix No 2

SAMPLING PROTOCOL OF ABORTIONS IN SHEEP AND GOATS

1. Purpose of Sampling

Detection of *C. burnetti* and/or other pathogenic causes of abortion with the PCR method.

2. Samplers

Staff trained in sampling procedures.

3. Basic principles

All appropriate measures must be taken to prevent contamination of the samples and the sampler including:

- Protective apron, disposable coveralls
- Disposable face mask
- Boots
- Gloves
- Disinfectant

4. Sampling devices

- Sterile forceps
- Sterile scissors
- Sterile containers

5. Sampling materials

- The entire fetus
- Placenta

6. Containers for sample collection

Each sample must be placed in sterile, durable container that closes tightly, and provide complete protection from infection, leaks that may occur during transport. I will use polyethylene bags.

7. Epidemiological bulletin sampling

The sampler after sampling should fill in, all the required information in the epidemiological form of sampling for each sample taken, and the original form must accompany the sample to the laboratory.

8. Transport of samples

The samples will be placed in cooling box (insulated transport containers) and send to the laboratory of the Veterinary Services as soon as possible. The temperature of the samples should not exceed 4 ° C.

Appendix No 3

DNA EXTRACTION PROTOCOL

Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol)

This protocol is designed for purification of total DNA from animal tissues

Important points before starting

- If using the Dneasy Blood & Tissue Kit for the first time, read "Important notes" page 15.
- For fixed tissues, refer to the pretreatment protocols "Pretreatment for Paraffin-Embedded Tissue" page, 41, and "Pretreatment for Formalin-Fixed Tissue", page 43.
- All centrifugation steps are carried out at room temperature (15-25C) in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5-10 s.
- Optional> RNase A may be used to digest RNA during the procedure. RNase A is not provided in the Dneasy Blood & Tissue Kit (see "Copurification of RNA", page 19).

Things to do before starting

- Buffer ATLS and Buffer Al may form precipitates upon storage. If necessary, warm to 56C until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96-100%) as indicated on the bottle to obtain a working solution.
- Preheat a thermomixer, shaking water bath, or rocking platform to 56C for use in step 2.
- If using frozen tissue, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.

Procedure

1. **Cut up to 25mg tissue into small pieces, and place in a 1.5 ml microcentrifuge tube. Add 180 ml Buffer ATL.**

   Ensure that the correct amount of starting material is used (see "starting amounts of samples", page 15).

   We strongly recommend to cut the tissue into small pieces to enable more efficient lysis. If desired, lysis time can be reduced by grinding the sample in liquid nitrogen* before addition of Buffer ATL and proteinase K. Alternatively, tissue samples can be effectively distrupted before proteinase K digestion using a rotor-stator homogenizer, such as the QIAGEN TissueLyser (see page 56 for ordering information). A supplementary protocol for simultaneous disruption of up to 48 tissue samples using the TissueLyser can be obtained by contacting QIAGEN Technical Services (see back cover).

2. **Add 20ml proteinase K. Mix thoroughly by vortexing, and incubate at 56C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform.**

   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1-3 h. If it is ore convenient, samples can by lysed overnight; this will not affect them adversely.

   Afterincubtion the lysate may appear viscous, but should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, see the "Troubleshooting Guide", page 47 for recommendations.

   Optional: If RNA-free genomic DNA is required, add 4ml RNase A (100 mg/ml, mix by vortexing, and incubate for 2 min at room temperature before continuing with step 3.

   Transcriptionally active tissues contain high levels of RNA, which will copurify with genomic DNA. For tissues that contain low levels of RNA, or if residual RNA is not a concern, RNase A digestion is not necessary.

3. **Vortex for 15 s. Add 200 ml Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 ml ethanol (96-100%), and mix again thoroughly by vortexing.**

   It is essential that the sample. Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

   A while precipitate may form on addition of Buffer AL and ethanol. This precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfe with the DNeasy procedure. Some tissue types may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case rigorously shaling or vortexing the preparation is recommended.

4. **Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifue at..6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.***

5. **Place the Dneasy Mini spin column in a new 2 ml collection tube (provided), add 500 ml Buffer AW1, and centrifuge for 1 min at >_6000 x g (8000rpm). Discard flow-through and collection tube.***

6. **Place the Dneasy Mini spin column in a new 2 ml collection tube (provided), add 500 ml Buffer AW2, and centrifuge for 3 min at 20,000 x a (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.**

   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

   Following the centrifugation step, remove the Dneasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carrover of ethanol occurs, empty the collection tube, and then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

7. **Place the Dneasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 ml Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min at >_6000 xx g (8000 rpm) to elute.**

   Elution with 100 ml (instead of 200 ml) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

8. **Recommended: For maximum DNA yield, repeat elution once as described in step 7**

   This step leads to increased overall DNA yield.

   A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

   Note: Do not elute more than 200 ml into a 1.5 microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

   *Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 8 for safety information.

Appendix No 4

GENOMIC PROPAGATION (PCR) PROTOCOL

Taq DNA Polymerase and Taq PCR Core Kit

Notes before starting

- Taq DNA Polymerase and the Taq PCR Core Kit are provided with Q-Solution, which facilitates amplification of templates that have a high degree of secondary structure or that are GC rich by modifying the melting behavior of DNA. When using Q-Solution for the first time for a particular primer-template pair, always perform parallel reactions with and without Q-Solution.
- Tag DNA Polymerase and Taq PCR Core Kits are provided with CoralLoad PCR Buffer, which contains a gel-loading reagent and gel-tracking dyes.
- The PCR Buffer and CorlLoad PCR Buffer provide a final concentration of 1.5 mM MgCI2 in the final reaction mix which will give satisfactory results in most cases. However, in some cases, reactions may be improved by increasing the final Mg2+ concentration. If a higher Mg2+ concentration is required, add the appropriate volume of 25mM Mgi2 to the reaction mix as described in the Taq PCR Handbook.
- High-quality, PCR-grade dNTP Mix (10 mM) is available separately from QIAGEN (cat. No. 201900) if needed.
- It is recommended that the PCR tubes be kept on ice until they are placed in the thermal cycler.
- A No Template Control (NTC) should always be included.

1. **Thaw 10x CoralLoad P~R Buffer or 10x PCR Buffer dNTP mix, primer solutions, Q-Solution (if required), and 25 mM MgCI2 (if required) at room temperature or on ice. Keep on ice after complete thawing and mix thoroughly before use to avoid localized differences in salt concentration.**
2. **Prepare a reaction mix according to Table 1. The reaction mix typically contains all the components needed for PR except the template DNA. Prepare**

a volume of reaction mix 10% greater than that required for the total number of PCR assays to be performed.

Table 1. Reaction setup using Taq DNA Polymerase

Component	Volume/reaction	Final concentration
Reaction Mix		
10x PCR Buffer* or	10ml	1x
Optional: 10x CoralLoad PCR Buffer*		
dNTP mix (10 mM of each)	2ml	200mM of each dNTP
Primer A	Variable	0.1-0.5mM
Taq DNA Polymerase	0.5 ml	2.5 units/reaction
RNAse free water	Variable	-
Optional: 5x Q-Solution **	20ml	1x
Template DNA	Variable	≤1 mg / reaction
(added at step 4)		
Total reaction volume	**100 ml ***	

*Contains 15 mM MgCI2

**For templates with GC-rich regions or complex secondary structure

***If using different reaction volumes, adjust the amount of each component accordingly.

3. **Mix the reaction mix gently but thoroughly, for example by pipetting up and down a few times. Dispense appropriate volumes into PCR tubes.**

4. **Add template DNA (_<1mg/reaction) to the individual PCR tubes containing the reaction mix. For RT-PCR, add an aliquot from the reverse trascriptase reaction. The volume added should not exceed 10% of the final PR volume.**

5. **Program the thermal cycler according to the manufacturer's instructions. A typical PCR cycling program is outlined in Table 2. A typical PCR cycling program is outlined in Table 2.**

Table 2. Optimized cycling conditions for Taq DNA Polymerase

Step	Time	Temperature	Comment
Initial denaturation	3 min	94C	
3-step cycling:			
Denaturation	0.5-1min	94C	
Annealing	0.5-1min	50-68C	Approximately 5C below T of primers
Extension	1 min	72C	For PCR products longer than I kb, use an extension time of approximately 1 min per kb DNA.
Number of cycles	25-35		
Final extension	10 min	72C	

6. **For a simplified hot start, proceed as described in step 6a.Otherwise, place the PCR tubes in the thermal cycler and start the cycling program.**

6a. Simplified hot start: Start the PCR program. Once the thermal cycler has reached 94C, place the PCR tubes in the thermal cycler in many cases this simplified hot start improves the specificity of the PCR. For highly and convenient hot-start PCR, use HotStar Taq Plus DNA Polymerase.

Note: After amplification, samples can be stored overnight at 2-8C, or at -20 C for longer storage.

7. **When using CoralLoad PCR Buffer, the PCR products can be directly loaded onto an agarose gel without prior addition of a PCRloading buffer and gel-tracking dyes. Refer to Table 3 to identify the gel tracking dyes present in CorlLoad PCR Buffer according to migration distance in different percentage agarose gels.**

Table 3. Migration distance of gel tracking dyes in CoralLoad PCR Buffer

%TAE (TBE) agarose gel	Red Dye	Orange Dye
0.8	500 (270)bp	80 (<10)bp
1.0	300 (220)bp	40 (<10)bp
1.5	250(120)bp	20 (<10)bp
2.0	100(110)bp	<10(<10)bp
3.0	50(100)bp	<10(<10)bp

Appendix No 5

GIEMSA PROTOCOL (Clinical Veterinary Microbiology)

A thin smear is taken from the biological sample.

The dried smear is first fixed in absolute methyl alcohol (Analar) for 3 minutes.

The fixated smear is stained with I part of Giemsa stain together with 9 parts buffer for 60 minutes.

The stained smear is washed with the buffer, drained and air- dried.