Sudan University of Science and Technology

College of Graduate Studies

Studies on Q Fever in Farm Animals in Kingdom of Saudi Arabia

دراسات عن **الحمى المجهولة** في حيوانات المزرعة في المملكة العربية السعودية

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DEDICATION

To the souls of my parents, to my family and to my brothers and sisters, and to the greatest man; my co-supervisor Professor Mansour Faris whom I will never forget what he did for me. To all I dedicate this work.

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ABSTRACT

The serological prevalence of *Coxiella burnetii* in domestic livestock in Saudi Arabia was studied using two serological tests: indirect enzyme linked Immunosorbent assay (ELISA) as the main test and indirect immunofluorescence assay (IFA) as a confirmatory test and for comparison with ELISA.

A total of 1970 farm animals of both sexes were tested serologically to determine the prevalence of *C. burnetii* specific IgG antibodies using indirect ELISA. The samples were collected from 489 camel, 428 cattle, 630 sheep and 423 goats. The animals were broadly divided into young and adult animals. All of them were clinically normal when sampled and none of the adult females was pregnant while some were lactating.

A total of 605 animals had anti-*C. burnetii* IgG antibodies in their sera, giving an overall serological prevalence of Q fever of 30.71% with a mean ELISA titre (S/P ratio or O.D.%) of 103.03%. These results indicate that *C. burnetii* is common in all species a of farm animals in Saudi Arabia. Camels showed the highest proportion of Q fever (*C. burnetii*) positive sera among all the species tested, with an overall prevalence of 51.53%. The second highest serological prevalence was recorded in goats (34.04%), followed by cattle (30.61%) and the least in sheep (12.38%). In all species, the serological prevalence of anti-*C. burnetii* antibodies was significantly higher in adult compared to young animals ($p<0.0001$). Females animals tended to be more commonly affected than males; however, statistical analysis revealed non-significant inter-sex difference ($p= 0.5847$). Antibodies against *C. burnetii* in domestic livestock were also investigated using ELISA assay in 285 defatted milk samples obtained from 48 she-camels, 90 cows, 60 ewes and 87 does. Milk samples from 30 camels (62.5%), 30 cows (33.3%), 24 goats (27.6%) and

3 ewes (5%) were positive for anti-*C. burnetii* antibodies. Serum samples from the same animals were simultaneously tested by ELISA. Of these, 32 camels (66.66%), 38 cows (42.2), 13 goats (14.9%) and 4 ewes (6.67%) were positive for anti-*C. burnetii* antibodies. Statistical analysis show a significant correlation between ELISA results in milk and serum. Serum samples from a total of 307 animals, comprising 92 camels, 72 cows, 72 sheep and 71 goats, were also subjected simultaneously to indirect immuno-fluorescence (IFA) and ELISA assays. A statistically significant correlation was found between the serological prevalence of Q fever as determined by these two assays. Using ELISA as a reference serological test, statistical analysis showed that both the sensitivity and specificity of IFA assays were good, indicating that either ELISA or IFA can be used for screening Q fever in farm animals or as confirmatory tests to one another. The shedding of *C. burnetii* by serologically positive animals was investigated by the polymerase chain reaction (PCR), using primers that amplify the repetitive transposon-like region of *C. burnetii*. The study was conducted on 82 whole blood, 72 milk, 29 faecal and 21 urine samples collected from camels. In addition, 29 milk samples and 7 whole blood samples from cattle, 38 whole blood, 29 milk and 20 faecal samples from goats and 22 blood samples from sheep were available for .PCR analysis

Out of a total of 149 whole blood samples collected from these different animal species, 13 samples (15.85%) from camels and 2 samples (5.6%) from goats showed positive amplification for *C. burnetii* DNA while all 22 sheep and 7 bovine blood samples were negative. Out of 144 milk samples collected from camels, cattle and goats, 5 samples (6.49%) from camels, 11 samples (28.94%) from cows and 0 samples from goats were positive for *C. burnetii* DNA. In addition, faecal samples collected from 29 camels and 20 goats revealed positive PCR products from 8 (27.59%)

and 12 (60%) samples, respectively. *C. burnetii* DNA was also demonstrated in 5 (23.81%) out of the 21 urine samples collected from camels. All sampled subjected to PCR analysis were from serologically positive animals with the exception of urine samples which were collected from slaughtered camels that were not serologically tested for anti-*C. burnetii* antibodies. Serum samples from known Q fever-positive and known Q-fever negative animals were used to study the possible effects of Q fever on various biochemical and electrolyte parameters. A total of 281 serum samples were collected from camels, sheep, goats and cattle. In all species, no significant differences were found between Q-fever positive and Q-fever negative animals. However, a few intra-specific differences existed within each species. The effect of Q fever was also investigated in the levels of anti-oxidant enzymes, namely thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GLUTH). TBARS level was determined in 239 known Q fever-positive and Q fever-negative animals while GLUTH level was determined in 188 known Q fever-positive and Q fever-negative animals. No significant differences in TBARS levels were found between Q fever-positive and Q fever negative animals in samples collected from each of goats, camels and sheep. However, the GLUTH level was found to be significantly reduced ($p<0.002$) in Q fever-positive camels as compared to Q fever negative camels. This enzyme is found in the cytoplasm of almost all mammalian cells, and a reduction in its activity could indicate some degree of cellular damage. However, further studies are needed to verify .this aspect

This study constitutes the first record of *C. burnetii* in cattle, sheep and goats in Saudi Arabia and the second, and more detailed, study on camels .in the country

ABSTRACT

اجريت هذه الدراسة لمعرفة الانتشار المصلي لبكتريا *Coxiella burnetii* المسببة للحمى المجهولة في الحيوانات الزراعية بالمملكة العربية السعودية. واستخدم في البحث نوعان من الاختبارات المصلية هما اختبار

الامصلص المناعي الأنزيمي (ELISA) التي استخدم كاختبار رئيسي واختبار اللصف المناعي (IFA) التي استخدم لتأكيد النتائج ومقارنتها.

تم اختبار عينات من مصل الدم لعدد 1970 حيوان من الجنسين لتحديد نسبة انتشار الأجسام المناعية من نوع IgGالمضادة لبكتريا *C. burnetii* بواسطة الاختبار المناعي الأنزيمي ELISA غير المباشر. وقد جمعت العينات من 489 رأس من الإبل و 428 رأس من الأبقار و 630 رأس من الضأن و 423 رأس من المعز. وعدد من الحيوانات البرية. وتم تقسيم الحيوانات إلي صغيرة (غير بالغة) وكبيرة (بالغة). وكانت جميع الحيوانات سليمة اكلينيكيا ولم تكن أي من الإناث البالغة حبلى ولكن بعضها كان منتجا للحليب.

أوضحت النتائج وجود أجسام مناعية من نوع IgG المضادة لبكتريا *C. burnetii* في 605 عينة من أمصل الحيوانات الزراعية التى تم اختبارها، أي بنسبة إيجابية عامة تبلغ 30.71 ٪. أما متوسط العيارية (%S/p Ratio or OD) فقد بلغ 103.03٪. وتدل هذه النتائج على انتشار ميكروب الحمى المجهولة في الحيوانات الزراعة بأنواعها المختلفة في المملكة العربية السعودية. وقد سجلت أعلى نسبة من الحالات الإيجابية في امصل الإبل حيث بلغت النسبة في هذه الحيوانات 51.53٪ تليها المعز بنسبة 34.04٪ فالأبقار بنسبة 30.61٪ وأقلها الضأن بنسبة 12.38٪.

وقد كانت نسبة الأجسام المضادة لميكروب الحمى المجهولة أعلى بكثير في الحيوانات البالغة مقارنة بالحيوانات الصغيرة وكان تأثير العمر عالي المعنوية ($P<0.0001$) في كل أنواع الحيوانات. كما أن نسبة الحالات الإيجابية مالت للارتفاع في الإناث مقارنة بالذكور إلا أن التحليل الإحصائي دل على عدم وجود فرق معنوي بين الجنسين ($P=0.5847$).

تم في هذه الدراسة أيضا رصد الأجسام المضادة لكوكسيلة *C. burnetii* في حليب الحيوانات الزراعية بواسطة اختبار ELISA. كما تمت مقارنة نتائج اختبار الحليب مع نتائج الاختبار في مصل الدم. وقد شملت الدراسة 285 عينة حليب منزوعة الدهن تم جمعها من 48 رأس من ألنوق 90 رأس من الأبقار 60 رأس من النعاج و 87 رأس من المعز. وقد سجلت أجسام المضادة لميكروب الحمى المجهولة في 30 (62.5٪) رأس من الإبل 30 (33.3٪) رأس من الأبقار 24 (27.6٪) رأس من المعز و 3 (5.0٪) رؤوس من النعاج. وتم في الوقت نفسه اختبار عينات مصل الدم من نفس الحيوانات التي اختبر حليبها حيث بلغ عدد الحالات الإيجابية في الاختبار المصلي 32 (66.7٪) رأس من الإبل 38 (42.2٪) رأس من الأبقار 13 (14.9٪) رأس من المعز و 4 (6.67٪) رؤوس من النعاج. وقد سجلت أعلى نسبة انتشار للأجسام المضادة بكل من الحليب ومصل الدم في النوق. وأظهرت نتائج التحليل الإحصائي توافقا معنويا بين النتائج المتحصل عليها في الحليب والمصل. تم كذلك فحص 307 عينة مصل من الحيوانات تمثل 92 رأس من الإبل و 72 من الأبقار و 72 من الأغنام و 71 من المعز تباعا وذلك بواسطة باختبار ELISA غير المباشر واختبار اللصف المناعي (immuno-fluorescence test IFA) غير المباشر. وقد بين التحليل الإحصائي وجود توافق معنوي بين نسبة الحالات الإيجابية للحمى المجهولة المتحصل عليها في الاختبارين. وباستخدام اختبار ELISA كاختبار مصلي مرجعي أثبتت التحاليل الإحصائية حساسية ونوعية عاليتين لاختبار اللصف المناعي في الكشف عن الأجسام المضادة لبكتريا *C. burnetii* في أمصل الحيوانات الزراعية.

تم أيضا استخدام اختبار تفاعل بوليمراز التسلسلي (PCR) لمعرفة بعض طرق إفراز ميكروب *C. burnetii*. واستخدمت بادئات IS1111 في الاختبار لتضخيم التفاعل. أجري التحليل في عدد 82 عينة دم غير متجلط و 72 عينة حليب منزوع الدهن و 29عينة براز و 21 عينة بول من الإبل إضافة إلى 29 عينة

حليب و 7 عينات دم من الأبقار علاوة على 38 عينة دم و 29 عينة حليب و 20 عينة براز من المعز و 22 عينة دم من الأغنام. ومن إجمالي عينات الدم البالغ عددها 149 عينة وجد حض دنا النووي لميكروب C. burnetiiفي 13 عينة (15.85٪) من الإبل وعينتين (5.6٪) من المعز بينما كانت عينات الدم من الأبقار والضأن سلبية. أما بالنسبة لعينات الحليب وإجمالي عددها 149 عينة فقد سجل وجود الحض النووي للميكروب في 5 عينات من الإبل (6.49٪) و 11 (28.94٪) عينة من الأبقار بينما كانت عينات المعز سلبية. وكانت 8 عينات (27.59٪) من براز الإبل و 12 عينة (60٪) من براز المعز إيجابية أيضا. كما وجد حض دنا النووي للميكروب في 5 (23.81٪) من عينات بول الإبل. وكانت جميع العينات قد جمعت من حيوانات إيجابية لاختبار باستثناء عينات البول التي جمعت من إبل غير مختبرة مصليا.

أخيرا تم جمع امصل من حيوانات ايجابية لاختبارات الحمى المجهولة وأخرى سلبية وذلك لدراسة تأثير العدوى على معايير الدم الكيموحيوية والشوارد الكهربائية حيث تم جمع وتحليل 281 عينة مصل من الإبل والضأن والمعز والأبقار. وقد أشارت النتائج إلى عدم وجود فروق معنوية بين أنواع الحيوانات المذكورة بالنسبة إلى معظم المعايير التي تم تحليلها بينما سجلت القليل من الفروق في بعض المعايير بين الحالات الإيجابية والحالات السلبية داخل النوع الواحد. تمت كذلك دراسة تأثير الحمى المجهولة على مستوى الأنزيمات المضادات للأكسدة تحديدا حمض الثايوباربتيورك (TABARS) والجلوتاثيون المختزل GLUTH)) ولوحظ انخفاض في تركيز الأنزيم الأخير في دم الإبل. وبما أن ذلك الأنزيم يوجد في سيتوبلازم الخلايا في جميع دولت الثني تقريبا فإن انخفاض نشاطه قد يشير إلي تلف الخلايا ولكن هنالك حاجة لإجراء المزيد من الدراسات حول هذا الموضوع.

تمثل هذه الدراسة أول تسجيل للعدوى بكوكسيلة الحمى المجهولة في الأبقار والضأن والمعز بالمملكة العربية السعودية وثاني تسجيل والأكثر تفصيلا للعدوى بذلك الميكروب في الإبل بالمملكة.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Q-fever or Coxiellosis, is one of the commonest zoonoses worldwide. Its causative agent, *Coxiella burnetii*, is an obligate intracellular bacterium that circulates in the wild between various species of ticks and small mammals such as rodents, wild rabbits, bandicoot and other macropods. The organism is characterized by its extremely high infectivity and tenacity in the environment (Maurin and Raoult 1999).

C. burnetii was first described in 1935 by Dr. Edward Derrick as a febrile illness of unknown origin among abattoir workers in Brisbani, Queensland, Australia; hence it was dubbed Q (Query?) fever (Derrick, 1937). The disease is also known as coxiellosis, abattoir Fever, Nine Mile Fever, Australian Fever, Queensland Fever and American Fever. Derrick recognized the infectious nature of Q fever and differentiated it from typhus, typhoid and leptospirosis. However, he was unable to isolate its causative agent in guinea pigs, and, hence, concluded that it might be an unidentified virus. Burnett and his colleagues (Burnett and Freeman, 1937; Burnett *et al.* 1939), on the other hand, succeeded in transmitting the disease experimentally from man to guinea pigs, rats and monkeys and suggested that it might be a Rickettsia. They described granule-filled vesicles in the spleen, and bacillary structures resembling rickettsia and stainable with Castaneda or Giemsa stains, in the tissues of these animals. Meanwhile, in the USA, Davies and Cox (1938) isolated an unknown microbe from a tick in Nine Miles Creek in Montana. An accidental laboratory infection showed

that this microbe - then known as the “Nine Miles agent” – was pathogenic to man. It was also pathogenic to experimentally infected guinea pigs. In the same year, Cox (1938) showed that the Nine Miles agent was in fact the same as that which caused Q fever in Australia. In 1948, Philips suggested creating a new genus, *Coxiella*, and renaming the Q-fever/Nine Miles agent *Coxiella burnetii* in honor of both Cox and Burnet.

1.2. Background

C. burnetii is a strict intracellular organism measuring 2 – 0.4 µm x 0.4 – 1.0 µm. It grows in invertebrates’ cells, vertebrate cells, yolk sac of chicken embryo and in vitro cell cultures. It has an outer membrane resembling that of gram negative bacteria. However, it is not stainable by Gram's stain but by the Gimenz method (Gimenz, 1964). Although *C. burnetii* was originally classified as a Rickettsia, subsequent genetic analysis showed that it was a member of the gamma *Proteobacteria* that include the genera *Lgionella*, *Francisella* and *Rickettsiella* (Weiss, 1989), and that a genetic organization exists between and among different *C. burnetii* isolates *(*Willems, *et al*. 1996). From a phylogenetic standpoint, *C. burnetii* is closest to *Legionella pneumophila* which causes Legionnaires disease in humans. In domestic animals as well as humans, *C. burnetii* primarily parasitizes monocytes and macrophages in which it multiplies. When the infection is acquired by inhalation, the organism invades pulmonary macrophages and when it is acquired by ingestion, the organism invades Kupffer cells surrounding the hepatic sinusoids. Entry of *C. burnetii* into the host's cells occurs by a complex process involving receptor-mediated phagocytosis. The phagosomes formed after internalization of the organism fuse rapidly with lysosomes forming phagolysosomes, which, in turn, fuse into larger vacuoles (Hackstadt and Williams, 1981) in which *C. burnetii* survives and

multiplies. This is attributed to the ability of *C. burnetii* to adapt to the acidic environment inside the vacuoles, a unique property shared only with the protozoan Leishmania, which enables *C. burnetii* to metabolize nutrients and synthesize the amino acids needed for its multiplication (Antoine *et al*., 1990).

C. burnetii exists in two distinct morphological forms: a “large cell variant,” which is large and pleomorphic, and a “small cell variant,” which consists of small, bacillary structures. The large cell variant is found in infected animals and humans and represents the vegetative form of *C. burnetii* which is capable of replicating within the host cells. The small cell variant is an inactive, spores-like form, capable of surviving outside the body, resisting heat and dryness and surviving for extended periods in the environment (Scott and Williams, 1990; McCaull, 1991).

Phase changes occur in the surface antigens of *C. burnetii*, known as Phase I or “pathogenic phase” and Phase II or” non-pathogenic phase.” In Phase I, the surface antigens are composed mainly of polysaccharides and in Phase II they are composed mainly of proteins. This phenomenon is used in serological tests to distinguish between acute and chronic forms of Q-fever in humans. In domestic animals, however, this distinction is unclear and the pathogenicity of the organism is not known to be phase-dependent.

Q fever has a pan-global distribution, being reported from virtually all parts of the world, with the exception of Antarctic regions. More than a hundred species of wild and domestic animals, including all farm animals, equines, pet animals and poultry can carry *C. burnetii*. The infection usually runs an asymptomatic course in animals (apart from occasional cases of mild fever, abortion or mastitis) and therefore infected animals may carry the organism and contaminate the environment for a long period of time without being

detected. *C. burnetii* also does not produce obvious changes in the tissues of infected animals or their meat and milk, while its isolation from the tissues and products of these animals is difficult, requiring special laboratory procedures. For this reason, the Infection in animals continues to pose a persistent public health hazard until detected and effectively dealt with. Apart from vertebrate animals, over 40 species of soft and hard tick serve as vectors of *C. burnetii* (Babudieri, 1959). Once infected, these ticks shed the organism in their feces for life, pass it transovarially to their offspring (Liebisch, 1979) and circulate it in nature among wild mammals and birds through tick bites or contact with tick feces (Herenda et al., 1994). In this manner, the organism survives almost permanently in the environment. It can also be wind-borne over long distances and is so infectious that exposure to only one organism can initiate disease in man. Hence, it is not surprising that after a long period of underestimation or misdiagnosis (Lang, 1990; Yoshii *et al*., 1991), Q fever is currently being increasingly recognized as a re-emerging disease of significant public health and veterinary concern in many countries. Its prevalence is rising in the wake of the AIDS epidemic and the increasing use of immunosuppressive drugs for organ transplantation on one hand, and increasing animal concentrations and agricultural industrialization on the other (Lang. 1998; Yoshiie *et al*., 1991). A large epidemic of Q fever affecting nearly 4,000 people was recently reported in the Netherlands, in which infected dairy goats were blamed as the most likely source of the infection (Van den Borm and Vellema 2009; Schimmer *et al*. 2009; CDC 2010).

1.3. Source of orgamism

Farm animals are universally considered to be the main source of infection to man. They usually contract the infection from other animals in the herd or from contaminated pastures through inhalation or ingestion. Occasionally they might be infected through tick bites. In humans, coxiellosis is primarily acquired by direct routes without need for an arthropod vector e.g., by inhalation of infected aerosols or exposure to dust in areas contaminated with the organism. They can also be infected by ingestion, especially consumption of raw or non-pasteurized milk and milk products of infected animals, and also via contaminated skin wounds and even by venereal and transplacental routes. Abattoir workers may also be infected as a result of handling infected meat and rarely through hides contaminated with the feces of infected ticks. Human to human transmission may also occur (Mann *et al*., 1986; Raoult, 2001).

C. burnetii may be present in large numbers in reproductive organs, udders, meat and various secretions of infected animals, which may carry that organism for life and secrete it in their milk, urine, placental and birthing fluids, both during abortion and normal birth. According to CDC (1997), up to 1 billion organisms may be shed per gram of placenta in goats. Due to the overwhelming concentration of *C. burnetii* in the placentae and birthing fluids of infected animals, most human infections are seen among farm workers, veterinarians and people living close to animal farms.

1.3.1. Clinical signs

1.3.1.1. In humans

In humans, *C. burnetii* has a wide spectrum of clinical manifestations, ranging from a silent or mild disease, with limited signs such as headache, to a fulminant infection affecting visceral organs and sometimes terminating

fatally. In general only about 50% of all infected individuals develop clinical disease, which is either acute or chronic. The acute form of Q fever is mainly flu–like (Baca and Paretsky, 1983; Marrie, 1988) with sudden fever, sweating, headache, anorexia, myalgia, chest pain, chills and cough. These symptoms usually clear up within 1-2 weeks, but occasionally complications may arise such as pneumonia (Sobradillo *et al*., 1989) or hepatitis (Fishbein and Raoult 1992). The chronic form, on the other hand, manifests as endocarditis, a serious and difficult-to-treat condition (Raoult 2001). The importance of the disease in man has increased significantly during the past few decades following the AIDS epidemic and the marked increase in organ transplantation with consequently increasing use of immunosuppressive drugs, and various other factors that compromise the immune system.

1.3.1.2. In farm animals

In farm animals, *C. burnetii* usually produces a latent infection. However, under conditions of stress, it might become clinically manifest, causing, in particular, late term abortion and neonatal mortality. Other reproductive disorders such as fetal dysplasias and mastitis may also occur (Crowther, *et al*., 1976; Baca *et al*., 1983; Herr, 1985, Palmer *et al*., 1983; Lang, 1990; Stalis *et al*. 1996; Schröder, 1998; Lloyd *et al.* 2010). The placenta of aborting animals may or may not show gross pathological lesions; when present, these lesions consist of inflammatory and necrotic changes, coupled with accumulation of thick, reddish brown exudate, in the cotyledons. Anorexia may also be observed (Spicer *et al*., 1977; Stalis, 1996).

1.3.1.3. In dogs and cats

Similar clinical manifestations including abortion, stillbirth, neonatal mortality, and less commonly transient fever, depression and anorexia, were reported in dogs and cats, which usually contract *C. burnetii* infection either by inhalation or by ingestion of milk, placenta and other tissues of infected animals and rarely through infected tick bites. These pet animals, in turn, continue shedding the organism in their milk, urine and feces for several weeks post-infection (Antonetti, 1952; Buhariwalla *et al*., 1996; Ayres, 1998).

1.3.1.4. In camels

The camel is suspected to play a key role in the transmission of Q fever to humans in Saudi Arabia, not only because of the high prevalence of coxiellosis in these animals but also because of the wide spread tradition of consuming raw camel milk throughout the Arabian Peninsula (Hussein *et al*., 2008). Several authors previously have detected antibodies against *C. burnetii* in camels' sera (reviewed by Wernery and Kaaden, 1995). The earliest record of coxiellosis in these animals dates back to 1948, when Blanc detected anti-*C. burnetii* antibodies in camels in Morocco. In the Sudan, serological prevalence ranging between 12 -14% was reported in camels by Harbi and Awad El Karim (1972) and Abbas *et al*. (1987). In Egypt, the serological prevalence of *C. burnetii* in camels was given as 13.9% by Elyan and Daoud (1955), 4% by Sabban *et al*. (1968) and 66% by Soliman *et al*. (1992). In India, prevalence rates ranging between 5.6 - 17% were reported (Kalra and Taneja, 1954; Veeraghavan Sukumaran, 1954; Pathak and Tanwani, 1969; Choudhury *et al*., 1971; Kulshreshtha *et al*., 1974; Ghosh *et al*., 1975; Mathur and Bhargave, 1979). Seroprevalence of coxiellosis was also reported in camels in several African countries, namely

20% in Kenya (Brown, 1956), from 13.6-22.2% (Giroud *et al*., 1954) to as high as 80% (Schelling *et al*., 2003) in Chad; 15.8% in Tunisia (Burgmeister *et al*., 1975), 12% in Nigeria (Ado, 1980) and 20.4% in Ethiopia (Richard, 1979). More recently, a much higher prevalence of Q fever antibodies was reported in Saudi camels (Hussein *et al*., 2008). Richard (1979) suggested that the pathogen city in camels might be similar to or greater than that recorded in other species of farm animals. Other camelidae, namely llama, alpaca, guanaco and vicuna were also found to be serologically positive for *Coxiella burnetii* antibodies but little is known about the pathogenesis of the infection in these species.

1.3.1.5. In wild animals

Antibodies against *C. burnetii* were also detected in several species of wild ungulates such as gazelles, deer and antelopes, indicating that they could disseminate the infection in the environment and also serve as sources of coxiellosis in domestic animals and humans (reviewed by Hussein *et al*., 2012). In earlier studies based on the complement fixation test (CFT), Enright *et al.* (1969) reported anti-*C. burnetii* antibodies in 22% of the black-tailed Columbia gazelle and noted that the prevalence in these gazelles was much higher in pastures shared with sheep as compared to sheep-free pastures. In a sero-epidemiological survey of *C. burnetii* in different species of animals in Bulgaria, Martinov *et al.* (1989), also using CFT, reported an overall prevalence of 32.3% in the Roe deer. On the other hand, in the Czech Republic, antibodies against *C. burnetii* were detected by microagglutination test (MAT) in the red deer, Dama gazelle, mouflon sheep and wild rabbits with an overall prevalence of 12% (Hubalek *et al*., 1993). In Japan, a high serological prevalence of *C. burnetii* was recorded in the Hokaido deer

(69%) and the Japanese deer (56%) using an enzyme linked immunosorbent assay (ELISA) (Ejertico *et al*., 1993). Anti- *C. burnetii* antibodies were also detected in other wild ungulates such as the river deer, the American deer (Schroder, 1998), the white-tailed or Virginia gazelle (Marrie *et al*., (1993) and the Cuvier gazelle (Stalis *et al.*, 1996). In addition, Hussein *et al*. (2012) carried out a preliminary screening of coxiellosis in captive gazelles and Oryx in Thumama, Riyadh, using the ELISA protocol in which a high serological prevalence of *C. burnetii* was observed in these animals. These findings imply the need to pursue further studies coxiellosis in wild animals particularly because transmission of *C. burnetii* from wild ungulates to humans has been documented in persons handling these animals and in hunters and others living near forested areas. During an outbreak of Q fever among cervids in Nüremberg Zoo, 26 zoo workers contracted the disease (Gaukler and Kraus 1974), while in the U.K., a family of seven developed Q fever after hunting a deer and feeding its liver to their pregnant dog, which, in turn, transmitted the infection to all family members (Laughlin *et al*. 1991). Also, during a wave of *C. burnetii*–associated abortions in a fallow deer farm in Stuttgart, Germany, 12 out of 13 in-contact people were infected, and two developed clinical disease (Simmert *et al*. 1998). Another incident of Q fever involving 25 out 117 workers was reported in a wildlife breeding station in Maldonado, Uruguay, in which the field deer, *Ozotoceros bezoarticus*, was identified as the main source of infection (Hernández *et al*. 2007). The possible role of wild ungulates such as gazelles in spreading *C. burnetii* in the Kingdom should therefore not be overlooked particularly as the populations of indigenous gazelles in Saudi Arabia are growing as a result of intensive breeding and re-introduction into the wild, as well as the

increasing number of people keeping gazelles as private collections in their farms, thus increasing the risk of disease transmission.

1.4. Epidemiology

Although Q fever has been described more than fifty years ago as being holo-endemic among the population of Saudi Arabia (Gelpi,1966; Lippe *et al*., 1968), extremely few publications on the disease have since been made, namely a report of a single case of acute Q fever leading to meningoencephalitis in a US soldier returning from Saudi Arabia after the first Gulf war (Ferranti and Dolan, 1993) and four other US soldiers who had apparently contracted subclinical infection in the Kingdom following exposure to animals (Byrne, 1997) in addition to 18 out of 51 persons recently shown by immunofluorescence to be positive for anti-*C. burnetii* in Riyadh, Saudi Arabia; no indication was given as to where they contracted the infection (Almogren *et al*., 2013). Similarly surprising is the lack of information on Q fever among indigenous animals in Saudi Arabia. Aside from sparse reports of anti-*C. burnetii* antibodies in camels (Hussein *et al*., 2008) and game animals (Greth *et al*., 1992; Hussein *et al*., 2012), no information has been published regarding the prevalence of this disease or its epidemiological features in animals in the Kingdom. This is surprising considering the huge volume of studies in other countries on the prevalence of *C. burnetii* and its pathogenesis and epidemiology in different species of farm animals such as cattle, sheep and goats, none of which has been investigated for coxiellosis in the Kingdom.

1.5. Pathophysiological changes

While it is known that overt clinical signs are rarely manifested in Q fever (coxiellosis) in farm animals, it is not known if the infection induces any pathophysiological changes such as changes in the blood proteins, enzymes, metabolites and inorganic constituents in infected farm animals. Another important aspect which is currently attracting attention is the role of oxidative stress in the biology and pathogenesis of *C. burnetii*. It has been proposed that survival of *C. burnetii* in phagolysosomal vacuoles requires specific iron uptake systems, with secretion of enzymes to detoxify the compartment (catalase and SOD), and down-regulation of an oxidative burst (acid phosphatase) (Samuel *et al*., 2003; Fernandes *et al*., 2009). Oxidative stress has been associated with other diseases in farm animals (Lykkesfeldt and Svendsen, 2007). For instance, oxidative stress due to *Babesia bigemina* and *Theileria annulata* has been shown to induce marked changes in selected metabolites (Saleh, 2009; Grewal *et al*., 2005). To the best of our knowledge, there are extremely few studies associating oxidative stress with Q fever. The study by Hill and Samuel (2010) is worth noting since it shows that replication of *C. burnetii* during infection is increased by decreasing oxidative stress. It is therefore, interesting to examine the possible association between oxidative stress and Q fever and to determine resultant changes that occur in blood metabolites in different species of animals. This could help to expand our knowledge of the pathogenesis of coxiellosis.

1.6. Diagnosis

Investigations on coxiellosis in farm animals to determine their role in the spread of the disease in in Saudi Arabia are thus long overdue. This could be attributed to the fact that animal infections neither produce overt clinical signs nor visible pathological changes in tissues, meat, milk and other

secretions of infected animals, making it impossible to diagnose *C. burnetii* on clinical or post-mortem basis, with the result that infected animals remain carrying the organism and contaminating the environment for extended periods of time without attracting attention. Laboratory tests are therefore required for the diagnosis of *C. burnetii* in animals. *C. burnetii* can be isolated in embryonated eggs, tissue cultures or experimental animals; however, this is a risky procedure that requires a special type of laboratory to avoid transmission to laboratory workers, besides being impractical for large scale surveys. In cases of abortion, suspected to be due to *C. burnetii*, a rapid preliminary diagnosis may be possible by examining stained placental smears but still this requires laboratory confirmation.Various immunological and immunohistochemical tests have been developed for the diagnosis of coxiellosis (Fournier *et al*., 1998). Earlier tests included microagglutination techniques, such as capillary tube agglutination, indirect hemolysis tests and allergic dermatological tests. Currently, the detection of *C. burnetii* antibodies by specific serological tests or the demonstration of *C. burnetii* DNA by the PCR are the most widely used tests for diagnosing infection and studying its prevalence in animals. The commonest serological tests presently used for the diagnosis of *C. burnetii* in animals are the compliment fixation test (CFT), immunofluorescence assay (IFA) and enzyme-linked immunosorbent assays (ELISA) (LaScola, 2002; Slaba *et al*., 2005). The CFT has lower sensitivity as a diagnostic test for Q-fever compared to IFA and ELISA tests which are highly sensitive and specific to both phase I and phase II antigens (Tokarovich *et al*., 1990; Slaba *et al*., 2005). Both IFA and ELISA tests were used in the present study and compared as herd screening tests. The IFA assays employ fluorescent markers conjugated to a specific antibody to detect antigen-antibody (Ag:Ab) reaction. In positive cases, the

fluorescent marker in the Ag:Ab complex emits a green light which is detected under the fluorescent microscope. There are direct and indirect types of the test. The latter is the one more commonly used. On the other hand, the most commonly used ELISA assay for screening *C. burnetii* infection in ruminants is an indirect test which utilizes a horseradish peroxidase-labeled monoclonal anti-ruminant IgG conjugate that reacts with a wide range of domestic and wild ruminant species (reviewed by Hussein *et al*., 2012).

1.6.1. Serological Tests:

Screening of animals for Q fever is largely based on serological methods. For this purpose, several serological tests have been developed (Fournier *et al.*, 1998) of which, the most widely used tests are enzyme linked immunosorbent assay (ELISA), indirect immunofluorescence assay (IFA) and complement fixation test (CFT) (Field *et al*., 1983; Kovacova *et al*., 1987; Fournier *et al*., 1998; Kovacova *et al*., 1998; Angelakis and Raoult, 2010). Two of these tests, namely ELISA and IFA, were used in the present study.

1.6.1.1. Indirect Enzyme-linked Immunosorbent assay (ELISA):

Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive and specific test for Q fever (Kittelberger *et al.*, 2009; Rousset *et al.*, 2007; 2009) and is economically feasible and easy to perform. Several authors reported that ELISA was more

Sensitive and more specific than either CFT or IFA for the diagnosis of Q fever (Peters *et al*., 1983; 1985; Kovacova *et al*., 1998; Berri *et al*., 2001; Kittelberger *et al*., 2007; Angelakis and Raoult, 2010). It is a convenient test

for large-scale screening of anti-*C. burnetii* antibodies in the serum and milk of different animal species The test was shown to have 100% specificity and 92–95% sensitivity relative to the indirect immunofluorescence tests (Jasper *et al*., 1994; Field *et al*., 2000, 2002; Bommeli 1997; Schalch *et al*., 1998; Arricau-Bouvery *et al*., 2005; Rousset *et al*., 2007).

Direct and indirect ELISA tests were developed for the diagnosis of *C. burnetii* and other infectious agents. In these tests, enzymes are used to detect the presence of either a specific antigen or a specific antibody in the test sample. Ready-to-use kits are commercially available that can detect anti-*C. burnetii* antibodies.

In the present study, an indirect ELISA assay was used to detect anti-*C. burnetii* antibodies in the sera and milk of animals. This method has been used extensively in serological surveys of Q fever in different species of domestic animals including camels (Schelling *et al.*, 2003; Hussein *et al*., 2008), cattle (Schelling *et al*., 2003; Cabassi *et al*., 2006; Seyitoğlu *et al*., 2006; Çekani *et al*., 2008; Banazis *et al*., 2009; Khalili and Sakhaee 2009; Agger *et al*., 2010; Angen *et al*., 2011), sheep (Berri *et al*., 2000, 2001; Schelling *et al*., 2003; Çekani *et al.*, 2008; Kennerman *et al*., 2008; Karaka *et al*., 2009; Banazis *et al*., 2009; Abed *et al*., 2010) and goats (Arricau-Bouvery *et al.*, 2005; Rousset *et al.*, 2007, 2009; Çekani *et al*., 2008; Khalili and Sakhaee 2009). The same test has also been used to screen *C. burnetii* antibodies in wild ungulates, including the field deer (*Ozotocerus bezoarticus*) (Hernández *et al*., 2007), Spanish mouflon (*Ovis aries musimon*) (López-Olvera *et al.*, 2009), Dama gazelle (*Dama dama*) (Lloyd *et al*., 2010), sand gazelles (Reem), mountain gazelles (Dim) and the Arabian Oryx (Hussein *et al*., 2012) as well as kangaroos (Banazis *et al*., 2009). It has also been recently used in humans (Nielsen *et*

al., 2012). The principle of the test is based on the interaction between *C. burnetii* antigen coating the bottom of micro-titration wells and anti-*C-burnetii* antibody (also designated primary antibody) in the test sample, resulting in the formation of an antigen-antibody complex. A second, enzyme-conjugated antibody (designated secondary antibody or anti-antibody) against the primary antibody is then added to the micro-titration wells where it reacts with the primary antibody resulting in a triple antigen-antibody complex. This complex is detected by adding an enzyme substrate which causes a change in color in serologically positive samples. The rate of substrate conversion is proportional to the amount of bound antibody. Hence, the intensity of the color, which is determined spectrophotometrically and expressed as S/P or percent optical density (O.D. %), is proportional to the antibody concentration (titre) in the sample versus known positive and known negative reference sera. An important advantage of enzyme-conjugated secondary antibodies is that they can detect several types of primary antibodies.

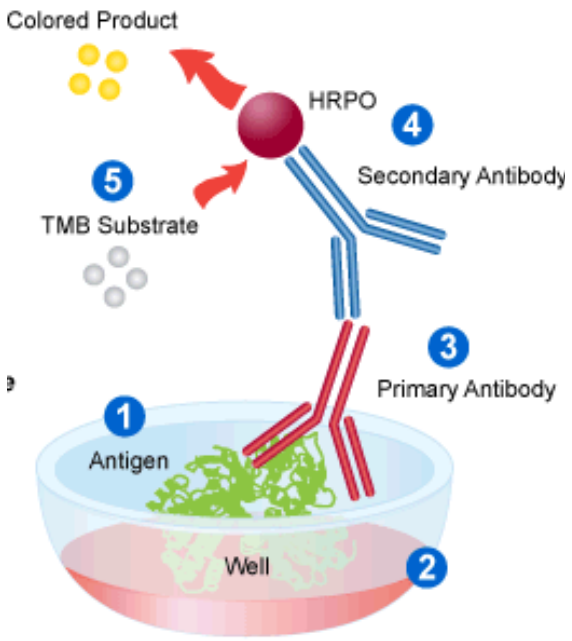

Figure 1: Schematic drawing of indirect ELISA test

1.6.1.2. Indirect immunofluorescence assay (IFA):

Immunofluorescence assay (IFA) is a simple, reliable and commonly used method for the serodiagnosis of Q fever in domestic and wild animals and especially in humans (Marrie *et al*., 1993; Tissot-Dupont *et al.*, 1994). The Q fever agent, *C. burnetii*, expresses two antigenic phases: phase I antigen which is expressed when the organism is isolated from man or animals and phase II antigen when it is isolated from cell culture (Tissot-Dupont *et al.*, 1994). Both phases are detectable by IFA. There are direct and indirect types of IFA. The direct test is used to detect an antigen by a specific fluorescein-isothiocyanate (FITC)-conjugated antibody against that antigen. In Q fever, the direct IFA is used primarily to detect the presence of *C. burnetii* antigen in samples such as fluids, tissues and tissue cultures and is

rarely used as a quantitative test. The indirect test employs a known antigen (in this case *C. burnetii* antigen) to detect specific antibodies against it in the tested samples. The principle of the test is based on the occurrence of a reaction between *C. burnetii* antigen and antibodies in the sample, resulting in the formation of an antigen-antibody complex which is then detected by reaction with a fluorescein-labeled anti-species immunoglobulin. The indirect test is more sensitive than the direct test and is a convenient quantitative test; hence, it was used in the present study to detect antibodies against Q fever and determine their titres in serum samples.

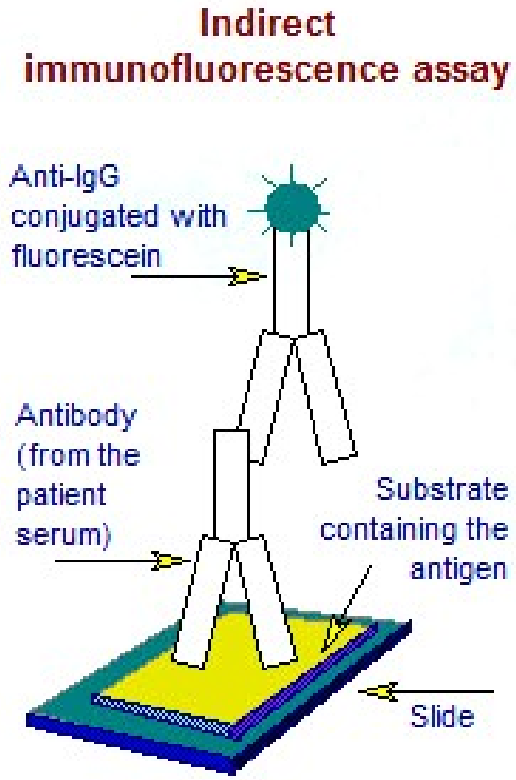

Figure 2: Schematic drawing of indirect immunofluorescence assay

1.6.1.3. Polymerase Chain Reaction (PCR):

PCR is one of the most sensitive and reliable means of direct detection and identification of *C. burnetii* shedders. Several PCR-based diagnostic methods have been developed to detect *C. burnetii* DNA in cell cultures and a wide range of clinical samples (Frazier *et al*., 1990; Stein and Raoult, 1992; Willems *et al*., 1994; Fournier *et al*., 1998; Klee *et al*. 2006) and the PCR is becoming an increasingly common technique in diagnostic laboratories with PCR capabilities (Berri *et al*., 2000; Nicollet and Valogenes, 2007). The sensitivity of any of the PCR tests developed depends, among other things, upon the chosen "target DNA." In Q fever, the most useful PCR targets are those that use the insertion sequence IS1111 (Hoover *et al*., 2002). Each *C burnetii* Nine Mile strain chromosome contains at least 19 copies of this sequence, and every *C burnetii* isolate tested so far has multiple copies of this element.

No reports are found in the literature indicating that PCR has ever been used to detect *C. burnetii* DNA in clinical materials from man or animals in Saudi Arabia. However, several studies have been reported in other countries dealing with the detection of *C burnetii* DNA in blood, milk and other clinical samples from different species of animals (Willems *et al*., 1994; Berri *et al*., 2001; 2003; Ongör *et al*., 2004; Kim *et al*., 2005; Guatteo *et al*., 2006; Rodolakis *et al*., 2007; Fretz *et al*., 2007; Garcia-Perez *et al*.,2009; Rouiz-Fons *et al*., 2008; Rousset *et al*., 2009; Rahimi *et al*., 2009; 2011; Rahimi, 2010; Angen *et al*., 2011; Doosti *et al*., 2014). During the present study, a preliminary PCR analysis was undertaken on 82 blood samples, 77 milk samples, 29 fecal samples and 21 urine samples collected from camels to determine the routes of shedding of *C. burnetii* by these animals.

Objectives of the study:

1. To investigate the serological prevalence of Q fever in indigenous camels, cattle, sheep and goats in Saudi Arabia.
2. To compare the use of ELISA as applied to milk samples versus serum samples for detecting the prevalence of *Coxiella burnetti* infection in farm animals.
3. To compare Q fever tests in farm animals using immunofluorescence as compared to ELISA procedures.
4. To investigate the shedding routes of *C. burnetii* in camels using polymerase chain reaction (PCR).
5. To compare *C. burnetii* shedding in samples of different animals using PCR.
6. To assess possible association between *C. burnetii* infection in farm animals and clinical laboratory findings, namely serum biochemical profiles, electrolytes and anti-oxidant enzymes.
7. To Propose ways for implementing the results and recommendations of the study.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Animals:

Male and female camels, sheep, goats and cattle were randomly sampled at farms, animal markets, slaughter-houses and free ranging herds in different localities in Saudi Arabia. The camels belonged to *Maghater*, *Majahim* and mixed (*Shu'l, Humr and Sufr*) breeds; the sheep belonged to *Najdi*, *Naimi* and *Harri* breeds and the goats to *Ardi* and *Demasqi* (*Dems*) breeds. All cattle were locally born and bred Friesian-Holstein cattle. In total, 1970 animals, comprising 489 camels, 428 cattle, 630 sheep and 423 goats were serologically tested for anti-*C. burnetii* antibodies using indirect enzyme-linked immunosorbent assay (ELISA). Milk samples were collected for ELISA testing from a total of 349 animals comprising 69 camels, 100 cattle, 102 sheep and 78 goats. Sera from the same animals were simultaneously tested with ELISA. In addition, serum samples from a total of 307 animals (92 camels; 72 cattle; 72 sheep and 71 goats were tested by ELISA and simultaneously tested by immunofluorescence assays (IFA) for confirmation of the results and comparison of the two tests.

The animals were broadly divided into young and adult animals. All of them were healthy when sampled and none of the adult females was pregnant while some were lactating.

A preliminary study using the polymerase chain reaction (PCR) was conducted on 82 whole blood, 72 milk, 29 faecal and 21 urine samples collected from camels, 38 blood, 29 milk and 20 faecal samples from goats, 29 milk samples and 7 whole blood samples from cattle and 22 whole blood samples from sheep.

2.2 Sampling:

Seven to ten ml blood sample was collected by jugular venipuncture from each animal into plain vacuotainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The samples were allowed to stand in a tilted position for 4 h at room temperature, and the sera were separated from clotted blood by centrifugation at 1,500×*g* for 10 min, dispensed into clean 1.5 ml plastic tubes and stored at −20°C until tested. Samples showing hemolysis were discarded and replaced.

For PCR analysis, 7 ml whole blood samples were collected from the jugular vein of 82 camels and 7 cattle into clean EDTA-K2 vacuotainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Whole blood was preferred to serum as it contains white blood cells which might harbor *C. burnetii*.10 ml milk samples were also collected aseptically from 77 lactating camels and 7 cows into sterile vials. Prior to sampling, the udders and teats were thoroughly cleaned and the first two streams of milk were discarded. Fecal samples were collected directly from the rectum of 29 camels, while urine samples were collected aseptically by needle aspiration from the urinary bladders of 21 camels slaughtered at Riyadh abattoir and dispensed into clean, sterile vials. All samples were promptly transported in ice to the laboratory and deep frozen at -96^{o} C until analyzed. No serological results were available for the camels from which urine was collected.

2.3 Methods

2.3.1 Serology

2.3.1.1 Indirect Enzyme-linked Immunosorbent assay (ELISA):

2.3.1.1 *a.Test Procedure:*

A commercial ELISA Q fever antibody test kit (CHEKIT-Q-Fever, IDEXX Laboratories, Bommeli Diagnostics, AG, Lieberfeld-Bern, Switzerland) **was used. Each kit contained** microtiter plates with 96 flat-bottomed wells pre-coated with inactivated *C. burnetii* antigen, monoclonal anti-ruminant immunoglobulin G (IgG) conjugated with horse-radish peroxidase (HRP), reference positive and negative control sera, tenfold concentration of phosphate buffered saline (PBS), TMB [tetramethylbenzidine] chromogen substrate N12, and stop solution (0.05 ml 2M H_2SO_4). The monoclonal anti-ruminant IgG conjugate was used for cattle, sheep and goats while camels were tested using an HRP-conjugated anti-camel IgG (Triple J. Farms, 777 Jorgensen Place, Bellingham, WA 98226, USA). Other test requirements included: 96-well microplate reader, microplate washer, shaker, incubator (+37°C), 8 and 12 channel precision pipettes with disposable plastic tips and distilled water.

The test sera, as well as the reference positive and negative control sera, were diluted to 1:400 with PBS, dispensed into the microtitre plates wells in amounts of 100 µl per well in duplicate and gently shaken. The plates were covered with a plastic lid, and incubated in a humid chamber at 37°C for 60 minutes, then washed three times in washing solution (~ 300 µL/well each time) at room temperature to remove any unbound material from the wells. After the final wash, the plates were gently tapped to remove any residual washing solution. 100 µl of freshly prepared conjugate was then added into each wells and the plates were covered with lids and incubated at 37°C for 60 minutes then washed three times in washing solution as before. 100 µl of freshly prepared chromogen substrate solution was then added to each well and the plates were gently shaken and incubated at room temperature for 15 min. Thereafter, the color reaction was stopped by adding 300 µl/well of the

stop solution and the absorbance of each well was measured in microplate reader (MTX Labsystem Inc., 8456 Tyco Road, Vienna, Va 22182, U.S.A) at 450 nm and the absorbance values were used to calculate the results.

Figure 3: ELISA unit

For milk testing, the milk samples were centrifuged and the fat-fraction was removed and discarded. The non-fat fraction was diluted 1:5 in wash solution and tested in the same way as serum samples (Agger *et al*., 2010).

2.3.1.1b. ***Interpretation of Results:***

The optical density (OD) of each sample was compared with the optical densities of the positive and negative reference sera. The following equation was used to express the OD of the test samples as a percentage of the positive control (S/P) which was taken to be 100%:

$$\text{Percent O.D. (or SP) of the sample} = \frac{100\,(S - N)}{(P - N)}$$

where S is the O.D. value of the test sample, while N and P are the O.D. values of the negative and positive reference sera, respectively. A good visual cut-off was observed at ≥40% O.D.; hence, the test sample was considered positive if the % O.D. value is ≥40 and negative if it is <40.

2.3.1.2. Indirect immunofluorescence assay (IFA):

2.3.1.2a. *Test Procedure*:

IgG antibodies against phase II *C burnetii* antigen were detected in the serum samples using an IFA kit (Vircell, S. L. Pza. Dominguez Ortiz I. Poligono Industrial Dose de Octubre. 18320 Santa Fe, Granada, Spain). Each slide in the kit had 10 spots coated with *C burnetii* phase II, Nine Mile strain (ATCC 616-VR) grown in MRC cells. The organisms were inactivated with formaldehyde and were fixed with acetone.

Each serum sample was initially diluted at 1:64 and 1:128 and then serial twofold dilutions were made in PBS for titrating positive samples as necessary up to a maximum titre of 1:8192; samples yielding 1:8292 titres were therefore recorded as ≥1:8192. Diluted serum samples along with known positive and negative control sera were overlaid onto the antigen spots and incubated at 37°C in a humid chamber for 30 minutes. After incubation, the slide was washed twice with PBS and once with distilled water. After washing, the slide was air dried, and 20 µl of the corresponding fluorescein isothiocyanate (FITC)–conjugated anti-species IgG was added to each antigen spot, namely FITC conjugated rabbit anti-bovine, rabbit anti-sheep and rabbit anti-goat IgG's (Gentex Inc., 2456 Alton Pkwy, Irvine, CA 92606, USA) and specific FITC conjugated goat anti-camel IgG (Triple

J. Farms, 777 Jorgensen Place, Bellingham, WA 98226, USA). The slides were incubated for 30 minutes at 37°C in a humid chamber and finally washed as described previously and dried in air. A drop of mounting medium of buffered glycerol plus Na azide was then added to the slide which was covered with a cover slip and examined immediately under 400 × magnifications using a fluorescence microscope (Axioskop 2 plus; Zeiss, Gottingen, Germany).

2.3.1.2b. ***Interpretation of results:***

Apple green fluorescence of coco-bacillary morphology detectable against a dark background at the serum dilution of 1:64 or more was considered a positive test in accordance with the recommendations of the manufacturers. The conjugate alone and the negative control serum gave negative results (no fluorescence).

Figure 4: Axioskop 2 plus fluorescence microscope

2.3.1.3 Polymerase Chain Reaction (PCR):

2.3.1.3.a. Samples:

A total of 367 blood, milk, faecal and urine samples were collected from some livestock which had antibodies against *C. burnetii* when serologically tested by ELISA (Table 1). 7 ml whole blood samples were collected into vacuotainer tubes containing EDTA as anti-coagulant. Milk, urine and faeces were collected into sterile clean universal bottles. 209 samples were collected from camels, comprising 82 blood, 77 milk, 29 faecal and 21 urine

samples. Goat samples comprised 38 blood, 29 milk and 20 faecal samples. From sheep, 22 blood and 4 milk samples were available while 7 blood and 38 milk samples were available from cows. None of the lactating animals from which milk samples were collected for DNA extraction showed clinically apparent signs of mastitis.

Table 1: Samples collected from different animal species and tested using PCR for the detection of *Coxiella burnetii* DNA

Species	Blood	Milk	Faeces	Urine	Total
Camels	82	77	29	21	**209**
Cattle	7	38	0	0	**45**
Goats	38	29	20	0	**87**
Sheep	22	0	0	0	**22**
Total	**149**	**144**	**49**	**21**	**363**

2.3.1.3.b. Test Procedure:

DNA Extraction: DNA was extracted from blood using QIAGEN DNeasy blood and tissue kit (GmbH, Hilden, Germany) according to manufacturer's instructions. 100 µl unclotted blood was pipetted into 1.5 ml microcentrifuge eppendorf tube and 20 µl of proteinase K was added to the blood. The volume was adjusted to 220 µl by adding 100 µl PBS. 200 µl AL buffer was then added and the tube was immediately vortexed thoroughly for 10 seconds, then incubated at 56^{o} C for 10 minutes. After incubation, 200 µl of ethanol (96-100%) was added to the tube and the contents again vogorously vortexed for 10 seconds. The contents of the tube were transfered to DNeasy Mini spin column fitted with 2 ml collection tube and the column was

cetrifuged at 6000 x*g* for 1 minute. The flow-through was discarded and the collection tube was also discarded and replaced with a new collection tube. 500 µl of the first washing buffer (AW1) was then added to the column and centrifuged at 6000 x*g* for 1 minute. The flow-through was discarded again with the collection tube and a new collection tube was used. A volume of 500 µl of the second washing buffer (AW2) was added to the Mini spin clumn and centrifuged at 20,000 x*g* (maximum speed) for 3 minutes to dry the DNeasy membrane and get rid of residual ethanol. The flow-through was discarded with the collection tube and the Mini spin column was placed into a new 1.5 ml microcntrifuge tube. 100 µl of the elution buffer (10 mMTris-HCl, 0.5 mM EDTA, pH 9.0) was added and the elute was kept ready for the PCR.

For DNA extraction from milk, 1 ml of milk was centrifuged at 8000 *g* for 60 minutes. The cream and milk layers were removed and the pellet was washed twice in distilled water. DNA was extracted using DNA extraction kit from QIAGEN using the QiaAmp mini kit (GmbH, Hilden, Germany) and the volume of the sedimented starting material would sediment, then the total volume of the starting material was adjusted to 200 µl. A total volume of 2.5 µl would subsequently be used in the polymerase chain reaction. DNA from urine samples was extracted also using the QiaAmp mini extraction kit. Briefly 1 ml of urine was centrifuged for 30 minutes and the supernatant was discarded and the sediment was used for DNA extraction. A total volume of 2.5 µl was used for PCR. DNA from faecal samples was extracted using a commercial kit from Bioline (London, UK) according to the manufacturer’s instructions.

Positive control DNA of *C. burnetii* was kindly provided by Professor K. Henning of the Friedrich-Loeffler-Institute in Wusterhausen, Germany.

Figure 5: Automated DNA extractor

*Polymerase chain reaction (PCR):*Amplification of the repetitive transposon-like region of *C. burnetii* was employed using 3 pairs of primers targeting the sequence, namely: IS111F1 (5'-TACTGGGTGTTGATATTGC-3') and IS111R1 (5'-CCGTTTCATCCGCGGTG-3') which amplify 485-bp fragment of the *htpAB*-associated repetitive element (GenBank accession number M80806); primers IS111F2 (5'-GTAAAGTGATCTACACGA-3'), and IS111R2 (5'-TTAACAGCGCTTGAACGT-3') which amplify 260 bp of the original PCR that resulted from the first pair of in a multiplex PCR (Fenollar *et al.*, 2004; Seshadri *et al.*, 2003). The third pair of primers comprise CoxP4 (TTAAGGTGGGCTGCGTGGTGATGG, nucleotide positions 222–245 in

GenBank accession M80806) and CoxM9 (GCTTCGTCCCGGTTCAACAATTGC, nucleotide position 669–648) which amplify 448 bp product of the transposase gene of *C. burnetii* (Panning *et al.*, 2008).

Each 25 µl reaction mixture was made of 5 µl PCR buffer (Bioline, UK), 0.2 µl taq polymerase (Bioline, UK), 1 µl of each of the four primers (10pm/µl), IS111F1, IS111R1, IS111F2 and IS111R2, sterile distilled water, and 2 µl of DNA. The IS111F1 and IS111R1 primers were used for the first amplification, while re-amplification was performed using the IS111F2 and IS111R2 primers (Seshadri et al., 2003). Following an initial denaturation step at 95°C for 8 minutes, the rapid PCR program was made of 35 cycles of denaturation at 95°C for 15 seconds, annealing at 52°C for 5 seconds, and extension at 72°C for 18 seconds. Re-amplification or second round PCR was performed using 35 cycles of denaturation at 95°C for 15 seconds, annealing at 48°C for 5 seconds and extension at 72°C for 18 seconds. The amplification was completed by holding for 10 minutes at 68°C to allow complete extension of the PCR products.

Figure 6: Thermo AMS 02G Thermocycler

2.3.1.3.c. Interpretation of results:

Amplicons from the second amplification were separated by electrophoresis on 1% agarose gels and digital images were taken after staining the gels using ethidium bromide and visualizing the PCR products on transilluminator.

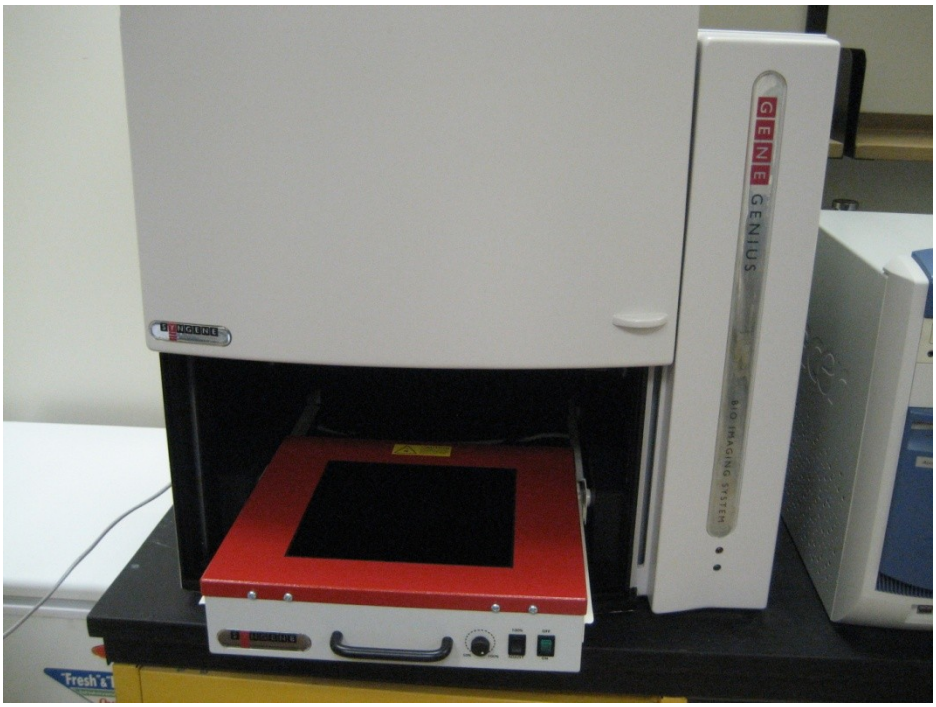

Figure 7: PCR gel documentation system

2.4 Chemical Analysis:

The concentrations of serum biochemical and electrolyte constituents were determined using UDICHEM-310 spectrophotometer (Fig 9) and commercial reagent kits (United Diagnostic Industries, Dammam, Saudi Arabia). The following constituents were determined: total serum proteins (PR), albumin (ALB), , glucose (GLU), urea (UR), blood urea nitrogen (BUN), triglycerides (TGL), creatinine (CRE), total lipids (TL), cholesterol (CHLO), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT) and inorganic constituents (Na, K and Ca). Total globulin (GLO) was taken as the difference between total protein and albumin, and the albumin globulin ratio (A/G) was calculated.

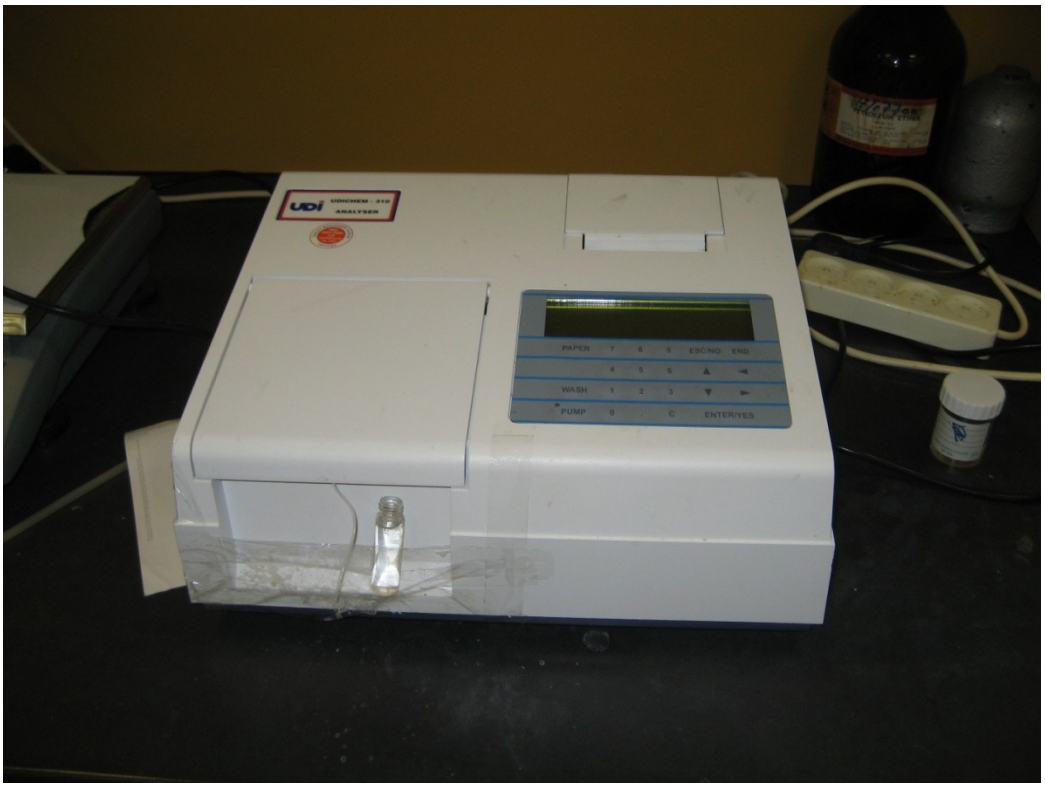

Figure 8: UDICHEM-310 spectrophotometer

The antioxidant status was assessed by measuring plasma thiobarbituric acid reactive substances (TBARS) and reduced glutathione peroxidase 1 (GLUTH) levels in Q-fever positive and Q-fever negative animals. The TBARS assay was carried out according to the modified method of Iqbal *et al.* (1996) while GLUTH was estimated according to the method of Jollow *et al.* (2001) using DTNB as a substrate; the yellow color which developed was read immediately at 412 nm and expressed as µmol/ml of serum.

2.5 Statistical Analysis:

The combined data were analyzed in this study with the incidence of Q-fever coded as a binary dependant variable (0 for non-affected and 1 for affected animals). Some descriptive statistics such as frequencies and means of Q-fever prevalence and ELISA titration were computed using SAS software

(V. 9.1, 2009). Different logistic models were utilized to examine the associations of independent variables of this study with the incidence of Q-fever. The general formula of the model was: Logit $P(x) = \beta_0+\beta_i(x_i)$, where β_0, β_1 are regression coefficients and x_i the effects of independent variables of species, breed, sex, age and location. Odd ratios were also directly estimated from the logistic models, and calculated as the rate of odds for x=1 to the odds of x=0, thus the outcomes revealed the probability of the risk to being positive to the Q-fever tests. Other statistical procedures were used to investigate the relationship between ELISA and IFA tests and determine the sensitivity, specificity and other related values.

CHAPTER THREE

RESULTS

3.1 Serological Prevalence of Q Fever (*C. burnetii*) in Animals Using ELISA:

Serum samples from a total of 1970 farm animals (camels, cattle, sheep and goats) were tested to determine the prevalence of *C. burnetii* specific IgG antibodies using indirect ELISA (Table 2). Of these animals, 605 revealed anti-*C. burnetii* IgG antibodies in their sera, giving an overall serological prevalence of Q fever of 30.71%. The highest proportion of Q fever positive sera was recorded in camels, with an overall prevalence of 51.53% (Table 1). The next highest prevalence was recorded in goats (34.04%), followed by cattle (30.61%) and least in sheep (12.38%). The S/P ratio, which is proportional to the concentration of antibodies, ranged between 40.04 – 375.13 with an overall mean value of 103.03. The highest S/P ratio was recorded in cattle followed, in descending order, by camels, goats and sheep. Comparison of serological prevalence and titration results of Q fever in adult versus young animals showed that the prevalence of *C. burnetii* specific antibodies in adult animals was nearly twice that recorded in young animals (Table 3). On the other hand, comparison of prevalence in male versus female animals (Table 4) showed non-significant inter-sex difference (P= 0.5847)

Table 2: Q-fever prevalence and titration means by species

Species	Results	No.	%	Titration		
				Mean	Min	Max
Cow	*-ve*	297	69.39	8.35	0	39.18
	+ve	131	**30.61**	126.49	41.33	228.02
Camel	*-ve*	237	48.47	14.60	0	38.97
	+ve	252	**51.53**	113.37	40.47	375.13
Sheep	*-ve*	552	87.62	10.48	0	39.65
	+ve	78	**12.38**	60.66	40.07	184.00
Goat	*-ve*	279	65.96	8.97	0	39.54
	+ve	144	**34.04**	99.38	40.11	226.19
total	*-ve*	1365	**69.29**	11.62	0	39.65
	+ve	605	**30.71**	60.67	40.07	375.13

Table 3: Q-fever prevalence and titration means by age

Age	Results	No.	%	Titration		
				Mean	Min	Max
Adult	*-ve*	994	65.92	12.02	0	39.65
	+ve	514	**34.08**	106.54	40.04	375.13
Young	*-ve*	371	80.30	6.25	0	39.26
	+ve	91	**19.70**	103.51	42.42	226.19

Table 4: Q-fever prevalence and titration means by sex

Sex	Results	No.	%	Titration		
				Mean	Min	Max
Male	*-ve*	244	85.92	6.46	0	38.97
	+ve	40	14.08	66.62	40.90	202.08
Female	*-ve*	1121	66.49	11.32	0	39.90
	+ve	565	33.51	108.91	40.07	375.13

The animals were also compared according to their geographical location, which showed that the serological prevalence of Q fever varied widely from one location to another, with highest prevalence in Harad and lowest in Riyadh, Tabrak, and Amariah (Table 5). Furthermore, they were also categorized into intensively and extensively reared animals (Table 6).

Table 5: Q-fever prevalence and titration means by location

Location	Results	No.	%	Titration		
				Mean	Min	Max
Amariah	*-ve*	308	86.52	7.835	0	39.65
	+ve	48	**13.48**	98.27	40.67	216.55
Hait	*-ve*	34	30.09	22.27	4.04	36.64
	+ve	79	**69.91**	90.78	40.52	191.04
Harad	*-ve*	-	-	-	-	-
	+ve	19	**100**	271.29	96.23	375.13
Jouf	*-ve*	66	58.41	18.08	0	37.91
	+ve	47	**41.59**	98.02	40.62	237.13
Kharj	*-ve*	426	57.97	10.71	0	39.54
	+ve	335	**42.03**	112.81	40.07	228.02
Riyadh	*-ve*	180	86.96	6.03	0	38.97
	+ve	27	**13.04**	70.36	42.42	202.08
Tabrak	*-ve*	315	86.30	12.48	0	39.50
	+ve	50	**13.70**	64.21	40.90	177.90

Table 6: Q-fever prevalence and titration means by rearing system

Rearing system	Results	No.	%	Titration		
				Mean	Min	Max
Intensive	*-ve*	982	79.26	10.08	0	39.65
	+ve	257	**20.74**	101.15	40.62	237.13
Extensive	*-ve*	383	5+2.39	11.37	0	39.54
	Ve	348	**47.61**	109.74	40.07	375.13

Logistic regression analysis and estimation of odd ratios (OR) for the overall results are presented in tables 7 and 8, respectively. The results indicated highly significant differences in the prevalence of Q fever between different species of animals ($p<0.0001$). Similarly, highly significant differences were observed between different geographical locations ($P< 0.0001$) and between young versus adult animals ($p<0.0001$). On the other hand, no significant differences were recorded between male and female animals ($p=0.5847$). The odd ratio was estimated from logistic models, and calculated as the rate of odds for x=1 to the odds of x=0, in order to reveal the probability of the risk of being positive to Q-fever. The results showed that the risk probability

of adult animals being affected with Q-fever was twice the probability of young animals (OR = 2.11). On the other hand, male and female animals showed similar risk probabilities with odd ratio of 1.3, which indicated that the sex of the animal had no significant effect on the incidence of Q-fever.

Table 7: Q fever in animals: Logistic regression analysis

Source	Df	Wald Chi-square	Probability
Species	3	148.29	<0.0001
Sex	1	0.30	0.5847
Age	1	28.26	<0.0001
Location	7	102.04	<0.0001

Table 8: Logistic Regression and Estimation of Odd Ratio

Source	Df	Wald Chi-square	Probability
Species	3	81.43	<0.0001
Sex	1	0.35	0.5527
Age	1	11.41	0.0007
Rearing System	1	14.74	<0.0001
Location	6	31.06	<0.0001

Prevalence data and analysis of the results at the species level are presented in Tables 9 – 23.

Proc frequency and independency test results for the prevalence of Q fever in camels using chi-square are summarized in Table 9, while the results of logistic regression analysis and odd ratio estimates are presented in Tables 8 and 10, respectively. The results indicated significant differences in the prevalence of Q fever between breeds (p=0.05) and between adult versus young camels (p<0.05) and a highly significant difference between different geographical locations (p=0.001). On the other hand, no significant difference was recorded between male and female camels (p>0.05).

Table 9: Q fever in camels; proc frequency and independency test using chi-square

Factors		**Animals**		**Prevalence of Q-Fever**				**x^2**	**Prob**
				+ve		-ve			
		No.	%	*No.*	%	*No.*	%		
Breed:	*Magater*	93	36.33	53	**56.99**	40	43.01	11.93	0.0026
	Majahe	100	39.06	49	**49.00**	51	51.00		
	*Other**	63	24.61	48	**76.19**	15	23.81		
Age:	*Adult*	322	65.85	205	**63.66**	117	36.34	55.56	<.0001
	Young	167	34.15	47	**28.14**	120	71.86		
Sex:	*Female*	360	73.62	227	**63.06**	133	36.94	72.53	<.0001
	Male	129	26.38	25	**19.38**	104	80.62		

Location [1]:	*A*	23	4.70	19	**82.61**	4	17.39	107.2	<.0001
	B	113	23.11	79	**69.91**	34	30.09		
	C	19	3.89	19	**100**	0	0		
	D	113	23.11	47	**41.59**	66	58.41		
	E	101	20.65	66	**65.35**	35	34.65		
	F	120	24.54	22	**18.33**	98	81.67		

Location[1]: A: Amaria, B: Hait , C:Harad, D:Jouf, E: Kharj and F: Riyadh.
* mixture of *shu'l*, *humr* and *sufr* camels in relatively small number each.

Table 10: Logistic regression analysis of factors affecting prevalence of Q-fever in camels

Effect	Df	Wald	Pr>chi-square
		Square	square
Breed	2	8.19	0.0167
Sex	1	2.09	0.1483
Age	1	4.26	0.0390
Location	4	13.80	0.0080

Table 11: Q fever in camels: odd ratio (OR) estimates

Effect	Comparisons	OR	95% confidence	
Breed	*Maghater vs Majahem*	1.17	0.59	2.29
	Maghater vs Others	0.404	0.190	0.856
	Majahem vs Others	0.345	0.160	0.744

Sex	Female vs Male	3.87	0.62	24.21
Age	Adult vs Young	3.03	0.92	9.09
Location	Amaria vs Hait	1.82	0.57	5.80
	Amaria vs Jouf	6.32	2.01	19.88
	Amaria vs Jouf	2.29	0.72	7.32
	Amaria vs Jouf	14.45	2.26	92.59
	Hait vs Jouf	3.47	1.96	6.17
	Hait vs Kharj	1.26	0.71	2.25
	Hait vs Riyadh	7.95	1.65	38.32
	Jouf vs Kharj	0.36	0.21	0.64
	Jouf vs Riyadh	2.29	0.53	9.87
	Kharj against Riyadh	6.30	1.31	30.35

The proc frequency and independency results of Q fever prevalence in cattle using chi-square are presented in Table 12. Logistic regression analysis and OR estimates are presented in Tables 13 and 14, respectively. A highly significant difference in the prevalence of Q fever was recorded between adult and young cattle ($p<0.005$), while no significant effects due to either sex or location were recorded in these animals.

Table 12: Q fever in cattle: proc frequency and independency test using chi-square

Factors	**Animals**		**Prevalence of Q-Fever**				**x^2**	**Prob**
			+ve		-ve			
	No.	%	*No.*	%	*No.*	%		

Age:	*Adult*	320	74.77	126	**39.38**	194	60.63	45.9	<.0001
	Young	108	25.23	5	**4.63**	103	95.37	0	
Sex:	*Femal*	352	82.24	128	**36.36**	224	63.64	30.9	<.0001
	Male	76	17.76	3	**3.95**	73	96.05	2	
Location	*A*	341	79.67	126	**36.95**	215	63.05	31.7	<.0001
	B	87	20.33	5	**5.75**	82	94.25	8	

Location[1]: A:Kharj, and B:Riyadh

Table 13: Logistic regression analysis; factors affecting the prevalence of Q-fever in cattle

Effect	Df	Wald square	Pr>chi-squar Square
Sex	1	0.26	0.6132
Age	1	9.74	0.0018
Location	1	1.95	0.1624

Table 14: Q fever in cattle: odd ratio (OR) estimates

Effect	Comparisons	OR	95% confidence	
Sex	Female vs. Male	0.54	0.05	5.96
Age	Adult vs. Young	10.05	2.36	42.83
Location	Kharj vs. Riyadh	3.02	0.64	14.20

Proc frequency and independency tests of Q fever prevalence in sheep are presented in Table 15, while the results of logistic regression and OR estimates are presented in tables 16 and 17, respectively. The corresponding data for goats are presented in Tables 18-20.

Table 15: Q fever in sheep: proc frequency and independency Test using chi-square

Factors		Animals		Prevalence of Q-Fever				x^2	Prob
				+ve		-ve			
		No.	%	*No.*	%	*No.*	%		
Breed:	*Najdi*	336	53.85	33	**9.82**	303	90.18	20.95	<0.0001
	Naimi	206	33.01	22	**10.68**	184	89.32		
	Harri	82	13.14	23	**28.05**	59	71.95		
Age:	*Adult*	542	86.58	75	**13.84**	467	85.79	6.89	0.0087
	Young	83	13.42	3	**3.61**	80	96.43		
Sex:	*Female*	571	91.36	70	**12.26**	501	87.74	0.29	0.5870
	Male	54	8.64	8	**14.81**	46	85.19		
Location	*A*	126	20.16	20	**15.87**	106	84.13	5.57	0.0619
	B	252	40.32	22	**8.73**	230	91.27		
	C	247	39.52	36	**14.**	211	85.43		

Location[1]: A:Kharj, B:Amaria and C:Tabrak

Table 16: Logistic regression analysis; factors affecting prevalence of Q- fever in sheep

Effect	Df	Wald chi-square	Pr>chi-square
		Square	square
Breed	2	15.61	0.0004
Sex	1	4.72	0.0298
Age	1	4.49	0.0341
Location	2	6.11	0.0471

Table 17: Q fever in sheep: odd ratio (OR) estimates

Effect	*Comparisons*	OR	95% confidence	
Breed	*Najdi vs. Naimi*	1.45	0.73	2.90
	Najdi vs. Harri	0.21	0.05	0.41
	Harri vs. Naimi	6.98	2.45	19.87
Sex	*Female vs. Male*	0.34	0.13	0.90
Age	*Adult vs. Young*	3.92	1.11	13.86
Location	*Kharj vs. Tabrak*	3.09	1.21	7.87
	Amaria vs.	1.59	0.70	3.61
	Kharj vs. Amaria	1.95	0.97	3.91

The results revealed a significant effect of age, sex and location ($p<0.05$) and a highly significant effect of breed ($p<0.0005$) on Q fever prevalence in sheep. On the other hand, a significant age effect ($P<0.05$) and highly significant ($p<0.0001$)

breed and location effects on the prevalence of Q fever were recorded in goats while no significant effect was found due to sex (Table 18).

Table 18: Q fever in goats; proc frequency and independency test using chi-square

Factors		Animals		Prevalence of Q-Fever				x^2	Prob
				+ve		-ve			
		No.	%	*No.*	%	*No.*	%		
Breed:	*Ardi*	235	56.90	109	**46.38**	126	53.62	31.84	<.0001
	Dems	178	43.10	35	**19.66**	143	80.34		
Age:	*Adult*	309	74.82	108	**34.95**	201	65.05	0.004	0.9504
	Young	104	25.18	36	**34.62**	68	65.38		
Sex:	*Female*	388	93.95	140	**36.08**	248	63.92	4.17	0.0411
	Male	25	6.05	4	**16.00**	21	84.00		
Locatio	*A*	214	51.82	123	**57.48**	91	42.52	100.19	<.0001
	B	81	19.61	7	**8.64**	74	91.36		
	C	118	28.57	14	**11.68**	104	88.14		

Location[1]: A:Kharj, B:Amaria and C:Tabrak

Table 19: Logistic regression analysis; factors affecting prevalence of Q-fever in goats

Effect	Df	Wald Square	Pr>chi-square
Breed	1	22.37	<.0001
Sex	1	0.21	0.6493
Age	1	6.28	0.0122
Location	2	50.67	<.0001

Table 20: Q fever in goats; odd ratio (OR) estimates

Effect	Comparisons	OR	95% confidence	
Breed	Ardi vs. Dems	4.99	2.57	9.73
Sex	Female vs. Male	0.72	0.18	2.94
Age	Adult vs. Young	2.25	1.19	4.25
Location	Kharj vs. Tabrak	4.31	2.03	9.17
	Amaria vs.Kharj	0.19	0.05	0.68
	Kharj vs. Amaria	23.07	8.17	65.22

3.2. Prevalence of Antibodies against *C. burnetii* in Milk: A Comparison with Serum Using ELISA Tests:

In addition to serum tests, the indirect ELISA technique can also be used to detect specific antibodies against *C. burnetii* in milk samples of lactating animals. In the present study, ELISA test was performed on a total of 285 defatted milk samples obtained from lactating camels, cows, ewes and does. Of the total number tested, 84 milk samples were positive for anti-*C. burnetii* antibodies, giving an overall prevalence of 29.47% (Table 21), with S/P values ranging between 40.28 - 284.54. As with the ELISA serum results, the highest prevalence of antibodies against Q fever in milk were recorded in camels (62.50%), followed by cows (33.33%) and does

(27.59%), with S/P values. The highest S/P values were also recorded in camels. No specific anti-*C. burnetii* antibodies were detected in the milk samples collected from sheep.

Serum samples were collected simultaneously from the same milk-sampled animals (cows, camels, does and ewes) and tested for the prevalence of Q fever by ELISA. Details of these animals, along with the results of their serological testing, are given in Table 22.

Table 21: Results of ELISA Test for Q Fever in Milk Samples

Species	No.	Positive	%	Titration			
				Mean	+ST	Min	Max
Cattle	90	30	**33.33**	83.19	53.6	46.05	164.65
Camels	48	30	**62.50**	143.20	13.5	40.84	284.51
Goats	87	24	**27.59**	75.37	6.30	41.42	168.15
Sheep	60	0	**0**				
Total	**285**	**84**	**29.17**	**102.39**	**6.43**	**40.84**	**284.51**

Table 22: Results of ELISA Test for Q Fever in Serum Samples

Species	No.	Positive	%	Titration			
				Mean	+SD	Min	Max
Cattle	90	38	**42.22**	120.30	5.85	42.02	169.92
Camels	48	32	**66.67**	186.65	19.24	44.83	375.13
Goats	87	13	**14.94**	60.89	3.72	41.94	90.96
Sheep	60	4	**6.67**	57.65	9.35	41.30	79.70
Total	**285**	**87**	**30.53**	**132.64**	**9.01**	**41.30**	**375.13**

As shown in Tables 21 and 22, the overall prevalence rates of Q fever antibodies were closely comparable in milk versus serum samples (29.17% and 30.53%, respectively), and in both cases, the highest prevalence of Q fever was recorded in camels followed by cattle, then goats. Spearman's correlation analysis revealed a highly significant positive correlation (0.58) between ELISA milk and ELISA serum results (Table 23).

Table 23: Spearman correlation for results of milk with serum

Species	Correlation coefficient	Probability
Cattle	0.38	0.0001
Camels	0.61	<0.0001
Goats	0.41	0.0002
Sheep	0.69	<0.0001
Total	**0.58**	**<0.0001**

It is to be noted, however, that no results were obtained in the 60 milk samples collected from ewes, while only four serum samples were positive

by the ELISA serum test. This indicates the need for further testing of a larger number of samples of milk and serum from sheep.

3.3. Serological Prevalence of Q Fever (*C. burnetii*) in Animals: A Comparison between Immunofluorescence (IFA) and ELISA Assays:

Serum samples from 92 camels, 72 cows, 72 sheep and 71 goats were subjected simultaneously to indirect immuno-fluorescence assay (IFA) and indirect ELISA assay. The aim was to compare the prevalence of antibodies against *C. burnetii* using either test, and to determine the level of agreement between them. A horse-radish peroxidase-conjugated anti-camel IgG was used for the ELISA test in camels, while the monoclonal HRP-conjugated anti-ruminant IgG, supplied with the kit, was employed for ELISA tests of cows, sheep and goats. On the other hand, specific FITC-conjugated anti-species IgGs were used in IFA assays, namely FITC-conjugated goat anti-camel IgG; and FITC-conjugated rabbit anti-bovine, anti-sheep and anti-goat IgGs. The sensitivity and specificity of IFA for serological screening of *C. burnetii* in different species of animals was also investigated.

The results of the ELISA and IFA assays are summarized in Tables 24 and 25, respectively. Statistical analysis using Chi square showed highly significant differences in the prevalence of anti-*C. burnetii* antibodies between different animal species using either of these assays. Furthermore, the serological prevalence of Q fever as determined by the two assays was fairly comparable in the case of cows and camels, while a higher proportion of positive cases was recorded in sheep and goats using IFA as compared to ELISA. However, Spearman correlation analysis showed that correlation

between ELISA and IFA tests was highly significant in all animal species except sheep (Table 26).

Table 24: Comparison between ELISA and IFA tests for Anti-Q fever antibodies in farm animals: ELISA results

Species	Negative		Positive		x^2
	N	%	N	%	
Cattle	36	50.00	36	50.00	37.90**
Camels	28	30.43	64	69.57	
Goats	49	69.01	22	30.99	
Sheep	45	62.50	27	37.50	
Total	158	51.46	149	48.53	

Table 25: Comparison between ELISA and IFA tests for Anti-Q fever antibodies in farm animals: IFA results

Species	Negative		Positive		x^2
	N	%	N	%	
Cattle	33	47.22	37	52.78	12.94**
Camels	35	38.04	57	61.96	
Goats	20	28.17	51	71.83	
Sheep	15	20.83	57	79.17	
Total	104	33.23	209	66.77	

The means and ranges of S/P ratios (O.D.%) in ELISA-positive sera versus the antibody titres in IFA-positive sera, based on serial dilutions, are summarized in Tables 26 and 27, respectively. As noted earlier, the highest mean ELISA titre (S/P ratio) was recorded in camels, followed, in descending order, by cows, goats and sheep (Table 26). On the other hand, the highest mean IFA titre was recorded in cows, followed by camels, sheep and finally goats (Table 27). In both camels and cattle very high IFA titres equal to or exceeding 1:8192 were recorded in some animals; similarly, high IFA titres up to 1:4096 were also recorded in sheep and goats. Such high titres suggest active recent infections in these animal.

Table 26: Comparison between ELISA and IFA assays for Anti-*C. burnetii* antibodies in farm animals: ELISA titration.

Species	N	Mean	SE	Min	Max
Cattle	36	122.13	5.76	55.75	169.92
Camels	64	147.73	12.99	40.47	375.13
Goats	22	75.39	5.73	40.11	126.67
Sheep	27	72.39	7.77	40.40	184.00

Table 27: Comparison between ELISA and IFA assays for Anti-*C. burnetii* antibodies in farm animals: IFA titration

Species	N	Mean Titer	±SE	Min	Max
Cattle	38	1785.34	328.53	64	≥8192
Camels	57	793.82	213.60	64	≥8192
Goats	51	735.37	158.47	64	4096
Sheep	57	787.09	134.36	64	4096

The sensitivity and specificity percentages of IFA test on cut-off point equal 40 in different species of the tested animals are presented in table 28. The coefficients of correlation between the two assays are presented in table 29.

Table 28: Sensitivity and Specificity Percentages of IFA Test on cut-off point equal to 40

Parameter	**Species**				
	Cow	**Camel**	**Goat**	**Sheep**	**All**
Positive Predictive Value	91.67	82.81	59.18	88.89	88.59
Negative Predictive	86.11	85.71	40.82	26.67	55.06
False Positive Rate	8.82	31.43	0	20.00	16.35
False Negative Rate	13.16	7.02	56.86	57.89	32.98
Sensitivity	**86.84**	**92.98**	**43.14**	**42.11**	**65.03**
Specificity	**91.18**	**68.57**	**100**	**80.00**	**83.65**
Prevalence	52.78	61.96	71.83	79.17	66.12

N=307, Cow=72, Camel=92, Goat=71 and Sheep 72

Table 29: Coefficient Correlations of Florescent and ELISA Tests

Species	**Coefficient Correlation**	**Probability**
Cattle	0.78	<.0001

Camels	0.65	<.0001
Goats	0.42	0.003
Sheep	0.19	0.1189
All	0.46	<.0001

The relationship between ELISA and IFA tests was achieved using the PROC CORR. Additionally, The receiver operating characteristics (ROC) curves of IFA compared to ELISA test in these farm animals were carried out to compare the sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, positive predictive value and negative predictive value for detecting Q-fever using the Sigma Plot software (SigmaPlotv12.0, Systat Software Inc., San Jose CA,USA). The probability value, which denotes statistical significance, was declared at P<0·05 (Figs 10 - 14).

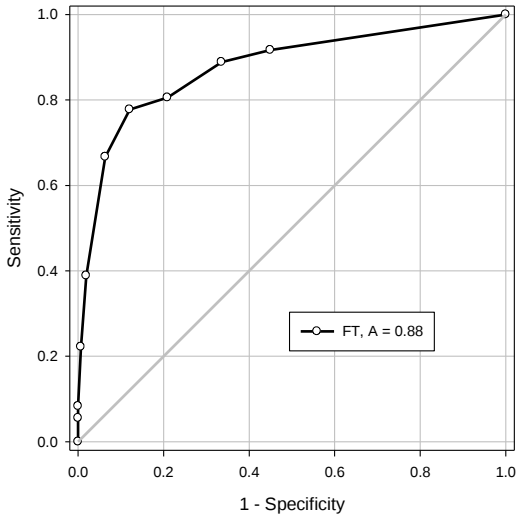

Figure 9: The sensitivity, specificity and area under the ROC curve of the IFA test to detect Q-fever in cattle.

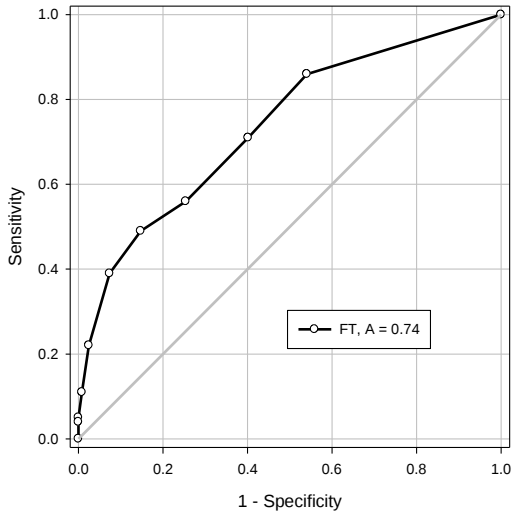

Figure 10: The sensitivity, specificity and area under the ROC curve of the IFA test to detect Q-fever in camels.

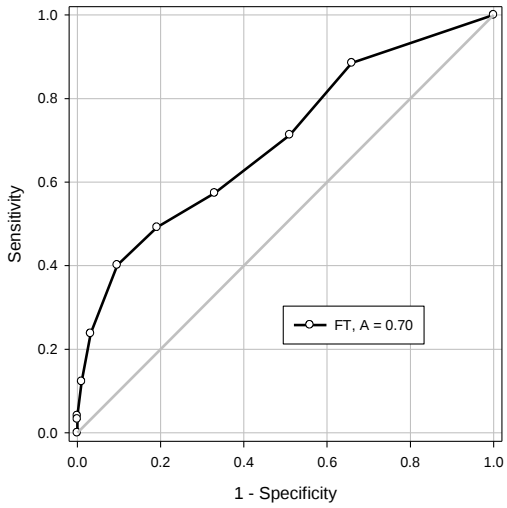

Figure 11: The sensitivity, specificity and area under the ROC curve of the IFA test to detect Q-fever in goats.

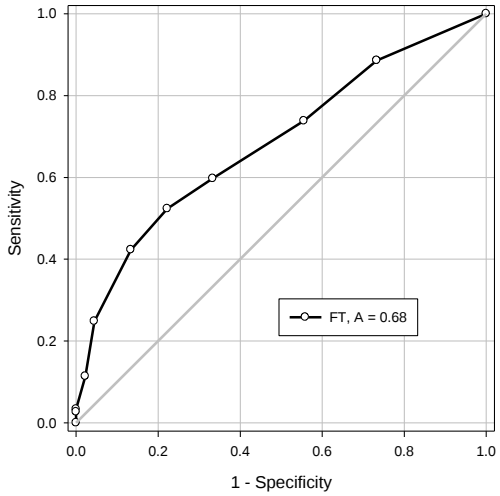

Figure 12: The sensitivity, specificity and area under the ROC curve of the IFA test to detect Q-fever in sheep.

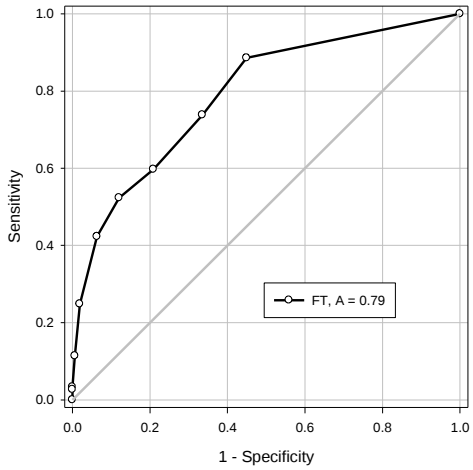

Figure 13: The sensitivity, specificity and area under the ROC curve of the IFA test to detect Q-fever in all data.

The reliability of the IFA test in detecting Q-fever in farm animals was also calculated using receiver operating characteristics (ROC). ROC Curve Area ± SE, 95% Confidence Interval, P Value, Sample Size and Negative Sample Size Positive are shown in table 30. The area under ROC curves were 0.88, 0.74, 0.70, and 0.68 for dairy cattle, camels, goats and sheep, respectively.

Table 30: Receiver operating characteristics (ROC) of fluorescence test (IFA) for detection of Q-fever in farm animals

Parameter	**Species**				
	Cow	**Camel**	**Goat**	**Sheep**	**All**
ROC Curve Area ±	0.88±0.04	0.74±0.03	0.70±0.04	0.68±0.04	0.79±0.02
95% Confidence	0.81-0.95	0.67-0.80	0.63-0.77	0.60-0.77	0.74-0.84
P Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Sample Size	158	122	94	45	158

Sample Size Positive	36	100	122	149	149

From these results, it is concluded that the indirect immuno-fluorescence (IFA) assay is highly sensitive and specific for testing Q fever in camels and cattle and less so in sheep and goats. It can therefore be used reliably for serological surveillance of Q fever in camels and cattle as an alternative to the ELISA test or for confirmation of ELISA results. On the other hand, it is suggested that a combination of IFA with another serological test, such as ELISA, be used for the screening of Q fever In small ruminants,

3.4. Detection of *C. burnetii* DNA Using Polymerase Chain Reaction (PCR):

Positive DNA amplification was obtained using primers that amplify the repetitive transposon-like region of *C. burnetii*, from blood, milk, faeces and urine of camels and from blood and milk of cattle (Table 31; Figs 15-18).

As shown in Table 30, out of 149 whole blood samples collected from different animal species, 13 samples (15.85%) from camels and 2 samples (5.6%) from goats showed positive amplification for *C. burnetii* DNA while all 22 sheep and 7 bovine samples were negative. Out of 144 milk samples collected from camels, cattle and goats, 5 samples (6.49%) from camels, 11 samples (28.94%) from cows and 0 samples from goats were positive for *C. burnetii* DNA. In addition, faecal samples collected from 29 camels and 20 goats also revealed positive PCR products from 8 (27.59%) and 12 (60%) samples, respectively. C. burnetii DNA was also demonstrated in 5 (23.81%) out of the 21 urine samples collected from camels.

Results of PCR results showed that *C. burnetii* shedding by camels was highest in faecal samples (27.6%) followed, in descending order, by urine, blood and milk. In goats, *C. burnetii* shedding was recorded in as high as 60% of the faecal samples tested. These findings suggest that faeces might be a major route for the shedding of this organism in both species. Bovine milk also appears to be an important source, with positive amplification of *C. burnetii* DNA being recorded in about 29% of samples tested. It should be pointed out, however, that these findings are of preliminary nature and should be corroborated by further analysis of samples from larger numbers of different species of animals. It is also imperative to evaluate *C. burnetii* shedding in birthing and fluids and cases of abortion in these animals.

Table 31: Detection of *Coxiella burnetii* DNA in samples from different animal species using PCR

Species	Sample	No Examined	No Positive	No Negative	% Positive
Camels	**Blood**	82	13	69	15.9
	Milk	77	05	72	6.5
	Faeces	29	08	21	27.6
	Urine	21	05	16	23.8
Cattle	**Blood**	07	00	07	00
	Milk	38	11	27	28.9
Goat	**Blood**	38	02	36	5.3
	Milk	29	0	29	0
	Faeces	20	12	8	60
Sheep	**Blood**	22	0	22	0

Figure 14. Electropherogram (2% agarose) showing amplification of the nested Polymerase Chain reaction of the *htpAB*-associated repetitive element of *Coxiella burnetii*. Lane (L) is the 100pb ladder, lanes 1-2 goat faeces, 3-4 camel faeces, 5-6 camel urine, 7-8 camel milk, 9-12 camel blood, lane 13 positive control.

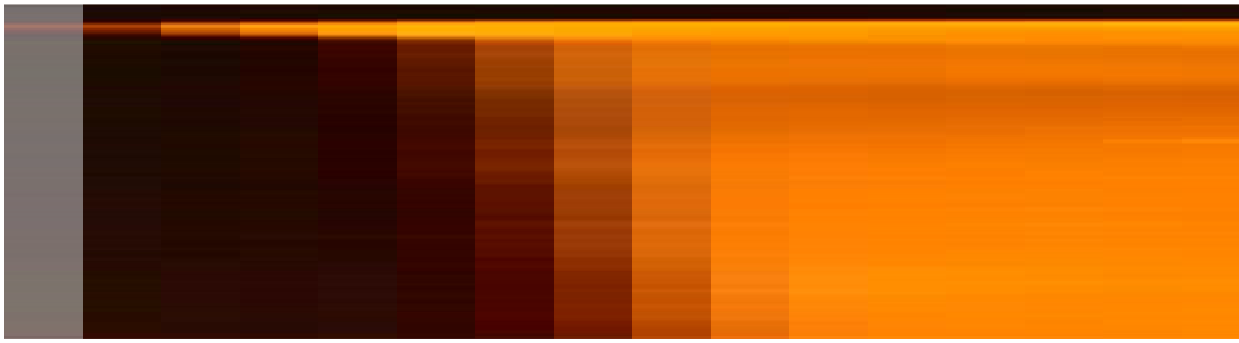

Figure 15. Electropherogram (2% agarose) showing results of the amplification of 448 bp of the transposase gene region of *Coxiella burnetii* using Cox P4 and CoxM9 primers. Lane (L) is the Hyper Ladder IV lanes 1-3 samples from camel blood; lanes 4-13 samples from camel milk.

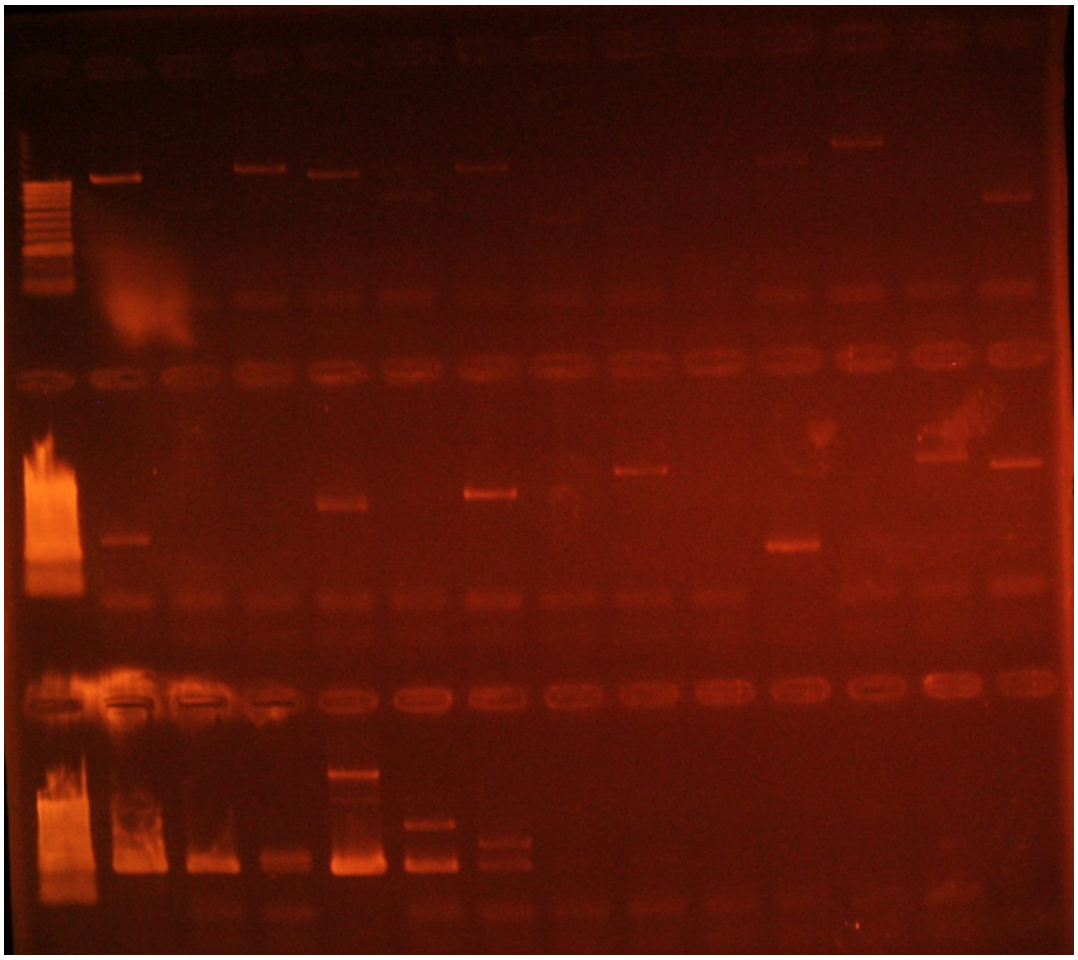

Figure 16. 2% agarose gel showing PCR products from the second round PCR of the *htpAB*-associated repetitive element of *Coxiella burnetii*. Some of the samples produced the expected fragment of DNA which is 260 bp whereas others failed to produce the expected PCR product which were regarded as negative. Even one which produced fragments which are longer than the expected fragments were regarded negative.

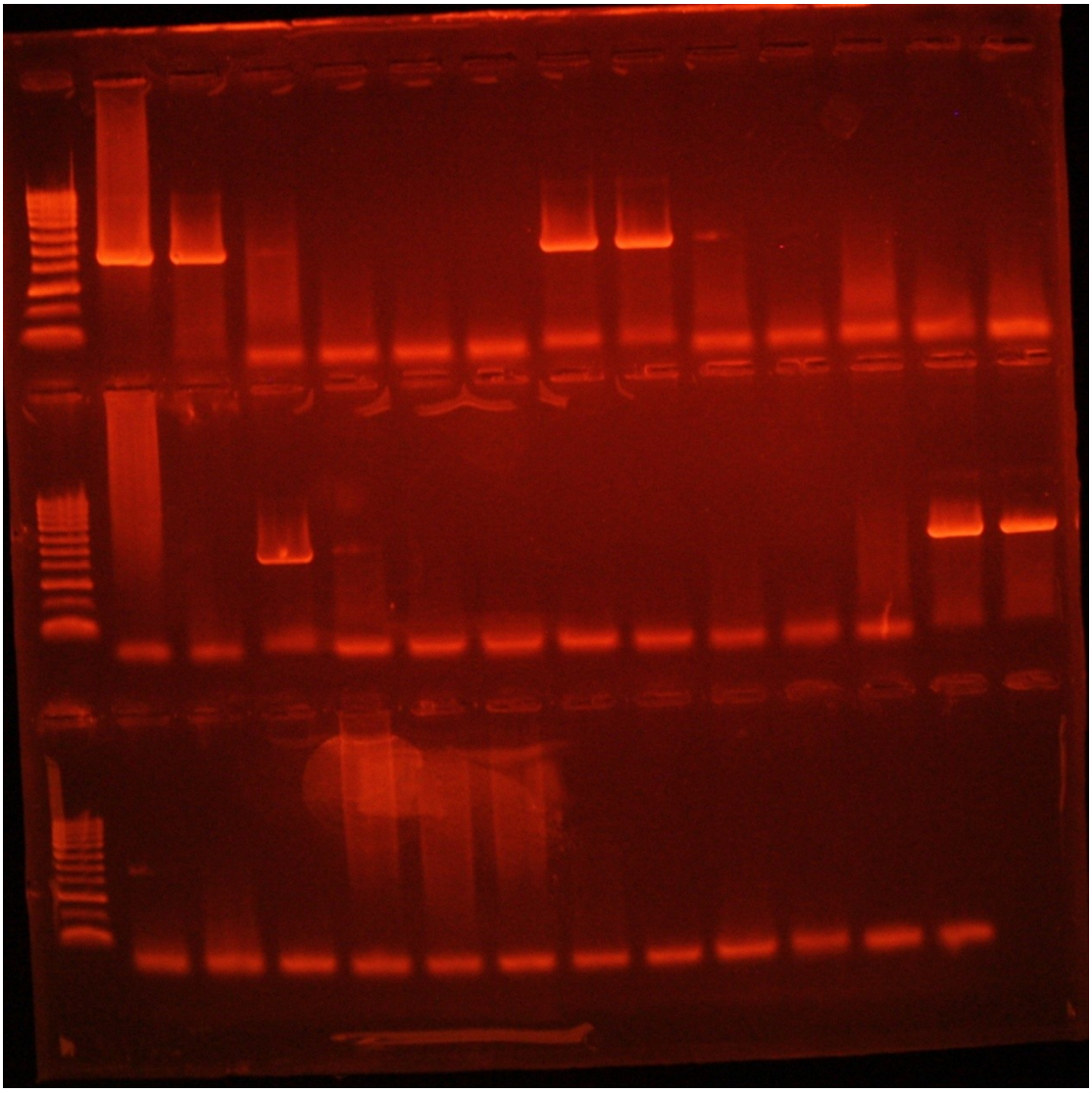

Figure 17. Electropherogram (2% agarose) showing results of the amplification of 448 bp of the transposase gene region of *Coxiella burnetii* using Cox P4 and CoxM9 primers. Samples from cow milk showing a PCR product of 448 bp.

3.5. Serum Biochemical and Inorganic Constituents and Anti-oxidant Enzymes:

Serum samples were collected from known Q fever-positive and known Q-fever negative animals for studying the possible effect of Q fever on biochemical and electrolyte parameters. A total of 281 serum samples were collected from camels, sheep, goats and cattle. Samples of cattle and goats were collected from 36 Q fever-positive and 36 Q fever-negative animals of each species. From camels, 36 samples were collected from positive and 30 samples from negative animals. From sheep 32 samples were collected from positive individuals while 39 samples were collected from negative individuals.

As stated under materials and methods, analysis of biochemical parameters and electrolytes was undertaken using a semi-automated biochemistry analyzer (UDICHEM-310 spectrophotometer) and commercial kits. Fourteen biochemical and electrolyte parameters were investigated. The studied parameters included TP, ALB, CREA, GLU, UR, BUN, TL, ALP, GGT, AST, CHOL, K, Na and Ca. GLOB concentration was derived by subtracting ALB concentration from TP concentration and the A/G ratio was thus calculated.

The results are summarized in Table 32. Generally, no significant differences were recorded in most of the studied parameters in all animal species. However, a few intra-specific differences existed within each species. Thus, in cattle significant differences existed between Q fever positive and Q fever negative animals regarding UR and BUN values, both of which were significantly higher in positive versus negative animals ($p<0.001$). On the other hand, CREA concentration was significantly lower in positive animals versus negative animals ($p<0.001$). Other values remained closely

comparable in positive and negative cattle. In camels, significant differences were observed in GGT, CHOL and Na values ($p<0.001$), both GGT and CHOL being higher and Na being lower in Q fever positive camels. In goats, significant differences were recorded between positive and negative animals in GLU, UR, BUN and GGT concentrations; all of these four parameters were lower in positive animals ($p<0.001$). In sheep, significantly higher CHOL and significantly lower CREA concentrations were recorded in positive as compared to negative animals ($p<0.001$).

Parameters	Cattle		Camel		Goat		Sheep	
	(NO.36)+ve	(NO.36) – ve	(NO 36) +ve	(NO. 30) ve	(NO. 32) +ve	(NO. 39) –ve	+ve (NO.36)	(NO.36) -ve
TP gm/dL	5.98 ± 0.11	6.38 ± 0.13	7.14 ± 0.11	6.99 ± 0.07	6.46 ± 0.16	6.66 ± 0.12	6.55 ± 0.12	6.37 ± 0.13
ALB gm/dL	2.97 ± 0.09	2.84 ± 0.08	4.30 ± 0.08	4.33 ± 0.07	3.63 ± 0.15	3.69 ± 0.13	3.70 ± 0.13	3.96 ± 0.11
GLOB gm/dL	3.04 ± 0.11	3.51 ± 0.16	2.84 ± 0.12	2.66 ± 0.09	2.83 ± 0.16	2.97 ± 0.15	2.85 ± 0.11	2.41 ± 0.11**
A/G Ratio	1.05 ± 0.07	0.90 ± 0.06	1.62 ± 0.09	1.71 ± 0.09	1.47 ± 0.13	1.41 ± 0.10	1.40 ± 0.09	1.79 ± 0.11**
CREA µmole/L	81.46 ± 2.35	97.41 ±2.91***	188.12±6.74	200.33±2.52	63.05 ± 1.79	58.26 ± 1.30*	66.00 ± 3.28	108.64±4.40**
GLU mmole/L	3.30 ± 0.19	3.08 ± 0.13	3.05 ± 0.26	3.54 ± 0.29	2.28 ± 0.12	2.96 ± 0.12***	1.89 ± 0.19	1.52 ± 0.17
UR mmole/L	7.27 ± 0.27	4.76 ± 0.16***	11.55±0.44	12.59 ± 0.29	4.19 ± 0.24	6.85 ± 0.51***	3.96 ± 0.27	4.37 ± 0.32
BUN mmole/L	7.30 ± 0.27	4.84 ± 0.15***	11.61 ± 0.45	12.74 ± 0.34*	4.21 ± 0.24	6.88 ± 0.51***	3.98 ± 0.27	4.39 ± 0.32
TL mmole/L	47.81 ± 3.30	48.55 ± 3.42	10.94 ± 0.65	19.92 ± 3.67*	20.19 ± 1.79	22.66 ± 1.92	1.88 ± 0.19	1.44 ± 0.15
ALP u/L	64.50 ± 4.46	62.01 ± 3.19	84.81 ± 7.58	73.07 ± 5.87	84.65±12.04	93.82 ± 7.23	116.35±14.41	144.34±13.29
GGT u/L	41.73 ± 3.25	49.22 ± 3.98	10.05 ± 1.47	5.60 ± 0.38**	34.40 ± 2.09	46.33 ± 1.90***	55.30 ± 1.85	51.28 ± 2.77
AST u/L	87.43 ± 4.78	94.09 ± 5.93	68.59 ± 2.98	62.39 ± 1.89	84.70 ± 3.89	87.96 ± 3.80	104.66 ± 5.34	98.31± 5.01
CHLO mmole/L	81.62 ± 2.37	88.80 ± 1.58*	114.73±4.02	135.54±2.72***	78.23 ± 1.21	82.86 ± 1.57*	99.76 ± 2.40	78.44 ± 1.31**
K mmole/L	4.88 ± 0.18	4.93 ± 0.15	11.36 ± 2.71	8.86 ± 0.21	9.00 ± 1.22	7.87 ± 0.23	6.34 ± 0.21	6.25 ± 0.23
Na mmole/L	138.75±3.03	130.96±5.91	113.88±7.57	157.86±6.30***	121.99±6.37	122.29 ± 4.79	136.48 ± 4.81	119.10 ± 3.46*
Ca mmole/L	3.36 ± 0.13	3.66 ± 0.16	4.0`2 ± 0.18	4.20 ± 0.18	3.04 ± 0.08	2.79 ± 0.06*	2.40 ± 0.09	2.26 ± 0.05

Table 32: Biochemical and inorganic parameters of negative and positive cases of Q fever in cattle, camel, goats and sheep in Saudi Arabia

With regards to anti-oxidant enzymes, thiobarbituric acid substances (TBARS) assay was carried out according to the method of Iqbal *et al*. (1998) on serum samples from a total of 239 known Q fever-positive and Q fever-negative animals, comprising 80 sheep, 80 camels and 79 goats. Reduced glutathione assays (GLUTH) were carried out using the method Jollo *et al*. (2003) on samples from a total of 188 Q fever-positive and Q fever- negative animals, also comprising goats (n= 72), camels (n= 60) and sheep (n= 56). No samples were available from bovines. The results obtained from these two antioxidant assays are presented in Table 33. No significant difference in the level of TBARS between Q fever-positive and Q fever negative was found in samples collected from each of goats, camels and sheep. On the other hand, while no significant difference in GLUTH level was recorded between positive and negative sheep and goat, the level of GLUTH was found to be significantly reduced ($p<0.002$) in Q fever positive camels as compared to Q fever negative camels. This enzyme is found in the cytoplasm of nearly all mammalian cells and is responsible for reducing hydrogen peroxide into water and hydroperoxides to alcohols. Hence, a reduction of its activity could be an indication of cellar damage.

Table 33: Antioxidants concentration in the blood of Q fever negative and positive goats, camels and sheep.

arameter	Goats		Camel		Sheep	
	+ ve	- ve	+ve	-ve	+ve	-ve
TBARS	(No. 45)	(No. 35)	(No 54)	(No. 26)	(No. 37)	(No. 42)
umol/ml	4.91 ± 0.29	5.07 ± 0.30	3.52 ± 0.19	3.43 ± 0.23	2.75 ± 0.30	3.31 ± 0.53
	+ve	-ve	+ve	-ve	+ve	-ve
GLUTH	(No. 40)	(No. 32)	(No. 30)	(No. 30)	(No. 28)	(No. 28)
umol/ml	1.98 ± 0.29	1.86 ± 0.29	0.96 ± 0.15***	3.22 ± 0.29	2.49 ± 0.40	2.49 ± 0.29

CHAPTER FOUR

DISCUSSION

The prevalence of Q fever in man and animals in the Kingdom of Saudi Arabia is unknown, and most people, including many veterinarians, are not even aware of its existence in that country. A thorough search of the literature revealed a dearth of information regarding the status of this important zoonotic disease in the Kingdom, even though it has been described as holoendemic among the inhabitants of the Kingdom since the 1960's. So far, only two reports, published more than 50 years apart, are found in the literature on Q fever in humans in Saudi Arabia (Gelpi, 1966; Almogren *et al*., 2013). Likewise, only two preliminary reports exist on the serological prevalence of Q fever in animals, one in camels (Hussein *et al*., 2008) and the other in three species of wild ruminants examined at KKWRC in Thumamah (Hussein *et al*., 2012). This paucity of information is striking given the astonishingly high infectivity of the Q fever agent (*Coxiella burnetii*), its ubiquitous distribution and its zoonotic nature. In other parts of the world, a large volume of literature is regularly published on human and animal coxiellosis (Q fever), and the disease is attaining increasing significance in many areas as a result of increasing agricultural activities, oversight of the infection in animals and its misdiagnosis or under-diagnosis in humans (Yoshii *et al*., 1991).

In the present study, detailed investigations into the prevalence and epizootiology of Q fever in indigenous farm animals, namely camels, cattle, sheep and goats, in Saudi Arabia have been undertaken, in which a combination of serological tests and PCR were used. Nearly 2000 serum samples were randomly collected from different species of animals and tested by the indirect ELISA procedure for antibodies against *C. burnetii*. Not unexpectedly an overall prevalence rate exceeding 26% was recorded in the Kingdom's farm animals, with species like the camel showing a

prevalence exceeding 50%. This high prevalence rate is a clear indication that Saudi Arabia is an important endemic focus of Q fever.

More than 51% of 489 camels tested during this study turned out to be serologically positive for Q fever, the highest prevalence rate among animals in the Kingdom. In a previous study by Hussein *et al*. (2008) an even higher Q fever seroprevalence, amounting to 62%, was recorded in Saudi camels. Similarly, a high prevalence rate (66%) was recorded in camels in Egypt (Soliman *et al*., 1992) while in Chad; the prevalence of Q fever in camels was as high as 80% (Schelling *et al*., 2003). Such alarmingly high prevalence rates, coupled with the wide spread tradition of consuming raw camel milk, underscore the leading role that camels appear to play in the transmission of Q fever to humans in this part of the world. Camel Q fever has virtually been reported wherever these animals are kept (Wernery and Kaaden, 1995; Scrimgeour *et al*., 2003; Mostafavi *et al*., 2012) indicating that their role in the transmission of Q fever to humans might extend beyond the Arabian peninsula, and the association between Q fever in camels and Q fever in their owners should be studied along with analysis of the different risk factors involved, particularly the consumption of raw camel milk.

The present study is the first record of Q fever in cattle, sheep and goats in Saudi Arabia and the second, and the only detailed, investigation of its prevalence among camels in that country. In goats, the serological prevalence reached around 34%, the second highest prevalence after camels, while in cattle, the overall prevalence was around 30%, more than double that recorded in sheep (12.8%). Q fever or coxiellosis in domestic ruminants has been widely reported in the Middle East (Wernery and Kaaden, 1995; Cetinkaya *et al*., 2000; Scrimgeour *et al*., 2003; Khalili and

Sakhaee, 2009; Sakhaee and Khalili, 2010; Kennerman *et al*., 2010; Mostafavi *et al*., 2012; Kshash, 2012; Asadi *et al*., 2013), Europe (Lyytikainen *et al*., 1998; Arricau-Bouvery *et al*., 2003; Masala *et al*., 2004; Parisi *et al*., 2006; Psaroulaki *et al*., 2006; Oporto *et al*., 2006; Rousset *et al*., 2007; Cekani *et al*., 2008; Pape *et al*., 2009; Czopowicz *et al*., 2010; McCaughey *et al*., 2010; Ruiz-Fons *et al*., 2010; Rodríguez *et al*., 2010; Schimmer *et al*., 2011; Cantas *et al*., 2011; Roest *et al*., 2012; Hogerwerf *et al*., 2013), Asia (Randhawa *et al*., 1973; To *et al*., 1998; Hirai and To, 1998; Giangaspero *et al*., 2012), Africa (Adesiyun *et al*., 1985; Reinthaler *et al*., 1988; Schelling *et al*., 2003; Mohammed *et al*., 2012), North America (Lang, 1988; Lang *et al*., 1991; McQuiston *et al*., 2002; Bjork, and Anderson, 2011) South America (Somma-Moreira *et al*., 1987; Hérnandez *et al*., 2007; Lemos, *et al*., 2011; Araujo-Meléndez *et al*., 2012) and Australia (reviewed by Cooper, 2011). In these different localities, the prevalence of Q fever among farm animals varies considerably between different animal species and for the same species in different countries. This suggests that the relative importance of each animal species as a reservoir of Q fever varies from one country to the other.

The occurrence of a significantly higher prevalence of Q fever in adult animals compared to young animals in the present study is concordant with previous observations in man and animals. In humans, the prevalence of Q fever was shown to be much higher in adult than young individuals (Marrie, 1995; Cardenosa *et al*., 2006; Gilsdorf, 2008; McCaughey *et al*., 2008), reaching its peak between the ages of 45 – 60 years (Marrie and Pollak, 1994). Similarly, higher prevalence of Q fever in different species of farm animals was recorded in adult versus young animals (Kilic *et al*., 2005; Hussein *et al*., 2008; Astobiza *et al*., 2012). It

should be pointed out; however, that *C. burnetii* infection can occur at any age. Among the animals presently tested and found positive for Q fever was a one day old lamb, which could have been infected *in uterus*. On the other hand, it was evident from the present study that no difference in the prevalence of Q fever existed between male and female animals in all the species except sheep. This is also concordant with previous observations in man and animals showing the absence of a statistically significant sex effect on the prevalence of Q fever (Tissot Dupont *et al*., 1992; Sanzo, 1993; Raoult, 1999; Nakaoune *et al*., 2004; CDC 2006; Gilsdorf *et al*., 2008; Hussein *et al*., 2008).

In general, the prevalence of Q fever in man and animals is increasing and the relative importance of each animal reservoir is also changing; in many countries, the disease is considered as an emerging or re-emerging disease (Arricaou-Bouvery and Rodolakis, 2005). Massive outbreak of Q fever, involving more than 4000 persons, have been reported in recent years in the Netherland in which goats were found to be the primary reservoirs of the infection (Speelman, 2010; Roest *et al*., 2011). The prevalence of Q fever not only varies between different countries but also between different geographical localities within the same country. This observation was confirmed in the present study which showed that the location from which the animal samples were obtained had a marked effect on the prevalence of Q fever in different species of animals.

In the present study, the absence of overt clinical signs of Q fever in the infected animals was not unexpected, since this disease is usually asymptomatic in animals (Maureen and Raoult, 1999). Occasionally, however, clinical signs of Q-fever might be observed, especially if the animals are subjected to stress, such as late pregnancy (Enright *et al*.,

1969); in that case stillbirth, retention of the placenta, placentitis, endometritis or inflammation of other parts of the female reproductive tract might be observed in cattle (Billdfell *et al*., 2000), sheep (Marmion and Watson, 1961; Crowther and Spicer, 1976; Palmer *et al*., 1983; Zeman *et al*., 1989; Hatchette *et al.*, 2001; Masala *et al*., 2004) and goats (Crowther and Spicer, 1976; Waldhelm *et al*., 1978; Palmer *et al*., 1983; Giovanna *et al*., 2004; Berri *et al*., 2005).

One of the objectives of the present study was to compare the use of ELISA in milk instead of serum for the detection of antibodies against *C. burnetii* in lactating animals. This was considered necessary since milk sampling is a non-invasive procedure compared to blood sampling and is therefore less likely to be resented by animal owners, particularly cattle and camel owners. It is also inexpensive and easier to perform and is less likely to be subjected to environmental contamination compared with other animal secretions such as urine, faeces and vaginal secretions (Roest *et al*., 2013). Also with milk sampling available, the collection of blood samples for Q fever serological screening will be limited to male animals and young, dry or non-lactating females. A number of authors previously investigated the use of ELISA and/or IFA for the detection of antibodies against *C. burnetii* in milk. Using ELISA, Astobiza *et al*. (2012) estimated the prevalence of *C. burnetii* in bulk milk samples from 178 dairy cattle herds in Spain. Anti-*C. burnetii* antibodies were recorded in about 57% of these samples. They also collected sera from 1,306 cows, 654 heifers and 502 calves from these herds for analysis by ELISA. Of these animals, 1019 (41.31%) were serologically positive for *C. burnetii*. A significantly higher serological prevalence was recorded in cows than heifers while none of the calves was positive. Statistical analysis showed a significant correlation between BMS and serum ELISA results. In the

United Kingdom, Paiba *et al*. (2012) used an ELISA test for the detection of IgG antibodies against *C burnetii* in randomly selected bulk milk samples from dairy cows and recorded serological evidence of infection in 21% of the samples.

The results of the present study agree with those reported by Guatteo *et al*. (2007) who compared the performance of an ELISA test as applied to either milk and serum for the detection of anti-*C. burnetii* antibodies in dairy herds. Out of a total of 448 cows tested, 264 serum samples and 257 milk samples were found to be positive. The level of agreement between the results of serum and milk testing, as determined statistically, was very good, with kappa=0.89. Based on these findings, these authors concluded that the ELISA test applied to milk offered a convenient tool for establishing the serological status of *C. burnetii* infection in lactating dairy cows. The present results also agree with Astobiza *et al.* (2012) in that a good level of agreement existed between the results of ELISA test for antibodies against *C. burnetii* in milk as compared to serum and that the prevalence of anti-*C. burnetii* antibodies was significantly higher in adult animals as compared to younger ones.

Also in the present study, a comparison was made between the performance of ELISA and IFA tests for serological diagnosis of Q fever in different species of farm animals. The IFA is commonly used for the serological diagnosis of Q fever in humans. Its use in animals is, however, limited (Berri *et al*., 2000; 2001; Rousset *et al*., 2009; Dogru *et al*., 2010) and in most cases, commercial fluorescene-conjugated antihuman immunoglobulin's were used instead of using species-specific FITC-conjugated immunoglobulin's, thus making the reliability of the results questionable. In the present study, we used FITC-labeled anti camel, anti-bovine, anti-sheep and anti-goat IgGs for IFA tests in these species. Using ELISA as a reference method, statistical analysis showed

high sensitivity and specificity of IFA, as well as a strong correlation between the IFA and ELISA tests, indicating that either test can be used to confirm or replace the other, provided that specific FITC-conjugated anti-species IgG are used.

Because of their high sensitivity and specificity, ease of performance and cost effectiveness, ELISA, IFA and other similar serological assays are the methods of choice for surveillance of Q fever in animals (Field *et al*., 2000; Kirkan *et al*., 2004; Angelakis and Raoult, 2009). However, the results of these tests are not necessarily correlated with active shedding of *C. burnetii*. Some serologically positive animals might reflect past infections and are no longer actively shedding *C. burnetii*, whereas some serologically negative animals might be actively shedding the organism. The detection of *C. burnetii* DNA in body fluids of animals by PCR is more reliable for identifying shedders. In a study on the relationship between *C. burnetii* shedding and serological response, Berri *et al.* (2001) tested serum and vaginal swabs from 36 pregnant ewes at parturition for *C. burnetii* antibodies using ELISA and IFA tests, respectively, and compared the results with PCR analysis. Serum samples from 8 ewes (24%) were positive by ELISA. Vaginal swabs were positive in 11 (32%) ewes by IFA and 15 (44%) by trans-PCR. Of the latter animals, the PCR analysis also showed that 8 (25%) ewes shed *C. burnetii* in their milk and 6 (18%) shed the organism in their feces, However, when the same animals were retested 4-5 weeks later by ELISA and PCR, 16 (44%) were ELISA-positive while only 2 (6%) were positive by PCR.

In the present study, *C. burnetii* DNA was detected in clinical samples of camels, sheep, goats and cows. DNA amplification of *C. burnetii* was obtained from blood, milk, faeces and urine of camels. *C. burnetii* DNA was also detected in the blood and faecal samples investigated from

goats. From cows PCR amplification was obtained from the milk but not from the blood. On the other hand, none of the blood samples available from sheep showed a positive amplification of *C. burnetii* DNA.

From the results obtained, it appears that the camel is likely to shed *C. burnetii* organisms through milk, blood, faeces and urine. Unfortunately, vaginal fluid was not available for testing from camels and other species. Vaginal swabs and birthing fluids have been reported by several authors as major routes of *C. burnetii* shedding, It is therefore important to carry out PCR analysis of these fluids for C. burnetii DNA in indigenous livestock in Saudi Arabia. As stated earlier, Q fever has been reported from camels in many countries (Hussein *et al*., 2008). These reports were based exclusively on serological evidence apart from a study conducted by Doosti *et al.* (2014) which showed evidence of *Coxiella burnetii* DNA in the blood of Iranian camels. The present study is superior and more comprehensive compared to Doosti *et al*. (2014). In the present study DNA of this organism was demonstrated from the blood, and, for the first time anywhere, from milk, faeces and urine of camels. Doosti *et al.* (2014) demonstrated prevalence of the organism in camel blood as (10.8%). In the present study, it has been shown that the highest level of organism was found in the faeces followed, in descending order, by urine, blood and milk. These results suggest that among the clinical samples tested, the most suitable route of discharge for the organism was the faeces followed by urine. We have tested the presence of *C. burnetii* DNA from different clinical samples of camels, because *C. burnetii* may, as already stated, be shed by routes other than blood e.g., vaginal mucus, urine, faeces and birth fluids (Maurin *et al.*, 1999; Kirkan *et al*., 2008). Therefore, testing animals only on blood samples can lead to misclassifying the status of the animal and misidentifying the commonest

route of excretion or discharge (Guatteo *et al*., 2006). Therefore the work of Doosti *et al*. (2014) is not telling exactly which route is preferred for the *C. burnetii* discharge. The differences between the prevalence of *C. burnetii* DNA in bovine, ovine, and caprine milk samples found in some studies may be due to the fact that there are different routes of shedding the organism in different animal species. According to Rodolakis *et al*. (2007), ovines shed the organism mainly in the faeces and vaginal mucus while bovines shed the organism in milk. Caprines shed the organism via vaginal discharges, faeces and milk. The absence of *C. burnetii* DNA from the sheep samples in the present study could be attributed to the fact that in this animal species the organism is shed primarily via vaginal mucus and faeces which have not been tested in the present study and this probably confirms that milk and blood are not the preferred routes of discharge for *C. burnetii* in sheep. It could also be partly attributed to the small number of samples tested. Goats were previously reported to shed the organism through vaginal discharge, faeces and milk (Rodolakis *et al*. 2007). In the present study, these animals seemed to have shed *C. burnetii* through blood and faeces but not the milk, with the faeces showing a high rate of discharge. Detection of *C. burnetii* DNA in the milk of cows confirmed the importance of this route of transmission in bovines as previously reported (Kim *et al*., 2006; Rahimi *et al*., 2010). Most of the recent PCR-based studies on the prevalence of *C. burnetii* in dairy animals are conducted on bulk milk samples (Kim *et al*., 2005; Fretz *et al*., 2007; Rodolakis *et al*., 2008; Rahimi *et al*., 2010).

Molecular studies in goats and other animal species are limited due to lack of simple and sensitive detection tools. Shedding of *C. burnetii* in goats via vaginal mucus, faeces, and milk lasted for 1-5 weeks, 2-5 weeks and 1 day to 6 weeks, respectively (Hatchette *et al*. 2003; Berri *et al*.

2005; 2007). Only techniques allowing the direct identification of *C. burnetii* shedders appear to be informative for assessing the actual route of transmission of the infection. Hence, detection of *C. burnetii* in milk or blood significantly depends on the sampling time. The use of repeated sampling can reduce the likelihood of erroneously identifying herds as Q fever negative (Guatteo *et al.* 2007). It is likely that the sample collection in the present study has coincided with the shedding period for some individuals and not for others; therefore, frequent sampling in affected herds is recommended in any epidemiological studies dealing with Q fever in goats. The present study has for the first time presented information about the direct detection of *C. burnetii* in camels, goats and cows in Saudi Arabia. It is also the first world record of *C. burnetii* in camel's milk, urine and faeces. The current study again shows clearly that camels are an important reservoir of *C. burnetii* and that they pose a significant public health hazard for the transmission of Q fever to humans in areas in which they are reared.

The PCR analysis showed that the shedding of *C. burnetii* by camels was highest in faecal samples (27.6%) followed, in descending order, by urine, blood and milk. In goats, *C. burnetii* shedding was recorded in as high as 60% of the faecal samples tested. These findings suggest that faeces might be a major route for the shedding of this organism in both species. Bovine milk also appears to be an important source, with positive amplification of *C. burnetii* DNA being recorded in about 29% of samples tested. By contrast, the present study yielded negative PCR results from 29 caprine milk samples. In Iran, only 1 of 56 caprine bulk milk samples obtained from 20 farms and none of 110 ovine bulk milk samples from 31 farms were reported (Rahimi *et al*., 2009) suggesting that milk might not be an important source of *C. burnetii* shedding in

small ruminants. The goat result are partly at variance with those of Rodolakis *et al.* (2007) in France who reported that goats excreted the bacteria mainly in milk while ewes, which came from flocks with abortions due to Q fever, shed the bacteria mostly in feces and in vaginal mucus. It is thus possible that in goats, and probably other species, the shedding is intermittent. According to Rodolakis *et al.* (2009) cattle, in contrast to sheep and goats, do not appear to shed *C. burnetii* in their faeces. Interestingly, these authors also noted that *C. burnetii* shedding in these different animals was not associated with parturition. The absence of *C. burnetii* DNA in 7 bovine blood samples tested with PCR in the present study suggests that blood might not ideal for PCR detection of *C. burnetii* in cattle. In this regard, Kirkan *et al*. (2008) recorded only 6 (4.2%) PCR-positive cases in 128 blood samples collected from 8 farms in Turkey.

It should be emphasized, however, that the above findings are of preliminary nature and should be corroborated by further extensive analysis of samples from different species of animals, especially since some studies indicated that PCR-positive rates of Q-fever infection varied widely (Ogawa *et al*., 2004). It is also imperative to evaluate *C. burnetii* shedding in birthing and fluids and cases of abortion in these animals.

The biochemistry results obtained in the present study showed variation in some biochemical parameters between Q fever positive and negative individuals from the animal species studied. An earlier study by Hussein *et al*. (2012) on the effect of Q fever on some biochemical parameters in wildlife showed that the values of total protein and creatinine increased while the value of ALP decreased in animals positive to Q fever compared to those showing negative reaction. In the present study there were no significant differences in the values of the total protein between positive and negative animals in all the species studied. The biochemical

values which have been recorded from camels, cattle and sheep during the present study agreed with some and varied with other values given for Saudi animals (Osman and Busadah, 2003). The latter authors, however, obtained their samples from few individuals and there could be several factors which affected the results.

Finally, the antioxidant status of Q fever positive versus Q fever negative animals was evaluated using Thiobarbituric Acid Reactive Substances (TBARS) and reduced glutathione assays. The level of TBARS was comparable in Q fever positive and negative animals, while glutathione was decreased in positive camels. Glutathione is thought to be among the fundamental antioxidant enzymes due to its close relationship to the direct elimination of reactive oxygen speices. Its level remained unchanged in positive and negative sheep and goat. However, the significant reduction of the activity of this glutathione in Q fever positive camels may result in a number of deleterious effects due to the accummulation of superoxidase radicals and hydrogen peroxide linked to neurodegenration (Fang *et al*., 2002). Reduction in GLUTH could indicate some degree of cellular damage. However, in the present study, it can not be concluded that the effect was due to the infection with Q fever since the mechnisms underlying the effect of the Q fever on this enzyme are still unknown. It is likely that the glutathione level is reduced due to its consumption to counteract free radical produced as a result of Q fever but this requires more biochemical, pthological, and pharmacokinetic research to establish the role of Q fever in reducing the level of glutathione in camels and not in sheep and goats.

CONCLUSION AND RECOMMENDATIONS

Conclusion:

- Q-fever is a major zoonosis in the Kingdom of Saudi Arabia. Its causative agent, *Coxiella burnetii*, is known for its high infectivity and resistance to environmental factors.
- The prevalence of *C. burnetii* is high among indigenous camels, cattle, goats and sheep in the Kingdom.
- Diagnosis and screening of *C. burnetii* is usually based on serological tests.

- Both indirect enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) is sensitive and specific, economically feasible and easy to perform. Therefore, either of them can be used for large-scale screening of *C. burnetii* in animals, and either can use as a confirmatory test for the other.
- Results of ELISA testing for anti-*C. burnetii* antibodies in milk match those obtained in serum. Since milk sampling is cheaper and less invasive than serum sampling, the prevalence of antibodies against this organism in lactating animals can be monitored using milk.
- Infected farm animals shed this organism in their milk, urine, feces, blood and birthing fluids.

Recommendations:

- Q fever is primarily an air-borne infection in man and farm animals, and its prevention is aimed at minimizing exposure to infected animal, animal products and environmental contamination. Several methods of prevention and control are currently aimed at cattle, sheep and goats; however, such measures can also be effective in preventing transmission to camel as well as wildlife at the interface with domestic livestock.
- People at high risk, such as farmers, animal owners, slaughter-house workers, milk and meat processing plants workers, as well as veterinarians and laboratory technicians must be educated about the importance of Q fever and ways to protect themselves and their animals. A very important aspect of prevention is the avoidance of drinking raw camel milk as it is a common practice in Saudi Arabia and most of the Arabian countries. Pasteurization of milk is essential and is considered a crucial factor in preventing transmission of the Q fever agent to humans.
- A national Q fever management program in the Kingdom of Saudi Arabia should be initiated in collaboration with the Ministry of

Agriculture as well as Wildlife Authorities in which screening of domestic livestock for Q fever should be performed on a regular basis and the implementation of strict control programs especially in dairy camel and cow farms.

- Further studies should be undertaken regarding the introduction of modern DNA-based methods for the diagnosis of Q fever for accuracy and determination of the animals' shedding patterns of the Q fever agent in animal herds.
- Infected fecal material from contaminated paddocks should never be spread to uninfected grounds. Fecal material from infected animals should be treated with 0.4% calcium cyanamide (Lime), to reduce the level of environmental contamination, and minimizing the chances of transmission to other co-grazing animal species as well as humans. Animal facilities and utensils especially used in milk storage should be also disinfected and kept clean all the time
- Strict quarantine measures should be applied when introducing new animals. This will significantly help in reducing the chances of Q fever agent being introduced to the original, uninfected herd.
- Separation of pregnant animals in an isolated facility due to increased risk of shedding the Q fever agent by this group of animals.
- Removal and deep burial of placenta/aborted fetus must be done immediately to prevent ingestion by domestic cats, dogs and wildlife and other susceptible animal species..

- People who work with animals or materials that may carry the Q fever agent should use appropriate protective equipment and be aware of the steps required to stop the spread of the bacteria. Such measures include:

- washing hands and arms thoroughly in soapy water after handling animals or animal products
- washing animal urine, feces, blood and other body fluids from the work site and equipment, and disinfecting equipment and surfaces where practicable
- minimizing dust and removal of rodents in abattoirs and animal housing areas
- keeping yard facilities for sheep and cattle well away from domestic living areas
- removing protective and/or other clothing that may carry the bacteria before returning to the home environment
- Proper disposing of animal tissues including birthing products. This usually involves burial under a half to one meter of soil or preferably incineration.
- Avoiding the consuming of unpasteurized milk.

- The status of Q fever among people in Saudi Arabia is unknown. Health authorities should therefore be encouraged to study the prevalence and epidemiology of the disease among the inhabitants of the Kingdom.
- Q fever cannot be eradicated but its prevalence can be reduced. In view of the high prevalence of *C. burnetii* in farm animals in Saudi Arabia, therefore, immunization of people who are occupationally exposed to Q fever should be seriously considered. Immunization of animals against *C. burnetii* should also be given due consideration.

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Figure 3: Q fever ELISA testing unit