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EPIDEMIOLOGICAL STUDY ON CONTAGIOUS BOVINE PLEUROPNEUMONIA AND FARMERS KNOWLEDGE, ATTITUDE AND PRACTICE TOWARDS THE DISEASE IN SELECTED DISTRICT OF EAST WOLLEGA AND WEST SHOWA ZONES, WESTERN ETHIOPIA

MVSc. Thesis

BY

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DEPARTMENT OF CLINICAL STUDIES

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EPIDEMIOLOGICAL STUDY ON CONTAGIOUS BOVINE PLEUROPNEUMONIA AND FARMERS KNOWLEDGE, ATTITUDE AND PRACTICE TOWARDS THE DISEASE IN SELECTED DISTRICT OF EAST WOLLEGA AND WEST SHOWA ZONES, WESTERN ETHIOPIA

A Thesis submitted to School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary epidemiology.

By

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June, 2017

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by: **Tesfaye Mersha** titled: **"Epidemiological Study on Contagious Bovine Pleuropneumonia and Farmers Knowledge, Attitude and Practice towards the Disease in Selected District of East Wollega and West Showa Zones, Western Ethiopia"** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary epidemiology.

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DEDICATION

I dedicate this research paper to my late father, ***Mersha Cherinnat,*** *who had pulled me away from the worst and close to the best of what life has to offer. If tears can resurrect, I could be the first man who gets his dad, but I am not fortunate!!*

STATEMENT OF AUTHOR

First, I declare that this thesis is my *genuine* work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AGID	Agar gel immunodiffusion test
AU-IBAR	African Union Inter-African Bureau for Animal Resources
BARC	Bako Agricultural Research Center
CBPP	Contagious Bovine Pleuropneumonia
C-ELISA	Competitive Enzyme-linked Immunosorbent Assay
CFSPH	Center of Food security and Public health
CFT	Compliment fixation test
CSA	Central Statistical Authority
DAGRIS	Domestic Animals Genetic Resources Information System
DGIT	Disk growth inhibition test
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra acetic acid
FAO	Food and Agriculture Organization
FAT	Fluorescent antibody test
GDP	Gross Domestic Product
HS	Haemorrhagic septicaemia
IAEA	International Atomic Energy Agency
IBT	Immunoblotting test
IFA	Indirect fluorescent antibody
IP	Percentage of inhibition
IU	International Units
KAP	Knowledge, attitude and practice
Mab	Monoclonal antibody
MOA	Ministry of Agriculture
NADH	Nicotinamide adenine dinucleotide
NVI	National Veterinary Institute
OIE	Office International Des Epizooties
PA	Peasant associations
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PPLO	Pleuropneumonia-like organisms
SAT	Slide agglutination test
SNNP	Southern nation and nationality people regional state
TADs	Srans-boundary animal diseases
USA	United States of America
UV	Ultraviolet

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ABSTRACT

Contagious bovine pleuropneumonia (CBPP) is a highly contagious disease of cattle caused by Mycoplasma mycoides subsp.mycoides small colony (*Mmm* SC). A cross-sectional study design was conducted in selected district of East Wollega and West Showa zones of western Ethiopia from December 2016 to April 2017 to assess the knowledge, attitude and practice (KAP) of farmers towards CBPP disease, to estimate seroprevalence and associated risk factors of CBPP, and to detect the causative agent of the disease from seropositive animals. A total of 113 households were purposively selected and interviewed with structured questionnaire. Consecutively, from interviewed households a total of 576 animals were randomly selected and serum samples were collected. The serum samples tested with c-ELISA and for further confirmation from 4 seropositive animals lung tissue were sampled and detection of *Mmm* SC was made using PCR. The overall animal and herd level seroprevalence of CBPP were 14.6% (95%CI: 11.80-17.73) and 54% (95%CI: 44.35-63.40), respectively. Among the potential risk factors considered, multivariate logistic regression analysis showed district, age, herd size, and history of respiratory disorder were statistically significant($p<0.05$). Cattle that found in Ilu Galan district were two times (OR=2.4, P=0.002) more likely to be affected by CBPP than Sibu Sire, adult cattle were two times (OR=2.1, p=0.009) more likely to have CBPP than young, large herd cattle were six times (OR=6.1, P= 0.000) more likely to be affected by CBPP than small herd, and animals that had history of respiratory problems were two times (OR=1.9, P=0.021) more likely to be seropositive than those hadn't. Of 4 seropositive animals lung tissue tested with PCR, *Mmm* SC was detected in all samples (100%), and the presence of the disease was confirmed in the area. However, the KAP questionnaire result indicated there were knowledge and attitude gap among the study farmers related to the disease in general. Besides, majority of farmers were practicing poor animal husbandry that created favorable conditions for the distribution of the disease in the community. Therefore, to deal with CBPP disease further study with large coverage using reliable tools like molecular technique is an essential, plus cattle herders should be made aware of about the disease and its importance through veterinary extension education.

Key words: *CBPP, seroprevalence, risk factors, KAP, c-ELISA, PCR, western Ethiopia*

1. INTRODUCTION

Ethiopia is heavily depending on agriculture sector which play a major role in overall economic development. Among the agricultural sectors, livestock is the one which is ranked first largest in Africa and tenth in the world (Hailu, 2014). Livestock provides a livelihood for 65% of the total population (Solomon *et al.,* 2003), and 80% of the rural population of the country and contributes 15-17% of Gross Domestic Product (GDP) and 35-49 % of agricultural GDP and 37-87% of the household incomes (Leta and Mesele, 2014). Therefore, an improvement this sector has the potential to contribute significantly to national income and to the welfare of the majority of rural families. Of the livestock species that are found in Ethiopia, cattle are the most important to the GDP of the country (Metaferia *et al.,* 2011). Cattle are used as source of draught power for the rural farming population, supply farm families with milk, meat, manure, and also as source of cash income, playing a significant role in the social, and cultural values of the society (Ulfina *et al*., 2005, Melaku, 2011; Tonamo, 2016). According to recent estimation, Ethiopia has 56.71 million of cattle population (CSA, 2015; Beyi, 2016) and this figure clearly indicated how much cattle are key important sectors of agriculture which take part in a potential pathway out of poverty of many, particularly for rural farmers.

Regardless of the large number of cattle we have and very important source of economy in the country, the sector is characterized by low productivity. Income derived from this sector of agriculture couldn't bear significant role in the development of the country's economy due to many constraints mostly diseases. Cattle disease problems were extremely exacerbated by drought, concentration of livestock at watering points and dry grazing grounds combined with reduced resistance, intensifies the spread of contagious and parasitic diseases which often cause higher losses than the forage or water shortages (World Bank, 2001). Ethiopia was ranked highest among Sub-Saharan countries in livestock disease burden (Grace *et al*., 2012), such as in 2014/2015 fiscal year deaths estimated for Ethiopia due to various diseases were 3.23 million cattle, 4.37 million sheep and 4.90 million goats (CSA, 2015). There are

numerous diseases of cattle that affect productivity and fertility of the sector in the country, however, in this study only put emphasis on contagious bovine pleuropneumonia (CBPP).

Contagious bovine pleuropneumonia (CBPP) is a highly contagious disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* small colony (*Mmm* SC). It has great potential for rapid spread and causes major impact on cattle production. The disease is manifested by anorexia, fever and respiratory signs such as dyspnoea, polypnoea, cough and nasal discharges in cattle (FAO, 2004; OIE, 2014). The principal route of infection is inhalation of infective droplets of diseased animals. Outbreaks tend to be more extensive in housed and in those in transit by train and on foot (Radiostits *et al*., 2007). Factors such as extremes of age, stress and concurrent infections may predispose to tissue invasion (Thomson, 2005). It is considered to be a disease of economic importance because of its high mortality rate, production loss, increased production cost due to cost of disease control, loss of weight and working ability, reduced fertility, and loss of cattle trade (Tambi, 2006; Radiostits *et al*., 2007). Due to its high economic impact, OIE declared as one of the most serious contagious animal disease and listed on the group of notifiable animal diseases of high socio-economic impact and regarded as major trans-boundary animal disease (TADs) (FAO, 2002).

Thus, CBPP is one of the great plagues that continue to devastate the cattle herds on which so many people are dependent in Africa and considered as the most serious infectious animal disease affecting cattle of the continent (Amanfu, 2009; Marobela-Raborokgwe, 2011). The disease spread alarmingly during the 1990, infecting several countries previously free from the disease and causing greater losses (OIE, 2001; Wade *et al.,* 2015). In recent years, the disease has emerged from areas where it has been persisting in endemic form to re-invade other areas from which it had previously been eradicated. In addition to these newly infected areas, the endemic areas are experiencing an upsurge in the incidence of CBPP (FAO, 2002).

With the imminent eradication of rinderpest, CBPP has becoming the most important cattle disease that hinders livestock development of Ethiopia. This is mainly caused due to the interruption of the consecutive yearly blanket vaccinations with combined rinderpest. Since 1992/93 which had certainly contained the disease to a relatively low level during the past

years by reducing the susceptible bovine population. However, CBPP is now re-emerging as one of the most economically important diseases that impede livestock production. Poorly understanding of pathogenesis, relatively ineffective vaccines or with adverse effects and poor diagnostic assays were further exacerbated the impact of the disease in the country (Gizaw, 2004). Ethiopia is experiences the largest number of cattle deaths, and reduction in cattle products under both endemic and epidemic conditions of CBPP compared to the other African countries probably due to large cattle population (Tambi and Maina, 2004). Therefore, CBPP was considered as one of the most important cattle disease and impediments to livestock development of the country (MOA, 2003; Admassu *et al.,* 2015).

In most continents, control strategies are based on the early detection of outbreaks, control of animal movements and stamping-out policy. However, in Africa control of the disease is only based on vaccination (T1/44 or T1SR) and antibiotic treatment (OIE, 2008; Radiostits *et al*., 2007). However, the consequences of antibiotic treatments in terms of clinical efficacy, emergence of resistant strains and persistence of chronic carriers have not been evaluated yet (Huschle *et al*., 2004). But, currently research work has shown that antibiotic treatment of cattle may greatly reduce the transmission to healthy contacts but this requires treatment of all affected cattle (CFSPH, 2015). Despite vaccination has been considered as a strategy for the control of CBPP in Ethiopia, the disease still persists in several regions of the country and its incidence increasing from year to year. This is, mainly due to lack of effective vaccine, irregular and low coverage of vaccination, lack of livestock movement control, and absence of systematic disease surveillance and reliable data (Gizaw, 2004).

Knowing the extent of diseases distribution like CBPP is valuable since used as an input for development of optimum prevention and controlling strategies and that will ultimately assist in poverty alleviation by improving the productivity of the sector. Therefore, to carry out an effective control of the disease, prerequisites such as understanding of the epidemiological scenario of the disease and farmer's knowledge, attitude and practices towards the disease should be well-known. However, currently there were several studies have been undertaken since 2006 on CBPP as a country with prevalence of 0.4%-31.8% (Kassaye and Molla, 2013; Alemayehu *et al.,* 2015; Teklue *et al.,* 2015; Ebisa *et al.,* 2015; Atnafie *et al.,* 2015; Mersha,

2016; Geresu *et al*., 2017). The previous studies were stressed mainly only on seroprevalence study, whereas there were no reported documents on farmers' knowledge, attitude and practices towards the disease to date in the country in general and western Oromia in particular. Moreover, further identification of the causative agent of the disease is great important, however, it is not well addressed so far and there was no previous study with regard in the current study areas. Hence, identifying of the causative agent of CBPP and knowing its epidemiological status, and more understanding the awareness of the society towards the disease were vital. Therefore, based on these key statements, the study was conducted with the objectives of assessing the knowledge, attitude and practice of farmers towards the disease, estimating seroprevalence and associated risk factors of CBPP, and detecting the causative agent of the disease from seropositive animals.

2. LITERATURE REVIEW

2.1. Definition

Contagious bovine pleuropneumonia (CBPP) is acute, sub-acute or a chronic disease that affects only cattle and occasionally water buffalo (*Bubalus bubalis*) and caused by *Mycoplasma mycoides* subspecies *mycoides* Small Colony (*Mmm* SC).Cattle suffering from the acute to sub-acute disease develop serofibrinous pleuropneumonia and severe pleural effusion (Penrith, 2014). It is also defined as an acute, sub-acute or chronic Mycoplasma disease of cattle which may cause high production losses and mortalities and characterized by fibrinous pneumonia, serofibrinous pleuritis, and oedema of the interlobular septa of the lungs (William and Amanfu, 2002).

2.2. Etiology, Classification and General Characteristics of CBPP

The causative organism of CBPP was isolated 111 years ago by the French scientists Nocard and Roux (Hamsten, 2009). It was classified as a mycoplasma in 1956 (Cottew and Yeats, 1978; Cottew *et al.*, 1987; Hamsten, 2009) and given its current name *Mycoplasma mycoides* subspecies *mycoides* small colony type in 1978 (Hamsten, 2009). *Mycoplasma mycoides* subsp. *mycoides* small colony belongs to the Class Mollicutes, order Mycoplasmatales, and family Mycoplasmataceae. The family Mycoplasmataceae contains two genera, Mycoplasma (urea negative) and Ureaplasma (urea positive) (Nicolet, 1996; Mtui-Malamsha, 2009).

Mmm SC shares many biochemicals, immunological and genetic properties with six other mycoplasmas grouped under the so called *Mycoplasma mycoides* cluster. Other members of the cluster are *Mycoplasma mycoides* subsp. *mycoides* large colony, *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma capricolum* subsp. *capripneumoniae*, *Mycoplasma mycoides* subsp. *Capri* and *Mycoplasma* sp. bovine group (Mtui-Malamsha, 2009). The closest relatives of *Mmm* SC are *Mycoplasma Mycoides* subsp. *Mycoides* Large Colony (*Mmm* LC) and *Mycoplasma mycoides* subsp.*capri* (*Mmc*) (Provost *et al.*, 1987; Thiaucourt *et al.*, 2000a). These two subspecies are frequent pathogens of small ruminants and indices a

‘contagious agalactia’ syndrome characterized by the development of mastitis, arthritis, keratitis, and, in kids pleuropneumonia and septicemia. Some strains might have specific pulmonary tropism and the disease caused by them has to be clearly differentiated from contagious caprine pleuropneumonia which is caused by *Mycoplasma capricolum* subsp. c*apripneumoniae* the former type F38 (Leach *et al.*, 1993; Penrith, 2014).

The distinction between *Mmm* SC, *Mmm* LC and *Mm*c has been difficult until recently but it can now be achieved by the use of specific monoclonal antibodies or specific polymerase chain reaction (PCR) techniques. *Mycoplasma mycoides* subsp. *mycoides* SC strains were considered to be very homogeneous but recent evidence has shown that various genotype can be distinguished by the use of molecular techniques such as restriction analysis of whole DNA, or southern blotting (Thiaucourt *et al.*, 2004). Recently described multi-locus sequence analysis distinguishes the three main lineages that correlate with their geographical origins (Europe, Southern Africa, and rest of Africa) (Cheng *et al.*, 1995; OIE, 2009). African and European *Mmm* SC strains can be distinguished by genetic as well as antigenic differences an indication that the outbreak occurring since 1980 in France, Spain, Portugal and Italy were not due to reintroduction of strains from Africa, but were more probably due to resurgence of CBPP from a region or regions where it has never been completely eradicated since the previous century. Within the ‘mycoides cluster’ *Mmm* SC strains have the peculiarity of possessing a number of ‘Insertion Sequence’ which may account for a great part of the *Mmm* SC genome variability (Penrith, 2014).

Mycoplasmas lack cell walls and are, therefore, pleomorphic and resistant to antibiotics of the beta lactamase group, such as penicillin. It is an extra-cellular pathogen that lives in close association with the host cells. The organism is capable of self-replication, has a genome size of 1,211 kb. Mycoplasmas are highly host specific and adapted to a main host in which they are commonly pathogenic, but may colonize other hosts (Nicolet, 1996). *Mmm* SC, for example, is commonly found in cattle suffering from CBPP but has also been isolated from sheep suffering from mastitis and goats suffering from contagious caprine (Westberg *et al.*, 2004). Growth of mycoplasma is relatively fastidious and requires special media rich in cholesterol (addition of horse serum). Grow readily under aerobic and anaerobic conditions.

Various types of serum broth are used as culture media, these should contain meat extract or peptone, or both, as the basic medium, as well as fresh extract of baker`s yeast and 10-20 percent serum, preferably from horses (Provost *et al.,* 1987). Various additives can be added, such as glucose, sodium pyruvate, DNA, sterol, and antibacterial and anti-fungal drugs in case of primary isolation from contaminated sample. Growth is relatively slow, and 24 to 48 hours are usually required before it is visible, but, primary isolation may take longer or necessitate the making of sub-cultures if the original sample was not of adequate quality (Thiaucourt *et al.,* 2004). Primary culture in liquid medium often yields the typical aspect of whitish 'comet', a kind of cloud floating in the medium that is easily dispersed by agitation. Solid media are prepared by the addition of 1% agar to the fluid medium, and *Mmm* SC colonies have the typical 'fried-egg' appearance of mycoplasmal colonies. The colony size seldom exceeds 1 mm in diameter and, in order to visualize them, a stereomicroscope must be used (Penrith, 2014).

Mycoplasma mycoides subsp.*mycoides* SC does not survive for long in the environment mean susceptible to environmental factors and on average only survives outside the host for up to three days in tropical areas and up to two weeks in temperate zones (Thiaucourt *et al.,* 2004; OIE, 2009). Culture of *Mmm* SC can be inactivated by ultra-violate radiation within few minutes. The organism is inactivated within 60 minutes at 56^0c and within two minutes at 60^0c, but can survive more than 10 years in frozen, infected pleural fluid. The organism inactivated by one percent phenol solution in three minutes, 0.05 percent formaldehyde solution in 30 seconds, and 0.01 percent mercuric chloride solution in one minute. The serial passage of *Mmm* SC in culture and in animals alters both its virulence and its pathogenicity; this formed the bases for development of attenuated vaccinal strains (Thiaucourt *et al.,* 2004).

2.3. Historical Origin and Geographical Distribution of CBPP

Historical accounts were not sufficiently precise to determine when and where CBPP emerged. Some statues dating from the Roman times suggest that traditional vaccinations were performed at that time. What known for sure is that CBPP was prevalent in Europe in 1770 and gained worldwide distribution during the second half of the nineteenth century

through cattle trade. The advent of new generation sequencing techniques as well as Bayesian statistical analysis allowed for the determination of the time of emergence of the common ancestor to all *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) strains: around 1700 (Thiaucourt, 2015).

However, it was supposed that contagious bovine pleuropneumonia was first described in Europe in 1564 and has with the exception of South America and Madagascar, occurred throughout the world. Until the 16th century, CBPP was restricted to the alpine region of Europe, from where it spread to the west and south of the continent, as a result of cattle movements caused by wars and importations. The disease spread to the United States of America (USA) in the second half of the 19th century. CBPP spread to Australia through cattle imported from England in 1858, and from Australia the infection was taken to Asia at the beginning of the 20th century. By the end of the 19th century, CBPP had been eradicated from most of Europe, but was still present in Germany and Austria in the 1910s and 1920s (Masiga *et al.,* 1996; Penrith, 2014).

CBPP has never been totally eliminated from the Iberian Peninsula. CBPP is widespread in Africa and is recognized to be present in some countries of Asia and Europe. In Asia, CBPP has been reported in recent times from Assam in India, Bangladesh and Myanmar. Sporadic outbreaks have been recognized in the Middle East, probably derived from importation of cattle from Africa. CBPP was eradicated from the United States of America in 1892, Zimbabwe in 1904, South Africa in 1924, Australia in 1972 and China in the 1980s. After virtual elimination from Europe in the nineteenth century, the disease reappeared in Portugal and Spain in 1951 and 1957, respectively. Outbreaks have been reported in southern France on a few occasions, the latest being in 1984. Australia eradicated the disease in 1973 after an intensive campaign of more than 10 years. In Italy, the disease reappeared in 1990 but was eliminated by 1993(FAO, 2002).

CBPP was introduced into South Africa in 1854 through importations of cattle from the Netherlands, and from there it spread to other countries in the region (Masiga *et al.*, 1996). However, Zimbabwe eradicated the disease in 1904, South Africa did so in 1924 and

Botswana completed eradication in 1939. Namibia and Angola have remained infected to this day, and the infection was reintroduced into Botswana in 1994. North African countries have been infected only on a sporadic basis, the most recent being Egypt in 1972, however, Egypt rapidly eradicated the disease. The origin of the disease in Central, West and East Africa is obscure. It has been suggested that the infection was introduced by zebu cattle when they first migrated to the African continent. There is a strong possibility that CBPP was introduced into East Africa from India by the army of Field Marshal Napier when he invaded Ethiopia in 1867-1868 (Masiga *et al.*, 1996; Penrith, 2014).

2.4. Epidemiological Situation of CBPP in Africa

In Africa, the control of CBPP greatly benefitted from the efforts toward the control of Rinderpest in the 1980s. At that time the geographical distribution of CBPP was limited and almost no outbreaks were observed thanks to annual combined vaccinations that given as a camping. CBPP gained a wider extension in Africa in the 1990s as it re-invaded countries, such as Botswana, Tanzania and Rwanda, that had eliminated the disease in the past (Ahmed, 2016). In Africa Botswana was the only country that succeeded in regaining a free status after very strict sanitary measures were applied. In the rest of the continent, CBPP continued to spread gradually to various countries such as Gabon, the Republic of Congo, the Gambia and Senegal. In 2015, CBPP was considered present in all countries of the south Sahara. The southern part of the continent is still free thanks to physical barriers (Thiaucourt, 2015). As the recent report revealed that based on data generated through reports submitted to AU-IBAR monthly by African union member states, CBPP is endemic in most pastoral areas of West, Central and East Africa, with at least 24 countries (45%) regularly reporting outbreaks every year for the last 10 years (Ahmed, 2016). The disease is also encroaching on new areas such as Gambia reporting an outbreak in 2013 for the first time after being free of the disease for 45 years. CBPP has also been reported in a few countries in Southern Africa (Angola, Namibia and Zambia). The reported morbidity and mortality as well as case fatality rates have been variable and there appears to be no clear seasonal pattern of outbreaks (no defined temporal trend). The reported fatality rates ranges between 17-20% (Thiaucourt, 2015;

Ahmed, 2016). African countries in which contagious bovine pleuropneumonia had been notified has shown in red color (Figure 1).

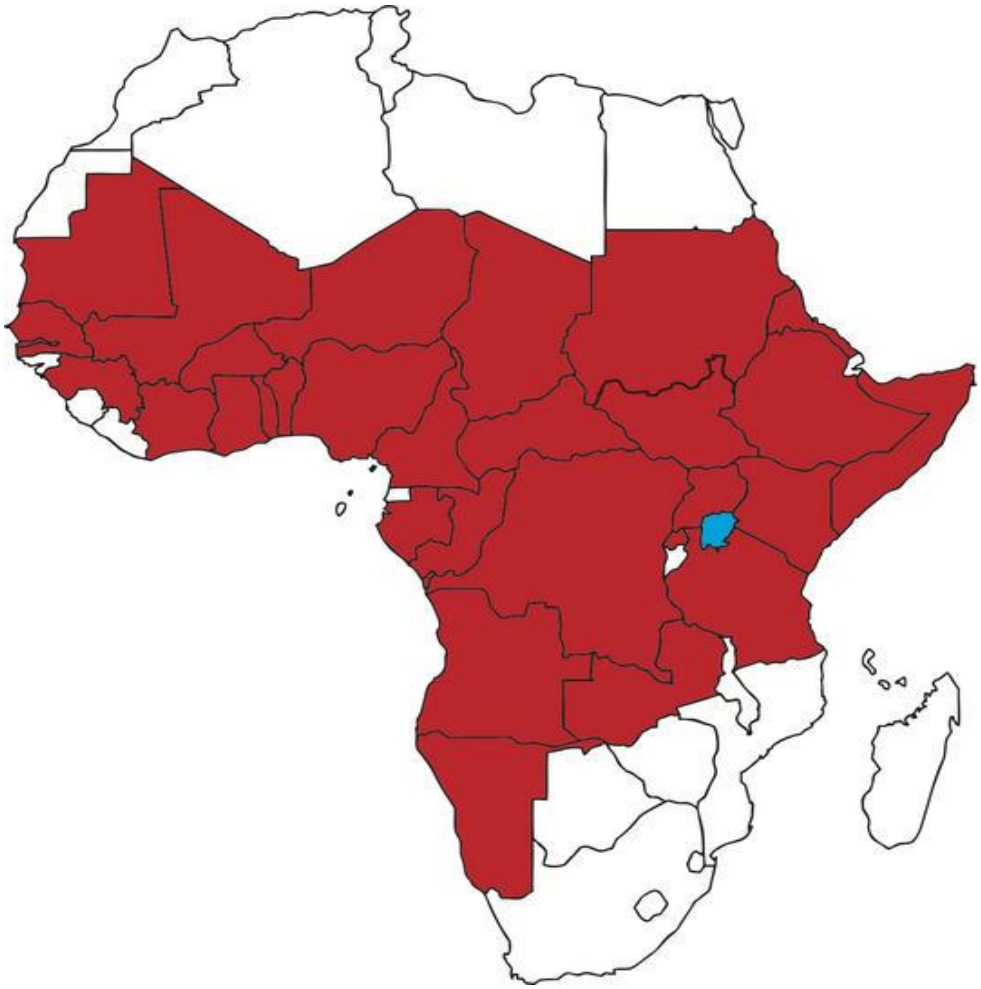

Figure 1: Geographic distribution of CBPP disease across African countries

Source:http://www.cirad.fr/var/cirad/storage/images/media/import-dossiers/images-et-fichiers-resultats-2015

2.5. Hosts

Under natural conditions, CBPP affects only cattle, both *Bos Taurus* (European) and *Bos indicus* (zebu) types. The domestic buffalo *(Bubalus bubalis)* is susceptible but the African wild buffalo *(Syncerus caffer)* is not. Some serological response may occur in other animal species, such as the gnu (*Gorgon taurinus),* and a case has been described of a Willems's

phenomenon in a roan antelope *(Hippotragus equinus).* It is considered that CBPP has no wild reservoir (Masiga *et al.,* 1996). Camels are resistant to the infection. *M. mycoides* can be isolated from cases of pneumonia in goats and very rarely in sheep (Brandau, 1995). But, the role of small ruminants as a reservoir for CBPP has not been demonstrated. Very rare infections in bison *(Bison bonasus)* and yak *(Bos grunniens)* have been detected in zoos (Masiga *et al.,* 1996).

2.6. Risk Factors of CBPP

CBPP is typical example of multifactorial diseases, where factors such as intercurrent infections, crowding, inclement climatic conditions, age, genetic constitution, and stress from transportation, handling and experimentation are important determinants of the final outcome of infection (Thiaucourt *et al.,* 2004).

2.6.1. Host related factors

Breeds: Among zebu, some breeds are remarkably resistant to CBPP like Somba, the breed of coastal lagoons of Benin and the small Côte d'Ivoire breed. The Maasai breed of Tanganyika is equally resistant (80-85%) of them recover without treatment whereas the European breeds and their crosses are more receptive (Radostitis *et al.,* 2007). In Zambia there is a high degree of infection among Barotse and Mashululumbive breeds, while in Sudan, by contrast almost 40% of zebus are resistant to experimental infection. Among cattle, it was found during the previous century that the Dutch and Flemish cattle of Europe were more susceptible than Swiss, while in present-day Kenya, Jersey cattle are affected more often than Friesians (Masiga *et al.,* 1996; Titus, 2003). N'Dama cattle of Guinea seem to be more susceptible than the zebu and crossbred N'Dama of Senegal. In Australia, dairy cows of Anglo-Normandy breeds were more susceptible to experimental infection than Hereford beef cattle (Provost *et al.*, 1987; Titus, 2003).

Age: Age of the animal also has various repercussions in the naturally occurring disease and response to vaccination. In the natural disease the susceptibility curve is sigmoid in shape

with three phases: an initial phase of low susceptibility in un weaned animals which develop only minor lesions of tendons and joints and not the severe pulmonary form; a subsequent phase of moderate susceptibility, gradually increasing until 12-18 months of age; and a final phase of full susceptibility which explains the choice of cattle over two years of age (Titus, 2003). The mechanism of these variations is unknown. If it is true, as stated by Willems, that calves up to six months of age respond poorly to the inoculation of virulent 'lymph', developing no more than slight, transient oedema, it is a fact that numerous cases of polyarthritis develop as a consequence. Heifers respond weakly to vaccination and become more resistant to experimental infection than adult cows (Provost *et al.,* 1987).

Individual animal factors: To start with individual factors in a group of animals of the same species breed or age cannot be predicted to the dismay of epidemiologists and hygienists. However, this rule includes numerous exceptions from group to group in each type of cattle. In general, some groups of animals show an evolutive receptivity in both directions according to the region and type of husbandry and may not contract a disease which decimates other herds at the same time, e.g. 30% in Tanganyika, 58% in Kenya, 10-30% in West Africa. In addition, the kinetics of the disease in a herd extends over about 20 weeks. Aerosol infection, as studied by Turner and Campbell in Australia, gave rise to the following divergent pattern: 8% developed the hyper acute form, 14% acute, 30% mild, 23% in apparent infection with serological conversion and 25% were fully resistant, 10% of survivors developed sequestered lesions (Provost *et al.,* 1987).

2.6.2. Pathogen related factor

*Mycoplasma mycoides s*ubspecies *mycoides* Small Colony is sensitive to all environment influences; do not ordinarily survive outside the animal body for more than a few hours. Restriction enzyme analysis of strains of the organism found that European strains have different patterns than African strains (Radiostits *et al.,* 2007). The organism can be grouped into two major, epidemiologically distinct, clusters. One cluster contains strains isolated from different European countries since 1980 and second cluster contains African. European strain lacks a substantial segment of genetic information which may have occurred by deletion

events. A variety of potential virulence factors have been identified, including genes of encoding putative, variables surface proteins, enzymes and transport proteins responsible for the production of H2O2 and the capsule which is thought to have toxic effect on the animal. Molecular epidemiology of CBPP by multilocus sequence analysis of *MmmSC* strains found a clear distinction between European and African strains. This indicates that the CBPP outbreaks which occurred in European were not introduction from Africa and confirms true reemergence (Radiostits *et al*., 2007; OIE, 2008; Mamo and Beshah, 2017).

2.6.3. Seasonal dynamics

Season seems to play a role in stimulating CBPP infection, particularly the rainy season, when animals are exposed to cold weather. Sudden changes of weather have been important factors in the spread of the disease and these may be more important than stable temperature and humidity, to which the animal may adapt (Titus, 2003). These changes may affect both the potential pathogen and the host. A dry climate diminishes the risk of spread, because infective aerosols from contaminated cattle evaporate rapidly, and the pathogen is inactivated by ultraviolet rays (Provost *et al.*, 1987; Titus, 2003).

2.6.4. Management related factor

The occurrence and incidence of CBPP influenced by management system, disease control policies and regulation of the country, knowledge of the disease by farmers, veterinarians and livestock field officers. The diagnosis capabilities of veterinary laboratory, disease surveillance and monitoring system, adequacy of vaccination programs, government budget allocated to control programs, desires of cattle owner and traders to control the disease are critically important management factors, which influence the effectiveness of controlling disease in a country (Radiostits *et al.*, 2007). This affects epidemiology of the disease and crucial factor since CBPP is essentially related to the movement of animals. Areas completely free from infection can adjoin endemic areas if there is no movement of cattle between them. A good example is the disease-free plateau of Adamaoua in Cameroon, separated by a 1000 meters drop from the infected plain of Benoue. In central Australia,

experience has shown that in a dry climate CBPP becomes extinguished spontaneously, provided the infected herds are kept in strict isolation, without introducing new animals. It is both true and false that CBPP is a disease of nomadic, or at least transhumant cattle. Compact grouping of herds during grazing, mixing with other herds at watering points, and confinement at night within small enclosures such conditions are eminently favorable for infection (Provost *et al.*, 1987).

2.7. Transmission and Clinical Signs

Mycoplasma mycoides sub.spp *mycoides* SC is mainly transmitted from animal to animal in respiratory aerosols. This organism also occurs in saliva, urine, fetal membranes and uterine discharges. Trans-placental transmission is also possible. Although there are a few subjective reports of transmission on fomites, mycoplasmas do not survive for more than a few days in the environment, hence indirect transmission is thought to be unimportant in the epidemiology of this disease (CFSPH, 2015). Moreover, in other report confirmed that the disease is transmitted almost exclusively by direct contact between infected and susceptible cattle, by means of infected aerosols from exhaled air. Airborne spread up to 200 meters is thought to be possible and conditions under which cattle are herded closely together is favored the rapid spread of the disease (FAO, 2002). Asymptomatically and chronically infected animals are very important in the spread of the disease to new areas. Chronic carriers are apparently healthy animals that have a localized focus of infection sequestered in a fibrous capsule in their lungs. Such animals are often referred to as “lungers” (CFSPH, 2015). The organism can persist in such lesions for many months and in time the fibrous capsule may break down, allowing viable organisms to escape by the bronchi and so infect susceptible in-contact animals. This is particularly prone to occur when chronic carrier animals are subjected to stress, such as when mustered or walked for long distance (OIE, 2015).

The clinical manifestations of CBPP in cattle range from hyper acute through acute, sub-acute and chronic forms. The hyper acute form is most often seen at the start of an outbreak and involves up to 10% of the infected animals. Death is sudden, within a week of respiratory

signs or without prior signs at all. The early stages of acute CBPP are indistinguishable from any severe pneumonia with pleuritis and acute CBPP affects approximately 20% of infected animals. Clinical signs start with fever, dullness, anorexia, irregular rumination and only slight respiratory distress (Hamsten, 2009). Respiratory symptoms including laboured and painful breathing and severe cough that increase in severity as the disease progress and lung lesions developed. An affected animal often present a typical stance with arched back, extended neck and forelegs spread apart in an effort to ease breathing. A nasal discharge and frothy saliva around the mouth is often seen. Sub-acute CBPP is the most common form (40-50%) and is a less severe form of the acute disease with only slight respiratory symptoms and intermittent fever (Hamsten, 2009). This form commonly progresses into chronic CBPP, which is a natural evolution of both acute and sub-acute CBPP. The chronic form, which can also be the initial stage of disease, is characterized by an apparently healthy state of the animal even though chronic lung lesions are present. These "silent" carriers of CBPP are infectious and thought to be an important factor in spreading the disease among cattle herds. It is estimated that up to 25% of affected cattle become chronic carriers (EMPRES, 2002; Hamsten, 2009)

Some experimental studies have shown that infection of cattle by endotracheal intubation resulted in chronic disease, while natural infection by close contact with infected animals induced severe infection that was more likely to result in mortality. However, other studies indicated that the only difference observed was in the incubation period, which was more variable in naturally infected cattle (Thiaucourt *et al.*, 2004). Typical respiratory lesions result from infection via the respiratory route, while subcutaneous inoculation results in the development of an invasive local oedema known as the 'Willems reaction' after the person who performed the first inoculation trials in 1852. Intraperitoneal inoculation results in peritonitis. In natural infection the lesions are confined to the lungs in both the acute and chronic forms of the disease, apart from the arthritis that has been described in calves (Penrith, 2014).

2.8. Pathogenesis

The result of the multiplication of *Mmm* SC in the lung is severe inflammation that causes respiratory distress and can result in the death of 30 percent or more of the affected cattle (Penrith, 2014). However, very little is known about the factors and mechanisms that affect the pathogenicity of *MmmSC*. No secreted toxins have been identified; neither receptor molecules on the bacterial surface that mediate binding to host epithelium or induce other cellular responses in the host tissues. Although, certain factors have been associated with the pathogenesis, the precise modes of action are still elusive (Persson *et al,* 2002; Mamo and Beshah, 2017). The pathogenesis has not been fully elucidated because of the high cost of experimental work in cattle and the lack of a laboratory animal model (Thiaucourt *et al.,* 2004). Actions involved in virulence include evasion of the host's immune system, tight adhesion to the surface of the host's cells, dissemination and persistence in the host, efficient importation of required nutrients and induction of cytotoxicity in the host. Mycoplasmas have the smallest genomes of all self-replicating organisms. Complete sequencing of the genome of *Mmm* SC in 2004 has revealed a lack of genes encoding primary virulence factors like toxins, cytolysins and invasins that are generally found in other bacteria, so that virulence depends on intrinsic metabolic and catabolic functions as well as surface proteins and their regulation (Thiaucourt *et al.,* 2004; Penrith, 2014)

Capsular polysaccharide: An important pathogenicity factor in *MmmSC* is the capsular polysaccharide (CPS), previously known as galactan (Woubit *et al*., 2007; Mamo and Beshah, 2017). It is made up of the carbohydrate galactose (90%) and to a lesser extent glucose (2-4%) and lipid. Injection of purified CPS to cattle produced severe respiratory collapse and even death. The CPS has been found to play a significant role in the pathogenesis of infection, binding to the host tissue surfaces and inducing resistance to phagocytosis. It has also been associated with the formation of auto reactive antibodies and consequently autoimmune responses. Toxic effects of *MmmSC* have also been associated with the capsule (Nicholas *et al*., 2000; Mamo and Beshah, 2017). Virulence appears to depend strongly on surface antigens that protect the organism and cause various reactions in the host. The capsular polysaccharide galactan has cytopathic and vaso-active effects and

contributes to the ability of the organism to spread and persist in the host, probably owing to its ability to protect the organism (Penrith, 2014). The galactan capsule is generally considered to contribute to pathogenicity by promoting binding to host tissue surfaces and enhancing resistance to phagocytosis. In addition, there is some evidence that the capsule of *M. mycoides* SC might have a direct toxic effect on host cells, and its structural similarity to bovine pneumogalactan further suggests that it might induce autoimmune reactions (Nicholas *et al.,* 2008).

Hydrogen peroxide: In a recent investigation reported by Woubit *et al*. (2007), there was indication that glycerol metabolism in *MmmSC* strains release hydrogen peroxide (H2O2) as a byproduct, resulting in disruption of host cell integrity. Hydrogen peroxide is produced by a membrane located enzyme L-glycerophosphate oxidase (GlpO) that is involved in glycerol metabolism (Pilo *et al.,* 2005). The production of large amounts of hydrogen peroxide (H2O2) due to a gene that facilitates active uptake of glycerol, which is phosphorylated and then metabolised further to release H2O2, which is then transported into the host cell, is important for virulence. Tight adhesion of the pathogen to the host cell is required for the H2O2 to be released into the host cell and produce a cytotoxic effect, since a strain that produced large amounts of H2O2 but did not adhere well to the surface was unable to cause cytotoxicity (Thiaucourt *et al.*, 2004; Penrith, 2014). The initial hypothesis was based on the fact that virulent *MmmSC* African strains possessed an active ATP-binding cassette (ABC) transport system for the utilization of glycerol, which is metabolized to dihydroxyacetone phosphate releasing H2O2, while European strains lacked part of the glycerol uptake genes due to deletion and are less virulent (Vile and Abdo, 2000; Mamo and Beshah, 2017). In conclusion, the production of hydrogen peroxide and other active oxygen species appears to be an important factor in mycoplasma pathogenicity. In mycoplasmas, hydrogen peroxide production may accompany the metabolism of sugars or certain organic acids to acetate plus carbon dioxide. There is a net reduction of NAD+ to NADH during this process and NAD+ is regenerated via NADH oxidase activity; this oxidation requires molecular oxygen, which is reduced to hydrogen peroxide or water. However, in *M. mycoides* SC, only traces of hydrogen peroxide are formed. An alternative means of hydrogen peroxide production in mycoplasmas is via the metabolism of glycerol as aforementioned (Nicholas *et al.,* 2008).

Variable surface protein: *MmmSC* express surface proteins which can undergo reversible changes to alter the antigenic repertoire in a cell population. The gene for these variable surface protein as they were termed encodes a lipoprotein precursor of 59 amino acids (aa), where the mature protein was predicted to be 36 aa and was anchored to the membrane by only the lipid moiety, as no trans membrane region could be identified (Woubit *et al.,* 2007). The protein was found to undergo reversible phase variation at a frequency of 9 x 10-4 to 5 x 10-5 per cell per generation and this variation enables the *Mycoplasma* organisms to escape the host immune defense mechanism of their host (Pettersson *et al*., 2002; Le Grand *et al.,* 2004; Citti *et al*., 2005 and Wise and Foecking, 2006; Mamo and Beshah, 2017). Surface lipoproteins have been identified that are strongly antigenic and are believed to have important roles in virulence. Several have been identified as promising candidates for mediation of adhesion, including a variable surface lipoprotein that appears to be specific to *Mmm* SC. Although adhesins have been found in other mycoplasmas and are considered to be central to their pathogenicity, enabling transport into the host cell of substances that induce the inflammatory reaction as well as being responsible for species specificity and tissue tropism, none have to date been found in *Mmm*SC (Penrith, 2014).

2.9. Post Mortem Lesions

In the absence of hard evidence for how the mycoplasma initiates infection, an immunopathological theory has been proposed in which autoimmune complexes are formed as a result of the host response against the pneumogalactan in the lung tissue, stimulated by the reaction to galactan produced by the mycoplasma; this causes thrombi in the lymphatic system, where lesions develop first. As a consequence, the lymph coagulates with distension of the interlobular septa and infiltration of lymphocytes and plasma cells around the blood vessels. The formation of these ‘cuffs’ or ‘organizing centres’ around the arterioles is the only histological pathognomic characteristic of CBPP. The secondary lesion involves the alveoli, which become full of exudates from the previous changes. Necrotic foci, surrounded by polymorphic granulocytes, develop and become sequestra (Nicholas *et al.,* 2008).

Except for young calves, pathological lesions are generally confined to the chest cavity. Usually one lung and pleura are affected and a large volume of pleural exudate containing clots of fibrin is common. Large fibrin "omelettes" make the lung adhere to the chest wall and at these sites, the pleura is thickened and opaque. The interlobular septa of affected lung regions are also thickened due to adsorption of the exudates and "frame" the lung lobules, which vary in colors of red, grey and yellow due to different stages of inflammatory lesions. This gives a characteristic "marbled" appearance when dissecting the lung, pleural exudate is rarely seen in chronic cases of CBPP, but adhesions between lung lobes and to the chest wall is more common (Masiga *et al.*, 1996; EMPRES, 2002). Necrotic lesions in the lung are surrounded by capsules of fibrous connective tissue forming structures called sequestra. Sizes of sequestra can vary from 1-30 centimeters (cm) in diameter. The capsule can be up to one centimeter thick and sequestra of different sizes can be found simultaneously in one lung. The inner parts of the sequestra often retain the (lung) structure of the acute lesion but the necrotic material may liquefy or become caseous over time. Fibrous scars can replace small sequestra over time, but large sequestra may persist for years. A sequestrum is believed to be a source of infection if it is ruptured or drained by a bronchus, but no evidence of this has been published (Egwu *et al.*, 1996; Hamsten, 2009)

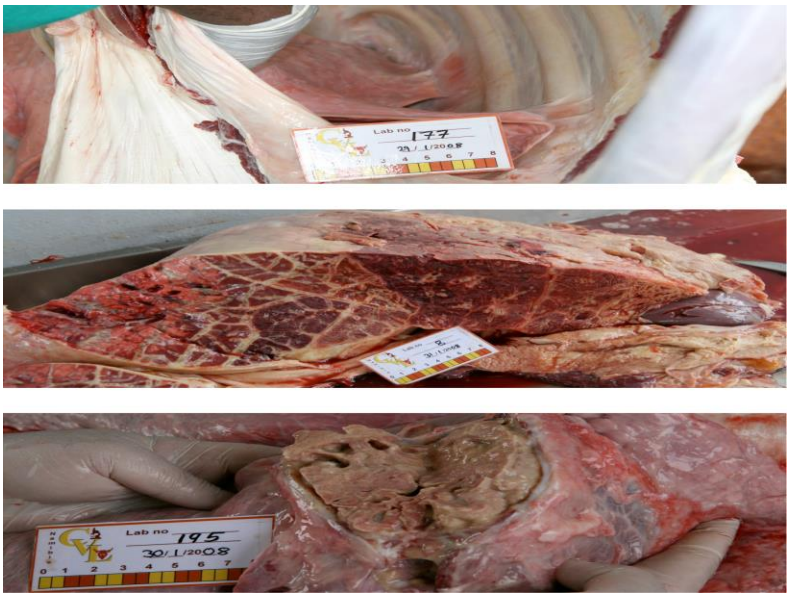

Figure 2: Typical lesion of CBPP that seen at post-mortem examination

Source: Hamsten (2009)

2.10. Morbidity and Mortality

The morbidity and mortality rates for CBPP are highly variable. In a naïve herd, the outcome varies from complete recovery of all animals to the death of the majority. Morbidity increases with close confinement, due to the increase in transmission, and infection rates can be as high as 50-80% in some situations. The mortality rate ranges from 10% to 80%, although mortality greater than 50% is reported to be uncommon (FAO, 2000; CFSPH, 2015). The severity of the illness can also be affected by the virulence of the strain, and secondary factors in the animal, such as nutrition and parasitism. As well as there may be breed-related differences in susceptibility. African and recent European isolates may differ in virulence because when they are first introduced into a naïve herd; African isolates usually cause acute disease severe, clinical signs and high mortality. Once the disease has become established, the mortality rates falls and the number of animals with chronic disease rises (Amanfu, 2009).

2.11. Differential Diagnosis

In carrying out CBPP diagnosis it is necessary to differentiate the disease from other diseases that may present similar clinical signs or lesions are listed below (FAO, 2002; Gizaw, 2004): Rinderpest: The confusion with rinderpest results from the fever and discharges observed from the eyes, nose and mouth. However, the characteristic lesions of rinderpest, which are essentially erosions in the mouth and throughout the digestive tract, together with the profuse, often bloody diarrhea in advanced cases, should enable easy differentiation from CBPP in which these are not seen. Lung lesions are seen in more chronic cases of rinderpest, consisting of red areas of collapse together with emphysema of lung lobules and the septa separating them. At this stage, the erosive lesions of rinderpest may have healed. Foot-and-mouth disease (FMD): Salivation, lameness and fever are the cause of confusion. Haemorrhagic septicaemia (HS): This is an acute disease and most affected animals die within 6 to 72 hours after the onset of clinical signs. Oedema of the throat and neck to the brisket is often very pronounced. The lung lesions seen in animals that survive the longest can appear very similar to the marbling lesion of CBPP. There may be yellow fluid in the chest cavity and the affected lung may adhere to the inside of the rib cage. Thus, in the

individual case distinguishing between HS and CBPP can be difficult. Bacterial or viral broncho-pneumonia: Post mortem examination shows usually both lungs to be affected, fibrinous exudate may be present but not to the same extent as in CBPP. While dark, solid areas of lung may be seen, these are usually restricted to the anterior lobes (not the diaphragmatic lobe as in CBPP) and marbled lungs are not often seen. Theileriosis (East Coast Fever): Coughing, nasal and ocular discharge and diarrhea are observed. Affected cattle show general enlargement of superficial lymph nodes and especially those of the head. The lungs contain much clear liquid, which is also present in the chest cavity. The airways in the lung may be filled with white froth. "cigarette burn-like" ulcers are seen in the abomasal folds. Neither pneumonia nor inflammation of the pleura is present. Ephemeral fever: Confusion with CBPP arises from the presence of fever, discharges from the eyes and dripping of saliva from the mouth, lameness and swollen joints (but in animals of all ages, unlike CBPP). Abscesses: They can be mistaken for sequestra. When cut open the content of abscesses is often offensive smelling, consisting of liquid purulent material. In abscesses a total destruction of the lung tissue occurs. Old, thickly encapsulated hydatid cysts can also cause some confusion. Tuberculosis: Tubercular nodules can superficially resemble sequestra, but they are degenerative cheese-like lesions, sometimes calcified. The lung tissue is destroyed and the same lesions are also seen in lymph nodes in the chest. The capsule of the tubercular nodules is not well defined when compared to that of sequestra. Similar lymph node lesions are always present. Actinobacillosis: The pulmonary lesions, when found, could be mistaken for sequestra. Lesions are generalized and seldom present in lungs alone. Echinococcal (hydatid) cysts: These cysts have a double wall and contain a clear liquid, often calcified when old. Foreign body reticulum pericarditis: Clinically similar to CBPP because of the dyspnoea associated with the disease (FAO, 2002; Gizaw, 2004).

2.12. Diagnostic Techniques

The *Mycoplasma mycoides* cluster consists of six Mycoplasma species or groups of strains, originating from bovines and goats (Thiaucourt *et al.,* 2000a; OIE, 2008).This cluster can be subdivided in two groups, capricolum and mycoides, comprising very closely related species. These 6 Mycoplasmas share serological and genetic characteristics and this causes taxonomic

and diagnostic problems (Persson *et al.*, 1999; OIE, 2008). A recently described technique that provides an easier way to perform molecular epidemiology of CBPP is a multi-locus sequence analysis (or typing). This technique allows the three main lineages that correlate with the geographical origins (Europe, Southern Africa, rest of Africa) to be distinguished (Lorenzon *et al.*, 2003). Quite interestingly, the strains of European origin can be clearly differentiated from African ones (Gonçalves *et al.*, 1998; Vilei and Frey, 2001). However, the oldest European strain kept in collection (1967) appears as a unique strain without the deletion and duplication. African strains seem to be more diverse. There is no doubt that further technical development will allow for a finer characterization of strains (Westberg *et al.*, 2004; OIE, 2008).

2.12.1. Identification of the agent

The causal organism can be isolated from samples taken either from live animals or at necropsy. Samples taken from live animals are nasal swabs or nasal discharges, broncho-alveolar lavage or transtracheal washing and pleural fluid collected aseptically by puncture made in the lower part of the thoracic cavity between the seventh and eighth ribs. Blood may also be cultured. Samples taken at necropsy are lungs with lesions, pleural fluid, lymph nodes of the broncho-pulmonary tract, and synovial fluid from those animals with arthritis. The samples should be collected from lesions at the interface between diseased and normal tissue (OIE, 2008). When dispatching samples to the laboratory, it is advisable to use a transport medium that will protect the Mycoplasmas and prevent proliferation of other bacteria (heart-infusion broth without peptone and glucose, 10% yeast extract, 20% serum, 0.3% agar, 500 International Units (IU)/ml penicillin, thallium acetate 0.2 g/litre) and the samples must be kept cool at 4°C if stored for a few days or frozen at or below -20°C for a longer period. For laboratory-to-laboratory transfer, lung fragments or pleural fluid can also be freeze-dried (OIE, 2008).

Culture: *Mmm* SC needs appropriate media to grow (Provost *et al*., 1987; OIE, 2008). Many attempts to isolate fail because the organism is labile, often present in small quantities, and demanding in its growth requirements. The media should contain a basic medium (such as heart-infusion or peptone), yeast extract (preferably fresh), and horse serum (10%). Several

other components can be added, such as glucose, glycerol, DNA, and fatty acids, but the effects vary with the strains. To avoid growth of other bacteria, inhibitors, such as penicillin, colistin or thallium acetate, are necessary. The media can be used as broth or solid medium with 1.0–1.2% agar. All culture media prepared should be subjected to quality control and must support growth of *Mycoplasma* spp. from small inocula. The reference strain should be cultured in parallel with the suspicious samples to ensure that the tests are working correctly (OIE, 2014).

After grinding in broth containing antibiotics, the lung samples are diluted tenfold to minimise contaminating bacteria and are inoculated into five tubes of broth and on to solid medium. The pleural fluid can be inoculated directly without previous dilution. Hermetic sealing of the petridishes or incubators with controlled humidity are recommended in order to avoid desiccation. To ensure the best conditions for mycoplasma growth, a CO2 incubator or candle jar should be used. The tubes and petridishes are inspected at day 5 and at day 10. In fluid medium, a homogeneous cloudiness usually appears within 2–4 days, frequently with a silky, fragile filament called a 'comet', which is characteristic of *Mmm* SC (*M. capricolum* subsp. *capripneumoniae*, the cause of contagious caprine pleuropneumonia). During the following days a uniform opacity develops with whirls when shaken. On agar media, the colonies are small (1mm in diameter) and have the classical appearance of 'fried eggs' with a dense centre. At this stage, the indirect fluorescent antibody (IFA) test or PCR can be performed (OIE, 2014).

Biochemical tests: For routine field use the immunological tests and PCR are sufficient, but where these give uncertain results, biochemical tests may be used. These biochemical tests should be carried out by a reference laboratory. For this purpose, after two or three subcultures, antibiotics should be omitted from the medium to check if the isolate is a mycoplasma or an L-form of a bacterium that will regain its original form in the medium without inhibitors. Once this test is done and after cloning (at least three colonies should be selected), the organism can be identified using biochemical tests (OIE, 2008).

*Mmm*SC is sensitive to digitonin does not produce 'film and spots', ferments glucose, reduces tetrazolium salts (aerobically or anaerobically), does not hydrolyse arginine, has no phosphatase activity, and has no or weak proteolytic properties. For these tests, special media have been developed that include the same basic ingredients (heart-infusion broth or Bacto PPLO (pleuropneumonia-like organisms) broth, horse serum, 25% yeast extract solution, 0.2% DNA solution), to which is added 1% of a 50% glucose solution for glucose hydrolysis, 4% of a 38% arginine HCl solution for arginine hydrolysis, and 1% of a 2% triphenyl tetrazolium chloride solution for tetrazolium reduction, plus a pH indicator (e.g. phenol red). (Note: a pH indicator should not be added to a medium containing triphenyl tetrazolium chloride.) For demonstration of proteolysis, growth is carried out on casein agar and/or coagulated serum agar. Once the biochemical characteristics have been checked, one of the following immunological tests can be performed to confirm the identification: disk growth inhibition test (DGIT), fluorescent antibody test (FAT), and the dot immunobinding on a membrane filter (MF-dot) test. The isolation and identification of the CBPP agent can be difficult and time consuming and depends on careful use of the appropriate procedures and media (Brocchi *et al*., 1993; OIE, 2008).

Polymerase chain reaction (PCR) methods: The PCR is sensitive, highly specific, rapid and relatively easy to perform, primers specific for the *M. mycoides* cluster (Taylor *et al.,* 1992; OIE, 2008) and for *Mmm* SC (Miserez *et al*., 1997; Persson *et al.,* 1999; OIE, 2008) have been reported. PCR has become the method of choice for the rapid and specific identification of *Mmm* SC when the organism is isolated from a clinical sample (Nicholas *et al*., 2012).

Using samples such as lung exudate allows the PCR to be performed directly after differential centrifugations to remove inflammatory cells and pellet mycoplasmas. For lung fragments, the PCR is applied after DNA extraction. The PCR can also be performed on urine or blood. The main advantage of the PCR technique is that it can be applied to poorly preserved samples (contaminated or without any viable mycoplasmas as may occur following antibiotic treatment). If direct detection of DNA from the organ under test fails, specimens should be enriched by culturing them in an appropriate medium for 24-48 hours, followed by attempted detection of DNA from the culture. The PCR has become the primary tool for

identification of *Mmm* SC. If a sample is PCR positive in a CBPP-free zone, the test should be confirmed by a second and different PCR while infection in endemic zone can be confirmed by the use of only one immunological test (Dedieu, *et al.,* 1994; OIE, 2008).

One of the problems with PCR is the possible occurrence of contamination if the necessary precautions and quality management system are not implemented correctly in the diagnostic laboratory. Great care must be taken to respect the strict separation between those parts of the laboratory that may be contaminated with PCR products (such as the electrophoresis room) and those parts of the laboratory devoted to preparing the PCR reagents (OIE, 2008). To avoid cross-contamination and carryover-contamination, strict separation of laboratory rooms, used for PCR preparation and handling of reactions, is needed. Various authors have developed a PCR system for *Mmm* SC identification and there is no preferred one, though the more sensitive, nested PCR should be avoided because of the higher risk of PCR product carryover, resulting in false positives (Nicholas *et al*., 2012), also the onset of real-time PCR assays should solve this possible troubleshooting as fluorescence resulting from genomic amplification is measured directly without opening the tubes. This technique has already been applied to *Mmm* SC detection (Gorton *et al.,* 2005). As the DNA target is not from a sample, where the number of cells vary and PCR inhibitors can be present, but from an isolate the sensitivity is not a critical point. Primers complementary to DNA regions CAP-21, lppA gene and 16S rRNA gene of the genome of *Mmm* SC have been designed by different authors and used in PCR systems, followed by restriction endonuclease analysis of the amplified product (amplicon) (PCR-REA) or by a second amplification (nested-PCR) (OIE, 2004; Nicholas *et al*., 2012).

2.12.2. Immunological tests (for antigen detection)

The etiological agent or its antigens can be demonstrated by immunochemical tests on infected tissues, tissue fluids and/or cultures of the organism. However, as some of these tests are dependent on a minimum number of organisms being present in the sample, only positive results are taken into account (OIE, 2008).

Indirect fluorescent antibody test: The IFA test can be performed on smears from clinical material using hyperimmune rabbit serum against *Mmm* SC and labelled anti-bovine IgG. Hyperimmune bovine serum has been used, but may have cross-reactive antibodies. The test is satisfactory when applied to pleural fluid smears, but is less satisfactory with lung smears due to considerable nonspecific fluorescence. However, good results can be obtained using lung smears counterstained with Erichrome black (OIE, 2014).

Fluorescent antibody test: The FAT is commonly performed on broth and agar cultures. It is slightly less specific than the IFA test. Broth culture: Place two drops on a microscope slide. Fix for 15 minutes with methyl alcohol, and leave in contact with the labeled hyperimmune serum for 30 minutes at 37°C in a humid chamber. Rinse three times with phosphate buffered saline (PBS, pH 7.2) and examine under an epifluorescence microscope (×80). Colonies grown on solid medium: Cut a block of agar supporting a number of young colonies and place on a slide with the colonies facing upwards. Place one or two drops of the labelled hyperimmune serum on the block and leave it in a humid chamber for 30 minutes. Place the block into a tube and wash twice for 10 minutes with PBS. Place the block on a slide with the colonies facing upwards and examine as before. Petri dish culture: the gel should not be too thick (no more than 3 mm) and should contain as little horse serum as possible. Rinse the gel three times with PBS, flood the surface with 1 ml of labeled serum and incubate for 30 minutes in a humid chamber. Rinse four times with PBS and examine directly under the microscope. The FAT in a Petri dish is used mainly just after isolation and before cloning, as it is very useful in the case of mixed infection with several mycoplasma species. Interpretation of the FAT: With broth culture, the mycoplasmas appear bright green on a dark background. However, experience is required for the FAT carried out with colonies on agar, because the background appears dark green (OIE, 2014).

Disk growth inhibition test: The DGIT is based on the direct inhibition of the growth of the agent on a solid medium by a specific hyperimmune serum. However, cross-reactions within the mycoides cluster are common and great care should be taken to differentiate *Mmm* SC (bovine biotype) from *Mmm* LC (caprine biotype; LC: large colonies). It is a simple test to perform, but some results require experience to be interpreted: small inhibition zones (less

than 2 mm wide), partial inhibition with 'breakthrough colonies', false-negative and false-positive reactions (very rare). The quality of the hyperimmune serum used in this test is critical for good results (OIE, 2008).

Agar gel immunodiffusion test: The agar gel immunodiffusion (AGID) test can detect the specific antigen present at the surface of *Mmm*SC and the circulating galactan invading the haemolymph system of sick animals. Pleural fluid, ground lung fragments or even sequestrae can be tested against a hyperimmune serum in two wells cut 5 mm apart in the gel. The gel is composed of Noble agar (12g) and thallium acetate (0.2 g/litre) in PBS, pH 7.2 (1000 ml). The test is considered to lack sensitivity and little is known about its specificity, but it has served as a screening test and only positive reactions should be taken into account. The results are better when the plate is incubated at 37°C and can be read within 24 hours, a simpler field test has been developed using impregnated paper discs instead of wells (OIE, 2008).

Dot immunobinding on membrane filtration: The MF-dot test can be used for routine identification tests in the laboratory (Poumarat *et al.*, 1991). Specific SC biotype specific MAbs have been developed to overcome cross-reactions within the mycoides cluster (Brocchi *et al.*, 1993; OIE, 2008).

Immunohistochemistry: *Mmm* SC immunoreactive sites can be detected in lung lesions using the peroxidase–antiperoxidase method on sections of paraffin-embedded material. Because the isolation of the agent is not always achieved from chronic cases and after treatment with antimicrobial drugs, this test is only supplementary to the diagnosis of CBPP and a negative result is not conclusive (Bashiruddin *et al.*, 1999; OIE, 2008).

2.12.3. Serological tests

Serological tests for CBPP are valid at the herd level only because false positive or false negative results may occur in individual animals. Tests on single animals can be misleading, either because the animal is in the early stage of disease, which may last for several months,

before specific antibodies are produced, or it may be in the chronic stage of the disease when very few animals are seropositive. False-positive results can occur (2%), of which an important cause is serological cross-reactions with other mycoplasmas, particularly other members of the *M. mycoides* cluster. The validity of the results has to be confirmed by post-mortem and bacteriological examination, and serological tests on blood taken at the time of slaughter (OIE, 2014). The CFT and ELISA are recommended for screening and eradication programmes. The highly specific immunoblotting test is useful as a confirmatory test but is not fit for mass screening (Le Goff and Thiaucourt, 1998; OIE, 2014).

The CFT and c-ELISA are the OIE prescribed herd level tests for CBPP and they are said to have specificity of 98% and 99.9%, respectively and sensitivity for both tests is said to be about 70%. Vaccinated animals are sero-negative by CFT within 3 months post vaccination. According to Thiaucourt and colleagues (2000), immune responses measured by c-ELISA following vaccination also wane after 3 months (Mtui-Malamsha, 2009).

Complement fixation test: a test suitable for determining freedom from disease and a prescribed test for international trade. The Campbell and Turner complement fixation (CF) test remains the recommended procedure (although the current method is slightly different from the original one), and it is widely used in all countries where infection occurs (Provost *et al*., 1987; OIE, 2008). For antigen titration and harmonization purposes, an international standard positive bovine serum is available from the OIE Reference Laboratory in Teramo, Italy. However, the CFT is still difficult to perform, requiring well trained and experienced personnel (OIE, 2014).

The limitations of the CF test are well known. With a sensitivity of 63.8% and a specificity of 98% (Bellini *et al.*, 1998; OIE, 2008), the CFT can detect nearly all sick animals with acute lesions, but a rather smaller proportion of animals in the early stages of the disease or of animals with chronic lesions. In addition, therapeutic interventions and improperly conducted prophylactic operations (partial slaughter of the herd) may increase the number of false-negative reactions. However, for groups of animals (herd or epidemiological unit) the CFT is capable of detecting practically 100% of infected groups. The nature of the

pathogenesis of the disease is such that the incubation period, during which antibodies are undetectable by the CFT, may last for several months (OIE, 2014).

Competitive Enzyme-linked Immunosorbent Assay(c-ELISA): c-ELISA) developed by the OIE collaborating centre for the diagnosis and control of animal diseases in tropical countries (Le Goff & Thiaucourt, 1998; OIE, 2008), has been validated internationally in accordance with OIE standards (Amanfu *et al.*, 1998; OIE, 2014). An indirect ELISA based on the use of a lipoprotein antigen is currently being validated by the IAEA (Abdo *et al.*, 2000; Bruderer *et al.*, 2002). Compared with the CFT, the c-ELISA has equal sensitivity and greater specificity. Advice on standard protocols and the availability of reagents can be obtained from the OIE reference laboratories for CBPP or the OIE collaborating centre for ELISA and molecular techniques in animal disease diagnosis (OIE, 2014).

Validation tests (Amanfu *et al.*, 1998; Le Goff and Thiaucourt, 1998) that have been carried out in several African and European countries would indicate: the true specificity of the c-ELISA has been reported to be at least 99.9%; the sensitivity of the c-ELISA and the CFT are similar; and antibodies are detected by the c-ELISA in an infected herd very soon after they can be detected by the CFT, and c-ELISA antibody persists for a longer period of time (Niang *et al*., 2006; OIE, 2014). To enhance its repeatability and the robustness, this c-ELISA is now provided as a ready-made kit that contains all the necessary reagents, including precoated plates kept in sealed bags. This kit can be obtained commercially and availability can be checked through the OIE reference laboratory in France. Sera are analyzed in single wells. The substrate has been modified and is now TMB (tetramethyl benzidine) in a liquid buffer and the reading is at 450 nm. The substrate colour turns from pale green to blue in the first place and becomes yellow once the stopping solution has been added. MAb controls exhibit a darker colour while strong positive serum controls are very pale. The cut-off point has been set at 50% and should be valid in every country (OIE, 2014).

Immunoblotting test: A field evaluation indicated a an immunoblotting test (IBT) is an immunoenzymatic test that is higher sensitivity and specificity than the CF test and has been developed to confirm doubtful CFT or c-ELISA results (Gonçalves *et al*., 1998). A core

profile of antigenic bands, present both in experimentally and naturally infected cattle are immunodominant. The more accurate picture of the immune status of animals given by this test is due to the possibility of a more precise analysis of the host's immune response in relation to the electrophoretic profile of *Mmm* SC antigens; thus the test overcomes problems related to nonspecific binding. It should be used primarily as a confirmatory test, after other tests and should be used in all cases in which the CF test has given a suspected false result (OIE, 2008).

Other tests: A rapid field slide agglutination test (SAT) with either whole blood or serum has been developed to detect specific agglutinins. The antigen is a dense suspension of stained mycoplasmas that is mixed with a drop of blood or serum. Due to a lack of sensitivity, the test detects only animals in the early stages (i.e. acute phase) of the disease. It should be used only on a herd basis. A latex agglutination test has been developed that is easier to interpret than the SAT (Ayling *et al.,* 1999; OIE, 2008). For CBPP, the CF test and ELISAs can be used in screening and eradication programmes, but the highly specific IB test should be used as a confirmatory test. However, the IB test is not fit for mass screening and may be difficult to standardize in countries with marginal laboratory facilities so IB testing should be performed in a reference laboratory (OIE, 2008).

2.13. CBPP Control and Prevention Methods

The USA, Japan and Western Europe managed to eradicate the disease through stamping out coupled with strict animal movement control (Masiga and Domenech, 1995; Provost *et al.*, 1987; Mtui-Malamsha, 2009). Recently, Botswana managed to control and eradicates CBPP through stampingout (Amanfu *et al.,* 1998). However, stamping out may not be economically feasible in endemic African countries and vaccination is the most frequently used control strategy in combination with animal movement control. To be effective, vaccination must be repeated initially at short intervals and thereafter annually over 3-5 years (FAO, 2002).

CBPP vaccination was initially (1920's to early 1970's) based on broth T1 vaccine which was later replaced by freeze-dried live attenuated *Mmm*SC vaccine T1/44 vaccine (OIE, 2008). A streptomycin resistant variant (T1SR) was developed and used in combination with

rinderpest vaccine (Rweyemamu *et al.,* 2000; Mtui-Malamsha, 2009). Re-assessment of efficacy of two predominant vaccine strains (T1/44 and T1/SR) by means of a challenge trial was carried out following vaccination failures in Botswana (Thiaucourt *et al.,* 2000b). The challenge trial was carried out in Cameroon, Kenya, and Namibia to cover the genetic diversity that exists among the pathogenic strains from different geographic areas. Susceptible cattle were vaccinated with either vaccine containing the minimum dose of 107 viable *Mmm* SC per dose, as recommended by the OIE (OIE, 2008). The efficacy varied from 33-67% regardless of the strain used. In a similar experiment, T1/44 provided higher protection (95%) than T1/SR (80.5%) in cattle vaccinated twice (Wesonga and Thiaucourt, 2000; Mbulu *et al.,* 2004). Based on a number of controlled experiments, CBPP vaccine efficacy ranged from 40% up to 95% (Thiaucourt *et al.*, 2000b; Mtui-Malamsha, 2009). Estimation of vaccine efficacy was based on the Hudson and Turner scoring system, however, the method has not been used consistently and comparison of results across trials may not be meaningful (Mariner *et al.,* 2006).

Antibiotic treatment against CBPP is widely used. It is not part of any official control strategy due to suspicion that its use could facilitate developments of sequestra, increase the number of carrier animals, increase development of resistant strains, and mask the occurrence of clinical disease (Provost *et al.*, 1987). Masking of clinical disease will make diagnosis difficult, which may contribute to unrecognized infections and CBPP transmission. Nevertheless, antibiotics are widely used in pastoralist communities (Msami *et al.,* 2001; Twinamasiko *et al.*, 2004; Mariner *et al.*, 2006). At a meeting of international experts organized by FAO in 2003 it was recommended that chemotherapy be reconsidered for CBPP control. Both in-*vivo* and in-*vitro* studies demonstrating usefulness of antibiotics for treating CBPP have been reported (Twinamasiko *et al.*, 2004; Yaya *et al.*, 2004; Hübschle *et al.*, 2006; Mtui-Malamsha, 2009). In an in-*vitro* experiment, tilmicosin, danofloxacin, oxytetracycline, florfenicol and spectinomycin were found to be effective against a variety of strains of *Mmm* SC isolated from CBPP cases that had occurred in Africa and Europe (Ayling *et al.,* 2000).

In a study carried out in Namibia, it was demonstrated that naïve animals kept in-contact to danofloxacin treated animals with CBPP had significantly fewer lesions, were less likely to die and to develop clinical disease than naïve animals kept in-contact to untreated animals with CBPP. In the same study, *Mmm* SC was isolated from a limited number of in-contact controls kept with the treated animals suggesting low spread of infection (Kusiluka and Sudi,2003; Hübschle *et al.*, 2006). In a different trial, long-acting tetracycline was demonstrated to be effective in limiting clinical severity of the disease but ineffective in the prevention of persistence of viable *Mmm SC* in treated animals (Yaya *et al.*, 2004; Niang *et al.*, 2007; Mtui-Malamsha, 2009).

Therefore, the direct effect of tetracycline on the individual is positive (less clinical damage), but the indirect effect on the population may be negative (masking of signs leading to transmission). Infection control methods act by reducing the effective reproductive number of the infectious agent in the population (Tambi *et al.*, 2006). The effective reproductive number is affected by contact between individuals, transmission probability per contact and duration of infectiousness. Animal movement control reduces the contact between infected and susceptible animals. Vaccination and treatment reduce susceptibility and infectiousness, respectively, at the individual level, thus reducing the probability of transmission per contact and reducing the prevalence of infection in the population. Because CBPP is a contagious disease, a reduction in prevalence will result in reduction in incidence thus further reducing prevalence in the population (Thompson, 2003; Mtui-Malamsha, 2009).

2.14. Socioeconomic of CBPP Disease

Estimates provided by indicate that animal diseases cause losses of up to 30% of the annual livestock output in developing countries. CBPP is considered as a disease of economic significance because of its ability to compromise food security through loss of protein and draft power, reduce output, increase production costs due to costs of disease control, disrupt livestock/product trade, inhibit sustained investment in livestock production and cause pain and suffering to animals (Windsor and Wood, 1998; Paskin, 2003; Tambi *et al*., 2006; Batu *et al.,* 2016). The OIE Terrestrial Animal Health Code provides standards for trade in live animals, semen and embryos but specifies that trade in milk and milk products, hides and

skins, and meat and meat products other than lung should be free of any restrictions. This international recommendation may, however, be overridden by more stringent national standards (Penrith, 2014).

Even though the economic burden of CBPP is generally difficult to compute, several studies were conducted, such as Thomson (2003) has stated that the cost of controlling CBPP using a mass vaccination programme in countries of Central, Eastern and Western Africa is quite high (300 million euros) and that even if half of this cost were to be recovered from cattle owners, many governments will still not be able to afford the rest. Tambi *et al*. (1999) estimated the cost of CBPP control by vaccination in 10 African countries during the PARC period and found unit costs to vary from 0.27 euro in Ethiopia to 0.71 euro in Côte d'Ivoire, with an average of 0.42 euro as well as in many countries, treatment of clinical disease is at the exclusive cost of cattle owners, despite the fact that the efficacy of treatment is largely unknown as are the epidemiological consequences (Tambi *et al*., 2006). A recent study in 12 countries in West, Central and East Africa that reported a total of 2,612 outbreaks to OIE during the period 1996 to 2002, accounting for 96% of the reports of CBPP for that period, suggested an amount of 44.8 million euros in direct losses and control costs. Based on mass vaccination for five years to attain 80% coverage and including an estimated cost of treating about the same percentage of animals, the cost-benefit ratios of controlling CBPP were favorable for the countries studied (Penrith, 2014). Moreover, in one study which conducted in Ethiopia reported that, the post-vaccinal reaction caused substantial economic loss and moral demotion to the resource poor livestock owning community (Sori, 2005).

2.15. The Epidemiology of CBPP in Ethiopia

The origin of CBPP in Central, West and East Africa is ambiguous and it has been suggested that the infection was introduced by zebu cattle when they first migrated to the African continent. After rinderpest has been brought under control, CBPP is considered to be among the most important cattle diseases and impediments to livestock development in Ethiopia, particularly in the lowlands of the country. The consecutive yearly blanket vaccinations with combined rinderpest and CBPP have certainly contained the disease to a relatively low level during the past years. But with the adoption of a strategy towards rinderpest eradication, the

vaccinations in the have ceased since 1992/93. Generally, the irregularity and low rate of vaccinations since 1993 seem to contribute to the increased incidence of the disease and its further spread. The usual blanket coverage was around 50% and never reached the desired 80-100% level (MOA, 2003). According to eleven years (1992-2002) disease outbreak reports by Federal Ministry of Agriculture, several CBPP epidemics have been recorded from the south, south-west, west, north-west and north-east regions of the country. The passive disease outbreak reports from 1992-2002 shows 587 outbreaks, 16,806 cases and 3,262 deaths. The highest record was in 1998 when 187 outbreaks with 5,652 cases and 1071 deaths were reported (MOA, 2002). Different studies conducted in Western Ethiopia, Northwest Ethiopia, Southern Ethiopia and different regions of the country revealed that CBPP is posing a major threat to cattle in many parts of the country thereby causing considerable economic losses through morbidity and mortality and warranting for serious attention (Afework, 2000; Admassu *et al*., 2015; Batu *et al.,* 2016). Gizaw (2004) reported in Somale region and the highest herd sero-prevalence was observed in Mieso district 100% followed by Qabribeyah 75% and Afdem 71.4% and according to the recent report of ministry of Livestock and Fishery resource development, there were outbreaks reports of CBPP disease in the current study areas (Table 1).

Table 1: CBPP outbreak reports to the Federal Ministry of livestock and fishery resource development during the years of 2011-2013 in East Wollega and West Showa zones

year	Number of outbreak	case	death	Slaughter	PAR	Number of vaccinated	Morbidity rate (%)	Mortality Rate (%)	CFR (%)
2013	6	158	10	0	165000	21785	0.365	0.023	7.26
2012	9	28	6	0	68823	4096	0.25	0.089	11.1
2011	13	222	34	3	191192	9650	0.28	0.056	31.69
Total	**28**	**408**	**50**	**3**	**425015**	**35531**	**0.3**	**0.056**	**16.68**

PAR=Population at Risk; CFR=Case Fatality Rate

Source: Ministry of livestock and fishery resource development

Furthermore, there are recent study reports on seroprevalence of CBPP in different parts of the country such as: Atnafie *et al.* (2015) reported 7.8% and 5.9% seropositive in two study

places of abattoirs at Bishoftu and export oriented feedlots around Adama, respectively; Mersha (2016) reported 28.5% seroprevalence in selected districts of western Oromia; Teklue *et al.* (2015) reported 11.9% seroprevalence in southern zone of Tigray region; Alemayehu *et al.* (2015) reported 0.4% seroprevalence in Borena pastoral; Kassaye and Molla (2013) reported 4% seroprevalence in and around export quarantine Adama town; Ebisa *et al.* (2015) reported (31.8%) in Amaro district of SNNP of Ethiopia, and Geresu *et al.* (2017) reported (6.51%) in Dello Mena and Sawena districts of Bale zone (Table 2).

Table 2: Recently reported sero-prevalence of CBPP in different parts of Ethiopia

Study Area	**Diagnostic test**	**Prevalence**	**References**
Western Oromia	c-ELISA	28.5%	Mersha (2016)
Bishoftu abattoir & Adama quarantine	c-ELISA	7.8% & 5.9%, respectively	Atnafie *et al.* (2015)
Southern zone of Tigray	CFT	11.9 %	Teklue *et al.*(2015)
Borena pastoral	c-ELISA	0.4 %	Alemayehu *et al.*(2015)
Export quarantine of in & around Adama	c-ELISA	4 %	Kassaye & Molla (2013)
Bako research dairy farm		71.8%	Almaw *et al*.(2016)
Amaro district, SNNPE	c-ELISA	31.8%	Ebisa *et al.*(2015)
Dello Mena & Sawena districts of Bale zone	c-ELISA	6.51%	Geresu *et al*.(2017)

CBPP diagnostic approaches like clinical examination, post-mortem examination to observe the characteristic lesions in organs of dead and\or slaughtered animals and laboratory examination to confirm the presence of infection, an outbreak investigations to isolate and identify the causative agent of *Mycoplasma* species through postmortem examination and sample collection, sero-prevalence studies, sick animals for autopsy and bacteriological specimen collection, and the clinical and pathological findings as well as the biochemical

tests performed so far were the major method that used CBPP diagnosis in Ethiopia (Gizaw, 2004; Regassa *et al*., 2005).

The clinical examinations of infected animals revealed nasal discharge, coughing, labored breathing, disinclination to move and postures that showed the animal was fighting to get enough oxygen. The profound lesions observed on postmortem showed adhesion of the pleura with the chest wall and the lung and consolidated lung tissues with characteristic marbling. The pleural cavity was full of copious, yellowish-colored clear fluid. Heavy deposits of fibrin flocculates were encountered (Gizaw, 2004; Regassa *et al*., 2005; Admassu *et al*., 2015).

Evidence of the growth of *Mycoplasma* organisms was based on a change in color of the growth medium from pink to yellow. Moderate turbidity with a whitish deposit at the bottom of the culture vessels were additional parameters used to determine *Mycoplasma* growth. Both the tissue sample processed and the pleural fluid cultured were positive for *Mycoplasma* growth after incubation for 72-120 hours in broth culture media. Gram-stained smears from these cultures showed the presence of gram-negative, pleomorphic organisms composed of coccoid, cocco-bacillary and filamentous organisms. Giemsa-stained preparations from the culture suspensions revealed coccoid, pear-shaped, and filamentous microorganisms. Growth on solid medium was characterized by the presence of micro colonies with a typical nipple-shaped appearance after 7 days of incubation. The colonies were observed under inverted microscope (32X) with transmitted light (Regassa *et al*., 2005). The compliment fixation test on serum is still the most useful methods of detecting infection. It is a rapid, simple and easy to perform and interpret the results. It is more specific than ELISA tests. It lacks sensitivity for serum samples having a very low antibody level. ELISA tests detect late and persistent infections while CFT detects early infections (Kassaye and Molla, 2013).

The major prevention and controlling methods of CBPP disease that practiced in Ethiopia so far are vaccination and antibiotic treatment. The control of CBPP by vaccination has been carried out for the last many years in Ethiopia. Previously the consecutive yearly blanket

vaccination with combined rinderpest and CBPP vaccine was adopted as a strategy to control CBPP. And this method was considered as a successful achievement in the control of CBPP. However, with the adoption of a strategy towards rinderpest eradication, the vaccinations in the highlands and most parts of the Somali region have ceased since 1992/93. Besides, the vaccination coverage was around 50% and did not reach the desired 80-100% level. Currently, CBPP control in Ethiopia was based on targeted and ring vaccination in the face of outbreaks (MOA, 2003; Admassu *et al*., 2015). In Ethiopia immunization of cattle against CBPP, has been practiced for the last many years using vaccines prepared from streptomycin-resistant vaccine seed strain T1SR which used previously and T1/44 which is the updated and currently used. However, it was reported that the improper usage of T1/44 leads post-vaccinal reaction and affected the vaccinated animals with an overall attack rate of 1.02% while the mortality and case-fatality rates were found to be 0.17% and 16.5% (Sori, 2005).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was carried out in selected districts (Sibu-Sire and Ilu-Galan) which were selected from two zones (East Wollega and West Showa) of Oromia regional state, western Ethiopia.

Sibu Sire district is found western part of Ethiopia in East Wollega zone of Oromiya regional state (Figure 3). The district is located on the Addis Ababa-Nekemte main road about 273 km from Addis Ababa (capital of the country) to the west of the country. Sire is the central town of the district and is located on geographical coordinates 9°04'59.9" N and 36°49'59.9" E. The district has altitude ranging from 1200-2400 with an average of 1830 meters above sea level (m.a.s.l). The agro ecologies of the area are 7.53% highland, 74.2% midland and 18.7% lowland type. It experiences a bimodal pattern of rainfall with the main rainy season extending from June to September (84% of rain is expected) and short rainy seasons from March to May. The area receives rainfall ranging from 1000-2400 mm with mean annual rainfall of 1700 mm. The minimum and maximum temperatures of the district were 10.9^0 C and 33.9^0 C, respectively. The livestock populations of the district were: Cattle (190,166), Sheep (35, 952), Goat (36,512), Poultry (193,155), Horse (12,249), Donkey (16,402) and Mules (1,196). According to the document of Sibu Sire veterinary clinic described the major livestock diseases that found in the district were trypanomosis, internal and external parasite, LSD, pasturellosis, mastitis, blackleg, NCD, anthrax and tuberculosis (Sibu Sire district Livestock and Fishery Resource Development Office, 2017).

Ilu-Gelan district is found in the western part of Ethiopia in West Showa zone of Oromiya regional state (Figure 3). The district is located on the Addis Ababa-Nekemte main road about 200 km from Addis Ababa (capital town of the country) to the west of the country. Ijaji is the central town of the district and the district is located on geographical coordinates 8°59'51" N and 37°19'49" E. The altitude of the district is ranging from 1565-1790 with an average of 1630 meters above sea level (m.a.s.l). The agro-ecology of the area is almost about 80% is lowland type. The mean annual rainfall of the area varies between 1600-2290

mm and the mean annual temperature is 27.3%. The livestock populations of the district based on species were 107,076 of cattle, 7,600 of sheep, 8,930 of goats, 41,285 of poultry, 650 of horses, 950 of mules, and 3,395 of donkeys. According to the report of Ilu Galan livestock health agency, the major endemic livestock diseases that found in the district were trypanomosis, pasturellosis, blackleg, salmonellosis, epizootic lymphagitis, African horse sickness, mengimites, and external parasite (ticks) (Ilu Gelan district Livestock and Fishery Resource Development Office, 2017).

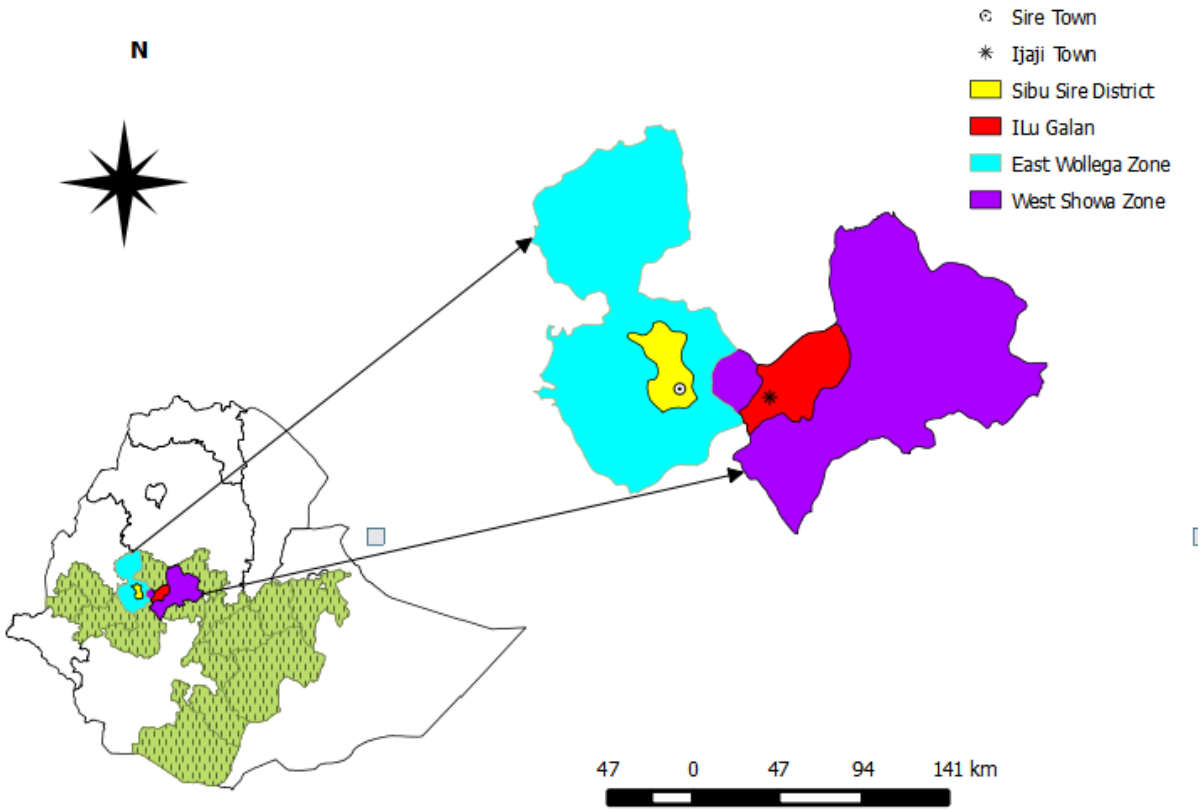

Figure 3: Map of the Study Area

3.2. Study Population

The study population comprises the dominant indigenous Horro cattle breeds distributed in the areas. Horro breed is zenga type cattle that are an intermediate breed type between the sanga and the zebu. Zenga breeds are often found in areas between the typical zebu areas in northern Africa and Sanga areas in the south (Rege, 1999; DAGRIS, 2007; Tola, 2015). The

Horro cattle are found in the western parts of Ethiopia, mainly in the eastern Wollega and western Shoa zones of the Oromia region and one of the common breed in Ethiopia. Therefore, indigenous horro cattle breed that managed under extensive production system (animals that are kept free-range for part or all of their production cycle) (Lawal-Adebowale, 2012) with no history of CBPP vaccination and animals above six months old age were considered.

3.3. Study Design and Methods

The cross-sectional study design was conducted from December 2016 to April 2017 in selected districts (Sibu Sire and Ilu Galan) of western Ethiopia and equal sample size were considered for the two districts. The purpose of the study was fully explained to the selected owners before conducting the study to have owners' agreement and questionnaire interviews were conducted during a face-to-face interview with the study participants. On each study household, questionnaire interviews of cattle owners (households) have been conducted and the knowledge, attitude and practice (KAP) of cattle owners or respondents' had towards respiratory disease in general and CBPP disease in particular was assessed. From each interviewed household, animals' blood samples collections were performed and the overall animal and herd level seroprevalence of CBPP were estimated as well as the associated potential risk factors were identified. For further confirmation of the presence of CBPP in the study area, identification of the causative agent of CBPP disease was made from lung tissue samples of seropositive animals.

3.4. Sampling and Sample Size Determination

3.4.1. Sample size determination

The sample size required for blood collection was determined depending on the expected overall prevalence of CBPP, 5% the desired absolute precision and 95% confidence interval using the formula given by Thrusfield (2007). Since there were recent previous reports of prevalence of CBPP disease in western part of the country such as 28.5% was reported by (Mersha, 2016) and 6.51% was reported by Geresu *et al*. (2017) and the average was 17.51%.

Thus, in this study an expected prevalence of 17.51% was considered to get the maximum sample size. Therefore, the sample size was calculated based on 17.51% expected prevalence and gives total sample size of 216 for each district. However, 288 animals were sampled from each district in order to increase the precision, which gives a total estimated sample size of 576 animals. Whereas an estimated sample size for households that participated in the questionnaire survey was calculated by dividing the total sample size (n=576) by the number of animals sampled within each herd or household (7) given an estimated of 83 households for inclusion in the questionnaire survey (Tadeg *et al.*, 2015). However, due to the inclusions of households that had less than seven animals, the total sample size of households was inflated to 113 (Gebremedhin *et al*., 2013). Therefore, in this study a total of 113 households or herds (29 from Lalisa and 26 from Cheri Jarso PAs of Sibu sire district while 31 from Ale Wara Ilu and 27 from Wadeyi Granche of Ilu Galan district) were selected. The sample size calculation formula:

$$n = \frac{(1.96)^2 (Pexp)\ (1-Pexp)}{d^2}$$

Where; n=Total number of sample size,

Pexp = Expected prevalence, d = Absolute precision (0.05)

3.4.2. Sampling techniques

To select the study districts non-probability sampling (purposive sampling) technique was used. In the first stage the two districts (Sibu Sire from East Wollega zone and Ilu Gelan from west Showa zone) were selected purposively based on nearby to Bako agricultural research center, easily accessible and densely populated area of cattle. In the second stage, after made of a brief discussion with the selected districts' of livestock and fishery resource development office and health agencies, peasant associations (PAs) which mean the smallest administrative unit of the districts were randomly selected from the two districts. Sibu Sire district has 22 PAs whereas Ilu Galan has 17 PAs, and from each district two PAs were randomly selected. Thus, from Sibu Sire (Lalisa and Cheri Jarso) and from Ilu Galan (Ale Wara Ilu and Wadeyi Granche) were sampled, accordingly.

To select households (herds) and individual animal two stage sampling methods were employed. The primary sampling unit was household that having at least one cattle in each selected PA and defined as herd. To select households or herds, since the total numbers of households that having cattle (sampling frame) in each PA were not well known, purposive sampling technique was used. Therefore, from each PA, households that having at least one cattle were recruited purposively based on households that found nearby to PA level veterinary clinics (type D clinics). And the secondary sampling unit was individual animals of households (animals that owned by the sampled households). Since between-cluster variance of CBPP disease of the area was unknown a simple random sampling method was applied to calculate the number of animals to be included from each herd in study (Tadeg *et al.,* 2015). From each list of herd (household that having animal) the maximum sample size sampled was seven (i.e. the average number of cattle per household of the area). From households those having greater than seven cattle, only seven animals were sampled using random sampling method. However, in case of households that have ≤ 7 cattle, all animals were sampled (Gebremedhin *et al*., 2013). Households (owners) of the study area were recognizes each cattle owned by name, thus, animals were randomly sampled using the name of animal as ID number and blood samples collections were conducted.

3.5. Data Collection

3.5.1. Questionnaire survey

A questionnaire survey was made to evaluate farmers' knowledge, attitude and practice (KAP) towards the general overview of cattle respiratory health problems with special emphasis to CBPP. Therefore, a detailed structured questionnaire (Appendix 3) was prepared in English before being reverse translated into Afaan Oromo, piloted and pre-tested. Information that contained in the body of developed questionnaire were: general household characteristics (gender, age, educational background, marital status, and house hold family size); cattle herd size and structure (herd size, sex, and age); to ascertain farmers knowledge, attitude and practices general overview of cattle's respiratory disorders such as presence or absence of respiratory disorders within the herds, farmers assumption related to the factors that causes the disorders, any infectious diseases known by farmers, major signs of CBPP

disease that recognized by farmers (chest pain, standing with elbow abducted, standing with back arched, head extended coughing, labored and painful breathing, frothy saliva at mouth, dilation of nostril and mucoid discharge, swelled throat and dewlap and polyarthritis particularly on young), any respiratory infectious diseases that are known by farmers, possible transmissions methods of CBPP or any contagious diseases, economic importance and farmers' routine cattle management practices that are associated with prevention and controlling methods of contagious infectious diseases like CBPP were the major issues that included in the questionnaire.

3.5.2. Blood sample collection

Blood samples of animals of interviewed famers were collected for diagnostic test. Therefore, from each sampled animal about 7 ml of whole blood were collected from the jugular vein of the randomly sampled animals using disposable plain vacutainer tubes and needles (BD Vacutainer Systems, Plymouth, UK) according to best practice of guidelines. Then, the samples were properly labeled and left for 24 hours at room temperature to allow clotting and serum samples were separated after 24 hours through gently transferring to other sterile vials and stored at -20^{O}C at Bako Agricultural Research Center animal health laboratory until submitted to National Veterinary Institute (NVI), Bishoftu. Finally, the serum samples were transported in to NVI with an ice box and submitted for laboratory analysis.

During blood sample collection variable that considered as risk factors were (Appendix 1): location (districts and PAs); age (grouped into two 6 months-3 years old called young and greater than 3 years old called adult based on owners information); sex (male and female); herd size (grouped in to three, since the interviewed minimum and maximum number of herd sizes ranges 2-25 categorized as 2-7 called small herds, 8-14 called medium herds and 15-25 large herds); previous history of respiratory disorder (yes/present or no/absent); body condition score (BCS) (characterized based on Nicholson and Butterwoth (1986) principles, then categorized in to three poor 0-3, medium 4-6 and good 7-9)(Appendix 2); and parity

(categorized as heifer or non parturated, primiparus or single and pluriparus or multiple parity).

3.5.3. Lung tissue sampling

After CBPP positive animals were screened using serological method, and trace backing of seropositive animal owners made to purchase seropositive animals for lung tissue sampling. Before starting the process of purchasing, the purpose of purchasing animals was clearly explained to the owners in order to have owners' agreement. Out of the total 84 seropositive animals which were resulted test positive using c-ELISA, four animals were purposively selected based on strength of seropositivity (bigger IP value) and bought. The purchased seropositive animals were transported to Bako Agricultural Research Center and post mortem examinations have been conducted. Upon post-mortem examination parts of suspected lesions of lung tissue samples were collected and immediately stored at -20^{O}C at Bako Agricultural Research Center, animal health laboratory until submitted to National Veterinary Institute (NVI), Bishoftu. Finally, the lung tissue samples were dispatched in to NVI with an ice box and submitted for PCR analysis.

Out of the four seropositive animals that that postmortem examination made 2/4(50%) had lesions of CBPP infection. Lung tissue was encapsulated and consolidated mean separated by a network of pale bands made thickening extensive fibrosis marbling and formed pleural adhesions, lung organ was strongly attached to chest walls (Figure 3), and the lung tissue became inflamed (Figure 4) upon post mortem examination while the other two lung samples were had no CBPP lesions.

Figure 4: (A) CBPP seropositive slaughtered cow of lung organ adhesion to the chest wall and (B) network of pale bands and made thickening extensive fibrin or fibrosis marbling appearance of lung tissue

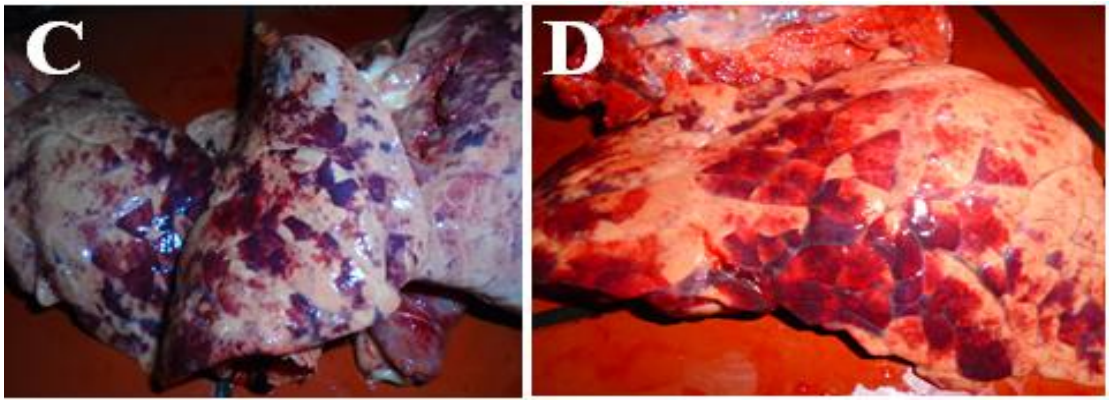

Figure 5: (C and D) the lung of CBPP seropositive young male bull slaughtered and its lung was severely inflamed upon postmortem examination as the picture indicates

3.6. Diagnostic Methods

3.6.1. Competitive ELISA

Micro plates are coated with *Mmm* SC purified lysate. Samples to be tested are premixed with a specific monoclonal Mab 117/5 in a separate plate (prelate) and content of the preplate

is transferred into the coated microplate. Any *Mmm* SC specific antibodies present in the sample will form an immune-complex with *Mmm* SC antigen coated on the microplate, competing with Mab 117/5 for the specific epitopes. After washing away unbound material, an anti-mouse antibody enzyme conjugate is added. In presence of immune-complex between *Mmm* SC antigen and antibodies from the sample, Mab 117/5 cannot bind to its specific epitopes and the conjugate is blocked from binding to Mab 117/5. Conversely in the absence of *Mmm* SC-Antibodies in the test sample, Mab 117/5 can bind to its specific epitopes and the conjugate is free to bind to Mab 117/5. Unbound conjugate is washed away and enzyme substrate (TMB) is added. In presence of the enzyme, the substrate is oxidized and develops a blue compound becoming yellow after blocking. Subsequent color development is inversely proportional to the amount of anti-*Mmm*SC antibodies in the test sample. The result is expressed in "percentage of inhibition" by comparing the optical density in the test well with the optical densities in the mAb control wells (Appendix 4).

3.6.2. Polymerase chain reaction (PCR)

The DNA extraction process from lung tissues samples were carried out as described by Johansson *et al.* (1998). The tissue samples were cut in small pieces using a sterile scalpel, grinded thoroughly and then homogenized in 2ml phosphate buffered saline (PBS). Then, the mixture was transferred to 2ml ml Eppendorf tubes containing 385 microliter STE buffer (100mM NaCl, 50mM Tris–HCl, pH 7.4, and 1mM EDTA), 5 microliter proteinase K (20 mg/ml), and 10 microliter SDS (20%). After extraction with 400 microliter phenol saturated with 1mM Tris–HCl (pH 7.4) and ethanol precipitation, the DNA was dissolved in 50 microliter of distilled H2O (Appendix 5).

Then DNA amplification was performed using *Mycoplasma mycoides* subsp.*mycoides* species-specific pair primers and 5µl of test DNA template in a total volume of 20 µl for 42 cycles. The reaction mixture consisted of RNase free water (3µl), Primer- MSC1-Fow-5pM/µl 5'-ATACTTCTGTTCTAGTAATATG-3' (2µl), Primer-MSC2-REV-5pM/µl 5'-CT GATTATGATGACAGTGGTTCA-3' (2µl), IQ Super mix (10µl), template (DNA) (3µl). The optimal amplification cycle for paired primers sets consisted of initial denaturation at 95^{o}c for 5 mints (1-Cycle), a 40 cycles of each denaturation at 95^{o}c for 30 sec, annealing at

50^{o}C for 30 sec, and initial elongation at 72^{o}c for 1 min, final elongation at 72^{o}c for 7 mints (1-Cycle) and the products were kept at 4^{o}C for electrophoresis. The positive control *Mmm* SC DNA used while nuclease free water and extraction control samples were used as negative control (Appendix 6). To confirm the targeted PCR amplification the PCR product was then subjected to electrophoresis in the 1.5% agarose gel containing 5µl gel red with 4µl dye and 10µL of DNA Ladder (Gene Ruler- Fermentas) was prepared and electrophoresis was carried out at 130 volts for one hr and the products were read under UV illumination adapted to molecular imaging software. The amplified product which produced specific bands with the molecular size of 260bp is observed in positive samples by ultraviolet trans-illumination (Appendix 7).

3.7. Data Management and Analysis

The questionnaire was entered into SPSS ver. 20 statistical software and finally analyzed using descriptive statistical tests (frequencies, proportions, means and ranges). Laboratory data were stored on Microsoft Excel spread sheet ver 2013, and analysis was done using STATA (version 13). Disease prevalence was analyzed using SPSS ver. 20 descriptive statistics (crosstabs) while degree of associations between the outcome variable and its potential risk factors were analyzed using STATA (version 13) using logistic regression model. CBPP sero positive was considered as the dependent variable and the risk factors as independent variables. Initially logistic regression of univariable analysis of measuring odds ratio made and the significant results of the univariate analysis were included in the final model using multivariate logistic regression analysis. Finally, the degree of association was computed using odds ratio (OR) signified by 95% confidence intervals with a p-value less than 0.05 taken as significant.

4. RESULTS

4.1. Knowledge Attitude and Practice (KAP) Questionnaire Result

4.1.1. Demographic characteristics, Herd size and structure of the respondents

A total of 113 households that had at least one cattle were involved in this study. The majority of the respondents were male (87.6%) and the rest female (12.4%). Similarly, the majority of respondents (86.7%) were married. The ages of respondents were ranges 15 to 80 with mean of 39.37±13 years old. Regarding education status of the participants (35.4%) of them had no formal education while (6.2%) of them college (university) level educated and the mean of household size of participants was 5.4±2.34. Total number of cattle per household was ranging from 2-25 with mean of 8.13±4.47. Out of a total 919 animals that 113 households had more than half of them were female cattle (53.2%) with mean of 4.3±2.51 per respondents and (46.79%) were male with mean of 3.8±2.47. Similarly, (26.66%) cattle population were young (1-3 years), (58.68%) adults (>3 years), and (14.04%) calves (less than one year) (Table 3).

Table 3: Socio-demographic characteristics, Herd size and structure of the respondents

Parameters	Mean±SD (Range)	Frequency	Proportion (%)
Gender of respondents			
Male	-	99	87.6
Female	-	14	12.4
Marital status			
Single	-	11	9.7
Married	-	98	86.7
Divorced		4	3.6
Educational Background			
Primary	-	5	45.1
Secondary	-	15	13.3
College or university	-	7	6.2
No formal education	-	40	35.4
Age of respondents	39.37±13.8(15-80)	-	-
Household family size	5.38±2.34(2-10)	-	-

Herd structure per household			
Sex			
Male	0-14	3.8±2.47	430(46.79)
Female	0-13	4.3±2.51	489(53.2)
Age			
Young (1-3 years)	0-11	2.2±1.93	245(26.66)
Adults (>3 years)	1-13	4.8±2.65	539(58.68
Calves (< 1 year)	0-4	1.14±1.13	129(14.04)
Over all	**2-25**	**8.13±4.47**	**919**

4.1.2. Farmers' knowledge and attitude related to CBPP disease in general

Of the total, (31.9%) of the respondents were knowledgeable with the cause of disorder while the majority of respondents had no any knowledge regarding to the factors that causes of respiratory disorders. (77.9%) of respondents were encountered respiratory problems of cattle, but (26.5%) of them recognized that the causes was due to infectious diseases while the majority of them had no reasons about the source of the problems and only (7%) of respondents had heard or knew CBPP while (93%) of the respondent hadn't knew (Table 4).

Table 4: overview of farmer's knowledge related to respiratory disorder of cattle and CBPP

Issue raised related to general overview of the respiratory disorder (RD) of cattle	**Response category (N=113)**		
	Yes (%)	No (%)	Don't know (%)
Have you encountered respiratory problems of cattle?	88(77.9)	25(22.1)	0
Has your neighbor had any problems with cattle RD?	73(64.6)	18(15.9)	22(19.5)
Do you know factor that cause respiratory disorder?	30(26.5)	24(21.2)	59(52.2)
Do you think infectious disease can cause the RD?	36(31.9)	10(8.8)	67(59.3)
Have your several animal experienced respiratory problems simultaneously?	44(38.9)	69(61.1)	0
Have you heard about CBPP disease?	8(7.1)	103(91.2)	2(1.8)

In this study the infectious diseases in general as well as respiratory diseases of cattle that known locally by farmers were assessed and the local names of the diseases were translated in to scientific or English name with the help of the nearby animal health worker of that area,

accordingly. As the result showed the predominant cattle diseases known by the respondents were trypanomosis (79.6%), blackleg (43.4%), pasturellosis (36.3%), anthrax (33.6%) and LSD (24.8%) whereas samba-beshita/dangila/ somba (25.7%) was the most known endemic respiratory disease of the area, however its scientific name couldn't identified yet (Table 5).

Table 5: Name of cattle diseases that commonly known by respondents in the study area

Issue raised to the respondent	Local name of disease that known by farmer	Scientific Name of the disease	Freq. (%)
Can you name any infectious diseases that found in your area or farm?	Hudha/Gororsisa	Pasturellosis	41(36.3)
	Bishoftu/Cacabsa/Aba-gurba	Blackleg	49(43.4)
	Desta	Rinderpest	6(5.3)
	Labobesa/Dukuba Alati	Milk fever	7(6.2)
	Aba-sanga/cita/Dingetegna	Anthrax	38(33.6)
	Gandi/Koksa	Trypanomosis	90(79.6)
	Masa(Maasaa)	FMD	11(9.7)
	Gara bokoksa	Bloat	17(15)
	Goga lukisa/Citesa	LSD	28(24.8)
	Samba beshita/Dangila/ somba	Could be CBPP or TB	29(25.7)
Can you name any respiratory infectious disease you know?	Sombaa/Danglaa/samba beshita	Could be CBPP or TB	13(11.5)
	koksa/Gandii	Trypanomosis	25(22.2)
	Don't know	Don't know	75(66.4)

The proportion of each major signs of CBPP disease that known by respondents were: grunting during coughing or exhaling (79.6%), head extended coughing (75.2%), dilation of nostril and mucoid discharge (71.7%), swelled of throat and dewlap (60.2%), standing with back arched (44.2%), laboured & painful breathing (54.9%), standing with the elbows abducted (38.9%), chest pain (19.5%), frothy saliva at the mouth (56.6%), and polyarthritis on young (23%). Generally in this study majority of the respondents were encountered or familiar with signs of CBPP disease (Table 6).

Table 6: Farmers knowledge assessment related to major symptoms of CBPP disease

Issue raised related to the major CBPP symptoms	Response category (N=113)		
	Yes (%)	No (%)	Don't know (%)
Have you know or encountered any of the following the major signs of CBPP disease?			
Chest pain	22(19.5)	56(49.6)	35(31)
Stand with the elbows abducted	44(38.9)	44(38.9)	25(22.1)
Standing with back arched	50(44.2)	37(32.7)	26(23.1)
Head extended coughing	85(75.2)	21(18.6)	7(6.2)
Laboured & painful breathing	62(54.9)	30(26.5)	21(18.6)
Grunting when exhaling (coughing)	90(79.6)	22(19.5)	1(0.9)
Frothy saliva at the mouth	64(56.6)	41(36.3)	8(7.1)
Dilation of nostril & mucoid discharge	81(71.7)	20(17.7)	12(10.6)
Swelled throat and dewlap	68(60.2)	35(31)	10(8.8)
Polyarthritis particularly on young	26(23)	55(48.7)	32(28.3)

Regarding knowledge of disease transmission, majority of the respondents hadn't aware of the possible transmission methods of contagious diseases like CBPP. However, 77% and 67.3% of the respondents recognized as diseases transmitted through close contact with diseased animals and through coughing of infected animals, respectively. Regarding to the contaminated diseased cattle fetal membrane and uterine discharge, only (8.8%) of the participants knowledgeable as it was associated with disease transmissions method. The table below is clearly summarized the knowledge of respondents had regarding the possible transmission methods of contagious diseases like CBPP (Table 7).

Table 7: Farmers knowledge regarding to the possible transmission methods of disease

Issue raised regarding to knowledge of disease transmissions methods	Response category (N=113)		
	Yes (%)	No (%)	Don't know (%)
What are the possible transmission methods of CBPP disease or any contagious diseases among cattle of the following?			
Through contaminated feed or water	29(25.7)	28(24.8)	56(49.6)
Transmitted through sexual contact	12(10.6)	44(38.9)	57(50.4)
Close contact with diseased animal	87(77)	9(8)	17(15)

Inhalation of infected droplets	56(49.6)	18(15.9)	39(34.5)
Through contaminated fomites/objects	27(23.9)	38(33.6)	48(42.5)
Can be transmitted through transplacental	30(26.5)	19(16.8)	64(56.6)
Through fetal membrane & uterine discharge	10(8.8)	30(26.5)	73(64.6)
Can be transmitted across long distance in air	19(16.8)	54(47.8)	40(35.4)
Through saliva or urine of diseased animal	31(27.4)	25(22.2)	26(23)
Through coughing of infected animal	76(67.3)	11(9.7)	26(23)

In the current study, majority of the respondents aware of the economic importance disease like CBPP for example (85.8%), (78.8%), (74.3%), and (57.5%), (61%) of respondents aware of as disease able to cause loss of body weight, loss of production, reduce working ability of cattle, mortality of cattle, and reduced growth rate, respectively. Regarding CBPP prevention and controlling methods, majority of the participants had basic knowledge on treatment of symptomized or diseased animal (93.8%) and vaccination (78.8%). Moreover, (32%) of the participants are aware of good management like decontamination of infected premises is used for disease prevention and controlling techniques. However, very few of participants were recognized test and slaughter/stamping out policy (16.8%) and isolation of new purchased animal from herd (19.5%) are used as disease prevention and controlling methods (Table 8).

Table 8: Farmers' knowledge and attitudes towards economic importance and disease prevention and controlling

Issue raised related to economic importance and prevention and controlling	**Response category (N=113)**		
	Yes (%)	**No (%)**	**Don't know (%)**
What are the economic importance' of CBPP/any respiratory related diseases in cattle production?			
Can cause mortality of cattle	65(57.5)	40(35.4)	8(7.1)
Can cause loss of body weight	97(85.8)	9(8)	7(6.2)
Reduced working ability of cattle	84(74.3)	4(3.5)	25(22.1)
Reduced fertility of cattle	54(47.8)	24(21.2)	35(31)
Reduced growth rate of cattle	69(61.1)	11(9.7)	33(29.2)
Can cause loss of production	89(78.8)	10(8.8)	14(12.4)

What do you think the effective way of disease prevention & controlling method of the following?			
Vaccination	89(78.8)	7(6.2)	17(15)
Treatment of symptomized animal	106(93.8)	5(4.4)	2(1.8)
Test and slaughter or stamping out policy	19(16.8)	71(62.8)	23(20.4)
Movement control or quarantine	24(21.2)	57(50.4)	32(28.3)
Isolation of new purchased animal from herd	22(19.5)	78(69)	13(11.5)
Decontamination of infected premises	36(31.9)	44(38.9)	33(29.3)

Cost of treatment of diseased animal (34.5%), death of animal due to the disease (29.2%), and loss of production (25.7%) are the foremost worries that respondents had felt related to respiratory disease of CBPP. Farmers preference ways of receiving knowledge related to animal diseases, the majority of the respondents (65.5%) preferred to receive knowledge through veterinarian/animal health workers followed by agricultural developmental agent (DA) (22.1%) whereas very few of respondents preferred to get through local cultural healers (2.7%). (60.2%) of the respondents preferred to know prevention and controlling methods followed by (22.1%) transmission methods while few farmers (4.4%) chosen diagnostic techniques (Table 9).

Table 9: Respondents attitudes regarding CBPP disease

Issue raised related to farmers attitude	Frequency	Percentage (%)
What worries you most felt if your animal diseased with respiratory disease of CBPP?		
Transmission to healthy animal	9	8
Cost of treatment	39	34.5
Death due to disease	33	29.2
Loss of production	29	25.7
No worries	3	2.7
Which ways do you want to receive knowledge about CBPP disease?		
Through district expert	6	5.3
Through PA DA	25	22.1
Veterinarian/animal health workers	74	65.5
Local cultural healers	3	2.7
Media	5	4.4

Which part of CBPP knowledge do you want to know more?		
About the causative agent of the disease	6	5.3
Symptoms of the disease	9	8
Transmission methods of the disease	25	22.1
Diagnostic methods	5	4.4
Prevention and controlling methods	68	60.2

4.1.3. Farmers' practices towards disease prevention and controlling techniques

In the present study result, there were very few farmers practiced good animal husbandry such as (3.5%) of respondents were practicing of communal grazing/watering, (15%) of respondents hadn't used common breeding bulls with the surrounding communities, (30.1%) of respondents hadn't purchased cattle from disease infected origin or outbreak area, (18.6%) performing isolation of new purchased animal from the herd and (8%) respondents were carry out restriction of freely cattle movement across the surrounding communities. Similarly, only 69% of the respondents were following up vaccination of cattle. Moreover, of the total respondents (45%) were familiar to selling of diseased animal to neighbors or local market/butchers, (52%) practicing treating of diseased animal with cultural medications and only (36.3%) of the farmers experienced separating or isolation of diseased animal from healthy herds whereas none of the respondent exercising stamping out policy of severely diseased animals. However, (99%) of respondents were carry out treating of diseased animals in nearby of veterinary clinics. Generally majority of the respondents were following up poor animal husbandry practices (Table 10).

Table 10: Farmers' practices towards animal disease prevention and controlling methods

Issue raised regarding respondents practices associated with disease prevention and controlling methods	**Response category(N=113)**	
	Yes (%)	**No (%)**
What are your practices you are doing during herd management to prevent contagious infectious diseases?		
Avoid using common breeding bulls in the community	17(15)	96(85)
Avoiding of communal grazing & watering	4(3.5)	109(96.5)
Avoiding of cattle purchase from disease outbreak area	34(30.1)	79(69.9)

Routine manure removal or changing of kraal	51(45.1)	62(54.9)
Follow up of regular vaccination of herds	78(69)	35(31)
Isolating new purchased animal from the herd	21(18.6)	92(81.4)
Restriction of freely cattle movement	9(8)	104(92)
What are the common practices you exercised when your animal suspected or diseased with respiratory disease?		
Selling to neighbors or local market/butchers	51(45.1)	61(54)
Presenting to veterinary clinic of area	112(99.1)	1(0.1)
Treating with cultural medications	59(52.2)	54(47.8)
Separating/isolation of diseased animal from health	41(36.3)	72(63.7)
Slaughtering for self-consumption	16(14.3)	97(85.8)
Follow up stamping out policy (culling)	0	113(100)

4.2. Sero-Prevalence and Associated Risk Factors of CBPP

4.2.1. The overall animal and herd level seroprevalence

Of the total 576 sampled animals from 113 cattle herds, 84 were seropositive with CBPP disease. The overall anima level seroprevalence CBPP was 14.6% (95% CI: 11.80-17.73). Out of the total 113 sampled herds, 61 herds were infected and the overall herd level seroprevalence of CBPP was 54% (95%CI: 44.35-63.40).

4.2.2. Animal level seroprevalence of CBPP across the risk factors

In this study different animal level seroprevalence was recorded across the study locations such as higher prevalence was observed in Ilu Gelan district (20.1%) than Sibu Sire district (9%). Similarly, highest prevalence was recorded in Ale Wara Ilu (22.1%) while lowest in Cheri Jarso (7%). In the present study also various prevalence were recorded across host related potential risk factors like sex, age, parity and body condition of the animals. As the result of the study described seropositivity was higher: in adult (18.1%) than in young (9.6%), highest in poor body condition (23.3%) than in medium (12.8%) and good body condition (6.4%) and highest seroprevalence in pluriparus (17.1%) while lowest in heifers (7.6%) were recorded. As well as highest seroprevalence was observed in large herd size

(30.6%) than small herd size (5.5%) and higher prevalence (18.8%) in animals with history respiratory disorder than healthy animals (10.2%) (Table 11).

Univariate Logistic regression analysis results showed that among the risk factors that considered district, age, history of respiratory disorder, herd size and body condition were spastically significant ($p<0.05$). The animal level sero prevalence was significantly higher in Ilu Galan district than in Sibu Sire such as cattle that found in Ilu Galan (OR=2.54, CI95%: 1.55-4.17, P = 0.000) were more than two times more likely to be affected by CBPP disease than Sibu Sire. Similarly, cattle that kept at Ale Wara Ilu (OR = 2.3, 95% CI: 1.2-4.4, P =0.012) were 2.3 times more likely to be CBPP seropositive than Lelisa. The likelihood of seropositivity with CBPP disease of adult cattle (OR=2.1, CI95%:1.24-3.46, P=0.005) were two times more seropositive than young, animals which had history of respiratory problems (OR=2.04, 95% CI: 1.26-3.31, P=0.004) were two times more likely to be seropositive than those hadn't, poor body condition animals (OR=4.4, 95% CI: 1.84-10.64, P=0.001) were four times more likely to be seropositive than good body condition and cattle that found in large herds (OR=7.6, 95%CI: 3.84-14.94, P = 0.000) were seven times more likely to be affected by CBPP disease than small herds. As well as the likelihood of getting risk of infection with CBPP disease of multiple parity (OR=2.5; 95% CI: 1.16-5.51, P=0.020) were two times more than heifers (Table 11).

Table 11: Animal level seroprevalence degree of association analysis across potential risk factors using univariate logistic regression

Risk factors	No Tested	% (95% CI)	Report of odds ratios		P-value
			OR (95% CI)	Std.Err.	
District					
Sibu Sire	288	9(5.98-12.95)	1		
Ilu Galan	288	20.1(15.66-25.24)	2.54(1.55-4.17)	0.64	0.000***
PA(kebele)					
Lelisa	146	11(6.4-17.19)	1		
Cheri Jarso	142	7(3.43-12.57)	0.62(0.27-1.41)	0.26	0.250
Wadeyi Granche	145	22.1(15.61-29.70)	1.8(0.92-3.53)	0.62	0.084
Ale Wara Ilu	143	18.2(12.23-25.49)	2.3(1.2-4.4)	0.74	0.012*
Sex					
Female	326	13.2(9.71-17.35)	1		

Male	250	16.4(12.03-21.58)	1.3(0.81-2.05)	0.31	0.280
Age					
Young	239	9.6(6.2-14.1)	1		
Adult	337	18.1(14.14-22.63)	2.1(1.24-3.46)	0.54	0.005**
Herd size					
Small	228	5.5(2.74-9.01)	1		
Medium	211	12.8(8.2-17.27)	2.5(1.24-5.1)	0.9	0.011*
Large	147	30.6(23.3-38.74)	7.6(3.84-14.94)	2.6	0.000***
History of RD					
No	292	18.8(14.52-23.80)	1		
Yes	284	10.2(6.95-14.34)	2.04(1.26-3.31)	0.5	0.004**
BCS					
Good	116	23.3(15.93-32.03)	1		
Medium	351	14.2(10.76-18.35)	2.42(1.06-5.51)		0.035*
Poor	109	6.4(2.62-12.78)	4.4(1.84-10.64)	1.98	0.001**
Parity					
Heifer	132	7.6(3.69-13.49)	1		
Primiparus	48	16.7(7.48-30.22)	2.44(0.9-6.61)	1.24	0.079
Pluriparus	140	17.1(11.30-24.42)	2.5(1.16-5.51)	1	0.020*

Coef=coefficient; CI=confidence interval; OR=Odd ratio; Std.Err=standard error; RD=respiratory disorder; BSC= body condition score; PA= peasant association; and *=statistically significant

Initially, univariate logistic regression was used to screen all potential risk factors for statistical significance at ($p < 0.05$) and variables that found statistically significant were subjected to multivariate logistic regression analysis with forward and backward variable selection approach based on the likelihood ratio statistic ($p<0.05$). The risk factors (district, age, and history of respiratory disorder, BCS and herd size) that statistically significant in univariable logistic regression analysis were included in the model and analyzed together. Therefore, the final multivariable logistic regression model analysis result showed that district, age, history of respiratory disorder and herd size were statistically significant association with CBPP seroprevalence ($P < 0.05$) (Table 12).

Table 12: The result of multivariate logistic regression potential risk factors of individual animal level seroprevalence degree of association analysis

Risk factors	Report of odds ratios		P-value
	OR (95% CI)	Std. Err.	
District			
Sibu Sire	1		
Ilu Galan	2.4(1.4-4)	0.64	0.002**
Age			
Young	1		
Adult	2.1(1.2-3.6)	0.58	0.009**
History of RD			
No	1		
Yes	1.9(1.1-3)	0.5	0.021*
Herd size			
Small	1		
Medium	2.4(1.2-5)	0.9	0.016*
Large	6.1(3-12)	2.2	0.000***

CI=confidence interval; OR=Odd ratio; Std.Err. =standard error *= statistically significant

4.2.3. Herd level seroprevalence of CBPP across the associated risk factors

In the present study the risk factors that considered at herd level seroprevalence were district, PA, herd size and history of respiratory health problems within the herd. The herd level CBPP seropositive was higher in Ilu Gelan (67.2%) than in Sibu Sire (30%). Among, the four sampled PAs the herd level prevalence was highest in Ale Wara Ilu (71%) while lowest in Cheri Jarso (30.8%). Similarly, highest herd level seroprevalence was observed in large herd size (83.9%) while lowest in small herd size (32.6%). Regarding to the history of occurrence of respiratory health problem, herds that encountered history of respiratory disorder had greater prevalence (56.9%) than those hadn't encountered (50.9%).

Univariate Logistic regression analysis results showed that among herd level risk factors (district, PA, herd size and history of respiratory problem and herd size) that considered, only district and herd size were statically significant effect on seropositivity ($p<0.05$). Such as the

likelihood of cattle herds infection with CBPP disease in Ilu Galan district (OR = 3.1, 95% CI: 1.43-6.64, p=0.004) three times more than Sibu Sire. The chance of seropositivity with CBPP disease in larger herd (OR=10.8, 95%CI: 3.4-34), p=0.000) ten times more likely than smaller herds (Table 13).

Table 13: Univariate logistic regression degree of association analysis of potential risk factors with herd level seroprevalence

Risk factors	Total Herd	% (95% CI)	Report of odds ratios		P-value
			OR (95% CI)	Std. Err.	
District					
Sibu Sire	55	30(27.22-54.1)	1		
Ilu Galan	58	67.2(53.66-78.99)	3.1(1.43-6.64)	1.21	0.004**
PA					
Lelisa	29	48.3(29.45-67.47)	1		
Cheri Jarso	26	30.8(14.33-51.79)	0.48(0.16-1.44)	0.27	0.189
Wadeyi	31	71(51.96-85.78)	1.82(0.63-5.3)	0.99	0.271
Granche					
Ale Wara Ilu	27	63(42.37-80.60)	2.62(0.9-7.59)	1.42	0.076
Herd size					
Small	31	83.9(66.3-94.5)	1		
Medium	39	53.8(37.2-69.9)	2.4(0.99-5.9)	1.1	0.05*
Large	43	32.6(19.1-48-.5)	10.8(3.4-34)	6.3	0.000***
History of RD					
No	55	50.9(37.07-64.65)	1		
Yes	58	56.9(43.23-69.84)	1.27(0.61-2.67)	0.48	0.524

Coef=coefficient; CI=confidence interval; OR=Odd ratio; Std.Err=standard error; RD=respiratory disorder; PA= peasant association; and *=statistically significant

4.3. Detection of *Mycoplasma Mycoides* Subsp. *Mycoides* (*Mmm* SC) Using PCR

Detection of *Mycoplasma mycoides* subsp. *mycoides (Mmm* SC) was made using conventional PCR assay directly from lung tissue (without culturing or enriching of lung samples that collected from asymptomatic seropositive) animals. The detections have been conducted using the primers specific to *Mmm* SC (Primer-MSC1-Fow-5pM/µl 5'-ATA

CTTCTGTTCTAGTAATATG-3' and Primer-MSC2-REV-5pM/µl 5'-CTGATTATGATGA CAGTGGTTC A-3'). Based on the result of PCR amplifications of the *Mmm* SC genomic DNA detection, of the four tested samples, *Mmm* SC antigen was detected in all four samples (100%). Thus, the PCR test result was indicated below as *Mmm* SC antigen was detected in all sampled animals lung tissues. The amplified product that produced specific bands with the molecular size of 260 bp was observed on agarose gel electrophoresis which indicated the presence of *Mmm* SC DNA in the samples (positive) (Figure 5). In conclusion, in this study out of 84 seropositive animals, four (two male and two female) seropositive animal were purposively selected based strength of seropositivity for further identification of the causative agent using molecular method (PCR) and *Mmm* SC was detected in all sampled animals as aforementioned and summarized on table below (Table 14).

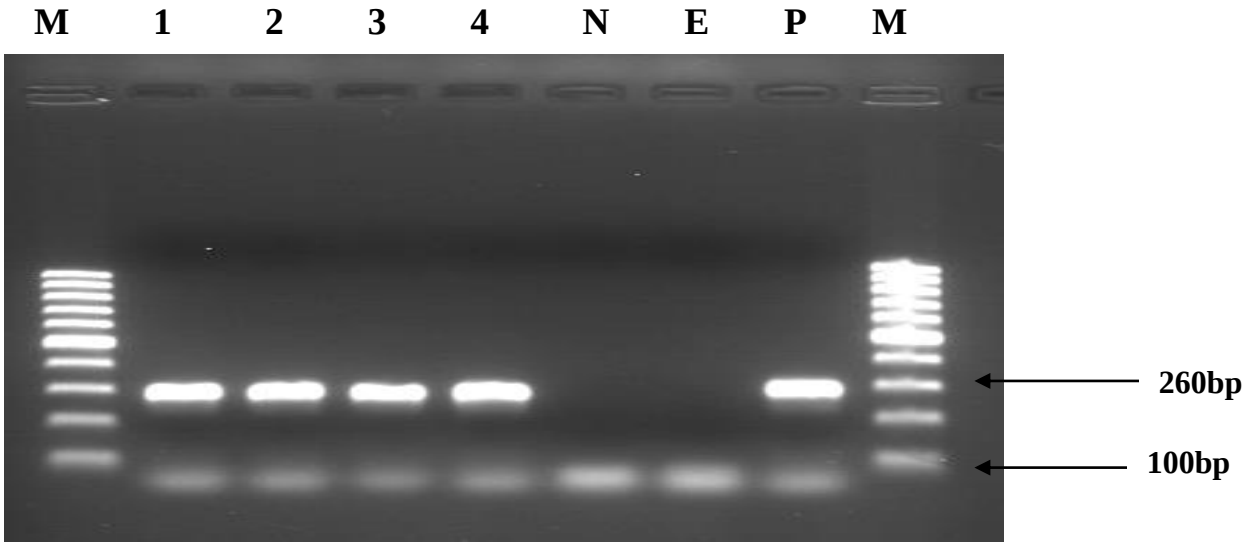

Figure 6: Gel electrophoresis of PCR products of samples which yielded bacterial genomic DNA

M= (Lanes1and 8) Molecular Ladder or molecular weight markers started 100bp (Feremantas) 1Kb; Lanes 1-4 = positive field samples extracted DNA; Lane N= RNase free water (negative Control); Lane E = Extraction control (negative control) and Lane P= Positive control.

Table 14: Summary of postmortem examination and PCR result

Ref. No	Age	Sex of animal	Life animal Serum c-ELISA result	Post mortem observation result	Type of sample used for molecular test	PCR-result
1	6	Female	++	No CBPP lesions	Lung tissue	+
2	7	Female	+++	Typical CBPP lesions	Lung tissue	+
3	8	Male	++	No CBPP lesions	Lung tissue	+
4	3	Male	++	Typical CBPP lesions	Lung tissue	+

5. DISCUSSION

Contagious bovine pleuropneumonia (CBPP) is an acute, sub-acute or chronic disease of cattle characterized by anorexia, fever and respiratory signs such as dyspnoea, polypnoea, cough, nasal discharges, fibrinous pneumonia, serofibrinous pleuritis, and oedema of the interlobular septa of the lungs (OIE, 2014). It is one of the great plagues that continue to devastate the cattle herds on which so many people are dependent and considered as the most serious infectious animal disease affecting African cattle (Amanfu, 2009; Marobela-Raborokgwe, 2011). Although the disease has great potential for rapid spread and causes major impact on cattle production, still nationwide epidemiological surveillance and control activities are often inadequate or unavailable in most African countries including Ethiopia (Ahmed, 2016).

Generally KAP study related to CBPP disease was found to be very important during the study of disease epidemiology. However, the researchers did not come across on KAP study related to CBPP disease except one paper which was reported by Kairu-Wanyoikea *et al*. (2014) on control of contagious bovine pleuropneumonia: knowledge, perceptions and practices in Narok district of Kenya. In our country Ethiopia many of the reported research findings related to CBPP were emphasized only on the seroprevalence of the disease except the report of Gizaw (2004) who had done participatory disease searches (PDS) in order to understand local perceptions of animal health situation in general and CBPP disease in particular, using semi-structured interviews, focus group discussions and visualization methods like mapping. In the present study even though the existence of the disease have been confirmed through the application of both serology and PCR techniques, large number of farmers had no awareness about CBPP disease in general. For instance, of the total participants (77.97%) were came across respiratory problems of cattle, on the other hand very few (7.1%) of the respondents have been heard about CBPP disease. However, among the respondents questioned to name any infectious disease they knew, 25.7% of farmers named samba beshita/dangla/ or somba and for further confirmation, the respondents again, questioned to name any respiratory infectious disease they knew, and 11.5% were responded sombaa/dangla/samba beshita, however, its scientific or English name couldn't identified yet.

As the present study indicated the aforementioned disease name was most probability might be CBPP disease, but, in order to know exact local name of the disease further assessment should be made in future. Even though the name of the disease is not well-known by the communities, majority of respondents were encountered CBPP disease and undoubtedly familiarized with symptoms of CBPP. For example, 79.6%, 75.2% and 71.7% of the respondents' well-informed each symptoms like grunting when exhaling (coughing), head extended coughing, and dilation of nostril & mucoid discharge, respectively.

As the result of KAP questionnaire indicated, majority of the participants do not aware of the possible transmission methods of contagious diseases like CBPP among cattle. However, transmission of through close contact with diseased animal (77%), and coughing of infected animal (67.3%) are the most well-known means of disease transmission methods in the area. On the contrary, majority of respondents were aware of the impact of any respiratory diseases including CBPP. Regarding disease prevention and controlling options only vaccination (78.8%) and treatment of diseased animal (93.8%) are the most recognized techniques of disease prevention and controlling option in the community. However, many of the controlling options like test and slaughter or stamping out policy of severely diseased cattle (16.8%), movement control or quarantine (21.2%), decontamination of infected premises (31.9%), and isolation of new purchased animal from herd (19.5%) were not recognized by farmers of the study area. Majority of farmers would not know what to do or would do nothing in the event of disease occurrence which agreed with the report of Kairu-Wanyoikea *et al*. (2014) in Narok district of Kenya. Therefore, such kind of knowledge gap among the society will be creating an ideal environment for contagious diseases like CBPP to easily distribute and infect large cattle population of the study area.

The result of this study showed regarding to farmers practice, majority of farmers were following up poor herd management practice that create favorable environment for disease multiplication and distribution across the surrounding communities of the area. Despite large number of peoples were following up poor management system of cattle production, many of farmers are voluntary or had positive attitude to apply any kind of prevention and controlling techniques of animal disease if the necessary education is afforded to them from

the government. Therefore, the present study result gave hope from farmer's point of view to create applicable disease prevention and controlling techniques through awareness creation in the communities. In a study in the UK with buffalo farmers, the authors reported that a change in practices among the farmers was necessary for the implementation of diseases prevention and controlling programs (Ellis-Iversen *et al.,* 2010). Therefore, optimum prevention and controlling of contagious animal diseases like CBPP can be easily achieved, through changing of farmers' poor animal husbandry practices.

The overall animal level seroprevalence of CBPP was (14.6%, 95% CI: 11.80 -17.73) which is comparably in agreement with the findings of various researchers who reported prevalence of 12% in southern zone of Tigray region of Ethiopia (Teklue *et al.*, 2015), 14% in Niger state of north central Nigeria (Alhaji and Babalobi, 2016), 14.3% in Kajiado district of Kenya (Matua-Alumira *et al.* 2006), 17.19% in Khartoum State of Sudan (Elhassan, 2012), and 17% in Turkana district of Kenya (Maritim, 2009). On the other hand, the finding of this study was higher than the results of Gizaw (2004) in Somali regional state (10.3%), Ahmed (2004) in Borena (9.4%), Atnafie *et al.*(2015) in Bishoftu abattoir and Adama quarantine (6.85%), Alemayehu *et al.*(2015) in Borena pastoral of Oromia (0.4%), Kassaye and Molla (2013) in export quarantine of in and around Adama (4%), Geresu *et al* (2017) in Dello Mena and Sawena districts of Bale zone (6.51%), Schnier *et al*. (2006) in south western Kenya (9.7%), Mbengue *et al.* (2013) in Senegal (0.43%), Mtui-Malamsha (2009) in the Maasai ecosystem of south-western Kenya (11.21%), and Zarina *et al*. (2016) in north east states of Peninsular Malaysia Pertanika (8%). On the contrary, it is by far much lower than the previous reports of Ebisa *et al.* (2015) with 31.8% in Amaro district of SNNP region, Mersha (2016) with 28.5% in selected districts of western Oromia, Soromou *et al.* (2014) with 21.05% in Guinea and Suleiman *et al.* (2015) with 30.2% in agro-pastoral areas of Nigeria.

The overall herd level seroprevalence of CBPP was (54%, 95% CI: 44.35-63.40). This overall herd level seroprevalence is closely in agreement with the finding of Suleiman *et al.* (2015) with 54.7% in agro-pastoral areas of Nigeria. However, higher than the previous report of (Bonnet *et al*., 2005) with 4.6% in the Ethiopian highlands, Gizaw (2004) with 30.4% in Somali regional state of Ethiopia, Zarina *et al*. (2016) with 17% in north east states

of Peninsular Malaysia Pertanika and Alhaji and Babalobi (2016) with 47.2% in Niger state of north central Nigeria. On the other hand the finding was lower than the report of Mtui-Malamsha (2009) with 85% in the Maasai ecosystem of south-western Kenya.

The rates of CBPP infection reported to vary from one region to another even within the region (Radostitis *et al*., 2007; OIE, 2014). CBPP occurrence is described to cover large area of the country. Different seroprevalences of CBPP were recorded across the study districts and peasant associations such as animal level seroprevalence was (20.1%, 95%CI: 15.66-25.24) in Ilu Gelan district while (9%, 95%CI: 5.98-12.95) in Sibu Sire ($P < 0.05$). Similarly, significantly higher prevalence was observed in Ale Wara Ilu (22.1%) PA than Cheri Jarso (7%) PA. The present result of variation seroprevalence within closely related study locations (districts and PAs) was agreement with the finding of different authors such as Ebisa *et al.*, (2015) in Amaro district of SNNP region, Mersha (2016) in selected districts of western Oromia, Teklue *et al.* (2015) in Southern zone of Tigray region, Gizaw (2004) in Somali regional state, Maritim (2009) in Turkana district of Kenya. This might indicate the presence of CBPP infection depends on certain associated risk factors like the epidemiology of the disease, agroecology, livestock population and movement, and different management system may be applied across the study sites such as the presence or absence of communal grazing and watering areas within locations and the probability of introduction new purchased animal from disease endemic area (Radiostits *et al*., 2007). Therefore, the probabilities of animals to be infected with CBPP disease as well as with other diseases are various across locations even it is different within similar agro-ecological locations.

There was no significant difference of CBPP seroprevalence among the sex which was (16.4%, 95%CI: 12.03-21.58) in male and (13.2%, 95%CI: 9.71-17.35) in female animal. This finding is in agreement with the work done by Ebisa *et al.,* (2015) in Amaro district of SNNP region, Mersha (2016) in selected districts of western Oromia, Teklue *et al.* (2015) in Southern zone of Tigray region of Ethiopia, Suleiman *et al.* (2015) in agro-pastoral areas of Nigeria, and Alhaji and Babalobi (2016) in Niger state of north central Nigeria. Therefore, the study result agreed with Provost *et al.* (1987) who reported sex has not been considered as the risk factors that affecting the susceptibility of cattle to CBPP disease infection. On the

other hand, contradicted with the finding of Schnier (2006) in in south western Kenya and Mtui-Malamsha (2009) in the Maasai ecosystem of south-western Kenya who reported statically significant difference among sex.

Age is supposed to have some association with occurrence of CBPP disease because young or calves become more resistant to experimental infection than adult cows (Provost *et al.* 1987; Titus, 2003). The result of this study also revealed there was statistically significant difference of CBPP disease among the age groups. Higher seroprevalence was recorded in adult animals (18.1%, 95%CI: 14.14-22.63) than in in young animals (9.6%, 95%CI: 6.2-14.1). The likelihood of seropositivity of adult cattle (OR=2.1, 95% CI: 1.24-3.46, P=0.005) was two times more seropositive to CBPP than young cattle. This result is in consistent with the reports of Elhassan (2012) in Khartoum state of Sudan, Alhaji and Babalobi (2016) in Niger state of north central Nigeria, Mtui-Malamsha (2009) in the Maasai ecosystem of south-western Kenya and Schnier *et al*. (2006) in south western Kenya. In contrast, there are different studies that reported insignificant associations such as Ebisa *et al.* (2015) in Amaro district of SNNP region, Teklue *et al.* (2015) in southern zone of Tigray region, Mersha (2016) in selected districts of western Oromia and Suleiman *et al.* (2015) in agro-pastoral areas of Nigeria. Calves were reported to be relatively more resistant to infection by *Mmm* SC than adult cattle (Lesnoff *et al.,* 2004; McKeever *et al.,* 2009), which may be explained by the fact that increasing age is a surrogate measure of repeated exposure (Boelaert *et al*., 2005) and similar findings were reported from Sudan Zessin *et al.* (1985) and McDermott *et al.* (1987). Moreover, the study also agreed with Thomson (2005) who reported that factor like extremes of age may predispose to tissue invasion of CBPP disease.

Seroprevalence of CBPP was highest in cattle with poor body condition (23.3%, 95%CI: 15.93-32.03) as compared to cattle with medium body condition (14.2%, 95%CI: 10.76-18.35) and good body condition (6.4%, 95%CI: 2.62-12.78). This finding is in agreement with the report of Ebisa *et al.*, (2015), Suleiman *et al.* (2015) and Mtui-Malamsha (2009).The poor body conditioned cattle were (OR=4.4, 95% CI: 1.84-10.64, P=0.001) four times more likely to have the CBPP compared to the good body conditioned cattle. This could due to related to the weak protective immune response in poor body conditioned cattle compared to

good ones. Loss of body condition is one of the indications for the presence of the infection in the animal. Mostly CBPP chronic carrier animals became emaciated because of the clinical characteristics of the disease. Besides, animals with good body condition have relatively good immunological response to the infectious agent than animals with medium and poor body condition score (Radostatit *et al.*, 2007). It was supposed that CBPP seropositive animals had poor body conditions than sero negative animals; thus, the present study result confirmed the assumption. This indirectly describes the impact of the disease associated with loss of productivity of cattle.

There was statistical significant between history of previous respiratory disorder/health problems and seroprevalence of CBPP disease (P=0.004). The prevalence was highest in respiratory disordered animals (18.8%, 95%CI: 14.52-23.80) compared to cattle that had not experienced respiratory problems (10.2%, 95%CI: 6.95-14.34). Respiratory disordered animals (OR=2.04, 95% CI: 1.26-3.31) were two times more seropositive of CBPP disease as compared to healthy animals which in line with the report of William and Amanfu (2002) and Mtui-Malamsha (2009). Among the clinical signs of CBPP respiratory disorders like laboured painful breathing, coughing and as severity of the disease progress lung lesions will be observed. CBPP is typically characterized by fibrinous pneumonia, serofibrinous pleuritis, and oedema of the interlobular septa of the lungs, frequently the symptoms of CBPP disease is associated with lung organs (EMPRES, 2002, Hamsten, 2009). In this study the reason of higher seroprevalence in cattle with history of respiratory disordered animal was due to the clinical characteristics of the disease. Hence, based on the present study result, if animals were exhibited any respiratory health problem in the study area; it could be an indication of CBPP infection.

The prevalence was highest in cattle with large herd size (30.6%, 95%CI: 23.3-38.74) as compared to cattle with small herd size cattle (5.5%, 95%CI: 2.74-9.01). The large herd size groups (OR=7.6, 95% CI: 3.84-14.94, P=0.000) were seven times more likely to have the CBPP infection compare to the small herd size cattle groups. The finding is in line with the report of Alemayehu *et al.* (2015) in Borena pastoral area of Southern Ethiopia, Suleiman *et al.* (2015) in agro-pastoral areas of Nigeria who reported large herd size cattle groups

significantly associated with CBPP seroprevalence. Compact grouping of herds during grazing or herding and confinement at night within small enclosures or kraal such conditions are eminently favorable for infection of CBPP disease (Provost *et al.,* 1987). Herds which had many animals were more likely to be CBPP positive compared to herds with few animals. The rate of effective contact between CBPP-infected and susceptible cattle is reported to be higher in larger herds (Lesnoff *et al.,* 2004), reason for which could be explained as the contagious nature of CBPP and its direct mode of transmission which might be increased by crowding and increased frequency of contacts as herd size increases. Furthermore, Majekodunmi *et al.* (2014) reported that CBPP prevalence may even be higher in parts of predominated by pastoral systems such as nomadic and transhumant where frequently larger herds are kept.

There is no any report on the issue of stage of parity susceptibility to CBPP disease so far. In this study, the seroprevalence was found to be higher in Pluriparus caws (17.1%, 95%CI: 11.30-24.42) as compared to with single parity (16.7%, 95%CI: 7.48-30.22) and heifers (7.6%, 95%CI: 3.69-13.49). As the current result showed the likelihood of seropositivity of CBPP was increased with increased cattle parity such as the likelihood of seropositivity with CBPP disease of multiple parity (OR= 2.5; 95% CI: 1.16-5.51, P=0.020) was 2.5 times more than heifer as well as the likelihood of seropositivity with CBPP disease of single parity cows (OR=2.4, 95% CI: 0.9-6.61, P=0.079) were 2.4 times more than heifers. The reason of higher prevalence in Pluriparous could be associated with age of animal because those multiple parity animals were older than single parity and heifers. According to the report of Provost *et al. (*1987), Titus (2003) and Lesnoff *et al.* (2004), heifers respond weakly to vaccination but become more resistant to experimental infection than adult cows.

Detection of *Mmm* SC was made directly from lung tissue that sampled from seropositive animals with the application PCR assay using the primers that specific to *Mmm* SC. Of the four animal lung tissues sampled, only in two samples (50%) lesions of CBPP observed while the other two lung samples were negative upon postmortem examination; however, with PCR amplifications of the genomic DNA results, *Mmm* SC was detected in all four

samples (100%). The positive CBPP pathology was taken as a definite mark of *Mmm*SC infection in the lungs of the animals. The absence of gross pathology was observed in positive both serological (c-ELISA) and molecular (PCR) tests which in line with the result of Bashiruddin *et al.* (2005). However, according to the report of Bashiruddin *et al.* (2005) the lack of appropriate gross pathology in the presence of positive serological or other detection tests like PCR may not be usual. The reason of absence of lesions on the two animals during post mortem examination could be the animals were treated with antibiotics, because antibiotic treatment is definitively stopped the extension of lesions as the report of (Thiaucourt *et al.*, 2004). In addition, Alhaji and Babalobi (2015) also reported that culturing and postmortem diagnosis of CBPP has not always have been successful in epidemiological surveillance especially where animals have been treated with antibiotics which is a common practice by animal health professionals. One of the unique advantage of PCR is the ability to detect died antigens, thus, the other probability of absence of lesion on the two samples could be due to died antigen, because died antigen is unable to induce lesions while capable to induce antibody production. According to the report of Bashiruddin *et al.* (2005) detection systems that do not rely on the viability of *Mmm* SC should be considered favorably. Collectively, PCR was the most successful detection method from tissues samples (Bashiruddin *et al.,* 1999; Bashiruddin *et al.,* 2005). Besides, this finding further validates the application of c-ELISA test for diagnosis of CBPP during epidemiological study of the disease, particularly in case of advance confirmatory diagnostic like culturing; biochemical test, molecular test and postmortem examination were not accessible.

6. CONCLUSIONS AND RECOMMENDATIONS

The output of this study has indicated that contagious bovine pleuropneumonia was endemic in the study area and confirmed by serology and polymerase chain reaction (PCR). Out of 576 sampled animals 84 were seropositive and an overall 14.6% seroprevalence was recorded using c-ELISA test and of which, 9% recorded from Sibu Sire while 20.1% from Ilu Galan district. The potential risk factors like location (district), age, history of respiratory health problem, body condition score and herd sizes were statistically significant effect on seroprevalence of the CBPP disease. Similarly, significantly higher prevalence was observed in multiple parity cows than heifers and in Ale Wara Ilu (22.1%) PA than Cheri Jarso (7%) PA. This indicates the presence of CBPP infection depends on certain geographical area preference of the pathogen, herd management system or animal husbandry, and host related potential risk factors. Of the four seropositive animals that tested with PCR *Mmm* SC was detected in all sampled animals. Even though the existence of the disease has been confirmed in the present study, there was knowledge and attitude gap among the community towards animal diseases in general and CBPP disease in particular. Besides, the majority of farmers were practicing poor animal husbandry that created favorable environment for CBPP disease multiplication and distribution across the surrounding communities. Thus, it is necessary to carry out careful herd management and control of animal movement within community and implementing regular vaccination of animals are good warranty. Therefore, based on the above conclusion the following recommendations were forwarded:

- Further investigation in wide geographical areas and large sample size using reliable tools like molecular technique and biochemical test are needed in order to know the exact epidemiological scenario of the disease.
- The farmers should be made aware of about CBPP disease particularly the economic importance, transmissions methods, and controlling techniques of the disease through veterinary extension education and possible means like media.
- The government has to apply controlling and prevention strategy of this economically devastating disease of cattle.

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8. APPENDICES

Appendix 1: Blood Sample Collection Format

Date_____/_____/2017

Ref.No	District	PA	Sex	Age	Herd size	Parity	BCS	History of respiratory disorders	CBPP result

Appendix 2: Description of Body Condition Scores (BCS)

Poor

- Clearly defined bone structure of shoulder, ribs, back, hooks and pins easily visible.
- Little muscle tissue or fat present.
- Small amount of muscling in the hindquarters. Fat is present, but not abundant.
- Space between spinous process is easily seen.
- Fat begins to cover loin, back and fore ribs.
- Upper skeletal structures visible. Spinous process is easily identified.

Medium/ Borderline (Optimum)

- Fore ribs becoming less noticeable. The transverse spinous process can be identified by palpation. Fat and muscle tissue not abundant, but increasing in fullness.

- Ribs are visible only when the animal has been shrunk. Processes not visible. Each side of the tail head is filled, but not mounded.
- Ribs not noticeable to the eye. Muscling in hindquarters plump and full. Fat around tail head and covering the fore ribs.

Good

- Spinous process can only be felt with firm pressure. Fat cover in abundance on either side of tail head.
- Animal smooth and blocky appearance; bone structure difficult to identify. Fat cover is abundant.
- Structures difficult to identify. Fat cover is excessive and mobility may be impaired.

Appendix 3: Knowledge, Attitude and Practice (KAP) Questionnaire Format

Participant ID Number: _____; Date of Interview: _____ /_____/ 2017

Section 1: Socio-demographic Characteristics of Respondents

1. Name of respondent: _____

1.1. Gender of the respondent: 1. Male 2. Female

1.2. Age (years): _____

1.3. Marital status: 1. Single 2. Married 3. Widowed 4. Divorced

1.4. Respondent's educational background:

1. Primary school 2. Secondary school 3. Vocational school
4. College / University 5. No formal Education

1.5. Respondent's position in the household (with respect to the head):

1. Husband 2. Wife 3. Daughter 4. Son
5. Relative living in a house 6. Farm laborer

1.6. Household size: 1. 1-3 2. 4-6 3. Greater than 6

Section 2: Size and Herd Structure of Cattle that Respondent Owned

2.1. Total number of cattle's of respondent owned _____

2.2. Sex: Male (No) _____and Female (No) _____

2.3. Age: young (*0.6-2 years) (No):*_____adult *(≥2 years)* (No):_____& calf (No):____

Section 3: Farmers Knowledge and Attitude on Respiratory Diseases of Cattle with Specific Emphasis to CBPP Infectious Disease

3.1. General overview of farmer's knowledge regarding respiratory disease and CBPP disease

No	Overview of cattle's respiratory disorder and CBPP	Yes	No	Don`t know
1	Have you encountered respiratory problems of cattle?			
2	Has your neighbor had any problems with cattle RD?			
3	Do you know factor that cause respiratory disorder?			
4	Do you think infectious diseases cause the disorder?			
5	Have your several animal experienced respiratory problems simultaneously?			
6	Have you heard about CBPP disease?			

3.2. Can you name any infectious diseases that found in your area/farm?

No	Local name	Scientific name
1		
2		
3		

3.3. Have you know or encountered any of the following MAJOR SIGNS of CBPP disease?

No	CBPP symptoms	Yes	No	Don`t know
1	Depression			
2	Anorexia			
3	Chest pain			
4	Stand with the elbows abducted			
5	Standing with back arched			
6	Head extended coughing			
7	Laboured & painful breathing			

8	Grunting when exhaling (coughing)			
9	Frothy saliva at the mouth			
10	Dilation of nostril & mucoid discharge			
11	Swelled throat and dewlap			
12	Epistaxis/bleeding			
13	Polyarthritis particularly on young			

3.4. Can you name a disease that shows all the above mentioned (QuestionNo.3.3) symptoms or any respiratory disease you know?

No	Local name	Scientific name
1		
2		

3.5. What are the possible transmission methods of RD or CBPP disease among cattle?

No	Transmission methods	Yes	No	Don`t know
1	Through contaminated feed or water			
2	Transmitted through sexual contact			
3	Close contact with diseased animal			
4	Inhalation of infected droplets			
5	Through contaminated fomites/objects			
6	Can be transmitted through transplacental			
7	Through fetal membrane & uterine discharge			
8	Can be transmitted across long distance in air			
9	Through saliva or urine of diseased animal			
10	Through coughing of infected animal			

3.5. Farmers' knowledge regarding importance of respiratory disease specific emphasis to CBPP.

No	Impacts of CBPP	Yes	No	Don't know
1	Can cause mortality of cattle			
2	Can cause loss of body weight			
3	Reduced working ability of cattle			
4	Reduced fertility of cattle			
5	Reduced growth rate of cattle			
6	Can cause loss of production			

3.6. What worries you most felt if your animal diseased with respiratory disease/CBPP?

1. Transmission to others 2. Cost of treatment

3. Death due to disease 4. Loss of production 5. No worries

3.7. Which ways do you want to receive knowledge about the disease?

1. Through Wereda expert 2. Through kebele DA

3. Veterinarian/animal health workers 5. Local cultural healers 4. Media

3.8. Which part of CBPP knowledge do you want to know more?

1. Its causes 2. Symptoms 3. Transmission methods

4. Diagnosis methods 5. Prevention and controlling methods

3.9. What do you think the effective way of prevention and controlling methods of CBPP?

No	Prevention and controlling methods	Yes	No	I don't know
1	Vaccination			
2	Treatment of symptomized animal			
3	Test and slaughter or stamping out policy			
4	Movement control or quarantine			
5	Isolation of new purchased animal from herd			
6	Decontamination of infected premises			

Section 4: Farmers General Practices to prevent Infectious Diseases with Specific Emphasis to CBPP disease

4.1. What are farmers' common practices that have been done during herd management to prevent CBPP or any diseases?

No	Activities	Yes	No
1	Avoid communal grazing & watering		
2	Avoid using of common breeding bulls		
3	Avoid purchase of cattle from infected origin		
4	Cleaning/ manure removal & disinfection of farm		
5	Follow up of regular vaccination of herds		
6	Restriction of freely cattle movement		
7	Isolating new purchased animal from the herd		

4.2. What are farmers practices when their animal suspected or diseased with CBPP disease?

No	Activities	Yes	No
1	Selling to neighbors or local market/butchers		
2	Treating with cultural medications		
3	Presenting to veterinary clinic of area		
4	Separating/isolation of diseased animal		
5	Slaughtering for consumption		
6	Follow up stamping out policy of diseased		

Appendix 4: C- ELISA principles and procedures

Reagents		Volume
1	MmmSC antigen coated Plate	10
2	Positive Control (Lyophilized)	1x1.0 mL
2a	Strong positive control (Lyophilized)	1x1.0 mL
3	Negative control (Lyophilized)	1x1.0 mL
4a	Conjugate concentrate (100x)	1x1.2 mL
5	Dilution Buffer N. 24	3x120 mL
A	TMB substrate N. 13	1x120 mL
B	Stop solution N. 13	1x120 mL
C	Wash Concentrate (20x)	2x100 mL
D	Detection Solution (Mab 117/5, Lyophilized)	1x1.0 mL

Note:- see table at the end of the insert for a description of symbols used on the insert and tables of this of this kit.

Storage

Store the reagents at 2-8^{0}c. Reagents are stable until expiration date, provided they have been stored properly.

Materials required but not provided

- Precision micropipettes or multi- dispensing micropipettes
- Dispensable pipette tips.
- Graduated cylinder for wash solution.
- 96-well micro plate reader (equipped with 450 nm filter)
- Micro plate washer (manual, semi- automatic system)
- Use only distilled or deionizer water for preparation of the reagents used in the test
- Micro plate covers (lid, aluminum foil or adhesive)
- Centrifuge (200xg)
- Vortex or equivalent
- Microplate shaker
- Agitator-Incubator capable of maintaining temperature of + 37^{0}C ($\pm$3^{0}c)

- uncoated micro plate for sample preparation

Precautions and Warnings

- Handle all biological material as potentially infectious.
- Wear protective gloves /protective clothing / eye or face protection when handling samples and reagents
- Refer to the product material safety data sheet for additional information
- See the end this insert for reagent hazard and precaution warnings.

Laboratory Practices

- Optimal results will be obtained by strict adherence to this protocol. Careful pipe ting, timing, and washing throughout this procedure are necessary to maintain precision and accuracy. Use a separate pipette tip for each sample and control.
- Do not expose TMB solution to strong light or any oxidizing agents. Handel TMB solution with clean glass or plastic ware.
- All wastes should be properly decontaminated prior to disposal. Dispose of contents in accordance with local, regional, and national regulations.
- Care should be taken to prevent contamination of kit components. Do not pour unused reagents back into containers.
- Do not intermix components from kits with different lot numbers

Wash Solution

The wash concentrate (20x) must be diluted 1.20 with distilled/demonized water before use (e.g. 15 ml of wash concentrate (20x) in285 ml of distilled water). This solution is hereafter called “wash solution”

Note: - the wash concentrate (20x) should be brought to 18-26^{0}c and well mixed to ensure dissolution of any precipitated salts. Wash solution is stable for up 10 3 days when stored at 2-8^{0}c.

Conjugate

The conjugate concentrate (100x) must be dilution buffer N. 24

Note: - diluted conjugate solution is stable for up to 8 hours at 18-260C

Controls

The lyophilized controls must be reconstituted one day in advance with 1 ml of sterile distilled water, liquated and kept at ≤ -160C. SPC = Strong positive control /PC = positive control / NC = Negative control.

Note:- reconstituted controls can be frozen and thawed no more than 3 times.

Tow SPC control wells can be replaced by your own internal reference control material (IRC).

Control wells:

CC = conjugate control wells (Dilution Buffer N.24 only).

MabC = detection solution (Mab 117/5) control wells (Dilution Buffer N.24 and detection Solution only).

Preparation of Samples

Samples and controls are pre-diluted on the prelate (uncoated) (see test procedure).

Note:-samples should not be de-complemented prior to the analysis.

Test procedure

All reagents must be allowed to come to 18-260C before use. Reagents should be mixed by gentle inverting or swirling. Controls may be dispensed anywhere on the micro plate (as an example CC =A1, A2,

SPC = B1,B2, IRC = C1, C2; PC =D1,D2,E1, E2; MabC =F1, F2, G1, G2; NC =H1, H2).

1. Obtain the required number of coated micro plates and uncoated micro plate for sample preparation and record the position of each sample.
2. Dispense the dilution buffer, controls, samples and detection solution:
    a. Dispense 100 µl of dilution N. 24 into each well of the prep late (S).
    b. Dispense another 110 µl of dilution buffer N.24 into two appropriate wells (CC)
    Note:-total volume of dilution buffer N.24 in CC wells: 210
    c. Dispense 11 µl of undiluted strong positive control in four appropriate wells.

Note: the supplied strong positive controls can be replaced in two wells by your own IRC.

d. Dispense 11 µl of undiluted positive control in two or four appropriate wells.
e. Dispense 11 µl of undiluted negative control in two appropriate wells.
f. Dispense 11 µl of undiluted sample per well into remaining wells of the prep late (s).
g. Dispense 11 µl of undiluted detection solution into each well of the preplate except in CC wells.

3. Homogenize the content of the wells and transfer 100 µl from each well of the prep late (S) to the appropriate wells of coated micro plate(S).

4. Cover the micro plate and incubate 1 hour (±5 min.) at +370C (±30C) under gentle agitation avoiding desiccation of the plates.

5. Remove the solution and wash each well with approximately 300 µl of wash solution 2 times. Avoid plate drying between plate washings and prior to the addition of the next reagent. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.

6. Add 100 of DILUTED conjugate in each well.

7. Cover the Microplate and incubate 30 minutes (± 3 min) at $+37^0$C (± 3^0C) under gentle agitation, avoiding desiccation of the plates.

8. Repeat step 5 but this time washing three times.

9. Add 100 µl of TMB substrate N.13 in each well.

10. Incubate 20 minutes (± 3 min) at $+37^0$C (± 3^0C) away from direct light.

11. Dispense 100 µl of stop solution N.3 per well.

12. Measure and record the absorbance value of samples and controls at 450 nm.

Note: when using robotics, incubation of micro plates in an incubation chamber allows working without plate covers. Use of robots is also not compatible with gentle microplate tapping or wiping. Plate can be held up to 1 hour in the dark prior to reading. The duration of substrate incubating can be adjusted to yield and) 0D of 1.000 in the MabC wells.

13. Calculation:

Controls

Calculate conjugate control mean Absorbance (CCx) and Mab control mean Absorbance (mabCx)

CCx= (CC1 A (450) + CC2 A(450)) /2

Mab = (MabCx = (MabC1 A(450) +MabC2 A (450) +MabC A(450)) /4

Samples and controls

Calculate the percentage of inhibition (S Pl) for each sample and control.

S PI % = 100x (MabCx –S A(450)) / (MabCx –CCx)

Validity criteria

$0.500 \leq$ MabCx≤ 2.000 CCx < 0.300

NC pl $\leq 35\%$ $50\% \leq$ pc pl $\leq 80\ \%$ $60\ \% \leq$ SPC pl $\leq 90\ \%$

For invalid assays, technique may be suspect and the assay should be repeated following a thorough review of the package insert

14. Interpretation:

Negative	Positive
S PI < 50 %	S PI ≥ 50 %

Note: for this test, the positivity threshold is set at 50% of inhibition. However, every measurement has a certain uncertainty which depends from the kit itself and of the capabilities of the testing laboratory. Sera with PI values within the range 50 % ± uncertainty of measurement should be consider with care and distinguished from the others that are positive or negative with certainty. It is advisable to perform this ELISA testing under quality assurance and, whenever possible, with an accreditation (i.e. ISO 17025).

Note: IDEXX has instrument and software systems available which calculate results and provide data summaries.

Appendix 5: DNA extraction procedures

Notes before starting

- Perform all centrifugation steps of room temperature (15-250C)
- Red dissolves any precipitates in Buffer Al and Buffer ATL.
- Add ethical to Buffer AW1 and Buffer AW2 concentrates.
- Equilibrate frozen tissue or cell pellets to room temperature.
- Preheat an incubator to 560C.
- Refer to the handbook for pretreatment of fixed tissue, insect, bacterial, or other material.

1a. Tissue: cut tissue (≤ 10mg spleen or ≤ 25 mg other tissue) into small pieces, and place in a 1.5 ml microcentrifuge tube. For rodent tails, use 1 (rat) or 2 (mouse) 0.4-0.6 cm lengths of tail. Add 180 Buffer alt. Add 20 proteinase K, mix by vortexing, and incubate at 560C until completely lysed. Vortex occasionally during incubation. Vortex 15 second directly before proceeding to step 2.

1b. on nucleated blood: pipet 20 proteinase K into a 1.5 ml or 2 ml micro centrifuge tube. Add 50-100 anticoagulant-treated blood. Adjust volume to 220 with PBS. Proceed to step2.

1c. nucleated blood: pipet 20 proteinase K into a 1.5 ml or 2 ml centrifuge tube. Add 5-10 anticoagulant-treated blood. Adjust volume to 220 with PBS. Proceed to step 2.

1d. Cultured cells: Centrifuge a maximum of 5x 106 cells for 5 min of 300x g (190 rpm). Resuspend in 200 PBS. Add 20 proteinase K. proceed to step 2.

2. Add 200 Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56^0C for 10 min.

3. Add 200 ethanol (96-100%). Mix thoroughly by vortexing.

4. Pipet the mixture into a DNeasy mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000rpm) for 1 min. discard the flow-through and collection tube.

5. Place the spin colum in a new 2 ml collection tube. Add 500 Buffers AW1. Centrifuge for 1 min at ≥ 600 x g. discard the flow-through and collection tube.

6. Place the spin column in a new collection tube, add 500 Buffer Aw2, and centrifuge for 3 min at 20,000 x g (14,00 rmp). Discard the flow-through and collection tube.

7. Transfer the spin column to a new 1.5 ml or 2 ml micro centrifuge tube.

8. Elute the DNA by adding 200 buffers AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15-250C). Centrifuge for 1 min at ≥ 6000 x g.

9. Optional Repeat step 8 for increased DNA yield.

Appendix 6: PCR amplification test protocols

NATIONAL VETERINARY INSTITUTE	Document No. NVI -QMS - QF - 41		
Title:- Master mix preparation and PCR work sheet	Effective Date 20/11/2016	Issue No. 1	Page No. 1of 1

Date: 22/05/2017

Conventional PCR for CBPP Isolation and Identity test procedure

1-Master mix preparation

Ser.no	Type of reagent	For one reaction	Total reaction 10	Remark
1	RNase free water	3µl	30	
2	Primer- MSC1 -Fow-5pM/ µl 5'-ATACTTCTGTTCTAGTAATATG-3'	2 µl	20	
3	Primer-MSC2 -REV-5pM/ µl 5'-CTGATTATGATGACAGTGGTTCA-3'	2 µl	20	
4	IQ Super mix	10µl	100	
5	Add Template (DN A)	3 µl		
	Total volume	20 µl		

2-Run PCR Reaction

	Temperature	Time	Cycle	Remark
Initial Denaturation	95^{o}c	5 mints	1-Cycle	

Denaturation	95oc	30 Sec	40 cycles	
Annealing	50^{o}c	30 Sec		
Elongation	72^{o}c	1 mint		
Final Elongation	72^{o}c	7 mints	1-Cycle	
Put at	4^{o}c	Until machine off		

Appendix 7: Agarose gel preparation and gel electrophoresis procedures

Protocol how to prepare and use Gel Red:

1. **For PCR products;**
    - 975 loading buffer –(960)
    - 25 Gel Red – (40)

Mix by vortex and add 4. For 20 of PCR product. Mix by pipe ting and load 10 into the Gel.

2. **For DNA Ladder**
    - 475 loading Buffer
    - 25 Gel Red

    Mix by vortex

Then prepare the following again;

- 265 PCR water
- 59 Pre-prepared (from No. 2 above)
- 27 DNA Ladder

Mix by vortex, and use 10 to load into the gel as molecular maker

- Prepare 1. 5% Ag agarose gel
- Add 5µl Gel red with 4µl dye and 10µl markers (Ladder)
- Run Electrophoresis for 1 hour at 130V
- Read the result by using UV –light
- It is around 260bp positive result

NB “When you prepare Agarose gel DO NOT add Gel Red!”